The mammalian mitochondrial Hsp70 chaperone system, new GrpE-like members and novel organellar substrates

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List of abbreviations

A..... absorbance
ADP..... adenosine 5'-diphosphate
ATP..... adenosine 5'-triphosphate
Azc..... L-azetidine-2-carboxylic acid
BAG-1..... Bcl-2-associated anti-death gene 1
Bcl-2..... B cell lymphoma 2
BiP..... immunoglobulin-binding protein
bis-acrylamide..... N,N'-methylene-bis-acrylamide
B. taurus ..... *Bos taurus*
BSA..... bovine serum albumin
bp..... base pair(s)
CCT..... chaperonin containing TCP-1
C-terminus..... carboxyl-terminus
cDNA..... complementary DNA
C. elegans ..... *Caenorhabditis elegans*
CH_3CN..... acetonitrile
CoA..... coenzyme A
Con. ..... control
cpm..... counts per minute
Cpn..... chaperonin
CyP..... cyclophilin
Da..... Dalton
DCR..... dienoyl CoA reductase
DHFR..... dihydrofolate reductase
D. melanogaster ... *Drosophila melanogaster*
DMEM..... Dulbecco's modified Eagle medium
DNA..... deoxyribonucleic acid
DTT..... 1,4-dithiothreitol
EB..... equilibration buffer
ECL..... enhanced chemiluminescence
E. coli ..... *Escherichia coli*

EDTA..... ethylenediaminetetracetic acid

ER..... endoplasmic reticulum

ESI-MS..... electrospray ionisation mass spectrometry

EST..... expressed sequence tag

GimC..... genes involved in microtubule biogenesis complex

Glycine..... aminoacetic acid

h..... hour(s)

Hap..... Hsp70/Hsc70-associating protein

HEPES..... N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

Hip..... Hsc70-interacting protein

Hop..... Hsc70/Hsp90-organising protein

HPLC..... high pressure liquid chromatography

Hs..... heat shock

Hsp..... heat shock protein

Hsc70..... constitutive (or cognate) isoform of Hsp70

Hsp70..... 70 kDa heat shock protein

HRP..... horseradish peroxidase

H. sapiens ..... *Homo sapiens*

IMAC..... immobilised metal affinity chromatography

IPTG..... isopropyl-1-thio-β-D-galactopyranoside

LB..... Luria-Bertani

min..... minute

MFE..... multifunctional enzyme

M. musculus ..... *Mus musculus*

Mops..... 3-[N-Morpholino]propanesulfonic acid

MPP..... mitochondrial processing peptidase

mRNA..... messenger RNA

MSF..... mitochondrial import stimulation factor

mt..... mitochondrial

mtTFA..... mitochondrial transcription factor A

NAC..... nascent polypeptide-associated complex

NADPH..... β-nicotinamide adenine dinucleotide, reduced form

NMR..... nuclear magnetic resonance

N-terminus..... amino-terminus

ORF..... open reading frame

PAGE..... polyacrylamide gel electrophoresis

PBS..... phosphate buffered saline

PCR..... polymerase chain reaction

PBF..... presequence binding factor

pI..... isoelectric point

PMSF..... phenylmethylsulphonyl fluoride

Ponceau S..... 3-hydroxy-4-[2-sulfo-4-(sulfophenylazo) phenylazo]-2,7-naphthalenedisulfonic acid

PPIase..... peptidyl-proyl *cis/trans* isomerase

PTS-1..... peroxisomal targeting sequence type-1

px..... peroxisomal

RACE..... rapid amplification of cDNA ends

RNA..... ribonucleic acid

R. norvegicus ..... *Rattus norvegicus*

RP..... reversed phase

RT..... reverse transcription

Rubisco..... Ribulose bisphosphate carboxylase/oxygenase

s..... second

S. cerevisiae ..... *Saccharomyces cerevisiae*

SCS..... succinyl CoA synthetase

SDS..... sodium dodecyl sulphate

SSC (20x)..... 3M NaCl, 0.3M Na_3citrate buffer

TAE (50x)..... 2M tris-HCl buffer, 1M acetic acid, 50mM EDTA, pH 8

TCP-1..... *t*-complex polypeptide 1

Tim..... Translocase of the inner membrane

TF..... trigger factor

TFA..... trifluoroacetic acid

Tom..... Translocase of the outer membrane

TRiC..... TCP-1 ring complex

Tricine..... N-Tris[Hydroxymethyl]methylglycine

Tris..... Tris[Hydroxymethyl]aminomethane

Tween-20..... polyoxyethylene-sorbitan monolaurate

U.....unit(s)

UTR..... untranslated region

v..... volume

w.....weight

v/v..... volume/volume

w/v..... weight/volume

WWW..... world wide web

ΔΨ.....*trans*-membrane potential

One and three letter codes for amino acids

A	Ala	alanine	L	Leu	leucine
R	Arg	arginine	K	Lys	lysine
N	Asn	asparagine	M	Met	methionine
D	Asp	aspartic acid	F	Phe	phenylalanine
C	Cys	cysteine	P	Pro	proline
E	Glu	glutamic acid	S	Ser	serine
Q	Gln	glutamine	T	Thr	threonine
G	Gly	glycine	W	Trp	tryptophan
H	His	histidine	Y	Tyr	tyrosine
I	Ile	isoleucine	V	Val	valine

Summary

The DnaK (Hsp70), DnaJ and GrpE heat shock proteins of *Escherichia coli* work synergistically in a diverse number of vital cellular processes including the folding of nascent polypeptides, assembly and disassembly of multimeric proteins, refolding of malfolded proteins, degradation of unstable and non-native polypeptides, regulation of the stress response and the mediation of protein translocation across membranes. Various biochemical and genetic studies have identified homologues of the DnaK, DnaJ, GrpE triad within all cells and the major compartments thereof that participate in similar functions. Thus the concept of a universally conserved Hsp70 chaperone 'system' ('machine' or 'team') has arisen and the *E. coli* triad is considered the prototype. In this study DnaK-affinity purification was employed to identify a mammalian mitochondrial GrpE homologue (mt-GrpE#1) for the first time. Isolation of a cDNA sequence encoding rat mt-GrpE#1 and deduction of its polypeptide sequence, permitted the generation of a consensus sequence for GrpE members from several biological kingdoms that revealed only six invariant residues at the amino acid level. Utilising this consensus sequence a second mammalian mt-GrpE homologue (mt-GrpE#2) was identified and shown to exhibit ~47 % positional identity to mt-GrpE#1 at the amino acid level. Following synthesis in *E. coli*, the functional integrity of mt-GrpE#1 and #2 was verified by their ability to stably interact with and stimulate the ATPase activity of mammalian mitochondrial Hsp70 (mt-Hsp70). A constitutive expression of both mitochondrial GrpE transcripts was observed in 22 distinct mouse tissues but the presence of putative destabilisation elements in the 3'-untranslated region of the mt-GrpE#2 transcript, which are not present in the mt-GrpE#1 transcript, may confer a different expression pattern of the encoded proteins. Evidence is also provided for the existence of a distinct cytosolic GrpE-like protein within mammalian cells.

In conjunction with these studies, several organellar polypeptides (from mitochondria, peroxisomes and the endoplasmic reticulum) were observed to be selectively retained on an immobilised Hsp70 member, the consequence of which is speculated to be of fundamental importance in maintaining proper organellar biogenesis and may constitute a new level of metabolic regulation. The cDNA sequence of one of the retained polypeptides was determined and subsequently characterised as a putative peroxisomal isoform of 2,4-dienoyl CoA reductase. Should this be the case, it is concluded that distinct isoforms of this protein exist in mammalian mitochondria and peroxisomes rather than the dual targeting of the known mitochondrial reductase to both organelles, this has been a subject of debate for several years.

Statement of authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis by which I have qualified for or been awarded another degree or diploma.

No other person's work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Dean J. Naylor August, 1999

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List of Publications

Parts of the work described in this thesis have been published as follows:

(1) Webster, T.J., **Naylor, D.J.**, Hartman, D.J., Høj, P.B., and Hoogenraad, N.J. (1994) "cDNA cloning and efficient mitochondrial import of pre-mtHsp70 from rat liver" *DNA and Cell Biology* **13,** 1213-1220

(2) Martinus, R.D., Ryan, M.T., **Naylor, D.J.**, Herd, S.M., Hoogenraad, N.J., and Høj, P.B. (1995) "The role of chaperones in the biogenesis and maintenance of the mitochondrion" *FASEB J.* **9,** 371-378

(3) **Naylor, D.J.**, Ryan, M.T., Condron, R., Hoogenraad, N.J., and Høj, P.B. (1995) "Affinity purification and identification of GrpE homologs from mammalian mitochondria" *Biochem. Biophys. Acta* **1248,** 75-79

(4) Ryan, M.T., **Naylor, D.J.**, Hoogenraad, N.J., and Høj, P.B. (1995) "Affinity-purification, overexpression and characterisation of Chaperonin 10 homologs synthesised with and without N-terminal acetylation" *J. Biol. Chem.* **270,** 22037-22043

(5) Martinus, R.D., Garth, G., Webster, T.J., Cartwright, P., **Naylor, D.J.**, Høj, P.B., and Hoogenraad N. J. (1996) "Mitochondrial chaperones responses in a mammalian cell line lacking mt-DNA: implications for mitochondrial-nuclear communication" *Eur. J. Biochem.* **240,** 98-103

(6) **Naylor, D.J.**, Hoogenraad, N.J., and Høj, P.B. (1996) "Isolation and characterisation of a cDNA encoding rat mitochondrial GrpE, a stress inducible nucleotide-exchange factor of ubiquitous appearance in mammalian organs" *FEBS Lett.* **396,** 181-188

(7) Ryan, M.T., **Naylor, D.J.**, Høj, P.B., Clarke, M.S., and Hoogenraad, N.J. (1997) "The role of molecular chaperones in mitochondrial protein import and folding" *Int. Rev. Cytol.* **174,** 127-193

(8) **Naylor, D.J.**, Hoogenraad, N.J., and Høj, P.B. (1997) "Mammalian mt-GrpE" In Molecular Chaperones and Protein Folding Catalysts (M-J Gething, ed.) pp. 142-143, Oxford University Press, New York

(9) Hoogenraad, N.J., Høj, P.B., **Naylor, D.J.**, and Webster, T.J.(1997) "Mammalian mt-Hsp70" In Molecular Chaperones and Protein Folding Catalysts (M-J Gething, ed.) pp. 67-69, Oxford University Press, New York

(10) **Naylor, D.J.**, Stines, A.P., Hoogenraad, N.J. and Høj, P.B. (1998) "Evidence for the existence of distinct mammalian cytosolic, microsomal and two mitochondrial GrpE-like proteins, the co-chaperones of specific Hsp70 members" *J. Biol. Chem.* **273,** 21169-21177

(11) **Naylor, D.J.**, Hoogenraad, N.J., and Høj, P.B. (1999) "Characterisation of several Hsp70 Interacting proteins from mammalian organelles" *Biochem. Biophys. Acta* **1431,** 443-450

(12) **Naylor, D.J.**, Koivurantac, K.T., Stines, A.P., Hiltunen, J.K., Hoogenraad, N.J., and Høj, P.B. (1999) "Mitochondria and peroxisomes may contain distinct isoforms of 2,4-dienoyl CoA reductase" To be submitted

Chapter 1

General introduction

1.1 Scope of chaperone existence and focus of this review

It has been argued that protein folding is the single most important process in biology, as it "adds functional flesh to the bare bones of genes" (Dobson and Ellis, 1998). An understanding of the mechanisms and principles guiding the folding of proteins into their biologically active states is desirable for scientists which frequently are confronted by the low natural abundance of proteins with biological, medical or industrial significance. In principle, the low natural abundance of such proteins can be overcome through their production by recombinant DNA technology. Unfortunately, despite extensive research, proteins produced by recombinant technology frequently accumulate as unfolded, insoluble aggregates known as "inclusion bodies" (Marston, 1986). Whilst Afinsen (1973) clearly demonstrated that some unfolded proteins can attain their native state spontaneously *in vitro*, it has now been recognised that many, if not most, unfolded proteins require a pre-existing protein machinery comprising so called "molecular chaperones" (often abbreviated as "chaperones") for their efficient folding (reviewed by Hartl, 1996; Bukau and Horwich, 1998).

The action of molecular chaperones is usually associated with the folding of nascent polypeptides, assembly and disassembly of multimeric proteins, and the refolding of denatured proteins (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). However, based on their ability to recognise and bind to non-native protein conformations in a promiscuous manner, it is now clear that molecular chaperones function in a variety of essential cellular processes. Some of these processes include the targeting of proteins to subcellular compartments, proteolysis, regulation of the stress response, antigen presentation, and activation of signal transduction molecules (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). As many aspects of these processes are sensitive to stresses that perturb the protein structure, such as high temperatures or "heat shock", many molecular chaperones are in fact stress inducible and consequently are frequently referred to as "stress proteins" or "heat shock proteins" (Hsps) (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). The involvement of chaperones in a multitude of cellular processes has led to an interest amongst medical researchers to study human molecular chaperones in order to uncover and treat diseases that may arise, at least in part, due to chaperone malfunction. The areas of medical research which have attracted most attention, and have a potential connection with molecular chaperone action, include prion diseases, autoimmune diseases (rheumatoid arthritis, multiple sclerosis), infectious diseases (tuberculosis, malaria, leprosy), cancer, mitochondrial diseases, certain hereditary diseases (cystic

fibrosis) and amyloid diseases (Alzheimer's) (reviewed by Morimoto *et al.*, 1990, 1994; Martinus *et al.*, 1995; Taubes, 1996; Ryan *et al.*, 1997).

Most knowledge concerning the biochemical and structural basis of molecular chaperone action has come from studying the Hsp70 (DnaK) and chaperonin (Hsp60/Cpn60, TRiC/CCT/TCP-1, TF55/Thermosome) systems (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). These families of chaperones transiently interact with non-native conformations of proteins in an ATP-dependent manner. Whilst these and other molecular chaperones can bind substrates on their own, the presence of "co-chaperones" is required for their efficient and correct functioning (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). Together, distinct molecular chaperones and their co-chaperones constitute chaperone "systems" ("machines" or "teams"), and often it can be a network of distinct chaperone systems that is responsible for carrying out a specific cellular process, such as *de novo* protein synthesis and folding. The conservation of chaperone systems amongst different organisms is striking at both a structural and functional level. Many of the components constitute essential gene products and are sometimes functionally interchangeable in heterologous systems (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). Nowhere has the degree of conservation in chaperone machines been more evident than that seen between eubacteria (eg. *Escherichia coli*) and mitochondria (reviewed by Ryan *et al.*, 1997). However this is perhaps not surprising, since mitochondria are believed to have originated as endosymbionts from a free living aerobic eubacterium that was engulfed by an anaerobic organism (the progenitor of present day eukaryotic cells) (reviewed by Gray, 1989, 1992).

The study of mitochondrial protein biogenesis has revealed an extensive involvement of both cytosolic and mitochondrial molecular chaperones, in particular those of the Hsp70 family, and the elucidation of their functions has provided a wealth of information about chaperones in general. As a consequence of the eubacterial origin of mitochondria, with the resultant transfer of genes to the nucleus of the present day eukaryotic cell, the vast majority of mitochondrial proteins must be synthesised on cytosolic ribosomes and successively be targeted to mitochondria, translocated across their membranes, folded, assembled (where appropriate), maintained in their functional state or disposed of with each step requiring molecular chaperone action (reviewed by Ryan *et al.*, 1997). The research reported in this thesis has primarily focused on the mammalian mitochondrial (mt-) heat shock protein 70 (Hsp70) system, which is comprised of the molecular chaperone mt-Hsp70, and the co-chaperones mt-GrpE and mt-DnaJ. Despite the existence of extensively characterised Hsp70 machines in both bacteria and yeast mitochondria, the comparatively small amount of research reported here already serves to

illustrate some fundamental differences between the evolution of the yeast and mammalian mt-Hsp70 machines. Accordingly, the aim of this introductory chapter is not to present a comprehensive review of the rapidly expanding area of molecular chaperone research, but will rather focus on a selection of the most important concepts, with particular emphasis on the mt-Hsp70 machine. For further information on molecular chaperones the reader is referred to two extensive reviews which I co-authored during my PhD candidature (Martinus *et al.*, 1995; Ryan *et al.*, 1997).

1.2 The discovery of heat shock (stress) proteins and molecular chaperones

In 1962 Fredrica Ritossa reported that exposure of isolated salivary glands of *Drosophila* to temperatures slightly above those which are optimal for the fly's normal growth and development resulted in a completely new pattern of puffing in their polytene chromosomes (Ritossa, 1962). This curious observation of chromosomal puffing, occurring within a minute or two after the temperature upshift, was later demonstrated by Tissières and colleagues (1974) to be accompanied by the rapid and high level expression of what are now known as "heat shock proteins" (Hsps). The process, was defined as the "heat shock response" and, was soon observed, by several researchers, to be a common property of all cells. Furthermore, evidence was accumulating that similar changes in gene expression occur when cells are subjected to a number of different and seemingly unrelated stresses, including exposure to amino acid analogues, puromycin, alcohols, heavy metal ions, glucose deprivation, the presence of calcium ionophores and viral infection (reviewed by Morimoto *et al.*, 1990). To account for the numerous stimuli that can elicit a heat shock response, the more general term "stress response" was introduced, and accordingly, it is associated with the expression of "stress proteins" (Thomas *et al.*, 1982).

Lawrence Hightower recognised that many of the agents that caused a stress response could perturb protein structure, and thus, he suggested that the common dominator for the induction of the stress response might be the accumulation of damaged or malfolded proteins in the cell (Hightower, 1980). The proposal was confirmed by Ananthan, Goldberg and Voellmy (1986) when they showed that the stress response could be elicited by injecting denatured, but not native, proteins into *Xenopus* oocytes. A picture was emerging whereby the cells of all organisms would respond to the accumulation of denatured and aggregated proteins, arising from heat shock and other metabolic insults, by initiating the stress response. The resultant synthesis and rapid increase in stress proteins was then believed to assist in the removal of such deleterious protein conformers.

In the quest to understand how the stress response works, several research groups isolated and sequenced the genes and cDNAs encoding stress proteins. As a result of this sequencing campaign, two unexpected but informative discoveries emerged, and in hindsight, these formed the foundations upon which chaperone research was rapidly built. First, comparisons of gene and cDNA sequences, that encoded stress proteins from a number of distant organisms, revealed that stress proteins are among the most highly conserved proteins in nature. In most organisms, Hsp70 members are the most prominent proteins induced by stress, thus it was not surprising that the first nucleotide sequence obtained from a gene encoding a stress protein corresponded to *Drosophila melangaster* Hsp70 (Ingolia *et al.*, 1980). In the next few years Elizabeth Craig and colleagues isolated and sequenced Hsp70 genes from *E. coli* (encoding DnaK) and *Saccharomyces cerevisiae* which were predicted to encode ~70 kDa proteins that were 48 % and 72 % identical to the *Drosophila* protein, respectively (Bardwell and Craig, 1984). Given the high degree of sequence conservation amongst Hsp70 members, several research groups soon isolated and reported the sequences of yet other genes and cDNAs encoding Hsp70 proteins. Furthermore, several researchers observed that antibodies raised against one Hsp70 protein could cross-react with stress proteins of a similar molecular mass in numerous and diverse organisms. Researchers were beginning to suspect that stress proteins performed essential and similar roles in all organisms and therefore have been highly conserved throughout evolution.

The second unexpected discovery to emerge from the sequencing of genes and cDNAs encoding stress proteins was that numerous stress proteins which were sometimes distinct, but still highly homologous, to those induced by stress could also be found in normal (or unstressed) cells. Furthermore, many of these constitutively expressed stress proteins were found to be essential for cell viability. Before the isolation and sequencing of the first Hsp70 gene in *Drosophila*, numerous research groups reported that the *D. melangaster* genome contained a Hsp70 gene family with multiple members. In subsequent years, several of these *Drosophila* Hsp70 genes were cloned and sequenced. In contrast to the first Hsp70 gene isolated, these were not inducible by stress but were expressed in an almost constitutive fashion during the normal development of fly cells (Ingolia and Craig, 1982; Craig, Ingolia and Manseau, 1983). These new genes encoded so-called heat shock constitutive (or cognate) proteins of 70 kDa (Hsc70) and were latter found to reside in the cell's cytosol. The abbreviation (Hsc70) has been widely adopted, and is now used to describe any constitutively expressed Hsp70 protein located in the cytosol of eukaryotic cells. Similar families of constitutive and stress inducible Hsp70 members have now been characterised in organisms as diverse as bacteria, yeast and man. In parallel to the characterisation of genes encoding Hsc70 proteins, the presence of a constitutively expressed

form of Hsp70 was independently discovered in mammalian cells by peptide mapping (Hightower and White, 1981) and later by immunological cross-reactivity (Welch and Feramisco, 1984).

Owing to the striking induction of Hsp70's expression in response to cellular stress, initial work on the Hsp70 family concentrated on its function following heat shock. Thus, when mammalian cells were subjected to heat shock, both the constitutive and stress inducible forms of Hsp70 are rapidly translocated to the nucleus with high levels accumulating within the region of the nucleolus involved in assembly of small ribonucleoproteins and pre-ribosomes (Pelham, 1984; Welch and Feramisco, 1984). Hsp70 then accelerates the restoration of functional nucleoli from heat damage (Pelham, 1984) in a process that is apparently dependent on ATP (Lewis and Pelham, 1985). Based on co-localisation of Hsp70 with pre-ribosomes, it is thought that the main role of Hsp70 is to assist in the re-activation of damaged pre-ribosomal complexes for their assembly into functional ribosomes during the cell's recovery from stress (Welch and Suhan, 1986). The first evidence that the energy of ATP might be required in the function of Hsp70 members was the reported weak intrinsic ATPase activity of *E. coli* Hsp70 (DnaK) (Zylicz *et al.*, 1983). Subsequently, Welch and Feramisco (1985) recognised that several mammalian Hsp70 proteins bound tightly to ATP and could be affinity purified on ATP-agarose. Thereafter, most studies utilising Hsp70 proteins have employed this facile purification technique.

While the function of Hsp70 members was initially realised from studies of the stress-inducible members, this was soon changed by the serendipitous discoveries of their roles in numerous cellular processes during non-stressing conditions. Thus, long before *E. coli* DnaK was recognised as an Hsp70 member, it was identified as a factor necessary for bacteriophage λ growth (Georgopoulos and Herskowitz, 1971). The exact role of DnaK was later defined to λ DNA replication where it was shown, in an ATP dependent manner, to disassemble the λO-λP-DnaB helicase complex at the origin of λ replication (*ori*λ). This liberates the DnaB helicase whose DNA unwinding activities permits initiation of λ DNA replication (reviewed by Georgopoulos *et al.*, 1990). In eukaryotic cells another Hsp70 member, the glucose regulated protein of 78 kDa molecular mass (Grp78), was originally identified as a protein synthesised at increased rates when cells are deprived of glucose (Pouyssègur *et al.*, 1977; Shiu *et al.*, 1977). The synthesis of Grp78 was subsequently found to be induced by numerous stresses but surprisingly, not significantly by heat shock (Welch *et al.*, 1983). In 1986 Munro and Pelham cloned and sequenced the cDNA encoding Grp78 which revealed the presence of an N-terminal presequence that targeted the protein to the ER lumen. In the same study, Munro and Pelham

(1986) showed that Grp78 was identical to the immunoglobulin heavy chain binding protein (BiP) and that the transient interaction of BiP with heavy chain immunoglobulins required hydrolysable ATP. BiP was originally discovered through its association with immunoglobulin heavy chains in pre-B cells, that do not synthesise light chains (Hass and Wabl, 1983). BiP is now recognised to interact with numerous ER resident and secretory proteins that require folding and oligomerisation. In the cytosol of mammalian cells, a protein originally termed the "clathrin uncoating ATPase", due to its ability to promote disassembly of clathrin lattices in an ATP dependent manner, was later recognised to be the constitutively expressed Hsp70 protein (Ungewickell, 1985; Chappell *et al.*, 1986). Later the cytosolic Hsp70s of yeast were shown to help maintain polypeptide preproteins in a competent state for translocation across the ER or mitochondrial membranes, the process was also dependent on hydrolysable ATP (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Zimmermann *at al.*, 1988; Murakami *et al.*, 1988).

Based on this early work with Hsp70, Pelham (1986) proposed a general mechanism for the action of Hsp70 members. He suggested that during heat shock or during *de novo* protein synthesis, the unfolded conformers of proteins expose hydrophobic surfaces and as a result have a tendency to form inappropriate intra- or inter-protein interactions that lead to aggregation. Hsp70 might recognise and shield such aggregation-prone surfaces in order to prevent unfavourable interactions and, should they occur, it was postulated that Hsp70 might promote disaggregation as well. Pelham further speculated that the energy released through ATP hydrolysis would promote a conformational change in Hsp70 that in turn would enable the release of a bound substrate. The released polypeptide then had the option of folding and assembling into its native, functional conformation or re-entering the Hsp70 cycle for another round of ATP-dependent binding and release. While an impressive majority of Pelham's predictions have been proven essentially correct and indeed can be applied to additional stress protein families, it is now recognised, at least for Hsp70 members, that the energy of ATP hydrolysis is required not for the release of substrate but is required for the tighter binding of substrate. As will be outlined in a latter section, it is in fact the binding, not the hydrolysis, of ATP by Hsp70 that triggers the release of bound substrate.

In a separate line of research, Laskey and colleagues (1978) describe the specialised nuclear protein nucleoplasmin, as a "molecular chaperone", due to its ability to promote nucleosome assembly from DNA and histones. The term molecular chaperone was later suggested to describe the function of a different protein implicated in the ATP-dependent assembly of Ribulose bisphosphate carboxylase/oxygenase (Rubisco) within chloroplasts (Barraclough and Ellis, 1980; Musgrove and Ellis, 1986). However, the idea that the so-called

"Rubisco binding protein" could function as a molecular chaperone originally attracted little attention presumably because of the well held belief in Christian Anfinsen's "self-assembly principle". Based largely on the spontaneous refolding of reduced and denatured ribonuclease A *in vitro*, the principle states that all of the information required for a polypeptide chain to fold and assemble into its native and functional conformation is contained solely within its amino acid sequence (reviewed by Anfinsen, 1973). Furthermore, the process requires neither an energy source nor additional macromolecules that convey steric information to guide the process. Nevertheless, researchers have over time recognised that the success of protein folding *in vitro* usually was restricted to small, single domain proteins and mostly required the use of low protein concentrations and low temperatures to decrease the probability that inappropriate interactions, between hydrophobic surfaces, would lead to aggregation. Given then that the inside of a cell represents a highly unfavourable environment for protein folding and assembly, and in light of Pelham's speculations on the role of Hsp70 in this process, Ellis (1987) proposed that proteins acting as "molecular chaperones" exist in all cells, and the compartments thereof, to ensure that protein folding and assembly proceeds correctly.

A year later support for Ellis' proposal came from yet another serendipitous discovery. In 1988, Hemmingsen *et al.* reported the cloning and sequencing of several cDNAs encoding the subunits of the Rubisco binding protein which, unexpectedly revealed ~50 % positional identity at the amino acid level with the *E. coli* GroEL protein. Like *E. coli* DnaK, the GroEL protein was discovered genetically in the early 1970's as a factor necessary for bacteriophage λ growth. However, unlike DnaK, the function of GroEL was pinpointed to the ATP-dependent assembly of oligomeric phage connector protein which is important for joining the head of the phage to its tail (reviewed by Georgopoulos *et al.*, 1990). In parallel with the study of Hemmingsen *et al.* (1988), independent work by McMullin and Hallberg (1988) established immunological similarity between GroEL and mitochondrial heat shock proteins of 60 kDa (Hsp60) in plants, humans, yeast, frogs and a protozoan. The high sequence similarity between these proteins lead Hemmingsen *et al.* (1988) to define them as a distinct family of molecular chaperones, termed the "chaperonins" or "Cpns" (Lubben *et al.*,1989 and Goloubinoff *et al.*,1989a). Each chaperonin is an oligomer with subunits of molecular mass ~60 kDa.

Initially it was believed that the chaperonins were only found in eubacteria, chloroplasts and mitochondria, in accordance with their common ancestry. Nevertheless, protein sequence searches later identified a distantly related entity, the cytosolic mouse *t*-complex polypeptide 1 (TCP-1), which exhibited low but significant amino acid similarity with the GroEL (Hsp60) family (Ellis, 1990; Gupta, 1990). Subsequently, TCP-1 related proteins were found in the

cytosol of numerous eukaryotes (collectively termed TRiC or CCT) and archaebacteria (termed TF55 or Thermosome) where they carry out similar ATP-dependent functions as the GroEL (Hsp60) family (reviewed by Willison and Horwich, 1996). Following the discovery of the distantly related TCP-1 family, the chaperonin family was divided into two distinct subclasses (Ellis, 1992): (1) the Group I chaperonins, found in plastids (Rubisco binding protein), mitochondria (Hsp60 or Cpn60), and eubacteria (GroEL); and (2) the Group II chaperonins, found in archaebacteria (TF55 or Thermosome) and the cytosol of eukaryotic cells (TRiC or CCT).

The discovery that cellular protein folding and/or assembly often required pre-existing protein machines, the molecular chaperones, soon led to the re-evaluation of Anfinsen's pioneer experiments on the spontaneous refolding of denatured proteins *in vitro*. Goloubinoff *et al.* (1989ab) reported that purified GroEL and it's co-chaperone GroES, in a process that requires ATP hydrolysis, could both increase the yield of correctly refolded bacterial Rubisco and reduce it's tendency to aggregate upon dilution from denaturing agents. Soon after this initial finding with the GroEL/GroES team, Skowyra *et al.* (1990) and Ziemienowicz *et al.* (1993) reported a similar finding with DnaK and it's co-chaperones DnaJ and GrpE. In this case though, the denatured *E. coli* RNA polymerase was utilised as a substrate and evidence was shown that both the DnaK/DnaJ/GrpE team and the GroEL/GroES team could dissociate and reactivate aggregates formed from this partially denatured protein in processes dependent on ATP (Ziemienowicz *et al.* 1993). Finally, in a well defined *in vitro* system, Langer *et al.* (1992) reported the synergistic and successive roles of DnaK and GroEL chaperone systems in the prevention of polypeptide misfolding and aggregation. Originally observed in mitochondria with Hsp70 and Hsp60 members, the synergistic and successive roles of various chaperone teams in protein folding and/or assembly has subsequently been outlined in the cytosol, ER and chloroplasts of eukaryotic cells (reviewed by Hartl, 1996; Frydman and Höhfeld, 1997; Johnson and Craig).

Early studies on the folding and assembly of newly imported mitochondrial matrix proteins identified a defective yeast strain (mif4) (Cheng *et al.*, 1989) which contained a temperature-sensitive lethal mutation in the essential Hsp60 gene (Reading *et al.*, 1989). Analysis of the mif4 mutant at non-permissive temperatures showed that whilst several preproteins were efficiently imported into mitochondria and whilst their N-terminal targeting peptides were removed, these proteins failed to assemble into their oligomeric complexes. In *Neurospora crassa* mitochondria, Ostermann *et al.* (1989) then observed that a monomeric protein, that could refold spontaneously *in vitro* with high efficiency, required a transient

interaction with Hsp60 for folding *in vivo*. This study was of particular interest for it provided the first compelling evidence that molecular chaperones are involved in protein folding per se rather than the subsequent assembly of folded monomers into oligomeric complexes. Prior to the characterisation of mitochondrial Hsp60 in fungi, Craig *et al.* (1987) identified an essential yeast gene (*SSC1*) and latter showed that it encoded a mitochondrial Hsp70 protein (Ssc1p or mt-Hsp70) (Craig *et al.*, 1989). Subsequently, crosslinking studies established that mt-Hsp70 could interact with a preprotein that was arrested in it's translocation across the mitochondrial membranes (Scherer *et al.*, 1990), while the analysis of an *SSC1* yeast mutant (*ssc*1-2) revealed a requirement for mt-Hsp70 in both the translocation and folding of mitochondrial preprotein proteins (Kang *et al.*, 1990). Manning-Krieg *et al* (1991) extended these observations when reporting that ATP is required by mt-Hsp70 to transiently associate with translocating preprotein proteins en route to the matrix. Furthermore, release of the subunits of two oligomeric proteins from mt-Hsp70 was observed to be insufficient in itself to ensure their correct folding and assembly; a subsequent ATP-dependent interaction with Hsp60 was required for this to occur.

1.3 How do proteins fold?

The process by which a linear polypeptide attains its single well-defined three dimensional structure from the astronomical number of folding pathways, that would otherwise lead to biologically inactive species, has perplexed researchers for many years (reviewed by Jaenicke, 1987, 1991, 1995). Most of our knowledge concerning the folding and assembly of proteins has come from *in vitro* test tube studies with purified globular proteins under well defined conditions which represent a much less complex environment than an intracellular milieu. In the seminal studies of Anfinsen and colleagues, it was observed that ribonuclease A, which had been unfolded by chemical denaturation, can spontaneously refold into it's correct functional conformation upon dilution of the denaturing agent—the "principle of self-assembly" (reviewed by Anfinsen, 1973). Similar *in vitro* experiments by subsequent investigators indicated that protein folding and assembly, where appropriate, followed an hierarchical mechanism (reviewed by Jaenicke, 1987, 1991, 1995). Successive elements of secondary/supersecondary structure and subdomains/ domains as intermediates are now agreed to be common to most, if not all, protein folding pathways and the driving force is thought to be the polypeptide's tendency to bury aggregation prone hydrophobic side chains to minimise their contact with polar water molecules (reviewed by Jaenicke, 1987, 1991, 1995).

Despite Anfinsen's principle of self assembly, researchers have over time recognised that the success of protein folding *in vitro* usually was restricted to small, single domain proteins, and more than often required the use of low protein concentrations and low temperatures to decrease the probability that inappropriate interactions, between exposed hydrophobic surfaces, would lead to aggregation. In contrast, protein folding and assembly *in vivo* is usually extremely efficient with greater than 95 % of *de novo* synthesised polypeptides reaching their functional states despite the requirement of many cell types to grow over a range of high temperatures and maintain very high protein concentrations, up to 500 mg/ml in mitochondria (Gething *et al.*, 1986; Copeland *et al.*, 1986; Hackenbrock *et al.*, 1968; Schwerzmann *et al.*, 1986). Clearly a large fraction of the intracellular space is occupied by macromolecules, and this may lead to a situation of "macromolecular crowding" (reviewed by Ellis and Hartl, 1996). One effect of macromolecular crowding, which can be particularly pronounced in specific locations of the cell, eg. on translating polysomes, might be a greater propensity of hydrophobic surfaces within nascent polypeptides to form inappropriate intermolecular associations that in turn would lead to unproductive aggregation. So, despite all these potential obstacles, why is protein folding and assembly *in vivo* so efficient ?

One explanation is that nascent polypeptides have the potential to fold co-translationally as they emerge from the ribosome, an option that does not exist *in vitro* where fully synthesised polypeptides are folded (Netzer and Hartl, 1997, 1998). Thus, the folding of a two-domain model protein, where each domain comprises of a continuous sequence of amino acids, occurs domain by domain as the polypeptide emerges from eukaryotic ribosomes (Netzer and Hartl, 1997). In comparison, in an *in vitro* system these N- and C-terminal domains fold concurrently and with low efficiency, presumably as a consequence of inappropriate interactions between hydrophobic surfaces of each unfolded domain (Netzer and Hartl, 1997). Interestingly, because bacterial translation does not support co-translational folding, this same problem might also be the reason why so many eukaryotic modular proteins misfold upon expression in *E. coli* (Netzer and Hartl, 1997). In the case of single domain proteins and two-domain proteins, whose domains are comprised of discontinuous amino acid sequences, these proteins have to fold post-translationally. For these proteins, it might therefore be undesirable for their emerging N-terminal regions to undergo premature folding or to form inappropriate interactions with themselves or other macromolecules prior to their complete synthesis. A similar situation exists for polypeptides which are translocated through narrow membrane channels en route to the lumen of an organelle such as the mitochondrion. The unfolded preprotein resembles an incompletely synthesised polypeptide during its vectorial (N- to C-terminus) and post-translational import into

mitochondria. What then prevents these *de novo* synthesised polypeptides, or even denatured pre-existing proteins, from associating inappropriately with themselves or other macromolecules during their translocation across membranes and/or their folding ? Numerous biochemical and genetic studies indicate that a pre-existing protein machinery, the "molecular chaperones", fullfil such a role.

1.4 The molecular chaperone concept

As will be outlined in detail later, molecular chaperones essentially prevent protein misfolding by binding to and stabilising non-native protein conformations before releasing them in a reaction often requiring ATP hydrolysis (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). Chaperones in general recognise and shield exposed hydrophobic side chains, which are usually buried within the protein's native conformation, and otherwise have a tendency to inappropriately associate with other interactive surfaces (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). The binding of chaperones to interactive surfaces in unfolded, but usually not native proteins, therefore not only protects unfolded protein conformers from aggregation, but most importantly, through timely release and rebinding cycles of chaperones, also allows productive interactions to occur which are required for correct folding. In the event that such productive interactions do not allow the native state to be reached, the same or a different type of chaperone can recognise and (re)bind the unfolded polypeptide, permitting another opportunity for productive interactions to occur that lead to the acquisition of the native and functional state (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). In this manner different types of chaperones can work sequentially with synergistic effects. Through this process of binding and release, molecular chaperones do not increase the rate of folding but instead increase the efficiency of protein folding. It should be noted chaperone action is not solely confined to "quality control" of protein biogenesis but also includes several essential cellular processes downstream of protein folding (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998).

The discovery of molecular chaperones by no means contradicts Anfinsen's principle of self-assembly (reviewed by Anfinsen, 1973). Molecular chaperones do not convey steric information specifying the correct folding pathway of a non-native protein and do not form a structural component of the folded and assembled states, instead they allow efficient self-assembly to proceed in the extremely unfavourable folding environment of a cell. It could be argued that the molecular chaperone machines constitute intracellular "Anfinsen tubes"—where

protein folding proceeds in the absence of other interfering molecules. Furthermore, molecular chaperones do not require metabolic energy to actively promote protein folding, instead the energy released through ATP hydrolysis by some chaperones is required to permit the tightly regulated and timely release of the chaperone from its substrate. This is entirely consistent with Anfinsen's finding that protein folding is an energetically favourable reaction.

Using the nomenclature first described in *Drosophila*, most molecular chaperones are referred to on the basis of their induction by heat shock (Hs), and their apparent subunit molecular mass (kDa) as revealed by SDS-PAGE analysis (reviewed by Georgopoulos and Welch, 1993; Martinus *et al.*, 1995; Gething, 1997). Thus the major families of heat shock proteins (Hsp) or molecular chaperones are: Hsp110, Hsp100, Hsp90, Hsp70, Hsp60 and sHsp (small Hsp, with molecular masses between 15-30 kDa) (Table 1). The amino acid sequence identity is high between members of each family but the identity between members within different families is usually insignificant. It should be noted, that although the problem of protein misfolding and subsequently aggregation increase with temperature, thus increasing the demand for molecular chaperones, not all heat shock proteins function as molecular chaperones. Some heat shock proteins are proteases which work synergistically with chaperones to remove unfolded or damaged proteins from the cell. Conversely, not all molecular chaperones accumulate at higher levels in response to high temperatures. Many molecular chaperones are in fact constitutively expressed to perform essential housekeeping functions in the cell which must occur at all temperatures encountered. One such function is obviously *de novo* protein synthesis.

1.5 The Hsp70 molecular chaperone system

1.5.1 *E. coli* constitutes a model system for the study of eukaryotic molecular chaperones

There is believed that mitochondria and chloroplasts originated as endosymbionts from free-living gram-negative eubacteria and accordingly (reviewed by Gray, 1989, 1992), their molecular chaperone complements, particularly the chaperonin (GroEL, Hsp60) and Hsp70 (DnaK) families, share extensive sequence and structural identity (reviewed by Gething, 1997). Whereas the creation of mitochondria and chloroplasts is believed to have occurred 1.5 billion years ago, the origin of the proeukaryotic cell probably dates back at least an additional billion years and as a result its ancestor(s) still remains rather elusive. Comparison of eukaryotic and gram-negative eubacterial Hsp70 sequences has revealed the presence of a unique 23-27 residue insert that is not present in Hsp70s from either archaebacteria or gram-positive eubacteria, suggesting the evolution of the proeukaryotic cell from a gram-negative eubacterium (Gupta and

Golding, 1993; Gupta *et al.*, 1994). On the other hand, the cytosolic Group II chaperonins (TRiC, TF55) have only been found in archaebacteria and eukaryotic cells, inferring that archaebacteria were in fact the ancestor of the proeukaryotic cell (reviewed by Willison and Horwich, 1996). To explain this apparent anomaly, a model has been proposed whereby the proeukaryotic cell is suggested to have a "chimeric origin", being derived from the primary fusion between a gram-negative eubacterium and a eocyte lineage of archaebacterium (reviewed by Gupta, 1996). Thus, according to this model, the genes encoding proteins of the eukaryotic cytosol could have either an archaebacterial origin (in the case of TRiC) or a gram-negative eubacterial origin (in the case of Hsp70).

The majority of bacteria today are derived from the eubacteria lineage, the most well studied being the gram negative bacterium *Escherichia coli*, which is a common inhabitant of human intestines. Unlike the highly compartmentalised and often highly differentiated eukaryotic cells, unicellular *E. coli* only contains a small number of molecular chaperones with seemingly redundant functions. The relatively simple cell structure and chaperone complement of *E. coli*, along with the ease with which mutants can be generated, has facilitated both the biochemical and genetic characterisation of *its* molecular chaperone functions *in vitro* and *in vivo*. Furthermore, given that *E. coli*, the eukaryotic cell and mitochondria may all share a common ancestor, and that chaperones have presumably been highly conserved throughout evolution to perform essential and similar roles, the study of *E. coli* chaperones should reveal invaluable information about their eukaryotic homologues. Indeed, as we will see in the following sections, the *E. coli* GroEL and DnaK chaperone teams are both structurally and functionally very similar to the mitochondrial Hsp60 and Hsp70 systems. Whilst the cytosol of eukaryotic cells contains a less conserved Hsp70 team, it does not contain a chaperonin closely related GroEL.

1.5.2 The *E. coli* DnaK (Hsp70) chaperone system

The DnaK chaperone machine of *E. coli* is comprised of the DnaK molecular chaperone (a Hsp70 member) and the co-chaperones DnaJ (a Hsp40 member) and GrpE. The genes encoding these proteins were first identified through mutations blocking the growth of bacteriophage λ due to the prevention of λ DNA replication (reviewed by Friedman *et al.*, 1984). The mutants were termed either *groP* (*gro* for blocking λ phage growth, and *P* because growth can be restored by secondary suppressor mutations in the λ *P* gene) or *grp* (for *groP*-like) and were classified into six groups, ie *grpA*, *B*, *C* (*groPC756*), *D*, *E* and *F* (*groPC259*). The *grpC* (*groPC756*) *and grpF* (*groPC259*) genes were later renamed *dnaK* and *dnaJ*, respectively, when

they were shown to play a role in *E. coli* DNA replication (Saito and Uchida, 1977, 1978; Sunshine 1977). Subsequently, the *grpE* gene product was also shown to be involved in *E. coli* DNA synthesis, however, it was not renamed (Ang *et al.*, 1986; Ang and Georgopoulos, 1989). In *E. coli* the *dnaK* and *dnaJ* genes constitute an operon, with the *grpE* gene found at a separate locus, but in some other prokaryotes the *grpE* gene has been identified as part of the *dnaK/dnaJ* operon (Saito and Uchida, 1978; Wetzstein and Schumann, 1990). All of these genes, along with *GroEL* and *GroES*, are components of the heat shock regulon (reviewed by Georgopoulos *et al.*, 1990, 1994). This regulon is under the transcriptional regulation of the *rpoH* (*htpR*) gene product σ^{32} and accordingly, the expression of these chaperone encoding genes is induced as part of the heat shock response which is elicited by numerous forms of stress, including temperature upshift (reviewed by Georgopoulos *et al.*, 1990, 1994). For example, DnaK is an abundant cytosolic protein constituting ~1.2 % of the total cellular protein in *E. coli* grown at 30°C, but its level is increased to ~3 % of the total cellular protein content only an hour after the *E. coli* growth temperature is increased from 30°C to 42°C (Herendeen *et al.*, 1979; Hesterkamp and Bukau, 1998). Despite the similar modes of regulation, the cellular concentrations of DnaK, DnaJ and GrpE, in non-stressed *E. coli* cells, are estimated at ~5 μM, ~1 μM and 1-2 μM, respectively (Bardwell *et al.*, 1986; Goodsell, 1991; Neidhart *et al.*, 1984; Diamant and Goloubinoff, 1998). Given DnaK (~70 kDa) probably functions as a monomer while both DnaJ (~41 kDa) and GrpE (~23 kDa) are most likely functional as dimers, the approximate molar ratio of the functionally active DnaK:DnaJ:GrpE components is ~20:2:3 (Schönfeld *et al.*, 1995a; Zylicz *et al.*, 1985; Osipiuk *et al.*, 1993; Harrison *et al.*, 1997). It should be noted though, all of these components can adopt a range of self-oligomers and as a result the form(s) in which these chaperones are functional is not clearly defined (Schönfeld *et al.*, 1997).

Detailed biochemical and genetic studies have established that the DnaK (Hsp70)/ DnaJ (Hsp40)/GrpE triad of *E. coli* functions synergistically in a diverse number of vital cellular processes, including the folding of nascent polypeptides (reviewed by Hartl, 1996; Ryan *et. al.*, 1997). In *E. coli*, the *grpE* gene is essential for cell viability (Ang *et al.*, 1986; Ang and Georgopoulos, 1989) while the requirement for the *dnaK* and *dnaJ* genes appears to be variable among *E. coli* K-12 strains (Teter *et al.*, 1999). Thus, while the *dnaK* and *dnaJ* genes cannot be deleted in *E. coli* W3110 genetic backgrounds, in the MC4100 background *dnaK* and *dnaJ* deleted strains grow slowly at 37°C with a tendency to rapidly accumulate extragenic suppressors and do not grow at all at 42°C (Bukau and Walker, 1989). The suppressor mutation was later ascribed to the σ^{32} factor which effectively prevents the uncontrolled overexpression of heat

shock proteins (Bukau and Walker, 1990). Interestingly, combined deletion of *dnaK* gene and the nonessential *tig* gene (encoding trigger factor (TF)) is lethal in the MC4100 background under normal growth conditions (Teter *et al.*, 1999). Together with studies on the DnaK and TF proteins, it appears that these proteins have important and partially overlapping functions in *de novo* protein folding (Teter *et al.*, 1999). In *dnaJ* deletion mutants part of DnaJ's function appears to be restored by the presence of second DnaJ-like homologue (ie CbpA) in *E.coli* cells, for *cbpA/dnaJ* double deletion mutants have a greater temperature restriction for growth (Ueguchi *et al.*, 1995). A third *dnaJ*-like gene (*YABH, djlA, rcsG*), encoding a type III inner membrane protein, also appears to function with DnaK and GrpE to positively activate the RcsA/B/C signal transduction pathway to induce capsular polysaccharide production (Kelley and Georgopoulos, 1997). An additional DnaK/DnaJ chaperone team, comprised of the gene products Hsc66 (HscA)/Hsc20 (HscB), exists in *E. coli* (Lelivelt and Kawula, 1995). These genes are induced by cold shock but not by heat shock (Lelivelt and Kawula, 1995). The Hsc66/Hsc20 team appears to operate independent of a GrpE-like member and is unable to complement a DnaK deletion mutant, implying that these Hsp70 members serve distinct cellular roles in *E.coli* (Hesterkamp and Bukau, 1998; Silberg *et al.*, 1998). A third *dnaK*-like gene encoding Hsc62 has recently identified in *E. coli* (Yoshimune *et al.*, 1998).

A multitude of studies have shown that homologous, and often multiple isoforms, of both DnaK and DnaJ exist in a large variety of procaryotic cells and in multiple compartments of eukaryotic cells, indeed DnaJ homologous even exist in several tumour viruses (reviewed by Laufen *et al.*, 1998; Kelley, 1998, 1999). The notable exception is the apparent absence of DnaK and DnaJ homologous in various archaebacteria (Gribaldo *et al.*, 1999). For those archaebacteria that do contain a DnaK system, they are suggested to have required them from bacterial donors through lateral gene transfer events (Gribaldo *et al.*, 1999; Nelson *et al.*, 1999). The conservation of the DnaK/DnaJ system is believed to have persisted throughout evolution for their cooperation is probably obligatory for the functioning of Hsp70 chaperones (Laufen *et al.*, 1999). In contrast, while GrpE homologues have been found in a large variety of procaryotic cells and at least in one archaebacterium, they have only been detected in mitochondria and possibly chloroplasts of eukaryotic cells (Schlicher and Soll, 1997; discussed in Naylor *et al.*, 1996, 1998) (Table 1). The absence of GrpE homologues from some Hsp70 systems might be explained by the action of additional co-factors that enable their Hsp70 partners to fulfil a broader range of tasks (see section 1.5.3.2). Taken together, these observations indicate that during the course of evolution, the amplification of components for Hsp70 systems has been accompanied by a diversification of individual chaperone functions, where different Hsp70 teams either work synergistically or

independently to perform essential roles during both normal conditions and when cells are exposed to stress (reviewed by Mayer and Bukau, 1998). Furthermore, numerous studies suggest that by increasing the number of DnaJ-like proteins that Hsp70 proteins can utilise, additional functional diversity can be generated (see section 1.5.3.2). This is probably the reason that *Saccharomyces cerevisiae* contain at least 17 DnaJ-like proteins and only 14 Hsp70 members (reviewed by Laufen *et al.*, 1998).

1.5.3 Structure-function relationship of the DnaK(Hsp70)/DnaJ(Hsp40)/GrpE system

1.5.3.1 DnaK(Hsp70) component

Hsp70 molecular chaperones consist of two major domains, a 44 kDa N-terminal ATPase domain and a 25 kDa C-terminal substrate binding domain that communicate to mediate efficient binding and timely release of substrates (Fig. 1). The X-ray crystallographic structure of the bovine brain Hsc70 ATPase domain has been known for some time (Flaherty *et al.*, 1990) and surprisingly, it is almost identical to that of the globular G actin monomer, and is similar to the structure of hexokinase (Flaherty *et al.*, 1991; Bork *et al.*, 1992; Holmes *et al.*, 1993). The Hsc70 ATPase domain consists of two lobes (I and II) and each is divided into a subdomain A and B (ie IA, IB and IIA, IIB). The two lobes are connected by two crossed α-helices and are separated by a large upper nucleotide binding cleft and a smaller lower cleft (the proposed binding site for the J-domain of DnaJ, see below). At the bottom of the nucleotide binding cleft ATP, coordinated to one Mg^{2+} and two K^{+} ions, is bound through interactions with both lobes and the connecting α-helices. At the amino acid level, the N-terminal ATPase domain is highly conserved amongst Hsp70 members, which is reflected in the nearly identical structures of the recently determined human Hsp70 (Sriram *et al.*, 1997) and *E. coli* DnaK ATPase domains (Harrison *et al.*, 1997).

The structure of the entire Hsp70 molecule is yet to be solved, and owing to the inherent oligomerisation behaviour of the C-terminal domain, the structural topology of this latter domain remained elusive for many years. The oligomerisation problem was recently overcome by allowing a C-terminal fragment of DnaK (Val 389-Ala 607) to bind a high affinity heptapeptide (NRLLLTG) (Gragerov *et al.*, 1994), which along with other (poly)peptide substrates, is known to promote and stabilise the monomeric (functional) form of Hsp70s. The X-ray crystallographic structure of this complex revealed that the C-terminal substrate binding domain is comprised of a further β-sandwich subdomain (Val 394-Lys 502) followed by an α-helical subdomain (Glu 509-Ala 607) (Zhu *et al.*, 1996) (Fig. 1). The compact β-sandwich subdomain is composed of eight antiparallel β-strands, where four lower strands form a regular β-sheet while four upper strands

form an unusual β-sheet with four connecting loops that protrude upwards from the β-sandwich to create a "substrate binding channel" ("pocket" or "cavity'). Two inner loops enclose the heptapeptide in an extended conformation while two outer loops stabilise the channel. Further stability of the channel is gained by critical interactions between these outer loops and helix B of the α-helical subdomain, where helix B appears to act as a "lid" to encapsulate the peptide in the channel but doesn't interact with the substrate directly. Interestingly, the substrate binding domain of DnaK was crystallised in two forms (type 1 and 2 crystals), and when superimposed reveal a kink half way along helix B (between residues Arg 536-Gln 538) of the type 2 crystal that causes a partial lifting of the lid and the subsequent destabilisation of the channel loops which grasp the heptapeptide. The type 2 crystal is suggested to represent an early conformational change, in the context of the whole molecule, that arises when DnaK binds ATP in its N-terminal domain which leads to the release of substrate in its C-terminal domain (reviewed by Rüdiger *et al.*, 1997a; Bukua and Horwich, 1998). The α-helical subdomain itself is composed of five successive helices (A-E) (Fig. 1). Helix A connects helix B to the β-sandwich subdomain while helices C-E, together with the C-terminal end of helix B, constitute a distinct segment (Gln 538-Ala 607) that may interact with DnaJ (Wawrzynów and Zylicz, 1995; Suh *et al.*, 1998).

Amongst Hsp70 members, both the ATPase domain and β-sandwich subdomain are highly conserved (~51-76 % positional identity between *E. coli* DnaK, bovine Hsc70 and hamster BiP), whilst the α-helical subdomain is considerably less conserved (~18-49 % positional identity for the region studied during X-ray crystallography and ~5-10 % for the remaining C-terminal segment) (Zhu *et al.*, 1996). Because of its relative variability, the α-helical subdomain is therefore a good candidate for providing at least some functional specificity to Hsp70 proteins, perhaps by coordinating Hsp70 interactions with cofactors such as DnaJ. In support of this idea, a regulatory EEVD motif has been identified at the extreme C-terminus of nearly all cytosolic eukaryotic Hsp70s (Freeman *et al.*, 1995). When deleted or certain changes are introduced into this motif, Hsp70's basal ATPase activity is dramatically increased, its affinity for unfolded substrates is reduced and its ability to interact with Hsp40 (Hdj1) in the refolding of denatured luciferase is inhibited (Freeman *et al.*, 1995). Consistent with study, the immobilised α-helical subdomain of rat Hsc70 quantitatively retained Hsp40 (Hdj1) only when the C-terminal EEVD motif was present (Demand *et al.*, 1998). Interestingly, a second co-factor of Hsc70 termed Hop was retained in similar manner to Hsp40 and in a non-competitive manner (Demand *et al.*, 1998). Further support for the contention that the α-helical subdomain provides

functional specificity comes from the findings that at least part of the functional uniqueness between two yeast Hsp70 members (Ssa and Ssb) is provided by the whole C-terminal substrate binding domain (Lopez-Buesa, *et al.*, 1998), and not by the β-sandwich subdomains alone (James *et al.*, 1997). Other studies suggest that the α-helical subdomain is dispensable for both the ATP-induced conformational changes and the subsequent release of substrates (Ha and McKay., 1995; Buckberger *et al.*, 1995; Wilbanks *et al.*, 1995), while it is not essential for the DnaJ stimulation of DnaK's ATPase activity (Gässler *et al.*, 1998; Leng *et al.*, 1998). Although these later results would appear to contradict the idea that DnaJ interacts with the α-helical subdomain, it should be noted that stimulation of ATPase activity represents only one consequence of the Hsp70-Hsp40 association. Thus, Zhang and Walker (1998) have shown that whilst DnaK, lacking residues 561-607 of the α-helical subdomain (ie helices C, D and E), has virtually the same affinity for small peptides, it can not stably bind them. A similar study with a fragment of rat Hsc70, lacking the entire α-helical subdomain, concluded that this destabilisation is even more pronounced with large, unfolded proteins, as exemplified with permanently unfolded *S*-carboxymethyl α-lactalbumin protein (Hu and Wang, 1996).

1.5.3.2 DnaJ(Hsp40) component

The unifying feature of the Hsp40 family is a conserved so-called "J-domain" (originally described for *E. coli* DnaJ) that is essential for orchestrating interactions with their Hsp70 partners which lead to ATP hydrolysis and tighter substrate binding (reviewed by Caplan *et al.*, 1993; Silver and Way, 1993; Laufen *et al.*, 1998; Kelley, 1998, 1999). The NMR solution structures of the *E. coli* DnaJ J-domain with the adjacent G/F-rich region (residues 2-108, Szyperski *et al.*, 1994; Pellecchia *et al.*, 1996) and the human Hsp40 (Hdj1) J-domain (residues Qian *et al.*, 1996) reveal very similar topologies. The J-domain is comprised of four α-helices, where the two amphipathic antiparallel helices 2 and 3 form a compact coiled-coil with helices 1 and 4 oriented perpendicular to it (Fig. 1). The structure is stabilised by hydrophobic residues on the amphipathic interior surfaces of helices 1, 2 and 3 which form a core. A flexible loop which connect helices 2 and 3 contains the highly conserved His/Pro/Asp motif that is implicated in the interaction of Hsp40 members with their Hsp70 partners (reviewed by Caplan *et al.*, 1993; Silver and Way, 1993; Laufen *et al.*, 1998). Indeed, a recent NMR study suggests that the flexible loop and helix 2 of the DnaJ J-domain are important for interaction with the ATPase domain of DnaK (Greene *et al.*, 1998). Helix 2 is predicted to contain a large positive surface potential which might mediate its electrostatic interaction with an oppositely charged surface of the DnaK

ATPase domain. This possibility is supported by studies with DnaK mutants which provided evidence that DnaJ (presumably its J-domain) interacts in an ATP-dependent manner with a lower cleft between the IA and IIA subdomains of the DnaK ATPase domain that is predicted to have negative surface potential (Suh *et al.*, 1998; Gässler *et al.*, 1998).

Studies with other DnaK mutants suggest that DnaJ may also require an interaction (presumably through domains other than the J-domain) at or near the substrate binding channel of DnaK to efficiently stimulate ATP hydrolysis and tighter substrate binding by DnaK (Suh *et al.*, 1998; Gässler *et al.*, 1998). This suggests that DnaJ can tightly couple functions within both the ATPase and substrate binding domains of DnaK, presumably by simultaneously interacting with these domains. In accordance, DnaK mutations that uncouple the interdomain communication, without causing structural or functional alterations, can not be stimulated by DnaJ (Laufen *et al.*, 1999). Despite the progress in mapping interactions between DnaK and DnaJ, further studies are required to identify if the J-domain does in fact bind to the lower cleft in the ATPase domain of DnaK, and to establish whether the additional interactions of DnaJ with the substrate binding domain of DnaK merely serve to stabilise the DnaJ-DnaK complex, or whether the interactions are integral to DnaK interdomain communication. As will be discussed below, certain DnaJ homologous (such as *E. coli* DnaJ) contain domains that permit them to bind protein substrates and target them to their Hsp70 partner, while other DnaJ-like proteins lack substrate binding domains and instead utilise their common J-domain to recruit their Hsp70 partner to the vicinity of a potential substrate. In light of this, the additional interactions that DnaJ forms with the substrate binding domain of DnaK probably serve to facilitate substrate transfer to DnaK and/or to permit the stable interaction of substrate with DnaK. Indeed, recent evidence suggest that alone DnaJ is unable to efficiently stimulate the ATP hydrolysis activity of DnaK, but in the presence of a protein substrate it can (Laufen *et al.*, 1999). This dual signal mechanism (ie DnaJ and substrate) for ATP hydrolysis is proposed to be conserved in all Hsp70/Hsp40 systems (Laufen *et al.*, 1999).

Apart from their ~70 residue signature J-domains, Hsp40 members typically contain different combinations of three additionally conserved domains, initially identified in *E. coli* DnaJ (Fig. 1). These are a glycine/phenylalanine- (G/F-) rich region, a zinc finger-like domain and a conserved C-terminal domain (reviewed by Caplan *et al.*, 1993; Silver and Way, 1993; Laufen *et al.*, 1998). The G/F-rich region is present in ~45 % of Hsp40 members, where it is believed to function as a flexible linker between the J-domain and other regions of Hsp40 members (reviewed by Laufen *et al.*, 1998). Although the NMR studies involving this region support its flexible nature, it is likely that G/F-rich region has less freedom when anchored to

adjacent Hsp40 segments (Szyperski *et al.*, 1994; Pellecchia *et al.*, 1996). The zinc finger-like domain and the conserved carboxyl terminus are present in different combinations in ~36 % of Hsp40 members (reviewed by Laufen *et al.*, 1998) and both regions have been shown to interact with non-native proteins (Szabo *et al.*, 1996; Banecki *et al.*, 1996; Lu and Cyr, 1998). Some members of the Hsp40 family only contain the signature J-domain, but these proteins appear to contain other specialised domains which permit them to bind specific substrates or localise them to a particular cellular milieu, examples include Tim44, Sec63p, Mdj2p, Zuotin, Dj1A, Auxillin, Hsj1a/b and the cysteine string protein (discussed below).

An important insight into the diversification of Hsp70 systems came from the finding that Hsp40 members can target or recruit substrates to their Hsp70 partners, for the utilisation of their chaperone function in cellular processes other than protein folding (review in Laufen *et al.*, 1998; Kelley, 1998, 1999). For DnaJ, which contains a zinc finger-like domain and a conserved carboxyl terminus, the outcome might be a broadened range of substrates for DnaK. For other Hsp40 members, such as eukaryotic auxillin which contains a clathrin binding domain, the effect is to target specific proteins (in this case clathrin) to Hsc70 (reviewed by Kelley, 1998, 1999). Still other Hsp40 members appear not to contain a polypeptide binding domain at all, and they utilise the J domain to recruit Hsp70 partners to the vicinity of a potential substrate. An example of such Hsp70 recruitment is evident in the translocation of proteins into mitochondria, where a matrix located J-domain of the inner membrane protein Tim44 permits an interaction with mt-Hsp70, thereby recruiting the power of the chaperone to the site of preprotein entry into the organelle (see section 1.6.1). Further functional diversity can be generated by the cooperation of several distinct Hsp40 members with the same Hsp70 member. Thus, three *E. coli* Hsp40 members (DnaJ, CbpA, Dj1A) can interact with DnaK (reviewed by Mayer and Bukau, 1998). Similarly, in the cytosol of mammalian cells, auxillin can recruit Hsp70 to uncoat clathrin coated endocytotic vesicles, the cysteine string protein cooperates with Hsp70 to promote the fusion of synaptic exocytotic vesicles with the plasma membrane (reviewed by Kelley, 1998, 1999), Hsp40 (Hdj1) associates with ribosomes and works together with Hsp70 during the folding of nascent polypeptides (Frydman *et al.*, 1994), while Hdj2 (but not Hsp40) cooperates with Hsp70 to facilitate mitochondrial protein import and the refolding of denatured proteins (Terada *et al.*, 1997). In yeast cells, the Hdj2 homologue (Ydj1p) contains a C-terminal farnesyl moiety which is implicated in its interaction with ER and mitochondrial membranes, where Ydj1p can presumably recruit the chaperone activities of Ssa1p (Hsp70) to facilitate protein translocation into these organelles, possibly by maintaining preproteins in an import competent state (Caplan *et al.*, 1992ab). Recently, Hdj2 has also been shown to be farnesylated (Kanazawa *et al.*, 1997).

1.5.3.3 GrpE component

The X-ray crystallographic structure of an N-terminally truncated (residues 34-197) GrpE bound to the ATPase domain of DnaK (residues 3-383) has been recently solved (Harrison *et al.*, 1997) (Figs 1 and 2). This confirmed earlier studies that a dimer of GrpE binds to a single molecule of DnaK in the absence of ATP (Osipiuk *et al.*, 1993; Schönfeld *et al.*, 1995b; Wu *et al.*, 1996). While the structural topology of each GrpE monomer is similar, the elongated dimer is asymmetrically bent towards DnaK. One proximal monomer of GrpE is closest and forms most of contacts with six distinct surface areas of DnaK (Harrison *et al.*, 1997). One of these areas (designated IV) constitutes a previously described conserved loop (residues 28-34 of DnaK and 56-62 of Ssc1) that was found to be essential for GrpE-DnaK (Buchberger *et al.*, 1994) and Mge1-Ssc1 (Miao *et al.*, 1997a) interactions, but may not be required for the function of at least Mge1 as a nucleotide release factor for Ssc1 (Sakuragi *et al.*, 1999). Within the GrpE-DnaK complex, the distal monomer of GrpE appears to function mostly in stabilisation of the proximal monomer and together they form an unusually long parallel α-helical structure (Harrison *et al.*, 1997). The long N-terminal α-helices (~100 Å) are followed by a shorter four-helix bundle, where each monomer of GrpE contributes two helices. The two monomers end in a six-stranded β-sheet, which extend from their respective four-helix bundle like a pair of arms. The proximal β-sheet arm is proposed to facilitate release of DnaK-bound ADP by essentially wedging out the IIB subdomain by 14 Å, relative to its position in the ADP-bound structure of the bovine Hsc70 ATPase domain. The wedging action displaces, by as much as 3 Å, three DnaK residues (Glu-267, Lys-270 and Ser-274) that provide important hydrogen bonds to the adenine and ribose rings of bound ADP. This explains the absence of bound ADP in the crystal structure defining the interaction between GrpE and the ATPase domain of DnaK compared with the ADP-bound structure of the bovine Hsc70 ATPase domain (Harrison *et al.*, 1997).

In addition to GrpE's role as a nucleotide release factor for DnaK, the crystal structure of dimeric GrpE bound to the ATPase domain of DnaK indicates that GrpE could also function in substrate release from DnaK (Harrison *et al.*, 1997). Thus, while the C-terminal β-sheet arms and the four-helix bundle of dimeric GrpE contribute most of the interactions with DnaK's ATPase domain necessary for nucleotide release, the long N-terminal α-helices of dimeric GrpE extend away from the C-terminal end of the ATPase domain to a location that might be in close proximity to DnaK's substrate binding domain. Here it is proposed that an N-terminal region of dimeric GrpE may mediate an interdomain communication for DnaK necessary for substrate

release (Harrison *et al.*, 1997). In support of this proposal, a GrpE mutation E53G which results in a temperature sensitive phenotype for bacteriophage λ replication (Wu *et al.*, 1994), maps to an area in the long α-helices that is not involved in their interface stability and which is located at least 22 Å from the ATPase domain of DnaK. This suggests that the N-terminal region of GrpE's long α-helices through an interaction with the substrate binding domain of DnaK, could contribute a distinct function to GrpE such as substrate release from DnaK. Other studies show that while full-length GrpE can cause the dissociation between a complex of DnaK and permanently unfolded reduced carboxymethylated α-lactalbumin, N-terminally truncated GrpE (lacking residues 1-33) failed to cause dissociation (Harrison *et al.*, 1997), even though this N-terminally truncated form of GrpE is functional in the renaturation of luciferase by the DnaK/DnaJ system (Harrison *et al.*, 1997). Based on these studies it is proposed that GrpE may be required for the efficient release of certain unfolded proteins that are tightly bound to DnaK. Further studies are required to understand whether GrpE has a direct role in the substrate release from DnaK and if so, how this is achieved.

1.5.4 Reaction cycle of the DnaK(Hsp70)/DnaJ(Hsp40)/GrpE system

A combination of detailed biochemical, genetic and structural studies have revealed that the chaperone activity of Hsp70 proteins is manifested through cycles of substrate binding and their timely release. Furthermore, such cycling requires large conformational changes in Hsp70 that are driven by the energy of ATP and are tightly controlled by substoichiometric amounts of co-factors (or "co-chaperones") (reviewed by Rüdiger *et al.*, 1997a; Bukau and Horwich, 1998). The process is often termed the Hsp70 "ATPase cycle" and in it's simplest form, the cycle is described as alternation between two conformational states: the ATP-bound state, with low affinity and fast association-dissociation rates for substrates (where the substrate binding channel is open), and the ADP-bound state, with high affinity and slow association-dissociation rates for substrates (where the substrate binding channel is closed) (reviewed by Rüdiger *et al.*, 1997a; Bukau and Horwich, 1998) (Fig. 3). An understanding of how these two conformational states interconvert to allow Hsp70 proteins to transiently interact with substrates is best understood for the bacterial DnaK system. In this case, substoichiometric amounts of DnaJ and GrpE are required for the proper functioning of DnaK and are believed to regulate DnaK's ATPase cycle in a highly dynamic manner (Liberek *et al.*, 1995; Diamant and Goloubinoff, 1998; Pierpaoli *et al.*, 1998) (Fig. 4). In fact together, DnaJ and GrpE can stimulate ATPase activity of DnaK at least

240-fold, which is probably more than sufficient to support its chaperone activity (Liberek *et al.*, 1991a; McCarty *et al.*, 1995; Gässler *et al.*, 1998).

Analysis of the kinetic properties for the two conformational states of DnaK reveals that, in order to support efficient protein folding, DnaK must rapidly bind to unfolded proteins in its ATP-bound state, for in the ADP-bound state, interaction would be too slow to prevent the aggregation of misfolded substrates. Furthermore, hydrolysis of ATP is then necessary to permit the transition of DnaK to the ADP-bound state, which is accompanied by a large conformational changes and subsequently the stabilisation of substrate interaction (Liberek *et al.*, 1991b; Schmid *et al.*, 1994; Palleros *et al.*, 1993, 1994; Greene *et al.*, 1995; McCarty *et al.*, 1995; Buchberger *et al.*, 1995). The intrinsic ATPase activity of DnaK (k_{cat} ~0.04 M ATP hydrolysed/ M DnaK/ min, at 30°C), however, isn't fast enough to allow efficient stabilisation of the substrate-DnaK interaction before the substrate dissociates (McCarty *et al.*, 1995; Theyssen *et al.*, 1996; Russell *et al.*, 1998). This problem is overcome, in probably all Hsp70 ATPase cycles, by the synergistic stimulation DnaK's ATPase activity by DnaJ and a polypeptide substrate (Laufen *et al.*, 1999). While earlier studies have shown that both DnaJ and polypeptide substrates alone are capable of stimulating DnaK's ATPase activity, the effects are much less efficient (Laufen *et al.*, 1999).

Following a suitable interaction time of the substrate with DnaK, the substrate is released in a process that requires the exchange of bound ADP for ATP and is accompanied by another large conformational change (reviewed by Rüdiger *et al.*, 1997a; Bukau and Horwich, 1998). Although the release of ADP from DnaK has been shown to be 10–20-fold faster than the rate of ATP hydrolysis in an unstimulated ATPase cycle, it becomes rate-limiting in the DnaJ-stimulated cycle (McCarty *et al.*, 1995; Theyssen *et al.*, 1996). In *E. coli* this limitation is overcome with the assistance of GrpE, which has a homologous component in the mitochondrial and probably chloroplast Hsp70 systems, that accelerate ADP release by up to 5000-fold (Packschies *et al.*, 1997) allowing the rapid binding of ATP (Theyssen *et al.*, 1996; Banecki and Zylicz, 1996). Unlike the original proposal (Liberek *et al.*, 1991b), conformational changes resulting from ATP binding, rather than hydrolysis, trigger substrate dissociation thereby completing the DnaK ATPase cycle and permitting DnaK to enter another (Buchberger *et al.*, 1995; Theyssen *et al.*, 1996). In purified systems, GrpE can stimulate the efficient release of both ADP and ATP from DnaK, however, several observations would suggest that GrpE predominantly exchangers ADP for ATP *in vivo*. Thus, GrpE forms a stable interaction with ADP-bound, but not ATP-bound, DnaK (Zylicz *et al.*, 1987), the intracellular levels of ATP are higher than the levels of ADP and ATP has a higher affinity for DnaK than does ADP (Theyssen *et al.*, 1996; Banecki and Zylicz, 1996; Russell *et al.*, 1998).

In summary, the action of DnaJ is to facilitate the association and stable binding of substrates to DnaK, whilst GrpE facilitates the release of substrates. Together, DnaJ and GrpE can tightly regulate the duration of substrate-DnaK interaction.

1.5.5 In some Hsp70 systems, GrpE may be replaced by additional co-factors that broaden the functions of Hsp70 chaperones

Given that the Hsp70 (DnaK)/Hsp40 (DnaJ) system has been highly conserved throughout evolution, it is intriguing that GrpE homologues may not be required for the efficient functioning of this system in the cytosol, the nucleus or the ER of eukaryotic cells. In agreement with this contention, it is known that the rate-limiting step in the prokaryotic (DnaK/DnaJ) and mitochondrial Hsp70/Hsp40 systems is the release of bound ADP, a process facilitated by GrpE (Liberek *et al.*, 1991a; Szabo *et al.*, 1994; Banecki and Zylicz, 1996), whilst in contrast, the rate-limiting step for the cytosolic/nuclear yeast (Ssa1p/Ydj1p) and mammalian Hsp70/Hsp40 systems is ATP-hydrolysis (Ziegelhoffer *et al.*, 1995; Höhfeld *et al.*, 1995; Minami *et al.*, 1996). In the latter cases, ADP is released spontaneously, and therefore GrpE may be dispensable for these systems. There are two problems with this scenario though. Firstly, the ADP-bound state of cytosolic/nuclear Hsp70 is too short-lived to permit an efficient interaction with a substrate (Höhfeld *et al.*, 1995; Minami *et al.*, 1996). Indeed, the same problem may exist for the DnaK system when the concentration of GrpE exceeds the normal physiological level (Diamant and Goloubinoff, 1998). As a consequence of elevated GrpE levels, the ATPase activity of the DnaK/DnaJ system turns over too rapidly to promote efficient protein folding. Presumably, GrpE increases the ADP/ATP exchange to the extent that substrate release from DnaK is too quick to ensure that the substrate is stabilised long enough to be partitioned down the correct folding pathway.

Secondly, recent studies have identified several co-factors that are capable of regulating the cytosolic/nuclear Hsp70 system although their precise role(s), is still largely unknown. For example, a Hsc70-interacting protein (Hip) (or p48) of ~41 kDa molecular mass prevents the release of ADP from Hsc70 and thereby stabilises substrate binding (Höhfeld *et al.*, 1995). Furthermore, a protein termed BAG-1 (Hap, Hap46, RAP46) competes with Hip for binding to the Hsc70 ATPase domain and once bound prevents the release of Hsc70 bound substrates even in the presence of ATP and Hsp40 (Hdj1) (Bimston *et al.*, 1998). The substrate in stable complex with Hsc70 and BAG-1 remains folding competent, suggesting that the complex represents an intermediate state in the pathway of Hsc70 mediated folding. BAG-1 stimulates the ATPase activity of Hsc70 several fold (Bimston *et al.*, 1998), but does not function as nucleotide

exchanger as originally proposed (Höhfeld and Jentsch, 1997). Recently, an evolutionary conserved family of BAG-1 proteins has been identified, with at least five members in humans that range in molecular mass from ~36-58 kDa (Froesch *et al.*, 1998; Takayama *et al.*, 1999). All members contain distinct N-terminal regions but share a conserved C-terminal BAG domain (of ~45 amino acids) which binds Hsp70 (Takayama *et al.*, 1999). Like many Hsp40 members, BAG-1 like proteins have been found to interact with numerous polypeptides and may also serve as adaptors for recruiting Hsp70 members to perform chaperone functions in numerous cellular processes (reviewed by Takayama *et al.*, 1999).

An additional Hsp70 adaptor is the ~60 kDa Hsc70/Hsp90-organising protein (Hop), alternatively named p60, Sti1, and RF-Hsp70 (reviewed by Frydman and Höhfeld, 1997). Hop appears to target Hsp90 to complexes of Hsp70 and steroid receptors or oncogenic protein kinases to facilitate the folding and functional maturation of these signal transduction proteins (reviewed by Frydman and Höhfeld, 1997; Buchner, 1999; Mayer and Bukau, 1999). A similar mechanism probably accounts for the synergistic action of Hop and Hsp90 in stimulating the Hsp70/Hsp40 system in the refolding of denatured firefly luciferase (Johnson *et al.*, 1998), although one study has reported that Hsp90 initially binds and maintains denatured luciferase in a state competent for refolding after the addition of Hsp70 and Hsp40 (Freeman and Morimoto, 1996). Hop purified from a rabbit reticulocyte lysate is reported to act as a recycling factor for Hsp70 by stimulating ADP release and permitting ATP binding (Gross *et al.*, 1999), while recombinant Hop is reported to be inactive in this regard despite its ability to facilitate the refolding of denatured luciferase (Johnson *et al.*, 1998). Hop can also interact directly with TRiC (CCT), where it promotes the ATP-bound or "open" state associated with substrate release, possibly by acting as a nucleotide exchange factor (Gebauer *et al.*, 1998). Recently, a putative 38 kDa DnaJ-like protein (ALA-D) was shown to stimulate, alone or synergistically with Hop, the renaturation of luciferase by Hsp70 (Gross *et al.*, 1999). Another potential regulator of Hsp70, a ~40 kDa protein termed HspBP1, inhibits the *in vitro* renaturation of luciferase by the Hsp70/Hsp40 system presumably by interacting with the Hsp70 ATPase domain and thereby possibly inhibiting ATP binding (Raynes and Guerriero, 1998). Finally, a 16 kDa protein (p16), which is a member of the Nm23/nucleotide diphosphate kinase family, can promote both the formation of monomeric Hsp70 (the active form) and the release of a Hsp70-bound substrate (Leung and Hightower, 1997).

1.5.6 The DnaK(Hsp70) substrate binding motif

Early studies established that DnaK and eukaryotic Hsp70 members preferentially bind heptameric peptides containing large internal hydrophobic residues in an extended conformation (Flynn, *et al.*, 1991; Landry, *et al.*, 1992; Blond-Elguindi, *et al.*, 1993; Fourie, *et al.*, 1994; Gragerov, *et al.*, 1994). More recently, a systematic peptide scanning approach was applied to more rigorously define the substrate specificity of DnaK (Rüdiger *et al.*, 1997a). A "high-affinity" motif is comprised of a 4-5 residue hydrophobic core enriched in Leu, but also in Ile,Val,Tyr and Phe, and flanked by two basic segments enriched in Arg and Lys. Acidic residues (Asp and Glu) are disfavoured throughout the motif (Zhu *et al.*, 1996; Rüdiger *et al.*, 1997a). The basic residues of the motif are thought to form electrostatic interactions with the negatively charged surroundings of the DnaK substrate binding channel. The DnaK binding motif is statistically predicted to occur every 33 residues within protein sequences, which is consistent with the promiscuity with which DnaK binds to protein substrates (Rüdiger *et al.*, 1997ab).

Co-crystallisation and structural determination of the DnaK substrate binding domain in association with the peptide NRLLLTG has also revealed that almost all of the interactions with the heptapeptide occurs through the five middle residues of the peptide (Zhu *et al.*, 1996) (Fig. 1). However, peptide binding was observed to be centred on Leu^4, which is completely buried in a deep pocket that is designated "site 0", and appears to be the crucial determinant for peptide binding though (Zhu *et al.*, 1996). Although Ile or Met are expected to fit the cental site 0 favourably, other residues would be expected to pay an enthalpic penalty for being too small or too big and charged residues are unlikely to be tolerated. Amongst Hsp70 members, the residues contributing to the interaction of the heptapeptide with DnaK and those forming β-subdomains in general (which house the substrate binding channel) are highly conserved (Zhu *et al.*, 1996; Rüdiger *et al.*, 1997b). In comparison, the residues contributing to the negative surface charges on either side of DnaK's substrate binding channel have been less conserved, and accordingly, differences in these surface charges have been predicted to influence substrate interactions with several Hsp70 members (Zhu *et al.*, 1996). It is therefore not surprising that, although some divergent peptide binding specificities have been observed amongst members of the Hsp70 family, the similarities are much more pronounced (Fourie, *et al.*, 1994, 1997). Indeed, domain shuffling experiments have shown that the β-subdomains from two distinct Hsp70 members (Ssa and Ssb) can be interchanged without significant effects, suggesting this subdomain plays little or no role in determining specific Hsp70 functions (James *et al.*, 1997).

As outlined earlier (section 1.5.3.2), the specificities of most, if not all, Hsp70 members are broadened by their association with one or more Hsp40 members. In addition, a recent study

has shown that DnaJ can permit DnaK to preferentially bind an unfolded protein rather than a peptide substrate, despite the fact that DnaK had at least a 10-fold higher affinity for the peptide and that the peptide was present at a five-fold molar excess over the unfolded protein (Laufen *et al.*, 1999). It is proposed that such discrimination imposed on DnaK may prevent its substrate binding channel from becoming jammed with peptides, which would be expected to be readily available in a cellular environment where proteolysis is constantly occurring. The capacity of certain Hsp40 members bind and target substrates to their Hsp70 partner may result from their abilities to recognise similar extended and unstructured binding motifs. This assumption is supported by their cooperation, which is probably obligatory, in numerous cellular processes such as *de novo* protein synthesis.

1.5.7 The contribution of molecular chaperones to the folding of newly synthesised proteins and those unfolded by stress

In both prokaryotic and eukaryotic cells, most of the molecular chaperone activity, involved in *de novo* protein folding, is contributed by the Hsp70 and chaperonin systems (reviewed by Hartl, 1996; Johnson and Craig, 1997; Bukau and Horwich, 1998). For example, in the cytosol of mammalian cells, a minimum of ~15-20% and ~9-15% of nascent polypeptides was observed to transiently interact with Hsc70 and TRiC, respectively (Thulasiraman *et al.*, 1999). A significant portion of *de novo* synthesised polypeptides, up to 65% in the cytosol of mammalian cells, would however be expected to reach their native state without the assistance of the Hsp70 and chaperonin systems (Thulasiraman *et al.*, 1999). Although it is apparent that certain substrates, such as inactive steroid receptors and oncogenic protein kinases, require an interaction with the highly conserved Hsp90 chaperone for their folding and functional maturation in eukaryotic cells, Hsp90 does not appear to have a general role in *de novo* protein folding (Nathan *et al.*, 1997, Johnson and Craig, 1997). Furthermore, these Hsp90 substrates require an initial interaction with Hsc70 (discussed below and in section 1.5.5). Some proteins such as small single domain polypeptides and larger proteins comprised of small independent domains may fold co-translationally without the assistance of chaperones in the eukaryotic cytosol (Netzer and Hartl, 1997, 1998). Other nascent polypeptides might be folded by the ribosomes themselves, which have recently been shown to posses an innate chaperone activity (Das *et al.*, 1996; Kudlicki *et al.*, 1997; Ellis, 1997; Caldas *et al.*, 1998). Regardless of chaperone involvement, further work is required to determine how newly synthesised polypeptides fold and assemble within the crowded environment of a cell without forming adverse interactions that would result in their aggregation.

Comparable to the proportion of newly synthesised polypeptides observed to associate with Hsc70 and TRiC in the mammalian cytosol, a minium of ~5-10 % and ~15 % of *de novo* synthesised polypeptides transiently interact with *E. coli* DnaK and GroEL respectively under normal growth conditions (Ewalt *et al.*, 1997; Teter *et al.*, 1999). Interestingly, while ~10 % of the polypeptides that associate with GroEL appear to have a strict dependence on GroEL for their folding, the remaining ~5 % are believed to constitute newly synthesised polypeptides that usually fold in a GroEL-independent manner but for an unknown reason become kinetically trapped during their folding (Ewalt *et al.*, 1997). For these latter polypeptides, a transient interaction with GroEL may be required to destabilise such trapped folding intermediates and this in turn permits their rapid folding. This proposal is supported by the observation, that the proportion of newly synthesised polypeptides associated with GroEL increases from ~15 % to ~30 % a few seconds after *E. coli* cells are shifted from growth at 30°C to 42°C (Ewalt *et al.*, 1997). It is tempting then to speculate, that the role of molecular chaperones in all cells might primarily be to fold a small proportion of proteins which face inherent difficulties folding into their native states. A secondary role is then to assist the folding of "self-sufficient" proteins in the event they experience occasional difficulties in their folding. This idea clearly awaits further investigations.

1.5.8 The cooperation of molecular chaperone systems during *de novo* protein folding and the refolding of stress-denatured proteins

During the folding of certain newly synthesised polypeptides, several studies have reported a successive and directional action of chaperone systems (ie Hsp70→chaperonin or Hsp70→Hsp90) that function synergistically by promoting early and later folding steps (Manning-Krieg *et al.*, 1991; Gaitanaris *et al.*, 1994; Saijo *et al.*, 1994; Rospert *et al.*, 1996; Frydman *et al.*, 1994; Frydman and Hartl, 1996; Heyrovská *et al.*, 1998; Johnson and Craig, 1997, Frydman and Höhfeld, 1997) (Fig. 5). A recent study employing a *dnaK* deletion mutant revealed that although certain proteins require the successive action of DnaK and GroEL *in vivo* for efficient folding, the initial interaction with DnaK is not obligatory for these proteins to reach GroEL (Teter *et al.*, 1999). However, it appears that in the absence of DnaK, another chaperone TF may be responsible for the transfer of such polypeptide chains to GroEL (Teter *et al.*, 1999). In prokaryotic and eukaryotic cells Hsp70 members interact co-translationally with a subset of nascent polypeptides as they emerge from ribosomes (Beckman *et al.*, 1990; Nelson *et al.*, 1992; Hansen *et al.*, 1994; Frydman *et al.*, 1994; Frydman and Hartl, 1996; Eggers *et al.*, 1997; Pfund *et al.*, 1998; Teter *et al.*, 1999). The initial interaction with Hsp70 is proposed to maintain the

folding competence of these translating polypeptides which in some cases may commence folding co-translationally (ie for multi-domain proteins comprised of independent modules) but in other cases requires the complete synthesis before folding can proceed (ie for single domain proteins, multi-domain proteins comprised of discontinuous amino acid sequences, and proteins that have to be post-translationally translocated across membranes). For a number of polypeptides the interaction with Hsp70 may be sufficient for folding and assembly, while for others the interaction is necessary but does not result in complete folding (Langer *et al.*, 1992; Gaitanaris *et al.*, 1994; Rospert *et al.*, 1996; Teter *et al.*, 1999). In the latter case, which often represents polypeptides that have difficulty reaching or maintaining their native conformations, these polypeptides require a further interaction with either a chaperonin or a Hsp90 systems for the completion of their folding and assembly (Nathan *et al.*, 1997; Netzer and Hartl, 1998) (Fig. 5). The sequential chaperone action is consistent with Hsp70 members interacting with earlier folding intermediates while the chaperonins and Hsp90 members are proposed to recognise intermediates which have native-like secondary structure and, in some cases, a global topology that resembles the completed tertiary state (reviewed by Hartl, 1996; Csermely *et al.*, 1998; Buchner, 1999). While the successive and directional action of the Hsp70→Hsp90 systems has only been studied in the eukaryotic cytosol (reviewed by Johnson and Craig, 1997; Frydman and Höhfeld, 1997), the action of the Hsp70→chaperonin systems has been well documented in the mitochondrial matrix, *E. coli* cytosol and eukaryotic cytosol (Manning-Krieg *et al.*, 1991; Mizzen *et al.*, 1991; Gaitanaris *et al.*, 1994; Saijo *et al.*, 1994; Rospert *et al.*, 1996; Frydman *et al.*, 1994; Frydman and Hartl, 1996; Heyrovská *et al.*, 1998; Teter *et al.*, 1999). Nevertheless, both pathways are known to involve a number of co-chaperones for their efficient functioning (see sections 1.5.4, 1.5.5) (reviewed by Hartl, 1996; Johnson and Craig, 1997; Bukau and Horwich, 1998).

In contrast to the directional association of newly synthesised polypeptides with chaperone systems during *de novo* folding, a retrograde passage of folding intermediates between chaperone systems has been proposed to be additionally required (Buchberger *et al.*, 1996; Bukau *et al.*, 1996; Ellis, 1999). In this latter case, the complete nascent polypeptide is believed to partition freely between the bulk cytosol and a "chaperone network" in order to reach its native state. Thus, *in vitro* denatured luciferase can be passed back and forth between the DnaK and GroEL systems (Buchberger *et al.*, 1996). However, it appears that there are differences between *de novo* protein folding and the refolding of denatured proteins and *in vivo* (Heyrovská *et al.*, 1998). Utilising a heterologous chaperonin trap (GroEL D87K) that irreversibly binds actin and other newly synthesised polypeptides, it was shown that expression of the trap in the cytosol of

yeast and mammalian cells did not interfere with protein folding (Thulasiraman *et al.*, 1999; Siegers, *et al.*, 1999). This indicates that *de novo* protein folding may proceed without the release of polypeptides into the bulk cytosol, although it is uncertain exactly how many polypeptides the trap can bind in these systems. More convincing data came from the observation that the trap could not interfere with folding of newly synthesised actin, which appears to require the successive action of Hsc70/Hsp40 and TRiC/GimC (alternatively named prefoldin) (Frydman *et al.*, 1994; Frydman and Hartl, 1996; Vainberg *et al.*, 1998; Thulasiraman *et al.*, 1999; Siegers, *et al.*, 1999). It is proposed that the sequential action of chaperone systems proceeds in a sequestered environment that protects actin polypeptides from forming unproductive interactions with macromolecules in the crowded cytosol (Ewalt *et al.*, 1997; Thulasiraman *et al.*, 1999; Siegers, *et al.*, 1999). In contrast, stress-denatured and folding-incompetent proteins are efficiently bound by the chaperonin trap and therefore are likely to require partitioning between chaperones and the bulk cytosol *in vivo* (Glover and Lindquist, 1998; Thulasiraman *et al.*, 1999; Siegers *et al.*, 1999). It is proposed that such partitioning or post-translational cycling between chaperone-bound and the bulk cytosol may facilitate refolding of denatured or malfolded proteins, and could assist in the degradation of damaged or folding-incompetent proteins (Buchberger *et al.*, 1996; Frydman and Hartl, 1996; Thulasiraman *et al.*, 1999; Ellis, 1999). In addition, post-translational cycling between chaperones and the bulk cytosol may assists the assembly of oligomeric proteins (Frydman and Hartl, 1996).

1.6 The components involved in mitochondrial protein import, folding and assembly

In metabolically active cells, such as those found in skeletal muscle, mitochondria are the most abundant organelles and have been estimated to contain up to 10-20 % of the total pool of intracellular proteins (Glover and Lindsay, 1992) at concentrations as high as 500 mg/ml in the matrix (Hackenbrock, 1968; Schwerzmann *et al.*, 1986). Furthermore, while there are ~1000 different mitochondrial proteins, only 8 from yeast (Borst and Grivell, 1978; Tzagoloff and Meyers, 1986) and 13 from vertebrates (reviewed by Shadel and Clayton, 1997) are encoded by mitochondrial genes. The remaining mitochondrial proteins are synthesised in the cytosol as nuclear encoded proteins and the vast majority are formed as preproteins (or precursors) destined for the mitochondrial matrix (reviewed by Schatz, 1996; Schatz and Dobberstein, 1996; Ryan *et al.*, 1997; Pfanner and Meijer, 1997; Pfanner *et al.*, 1997; Neupert, 1997; Pfanner, 1998). In the cytosol, these preproteins must be maintained in an unfolded "import component" conformation to ensure their efficient entry into and subsequent folding within the mitochondrial matrix

(reviewed by Lithgow *et al.*, 1997; Ryan *et al.*, 1997; Pfanner *et al.*, 1997; Neupert, 1997). Not surprisingly, this process requires numerous cellular components, including many molecular chaperones (Table 1). Despite the involvement of numerous cytosolic molecular chaperones and translocases of the outer (Toms) and inner mitochondrial membranes (Tims) (Pfanner *et al.*, 1996), for the efficient import of preproteins, the following sections will mostly be concerned with roles that the matrix located mt-Hsp70 system fulfils in the biogenesis and turn-over of mitochondrial proteins (Fig. 5). These roles include: the vectorial import of translocating preproteins into the matrix, folding and assembly of both newly imported and mitochondrial synthesised polypeptides, the proteolytic degradation of abnormal proteins, stabilisation of stress denatured proteins against aggregation and their reactivation.

1.6.1 The translocation of preproteins across the mitochondrial inner membrane requires the matrix located mt-Hsp70

Mitochondrial preproteins characteristically contain an N-terminal targeting sequence known as the presequence (or leader sequence). Typical presequences are 15-40 residues in length and generally have the ability to form an amphiphilic α-helix with positive charged residues on one side and hydrophobic residues on the other (von Heijne, 1986; Hartl *et al.*, 1989; Jarvis *et al.*, 1995) . It is generally believed that an inner membrane potential ($\Delta\psi$) electrophoretically drives the translocation of positively charged presequences through a putative proteinaceous membrane pore towards the negatively charged matrix (Martin *et al.*, 1991) (Fig. 5). The ~90 kDa inner membrane translocase is probably formed by two copies each of the integral membrane proteins Tim17 and Tim23, the latter of which may attach the peripheral membrane protein Tim44 (Blom *et al.*, 1995; Berthold *et al.*, 1995; Bauer *et al.*, 1996; Dekker *et al.*, 1997; Bömer *et al.*, 1997). The association of Tim44 with the inner membrane translocase appears to serve two functions, firstly to anchor the otherwise soluble mt-Hsp70 (Ssc1) protein (in a 1:1 complex) at the site of preprotein entry into the matrix and secondly, to permit the efficient interaction of the preprotein with mt-Hsp70 (Schneider *et al.*, 1994, 1996; Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Ungermann *et al.*, 1994, 1996). Both processes require the presence of an 18 amino acid segment within Tim44 (residues 185-202 of yeast protein) which shows similarity with the conserved J-domain of Hsp40 members (Rassow *et al.*, 1994; Merlin *et al.*, 1999). Upon entry of the presequence into the matrix, it is often removed by the mitochondrial processing peptidase (MPP) to generate a "mature" polypeptide (Kalousek *et al.*, 1993; Gavel and von Heijne, 1990). Further import of the mature polypeptide is independent of

the $\Delta\psi$ and instead requires an association with mt-Hsp70, which is thought to occur as soon as an epitope becomes available in the matrix (Gambill *et al.*, 1993; Glick *et al.*, 1993; Voos *et al.*, 1993, 1996; Stuart *et al.*, 1994; Ungermann *et al.*, 1994, 1996). In fact, DnaK binding motifs have been observed in almost all known mitochondrial presequences (~147), across several biological kingdoms, and have been suggested to serve as efficient binding sites for mt-Hsp70, as these Hsp70 systems have been the most conserved during evolution (see section 1.5.6) (Rüdiger *et al.*, 1997a; Zhang *et al.*, 1999). Complete translocation of the polypeptide is achieved by cycles of ATP-dependent preprotein binding and release by the mt-Hsp70/Tim44 system, which analogous to the *E. coli* DnaK/DnaJ system, is tightly regulated by a GrpE homologue termed Mge1 (or mt-GrpE) (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Nakai *et al.*, 1994; Laloraya *et al.*, 1994, 1995; Voos *et al.*, 1994; Westermann *et al.*, 1995; Schneider *et al.*, 1996). While these general functions of the mt-Hsp70/Tim44/ Mge1 system are undisputed, two opposing models have prevailed to explain how the mt-Hsp70 systems drives the forward movement of mature polypeptides into the mitochondrial matrix (reviewed by Glick, 1995; Pfanner and Meijer, 1995; Horst *et al.*, 1997a; Pilon and Schekman, 1999).

In the first model, the "Brownian ratchet or trapping model", the translocating mature polypeptide is proposed to slide back and forth through the proteinaceous membrane channel by Brownian motion (Simon *et al.*, 1992; Ungermann *et al.*, 1994, Gaume *et al.*, 1998). Association of a polypeptide with mt-Hsp70 (or trapping) in the matrix prevents its retrograde movement out of the membrane channel and in turn confers unidirectionality to the import process (reviewed by Neupert *et al.*, 1990). As the translocating polypeptide diffuses further into the matrix, additional mt-Hsp70 molecules may bind, independent of Tim44, to further prevent retrograde movement (Ungermann *et al.*, 1996). The process is said to resemble a ratchet action and results in the complete translocation of the polypeptide into the matrix (Simon *et al.*, 1992). In the second model, the "force-generated motor or pulling model", it is proposed that mt-Hsp70 exerts a more active role on polypeptide translocation (Voos *et al.*, 1996; Horst *et al.*, 1996, 1997a; Matouschek *et al.*, 1997; Bömer *et al.*, 1998; Chauwin *et al.*, 1998; Voisine *et al.*, 1999). Anchored via Tim44 to the membrane pore, mt-Hsp70 is thought to undergo a large conformational change to impose an inward "pulling force or power stroke" on the bound polypeptide. In this manner, cycles of ATP-dependent binding and release would effectively pull the mature part of the translocating preprotein into the matrix. Most of the controversy between these models arises about how folded domains of the preprotein are unfolded on the cytosolic surface of mitochondria to permit further passage of the polypeptide through the translocation channel, which can only accommodate unfolded or largely unfolded structures (Schleyer and

Neupert, 1985; Eilers and Schatz, 1986; Schwartz *et al.*, 1999). On one hand, partial unfolding and refolding (thermal breathing) of cytosolic domains is proposed to occur spontaneously, and in the partially unfolded state, movement of extended segments of the preprotein may enter the membrane translocase by Brownian motion (Gaume *et al.*, 1998). Binding of mt-Hsp70 to the emerging preprotein (or trapping) in the matrix would then efficiently arrest the partially unfolded state in the cytosol and thereby favour further unfolding and in turn inward translocation. On the other hand, the force-generated motor model proposes that tightly folded cytosolic domains of the preprotein are mechanically unfolded by an inwards pulling force exerted by mt-Hsp70 at the matrix side of the inner membrane (Matouschek *et al.*, 1997; Bömer *et al.*, 1998; Voisine *et al.*, 1999). Based on theoretical calculations, the pulling force model may also be required to overcome strong interactions formed between the translocation channel and the preprotein (Chauwin *et al.*, 1998).

Recent evidence suggest that the trapping and pulling actions are not mutually exclusive, but are in fact distinct and separable functions of mt-Hsp70 during preprotein import into mitochondria (Voisine *et al.*, 1999). Studies in yeast mitochondria with mutant forms of mt-Hsp70 and Tim44 have shown, that while trapping appears to be largely sufficient to explain the import of unfolded and loosely folded preproteins, the unfolding of preproteins appears to require a pulling force that is attributed to mt-Hsp70 only when it is anchored to the translocation pore through an interaction with Tim44 (Bömer *et al.*, 1997, 1998; Voisine *et al.*, 1999; Merlin *et al.*, 1999). Interestingly, the import of several loosely folded preproteins was observed to proceed in the absence of functional Tim44, implying that the interaction of mt-Hsp70 with Tim44 might not be entirely required in the trapping model (Bömer *et al.*, 1998; Merlin *et al.*, 1999). In support of this idea, the inner membrane translocase contains at least two mt-Hsp70 binding sites, one site at Tim44 and a second site directly at the Tim23/Tim17 complex (Rassow *et al.*, 1995; Voos *et al.*, 1996; Bömer *et al.*, 1997). Only a proportion of Tim23/Tim17 complexes were observed to contain Tim44, which is consistent with there being five to ten times less Tim44 than Tim17 or Tim23 in mitochondria (Dekker *et al.*, 1997; Blom *et al.*, 1995).

1.6.2 The requirement of a distinct mt-Hsp70 system for protein (re)folding and assembly

During mitochondrial protein translocation into matrix, mt-Hsp70 is anchored to the translocation channel by an association with Tim44 (see section 1.6.1). In contrast, it appears that a distinct DnaJ homologue (Mdj1 or mt-DnaJ) assists mt-Hsp70 during *de novo* protein folding, assembly and the refolding of stress denatured proteins (Kang *et al.*, 1990; Herrmann *et al.*, 1994; Rowley *et al.*, 1994; Voos *et al.*, 1994; Laloraya *et al.*, 1995; Rospert *et al.*, 1996;

Westermann *et al.*, 1995, 1996; Prip-Buus *et al.*, 1996; Horst *et al.*, 1997b; Deloche *et al.*, 1997; Kubo *et al.*, 1999) (Fig. 5). Regardless of which DnaJ homologue is utilised by mt-Hsp70, it appears that a cooperation with Mge1 (or mt-GrpE), which functions as a nucleotide release factor (Deloche and Georgopoulos, 1996; Dekker and Pfanner, 1997; Miao *et al.*, 1997a), is probably indispensable for all mt-Hsp70 functions (Bollinger *et al.*, 1994; Ikeda *et al.*, 1994; Nakai *et al.*, 1994; Voos *et al.*, 1994; Schneider *et al.*, 1994, 1996; Kronidou *et al.*, 1994; Laloraya *et al.*, 1994, 1995; Westermann *et al.*, 1995; Horst *et al.*, 1997b; Deloche and Georgopoulos, 1996; Azem *et al.*, 1997; Kubo *et al.*, 1999). Endogenous Mdj1 was found predominantly associated with the matrix side of the inner mitochondrial membrane as a peripheral protein, although Mdj1 appears to be active as a soluble entity both *in vitro* and *in vivo* (Westermann *et al.*, 1996; Horst *et al.*, 1997b; Kubo *et al.*, 1999). In analogy to the *E. coli* DnaJ protein, Mdj1 is a chaperone in its own right, for Mdj1 can bind to unfolded polypeptides and thereby prevent their misfolding and aggregation (Prip-Buus *et al.*, 1996, Westermann *et al.*, 1996). An initial association with Mdj1 appears to be required for the efficient binding of numerous unfolded polypeptides to mt-Hsp70 (Wagner *et al.*, 1994; Prip-Buus *et al.*, 1996, Westermann *et al.*, 1996). Mdj1 probably targets substrates to ATP-bound mt-Hsp70 and promotes ATP-hydrolysis resulting in a concomitant conformational change to the ADP-bound state which in turn permits the stable binding of substrates. The ATPase cycle of mt-Hsp70 during the processes of protein import, (re)folding and assembly, appears to very similar to that discussed earlier for DnaK (section 1.5.4) (Bollinger *et al.*, 1994; Nakai *et al.*, 1994; Voos *et al.*, 1994, 1996; Schneider *et al.*, 1994, 1996; Rassow *et al.*, 1994; Kronidou *et al.*, 1994; Westermann *et al.*, 1995; Laloraya *et al.*, 1995; von Ahsen *et al.*, 1995; Horst *et al.*, 1996, 1997ab; Deloche and Georgopoulos, 1996; Deloche *et al.*, 1997; Azem *et al.*, 1997; Dekker and Pfanner, 1997; Miao *et al.*, 1997a; Kubo *et al.*, 1999). Interestingly, like *E. coli* (see section 1.5.2), the yeast mitochondria matrix contains at least two and probably three Hsp70 members (Schilke *et al.*, 1996; Knight *et al.*, 1998; Miao *et al.*, 1997b). The *SSH1* (or *SSQ1*) gene encodes a ~70 kDa protein (Ssh1 or Ssq1) that shares 53% positional identity with mt-Hsp70 (or Ssc1) (Schilke *et al.*, 1996) and is one thousand-fold less abundant than mt-Hsp70 (Knight *et al.*, 1998). In comparison to mt-Hsp70, Ssh1 does not appear to play a general role in the import of mitochondrial preproteins but is suggested to play a critical role in iron metabolism by assisting the maturation of the Yfh1p protein (known as frataxin in humans) (Knight *et al.*, 1998). A third putative mitochondrial Hsp70 gene (*SSC2/J1*) has been identified following the complete DNA sequencing of the *Saccharomyces cerevisiae* genome (Miao *et al.*, 1997b) but the function of this gene's product is yet to be established in any detail.

Whereas the mt-Hsp70, Tim44, and Mge1 components are essential for yeast viability, Mdj1 is only essential at temperatures above 35°C. This may reflect the non-essential role of Mdj1 in mitochondrial protein import (Rowley *et al.*, 1994; Westermann and Neupert, 1997). The phenotype of *mdj1* deletion mutants is partly explained by a second DnaJ-like homologue (Mdj2) in yeast mitochondria of unknown function (Westermann and Neupert, 1997). A single *mdj2* deletion has no detectable growth defects, but double deletion mutants of *mdj1* and *mdj2* can not grow at 35 °C (Westermann and Neupert, 1997). This suggests that while Mdj2 has a relatively limited number of functions compared with Mdj1, there is at least a partial overlap in their functions. Unlike Mdj1 though, Mdj2 is an integral inner membrane protein with a J-domain that protrudes into the matrix (Westermann and Neupert, 1997). The domain structure is very similar to the *E. coli* Dj1A (RcsG) protein which appears to recruit DnaK, and in turn GrpE, to membrane associated substrates that are involved in a signal transduction pathway (Kelley and Georgopoulos, 1997). Interestingly, mt-Hsp70 appears to mediate the assembly, rather than folding, of the mitochondrial encoded subunits 6 and 9 into the F_o-ATP synthase complex (Herrmann *et al.*, 1994). Translation of these subunits is initially assisted by both mt-Hsp70 and Mdj1, presumably in cooperation with mt-GrpE (Herrmann *et al.*, 1994; Westermann *et al.*, 1995). It is tempting then to speculate that Mdj2, as opposed to Mdj1, is required for the insertion of these protein subunits into the inner membrane.

Analogous to the situation at the ribosomes, the folding of translocating polypeptide is not always Hsp70-dependent (Rospert *et al.*, 1996; Netzer and Hartl, 1998). Investigation of the chaperone requirements for the folding of four newly imported monomeric preproteins, revealed that two of these, cyclophilin 20 and a matrix-targeted variant of barnase, could fold independent of mt-Hsp70 and Hsp60 functions (Rospert *et al.*, 1996). For these preproteins folding appears to occur in a co-translocational manner and therefore may not require chaperone assistance (Rospert *et al.*, 1996, Netzer and Hartl, 1998). For the remaining preproteins, folding appears to proceed in a post-translocational manner (Rospert *et al.*, 1996). Where one preprotein, a matrix-targeted variant of dihydrofolate reductase (DHFR), was dependent on an association with mt-Hsp70 for folding, while the other, rhodanese, was dependent on an association with Hsp60 for folding (Rospert *et al.*, 1996). In both cases, chaperone assisted folding probably requires multiple ATP-dependent interactions and a number of co-chaperones (ie Mdj1, Mge1 and Hsp10). As discussed earlier (section 1.5.8), the successive and directional action of the mt-Hsp70→Hsp60 systems is probably required for the *de novo* folding of a subset of inherently aggregation prone polypeptides, while a retrograde action may be additionally required during the refolding of stress-denatured proteins (Manning-Krieg *et al.*, 1991; Mizzen *et al.*, 1991; Saijo *et al.*, 1994;

Rospert *et al.*, 1996; Heyrovská *et al.*, 1998; Ellis, 1999). Accordingly, upon inactivation of cyclophilin 20, folding intermediates of DHFR reversibly accumulate in association with both mt-Hsp70 and Hsp60 (Rassow *et al.*, 1995; Rospert *et al.*, 1996). It appears that cyclophilin 20, by exerting a peptidyl-proyl *cis-trans* isomerase activity, can cooperate with these chaperones to accelerate the rate of DHFR folding in mitochondria (Matouschek *et al.*, 1995; Rassow *et al.*, 1995). In summary, during the import by or following the release of preproteins from the translocation channel anchored mt-Hsp70/Tim44/Mge1 system, folding may occur by a number of different pathways in a chaperone dependent or independent manner.

1.6.3 Protein import and folding in mammalian mitochondria

The information described in the previous sections (1.6.1 and 1.6.2) has mostly been obtained using the fungi *Saccharomyces cerevisiae* and *Neurospora crassa* as model systems (reviewed by Schatz, 1996; Schatz and Dobberstein, 1996; Ryan *et al.*, 1997; Pfanner and Meijer, 1997; Pfanner *et al.*, 1997; Neupert, 1997). This is primarily due to the ease with which mutants of the components involved in mitochondrial preprotein import and folding can be generated and characterised. In addition, because mitochondria are organelles of endosymbiotic origin, the study of *E. coli* molecular chaperones has also provided us with some important insights into the roles of their mitochondrial counterparts. Recently sequencing of the entire genomes of *S. cerevisiae* and *E. coli* have been completed, allowing us for the first time to analyse a complete set of components involved in protein targeting and folding within an organism. There is increasing evidence that the cellular machinery involved in the import and subsequent folding of mitochondrial proteins has been conserved during the evolution of higher eukaryotes such as mammals (reviewed by Ryan *et al.*, 1997). However, there is also likely to be unique requirements for cells that experience developmental- and tissue-specific gene expression. Thus, cells that divide infrequently or not all, such as adult neuronal cells, may differ in some key aspects from those of unicellular eukaryotes which divide constantly. In mammalian mitochondria, single homologues of Tim44 (Wada and Kanwar, 1998) and mt-Hsp70 (reviewed by Hoogenraad *et al.*, 1997) have been identified, but surprisingly two Mge1-like proteins (mt-GrpE#1 and #2) exist (see chapters 2, 3 and 4) (Naylor *et al.*, 1995, 1996, 1997, 1998). A novel human DnaJ-like cDNA has also been cloned and the encoded protein (hTid-1) is very homologous to the *D. melangastor* mitochondrial Tid50 protein (Schilling *et al.*, 1998; Kurzik-Dumke *et al.*, 1998). Surprisingly the authors (Schilling *et al.*, 1998) failed to identify a characteristic mitochondrial presequence on hTid-1, suggesting that it also resides in the mitochondrial matrix. The yeast complement of mitochondrial matrix protein import and folding

components, therefore, appears to have been highly conserved in mammalian mitochondria, but interestingly, at least one difference, namely the presence of two functional mt-GrpE members, has so far been identified. Further work is required to determine if additional differences are present. This will obviously be facilitated by the completion of the human, rat and mouse genome sequencing projects. Investigations are also required to examine if the known mammalian components, involved in mitochondrial protein import and subsequent folding, function in a manner analogous to their yeast counterparts and whether the greater functional diversity of mammalian mitochondria requires the presence of additional components, such as an extra GrpE homologue.

1.7 Roles of chaperones in protein degradation within *E. coli* and mitochondria

1.7.1 Many stress proteins are proteases

The common consequence of many stress conditions appears to be the accumulation of unfolded or malfolded proteins in the cell. As discussed earlier, several stress inducible proteins facilitate cell recovery through chaperone action which restores damaged proteins into their native, functional state. However, should a polypeptide be unable to attain its native conformation, it is rapidly degraded by another set of stress inducible proteins which act as proteases alone or participate in protease action (Parsell and Lindquist, 1993) (Fig. 5).

The "refold or degrade" model also applies to the action of stress proteins that are constitutively expressed within the cell. The co-operative action of chaperones (DnaK/ DnaJ/ GrpE and GroEL/ GroES) in cellular processes, includes the degradation of inactive proteins with non-native conformations. Indeed, abnormal proteins exhibit an increased half-life in *E. coli rpoH* mutants which contain reduced levels of molecular chaperones (Goff *et al.*, 1984; Grossman *et al.*, 1984) and in *E. coli* strains with mutations in individual molecular chaperone genes (Straus *et al.*, 1988). Under both normal and stressed cellular conditions, it is now believed that the molecular chaperones aid in the removal of abnormal proteins by presenting them to proteases for degradation and thus prevent the formation of aggregates which may be difficult to degraded and therefore harmful to cells (Hayes and Dice, 1996).

In this section an overview of the current knowledge about the possible relationship between mitochondrial proteolysis machineries and chaperone teams will be presented. The proteolytic mechanisms thus far discovered in mitochondria all have homologues which have been better characterised in *E. coli*. Therefore, examples of certain events in protein turnover within *E. coli* cells will be used to exemplify the possible functions of mitochondria counterparts.

1.7.2 Protein degradation within the mitochondrion

Proteolysis plays a key role in the maintenance of mitochondrial functions by potentially regulating the availability of certain short-lived regulatory proteins, ensuring the proper stoichiometry of multi-protein complexes and removing abnormal proteins. Earlier investigations on the turnover of proteins within mitochondria assumed that the organelle is degraded within the lysosome, following the observation of whole mitochondria within autophagic vacuoles (autophagosomes) (reviewed by Hare, 1990; Tanner and Dice, 1996). However, later studies have shown that the average half-lives of proteins differ in various mitochondrial compartments and that different proteins are degraded at distinct rates within the same compartment (reviewed by Hare, 1990; Tanner and Dice, 1996). This suggests that a distinct and selective mitochondrial proteolytic system exists. This notion is in accord with the recent discovery of several ATP-dependent proteases in yeast and mammalian mitochondria and with their significant homology to characterised bacterial counterparts (reviewed by Ryan *et al.*, 1997; Suzuki *et al.*, 1997).

1.7.3 Chaperone assisted proteases of *E. coli*

In *E. coli*, the transcription of at least 20 heat shock genes requires an alternative RNA polymerase sigma factor (σ^{32}). In addition to the heat shock genes that encode molecular chaperones, several encode proteases or proteins that assist in protease action, namely La (Lon), ClpP, ClpA, ClpB, ClpX, FtsH (HflB), HflX, HflK and HflC (reviewed by Georgopoulos *et al.*, 1990; 1994).

1.7.3.1 The La (Lon) protease

Most of the work performed on *E. coli* heat shock proteins that are involved in protein degradation has concentrated on the La (Lon) protease, the product of the *lon* gene (Gottesman and Maurizi, 1992; Goldberg, 1992). La is an ATP-dependent serine endoprotease which catalyses the rate-limiting steps in the specific degradation of highly unstable and non-native proteins. La exists as a homotetramer of 90 kDa subunits (reviewed by Goldberg, 1992).

An intriguing feature of the La protease is its ability to form a complex *in vivo* with DnaK, GrpE and a short-lived mutant protein, phoA61, shortly before its degradation (Sherman and Goldberg, 1992). The isolation of several mutants has helped characterise the function of this complex and the roles of each member within the proteolytic process. Deletion of the *dnaK* or *lon* gene renders phoA61 less susceptible to proteolysis, implying that both DnaK and the La

protease are required for its efficient degradation. In the *dnaJ259* mutant, degradation of phoA61 was inhibited and less DnaK was found in complex with phoA61, while in the *grpE280* mutant phoA61 degradation was accelerated and more DnaK was found in complex. The DnaJ chaperone, may promote phoA61 binding to DnaK (Jubete *et al.*, 1996) while GrpE, by acting as a nucleotide exchange factor, would be expected to promote phoA61 release from DnaK. Therefore, in the *grpE280* mutant, phoA61 is expected to associate longer with DnaK and thereby increase its chances of being degraded by the La proteases. In support of this idea, phoA61 degradation is accelerated in a *dnaK756* mutant strain which encodes a DnaK protein that is defective in its release of bound polypeptide substrates (Liberek *et al.*, 1991b).

1.7.3.2 The Clp proteases

In *lon* deletion mutants, residual proteolysis of highly unstable and non-native proteins still occurs. This is largely due to another ATP-dependent protease, Clp (Ti), which shares a number of characteristics with the La protease (Gottesman and Maurizi, 1992; Goldberg, 1992). The Clp protease is an ATP-dependent serine endoprotease which catalyses the rate-limiting steps in the specific degradation of highly unstable and non-native proteins. Clp is comprised of two distinct subunits of molecular masses 81 kDa (ClpA) and 21 kDa (ClpP) (Gottesman and Maurizi, 1992; Goldberg, 1992). The hexameric ClpA component is the regulatory unit and contains the sole ATPase domain, while ClpP is the proteolytic subunit existing as a tetradecamer of two heptameric rings. The arrangement of the ClpP subunits is similar to that of the inner (β-type) subunits of both the eukaryotic and archael proteasomes, and is reminiscent of the seven-fold symmetric structures of the tetradecameric chaperonin GroEL (Kessel *et al.*, 1995). The conservation of this structural organisation, in seemingly unrelated proteins, may be advantageous for the shielded processing of polypeptide substrates within cavities where repeated contacts with the various subunits of these multimeric proteins are made.

Recently, several homologues of the ClpA subunit have been identified, namely ClpB, ClpC, ClpX and ClpY. Together they comprise the Clp or Hsp100 family (reviewed by Schirmer and Lindquist, 1997) (see Table 1). In addition to their regulatory roles with the ClpP protease, ClpA and ClpX can independently carry out molecular chaperone functions (Wickner *et al.*, 1994; Wawrzynów *et al.*, 1995) and as a result can target different proteins substrates to ClpP for degradation (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993). Therefore, selective degradation by the ClpP protease appears to be determined by its interaction with different regulatory ATPase subunits.

A short-lived fusion protein (CRAG) is degraded *in vivo* by the ClpP protease, in an ATP-dependent process which is independent of ClpA, X, and C ATPase subunits but involves the chaperonins GroEL and GroES (Kandror *et al.*, 1994). An additional component of this process was latter found to be the Trigger Factor (TF) protein (Kandror *et al.*, 1995). TF enhances the capacity of GroEL to bind many unfolded proteins, including CRAG, fetuin and histone, which at least for CRAG is the rate-limiting step in its degradation (Kandror *et al.*, 1994, 1995, 1997). It appears that an initial association between TF and GroEL is essential for the targeting and the binding of CRAG to the ClpP protease for degradation. TF has been predicted to possess a domain belonging to the FK506-binding protein (FKBP) family and a domain predicted to exhibit peptidyl-prolyl isomerase activity (Callebaut and Mornon, 1995; Stoller *et al.*, 1996). A peptidyl-prolyl isomerase activity of TF may be important for its ability to stimulate the binding of unfolded proteins to GroEL if isomerisation of critical peptidyl proline segments is required.

1.7.3.3 The FtsH (HflB) protease

Upon disappearance of stress, the RNA polymerase σ^{32} subunit is degraded rapidly, with a half life of about 1 min, by another ATP-dependent protease named FtsH (HflB) (Tomoyasu *et al.*, 1995; Herman *et al.*, 1995). FtsH is a member of a novel ATPase family, referred to as the AAA-protein family, members which are characterised by a highly conserved ATP binding site (Kunau *et al.*, 1993; Patel and Latterich, 1998). Members of the family have been found to be involved in a number of diverse cellular processes including control of the cell cycle, regulation of transcription, insertion of proteins into membranes, secretion of protein, biogenesis of organelles and degradation of proteins. Two features distinguish the FtsH protease from the La and Clp proteases: it is a metalloprotease with a conserved zinc-binding motif (HEXXH) and it is active as an integral plasma-membrane protein. However, like the protease La, FtsH appears to require the cooperative action of DnaK, DnaJ and GrpE to degrade σ^{32} (Tomoyasu *et al.*, 1995). The exact mechanism of this cooperation is still unclear but it is believed that, with the disappearance of stress and thereby the disappearance of denatured proteins, DnaK is permitted to bind σ^{32}. This not only prevents rebinding of σ^{32} to the RNA polymerase apoenzyme, but could also facilitate the presentation of σ^{32} to the FtsH protease for selective degradation.

1.7.4 Chaperone assisted proteases within the mitochondrion

1.7.4.1 Homologues of the La (Lon) protease

The existence of an ATP-dependent protease, resembling the protease La, within rat liver and bovine adrenal cortex mitochondria has been known for some time (Desautels and Goldberg 1982; Watabe and Kimura, 1985a). The corresponding proteases have been purified from bovine adrenal glands (Watabe and Kimura, 1985b), rat liver and yeast (Kutejová *et al.*, 1993). Compared with the *E. coli* protease La (87 kDa subunit molecular mass) which is active as a homotetramer, both the rat (105 kDa subunit molecular mass) and yeast (120 kDa subunit molecular mass) proteases are apparently active as homohexamers (Kutejová *et al.*, 1993). An expressed sequence tag corresponding to a partial human clone with considerably homology to the *E. coli* protease La has appeared in nucleotide databanks (Adams *et al.*, 1992). This permitted the cloning of a cDNA and gene encoding full length protease La homologues from humans (Wang *et al.*, 1993; Amerik *et al.*, 1994) and yeast (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994). The human La protease preprotein is synthesised with a transient signal peptide and is imported efficiently into isolated mitochondria, where it is processed within the matrix into its soluble, mature form. Immunofluorescence microscopy has revealed an exclusive mitochondrial distribution for this protein (Wang *et al.*, 1994). Disruption of the yeast *PIM1* gene, encoding the yeast La protease (Pim1p), results in an inability of cells to grow on nonfermentable carbon sources, a deficiency in respiration, extensive deletions in mtDNA and accumulation of electron dense inclusions which probably represent aggregated mitochondrial proteins (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994). Like its *E. coli* homologue, expression of the yeast gene is induced by heat shock (Van Dyck *et al.*, 1994), implicating a role for this protease in the degradation of misfolded proteins. Indeed, Pim1p is required for the ATP-dependent and selective degradation of the β-subunits of the general matrix peptidase and the F_1-ATPase *in vivo*. Reminiscent of the situation for phoA61 degradation in *E. coli*, recent studies have shown that Pim1p, with the assistance of mt-Hsp70, Mdj1p (mt-DnaJ) and probably Mge1p (mt-GrpE), can degrade two unstable proteins mistargeted to the mitochondrial matrix (Wagner *et al.*, 1994). A question that remains unresolved is whether molecular chaperones are required for the efficient degradation of authentic mitochondrial proteins which denature naturally during the normal operation of the organelle.

1.7.4.2 Homologues of the Clp family and ClpP proteolytic subunit

Members of the Clp family seem to be ubiquitous across biological kingdoms (Squires and Squires, 1992). DNA sequences encoding Clp homologues have been obtained from at least

11 different organisms and the corresponding proteins can be found in several compartments of the eukaryotic cell, including the chloroplast. In yeast cells, members of the Clp family have been identified in the mitochondrial matrix (Hsp78) and cytosol (Hsp104) (Leonhardt *et al.*, 1993). Both of these proteins are closely related to the *E. coli* ClpB protein as indicated by the presence of two consensus ATP-binding sequences in their primary structures. Surprisingly, disruption of the *HSP78* gene had no apparent phenotypic trait, hence the function of Hsp78 remains obscure (Schmitt *et al.*, 1995; Moczko *et al.*, 1995). Only after making double mutants which combined a deletion of the *HSP78* gene with a temperature-sensitive mutation in the *SSC1* (mt-Hsp70) gene, was a possible function for Hsp78 recognised (Schmitt *et al.*, 1995; Moczko *et al.*, 1995). In both studies of these double mutants, a loss of mtDNA was observed (Moczko *et al.*, 1995), suggesting functional overlap between Hsp78 and mt-Hsp70. Furthermore, the double mutants had a strongly reduced mitochondrial membrane potential, which in part explains the observed defect in the rate of preprotein import (Moczko *et al.*, 1995). Schmitt *et al.* (1995) suggested that Hsp78 can act as a molecular chaperone in mitochondrial protein import by preventing aggregation of malfolded proteins under conditions of impaired mt-Hsp70 function. Alternatively, Moczko *et al.* (1995) suggested that Hsp78 functions by maintaining mutant mt-Hsp70 in a soluble state, thereby regulating its activity and that Hsp78 becomes more important in situations where the activity of mt-Hsp70 is limiting.

Initial investigations into the presence of ClpP homologues in animal cells used antibodies to the *E. coli* ClpP proteolytic subunit. Cross-reacting proteins with molecular masses of 20-30 kDa were observed in bacteria, lower eukaryotes, plants and animal cells, indicating a universal conservation of the protein (Maurizi *et al.*, 1990). Three overlapping human expressed sequence tags with significant homology to the *E. coli* ClpP amino acid sequence have been identified and the cloning of a full length human ClpP homologue has been accomplished (Bross *et al.*, 1995). Northern blotting showed the presence of the ClpP transcript in several organs whilst the cDNA sequence revealed a mitochondrial transient signal-peptide. Following the characterisation of the encoded product as a functional mitochondrial ClpP protease, it will be intriguing to see if it can work in conjunction with the mitochondrial chaperonin Hsp60. If this is found to be the case, it is likely that a mitochondrial homologue of trigger factor will also be present.

1.7.4.3 Homologues of the FtsH (HflB) protease

In yeast mitochondria, five members of the AAA-protein family have been identified, namely Bcs1p, Msp1p (Yta4p), Yta10p (Afg3p), Yme1p (Yta11p) and Yta12p (Rca1p). One of

these, Bcs1p appears to be an integral protein of the mitochondrial inner membrane, consistent with its inferred role in the assembly of the ubiquinol-cytochrome C reductase (bc_1) complex (Nobrega *et al.*, 1992). In contrast, Msp1p (Yta4p) is an integral protein of the mitochondrial outer membrane and functions in intramitochondrial protein sorting (Nakai *et al.*, 1993). The others, Yta10p (Afg3p), Yme1p (Yta11p) and Yta12p (Rca1p) constitute a subfamily of AAA-proteins that have metal-dependent peptidase activity, reside as integral proteins of the mitochondrial inner membrane, and share extensive homology with the *E. coli* FtsH protease (Pajic *et al.*, 1994; Leonhard *et al.*, 1996). Yme1p, has a functional domain that protrudes into the **i**nner membrane space and is the only identified component of the *i*-AAA protease (Thorsness *et al.*, 1993; Leonhard *et al.*, 1996, 1999). In comparison, Yta10p and Yta12p have functional domains that protrude into the **m**atrix and together comprise the *m*-AAA protease (Schnall *et al.*, 1994; Tauer *et al.*, 1994; Leonhard *et al.*, 1996; Arlt *et al.*, 1996, 1998). Both of these ATP-dependent proteases can degrade unassembled inner membrane proteins and appear to be essential for the assembly of respiratory chain complexes (Leonhard *et al.*, 1996, 1999; Arlt *et al.*, 1996, 1998). Analogous to the ability of DnaK to present unfolded substrates to both the La and FtsH proteases for selective degradation, a recent study has shown that the Pim1p and *m*-AAA protease have overlapping substrate specificities and function in cooperation with mt-Hsp70 in yeast mitochondria (Savel'ev *et al.*, 1998).

1.8 Future directions

Following the recent completion of the *S. cerevisiae* and *E. coli* genome sequencing projects, scientists have been permitted, for the first time, to analyse a complete set of molecular chaperones involved in protein targeting and folding within an organism. While the extensive characterisation of chaperones from bacteria and fungi has proven instrumental to the investigation of their counterparts in higher eukaryotes such as mammals, the relatively little amount of research undertaken on mammalian chaperones has already identified some novel components. The future completion of the human, rat and mouse genome sequencing projects will allow scientists to identify differences in the chaperone complements of mammals, bacteria and fungi. These differences may ultimately explain how greater specialisation and diversity of cellular functions, can be generated in mammalian cells. An understanding of how human molecular chaperones function may also assist the treatment of a number of human diseases that are expected to result, at least in part, as a consequence of molecular chaperone malfunction.

Table 1. **An overview of the distribution and subcellular location of molecular chaperones, co-chaperones, protein folding catalysts and proteases with homologues important to mitochondrial protein biogenesis.**

Family	Prokaryotes (*E. coli*)	Eukaryotes		
		Cellular location	Fungi	Mammals
Hsp110 (similar to Hsp70)	?	?	Sse1p (Msi3p), Sse2p	Apg-1(Osp94), Apg-2(Hsp70 RY)
		nucleus	?	Hsp110 (Hsp105,107,112)
		cytosol	Hsp88 (+ mito memb. bound)	Hsp110 (Hsp105,107,112)
		ER	?	Grp170
Hsp100 (Clps)	Class I: Clp A,B,C,D,L Class II: Clp M,N,X,Y	?	ClpX-like	ClpM-like (SKD3), ClpX-like
		cytosol	Hsp104 (ClpB homolog)	?
		nucleus	Hsp104 (ClpB homolog)	?
		mito.	Hsp78 (ClpB homolog)	?
Hsp90	HtpG (C62.5)	cytosol	Hsp82, Hsc82	Hsp86 (Hsp90α), Hsp84 (Hsp90β)
		nucleus	Hsp82	Hsp84 (Hsp90β)
		ER	?	Grp94 (ERp90, gp96, endoplasmin)
Hsp70	DnaK, Hsc66 (HscA), and Hsc62	cytosol	Ssa1-4p, Ssb1,2p,	Hsp72 (Hsp70), Hsp73 (Hsc70, Prp73)
		nucleus	?	Hsp72, Hsp73
		ER	Ssd1p (Kar2p),Ssi1p(Lhs1p)	BIP (Grp78)
		ER memb.	?	Stch
		mito	mt-Hsp70 (Ssc1p), Ssh1p(Ssq1p), Ssc2p(Ssj1p)	mt-Hsp70 (Grp75, Pbp74)
		mito. OM PA	?	Hsp73
Group I Chaperonins	eubacteria GroEL	mito.	Hsp60 (Mif4p, Cpn60)	Cpn60 (Hsp60)
Group I co-chaperonins	eubacteria GroES	extracellular	?	EPF (Cpn10, Hsp10)
		mito.	Cpn10 (Hsp10)	Cpn10 (Hsp10)
Group II Chaperonins	archaebacteria TF55	cytosol + nucleus	TRiC (CCT)	TRiC (CCT)
Group II co-chaperonins	?	cytosol + nucleus	GimC (Prefoldin)	Prefoldin (GimC)
Small Hsp (sHsp) 15-30 kDa	IbpA (14 kDa) IbpB (16 kDa)	mito. OM PA	Hsp30 (+ cytosol)	?
		cytosol	Hsp12,26	Hsp24(25,27,28) + α-crystallin
		nucleus	Hsp26	Hsp24(25,27,28) + α-crystallin
Hsp40 (DnaJ)	DnaJ, CbpA, Hsc20 (HscB), DjlA (RcsG)	?	Xdj1 (possibly a pseudogene), HLJ1	
		nucleus	Zuotin, Sis1p	Hdj1 (Hsp40), Hdj2 (HSDJ), Hsj1a/b (neurone specific)
		cytosol	Ydj1p (Mas5p), Sis1p, Caj1 (+ memb. bound), Zuotin, Djp1p	Hdj1 (Hsp40), Hdj2 (HSDJ), Auxillin, ALA-D, Hsj1a/b (neurone specific), cysteine string protein
		ER	Scj1p	? Hdj1 (Hsp40)
		ER memb.	Sec63p (Npl1p), Jem1p	? Mtj1 (Sec63p-like)
		mito.	Mdj1p	? hTid-1
		mito. IM	Tim44, Mdj2p	mTim44
GrpE	GrpE	mito.	Mge1p (Yge1, GrpEp)	mt-GrpE#1 +mt-GrpE#2
La(Lon)	La	mito.	Lon (Pim1p)	Lon
Immunophilins (PPIases)	10.1kDa PPI CyP18,21, WHP, FKBP33	nucleus	?	CyP-40,FKBP25, FKBP52(?)
		cytosol	CYP1,2, FKB1 (RBP1)	CyP-40, CyPA, FKBP12,52
		ER	CYP2	CyPB, CyPC, FKBP13
		mito.	CYP3 (CPR3, cyclophilin 20)	CyPD
		Cell Surface	?	NK-TR
NAC and Trigger Factor	Trigger Factor (TF)	cytosol	αNAC and βNAC	αNAC and βNAC
PBF, MSF, Mft52, and SecB	SecB	cytosol	Mft52, MSF (BMH1 + BMH2)	PBF + MSF
MPP	?	mito.	α-MPP + β-MPP	α-MPP + β-MPP

The members of each family have been grouped according to their sequence and functional similarities, except for the NAC/TF and the PBF/MSF/SecB families whose members fulfil similar roles in prokaryotes and eukaryotes despite the lack of sequence similarity. Alternative abbreviations for otherwise identical components are shown in brackets. Extra information is also shown in brackets. Key abbreviations are: TCP-1, T-complex polypeptide 1; TRiC, TCP-1 ring complex; CCT, chaperonin containing TCP-1; PPIases, peptidyl-prolyl *cis/trans* isomerase; NAC, nascent polypeptide associated complex; PBF, presequence binding factor; MSF, mitochondrial import stimulating factor; MPP, mitochondrial general processing peptidase; mito., mitochondria; ER, endoplasmic reticulum; memb. membrane; OM, outer membrane; IM, inner membrane; PA, peripheral associated. All of the references from which this data was derived can be found in Martinus *et al.*, 1995 and in the "Guidebook to Molecular Chaperones and Protein-Folding Catalyst" edited by M.-J. Gething, 1997. It should be noted that a recent review has speculated that several mitochondrial matrix chaperones and co-chaperones may be exported to other cellular compartments by specific transport mechanism (Soltys and Gupta, 1999).

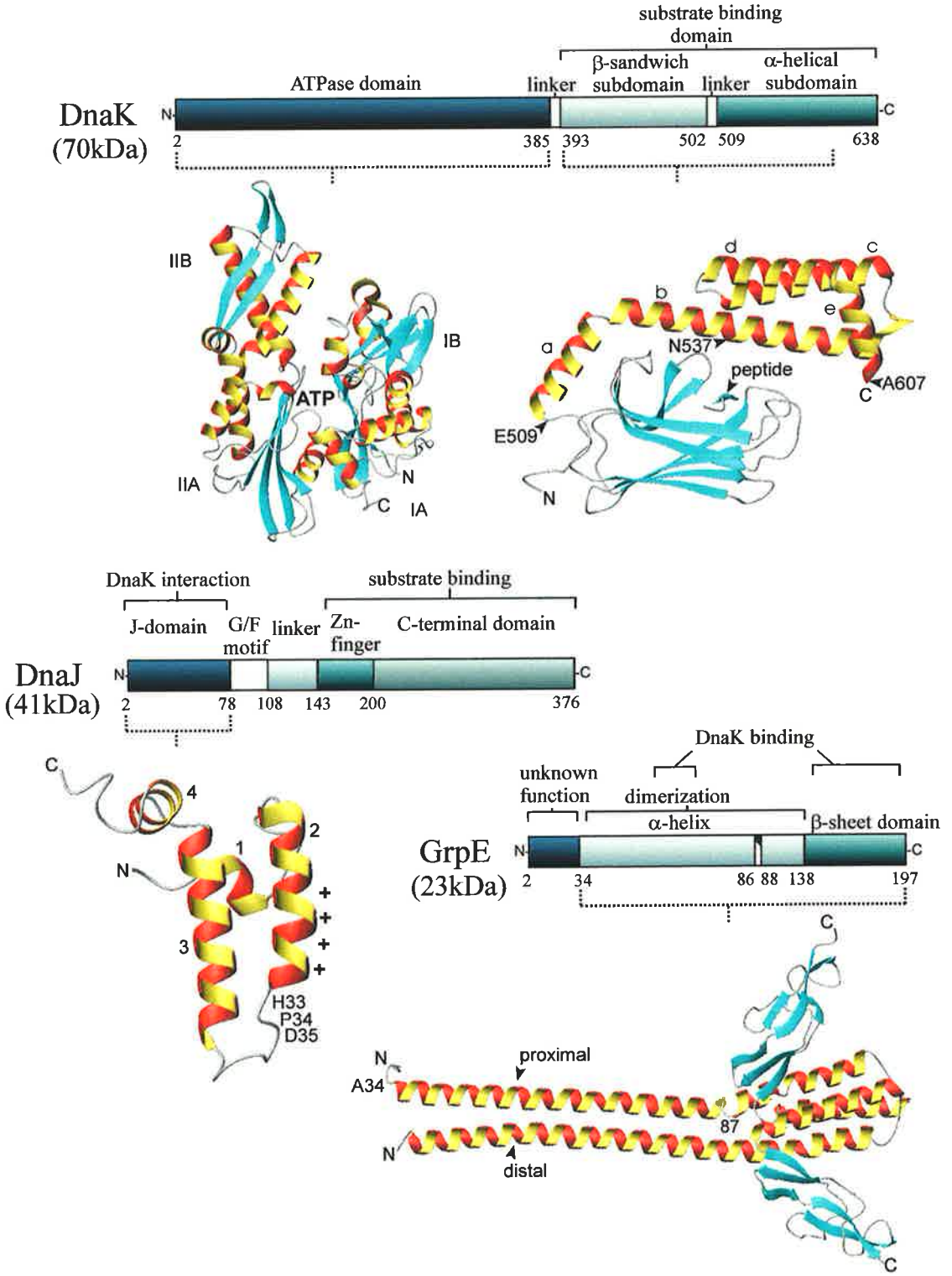


Fig. 1. **Structural and functional features of DnaK, DnaJ and GrpE.** Linear representations reveal the domain organisations of the DnaK, DnaJ and GrpE polypeptides. Beneath the linear representations, ribbon diagrams show the known structures for fragments of each polypeptide, where β-strands are coloured blue while α-helices are coloured red and yellow. The X-ray diffraction coordinates for the N-terminally truncated GrpE dimer (Harrison *et al*, 1997; PDB ID code 1DKG), the ATPase domain of DnaK (Harrison *et al*, 1997; PDB ID code 1DKG) and the substrate binding domain of DnaK (Zhu *et al*., 1996; PDB ID code 1DKX) were obtained from the Brookhaven **P**rotein **D**ata **B**ank. The nuclear magnetic resonance (NMR) coordinates for the J-domain of DnaJ (Pellecchia et al., 1996; PDB ID code 1XBL) were also obtained from the Brookhaven Protein Data Bank. The NMR structure numbered 1, from a collection of 20 similar structures, is shown. Ribbon diagrams were generated using the MOLMOL molecular graphic program, version 2.6 (Koradi *et al*., 1996).

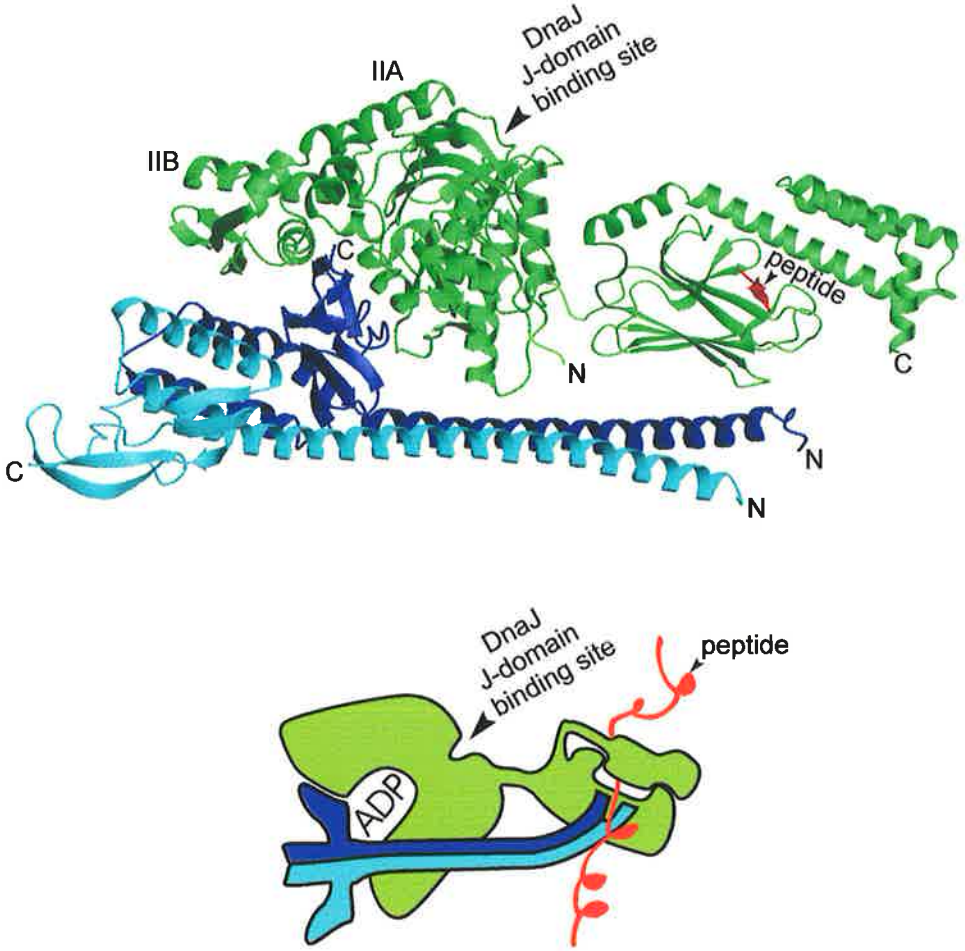


Fig. 2. **Ribbon diagram illustrating the complex of a GrpE dimer (the proximal monomer is shown in dark blue and the distal monomer is shown in light blue) and the ATPase domain of DnaK (green).** A ribbon drawing of the C-terminal substrate binding domain of DnaK (green) is also shown but its true orientation with respect to the N-terminal ATPase domain is currently unknown (see text for details). The X-ray crystallographic structures of the complexes between (i) an N-terminally truncated GrpE dimer (residues 34-197) and the ATPase domain of DnaK (residues 3-383) (Harrison *et al*, 1997; PDB ID code 1DKG) and (ii) the C-terminally truncated substrate binding domain of DnaK (residues 389-607) with a bound peptide substrate (red) (Zhu *et al*., 1996; PDB ID code 1DKX) were obtained from the Brookhaven Protein Data Bank. The exact details of the contacts between the GrpE dimer and the ATPase domain of DnaK are available at www.rockefeller.edu/kuriyan. Ribbon diagrams were generated using the MOLMOL molecular graphics program, version 2.6 (Koradi *et al*., 1996). Beneath the ribbon diagrams is shown a schematic diagram of the DnaK/GrpE/peptide substrate complex that helps to describe the function of each component in figures 3, 4 and 5.

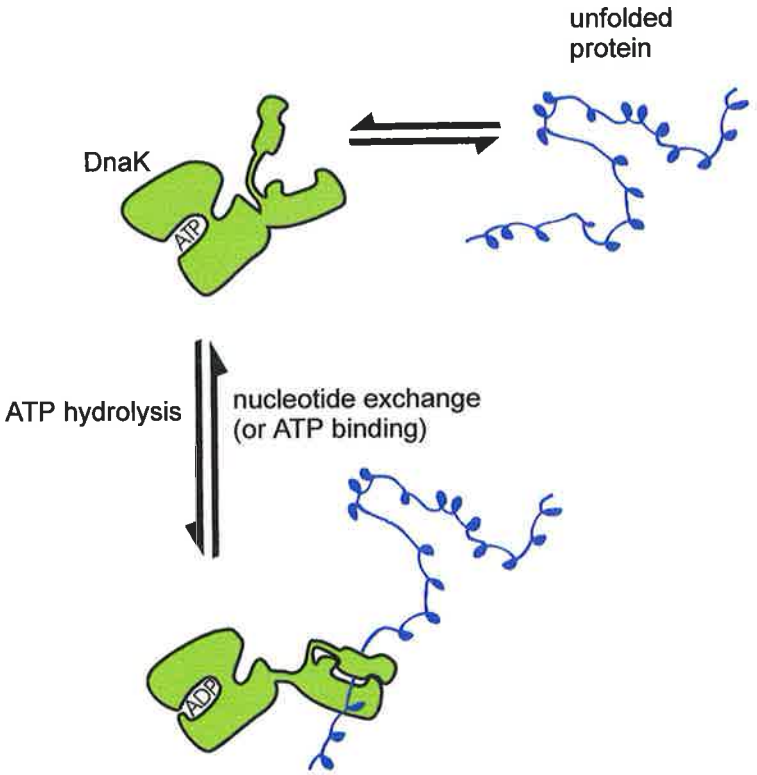


Fig. 3. **The DnaK chaperone alternates between two major conformational states.** Schematic diagrams of DnaK, depict the ATP- and ADP-bound conformations. Substrates initially associate with the ATP-bound state, the energy liberated by ATP hydrolysis then permits a transition to the ADP-bound state which in turn stabilises the interaction with substrate. Nucleotide exchange, or ATP binding, permits the transition back to the ATP-bound state and allows the substrate to dissociate from DnaK and rebind if further chaperone action is required. Alternations between the ATP- and ADP-bound states are regulated by DnaJ and GrpE which facilitate ATP hydrolysis and nucleotide exchange, respectively (see Fig. 4).

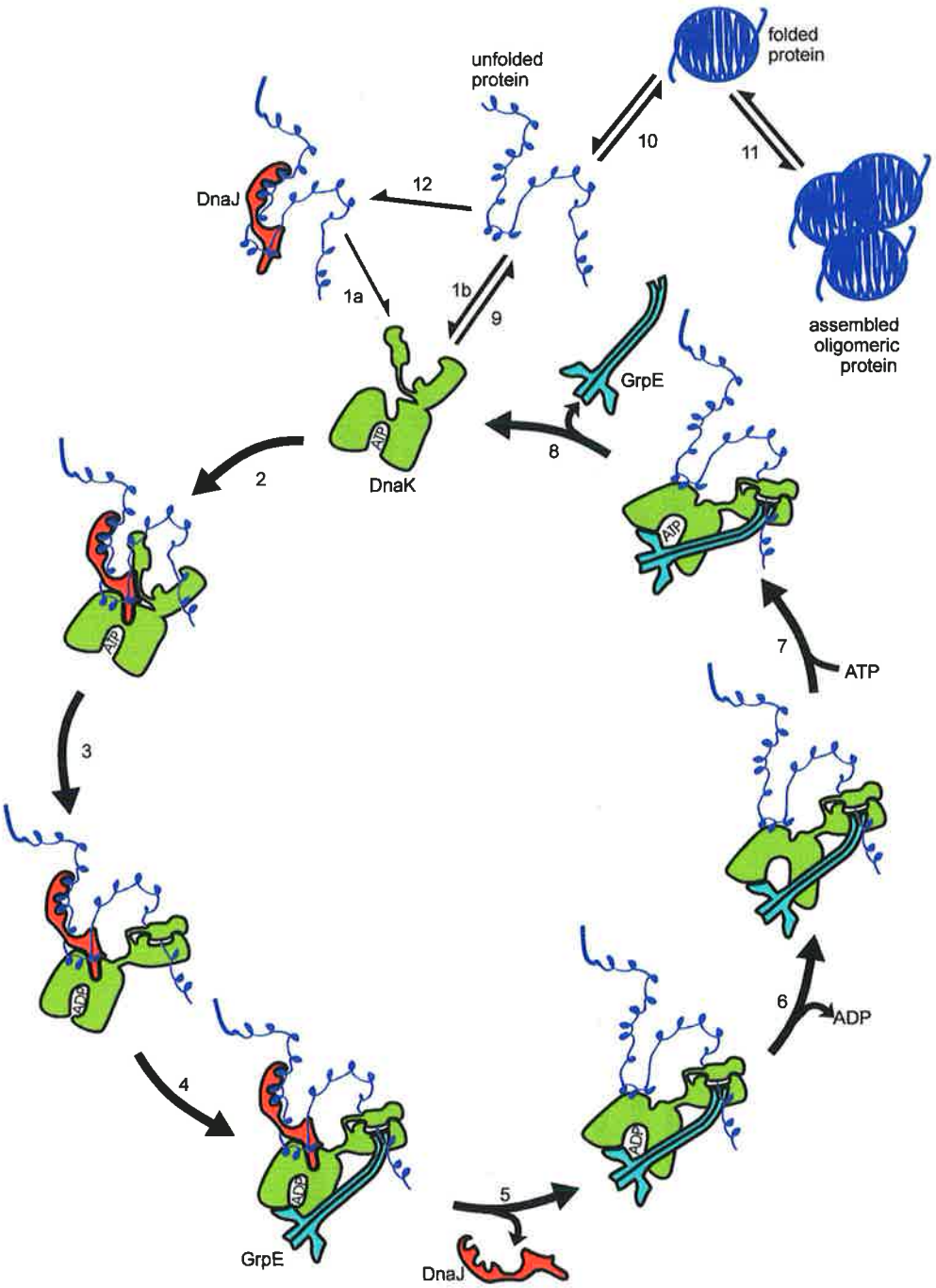
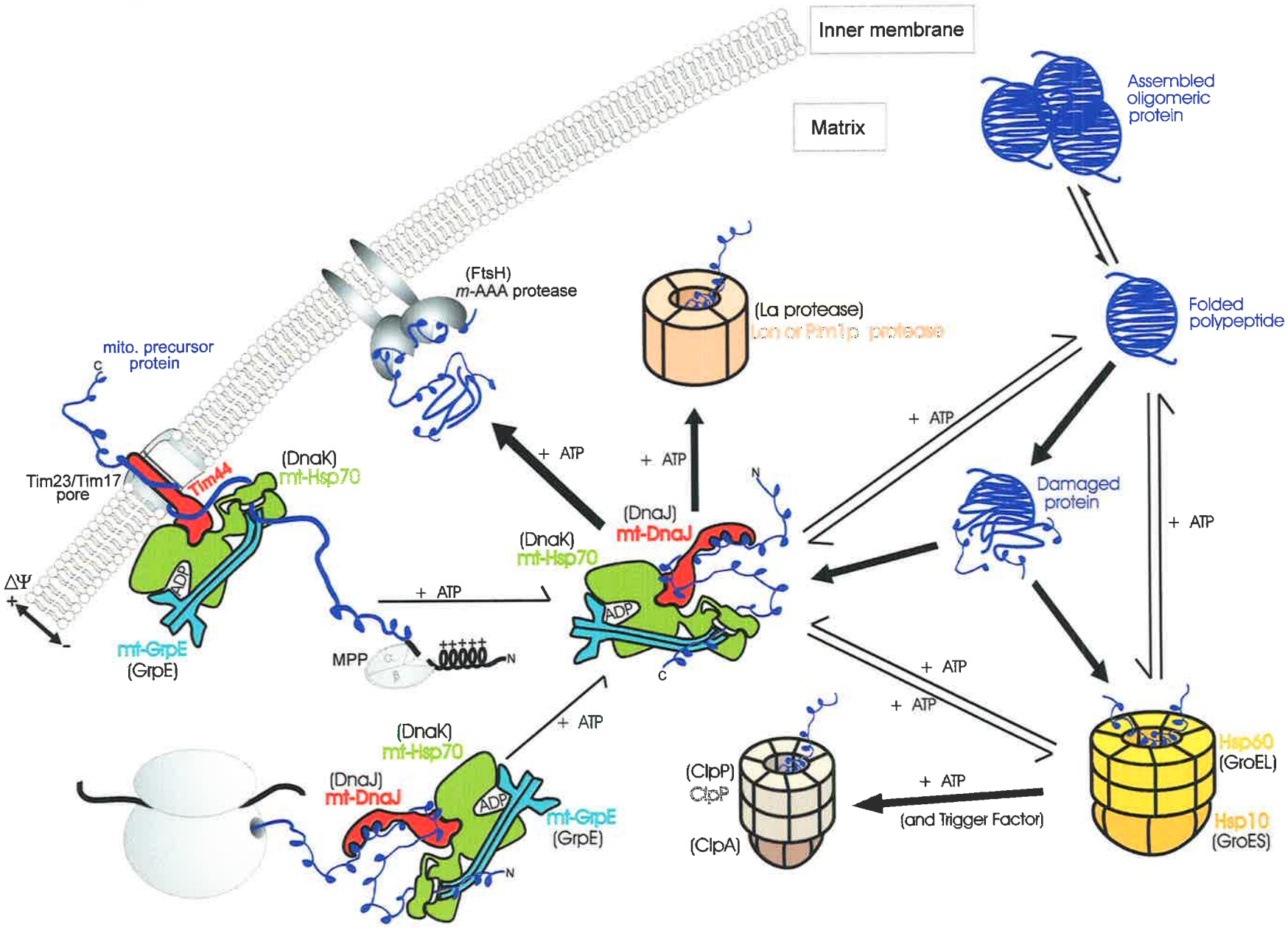


Fig. 4. **Proposed mechanism by which DnaJ and GrpE regulate the function of DnaK.** The cycle begins with the association of a protein substrate with either (1a) DnaJ or (1b) ATP-bound DnaK. Regardless of which chaperone initially binds the substrate, (2) the interaction of both the substrate and DnaJ with ATP-bound DnaK is required for efficient stimulation of DnaK's ATPase activity. (3) ATP hydrolysis is accompanied by a large conformational change in DnaK which enables the substrate to be bound more tightly. (4, 5, 6) GrpE interacts with the stable ADP-bound DnaK-substrate-DnaJ complex and induces the release of ADP by wedging apart DnaK's nucleotide binding pocket. DnaJ leaves the complex as a result of GrpE's action. (7) The rapid binding of ATP to DnaK is accompanied by another large conformational change (8) which permits GrpE to leave the complex and reduces DnaK's affinity for substrate. (9) The substrate is released and partitions between folding (10) and assembly (11) or re-enters the cycle by associating with either DnaJ (12) or DnaK (1b).

Fig. 5. **Proposed model of molecular chaperone assisted protein import, folding, assembly and degradation within mitochondria.** In accordance with the endosymbiotic hypothesis, several of the well studied *E.coli* chaperone and proteolysis machines (in parenthesis) have been conserved in mitochondria to fulfil similar coordinated tasks. Nascent polypeptides are initially bound by the mt-Hsp70 chaperone machine during their translation from mitochondrial ribosomes or during their import into mitochondria. For some proteins the initial interaction with the mt-Hsp70 machine may be sufficient to ensure proper folding and assembly to a functional state, although in some cases, the further action of the Hsp60/Hsp10 chaperone machine is required. In an analogous fashion, denatured proteins are readily rebound by these chaperone machines and can either be refolded and assembled or targeted for rapid degradation by specific proteases. A mitochondrial cyclophilin 20, with peptidyl-prolyl *cis-trans* isomerase activity, may participate in several steps of the folding cascade.


Inner membrane
Matrix
Assembled oligomeric protein
Folded polypeptide
Damaged protein
(FtsH) m-AAA protease
(La protease)
Lon or Pim1p protease
mito. precursor protein
C
Tim23/Tim17 pore
Tim44
(DnaK) mt-Hsp70
ADP
ΔΨ
+
−
mt-GrpE
(GrpE)
MPP
N
C
(DnaJ) mt-DnaJ
+ ATP
(ClpP) ClpP
(ClpA)
(and Trigger Factor)
Hsp60 (GroEL)
Hsp10 (GroES)

Chapter 2

Affinity-purification and identification of a mammalian mitochondrial GrpE-like protein (mt-GrpE#1)

2.1 INTRODUCTION

Hsp70 homologues are ubiquitous stress proteins and integral components of chaperone machineries in both prokaryotic and eukaryotic cells where they are essential for a multitude of functions, including protein translocation and folding (Chapter 1; Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). Central to the function of Hsp70 homologues is their ability to bind unfolded proteins and release them in an ATP dependent manner. All members of the Hsp70 family exhibit a weak intrinsic ATPase activity, which in the case of the *Escherichia coli* homologue DnaK, can be stimulated at least 240-fold by the co-chaperones DnaJ and GrpE (Liberek *et al.*, 1991a; McCarty *et al.*, 1995; Gässler *et al.*, 1998). Various biochemical and genetic studies have identified homologues of this triad within all cells and the major compartments thereof. Irrespective of the origin of the Hsp70/DnaJ/GrpE triad, their involvement in similar functions has led to the concept of a universally conserved Hsp70 chaperone "machine" ("system" or "team") of which the *E. coli* triad is considered the prototype (reviewed by Hartl, 1996; Gething, 1997; Bukau and Horwich, 1998). Nowhere has the degree of conservation in chaperone machines been more evident than that seen between eubacteria (eg. *E. coli*) and mitochondria (reviewed by Ryan *et al.*, 1997). The mitochondrial matrix Hsp70 homologue (mt-Hsp70) functions in the unidirectional import of proteins from the cytoplasm (reviewed by Pilon and Schekman, 1999; Dekker and Pfanner, 1999). Once translocated, certain incoming proteins appear to be transferred to Hsp60 (Cpn60) which in association with Hsp10 (Cpn10) completes their folding (reviewed in Dekker and Pfanner, 1999). The factors required to ensure the transfer from mt-Hsp70 to Hsp60 have not been established, but it may involve DnaJ and GrpE homologues as has been indicated from *in vitro* studies employing purified bacterial chaperones (Langer *et al.*, 1992). Indeed, recent reports have established the presence of both a GrpE and a DnaJ homologue in mitochondria of *S. cerevisiae* where they facilitate protein import and folding (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Nakai *et al.*, 1994; Herrmann *et al.*, 1994; Rowley *et al.*, 1994; Laloraya *et al.*, 1994, 1995; Voos *et al.*, 1994; Westermann *et al.*, 1995, 1996; Schneider *et al.*, 1996; Prip-Buus *et al.*, 1996; Horst *et al.*, 1997b; Deloche *et al.*, 1997; Kubo *et al.*, 1999)

Over the last decade, our laboratory has addressed the role of chaperones in the biogenesis of mitochondrial proteins from higher eukaryotes (reviewed by Ryan *et al.*, 1997). We have thus identified and cloned cDNAs encoding rat mt-Hsp70 (Webster *et al.*, 1994), rat Hsp60 (Peralta *et al.*, 1990) and rat Hsp10 (Ryan *et al.*, 1994), but when this work commenced

neither GrpE nor DnaJ homologues had been identified in organelles of higher eukaryotes. To facilitate the biochemical studies of protein folding in eukaryotes, I set out to identify and purify mitochondrial GrpE homologues from rat, bovine and porcine mitochondria by exploiting their strong interaction with DnaK in the absence of ATP.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of a DnaK-affinity column—*E. coli* DnaK was purified by ATP-agarose chromatography (C-8 linkage with a 9 atom spacer, Sigma Cat.# A2767) and coupled to Affi-Gel 10 (BIO-RAD) essentially as described by Zylicz *et al.* (1987). Approximately 100 mg of DnaK in 0.1 M MOPS (pH 7.5) and 80 mM $CaCl_2$ was coupled to 8 ml Affi-Gel 10, packed into a column (1.6 x 20 cm) and washed with equilibration buffer (EB) (20 mM MOPS at pH 7.5, 1 mM DTT, 10 mM $MgCl_2$). These and all other procedures were carried out at 4 °C unless otherwise stated.

2.2.2 Isolation of soluble mitochondrial proteins—Fresh bovine, porcine or rat livers were homogenised using 2.5 L per kg liver of mitochondrial isolation buffer (20 mM HEPES at pH 7.5, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) fortified with 2 mg/ml BSA. The homogenate was centrifuged at 1000 x *g* for 30 min. The supernatant was recentrifuged at 10,000 x *g* for 30 min giving a cytosolic supernatant and a crude mitochondrial pellet. The mitochondrial pellet (from 4 kg starting material) was washed by resuspension in 4 L of mitochondrial isolation buffer and pelleted by centrifugation at 10,000 x *g* for 20 min. The mitochondrial pellet was again resuspended in 1 L of mitochondrial isolation buffer and further purified on ninty six 30 ml sucrose density gradients using swing-out rotors at 100,000 x g for 1 h (Vijayasarathy *et al.*, 1989). Mitochondria purified from the sucrose density gradients were pelleted at 20,000 x *g* for 20 min following 2.6 times dilution with 20 mM MOPS at pH 7.5 containing 1 mM DTT and 1mM EDTA. The pellet was resuspended in 400 ml lysis buffer (20 mM HEPES at pH 7.5, 1 mM DTT, 0.4 mg/ml PMSF, 1 mM EDTA and 0.5% (w/v) Lubrol PX) and incubated up to 5 h before centrifugation at 80,000 x g for 1.5 h to give a supernatant defined as total mitochondrial proteins.

2.2.3 DnaK-affinity chromatography—Mitochondrial proteins were passed over a ~100 ml ATP- agarose column (C-8 linkage with a 9 atom spacer, Sigma Cat.# A2767) that was pre-equilibrated in EB. The unbound fraction, depleted of mt-Hsp70, was fortified with apyrase (62 units/500 ml) to degrade endogenous ATP and then loaded onto the DnaK-affinity column at a flow rate of 0.3 ml/min followed by extensive washing with the EB. After washing, bound proteins were eluted firstly with EB containing 1 M KCl (15 ml) and then with EB containing 5

mM ATP and 0.5 M KCl (50 ml). The polypeptide content of all fractions (1.4 ml) was determined by SDS-PAGE in 16.5 % (w/v) Tris-Tricine gel (section 2.2.5). Fractions eluted with ATP contained a prominent 24 kDa polypeptide (ie mt-GrpE#1). This material was prepared for HPLC on a 5 μm C_4 reversed phase (RP) column (4.6 mm x 250 mm, VYDAC Cat.# 214TP54) by adding an equal volume of 6 M guanidine-hydrochloride. The column was operated in 0.1% (v/v) TFA at 0.6 ml/min using an CH_3CN gradient composed of two linear segments (0-40 min: 0-48% (v/v) CH_3CN; 40-100 min: 48-80% (v/v) CH_3CN). The 24 kDa polypeptide was eluted at 59% (v/v) CH_3CN.

2.2.4 Protein and peptide sequence analysis—Protein and peptide sequencing was performed on an Applied Biosystems Model 470A gas phase sequencer with a model 120A on-line analyser. N-terminal sequencing was performed on the C_4 reversed phase HPLC purified 24 kDa protein (mt-GrpE#1) by Peter Høj and Rosemary Condron (School of Biochemistry, La Trobe Uni., Bundoora, Vic., Australia). Tryptic peptides of the SDS-PAGE purified 24 kDa protein were obtained by *in situ* digestion and microbore-HPLC on a 5 μm C_8 reversed phase column (1.0 mm x 250 mm, VYDAC, Cat.# 208TP51) as detailed by Tetaz *et al.* (1993). The methods for the extraction of tryptic peptides from a 10 % (w/v) Tris-Glycine gel (section 2.2.5) and the operation of the C_8 microbore RP-HPLC column are summarised in figure 1. N-terminal sequencing of the tryptic peptides was carried out by Tim Tetaz (Baker Medical Research Institute, Melbourne, Vic., Australia).

2.2.5 SDS-PAGE—SDS-PAGE was carried out according to the method of either Schägger and von Jagow (1987) using a Tris-Tricine buffer system and a 16.5 % (w/v) separating gel without urea, preceded by a 10 % (w/v) spacer gel (acrylamide/bisacrylamide 32:1), or according to Fling and Gregerson (1986) in Tris-glycine buffers using a 12.5 % (w/v) separating gel (acrylamide/bisacrylamide 50:1.35).

2.2.6 Coomassie Brilliant Blue staining of proteins after SDS-PAGE—Polyacrylamide gels were soaked in 0.1 % (w/v) Coomassie Brilliant Blue R-250, 40 % (v/v) ethanol and 7 % (v/v) acetic acid for 1-2 h with gentle shaking. Gels were destained by washing in 20 % (v/v) ethanol and 7 % (v/v) acetic acid.

2.3 RESULTS AND DISCUSSION

The aim of this study was to determine whether homologues of *E.coli* GrpE exist in mammalian mitochondria. Our laboratory had previously identified mt-Hsp70, Hsp60 and Hsp10 as stress inducible proteins (Hartman *et al.*, 1992). Since bacterial GrpE is a heat shock protein with a molecular mass of ~24 kDa (Ang *et al.*, 1986), We therefore attempted to employ heat shock and metabolic labelling (Hartman *et al.*, 1992) for the identification of a 24 kDa stress-inducible protein in rat hepatoma cells, but without success (see Chapter 3, Fig. 5*A*).

As an alternative, a DnaK-affinity column was prepared. It had previously been shown that GrpE from *E.coli* binds strongly to immobilised DnaK and can be eluted specifically with 10 mM ATP but not with 2 M KCl (Zylicz *et al.*, 1987). Since DnaK and mammalian mt-Hsp70 exhibit ~51% positional sequence identity (Webster *et al.*, 1994), it was therefore likely that a mitochondrial GrpE homologue could be purified by DnaK-affinity chromatography (outlined in Fig. 2). Thus, a large quantity of bovine liver mitochondria was purified by sucrose density gradient centrifugation and following lysis with Lubrol PX, the soluble protein fraction (approx. 2,800 mg) was loaded onto an ATP-agarose column to remove endogenous mt-Hsp70. The depleted lysate was subsequently treated with apyrase and loaded onto the DnaK-affinity column. Following washing, the bound material was eluted successively with 1 M KCl and 5 mM ATP/0.5 M KCl. SDS-PAGE analysis (Fig. 3) showed that the majority of matrix proteins had no affinity for the column (compare *lanes 2* and *3* of Fig. 3) whilst a limited set of proteins were eluted with 1 M KCl (*lane 4*). Upon elution with 5 mM ATP/0.5 M KCl, a 24 kDa component was obtained along with a small amount of lower molecular mass material (*lane 5*). The yield of this protein was ~500 µg. Binding did not take place on a plain Affi-Gel 10 column and omission of the initial ATP-agarose step diminished the yield considerably, presumably because the 24 kDa protein preferentially associates with the soluble and more abundant mt-Hsp70 (data not shown). The initial ATP-agarose step was not included for the isolation of the 24 kDa protein from the rat extract and the porcine extract was only partially depleted of mt-Hsp70. As a result, the yield of the 24 kDa component was considerably diminished from the rat extract (Fig.3, *lane 7*), while both the rat and porcine (Fig.3, *lane 6*) preparations were contaminated with mt-Hsp70 presumably due to dimerisation with DnaK. Interestingly, I never saw co-elution of the 24 kDa polypeptide with mt-Hsp70 during ATP-agarose chromatography. This is presumably because binding of mt-Hsp70 to the ATP-agarose matrix abolishes

productive interaction with the 24 kDa polypeptide through a steric exclusion or a conformational change.

The bovine 24 kDa protein was further purified by reversed-phase HPLC (Fig. 4). Only one major protein containing peak was observed, underscoring the high selectivity of the DnaK column. Direct amino acid sequencing did, however, indicate the presence of both a major and a minor sequencing species. 28 N-terminal residues of a major sequencing species (Fig. 5) were obtained and the sequence of the minor species was contained therein. This suggests that the minor species represented a proteolytic breakdown product of the 24 kDa component and hence explains the presence of a small amount of lower molecular mass material observed on SDS-PAGE (Fig. 3). Further sequence data was obtained from tryptic peptides that were isolated from the SDS-PAGE resolved and then *in situ* digested 24 kDa component. A total of 85 residues representing ~40% of the total protein were identified (Fig. 5). A search of the relevant databases did not reveal any identical proteins. However, extensive sequence identity is evident when compared to prokaryotic GrpE homologues and in particular with a then newly discovered mitochondrial Mge1p (GrpEp) protein from *S. cerevisiae* which exhibits 42% positional identity (Fig. 5) (Bollinger *et al.*, 1994; Ikeda *et al.*, 1994). Based on the sequence similarities and the affinity of the 24 kDa polypeptide for DnaK, I therefore conclude that the purified 24 kDa protein represents a mammalian mitochondrial homologue of GrpE and is designated mt-GrpE#1.

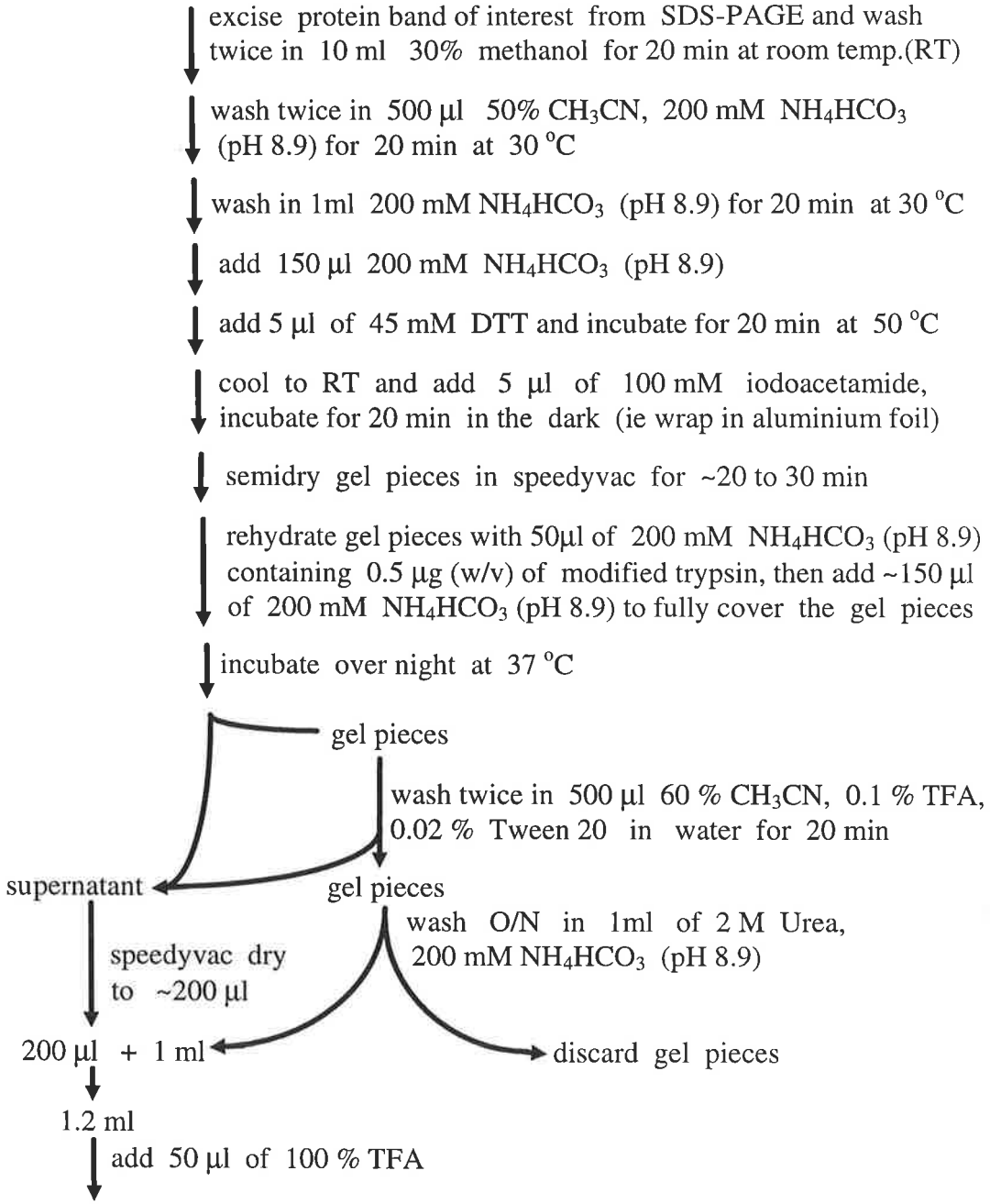
In bacteria (Ang *et al.*, 1986; Ang and Georgopoulos, 1989) and yeast mitochondria (Bollinger *et al.*, 1994; Ikeda *et al.*, 1994), GrpE counterparts are essential for cell survival under a wide range of growth conditions. I therefore expect that mammalian mt-GrpE#1 also undertakes important functions in the biogenesis and maintenance of the mitochondrion. By analogy to the proven function of GrpE in bacteria and yeast, mammalian mt-GrpE#1 most likely acts an ATP/ADP nucleotide exchange factor which modulates the binding of mt-Hsp70 to substrate proteins during mitochondrial protein import and (re)folding in the matrix (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Nakai *et al.*, 1994; Laloraya *et al.*, 1994, 1995; Voos *et al.*, 1994; Kronidou *et al.*, 1994; Westermann *et al.*, 1995; Schneider *et al.*, 1994, 1996; Horst *et al.*, 1997b; Deloche and Georgopoulos, 1996; Azem *et al.*, 1997; Dekker and Pfanner, 1997; Miao *et al.*, 1997a; Kubo *et al.*, 1999). Furthermore, during the process of protein folding, mammalian mt-GrpE#1 would be expected to stimulate mt-Hsp70 ATPase activity in conjunction with a DnaJ homologue (Rowley *et al.*, 1994; Herrmann *et al.*, 1994; Voos *et al.*, 1994; Laloraya *et al.*, 1995; Westermann *et al.*, 1995, 1996; Prip-Buus *et al.*, 1996; Horst *et*

al., 1997b; Deloche *et al.*, 1997; Kubo *et al.*, 1999). To address these problems biochemical assays with purified components are required. Whilst mt-Hsp70 and both yeast GrpEp and mammalian mt-GrpE can be readily purified (Bolliger *et al.*, 1994, this study), mitochondrial DnaJ has not yet been purified from any source although its presence has been clearly established in *S.cerevisiae* (Rowley *et al.*, 1994; Herrmann *et al.*, 1994; Kronidou *et al.*, 1994; Westermann *et al.*, 1996; Prip-Buus *et al.*, 1996; Horst *et al.*, 1997b; Deloche *et al.*, 1997; Kubo *et al.*, 1999)

Since bacterial DnaJ is eluted specifically from DnaK columns with 0.5 M KCl (Georgopoulos *et al.*, 1990, *Table 2*), I therefore investigated by sequence analysis whether any of the predominant proteins eluted in the 1 M KCl wash (Fig. 3, *lane 4*) represented DnaJ homologues. As discussed in Chapters 5 and 6 these selectively retained proteins probably constitute novel Hsp70 substrates, some of which fulfil essential cellular functions, but none appear to not be DnaJ homologous. I considered the possibility that bovine mt-DnaJ, like yeast mitochondrial Mdj1p (Rowley *et al.*, 1994), is a peripheral inner membrane protein which had not been released during preparation of the matrix. The membrane pellet obtained after Lubrol PX treatment was therefore further incubated with 1% Triton X-100 overnight to thoroughly solubilise the membrane proteins. While chromatography of this supernatant on the DnaK column failed to reveal a DnaJ component, consistent with the function of Mge1p in mitochondrial import, a distinct membrane bound pool of mt-GrpE#1 was identified (data not shown). It therefore appears that purification of mitochondrial DnaJ may best be achieved using traditional chromatography and mt-Hsp70 ATPase assays in the presence of mt-GrpE#1. The relatively easy purification of mt-GrpE#1 described here, combined with the availability of optimised procedures for purification of mt-Hsp70, Hsp60 and Hsp10 (Ryan *et al.*, 1995), will facilitate this process and thus further help define the role of mitochondrial chaperones in protein folding.

As a result of the information gathered and the tools generated in this study, I have been able to undertake the further characterisation of mammalian mt-GrpE#1 (Chapter 3) and unexpectedly, have identified and subsequently characterised a second mammalian mitochondrial GrpE-like protein (mt-GrpE#2) (Chapter 4).

Protein (at least 100 pmol but preferably 200 to 300 pmol/ species) is subjected to SDS-PAGE, and visualised by staining with Coomassie Brilliant Blue and destaining as described in the Materials and Methods (section 2.2.6). The following steps are then executed:



Peptides are separated on a 5 µm C_8 microbore RP-HPLC column (1.0 mm x 250 mm, VYDAC, Cat.# 208TP51) as detailed by Tetaz *et al.* (1993). The column was equilibrated in 0.1% (v/v) TFA and the sample was loaded at 0.05 ml/min in 0.08 % (v/v) TFA and 6% (v/v) CH_3CN. Following loading, an CH_3CN gradient composed of four linear segments (0-15 min: 4.2-10.5% (v/v) CH_3CN; 15-130 min: 10.5-35% (v/v) CH_3CN; 130-140 min: 35-49% (v/v) CH_3CN; 140-150 min: 49-70% (v/v) CH_3CN) was developed in the presence of 0.08 % (v/v) TFA.

Fig. 1. *In situ* digestion, RP-HPLC purification and N-terminal sequencing of proteins separated by SDS-PAGE.

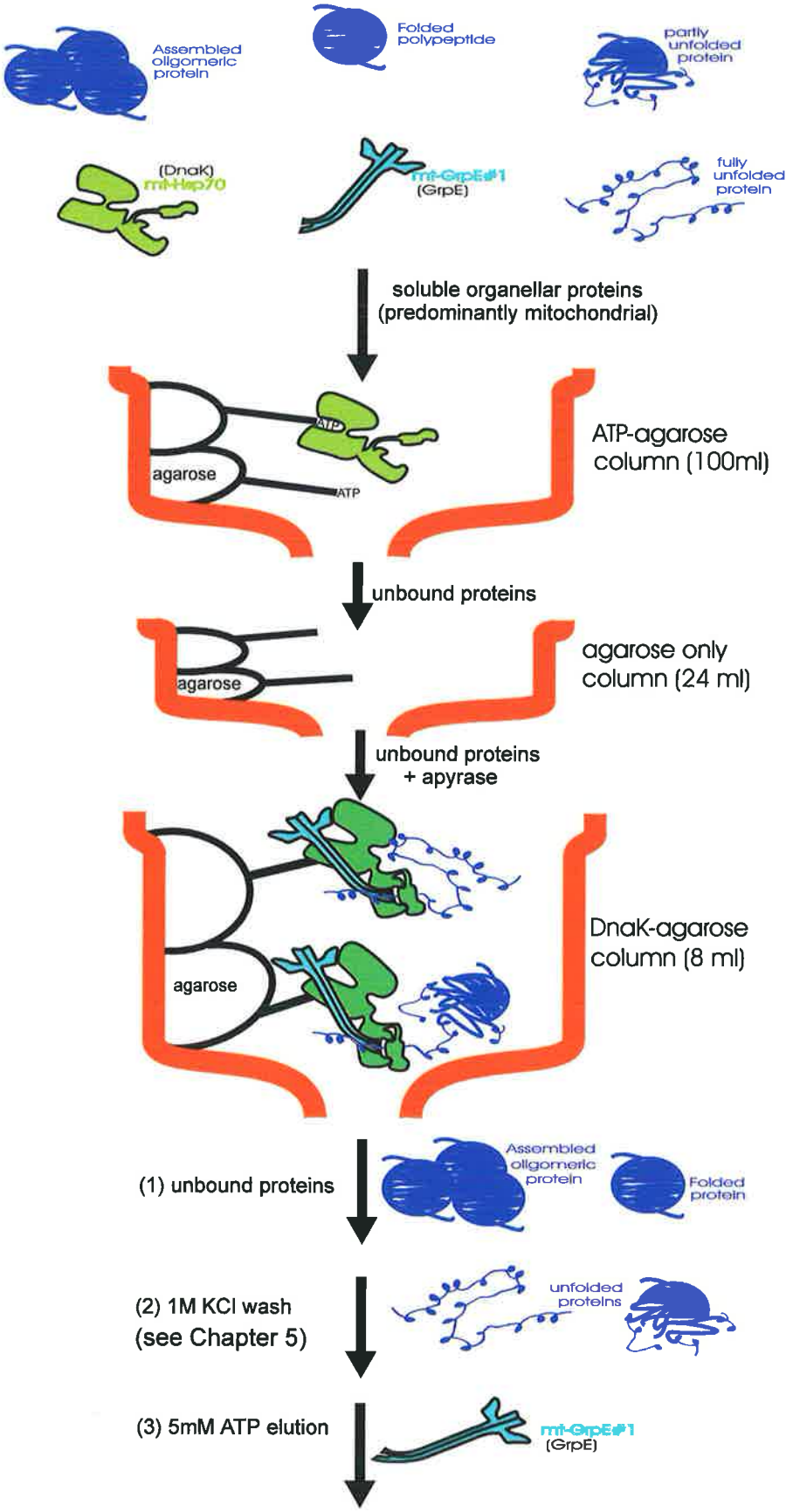


Fig. 2. **Flow diagram of the purification procedure for mt-GrpE#1.** See text and figure 3 for further details.

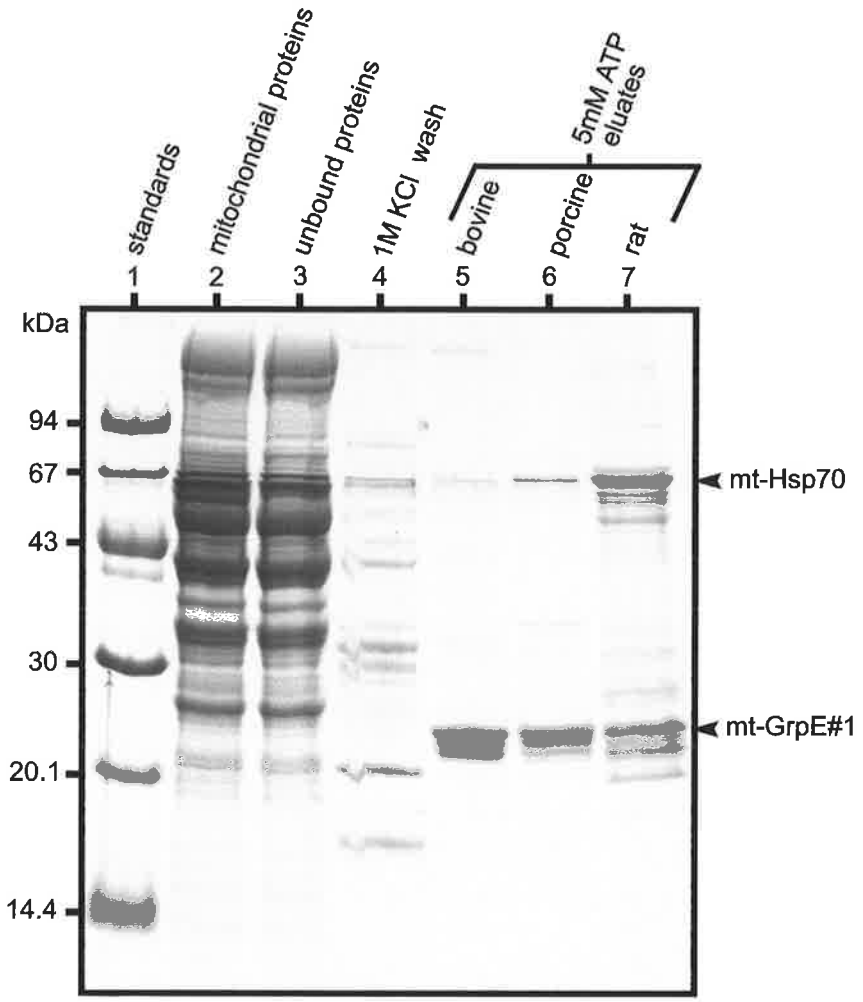


Fig. 3. Purification of mt-GrpE#1 from bovine, porcine and rat liver. Soluble proteins extracted from bovine liver mitochondria were firstly applied to an ATP-agarose column in order to remove exogenous mt-Hsp70. The unbound fraction from the ATP-agarose column was then supplemented with apyrase and loaded onto a DnaK-column (*lane 2*). After the unbound material had passed through the column (*lane 3*), the column was extensively washed with equilibration buffer (EB). Bound proteins were eluted firstly with EB containing 1 M KCl (*lane 4*) and then with EB containing 5 mM ATP and 0.5 M KCl (*lane 5*). The ATP-eluate obtained from porcine (*lane 6*) and rat liver (*lane 7*) extracts containing residual mt-Hsp70 was also analysed. The polypeptide content of all fractions was analysed by SDS-PAGE in a 16.5 % Tris-Tricine gel followed staining with Coomassie Brilliant Blue. *Lane 1* contains molecular mass standards.

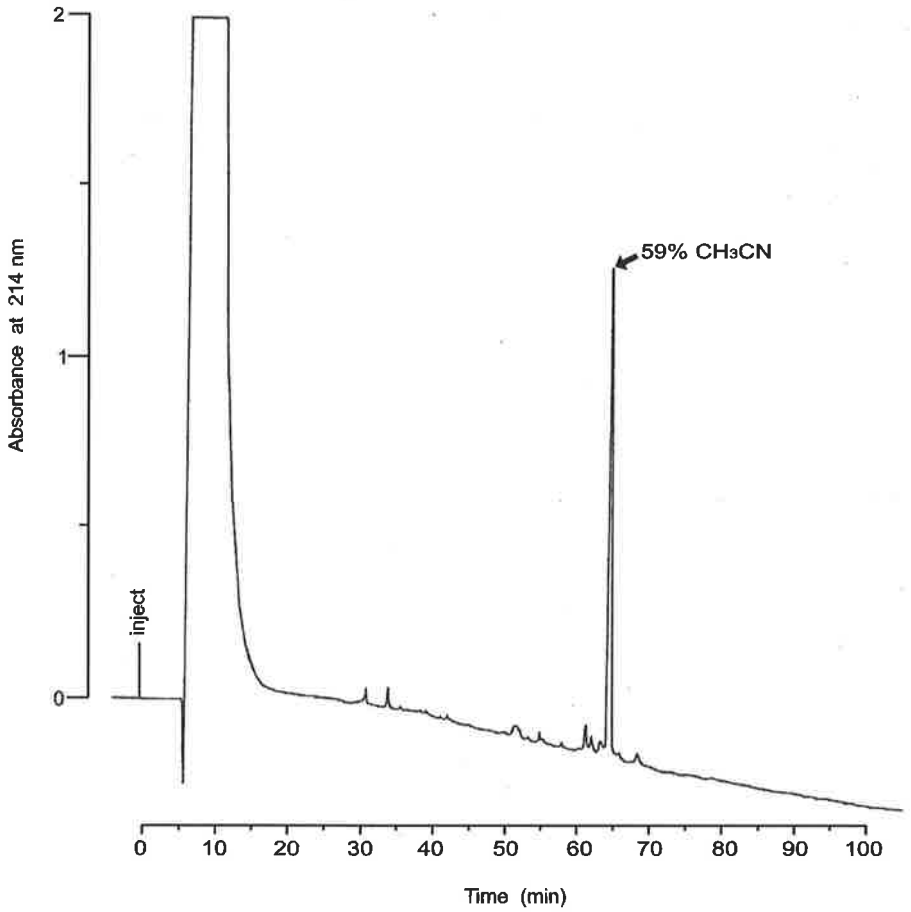


Fig. 4. HPLC chromatography of bovine mt-GrpE#1 purified by DnaK-affinity chromatography. The material obtained in the ATP eluate from the DnaK-column (Fig. 3, *lane 5*) was further purified by C_4 reversed phased HPLC chromatography as described in the Materials and Methods (section 2.2). The 24 kDa polypeptide eluted at 59% (v/v) CH_3CN was used for N-terminal sequence analysis.

Fig. 5. **Multiple amino acid sequence alignment showing the similarity between bovine mt-GrpE#1 and several GrpE homologues.** The peptide sequences obtained herein for bovine mt-GrpE#1 were compared to the polypeptide sequences of *Saccharomyces cerevisiae* Mge1p (GenBank™ accession no. D26059), *Escherichia coli* GrpE (GenBank™ accession no. X07863), *Mycobacterium tuberculosis* GrpE (GenBank™ accession no. X58406) and the methanogenic Archaean *Methanosarcina mazei* GrpE (GenBank™ accession no. X74353). Identical residues are shaded yellow, while gaps generated to maximise homology between sequences are indicated (-). The N-terminal mitochondrial signal sequence from yeast Mge1p is underlined (Azem *et al.*, 1997)

Chapter 3

Isolation and characterisation of a cDNA encoding mammalian mt-GrpE#1

3.1 INTRODUCTION

The *E. coli* DnaK(Hsp70)/ DnaJ/ GrpE and GroEL(Hsp60)/ GroES(Hsp10) molecular chaperone systems have been reported to be involved in a number of essential cellular processes (Chapter 1; Hartl, 1996; Johnson and Craig, 1997; Bukau and Horwich, 1998). Furthermore, during nascent polypeptide folding or the refolding of denatured proteins, these chaperone systems have in some cases been observed to function successively and synergistically (Chapter 1; Hartl, 1996; Johnson and Craig, 1997). In yeast mitochondria these chaperone systems have been highly conserved and reported to fulfil essential functions during protein import and folding (Chapter 1; Hartl, 1996; Ryan *et al.*, 1997; Dekker and Pfanner, 1999) whilst relatively little is known about the homologues from mitochondria of higher eukaryotes (Chapter 1; Ryan *et al.*, 1997). While cDNAs encoding mitochondrial homologues of *E. coli* DnaK, GroEL and GroES have been isolated from higher eukaryotic sources (Webster *et al.*, 1994; Peralta *et al.*, 1990; Ryan *et al.*, 1994), cDNAs encoding mitochondrial homologues of DnaJ and GrpE are yet to be reported. In chapter 2 it was outlined how affinity chromatography on DnaK columns was used to identify a mitochondrial GrpE homologue (mt-GrpE#1) from bovine, porcine and rat liver mitochondria (Naylor *et al.*, 1995). Furthermore, I obtained several peptide sequences from the affinity purified bovine mt-GrpE#1 protein (Chapter 2; Naylor *et al.*, 1995). Utilising these peptide sequences, I have now isolated a cDNA encoding a 217 residue nuclear encoded precursor of rat mt-GrpE#1 including a typical mitochondrial presequence of 27 residues. Western blotting revealed that the 21 kDa GrpE homologue is present exclusively in the mitochondrial fraction where it comprises only ~0.03% of the total soluble protein, while Northern blotting showed that the mt-GrpE#1 transcript is present in most if not all rat organs. By contrast to other mitochondrial chaperones, the levels of mt-GrpE#1 and its transcript in cultured cells are only marginally increased in response to the proline analog L-azetidine 2-caboxylic acid and are not increased in response to heat shock. Furthermore, members of the GrpE family exhibit a much lower degree of sequence identity than do the well studied members of the Hsp70, Hsp60 and Hsp10 families.

3.2 MATERIALS AND METHODS

3.2.1 Synthesis of oligonucleotide primers—Based on peptide sequences obtained from bovine mt-GrpE#1 (Chapter 2; Naylor *et al.*, 1995), degenerate oligonucleotides representing all possible codon usages were synthesised on an Applied Biosystems DNA Synthesiser 381 by Rosemary Condron (School of Biochemistry, La Trobe Uni., Bundoora, Vic., Australia). The amino acid sequences and corresponding oligonucleotide sequences (in parenthesis) are: ^{4}AKQKNDG^{10} [primer #1: 5'-GCNAA(A/G)CA(A/G)AA(A/G)AA(C/T)GA(C/T)GG-3'] and ^{143}FDPYEHEA^{150} [primer #2: 5'-A(A/G)CT(A/G)GGNAT(A/G)CT(C/T)GT(A/G)CT-(C/T)CG-3'] (see Fig. 1).

The oligonucleotides employed in 5'-RACE analysis were those designed by Frohman *et al.* (1988) combined with those designed from a partial cDNA clone (nucleotides 40 to 947, Fig. 1) of mt-GrpE#1, namely the antisense primer #3 [5'-CCCCTCCACAGGGGTGTGGA-3'] and nested antisense primer #4 [5'-GCAACCTCCAGCAAGTCCTT-3'] (Life Technologies, Inc.).

The primer sets used in semi-quantitative RT-PCR of various chaperone transcripts were: for mt-Hsp70 RT-70a [5'-ATGGATCCATGGCGTCAGAAGCAATCAAGGGTGC-3'] and RT-70b [5'-TCACAGCGACAGGCCACTAAG-3'], for Hsp60 RT-60a [5'-CAAATG-AAGAGGCTGGGGATGGCA-3'] and RT-60b [5'-GAGCAGGTACAATGGA-CTGAACAC-3'], for Hsp10 RT-10a [5'-ATGGCTGGACAAGCTTTT-3'] and RT-10b [5'-GCTTCATGTGACACCATTTCAA-3'] and for mt-GrpE#1 RT-Ea [5'-TGCACAGCTAC-AAAACAA-AAG-3'] and RT-Eb [5'-CAAGCCCGCCCACCCTTTGA-3'] (Life Technologies, Inc.).

3.2.2 Isolation of mRNA, RT-PCR, cDNA library screening and 5'-RACE analysis—mRNA was isolated from 5 x 10^7 clonal rat hepatoma H4 cells (section 3.2.6; Hartman *et al.*, 1992) using a Fast Track® mRNA isolation Kit (Invitrogen®) and partial first strand cDNA was generated with primer #2 and SUPERSCRIPT^{TM} II RNase H$^-$ Reverse Transcriptase (Life Technologies, Inc.) according to the manufacturer's protocol. Using the partial first strand cDNA and primers #1+2, a 440 bp PCR product was produced using a 30 cycle protocol (94°C for 60 sec, 50°C for 90 sec and 72°C for 90 sec) according to the manufacturer's specifications. The PCR product was recovered and used to screen an adult rat liver cDNA library constructed in the λZAP II vector (Stratagene). Recombinants (5 x 10^5) were screened

essentially as described previously (Høj *et al.*, 1989) and two independent clones were isolated. Both strands of the two clones were sequenced fully by the dideoxynucleotide chain termination method of Sanger *et al.* (1977) using a Sequenase™ Version 2.0 sequencing kit. The products of sequencing reactions were electrophoresed in 6 % (w/v) denaturing polyacrylamide gels using the Bio-Rad Sequi-Gen nucleic acid sequencing cell according to the manufacturer's specifications.

Cloning of nucleotides -14 to 39 of the final cDNA (see Fig. 1) was performed with the aid of primers #3 and #4 by 5'-RACE PCR according to Frohman *et al.* (1988).

*3.2.3 **Production of anti-mt-GrpE#1 and anti-Hsp60 polyclonal antibodies***—100 µg of both bovine mt-GrpE#1 (Chapter 2; Naylor *et al.*, 1995) purified by affinity chromatography and recombinant rat Hsp60 (Peralta, 1993) were mixed with an equal volume of Freunds complete adjuvant (Life Technologies, Inc.) and injected subcutaneously into seven month old New Zealand white rabbits (Animal house, Latrobe University, Bundoora, Vic., Australia). The rabbits were then boosted, three times at six week intervals, with approximately 50µg of the corresponding antigen excised from SDS-PAGE 10 % (w/v) Tris-Glycine gels (section 2.2.5), developed into a slurry and mixed with an equal volume of Freunds incomplete adjuvant as described by Harlow and Lane (1988). Ear bleeds were performed ten days after the third boost and sera containing polyclonal antibodies to mt-GrpE#1 and Hsp60 were collected and processed (Harlow and Lane 1988). The sera were supplemented with 0.02% (v/v) sodium azide and stored at -70°C. Dilutions of 1 in 10,000 were used in Western blot analysis.

*3.2.4 **Western blot analysis***—Cellular protein fractions were resolved by SDS-PAGE in a 16% (w/v) Tris-Tricine gel (section 2.2.5) and transferred onto a nitrocellulose membrane (MSI Laboratories, Westboro, MA) using a semi-dry transfer unit (LKB-Pharmacia Biotech.) according to Harlow and Lane (1988). The transfer buffer consisted of 48 mM Tris, 39 mM Glycine, 0.037 % (w/v) SDS, 20 % methanol and 2 mM DTT. Transfer was performed at ~1 mA/cm^2 for 1 h prior to blocking of the membrane from 1 h to overnight in 5 % (w/v) skim milk powder in TNBS (25 mM Tris-HCl pH 8.0, 200 mM NaCl, 2.5 mM KCl, 0.5 % (v/v) Tween-20). After washing the membrane in TNBS (3 x 10 min), the filter was incubated with the rabbit anti-mt-GrpE#1 serum (1 in 10, 000 dilution in TNBS containing 3 % (w/v) BSA) for 1 h prior to 4 x 10 min washes in TNBS. The membrane was then incubated with Horse-radish peroxidase—labelled goat anti-rabbit IgG (1 in 10,000 dilution in TNBS containing 3

% (w/v) BSA; Amersham) for 1 h prior to 4 x 10 min washes in TNBS. mt-GrpE#1 was visualised by incubating the membrane with enhanced chemiluminescence (ECL™) detection reagents (Amersham) and subsequent exposure to Hyperfilm™-MP (Amersham) according to the manufacturer's instructions. Following re-blocking and washing of the membrane, the membrane was reprobed with serum raised against Hsp60 (1 in 10,000 dilution) and bound antibodies were detected as described above.

Rat liver mitochondria were prepared as described in section 2.2.2 (Hartman *et al.*, 1992). Microsomes were pelleted by centrifugation of the post-mitochondrial supernatant at 120,000 x *g* for 1 h and the final supernatant was defined as the cytosolic fraction. Soluble microsomal proteins were prepared using the same method for the preparation of soluble mitochondrial proteins (section 2.2.2; Hartman *et al.*, 1992).

*3.2.5 **Northern analysis***—For identification of the tissue distribution of mt-GrpE#1 transcripts, a Northern blot with mRNA (~2μg each) from eight different rat tissues was purchased from Clontech (Cat.# 7764-1) and probed with a ^{32}P-labelled mt-GrpE#1 cDNA probe (nucleotides 82 to 719, Fig. 1) in Express Hyb hybridisation solution (Clontech) for 1 h at 68°C. Final washes were performed twice for 20 min at 65°C in 0.1× SSC supplemented with 0.5% (w/v) SDS and filters were analysed using a Storm PhosphorImager and ImageQuaNT software (Molecular Dynamics).

*3.2.6 **Growth of tissue culture cells***—Clonal rat hepatoma H4 cells were gift from Dr. Gary Brown (Royal Children's Hospital, Melbourne, Vic., Australia). H4 cells were grown by Joan Hoogenraad (School of Biochemistry, La Trobe Uni., Bundoora, Vic., Australia) as monolayers in Dulbecco's modified Eagle's medium (DMEM; Multi Cell, Trace Biosciences Pty. Ltd.) with 10 % (v/v) fetal calf serum (Multi Cell, Trace Biosciences Pty. Ltd.) at 37 °C in an atmosphere of 5 % CO_2.

*3.2.7 **Stress treatments, metabolic labelling and fractionation of tissue culture cells***—The growth of H4 cells in the presence of the amino acid analog L-azetidine-2-carboxylic acid (Azc) (Sigma), the conditions for heat shock and the isolation of ^{35}S-labelled cytosolic and mitochondrial proteins were as described by Hartman *et al* (1992). For Azc stress, H4 cells (5 x 10^7 H4 cells) at 70-80% confluence were grown for 10 h in the presence (stressed cells) or absence (control cells) of 5 mM Azc and then labelled for 2 h in methionine- and Azc-free

DMEM (Sigma) supplemented with 10% dialysed fetal calf serum, [^{35}S]methionine and [^{35}S]cysteine at 10 μCi/ml (Tran^{35}S-label, New England Nuclear-Dupont, 1165 Ci/mmol). For heat shock treatments, cells were heat shocked at 45°C (stressed cells) for 17 min and then metabolically labelled for 12 h at 37°C in fresh, prewarmed methionine free DMEM supplemented with 10% (v/v) dialysed fetal calf serum, [^{35}S]methionine and [^{35}S]cysteine at 10 μCi/ml. Control cells were labelled in an identical fashion for both treatments.

Soluble cytosolic and mitochondrial proteins were extracted from the H4 cells as described by Hartman *et al.* (1992). A crude cellular lysate was prepared by hypotonic lysis and Dounce homogenisation. Cellular membranes and nuclei were removed as a pellet following a 754 x *g* centrifugation and a crude mitochondrial pellet was obtained by recentrifugation of the supernatant at 10,000 x *g*. The remaining supernatant represented the cytosolic fraction. Mitochondria were further purified on sucrose gradients and following incubation with 0.5% (w/v) Triton X-100 Reduced (Sigma) at 4°C for 1 h, a mitochondrial supernatant was obtained after centrifugation at 80,000 x *g*.

3.2.8 ***Semi-quantitative RT-PCR***—H4 cells (5 x 10^7) grown to 70-80% confluence at 37°C were either (i) grown a further 12 h at 37°C (control cells), (ii) supplemented with 5 mM Azc and grown an additional 12 h (Azc stress) at 37°C or (iii) grown an additional 7 h at 37°C followed by 20 min at 45°C and then at 37°C for 5 h (heat shock). Cells were harvested at the same time point and mRNA isolated as described above.

First strand cDNA was generated using 1μg of mRNA and oligo$(dT)_{15}$. 10 % of each cDNA batch was used in separate PCRs with the primer pairs for mt-Hsp70 [RT-70a and RT-70b], for Hsp60 [RT-60a and RT-60b], for Hsp10 [RT10a and RT10b] and for mt-GrpE#1 [RT-Ea and RT-Eb] in a 30 cycle PCR protocol as described above.

3.3 RESULTS AND DISCUSSION

*3.3.1 **Cloning of rat mt-GrpE#1 cDNA***—Based on peptide sequences I previously obtained for bovine mt-GrpE#1 (Chapter 2; Naylor *et al.*, 1995), degenerate oligonucleotides were designed and employed in RT-PCR to generate a cDNA fragment of expected size (440 nucleotides). This PCR product was used to probe a rat liver cDNA library and a mt-GrpE#1 cDNA clone (nucleotides 40 to 947, Fig. 1) lacking an ATG initiation codon was thus obtained. As several attempts to isolate a full length cDNA from two different libraries failed, 5'-RACE PCR was employed to define 53 nucleotides corresponding to the 5'-end of the rat mt-GrpE#1 transcript (nucleotides -14 to 39, Fig. 1). An ATG codon (nucleotides 1 to 3) that fulfils the Kozak requirements for initiation of translation in eukaryotes (Kozak, 1987) was evident in three independently cloned 5'-RACE PCR products. The nucleotide sequences of these products were all identical in a 305 bp overlap with the sequence of the cDNA clone initially isolated from the library. I am therefore confident that the composite cDNA represented in Fig. 1 corresponds to a single GrpE transcript.

Search of databases (June, 1996) revealed a rat (GenBank accession no. H32411), three mouse (GenBank accession nos. W08216, W55413, W30513) and sixteen human expressed sequence tags (ESTs) with a high degree of positional identity to parts of the rat mt-GrpE#1 cDNA. None of these ESTs encode a full-length mt-GrpE#1 and most if not all contain numerous and obvious sequencing mistakes, such as stop codons within the open reading frame. Of the twenty ESTs, only three human sequences [1](TIGR HCD assembly no. THC94455) contain an ATG start codon and none of the mt-GrpE#1 transcripts contain a poly(A) tail despite the presence of a putative polyadenylation signal in the rat transcript sequenced in this report (Fig. 1). Together, these results indicate that the mt-GrpE#1 transcript may be somewhat unstable or alternatively difficult to reverse transcribe. This may explain why screening of cDNA libraries failed to produce full length clones.

The combined information, obtained from sequencing a partial rat cDNA clone and 5'-RACE clones reveals a complete rat mt-GrpE#1 cDNA comprising a coding region of 651 nucleotides and a minimal transcript size of 961 nucleotides (Fig. 1). Comparison of the deduced amino acid sequence with the N-terminal sequence of purified bovine mt-GrpE#1 (Fig. 1) reveals a 27 residue N-terminal targeting sequence which, upon mitochondrial import,

[1] The TIGR Human cDNA Database (HCD) assembly no. THC94455 has been revised, the information is now part of the TIGR Human Gene Index (HGI) assembly no. THC208554 which can be found at http://www.tigr.org

is proteolytically removed from the 217 residue primary translation product (~24.3 kDa) to generate the mature protein of 190 amino acids (~21.3 kDa). The predicted isoelectric point (pI) of the mature rat mt-GrpE#1 protein is 6.5 whilst the precursor, due to its basic mitochondrial targeting sequence, has a predicted pI of 8.5.

3.3.2 Members of the GrpE family exhibit a relatively low degree of sequence identity at the amino acid level—The deduced amino acid sequences of rat and human mt-GrpE#1 exhibit 88.9% positional identity, while positional identities with other GrpE family members are: 41.8% for mitochondrial Droe1p form *D. melanogaster* (GenBank accession no. U34903), 33.3% for *C. elegans* GrpE (EMBL accession no. Z46996), 25.9% for mitochondrial Mge1p (Yge1p, GrpEp) from *S. cerevisiae* (Ikeda *et al.*, 1994; Bolliger *et al.*, 1994), 20.8% for *E.coli* GrpE (Lipinska *et al.*, 1987) and 19.1% for archaebacterial *M. mazei* GrpE (Conway de Macario *et al.*, 1994). Comparison of the deduced amino acid sequences of nineteen GrpE family members (ie all known members in June 1996) revealed that six residues are strictly conserved, four of which reside in the C-terminal third of GrpE (Fig. 2). This region of *E. coli* GrpE forms a six-stranded β-sheet and, as part of GrpE dimer, the β-sheets extend out from the remaining long α-helical structures like a pair of arms (Chapter 1; Harrison *et al.*, 1997). A complex between dimeric GrpE and the ATPase domain of DnaK reveals that most contacts are made within the proximal β-sheet arm which is proposed to facilitate ADP release from DnaK (Chapter 1; Harrison *et al.*, 1997).

Members of the GrpE family exhibit a much lower degree of sequence identity than do the well studied members of the Hsp70, Hsp60 and Hsp10 families. Thus the 21 % positional identity of rat mt-GrpE#1 with *E.coli* GrpE can be compared with the 51 % (Webster *et al.*, 1994), 49 % (Peralta *et al.*, 1990) and 45 % (Hartman *et al.*, 1992) positional identities that have been determined for the rat mitochondrial homologues of *E.coli* DnaK (mt-Hsp70), GroEL (Hsp60) and GroES (Hsp10), respectively. The low conservation of the primary structures between the eukaryotic and bacterial GrpE homologues appears not to have changed structural features to a significant extent. Thus, in keeping with the functional interchangeability of the rat and bacterial chaperonins (Hartman *et al.*, 1992, Ryan *et al.*, 1995), mammalian mt-GrpE#1 like *E.coli* GrpE binds with high affinity to immobilised *E.coli* DnaK, the interaction being readily terminated in the presence of 5 mM ATP but persisting in the presence of 1 M KCl (Chapter 2; Naylor *et al.*, 1995).

3.3.3 mt-GrpE#1 is a low abundance mitochondrial protein of ubiquitous appearance in mammalian organs—As seen for yeast Mge1p (Nakai *et al.*, 1994), polyclonal antibodies raised against mammalian mt-GrpE#1 revealed an exclusive mitochondrial location for the 21 kDa protein (Fig. 3). Thus by contrast to the appearance of DnaK and DnaJ homologues in a number of compartments of both yeast and mammalian cells, GrpE homologues appear confined to mitochondria. It is somewhat surprising that the role of GrpE like molecules may not be required for the proper functioning of Hsp70 homologues in other compartments whilst recognisable homologues of DnaJ have been found in the cytosol, mitochondria and endoplasmic reticulum of yeast and mammalian cells (Chapter 1; Gething, 1997). For these Hsp70 systems, GrpE may be replaced by additional co-factors that broaden the functions of Hsp70 chaperones (Chapter 1, section 1.5.5).

We have previously noted a relatively high concentration of the mitochondrial chaperones mt-Hsp70 (Webster *et al.*, 1994), Hsp60 (Peralta, 1993) and Hsp10 (Hartman *et al.*, 1992, Ryan *et al.*, 1995). By contrast, our previous attempts at purifying mt-GrpE#1 revealed a very modest concentration for this protein (Chapter 2; Naylor *et al.*, 1995). This inference is supported by the Western blot analysis shown in Fig. 5*B* from which it is concluded that mt-GrpE#1 constitutes about 0.03 % of the Triton X-100 soluble mitochondrial protein fraction as also determined for *S. cerevisiae* Mge1p (Nakai *et al.*, 1994). This value can be compared with a figure of about 1 % for mt-Hsp70 (Webster *et al.*, 1994; Nakai *et al.*, 1994) and 1.3 % for Hsp60 (Fig. 5*B*). Assuming that GrpE functions as a dimer, mt-Hsp70 as a monomer and Hsp60 as a tetradecamer, mt-GrpE#1 therefore appears about 20 times and 2-4 times less abundant than mt-Hsp70 and Hsp60, respectively. This is in accordance with the function of Mge1p as an ADP/ATP exchange catalyst during the cycles of protein binding and release by mt-Hsp70 which is an integral part of mitochondrial protein import and subsequent folding (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Nakai *et al.*, 1994; Voos *et al.*, 1994; Kronidou *et al.*, 1994; Laloraya *et al.*, 1994, 1995; Westermann *et al.*, 1995; Schneider *et al.*, 1994, 1996; Deloche and Georgopoulos, 1996; Horst *et al.*, 1997b; Azem *et al.*, 1997; Dekker and Pfanner, 1997; Miao *et al.*, 1997a; Kubo *et al.*, 1999). Consistent with this function of Mge1p I have further shown that a small fraction of rat mt-GrpE#1 (but not Hsp60) resides in the membrane fraction of lysed mitochondria (Chapter 2). By analogy to fungal mitochondria (Chapter 1; Dekker and Pfanner, 1999), it is therefore tempting to speculate that the membrane association of mt-GrpE#1 occurs through complex

formation with mt-Hsp70 which in turn may bind to a recently described mammalian homologue of Tim44 (Wada and Kanwar, 1998).

By contrast to the restricted location of mt-GrpE#1 within cells, Northern blot analysis revealed the presence of a ~1.2 kilobase mt-GrpE#1 transcript mRNA in all organs studied (Fig. 4). At equal mRNA loading, the mt-GrpE#1 transcript was particularly abundant in heart, kidney and liver tissues, perhaps because mitochondria are abundant in these organs. This widespread expression is expected since the *S. cerevisiae* MGE1 (YGE1) gene is essential for cell viability (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994) and is further underscored by the fact that the twenty ESTs (June, 1996) related to mt-GrpE#1 were obtained from sources as divergent as white blood cells, testis, brain, liver, spleen, melanocytes, ovary and pheochromocytoma (PC)-12 cells.

3.3.4 mt-GrpE#1 synthesis is induced slightly by amino acid analogue treatment but not by heat shock—Consistent with their role as facilitators of protein folding, the synthesis of many chaperones have been noted to increase under conditions which are not conducive to protein folding (Chapter 1). In *E. coli* the GrpE gene, like the DnaK/DnaJ and GroEL/GroES operons, is part of the heat shock regulon and thus transcribed/translated at a higher rate in response to heat shock (reviewed in Georgopoulos *et al.*, 1990, 1994). Whilst mammalian Hsp60 (Mizzen *et al.*, 1989) and Hsp10 (Hartman *et al.*, 1992) are clearly heat shock inducible, surprisingly, neither mammalian mt-Hsp70 nor *S. cerevisiae* Mge1p appear to be inducible by heat shock (Mizzen *et al.*, 1989; Nakai *et al.*, 1994). It was therefore of interest to investigate whether rat mt-GrpE#1 is synthesised at elevated rates in response to stress. Rat hepatoma cells were either heat shocked or grown in the presence of the proline analogue Azc and following metabolic labelling, cellular fractions were analysed by SDS-PAGE and PhosphorImaging to detect *de novo* synthesised proteins (Fig. 5*A*). Inspection of the cytosolic fractions clearly indicated a sharp induction of Hsp72 (Hsp70) by both types of stress and similarly a significant but less pronounced increase in mitochondrial Hsp60. Under no circumstances was it possible to detect metabolically labelled mt-GrpE#1, let alone observe an increase in its intensity. In contrast a cytosolic 28 kDa protein, putatively identified as the cysteine containing rat Hsp28 (GenBank accession no. S67755), was clearly accumulated at a higher rate following heat shock. When these observations were backed up by Western blot analysis (Fig. 5*B*) similar results were obtained, except now a slight increase in the total

amount of both Hsp60 and mt-GrpE#1 was noticeable upon close inspection of the corresponding filters.

In an attempt to further investigate whether such perceived increases in mt-GrpE#1 levels could be correlated with an increase in mRNA steady-state levels, semi quantitative RT-PCR was performed in triplicate and each time a similar relative intensity of the various chaperone transcripts was observed (Fig. 5*C*). As expected from previous metabolic labelling experiments (Mizzen *et al.*, 1989; Hartman *et al.*, 1992), mt-Hsp70 mRNA levels increased in response to Azc treatment but not in response to heat shock whilst Hsp60 and Hsp10 mRNA levels increased in response to both treatments. These responses are in accord with nuclear run off experiments and Northern analysis we have performed in a parallel study (Martinus *et al.*, 1996) and indicates that the RT-PCR procedure, when performed carefully, will give a reliable indication of comparative transcript levels. The RT-PCR results obtained with the mt-GrpE#1 specific primer set mirrored that obtained for mt-Hsp70 and thus indicated that the levels of mt-GrpE#1 do not increase in response to heat shock but marginally in response to amino acid analogue treatment. Consistent with this observation, the yeast MGE1 (YGE1) gene is not induced by heat shock (Ikeda *et al.*, 1994) and it appears that neither the *S. cerevisiae* nor the *C. elegans* grpE gene contains heat shock elements (Ikeda *et al.*, 1994; Wilson *et al.*, 1994).

In conclusion, I have cloned a cDNA encoding a ubiquitously expressed mt-GrpE#1 from a higher eukaryote. I have shown that the degree of conservation within the GrpE family is much lower than that seen for other chaperone/co-chaperone members such as Hsp70, Hsp60 and Hsp10. However, it appears that this relatively low degree of sequence conservation has not been accompanied by a parallel diversification of function. The very specific interaction between mammalian mt-GrpE#1 and DnaK combined with its stress inducibility signifies that the essential features of the bacterial DnaK/GrpE system have been maintained during the evolution of mitochondria in higher eukaryotes. A more rigorous test of this assumption will be facilitated by the availability of a mt-GrpE#1 clone and corresponding antibodies.

Utilising the consensus sequence generated in this study for all known GrpE members across several biological kingdoms, I have identified and subsequently characterised a second mammalian mitochondrial GrpE-like protein (mt-GrpE#2) (Chapter 4). This amplification in mammalian mt-Hsp70 system components may ultimately be related to the greater specialisation and diversity of mitochondrial functions in mammalian cells compared with those of lower eukaryotes, such as yeast.

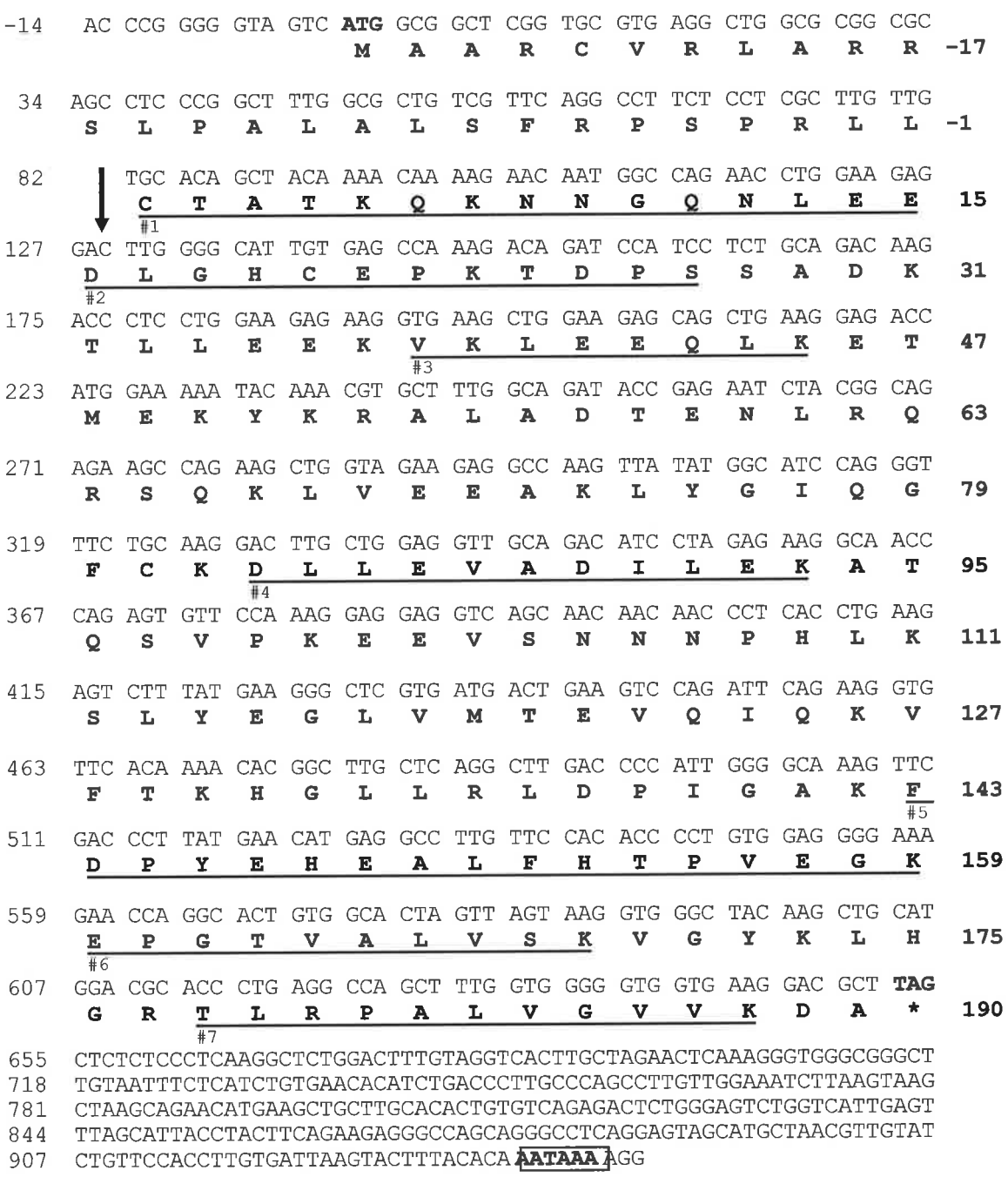
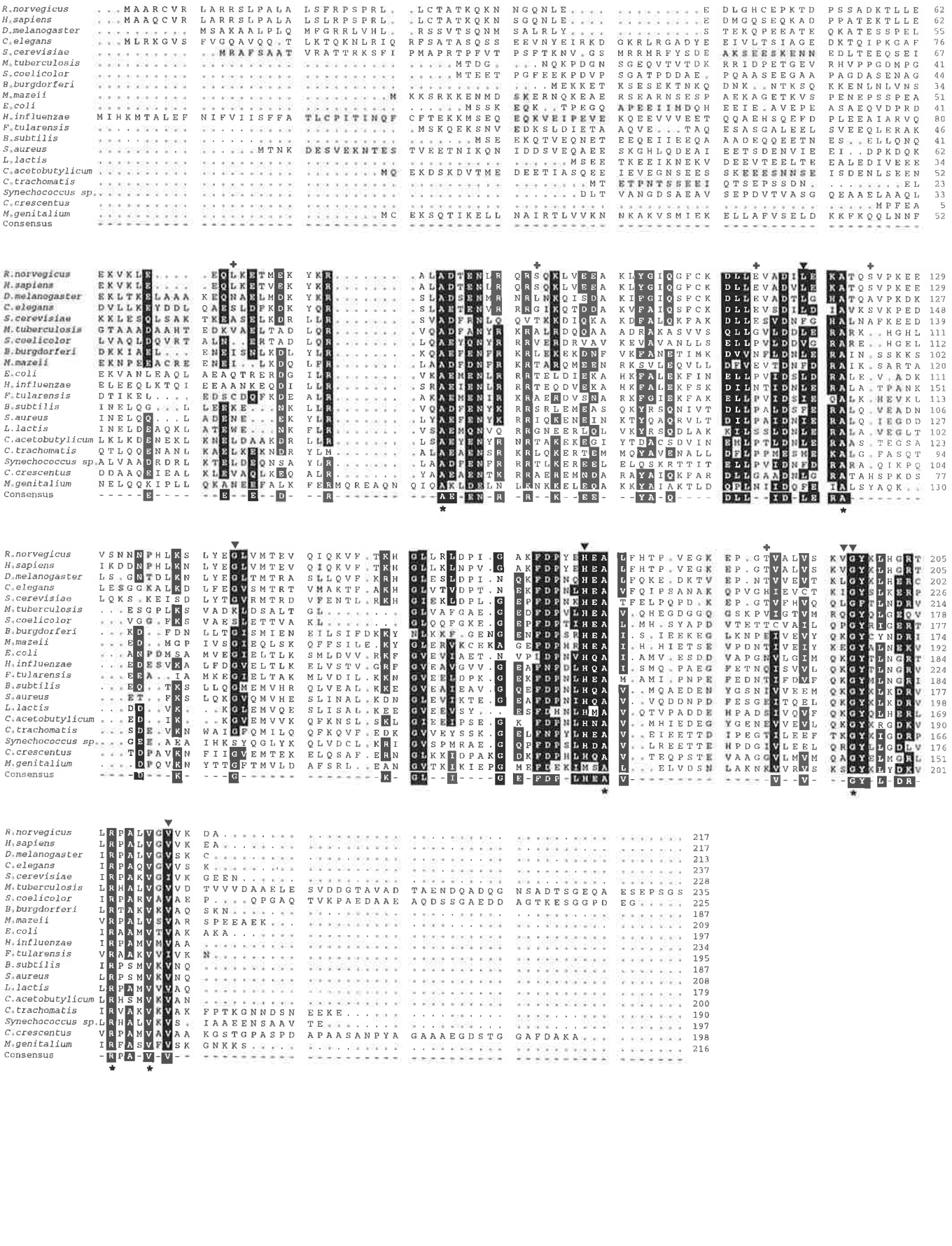


Fig. 1. **The cDNA and deduced amino acid sequence of rat mt-GrpE#1.** The numbering of nucleotides is shown on the left-hand side and the first base of the putative initiator codon is numbered +1. Amino acids are given by their single letter code and they are numbered on the right-hand side. The first residue of the mature protein is numbered +1 and the cleavable presequence is defined as amino acids -27 to -1 with the point of cleavage indicated with an arrow. Regions of the deduced amino acid sequence that are almost identical to peptide sequences obtained from purified bovine mt-GrpE#1 (Chapter 2, Fig. 5; Naylor *et al.*, 1995) are underlined and labelled #1 to 7. A putative polyadenylation site is boxed. This sequence has been submitted to GenBank and given accession number U62940.

Fig. 2. **Multiple sequence alignment of the predicted amino acid sequences of GrpE proteins (or homologues)** from: *Rattus norvegicus* (mt-GrpE#1 this study, GenBank™ accession no. U62940), *Homo sapiens* (mt-GrpE#1 compiled and edited from GenBank™ accession nos. AA252446 and N28384), *Drosophila melanogaster* (GenBank™ accession no. U34903), *Caenorhabditis elegans* (EMBL accession no. Z46996), *Saccharomyces cerevisiae* (Mge1p, GenBank™ accession no. D26059), *Mycobacterium tuberculosis* (GenBank™ accession no. X58406), *Streptomyces coelicolor* (EMBL accession no. X77458), *Borrelia burgdorferi* (GenBank™ accession no. M96847), *Methanosarcina mazeii* (GenBank™ accession no. X74353), *Escherichia coli* (GrpE, GenBank™ accession no. X07863), *Haemophilus influenzae* (GenBank™ accession no. L44715), *Francisella tularensis* (GenBank™ accession no. L43367), *Bacillus subtilis* (GenBank™ accession no. M84964), *Staphylococcus aureus* (GenBank™ accession no. D30690), *Lactococcus lactis* (GenBank™ accession no. X76642), *Clostridium acetobutylicum* (GenBank™ accession no. M74569), *Chlamydia trachomatis* (GenBank™ accession no. M62819), *Synechococcus sp.* (partial sequence, EMBL accession no. D28550), *Caulobacter crescentus* (EMBL accession no. U33324) and *Mycoplasma genitalium* (EMBL accession no. U39697). The alignment was made using the PILEUP program (Genetic Computer Group, Madison, WI) and the initiating methionine residue was numbered 1 as the processing sites are not known for most of the primary translation products. Residues that are conserved in at least fourteen of the twenty sequences are highlighted. Conserved residues are defined as A/G, Y/F, S/T, I/V/L, R/K/H and D/E/N/Q. The asterisks under the consensus sequence indicate the positions of six strictly conserved residues. The symbols (▼) and (✚) above the sequence show the positions of amino acid changes in characterised mutants from *E.coli* (Wu *et al.*, 1994) and *S.cerevisiae* (Westermann *et al.*, 1995), respectively. Residues denoting the yeast Mge1p (Azem *et al.*, 1997) and the suspected mammalian mt-GrpE#1 (Chapter 2; Naylor *et al.*, 1995) N-terminal mitochondrial targeting sequences are underlined. Since the N-terminal mitochondrial targeting sequences of most mt-GrpE homologues is yet to be determined, the suspected initiating methionine of all mt-GrpE proteins has been numbered +1 to facilitate comparison.



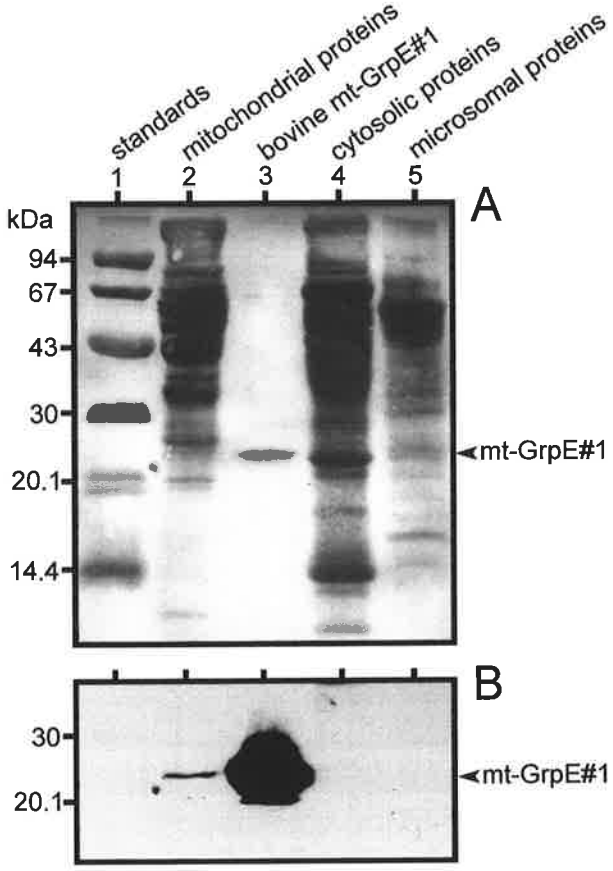


Fig. 3. **Western blot analysis confirms a mitochondrial location of mt-GrpE#1.** *(A)*: Molecular mass standards (*lane 1*), mitochondrial extract (50 µg; *lane 2*), bovine mt-GrpE#1 (1.6 µg; *lane 3*), cytosolic extract (70 µg; *lane 4*) and microsomal extract (55 µg; *lane 5*) were electrophoresed in a 16.5 % Tris-Tricine gel, transferred to nitrocellulose and stained with Ponceau S. *(B)*: The filter was probed with rabbit anti-mt-GrpE#1 antiserum followed by detection of bound antibodies with a secondary HRP-conjugated antibody.

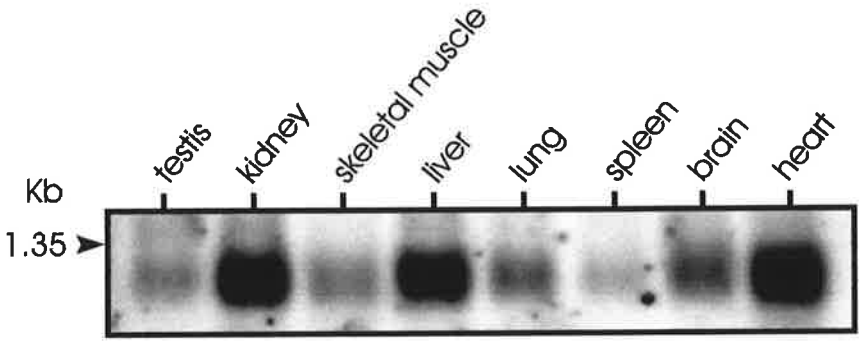
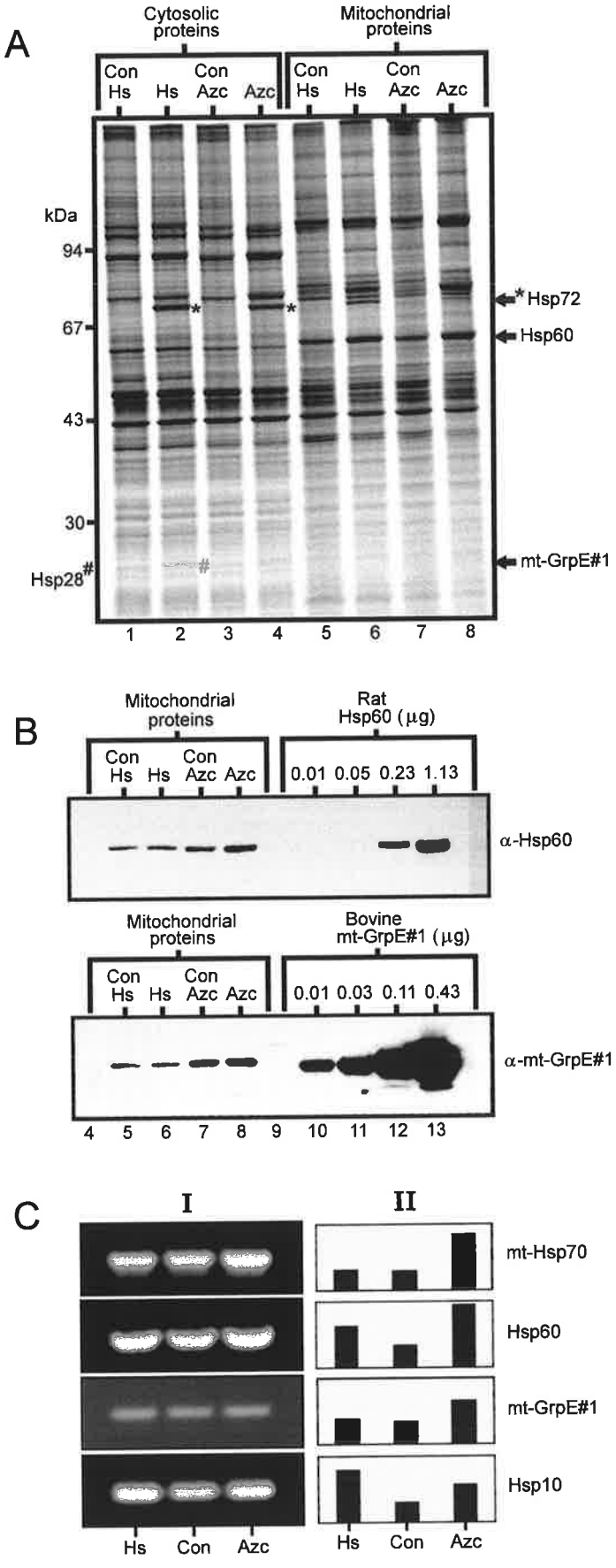


Fig. 4. **Northern blot analysis indicates a ubiquitous but varying level of mt-GrpE#1 mRNA in several rat organs.** Each lane contained approximately 2 μg of mRNA isolated from the indicated tissues and loadings were adjusted to contain equal amounts of β-actin mRNA.

Fig. 5. **mt-GrpE#1 is a low abundance protein and its synthesis appears to be only slightly stress inducible.** (***A***) Cytosolic (*lanes 1* to *4*) and mitochondrial (*lanes 5* to *6*) proteins metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine were recovered either from untreated cells (Con; *lanes 1,3,5* and *7*), cells subjected to heat shock (Hs; *lanes 2* and *6*) or cells grown in the presence of Azc (*lanes 4* and *8*). Equal amounts of trichloroacetic acid-insoluble radioactive material (2 x 10^5 cpm) were loaded in each sample well, separated in a 16.5 % Tris-Tricine gel and analysed using a Storm PhosphorImager and ImageQuaNT software (Molecular Dynamics). The indicated positions of Hsp70, Hsp60 and mt-GrpE#1 were determined by electrophoresis of purified stress proteins in adjacent lanes followed staining with Coomassie Brilliant Blue. A protein believed to represent Hsp28 is also indicated. (***B***) A 16.5 % Tris-Tricine gel was loaded in the order described above with equal amounts of trichloroacetic acid-insoluble radioactive material (4 x 10^5 cpm). Standard amounts of purified rat Hsp60 and bovine mt-GrpE#1 were loaded in adjacent lanes 10-13 (as indicated). Following blotting to nitrocellulose, the filter was sequentially probed with antiserum against mt-GrpE#1 and against Hsp60. The amount of mitochondrial supernatant proteins loaded were: 7.4μg for control heat shock (Con Hs), 5.6μg for heat shock (Hs), 15.6μg for control Azc and 15.1μg for Azc treatment. (***C***) **Semi-quantitative RT-PCR analysis confirms a slight stress-inducibility of mt-GrpE#1 by Azc but not by heat shock.** (***I***) RT-PCR was performed on several chaperone mRNA transcripts isolated from untreated (Con), heat shocked (Hs) or Azc treated clonal rat hepatoma cells (as indicated). PCR products were separated on a 1 % agarose gel supplemented with 0.02 % (w/v) Ethidium bromide. (***II***) The gel was scanned then quantitated with an ImageQuaNT program (Molecular Dynamics). The histograms indicate the relative amounts of pixels counted, for each transcript, in terms of the three cellular treatments indicated. The complementary primer sets were tested thrice and each time similar relative intensities of the various chaperone transcripts were observed.


A
Cytosolic proteins
Mitochondrial proteins
Con Hs
Hs
Con Azc
Azc
kDa
94
67
43
30
Hsp28#
*Hsp72
Hsp60
mt-GrpE#1
1 2 3 4 5 6 7 8
B
Mitochondrial proteins
Rat Hsp60 (μg)
0.01 0.05 0.23 1.13
α-Hsp60
Mitochondrial proteins
Bovine mt-GrpE#1 (μg)
0.01 0.03 0.11 0.43
α-mt-GrpE#1
4 5 6 7 8 9 10 11 12 13
C
I
II
mt-Hsp70
Hsp60
mt-GrpE#1
Hsp10
Hs Con Azc
Hs Con Azc

Chapter 4

Definition of two functional mitochondrial GrpE-like proteins in mammalian cells

4.1 INTRODUCTION

Homologues of DnaK and DnaJ have been identified in several major compartments of the eukaryotic cell, including the cytosol, nucleus, endoplasmic reticulum (ER), mitochondria and chloroplasts and in many cases these chaperones exist in multiple isoforms (Chapter 1; Gething, 1997). In comparison, eukaryotic homologues of GrpE have only been detected in mitochondria (Chapters 2 and 3; Ikeda *et al.*, 1994; Bollinger *et al.*, 1994; Laloraya *et al.*, 1994; Naylor *et al.*, 1995, 1996, 1998; Mehta *et al.*, 1997) and possibly chloroplasts (Schlicher and Soll, 1997), but in no case have isoforms been detected. While a chloroplast GrpE-like protein has been inferred through cross-reactivity with antibodies to *E. coli* GrpE (Schlicher and Soll, 1997), mitochondrial GrpE homologues from *Saccharomyces cerevisiae* (Mge1p, Yge1p, GrpEp, mGrpE), *Drosophila melanogaster* (Droe1p) and mammals (mt-GrpE#1) were identified by their abilities to bind specifically to Hsp70 members and the complexes formed are not disrupted in the presence of 1 M salt but readily dissociate in the presence of 5 mM ATP (Chapter 2; Bollinger *et al.*, 1994; Naylor *et al.*, 1995; Mehta *et al.*, 1997). Such a specific interaction was first observed between the *E. coli* members of GrpE and DnaK (Zylicz *et al.*, 1987).

It is somewhat surprising then that the role of a GrpE-like protein may not be required for the proper functioning of Hsp70 homologues outside mitochondria and chloroplasts whilst DnaJ homologues exist in all additional compartments that contain Hsp70 members (Chapter 1; Gething, 1997). It may, however, be possible that the three-component chaperone team of Hsp70, DnaJ and GrpE is conserved in numerous compartments of eukaryotic cells but sequence similarities have been too low to allow facile identification through comprehensive searches of data bases (Chapter 3; Naylor *et al.*, 1996). Thus, as opposed to members of the Hsp70 (DnaK), Hsp60 (GroEL) and Hsp10 (GroES) families from rat mitochondria and *E. coli* which exhibit 51%, 49% and 45% positional identity at the amino acid level respectively, the GrpE family only exhibits 21% positional identity and comparison of twenty GrpE sequences identified only six invariant residues (Chapter 3; Naylor *et al.*, 1996). Another likely reason for the paucity of identified GrpE-like proteins is their very low abundance in most systems, for example, Mge1p and mt-GrpE#1 comprise only ~0.03 % of the total soluble mitochondrial proteins (Chapter 3; Naylor *et al.*, 1996; Nakai *et al.*, 1994). Taken together, the low degree of sequence similarities and the low abundance of at least some GrpE members calls for both a sequence based and a functional approach to search for new GrpE-like chaperones. Indeed, recently a cytosolic/nuclear located Hsp70/Hsc70-associating protein

BAG-1 (alternatively named RAP46 or Hap) with GrpE-like activity[1], but a seemingly unrelated primary structure, has been reported (Zeiner *et al.*, 1997; Takayama *et al.*, 1997; Höhfeld and Jentsch, 1997). Alternative translation initiation from a single BAG-1 transcript in both human and mouse cells is believed to generate either a cytosolic ~36 kDa BAG-1 protein or a ~50 kDa BAG-1 protein with an N-terminal extension thought to be important in nuclear targeting (Packham *et al.*, 1997). The finding of a cytosolic/nuclear located protein with GrpE-like activity lends further support to the supposition that Hsp70 members may operate as components of DnaK/DnaJ/GrpE-like chaperone teams irrespective of the organism or cellular location.

In this study I have now further explored the complexity of the mammalian GrpE complement. I provide evidence that mammalian cells, in addition to their previously identified mt-GrpE#1 and cytosolic/nuclear located BAG-1 members, contain a second distinct mitochondrial GrpE member (mt-GrpE#2) which functions as a co-chaperone for mt-Hsp70. I also report either the rediscovery of cytosolic BAG-1 or the possible existence of a distinct cytosolic ~40 kDa GrpE-like protein on the basis of cross-reactivity with antibodies to mt-GrpE#1.

[1] Very recent studies reveal that while a central region (residues 62-138) of BAG-1 may share three-dimensional structural homology with *E. coli* GrpE (Stuart *et al.*, 1998) and whilst BAG-1 can stimulate the ATPase activity of Hsp70 several fold, it does not function as a nucleotide exchange factor as originally proposed (Bimston *et al.*, 1998).

4.2 MATERIALS AND METHODS

4.2.1 Immunological techniques—For Western blotting, cellular protein fractions were resolved by SDS-PAGE in a 16% (w/v) Tris-Tricine gel (section 2.2.5) and transferred to nitrocellulose using a semi-dry transfer unit (section 3.2.4). Blots were probed with the relevant rabbit antiserum and HRP-labelled goat anti-rabbit IgG, followed by incubation with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech) and subsequent exposure to Hyperfilm™-MP (Amersham Pharmacia Biotech) (section 3.2.4.). Mitochondria (bovine liver) were prepared according to Hartman *et al.* (1992) (section 2.2.2), microsomes (porcine liver) were pelleted by centrifugation of the post-mitochondrial supernatant at 100,000 x g for 1 h and the final supernatant was defined as the cytosolic (rat liver) fraction (section 3.2.4). The purification of bovine mt-GrpE#1 on a DnaK-column and the preparation of an antiserum against bovine mt-GrpE#1 was described in chapters 2 and 3, respectively (Naylor *et al.*, 1995, 1996). An antiserum against recombinant mouse mt-GrpE#2 (section 4.2.4) was prepared from the SDS-PAGE purified protein as described in section 3.2.3 (Naylor *et al.*, 1996). The serum was supplemented with 0.02% (v/v) sodium azide and stored at -70 °C. Dilutions of 1 in 10,000 were used in Western blotting analysis.

4.2.2 Nucleotide sequence analysis—The verified nucleotide sequence encoding mouse mt-GrpE#2 was obtained by sequence analysis of two independently isolated ESTs from the I.M.A.G.E. Consortium Lawrence Livermore National Laboratory cDNA clones collection (I.M.A.G.E. Consortium CloneIDs 482996 and 478162 corresponding to GenBank™ accession nos. AA060861 and AA049605 respectively, see Results and Discussion section 4.3) (Lennon *et al.*, 1996). Both strands of the two clones were sequenced using the dideoxynucleotide chain termination procedure of Sanger *et al.* (1977) (section 3.2.2).

4.2.3 Southern blot analysis—Genomic DNA was prepared from 0.5 g of rat liver according to Sambrook *et al.* (1989). Approximately 10 µg of genomic DNA was digested with the appropriate restriction enzyme (40 U at 37°C for 8 h) and electrophoresed in a Tris-Acetate-EDTA (TAE) buffered 0.8% (w/v) agarose gel. The digested DNA was transferred onto a Hybond™ -N+ nylon membrane (Amersham Pharmacia Biotech) and the blot was prehybridised for 6 h at 65°C in 5x SSC, 0.5% (w/v) SDS, 100µg/ml sheared salmon sperm DNA and 5x Denhardt's solution (0.1%(w/v) Ficoll™ 400, 0.1% (w/v) polyvinylpyrrolidone

and 0.1% (w/v) BSA). The membrane was probed with a ^{32}P-labelled mouse mt-GrpE#2 cDNA fragment (nucleotides 65 to 690, Fig. 1), then stripped by incubation in 0.5 % (w/v) SDS at ~90 °C for 10 min and reprobed with a ^{32}P-labelled rat mt-GrpE#1 cDNA fragment (GenBank™ accession no. U62940, nucleotides 96 to 733). The denatured probes were added to the prehybridisation solution and hybridisations were carried out for 12 h at 65°C. Final washes were performed for 10 min at 65°C in 0.1× SSC supplemented with 0.1% (w/v) SDS and the blots were analysed using a Storm PhosphorImager and ImageQuaNT software (Molecular Dynamics).

4.2.4 Expression of mouse mt-GrpE#2, rat mt-GrpE#1 and rat mt-Hsp70 in E. coli—The cDNA coding regions specifying the mature translation products of mouse mt-GrpE#2 (579 bp), rat mt-GrpE#1 (570 bp) and rat mt-Hsp70 (1899 bp, GenBank™ accession no. S75280) were amplified with Vent$_R$®DNA polymerase (New England BioLabs) utilising specific primer pairs in a 30 cycle PCR protocol (94 °C for 60 sec, 55 °C for 90 sec and 72 °C for 90 sec). The primer pairs used were: for mouse mt-GrpE#2 (5'-GTTAACATATGAGCA-CTGCCACCCAAAGAACT-3' + 5'-AGCCGGATCCTTAGAGTCTTCTCTGAGACTC-TAC-3'), for rat mt-GrpE#1 (5'-CCATTGGGATCCTAAGGAGGTTAACATATGTGCAC-AGCTACAAAACAAAAG-3' + 5'-CGGAATTCAAGCTTGGATCCTTAAGCGTCCTTC-ACCACCCCCAC-3') and for rat mt-Hsp70 (5'-ATGGATCCATATGGCGTCAGAAGCA-ATCAAGGGTGC-3' + 5'-TTGGATCCTTAATGATGATGATGATGATGAGAACCCCG-CGGAACTAA-3'). PCR products were digested with *Nde*I and *Bam*HI then ligated individually into the same predigested sites of the pET-14b vector (for mt-GrpE#1 + #2) or pET-3a vector (for mt-Hsp70) as specified in the manufacturer's manual (4th Ed., Novagen). Note, the mt-Hsp70 construct was designed to remove a pre-existing N-terminal T7-Tag™ and introduce a C-terminal hexahistidine tag preceded by a thrombin cleavage site. Constructs under the selection of ampicillin were then separately transformed into BL21(DE3) cells co-harbouring the pLysS plasmid under the selection of chloramphenicol (Novagen). The authenticity of each construct was verified by sequence analysis of ~300 bp from either end of each cDNA insert within the pET-14b or pET-3a vectors.

For expression of the recombinant mt-GrpE#1 and #2 proteins bearing an N-terminal hexahistidine tag (removable with thrombin), typically 1.5 L of Luria broth containing 100μg/ml ampicillin and 40μg/ml chloramphenicol was inoculated with the transformants and shaken at 30 °C until the A_{600nm} was equal to 0.5. Synthesis of the recombinant protein was

initiated by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM and incubation continued at 30 °C for 3 h. Cells were then harvested and lysed by the lysozyme method according to Sambrook *et al.* (1989). After centrifugation (16,000 x *g* for 10 min) the supernatant was applied at 4 °C to a 2 ml Talon™ metal affinity column (CLONTECH) equilibrated in 25 mM Tris-Cl (pH 8.0), 200 mM NaCl and 10 mM β-mecaptoethanol. Following extensive washing with equilibration buffer (EB), the column was washed successively with 0.3 M NaCl in EB (ie 0.5 M NaCl final concentration), 10 mM imidazole in EB, 5 mM ATP, 10 mM $MgCl_2$ and 50 mM KCl in EB, 2.5 mM $CaCl_2$ in EB and finally an essentially pure preparation of recombinant protein (~40 mg for mt-GrpE#1 and ~14 mg for mt-GrpE#2) was recovered by recirculating through the column a 20 ml solution of EB fortified with 2.5 mM $CaCl_2$ and 10 U human thrombin (Boehringer Mannheim) for 5 h. Uncleaved recombinant protein retained on the IMAC columns was eluted with 100 mM imidazole in EB (~20 mg for mt-GrpE#1 and ~7 mg for mt-GrpE#2) and subjected to gel filtration on a Superdex-200 (26/60, Pharmacia) column equilibrated in 0.1 M NaPi (pH 7.3) and 100 mM NaCl at 4 °C. The masses of hexahistidine tagged recombinant mt-GrpE#1 + #2 were determined by Yogi Hayasaka (Australian Wine Research Institute, Glen Osmond, SA., Australia) on an electrospray ionisation-triple quadrupole mass spectrometer (PE SCIEX API300) essentially as described by Hartman *et al.* (1992). A proteolytic fragment of recombinant mouse mt-GrpE#2 was subjected to SDS-PAGE in a 12.5% Tris-Glycine gel (34), recovered with a Gel-Spin Protein Recovery Kit (ScimaR) then taken for direct N-terminal protein sequencing as described previously (Hartman *et al.*, 1992).

Expression and subsequent purification of the recombinant rat mt-Hsp70 protein was carried out as described for mt-GrpE#1 and #2 except expression was performed for 16 h at 16 °C (instead of 30 °C). Typically ~10 mg of soluble mt-Hsp70 was purified from 1L of culture. In comparison to the native rat liver mt-Hsp70 protein, purified by ATP-agarose chromatography (Webster *et al.*, 1994), recombinant mt-Hsp70 protein readily precipitated upon freeze-thawing.

The *E. coli* strain BB4041 which synthesises N-terminal hexahistidine tagged *E. coli* GrpE was obtained from Bernd Bukau and the expression and subsequent purification of the recombinant protein was performed as described previously (Gamer *et al.*, 1992).

4.2.5 Native PAGE—Nondenaturing PAGE was performed in 6% Tris-Glycine gels by omitting SDS from the PAGE system (see section 2.2.5). The protein samples were prepared in 25 mM Tris-Cl (pH 8.0), 50 mM NaCl, 50 mM KCl,10 mM $MgCl_2$, 10 mM β-mecaptoethanol and 50 U/ml apyrase and incubated at 4 °C for 6 h prior to electrophoresis at 30 mA for 12 h at 4 °C. Rat liver mt-Hsp70, mouse RIF-1 cell Hsc70 and pig liver BiP were purified by ATP-agarose chromatography (C-8 attachment with a 9 atom spacer, Sigma Cat.# A2767), from mitochondrial, cytosolic and microsomal soluble protein extracts (see section 3.2.4), essentially as described by Webster *et al.* (1994).

4.2.6 ATPase assay—The ATPase activity of recombinant rat mt-Hsp70 was measured by the conversion of [γ-^{32}P]ATP (1 μCi; 4000 Ci/mmol, Bresatec) to γ-^{32}Pi essentially as described previously (Liberek *et al.*, 1991a). Assays were carried out for 30 min at 30 °C in 50 μl reaction mixtures containing 50 mM Tris-Cl (pH 8), 50 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 40 μM ATP and the appropriate proteins. Reactions were terminated by spotting 2 μl samples onto Polygram CEL 300 polyethyleneimine cellulose plates (Macherey-Nagel) where [γ-^{32}P]ATP was separated from γ-^{32}Pi by one-dimensional chromatography using a solution of 1 M formic acid and 1 M LiCl. Hydrolysed ATP was then quantified on a Storm PhosphorImager with an ImageQuaNT program (Molecular Dynamics). The *E. coli* DnaJ protein was purchased from StressGen Biotechnologies Corp. while the recombinant rat mt-Hsp70, rat mt-GrpE#1 and mouse mt-GrpE#2 proteins were prepared without their hexahistine tags as described in section 4.2.4.

4.2.7 RNA dot blot analysis—For identification of the tissue distribution of the mt-GrpE transcripts, an RNA dot blot with mRNA from 22 different mouse tissues (100-500 ng each) was purchased from CLONTECH (Cat.# 7771-1) and prehybridised, hybridised and analysed as described for the Southern blot analysis above (section 4.2.3).

4.3 RESULTS AND DISCUSSION

4.3.1 Evidence for the existence of a distinct cytosolic GrpE-like protein—It has previously been established that *E. coli* GrpE binds strongly to immobilised DnaK in the absence of ATP (Zylicz *et al.*, 1987). Utilising this specific interaction I previously reported the DnaK-affinity purification of a single mitochondrial GrpE homologue (mt-GrpE#1) from rat, porcine and bovine liver mitochondrial extracts (Chapter 2; Naylor *et al.*, 1995). In the present work I employed an antiserum to mt-GrpE#1 to investigate whether additional GrpE-like members may exist in extra-mitochondrial locations of mammalian cells. Fig. 1*A* shows the Western blotting pattern observed when the mt-GrpE#1 antiserum was used to probe the protein constituents from several cellular compartments and, as a positive control, the DnaK-column purified mt-GrpE#1 protein. As suspected, the antiserum detected predominantly the DnaK-column purified mt-GrpE#1 protein (Fig. 1*A*, *lane 2*) which co-migrated with a single species in the mitochondrial fraction (Fig. 1*A*, *lane 1*). The detection of a small amount of a slightly lower molecular mass species was previously suspected to be due to a proteolytic breakdown of the mt-GrpE#1 protein (Fig. 1*A*, *lane 2*) (Chapter 2; Naylor *et al.*, 1995).

The mt-GrpE#1 antiserum also appeared to detect a minor amount of a ~50 kDa protein in the DnaK-column purified mt-GrpE#1 material (Fig. 1*A*, *lane 2*) which co-migrated with a single species in the microsomal fraction (Fig. 1*A*, *lane 4*). I initially thought that this protein could constitute a putative microsomal GrpE homologue (Naylor *et al.*, 1998) and subsequently invested a large effort into purifying the cross-reactive ~50 kDa protein. N-terminal protein sequencing, however, revealed that it constitutes an immunoglobulin (IgG) heavy chain member (data not shown). Subsequent Western blot analysis on the microsomal fraction with only the secondary goat anti-rabbit IgG conjugated to HRP also detected the ~50 kDa protein (data not shown) and thereby confirmed the identity of the cross-reactive ~50 kDa protein as an IgG. The cross-reactivity seen during Western blot analysis with the mt-GrpE#1 antiserum (Fig. 1*A* *lanes 2* and *4*) is therefore not likely to be due to structural similarity of the ~50 kDa protein with GrpE members. In hindsight, it is clear that IgG binding to and ATP induced release from DnaK (a BiP homologue) is likely to have occurred.

Surprisingly, in addition to mt-GrpE#1 and the IgG heavy chain, a single ~40 kDa protein was recognised by the mt-GrpE#1 antiserum in the cytosolic fraction but was not recognised by the secondary goat anti-rabbit IgG conjugated to HRP (Fig. 1*A*, compare *lanes 2* and *3*). I suspect that this protein corresponds to a recently characterised cytosolic ~36 kDa protein from humans and mice with GrpE-like activity (BAG-1, RAP46, Hap (Zeiner *et al.*,

1997; Takayama *et al.*, 1997; Höhfeld and Jentsch, 1997; Packham *et al.*, 1997). Despite the former report that the primary sequence of BAG-1 does not display regions of significant similarity to previously identified members of the GrpE family (Höhfeld and Jentsch, 1997), the cross-reactivity displayed here does indicate a structural similarity to mt-GrpE#1 and accordingly a small segment within the BAG-1 protein that is conserved in several members of the GrpE family can readily be identified (Fig. 1*B*). The presence of such a conserved segment supports the likelihood that BAG-1 cross-reacts with antibodies raised against mt-GrpE#1, however, I can not dismiss the possibility that the ~40 kDa cytosolic protein represents an as yet uncharacterised polypeptide with GrpE-like structural and biochemical characteristics.

4.3.2 Identification of a second mammalian mt-GrpE cDNA—Based on a consensus sequence for the GrpE family (Chapter 3; Naylor *et al.*, 1996) a search of databases revealed a previously uncharacterised mouse expressed sequence tag (EST), from both 13.5 to 14.5 (GenBank™ accession no. AA049605) and 19.5 (GenBank™ accession no. AA060861) days post conception embryo cDNA libraries. These ESTs exhibit significant homology to our earlier reported rat mt-GrpE#1 cDNA sequence (Chapter 3; Naylor *et al.*, 1996) but represent a distinct entity. Complete sequencing of the two EST clones revealed a 1445 bp cDNA (Fig. 2), where GenBank™ accession no. AA049605 (Fig. 2, nucleotides 4 to 1448) and GenBank™ accession no. AA060861 (Fig 2, nucleotides 27 to 1448) were identical in a 1422 bp overlap. By comparison of the predicted amino acid sequence (Fig. 2) with several GrpE homologues (Fig. 3 and Table I), it is concluded that the cDNA encodes a second mouse mitochondrial GrpE protein (named mt-GrpE#2) and in all likelihood only the initiation ATG codon is missing from the coding region of the cDNA clone. In support of this prediction, two additional mouse ESTs have recently been deposited in GenBank™ (accession nos. AI116966 and AI116955). These are identical in a 267 nucleotide overlap with the cDNA clone described above and each EST contains additional 5' sequence (Fig. 2, nucleotides -19 to 3) including an ATG codon (Fig. 2, nucleotides 1 to 3). I am therefore confident that the composite cDNA represented in Fig. 2 corresponds to a single GrpE transcript.

Southern analysis on restriction endonuclease digested rat genomic DNA (Fig. 4) showed that mt-GrpE#1 and #2 are encoded by distinct genes which probably exist as single copies within the rat genome. Comparison of the N-terminal amino acid sequence of purified bovine mt-GrpE#1 (Chapter 2; Naylor *et al.*, 1995) with the deduced amino acid sequences of mouse mt-GrpE#2 and several mt-GrpE#1 members predicts the presence of a conserved

cleavable mitochondrial targeting sequence, defined as residues 1 to 31 (where residue 1 is the predicted initiation methionine) for mouse mt-GrpE#2 and residues 1 to 27 for rat, mouse and human mt-GrpE#1 (Fig. 3). Characteristic of mitochondrial targeting sequences, residues 8-19 of mouse mt-GrpE#2 and 4-21 of rat, mouse and human mt-GrpE#1, have a strong potential to form amphiphilic alpha-helices with a high content of serine and basic residues but seldom acidic residues (von Heijne, 1986). Upon mitochondrial import, these putative N-terminal targeting sequences are expected to be proteolytically removed from the 224 residue mouse mt-GrpE#2 (~25.0 kDa) and 217 residue rat mt-GrpE#1 (~24.3 kDa) precursors to generate the mature proteins of 193 (~21.5 kDa) and 190 (~21.3 kDa) amino acids, respectively. The predicted isoelectric points (p*I*s) of the mature mouse mt-GrpE#2 protein is 6.8 and that of the mature rat mt-GrpE#1 protein is 6.5 whilst the precursors, due to their basic mitochondrial targeting sequences, have predicted p*I*s of 7.7 for 8.5, respectively.

The C-terminal tripeptide RRL of mouse mt-GrpE#2 (Fig. 2) is reminiscent of a peroxisomal targeting sequence type1 motif (PTS1) , ie (S,A,C)-(K,R,H)-(L,M) and while an analogous C-terminal tripeptide (KKL) is necessary for targeting human alanine/glyoxylate aminotransferase-1 (AGT-1) to peroxisomes, it is unable to direct the import of the bona fide peroxisomal protein firefly luciferase (Motley *et al.*, 1995). Furthermore, marmoset AGT-1 (bearing the same C-terminal tripeptide KKL) can be synthesised with and without a cleavable (N-terminal) mitochondrial targeting sequence, the presence of a mitochondrial targeting sequence being dominant over the PTS1. This allows the efficient targeting of the protein to both organelles (Purdue *et al.*, 1992). It would be interesting to see if the mature mouse mt-GrpE#2 protein, without it's mitochondrial targeting sequence, can be efficiently targeted to peroxisomes via a putative C-terminal PTS1. Recently a watermelon cotyledon Hsp70 has been shown to be targeted to both the matrix of plastids and glyoxysomes by a similar mechanism to that described for AGT-1 (Wimmer *et al.*, 1997), thus increasing the likelihood that such dual targeting for mt-GrpE#2 could exist.

4.3.3 mt-GrpE#2 is present in mitochondria—The specific detection of mt-GrpE#1 in mitochondria (Fig. 1*A*, *lane 1*) correlates well with the prediction that newly synthesised mt-GrpE#1 contains an N-terminal mitochondrial targeting sequence (Fig. 2 and 3). In order to evaluate whether mt-GrpE#2 could also be detected in mitochondria, I prepared an antiserum to recombinant mt-GrpE#2 synthesised in *E. coli* (Fig. 5*A*, *lanes 2-4*). Figure 5*B* reveals that the antiserum to recombinant mt-GrpE#2 doesn't cross-react with *E. coli* GrpE or rat mt-GrpE#1, thereby making it an effective tool to establish whether mt-GrpE#2 is located within

mitochondria. Utilising this specific antiserum to probe a rat liver mitochondrial extract, I observed that, like mt-Grp#1, mt-GrpE#2 also resides within mitochondria (Fig. 6). I suspect that mt-GrpE#2 is less abundant than mt-GrpE#1 (Chapter 3, ~0.03% of the total soluble mitochondrial proteins) for I could not detect mt-GrpE#2 within the DnaK-column purified bovine mt-GrpE#1 preparation by N-terminal sequence analysis (Chapter 3, Fig. 5). Surprisingly, Western Blot analysis revealed that the mature mt-GrpE#2 protein (Fig. 6*B*) migrated significantly further than the mature mt-GrpE#1 (Fig. 6*C*) protein during SDS-PAGE, implying that the presequence of mt-GrpE#2 may be longer than expected and is probably cleaved at a site further C-terminal within the protein sequence than that predicted in Fig. 3.

4.3.4 Properties of mt-GrpE#1 and #2 and interaction with DnaK—In order to evaluate whether mouse mt-GrpE#2 might function as a co-chaperone for an Hsp70 member, I firstly investigated whether mt-GrpE#2 could form a stable complex with DnaK in the absence of ATP, a characteristic of several other GrpE family members (Bolliger *et al.*, 1994; Naylor *et al.*, 1995; Mehta *et al.*, 1997; Zylicz *et al.*, 1987; Nakai *et al.*, 1994; Voos *et al.*, 1994). The mature portions of both the rat mt-GrpE#1 and mouse mt-GrpE#2 proteins (predicted in Fig. 3) with N-terminal hexahistidine tags were thus synthesised in *E. coli* and then purified by immobilised metal affinity chromatography (IMAC) (Fig. 7*A*, *lanes 3* and *5* respectively). As seen in Figure 7, both mt-GrpE#1 and mt-GrpE#2 form a specific complex with *E. coli* DnaK. This complex is stable in the presence of 0.5 M NaCl but readily dissociated in the presence of 5 mM ATP (Fig. 7*A*, *lanes 2* and *4*, respectively). Both N-terminal protein sequencing (of the first 10 residues) and mass spectrometric analysis unequivocally revealed the identity of the interacting protein recovered in the 5 mM ATP eluate as *E. coli* DnaK. Specifically, the predicted mass of mature *E. coli* DnaK is 68,983.20 Da, whilst those obtained by mass spectrometry were 68,976 +/- 5 Da (Fig. 7*A*, *lane 2*) and 68,983 +/- 4 Da (Fig. 7*A*, *lane 4*). To dismiss the possibility that DnaK might bind directly to the IMAC resin rather than mt-GrpEs, IMAC purification was also performed on lysates prepared from *E. coli* not subjected to prior induction of mt-GrpE#1 and #2 synthesis. In this case, DnaK was not detected in the 5 mM ATP eluates (data not shown).

The unequivocal demonstration of *E. coli* DnaK interaction with the recombinant mt-GrpEs suggested a functional integrity. To further characterise the properties of the two mammalian mt-GrpEs, they were subjected to gel filtration and their elution times compared to those of known standards. It has previously been determined that alone, both *E. coli* GrpE

and *S. cerevisiae* Mge1p predominantly exist as dimers and to a lesser extent as higher oligomers (Osipiuk *et al.*, 1993; Schönfeld *et al.*, 1995b; Wu *et al.*, 1996; Deloche and Georgopoulos, 1996; Azem *et al.*, 1997). Likewise, both mt-GrpE#1 and #2 were observed to exist mostly in a dimeric form (with a native molecular mass of ~57 kDa) but higher oligomeric forms were also evident (Fig. 7*B*). In further support of a dimeric (or higher oligomeric) state of mt-GrpE#2, I have observed that a proteolytic fragment of recombinant mt-GrpE#2 (lacking the hexahistidine tag and beginning with the N-terminal sequence PDGLGPSLAE...) copurifies with the intact protein (containing the hexahistidine tag) through IMAC purification and subsequent gel filtration (Fig. 7*A*, *lane 5*). The fragment is not a result of non-specific thrombin cleavage for it persists in the eluate when the thrombin cleavage step is omitted and alternatively the hexahistidine tagged protein is eluted with 100 mM imidazole (data not shown).

4.3.5 mt-GrpE#1 and #2 are functional co-chaperones for mt-Hsp70—The evidence presented thus far would strongly suggest that both mt-GrpE#1 and #2 are authentic GrpE homologues which function as co-chaperones for mammalian Hsp70 members. I therefore investigated whether mt-GrpE#1 and #2 could interact in stable fashion with any of the well known mammalian Hsp70 members (ie Hsc70, BiP and mt-Hsp70, Fig. 8*A*) in the absence of ATP. Figure 8*B* shows that both mt-GrpE#1 and #2 interact specifically with mt-Hsp70 during native PAGE analysis. This interaction is not observed with cytosolic/nuclear Hsc70 nor ER BiP (Fig. 8*B*). Such specificity in the interaction between an Hsp70 member and its GrpE partner is not uncommon as *E. coli* GrpE neither interacts with nor is capable of stimulating the ATPase activity of the cytosolic *S. cerevisiae* Hsp70 member (Ssa1p) (Levy *et al.*, 1995). Of further interest here is the observation that mt-Hsp70 oligomers are apparently dissociated by mt-GrpE#1 and #2 to permit the formation of a stable heterologous complex (Fig. 8*B*). An apparent DnaK oligomer dissociating activity has been ascribed to *E. coli* GrpE previously (Schönfeld *et al.*, 1995b). In that case it is suggested dimeric GrpE serves to stabilise monomeric DnaK in a 2:1 complex rather than actively disrupting DnaK oligomers. In the ER, it has been suggested that BiP interconversion between the oligomeric (inactive) and monomeric (active) state allows the cell to maintain a constant level of functionally active BiP available (Freiden *et al.*, 1992). Thus GrpE molecules, in addition to their role as nucleotide exchanger factors for the stimulation of Hsp70 ATPase activity, may also serve to maintain adequate cellular levels of their functionally active Hsp70 counterpart by modulating

the interconversion of oligomeric (inactive) and monomeric (active) forms of these chaperones.

I further tested the functional integrities of mt-GrpE#1 and #2 by investigating whether they, in the presence of a DnaJ member, could stimulate the intrinsic ATPase activity of mt-Hsp70 synergistically. Previous studies have shown that together *E. coli* GrpE and DnaJ can stimulate the low intrinsic ATPase activity of DnaK at least 50-fold, while alone GrpE and DnaJ stimulate the activity 2 and 13-fold, respectively (Liberek *et al.*, 1991a; McCarty *et al.*, 1995). As a mammalian mitochondrial homologue of DnaJ has not been identified, I employed *E. coli* DnaJ in the following ATPase assays as it has been observed to be functionally equivalent to *S. cerevisiae* Mdj1p (mt-DnaJ) in the synergistic stimulation of the Ssc1p (mt-Hsp70) ATPase activity with Mge1p (mt-GrpE) (Horst *et al.*, 1997b). Figure 8*C* shows that *E. coli* DnaJ alone can indeed stimulate the ATPase activity of mt-Hsp70 (~6.5-fold), while together DnaJ and either mt-GrpE#1 or #2 synergistically stimulate the ATPase activity of mt-Hsp70 ~15-fold. The mt-Hsp70 ATPase activity was stimulated only weakly in the presence of either mt-GrpE#1 or #2 alone (~ 1.5-fold, Fig. 8*C*). Neither the mt-GrpE#1 or #2 preparations, nor the DnaJ preparation, exhibited any apparent ATPase activity (data not shown). It is concluded that mt-GrpE#1 and #2 both constitute active co-chaperones and function in a similar manner to *E. coli* GrpE in both the stimulation of the ATPase activity of mt-Hsp70 and the specific dissociation of the oligomeric states of mt-Hsp70.

4.3.6 Expression of mt-GrpE#2—A ubiquitous but varying level of expression for the rat mt-GrpE#1 transcript (~1.2 kbp) was previously seen in eight different tissues (Chapter3; Naylor *et al.*, 1996). Using a more expansive dot blot of murine mRNAs obtained from 22 different sources (Fig. 9*A*) and normalised to the mRNA expression levels of eight different housekeeping genes, the expression of both the mt-GrpE#2 and #1 transcripts appeared to be ubiquitous (Fig. 9*B* + 9*C*, respectively). I rule out non-specific hybridisation as negative controls such as yeast total RNA and tRNA (grid position = F1 + F2, respectively); *E. coli* rRNA and DNA (grid position = F3 + F4, respectively); synthetic poly r(A) (grid position = G1) and C_0t 1 DNA (representing the most abundant repetitive sequences, grid position = G2) did not give rise to any hybridisation signal. Furthermore, since the stringency of the hybridisation was identical to that seen for the Southern blot in Fig. 4, cross hybridisation of the mt-GrpE#2 probe with mt-GrpE#1 transcript is most unlikely. Thus, it appears that both transcripts encoding mt-GrpE#1 and mt-GrpE#2 are expressed ubiquitously in mammalian tissues, which is consistent with the essential cellular functions of both the *E. coli* GrpE (Ang

et al., 1986; Ang and Georgopoulos, 1989) and yeast Mge1p (Bollinger *et al.*, 1994; Ikeda *et al.*, 1994) counterparts.

Interestingly, the 3'-UTR of the mt-GrpE#2 contains two copies of the nonameric sequence UUAUUUA(U/A)(U/A) which are not present in the mt-GrpE#1 transcript (Fig. 2). These AU-rich elements are known to play a crucial role in the turnover of certain mammalian mRNAs, thus enabling an effective level at which gene expression can be controlled (Lagnado *et al.*, 1994; Zubiaga *et al.*, 1995). Perhaps more importantly, at least in this context, removal of the 3'-UTR from the granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA, which harbours the very same elements, effectively increases the stability of the transcript but decreases the efficiency of translation, implicating a role for 3'-UTR of GM-CSF in translational control (review by Rajagopalan *et al.*, 1997). Whether this form of post-transcriptional regulation is imposed on mt-GrpE#2 mRNAs is not known, but it may suggest that different post-translational regulation of the mt-GrpE#1 and mt-GrpE#2 transcripts may confer a different expression pattern of the encoded proteins. Nevertheless, it is clear that mt-GrpE#2 has all the hallmarks of a true Hsp70 co-chaperone and it shall be intriguing to elucidate its exact role in future studies.

In conclusion I have demonstrated that isoforms of GrpE-like molecules, as seen for both DnaK and DnaJ-like chaperones, can co-exist in a single compartment of a higher eukaryotic cell. This has not previously been found for GrpE family members from any other species[2], even where complete genome sequences are known (ie yeast and bacteria), and its significance is yet to be determined in terms of mitochondrial chaperone activities and functions. The tools generated during this study will assist in elucidating the importance or otherwise of these components for the biogenesis and maintenance of proteins in higher eukaryotic cells. They may also help to ultimately explain how mitochondria from mammalian sources have evolved with greater specialisation and diversity compared with those of lower eukaryotes, such as yeast.

[2] A year after this work was published Naylor *et al.* (1998) *J. Biol. Chem.* **273**, 21169-21177, another study reported the cloning of two distinct cDNAs that encode mitochondrial GrpE-like proteins from *Nicotiana tabacum* (Padidam *et al.*, (1999) *Plant Mol. Biol.* **39**, 871-881).

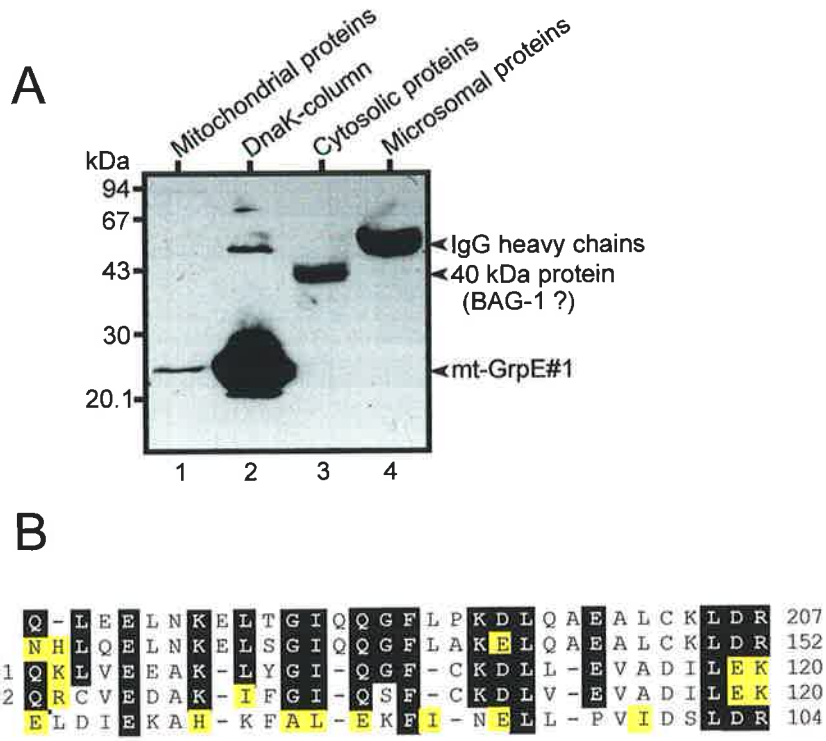


Fig. 1. **Evidence for the exsistence of a distinct cytosolic GrpE homologue in mammalian cells. (*A*)** A bovine mitochondrial extract (50 µg; *lane 1*), bovine mt-GrpE#1 eluted from a DnaK column by 5 mM ATP (1.6µg; *lane 2*), rat cytosolic extract (70µg; *lane 3*) and a porcine microsomal extract (55µg; *lane 4*) were electrophoresed in a 16.5 % Tris-Tricine gel, then transferred to nitrocellulose. The filter was probed with antiserum raised against bovine mt-GrpE#1 followed by detection of bound antibodies with a secondary horse radish peroxidase conjugated antibody. **(*B*) An amino acid segment within the mammalian BAG-1 protein is conserved in several members of the GrpE family.** Comparison of an amino acid segment from *Homo sapiens* (SWISS-PROT accession no. Q99933) and *Mus musculus* (SWISS-PROT accession no. Q60739) BAG-1(Hap, RAP46) with an analogous region in *Rattus norvegicus* mt-GrpE#1 (GenBank™ accession no. U62940), *Mus musculus* mt-GrpE#2 (GenBank™accession no. AF041060) and *Escherichia coli* GrpE (GenBank™ accession no. X07863). Residues identical to those of human (h) and mouse (m) BAG-1 sequences are highlighted in black whilst conserved residues (defined as A/G, I/ L, R/K/H, and D/E/N/Q) are shaded yellow. The numbering of the C-terminal residue (on the right hand side) of each segment indicates its position within the entire protein sequence.

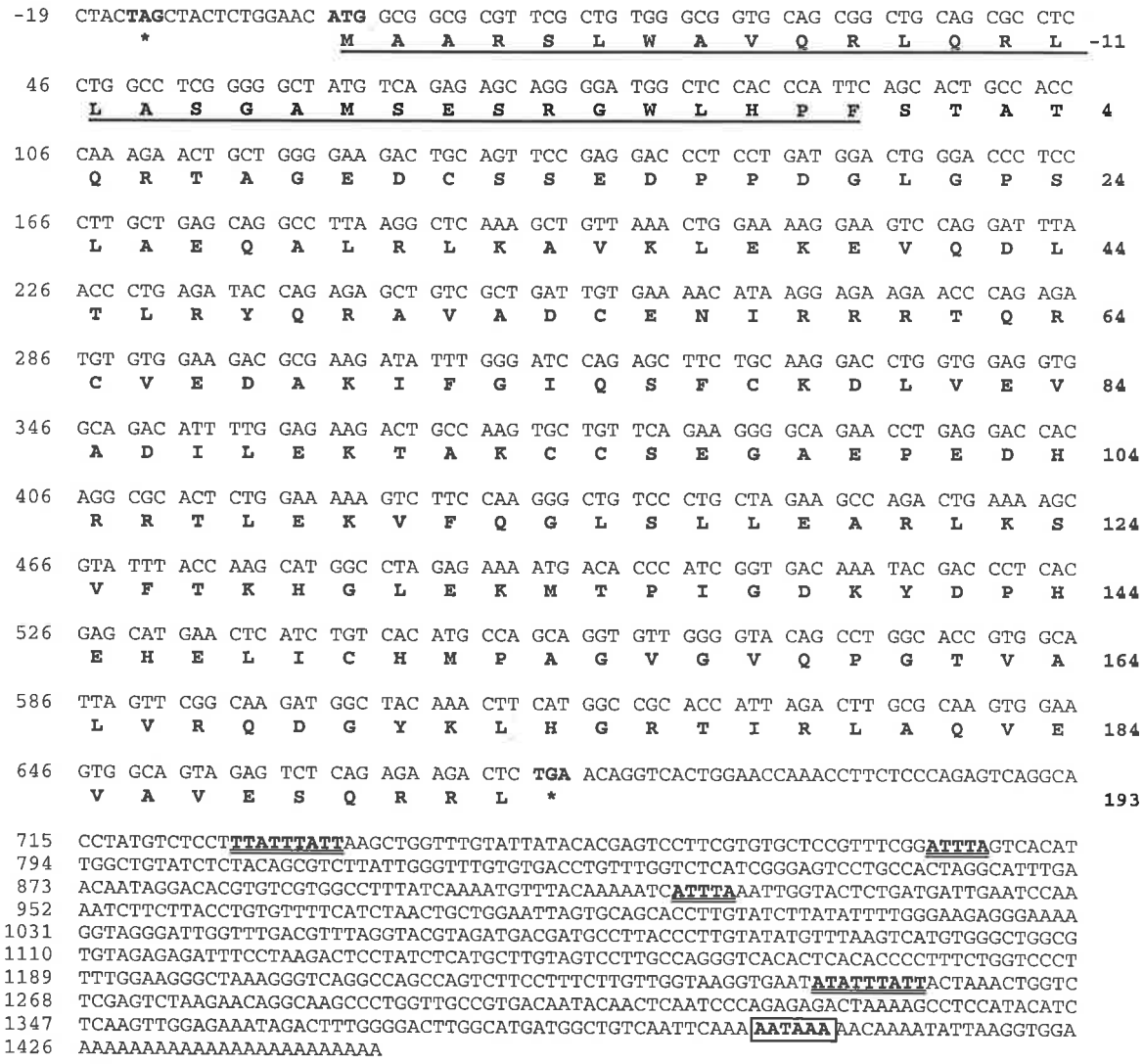


Fig. 2. **The cDNA and deduced amino acid sequence of mouse mt-GrpE#2.** The numbering of nucleotides is shown on the left-hand side and the first base of the predicted initiator codon is numbered +1 (see text for details). Amino acids are given by their single letter code and they are numbered on the right-hand side. The predicted first residue of the mature protein is numbered +1 and the cleavable presequence (underlined) is thus defined as amino acids -31 to -1. A putative polyadenylation site is boxed and possible RNA destabilisation sequences are double underlined. This sequence has been submitted to GenBank™ and given accession number AF041060.

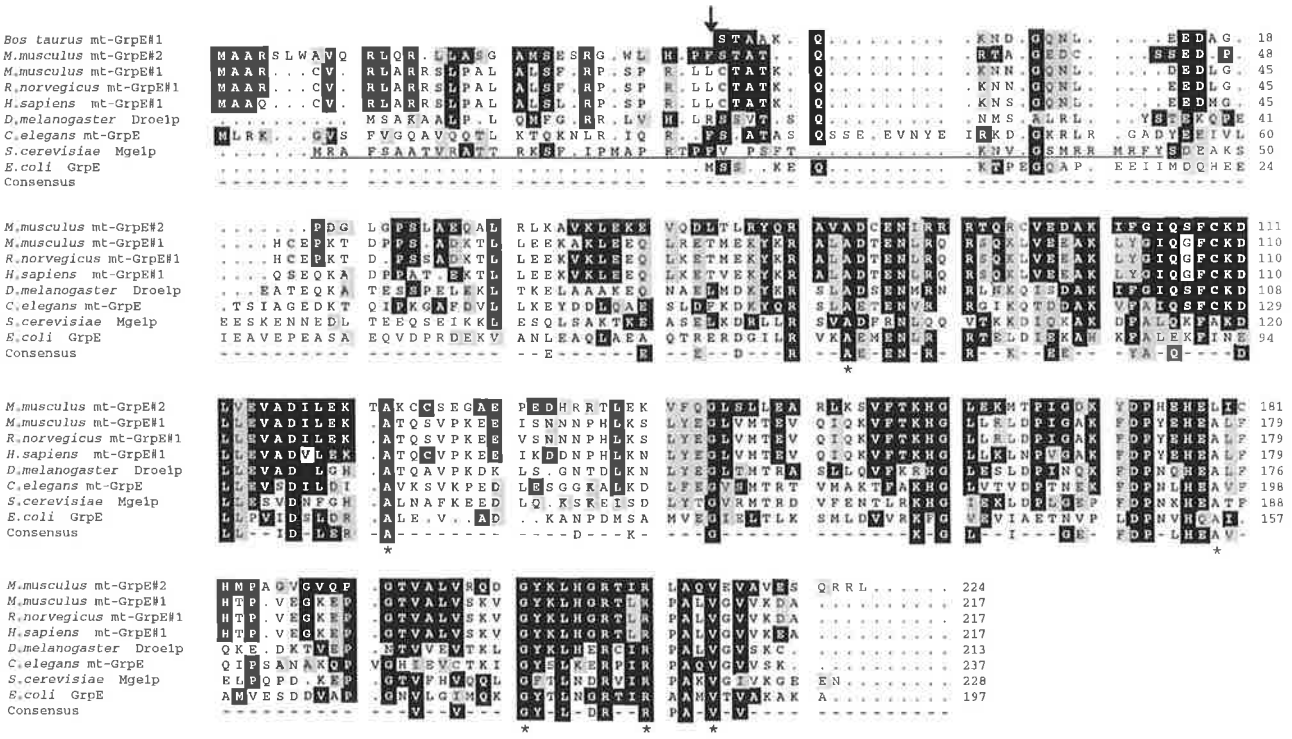


Fig. 3. **Multiple amino acid sequence alignment showing the similarity between mouse mt-GrpE#2 and selected GrpE homologues** from: *Bos taurus* (N-terminal peptide, see Chapter 2; Naylor *et al.*, 1995), *Mus musculus* (compiled and edited from GenBank™ accession nos. AA269811 and W70459), *Rattus norvegicus* (GenBank™ accession no. U62940), *Homo sapiens* (compiled and edited from GenBank™ accession nos. AA252446 and N28384), *Drosophila melanogaster* (GenBank™ accession no. U34903), *Caenorhabditis elegans* (EMBL accession no. Z46996), *Saccharomyces cerevisiae* (GenBank™ accession no. D26059) and *Escherichia coli* (GenBank™ accession no. X07863). The alignment was made using the PILEUP program (Genetic Computer Group, Madison, WI) and the suspected initiating methionine was numbered +1 to facilitate comparison since the processing sites are not known for most of the primary translation products. The N-terminal mitochondrial signal sequence from yeast Mge1p is underlined (Azem *et al.*, 1997) and based on the mature N-terminal sequence of bovine mt-GrpE#1, the suspected site of signal sequence cleavage is indicated with an arrow for the other mt-GrpE precursors. Residues that are identical to the mouse mt-GrpE#2 sequence are highlighted in black whilst conserved residues (defined as A/G, Y/F, S/T, I/V/L, R/K/H, and D/E/N/Q) are shaded grey. The asterisks under the consensus sequence (generated by alignment of 20 GrpE sequences) indicate the positions of six strictly conserved residues (see Chapter 3; Naylor *et al.*, 1996,1998).

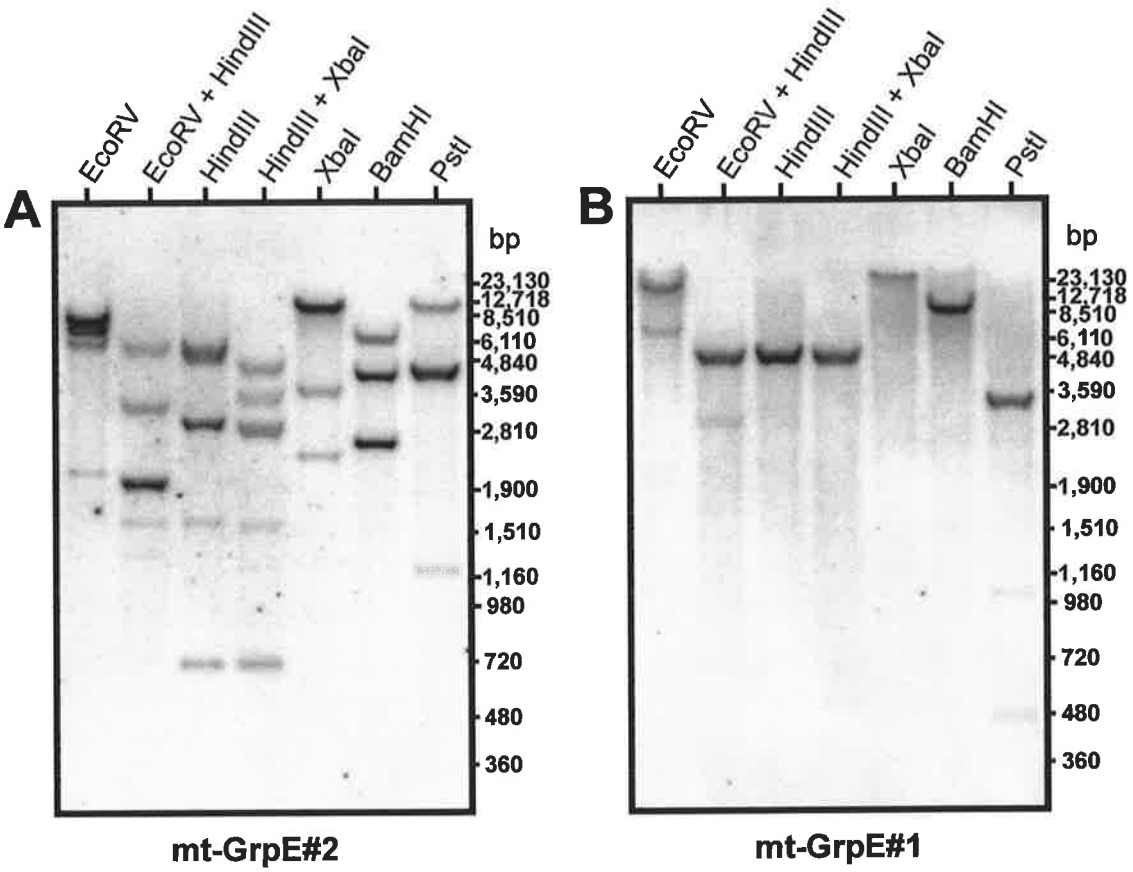


Fig. 4. **Southern blot analysis reveals mt-GrpE#1 and mt-GrpE#2 are encoded by distinct genes within the rat genome.** Rat liver genomic DNA digested with the indicated restriction enzymes was applied to lanes as indicated (10μg/ lane) and subjected to Southern blot analysis. The filter was first probed with the ^{32}P-labelled (***A***) mouse mt-GrpE#2 cDNA then stripped and reprobed with the ^{32}P-labelled (***B***) rat mt-GrpE#1 cDNA.

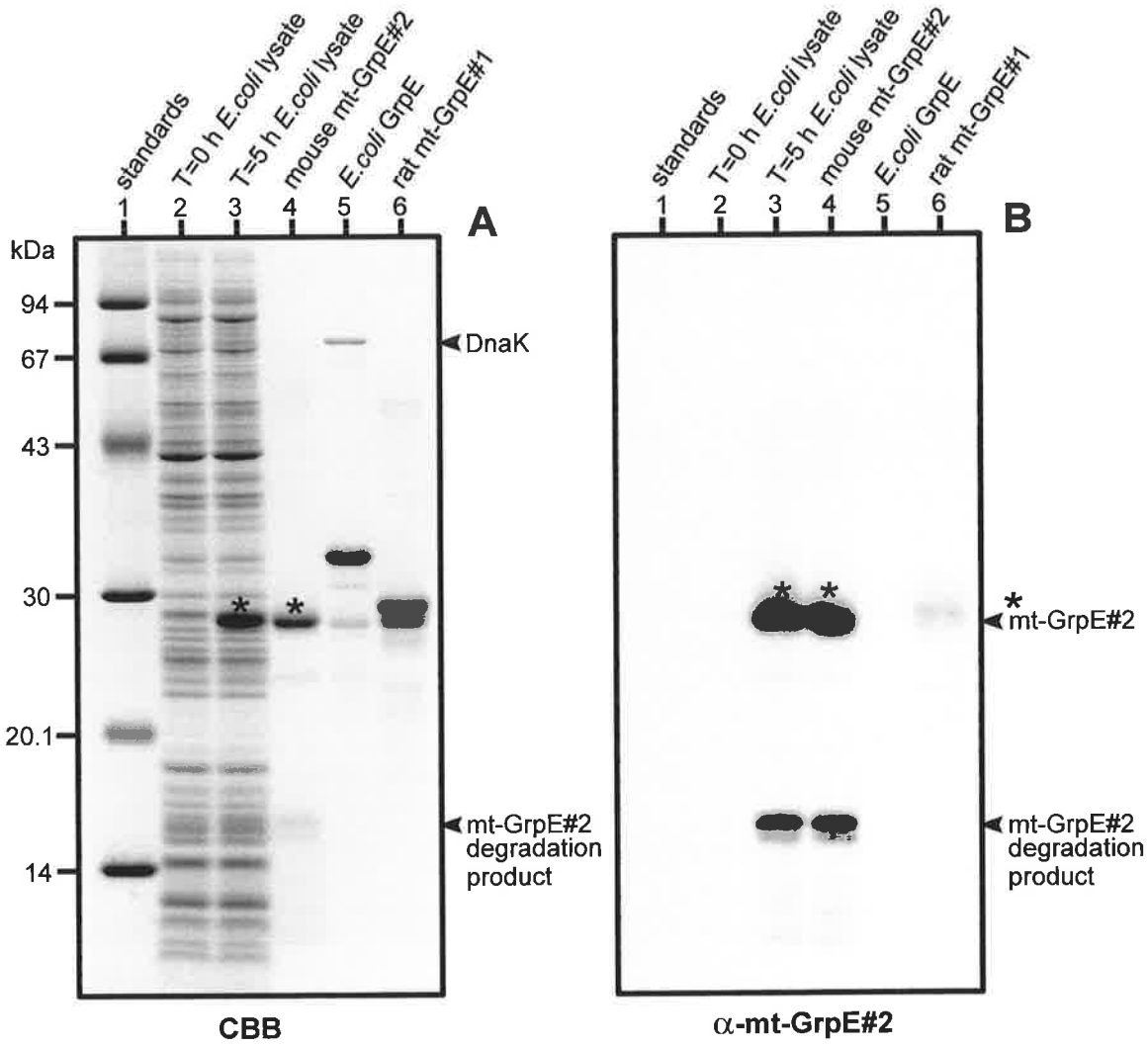


Fig. 5. ***Production of a specific antiserum to recombinant mt-GrpE#2.*** (*A*) The mature portion of the mouse mt-GrpE#2 protein (Fig. 2, residues 1 to 193) with an N-terminal hexahistidine tag was synthesised in *E. coli* for 5 h (*lane 3*) and purified by immobilised metal affinity chromatography (IMAC) (*lane 4*) as described in section 4.2.4. N-terminal hexahistidine tagged *E. coli* GrpE (*lane 5*) and the mature portion of rat mt-GrpE#1 (*lane 6*) (Chapter 3, Fig. 1, residues 1 to 190) were similarly synthesised in *E. coli* and purified by IMAC. Unlike the IMAC purification of *E. coli* GrpE, the IMAC purification of mouse mt-GrpE#2 and rat mt-GrpE#1 involved a pre-elution wash in a buffer containing 5 mM ATP which efficiently dissociates any bound *E. coli* DnaK (see section 4.3.4). The polypeptide content of the indicated fractions was analysed by SDS-PAGE in a 13 % Tris-Glycine gel followed by staining with Coomassie Brilliant Blue (CBB). *Lane 1* contained molecular mass standards. (***B***) An identical gel to that described in panel *A* was electrophoresed and the proteins were transferred to nitrocellulose. The filter was probed with an antiserum prepared against gel purified recombinant mt-GrpE#2 (*lane 4* of panel *A*, see section 4.2.1) and bound antibodies were detected with a secondary HRP-conjugated antibody. The position of a mt-GrpE#2 degradation product and/ or a product synthesised as a result of mRNA initiation from an internal ATG codon within the mt-GrpE#2 cDNA is shown.

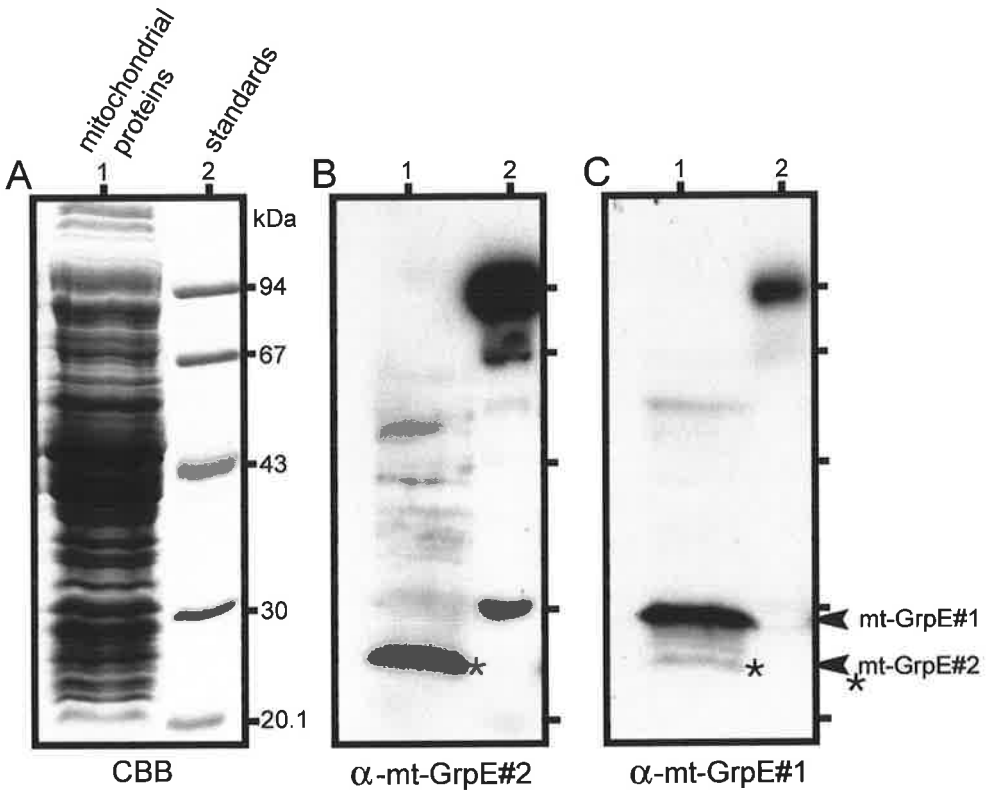


Fig. 6. **Western blot analysis confirms a mitochondrial location for both mt-GrpE#1 and mt-GrpE#2. (*A*)** A rat liver mitochondrial extract (80μg; *lane 1*) and molecular mass standards (*lane 2*) were electrophoresed in a 16.5 % Tris-Tricine gel and stained with Coomassie Brilliant Blue (CBB). **(*B*, *C*)** An identical gel to that described in panel *A* was electrophoresed and the proteins were transferred to nitrocellulose. The filter was sequentially probed with antiserum against **(*B*)** mt-GrpE#2 and against **(*C*)** mt-GrpE#1. Bound antibodies were detected with a secondary HRP-conjugated antibody.

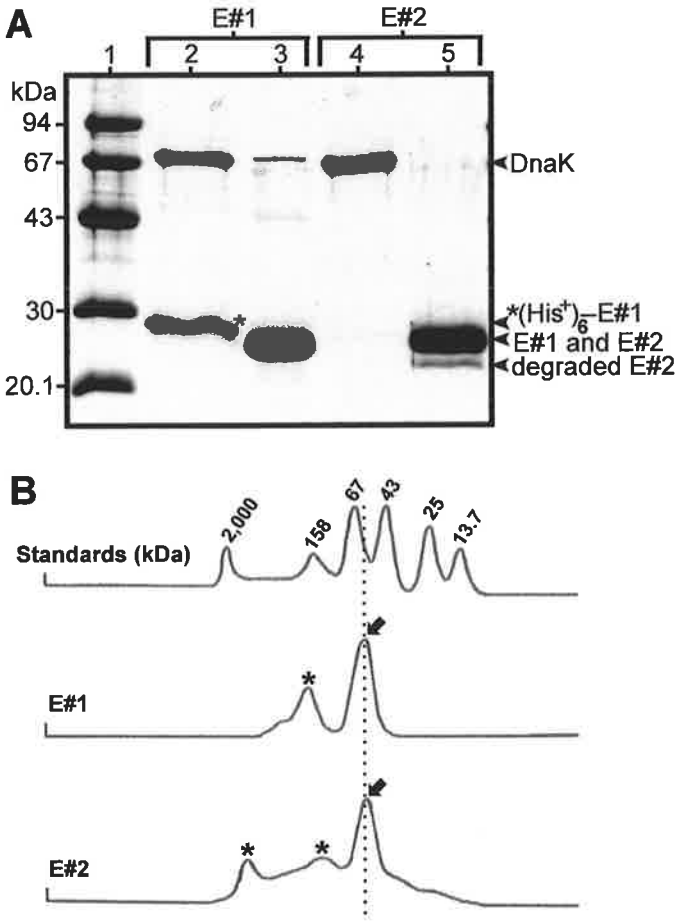


Fig. 7. **mt-GrpE#1 and #2 are oligomers which form stable complexes with DnaK.** **(*A*)** The mature portion of both the rat mt-GrpE#1 (E#1) and mouse mt-GrpE#2 (E#2) proteins were synthesised in *E. coli* with N-terminal hexahistidine tags and following cell lysis were retained on ~2 ml immobilised metal affinity chromatography (IMAC) columns pre-equilibrated in 25 mM Tris-Cl (pH 8.0), 200 mM NaCl and 10 mM -mercaptoethanol (see Fig. 5). After extensive washing in equilibration buffer (EB), the columns were successively washed with 100 ml of EB containing 0.3 M NaCl (ie 0.5 M NaCl in total), 100 ml of EB containing 5 mM ATP, 10 mM $MgCl_2$ and 50 mM KCl (*lanes 2* and *4*), 100 ml of EB containing 10 mM $CaCl_2$ and 20 ml of EB containing 10 mM $CaCl_2$ and 10U thrombin (*lanes 3* and *5*). The polypeptide content of the indicated fractions were analysed by SDS-PAGE in a 13% Tris-Glycine gel followed by staining with Coomassie Brilliant Blue. *Lane 1* contained molecular mass standards. **(*B*)** The retention times (increasing from left to right) of several standard proteins, recombinant rat mt-GrpE#1 (E#1) and recombinant mouse mt-GrpE#2 (E#2) were measured on a Superdex-200 (26/60) gel filtration column equilibrated in 0.1 M NaPi (pH 7.3) containing 100 mM NaCl. Eluates were monitored at 280 nm. A vertical dotted line is provided to help compare the three chromatograms whilst arrows and asterisks indicate the position of dimeric and higher oligomeric states of mt-GrpE#1 and mt-GrpE#2, respectively. The standards used were: blue dextran (2,000 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

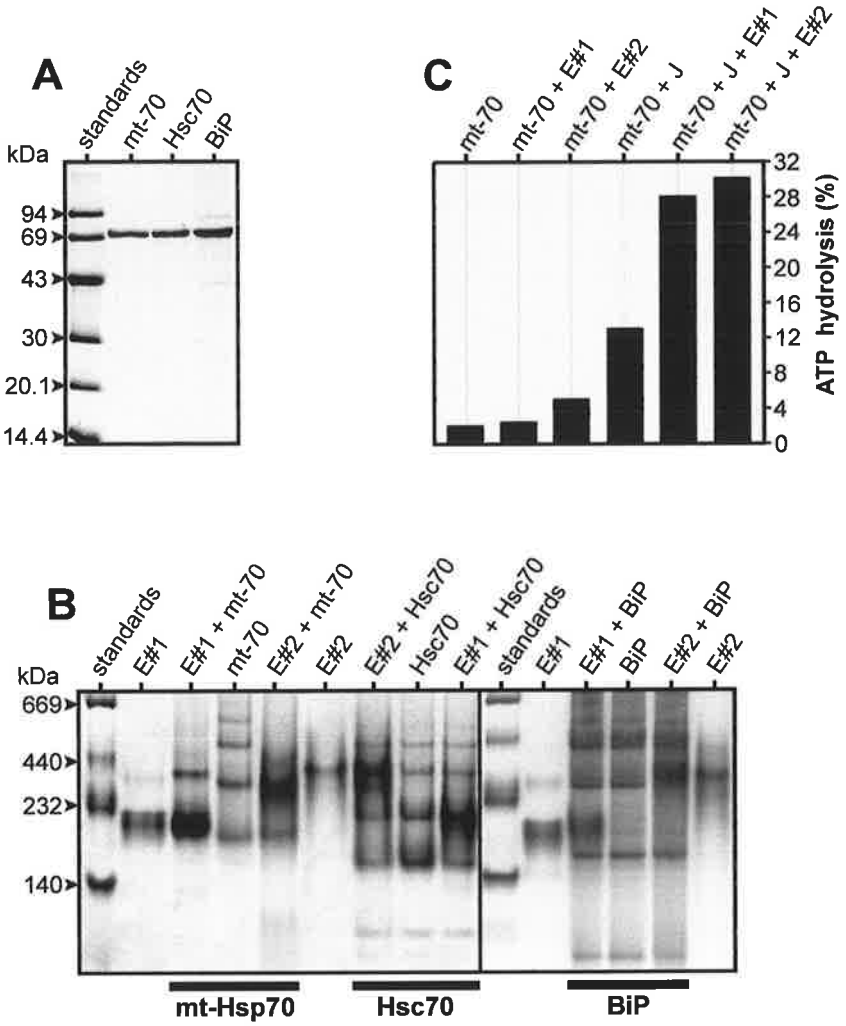


Fig. 8. **mt-GrpE#1 and #2 interact specifically with and stimulate the intrinsic ATPase activity of mt-Hsp70.** (***A***) Purified mammalian mt-Hsp70 (mt-70, 2μg), Hsc70 (2μg) and BiP (2μg) were analysed by SDS-PAGE in a 13% Tris-Glycine gel followed by staining with Coomassie Brilliant Blue (CBB). (***B***) Mixtures of these Hsp70 proteins (30μg each) and either mt-GrpE#1 (E#1, 20 μg) or mt-GrpE#2 (E#2, 30 μg) were depleted of ATP and the complexes formed were analysed by Native-PAGE in 6% Tris-Glycine gels followed by staining with CBB. In panels *A* and *B* molecular mass standards are shown for comparison. (***C***) Evaluation of the synergistic stimulation of mammalian mt-Hsp70 (mt-70, 1 μM) ATPase activity by *E. coli* DnaJ (J, 1 μM) and mt-GrpE#1 (E#1, 2 μM) or mt-GrpE#2 (E#2, 2 μM) was performed at 30°C with 40 μM ATP for 30 min as described in section 4.2.6.

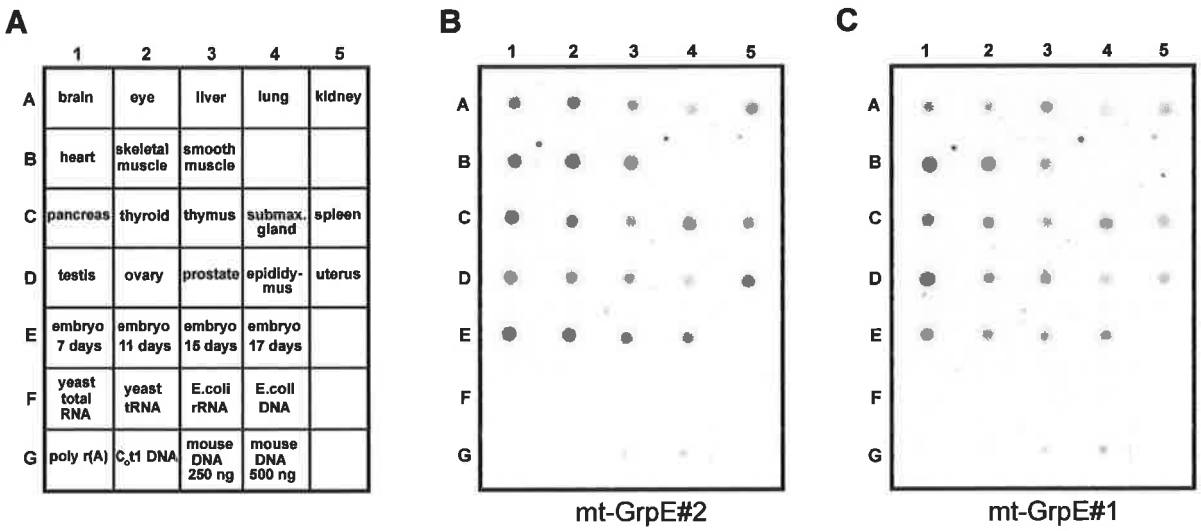


Fig. 9. **mRNA dot blot analysis indicates a ubiquitous but varying level of the mt-GrpE#1 and mt-GrpE#2 transcripts in mouse tissues.** Each dot contained approximately 100-500 ng of mRNA isolated from the indicated tissues and loadings were normalised to the mRNA expression levels of eight different housekeeping genes. (***A***) The diagram shows the type and position of mRNA and controls dotted onto the membrane. (***B***) The filter was probed with the ^{32}P-labelled mouse mt-GrpE#2 cDNA then stripped and (***C***) reprobed with the ^{32}P-labelled rat mt-GrpE#1 cDNA.

Chapter 5

Characterisation of several Hsp70 interacting proteins from mammalian organelles

5.1 INTRODUCTION

It has been well documented that DnaK and eukaryotic Hsp70 members preferentially bind to short extended peptides containing large internal hydrophobic. (Chapter 1; Hartl, 1996; Rüdiger *et al.*, 1997ab; Bukau and Horwich, 1998). Furthermore, such binding sites have been shown to arise during *de novo* protein synthesis and after protein denaturation, but are usually buried in native, functional proteins (Chapter 1; Hartl, 1996; Rüdiger *et al.*, 1997ab; Bukau and Horwich, 1998). These conclusions have predominantly been drawn from the studies with biologically irrelevant synthetic peptides and chemically denatured proteins. While the interaction of Hsp70 chaperones with proteins most likely occurs via short stretches of amino acids, both the spectrum and characteristics of *in vivo* substrates with affinity for Hsp70 chaperones are still largely unknown. In particular, little attention has been paid to the extent to which fully synthesised and folded proteins serve as substrates for Hsp70 members *in vivo*. Three processes which involve seemingly native-like Hsp70 substrates are the disassembly of clathrin coated vesicles, bacteriophage λ DNA replication, and immunoglobulin G (IgG) assembly from light and heavy chains (reviewed by Gething, 1997). In the first case, Hsp70 is proposed to bind an internal epitope of clathrin light chains that becomes exposed when the calcium levels increase within the endocytosed vesicle (DeLuca-Flaherty, *et al.*, 1990; Brodsky, *et al.*, 1991). The Hsp70 binding site(s) is proposed to be within a conformationally flexible structure, such as loops or turns, and probably forms the basis for the interaction of Hsp70 with other seemingly native-like proteins. Analogous to Hsp70's involvement in the disassembling clathrin triskelions, DnaK disassembles the protein complex λO-λP-DnaB helicase at the origin of λ replication, thereby liberating DnaB helicase whose DNA unwinding activities permit λ DNA replication to begin (reviewed by Georgopoulos, *et al.*, 1990).

Another likely class of fully folded proteins which could serve as substrates for Hsp70 chaperones are the subunits of multimeric proteins, not because they are denatured per se but since, in a monomeric state, they may expose aggregation prone structures which need to be shielded from the aqueous environment until they are fully assembled into their multimeric state. Such associations could be particularly relevant in the case of hetero-oligomers, such as IgGs, for which the rates of subunit synthesis may differ. In this case, a temporary metabolic retention ("parking") mechanism could be envisaged to maintain rapidly synthesised subunits

in an "assembly competent" or soluble "predegradation" form during the absence of slowly synthesised subunits (reviewed by Hartl, 1996; Ryan *et al.*, 1997).

In this study I have investigated the range and type of mature proteins from mammalian organelles which selectively interact with immobilised *Escherichia coli* Hsp70 (DnaK). Amongst a subset of organellar proteins selectively retained on DnaK, the major constituents represent unstable proteins and subunits of oligomeric proteins. The interactions with DnaK were diminished in the presence of mt-Hsp70 and BiP, while the complexes formed with DnaK were dissociated in the presence of K^+ and GrpE-like co-chaperones, suggesting that these organellar proteins constitute general Hsp70 substrates. Identification of six major constituents revealed that they represent novel Hsp70 interacting proteins and that their polypeptide sequences contain potential DnaK binding motifs which may serve as genuine binding sites for related organellar Hsp70 members. The selective retention of these fully synthesised proteins on Hsp70 most likely reflects the function of this molecular chaperone in protein biogenesis, but additionally, could extend the known functions of Hsp70 to include modulating the activities of certain proteins or enzymes which are important in cellular homeostasis.

5.2 MATERIALS AND METHODS

5.2.1 DnaK- and ATP-affinity chromatography—Preparation and use of a DnaK-affinity column was described in chapter 2 (Naylor *et al.*, 1995). Rat, porcine and bovine organelles were prepared from liver homogenates by differential centrifugation followed by sucrose density-gradient centrifugation with all steps performed at 4 °C (see section 2.2.2). The purified organelles were recovered from a broad region of the gradient where mitochondria predominantly reside with significant amounts of endoplasmic reticulum and peroxisomes present (see section 2.2.2). The purified organelles were lysed by incubation at 4 °C for 1 h in 20 mM HEPES pH 7.5, 1 mM DTT, 0.2 mg/ml phenylmethylsulfonyl fluoride, and 0.5 % (w/v) Lubrol PX followed by centrifugation at 80,000 x *g* for 1 h to give a supernatant representing the soluble organellar proteins (see Hartman *et al.*, 1992). Following DnaK-affinity chromatography of the protein extract prepared from rat organelles, several of the major constituents from the 1M KCl eluate (see Chapter 2, Fig. 3) were further purified on a 5 µm C_4 reversed phase (RP)-HPLC column (4.6 mm x 250 mm, VYDAC Cat.# 214TP54). The column was operated in 0.1% (v/v) TFA at 1 ml/min using an CH_3CN gradient composed of two linear segments (0-20 min: 0-48% (v/v) CH_3CN; 20-80 min: 48-72% (v/v) CH_3CN) and monitored at 214 nm. Fractions corresponding to each major polypeptide peak on the chromatogram were collected and concentrated in a speedy vac (Savant). The polypeptide content of all fractions was analysed by SDS-PAGE in 12.5 % Tris-Glycine gels (section 2.2.5).

ATP-agarose (linked through C-8 with a 9 atom spacer, Sigma Cat.# A2767) affinity chromatography with soluble rat, porcine and bovine liver organellar protein extracts was carried out as described in section 2.2.3 (Welch and Feramisco, 1985). In both DnaK- and ATP-affinity chromatography the equilibration buffer (EB) consisted of 20 mM Mops (pH 7.5), 1 mM DTT and 10 mM $MgCl_2$.

5.2.2 Protein sequence analysis—Protein sequencing was performed by Peter Høj and Rosemary Condron (School of Biochemistry, La Trobe Uni., Bundoora, Vic., Australia) on an Applied Biosystems Model 470A gas phase sequencer with a model 120A on-line analyser. N-terminal sequencing was performed on the RP-HPLC purified rat polypeptides from the 1M KCl eluate of the DnaK column. N-terminally blocked polypeptides (~50µg) were digested with endoproteinase Lys-C (3µg), according to the manufacturer's specifications (Promega),

and peptide fragments were isolated by RP-HPLC using a Brownlee RP-300 Aquapore C_8 guard column (4.6 x 30 mm) connected to a 5µm C_8 VYDAC column (4.6 x 250 mm; Cat.# 208TP54). The columns were operated in 0.1% (v/v) TFA at 1 ml/min using a linear gradient of 0-72% CH_3CN developed in 130 min and monitored at 214 nm.

Rat liver mt-Hsp70 and BiP, recovered in the 5 mM ATP eluates from the ATP-agarose column, were identified following further purification on a C_4 RP-HPLC column (as described in section 5.2.1) prior to N-terminal sequencing of the first eight residues.

The algorithm used to predict DnaK binding sites within the polypeptide sequence of each identified DnaK interacting protein was provided by Stefan Rüdiger and Bernd Bukau (Rüdiger *et al.*, 1997b).

5.3 RESULTS

It has previously been established that *E. coli* GrpE binds specifically to DnaK and the complex formed cannot be disrupted in the presence of 2 M KCl but is readily dissociated in the presence of 10 mM ATP (Zylicz *et al.*, 1987). Utilising this specific interaction I reported in chapter 2 the highly specific DnaK-affinity purification of a mitochondrial GrpE homologue (mt-GrpE#1) from several mammalian sources (Naylor *et al.*, 1995), and subsequently in chapter 3, the cloning of the corresponding rat cDNA (Naylor *et al.*, 1996, 1998). From these initial DnaK-affinity purifications, it became apparent that only a subset of the organellar proteins loaded onto the DnaK columns was routinely recovered in a 1M KCl eluate prior to the elution of GrpE homologous with 5 mM ATP. Importantly, the proteins recovered in 1M KCl eluate were not the most abundant proteins of the starting material. Other reports have shown that (i) K^+ (but not Na^+) is required for the ATP-induced dissociation of substrate proteins from DnaK (Palleros *et al.*, 1993), (ii) K^+ is an indispensable cofactor for GrpE stimulation of the ATPase activity of the DnaK-DnaJ complex (Motohashi *et al.*, 1997) and (iii) in the presence of K^+ but absence of ATP, the binding of GrpE to DnaK causes the dissociation of a complex between DnaK and reduced carboxymethylated α-lactalbumin (Harrison *et al.*, 1997). Hence, the subset of organellar proteins recovered in the 1 M KCl eluate most likely represent genuine DnaK substrates and identification of these proteins could provide important clues concerning the nature of cellular substrates recognised by members of the Hsp70 family. In the present work I have therefore extended our previous studies to identify several of the major rat protein constituents eluted from a DnaK column with a 1 M KCl wash in the presence of GrpE homologues. Fig. 1*B* reveals that there are at least two distinct subsets of fully synthesised organellar polypeptides that are selectively retained on immobilised DnaK, firstly those that elute in the presence of 1 M KCl (*lane 3*) and secondly the mammalian GrpE-like co-chaperones which form a complex with DnaK that is readily dissociated in the presence of 5 mM ATP (*lane 4*) but not in the presence of 1 M KCl. Under identical chromatographic conditions or by lowering the KCl concentration to 0.1 M, similar subsets of DnaK interacting proteins were also recovered from rat, porcine and bovine extracts, and importantly, retention of the polypeptides was not observed on the coupling resin alone (data not shown). Prior to DnaK-affinity chromatography, organellar extracts were passed over an ATP-agarose column to efficiently remove exogenous Hsp70 members,

namely mt-Hsp70 and BiP, which because of their high abundance might preferentially associate with DnaK interacting proteins (Fig. 1*A*, *lane 2*). Indeed, omission of this initial ATP-agarose step significantly diminished the yield of the two distinct protein subsets eluted from the DnaK-column, thus indicating that these organellar proteins probably constitute general Hsp70 substrates (data not shown). Interestingly, while both pig and bovine Hsp60 were efficiently co-purified with mt-Hsp70 and BiP during ATP-agarose chromatography (data not shown), relatively less rat Hsp60 could be co-purified (Fig 1*A*, *lane 2*) and was only detected following Western blot analysis (data not shown).

In order to identify the polypeptides recovered in the 1 M KCl eluate of the DnaK-affinity column, the major constituents were firstly separated by RP-HPLC (Fig. 1*C,D*) then subjected to N-terminal protein sequencing (Table 1). The identities of several constituents were determined as the endoplasmic reticulum cyclophilin-B (CyP-B), mitochondrial transcription factor A (mtTFA), mitochondrial alpha subunit of succinyl CoA synthetase (α-SCS) (isoform 1) and mitochondrial 2,4-dienoyl CoA reductase (mt-2,4-DCR#1). Several other constituents were N-terminally blocked to protein sequencing. The identities of these blocked polypeptides were determined following digestion with a protease and sequence analysis of purified peptides. They include the mitochondrial alpha subunit of succinyl CoA synthetase (α-SCS) (isoform 2), peroxisomal multifunctional enzyme (px-MFE-1) and a peroxisomal enzyme suspected to be an isoform of 2,4-dienoyl CoA reductase (px-2,4-DCR#1) (see Chapter 6) (Table 1). As observed in a previous study (Osumi *et al.*, 1985), px-MFE-1 is N-terminally modified, however the N-terminal modification of α-SCS has not been reported before. The significance of this finding is unclear, but interestingly I confirmed the presence of both N-terminally modified and unmodified species of α-SCS, as seen in the 1 M KCl eluate of the DnaK column (Fig. 1*C*, *lanes 3* to *5*), in a commercial preparation of pig heart SCS (Boehringer Mannheim, Cat.# 161543; data not shown). The peptide sequences derived from px-2,4-DCR#1 have not previously been determined. In chapter 6 I employed these peptide sequences for the isolation of corresponding cDNA clones from both rat and human sources, which in turn, allowed us to determine that rat px-2,4-DCR#1 is N-terminally acetylated. Furthermore, a clue to the mechanism underlying the KCl mediated elution of a subset of proteins retained on immobilised DnaK in the presence of GrpE homologues, comes from the observation that DnaK and GrpE form a stable interaction with rat px-2,4-DCR#1 expressed in *E. coli* (Chapter 6). The complex formed between recombinant px-2,4-DCR#1, DnaK and GrpE cannot be disrupted in the presence of 200 mM NaCl but is readily

dissociated in the presence of 50 mM KCl and 5 mM ATP. This suggests that the K^+ mediated dissociation is specific because Na^+ cannot substitute for K^+ in this regard and further underscores the authenticity of the observations reported herein.

Once the identities of several proteins recovered in the 1 M KCl eluate of the DnaK column were established, I searched their entire amino acid sequences with an algorithm designed to predict 95% of high affinity DnaK binding sites in linear peptide sequences at a certain threshold (ie $\Delta\Delta G_K < -5$) (Rüdiger *et al.*, 1997b). As expected, several high affinity DnaK binding sites were predicted within the sequence of each polypeptide (Fig. 2).

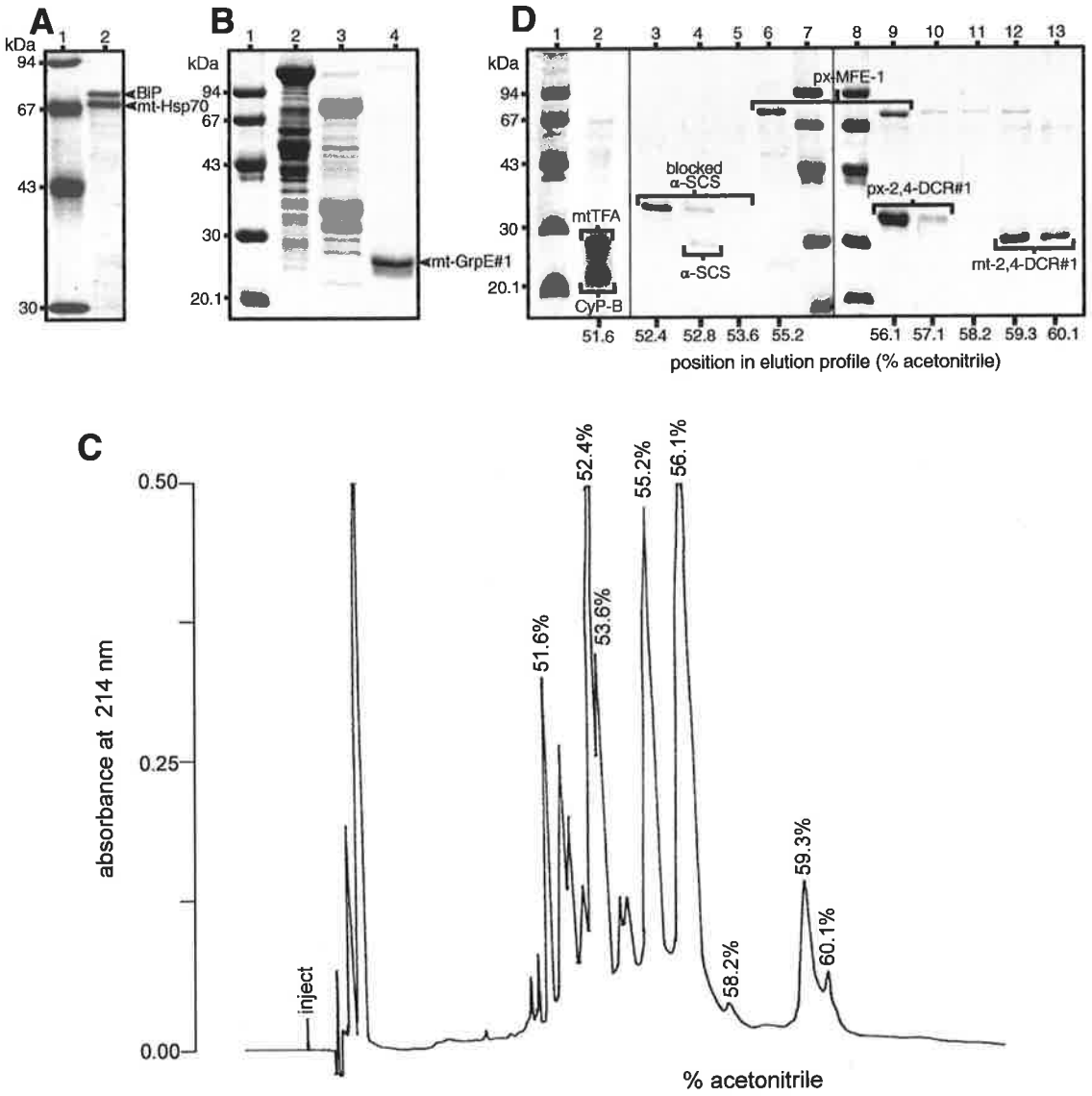


Fig. 1. **Several organellar polypeptides are selectively retained on immobilised DnaK.** **(*A*)** Soluble proteins extracted from rat liver organelles were firstly applied to an ATP-agarose column in order to remove exogenous Hsp70 members. The column was then successively washed in equilibration buffer (EB), EB containing 1M KCl, and finally with EB containing 5 mM ATP and 0.5 M KCl (*lane 2*). **(*B*)** The unbound fraction from the ATP-agarose column was supplemented with apyrase and loaded onto a DnaK-affinity column. Following extensive washing with EB, several bound proteins were firstly eluted with EB containing 1M KCl (*lane 3*) and then an essentially pure preparation of mt-GrpE#1 was eluted with EB containing 5 mM ATP and 0.5 M KCl (*lane 4*). The polypeptide content of each fraction in panels *A* and *B* was analysed by SDS-PAGE in 12.5% gels followed by staining with Coomassie Brilliant Blue **(*C*)** The major polypeptide constituents recovered in the 1M KCl eluate were further purified on a C_4 RP-HPLC column prior to their identification by N-terminal protein sequencing as described in section 5.2. The elution time of each fraction increases from left to right and is indicated above each peak on the chromatogram as the percentage of acetonitrile required for elution. **(*D*)** The polypeptide content of each peak on the chromatogram was analysed by SDS-PAGE in 12.5% gels followed by staining with Coomassie Brilliant Blue. *Lane 1* of panels *A*, *B*, and *D* contained molecular mass markers.

Table 1

Identification of several organellar proteins recovered in the 1 M KCl eluant of the DnaK-column. Peptide sequences were generated from either the N-terminus and/or internal fragments of the rat organellar polypeptides, where X denotes any amino acid and residues in brackets indicate the several possibilities at that position. Sources of the peptide sequences were identified by performing BLAST (Genetic Computer Group, Madison, WI) searches of numerous databases and the position of the peptide sequence(s) within the known (mature) polypeptide is shown. The predicted molecular mass (kDa) of each (mature) subunit polypeptide together with its known cellular location is given.

name of known protein or homologue	sequence of rat peptides	position in polypeptide	subunit mass (kDa)	cellular location	acccession number
rat cyclophilin B (CyP-B)	NDKKKGPKVTVKVYFDFQIGD-EPVGRVTFXLT(Q/S)KTRPXT	1 to 39	20.3	ER	SWISS-PROT P24368[a]
mouse mitochondrial transcription factor A (mtTFA)	XXXGXYPK(K/A)PMSSYLRFST	1 to 19	23.6	mt	GenBank™ U57939
alpha subunit of rat succinyl-CoA synthetase (α-SCS)	GSYTASRKNIYIDKNXKV TRLIGPNCPGIINPGECK	1 to 18 152 to 169	32.2	mt	GenBank™ J03621
rat peroxisomal multifunctional enzyme-1 (px-MFE-1)	XXVGLPEVTLGILPGA	116 to 132	78.6	px	GenBank™ K03249
rat mitochondrial 2,4-dienoyl-CoA reductase #1 (mt-2,4-DCR#1)	SIDAPQSKFFPPIL	1 to 14	32.4	mt	PIR S11021
rat peroxisomal 2,4-dienoyl-CoA reductase #1 (px-2,4-DCR#1)	SGQSYLAAGLLQNQVAVVT AISRELLHLGXNVVIASRK LXRLTAAVDELRASXPPSSSTQ EEEVNNLVK XXLAXYGK INFLVNNAGGQFMAPAEDITAK TMALTWASSGVRINXVAPXTI-YXQXA QARL	5 to 23 32 to 50 51 to 72 83 to 91 92 to 99 100 to 121 183 to 208 299 to 302	32.3	px	GenBank™ AF021854

[a] The SWISS-PROT accession number P24368 entry was generated by translation of the cDNA which contained two frame shift errors in the coding region. The correct primary sequence has been predicted by Hasel *et al.* (1991) and confirmed in this study.

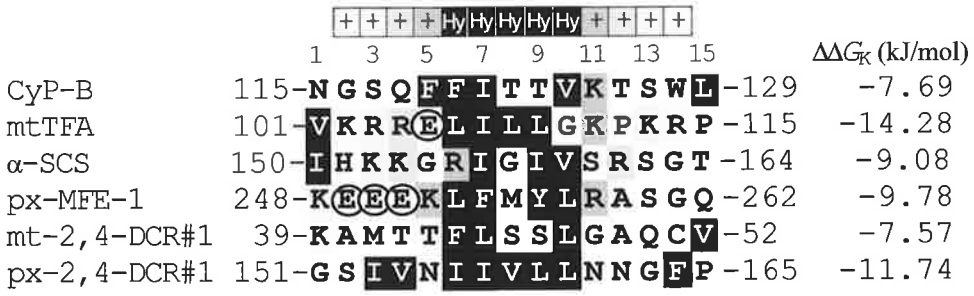


Fig. 2. **Prediction of DnaK binding sites within the polypeptide sequences of several DnaK interacting proteins from mammalian organelles.** A previously published algorithm (Rüdiger *et al.*, 1997b) was employed to predict high affinity DnaK binding sites within the entire sequence of each polypeptide identified in the 1 M KCl eluate of the DnaK column (see Table 1). A sequence alignment of the best fitting DnaK binding sites predicted and their position within the entire polypeptide sequence is shown. The algorithm was established from a DnaK binding motif (indicated above the alignment) which consists of a hydrophobic core (Hy) of 4 to 5 residues enriched particularly in L, but also in I, V, Y and F, and two flanking segments comprised of 4 amino acids enriched in the basic (+) residues R and K. In most cases, the core consists of 2 to 4 hydrophobic residues. The basic residues decrease in importance with increasing distance from the core while acidic residues (E and D) are disfavoured throughout the motif. The overall average energy contributions ($\Delta\Delta G_K$) for each amino acid within a 13 residue DnaK binding motif was calculated and is shown adjacent to the corresponding sequence, where motifs are predicted to have highest affinity to DnaK when they have the lowest $\Delta\Delta G_K$ value.

5.4 DISCUSSION

In the present study I utilised the well documented features of *E. coli* Hsp70 (DnaK) to identify a number of fully synthesised organellar proteins which, in addition to GrpE-like co-chaperones, are selectively retained on immobilised DnaK in the absence of mt-Hsp70 and BiP, and therefore, probably constitute general Hsp70 substrates. Whilst it would also have been desirable to perform the study with immobilised mt-Hsp70 and BiP, this was not feasible due to the very large amounts of protein required (~100 mg) and the inability to obtain such large amounts either from recombinant technology in *E. coli* (data not shown) or from isolated mitochondria and microsomes, respectively. The selective retention of these Hsp70 interacting proteins was underscored by the fact that they were present at relatively low abundance within the organellar extracts used and indeed constitute only a small subset of the total organellar proteins. Protein sequence analysis, and in one instance cDNA sequence analysis, identified the major constituents as the mitochondrial transcription factor A (mtTFA), mitochondrial alpha subunit of succinyl CoA synthetase (α-SCS), mitochondrial 2,4-dienoyl CoA reductase (mt-2,4-DCR#1), endoplasmic reticulum cyclophilin-B (CyP-B), peroxisomal multifunctional enzyme (px-MFE-1) and an undescribed peroxisomal enzyme, bearing a typical C-terminal type-1 peroxisomal targeting signal, which is suspected to represent an isoform of 2,4-dienoyl CoA reductase (px-2,4-DCR#1) (see Chapter 6). While the exact mechanism governing the dissociation of these organellar proteins from DnaK is not fully understood, our results and those of others would suggest that K^+ ions are an indispensable cofactor for the GrpE mediated release of these organellar and previously reported substrate proteins from DnaK (Palleros *et al.*, 1993; Harrison *et al.*, 1997; Motohashi *et al.*, 1997). Thus, retention of these polypeptides on immobilised DnaK is most likely associated with the tightly modulated ATPase cycle and further studies involving these organellar proteins as substrates may assist in elucidating the exact role of GrpE co-chaperones. Indeed, in chapter 6 I have shown that the interaction of px-2,4-DCR#1 with DnaK is linked to the chaperone's ATPase cycle.

There are several possible reasons for the observed binding of these organellar proteins to immobilised DnaK and hence by implication to mt-Hsp70 or BiP. The most likely reason is that chaperone binding fulfils a "quality control" mechanism, thus

recognising and shielding aggregation prone hydrophobic surfaces of protein substrates which may arise as part of their biogenesis or subsequent breakdown. Consistent with this scenario, multiple high affinity DnaK binding sites, which are accessible throughout the biogenesis and breakdown of proteins, were predicted within the sequence of each of the identified DnaK interacting proteins. The unstable regulatory protein mtTFA (Shadel and Clayton, 1997) and peroxisomal protein px-MFE-1 (Osumi and Hashimoto, 1979) would both be expected to expose aggregation prone surfaces as recognition sites for chaperones. Other prime targets could include the subunits of oligomeric enzymes which may expose hydrophobic surfaces to an aqueous cellular environment that are not exposed in the assembled oligomeric state. Indeed, a high affinity DnaK binding site has been identified within the dimerisation interface of human p53 (Rüdiger *et al.*, 1997b; Clarke *et al.*, 1988). This observation has led to the speculation that Hsp70, through binding such subunit interfaces, may be able to modulate the oligomerisation of multimeric proteins (Rüdiger *et al.*, 1997b). Consistent with this idea, in a study of BiP binding sites within immunoglobulin heavy chains, the majority of binding sites were mapped to the regions of contact made with the light chain subunits (Knarr *et al.*, 1995). Several of the DnaK interacting proteins identified in this study can adopt an oligomeric state, namely the α-SCS (αβ-dimer), mtTFA (homotetramer), mt-2,4-DCR#1 (homotetramer) and px-2,4-DCR#1 (homodimer) (see Chapter 6) (Wolodko *et al.*, 1986; Antoshechkin *et al.*, 1997; Hakkola *et al.*, 1993; Gurvitz *et al.*, 1997). The finding that only the α-subunit and not the β-subunit of succinyl CoA synthetase was recovered from the DnaK-affinity column could be explained if the α-subunit is synthesised more rapidly than the β-subunit and the α-SCS:Hsp70 complex allows the α-subunits to be temporarily retained ("parked") in an "assembly competent" form until β-subunits become available for assembly into the αβ-dimer. Alternatively, the α-SCS:Hsp70 complex could allow the α-subunits to be retained ("parked") in a soluble "predegradation" form to facilitate its rapid degradation in the prolonged absence of β-subunits. Indeed, *Saccharomyces cerevisiae* mt-Hsp70 has been observed to maintain a subunit of mitochondrial ribosomes (var1) in an "assembly competent" state during the absence of the other nuclear-encoded components while a prolonged interaction of var1 with mt-Hsp70 appears to facilitate var1 degradation (Herrmann *et al.*, 1994). As first observed in *E. coli* with DnaK, the prolonged interaction of substrates with mt-Hsp70 facilitates substrate presentation to the Lon (Pim1p) protease

for degradation, presumably by increasing the accessibility of the substrate to the protease (Chapter 1; Wagner *et al.*, 1994; Herrmann *et al.*, 1994; Ryan *et al.*, 1997).

At least one of the identified DnaK interacting proteins may be retained on DnaK (or an organellar Hsp70 member) in a functional conformation via interaction with a region(s) of the chaperone outside the "substrate binding channel". In this context, it is possible cyclophilin-B (CyP-B) may be selectively retained on immobilised DnaK (or BiP) in its functional state because it is likely to work synergistically as a co-chaperone. Thus, CyP-B is a protein folding catalyst that accelerates rate limiting steps in the ER through its peptidyl-prolyl isomerase (PPIase) activity and accordingly can be coprecipitated in complex with several ER chaperones (ie Hsp47, BiP, PDI, Grp94) and procollagen (Smith *et al.*, 1995). The selective retention of PPIases on molecular chaperones for their coordinate action in protein biogenesis and/or degradation has previously been observed in both *E. coli* with GroEL and Trigger Factor and in the eukaryotic cytosol where cyclophilin 40 or FKBP52 can be found in complex with Hsp70 and Hsp90 via direct interaction with Hsp90 (Gething, 1997). It can not be dismissed however, that CyP-B in its functional state interacts indirectly with DnaK via an association with a DnaK substrate or alternatively, interacts directly as a substrate in an unfolded state.

As seen for the interactions of PPIases with molecular chaperones, a precedent for a specific interaction between DnaK and the oligomeric protein mtTFA has been demonstrated in bacteria. In this case, the interaction could serve regulatory purposes at the transcriptional level brought about by the selective interaction of a molecular chaperone with a regulatory molecule. This is probably best exemplified by the tight regulation imposed on the *E. coli* heat shock (stress) response by the DnaK chaperone machine selectively binding, and thereby modulating the activity of, the heat shock promoter-specific σ^{32} subunit of RNA polymerase (reviewed by Bukau, 1997). Reminiscent of the *E. coli* σ^{32} factor, mt-TFA is a mitochondrial promoter-binding subunit of mitochondrial RNA polymerase and probably constitutes the principle activator of mitochondrial gene expression in vertebrates (Larsson *et al.*, 1998; Shadel and Clayton, 1997). Furthermore, it has previously been demonstrated that nuclear respiratory factors 1 and 2 can activate the expression of numerous nuclear genes that contribute to mitochondrial respiratory function, including mtTFA (Virbasius *et al.*, 1994). Therefore,

in an analogous manner to the tight regulation imposed on σ^{32} by DnaK binding, the binding of mtTFA to mt-Hsp70 could constitute a potential regulatory link for the coordinate expression of both nuclear and mitochondrial genes during organellar biogenesis. In fact, recently mtTFA (also known as Tfam) was found to regulate mitochondrial DNA copy number *in vivo* and to be essential for mitochondrial biogenesis and embryonic development (Larsson *et al.*, 1998). By further analogy with the fate of σ^{32} in *E. coli*, excessive mtTFA could be captured by mt-Hsp70 and presented to the mitochondrial inner membrane located *m*-AAA protease for rapid degradation (Leonhard *et al.*, 1996; Savel'ev *et al.*, 1998). The *m*-AAA protease is a homologue of the *E. coli* FtsH (HflB) protease, the latter is apparently involved in degrading excess amounts of σ^{32} being presented by DnaK (reviewed by Akiyama *et al.*, 1997).

In conclusion, I have identified a small subset of fully synthesised organellar proteins which can be selectively retained on immobilised DnaK and have observed that their interactions with DnaK are significantly diminished in the presence of mt-Hsp70 and BiP. Although, at this point in time, I cannot decipher exactly why these organellar proteins are selectively bound by either mt-Hsp70 or BiP, this study does indicate that the chaperone action of Hsp70 may not be confined to "quality control" of protein biogenesis alone, but may well extend into cellular homeostasis. Therefore, the list of proteins identified herein should now be considered prime candidates for further elucidation of Hsp70 function beyond that of protein biogenesis alone.

Chapter 6

Mitochondria and peroxisomes may contain distinct isoforms of 2,4-dienoyl CoA reductase

6.1 INTRODUCTION

Homologues of Hsp70 (DnaK), DnaJ and GrpE have been identified in several major compartments of the eukaryotic cell, including the cytosol, nucleus, endoplasmic reticulum (ER), mitochondria and chloroplasts, and in many cases, these chaperones or co-chaperones exist in multiple isoforms (Chapter 1; Hartl, 1996; Ryan *et al.*, 1997; Gething, 1997; Bukau and Horwich, 1998; Naylor *et al.*, 1998). In comparison, little is known about the peroxisomal complement of molecular chaperones. In the cytosol, several chaperones including Hsp70, a DnaJ homologue (Djp1p), and possibly Hsp90 are involved in the targeting of proteins to the peroxisomal matrix (Walton *et al.*, 1994; Hettema *et al.*, 1998; Crookes and Olsen, 1998), but only recently has a chaperone been identified within the matrix of a microbody (peroxisomes, glyoxysomes, and glycosomes) (Wimmer *et al.*, 1997). Alternative translational initiation, from a single Hsp70 transcript in watermelon cotyledons, permitted the targeting of the previously described plastid Hsp70 to both the matrix of plastids and glyoxysomes by the addition of long or short N-terminal presequence to the respective polypeptides (Wimmer *et al.*, 1997). By analogy to known functions of ER Hsp70 (BiP) and mitochondrial Hsp70 (mt-Hsp70), the location of glyoxysomal Hsp70 infers that it could participate in the import and the subsequent folding and assembly of glyoxysomal proteins in the matrix (reviewed by Hartl, 1996; Ryan *et al.*, 1997; Gething, 1997). In contrast to the ER and mitochondria though, peroxisomes can translocate at least some fully folded and assembled oligomeric proteins across their membranes (reviewed by Subramani, 1996; McNew and Goodman, 1996). The function, therefore, of microbody matrix located Hsp70 members still remains largely unknown. In Chapter 5 it was observed that the interaction of several mammalian organellar proteins with immobilised DnaK was diminished in the presence of mt-Hsp70 and BiP, suggesting that these proteins may be genuine substrates for mt-Hsp70 or BiP (Naylor *et al.*, 1999). Amongst these organellar proteins were the monomeric peroxisomal multifunctional enzyme isoform-1 (px-MFE-1), the 120 kDa homotetrameric isoform of mitochondrial 2,4-dienoyl CoA reductase (mt-2,4-DCR#1)(EC 1.3.1.34), and an undescribed ~33 kDa N-terminally modified protein. Isolation and sequencing of several internal peptides derived from the unknown ~33 kDa protein revealed significant similarities with the polypeptide sequence of mt-2,4-DCR#1.

The β-oxidation of unsaturated fatty acids requires the auxiliary enzymes Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8, an integral activity of px-MFE-1) and 2,4-dienoyl-CoA reductase,

in addition to the enzyme complement responsible for degradation of saturated fatty acids (reviewed by Hiltunen *et al.*, 1996; Kunau *et al.*, 1995). The importance of 2,4-dienoyl-CoA reductases is exemplified in both a reductase-deficient *E. coli* mutant and a *Saccharomyces cerevisiae* null mutant which are unable to grow on petroselineate (*cis*-6-octadecenoate) as the sole carbon source (You *et al.*, 1989; Gurvitz *et al.*, 1997). In addition, the report of a lethal inborn 2,4-dienoyl-CoA reductase deficiency in a human patient again underscores the essential requirement of this enzyme (Roe *et al.*, 1990). At least three isoforms of 2,4-dienoyl CoA reductase exist in mammals, two mitochondrial reductases (native molecular masses of 120 kDa and 60 kDa) and a third peroxisomal reductase (Dommes *et al.*, 1981; Hakkola and Hiltunen, 1993). Although the human gene (DECR) (Helander *et al.*, 1997) and the rat and human cDNAs encoding the 120 kDa mitochondrial isoform have been cloned and sequenced (Hirose *et al.*, 1990; Koivuranta *et al.*, 1994), no sequence information exists for either the second 60 kDa mitochondrial isoform or the peroxisomal reductase, and to date these later isoforms have not been purified to homogeneity. The 120 kDa mitochondrial reductase has been purified from rat and bovine liver, and has been shown to be active as a homotetramer comprised of 32.4 kDa subunits (Kimura *et al.*, 1984; Dommes *et al.*, 1982; Dommes and Kunau, 1984). One study has reported the purification of the peroxisomal isoform from rat liver (Kimura *et al.*, 1984), but further investigations indicated that it most likely represented the previously characterised 120 kDa mitochondrial isoform (Hakkola *et al.*, 1989). In yeast, β-oxidation of fatty acids is confined to peroxisomes (Kunau *et al.*, 1988). Therefore, the 2,4-dienoyl CoA reductases purified from yeasts constitute the only peroxisomal reductases so far characterised at the protein level (Gurvitz *et al.*, 1997; Mizugaki *et al.*, 1985; Dommes *et al.*, 1983). In a recent study, the *Saccharomyces cerevisiae* peroxisomal reductase (Sps19p) was shown to be encoded by the sporulation-specific *SPS19* gene (Gurvitz *et al.*, 1997). The cDNA deduced sequence of Sps19p shares ~34% similarity with the rat and human 120 kDa mitochondrial reductases (Gurvitz *et al.*, 1997). Purified Sps19p was shown to be active as a homodimer (native molecular mass 69 kDa) and to catalyse the NADPH-dependent reduction of 2,4-dienoyl-CoAs into 3-enoyl-CoAs, the same end products generated by the mammalian mitochondrial reductases (Gurvitz *et al.*, 1997; Hakkola and Hiltunen, 1993; Dommes and Kunau, 1984; Kunau and Dommes, 1978). In contrast, bacterial 2,4-dienoyl-CoA reductase generates 2-enoyl-CoAs as the end products (Dommes and Kunau, 1984; Mizugaki *et al.*, 1983) whilst the end products generated by mammalian peroxisomal 2,4-dienoyl CoA reductases remains unknown.

The evolutionary reason for the compartmentalisation of β-oxidation to both mitochondria and peroxisomes in mammals has undoubtedly been obscured by the lack of information on peroxisomal 2,4-dienoyl CoA reductase, and is even further clouded by the argument for a distinct peroxisomal isoform or the dual targeting of a mitochondrial isoform to both organelles (Hakkola *et al.*, 1989; Mizugaki *et al.*, 1996). In the present study I have further characterised a previously undescribed ~33 kDa DnaK interacting protein (see Chapter 5, Naylor *et al.*, 1999) as a putative peroxisomal isoform of 2,4-dienoyl CoA reductase (px-2,4-DCR#1) which is distinct from the 120 kDa mitochondrial reductase (mt-2,4-DCR#1). The selective interaction of both px-2,4-DCR#1 and mt-2,4-DCR#1 with DnaK is discussed in the context of an analogous interaction with mitochondrial or a peroxisomal Hsp70, which could constitute a new level of metabolic control.

6.2 MATERIALS AND METHODS

6.2.1 Purification of the 2,4-dienoyl-CoA reductases—A partial purification of rat liver mt-2,4-DCR#1 and px-2,4-DCR#1 was achieved by DnaK-affinity chromatography with soluble organellar proteins as described previously (Chapter 5, Naylor *et al.*, 1995, 1999). Following DnaK-affinity chromatography, ~250 μg of protein from the 1 M KCl eluate was loaded onto a Superose-12 FPLC gel filtration column (Pharmacia) equilibrated in 35mM sodium acetate (pH 5.0) containing 100 mM sodium chloride. The gel filtration column was operated at 0.5 ml/min and the effluent was monitored at 225 nm. The polypeptide content of all fractions was subjected to SDS-PAGE in 12.5% Tris-glycine gels (Fling and Gregerson, 1986) and visualised by the ammoniacal silver staining method described by Harlow and Lane (1988). The identities of several proteins were revealed by direct N-terminal sequencing or following digestion with endoprotease Lys-C and subsequent purification of the peptide fragments by RP-HPLC as described in Chapter 5 (Naylor *et al.*, 1999).

The mass of native rat liver px-2,4-DCR#1 (containing a blocked N-terminus) was determined by Yogi Hayasaka (Australian Wine Research Institute, Glen Osmond, SA., Australia) on an electrospray ionisation-triple quadrupole mass spectrometer (PE SCIEX API300) essentially as described by Hartman *et al.* (1992).

6.2.2 Synthesis of oligonucleotide primers—The oligonucleotides employed in 5'-RACE analysis were those designed by Frohman *et al.* (1988) combined with a degenerative antisense primer #1 [5'-CAT(A/G)AA(T/C)TGNCCNCCNGC(A/G)TT-3'] designed on the basis of a peptide sequence (Fig.3, #6 NAGGQFM) obtained from rat px-2,4-DCR#1 (see Chapter 5, Naylor *et al.*, 1999) and a nested antisense primer #2 [5'-CCACAGC-AGCGGTTAATCTGTCCA-3'] designed on the basis of a rat EST (GenBank accession no. H35326). Primer #3 [5'-GATAGTCACGTGGCCGCAGACTGC-3'], which corresponds to the first twenty four nucleotides of the full length rat px-2,4-DCR#1 cDNA (Fig. 4), was synthesised on the basis of the sequence obtained from a 5'-RACE product.

Construction of the expression vector for the synthesis of rat px-2,4-DCR#1 utilised primer #4 [5'-GTTAACATATGGGCTCTTGGAAGAGTGGTCAG-3'] and primer #5 [5'-AGCCGGATC-CTTAGAGCCTGGCTTGCTTCTTCAA-3'] as outlined below.

6.2.3 Southern blot analysis—Rat genomic DNA (10 μg) was digested with the appropriate restriction enzyme (40 U at 37 °C for 8 h) in the recommended buffers (Promega) and then electrophoresed in a Tris-acetate-EDTA (TAE) buffered 0.8% (w/v) agarose gel. The digested DNA was transferred onto a Hybond™ -N+ nylon membrane (Amersham) and the blot was prehybridised for 6 h at 65 °C in 5x SSC, 0.5% (w/v) SDS, 100 μg/ml sheared salmon sperm DNA and 5 x Denhardt's solution (0.1% (w/v) Ficoll™ 400, 0.1% (w/v) polyvinylpyrrolidone and 0.1% (w/v) BSA). The membrane was first probed with a ^{32}P-labelled rat px-2,4-DCR#1 cDNA fragment (nucleotides -96 to 412, Fig. 4), then stripped and reprobed with a ^{32}P-labelled rat mt-2,4-DCR#1 cDNA fragment (GenBank accession no. D00569, nucleotides 356 to 944). The denatured probes were added to the prehybridisation solution and hybridisations were carried out for 12 h at 65 °C. Final washes were performed for 10 min at 65 °C in 0.1 x SSC supplemented with 0.1% (w/v) SDS and filters were analysed using a Storm PhosphorImager and ImageQuaNT software (Molecular Dynamics).

6.2.4 Isolation of mRNA, RT-PCR, cDNA library screening and 5'-RACE analysis— mRNA was isolated from 5 x 10^7 clonal rat hepatoma cells (H4) using a Fast Track® mRNA isolation Kit (Invitrogen®) (Chapter 3, Naylor *et al.*, 1996) and partial first strand cDNA was generated with primer #1 and SUPERSCRIPT™ II RNase H$^-$ Reverse Transcriptase (Life Technologies, Inc.) according to the manufacturer's protocol. Using the partial first strand cDNA as a template, cloning of nucleotides -96 to 177 of the final cDNA (see Fig. 4) was performed with the aid of primers #1 and #2 by 5'-RACE PCR according to Frohman *et al.* (1988). A subsequent 273 bp PCR product (excluding the 5'-RACE sense-strand primer sequence) was produced with primers #2, #3 and *Taq* DNA polymerase (Life Technologies, Inc.) in a 30 cycle protocol (94 °C for 60 sec, 50 °C for 90 sec and 72 °C for 90 sec) according to the manufacturer's instructions. The PCR product was recovered and used to screen a cDNA library constructed in the UNI-ZAP XR vector using mRNA from livers of 7-8 week old rats (Stratagene). Recombinants (1 x 10^5) were screened essentially as described previously (Høj *et al.*, 1989) and two independent clones were isolated. Both strands of the two clones were sequenced using the dideoxy method of Sanger *et al.* (1977) (see section 3.2.2).

6.2.5 Immunological techniques—An antiserum against recombinant rat px-2,4-DCR#1 was prepared from the SDS-PAGE purified protein as described previously (Chapter 3, Naylor

et al., 1996). The serum was supplemented with 0.02% (v/v) sodium azide and stored at -70 °C. Dilutions of 1 in 10,000 were used in immunostaining analysis.

6.2.6 Expression of rat px-2,4-DCR#1 in E. coli—The coding region of the rat px-2,4-DCR#1 cDNA was amplified with $Vent_R$® DNA polymerase (New England BioLabs) utilising primers #4 and #5 in a 30 cycle PCR protocol as described above. The 927 bp PCR product was digested with *Nde*I and *Bam*HI, then ligated into the same predigested sites of the pET-14b vector as specified in the manufacturer's manual (4th Ed., Novagen). The construct under the selection of ampicillin was transformed into BL21(DE3) cells co-harbouring the pLysS plasmid under the selection of chloramphenicol (Novagen). Both strands of the rat px-2,4-DCR#1 cDNA within the pET-14b vector were sequenced using the dideoxy method of Sanger *et al.* (1977) to verify the authenticity of the construct.

For expression of the recombinant protein bearing an N-terminal hexahistidine tag (removable with thrombin), typically 1 L of Luria broth containing 100 μg/ml ampicillin and 40 μg/ml chloramphenicol was inoculated with the transformants and shaken at 30 °C until the A_{600nm} was equal to 0.5. Synthesis of the recombinant protein was initiated by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM and incubation continued at 30 °C for 5 h. Cells were then harvested and lysed by the lysozyme method according to Sambrook *et al.* (1989). After centrifugation (16,000 x *g* for 10 min) the supernatant was applied at 4 °C to a 2 ml Talon™ metal affinity column (Clontech) equilibrated in 25 mM Tris-Cl (pH 8.0), 200 mM NaCl and 10 mM β-mercaptoethanol. Following extensive washing with equilibration buffer (EB), the column was washed successively with 10 mM imidazole in EB, 5 mM ATP, 10 mM MgCl and 50 mM KCl in EB, before an essentially pure preparation of rat px-2,4-DCR#1 (50 mg) was eluted with 100 mM imidazole in EB. The identities of *E.coli* DnaK and GrpE (recovered in the 5 mM ATP eluate) were determined by direct N-terminal protein sequencing as described previously (Hartman *et al.*, 1992).

Removal of the N-terminal hexahistidine tag was performed at 22 °C for 2 h with a 1:50 (w/w) ratio of human thrombin (Boehringer Mannheim): recombinant rat px-2,4-DCR#1 in 12.5 mM Tris-Cl (pH 8.0), 100 mM NaCl, 10 mM β-mecaptoethanol, 100 mM imidazole and 2.5 mM $CaCl_2$. Cleavage of recombinant rat px-2,4-DCR#1 into two distinct domains was achieved by carrying out the digestion as described above at 22 °C for 2 h, followed by a

further incubation at 4 °C for 14 days. The masses of recombinant rat liver px-2,4-DCR#1 (minus the N-terminal hexahistidine tag) and the two thrombin derived domains were determined by Yogi Hayasaka (Australian Wine Research Institute, Glen Osmond, SA., Australia) on an electrospray ionisation-triple quadrupole mass spectrometer (PE SCIEX API300) essentially as described by Hartman *et al.* (1992)

*6.2.7 **Enzyme assays***—2,4-dienoyl-CoA reductase activity was measured spectrophotometrically at 340 nm by monitoring the substrate-dependent oxidation of NADPH at 22 °C as described previously (Kunau and Dommes, 1978). The assay mixture contained 0.1 M NaPi (pH 7.3), 125 μM NADPH (Boehringer Mannheim), 50 μg/ml BSA and either 60 μM 2,4-hexadienoyl-CoA or 60 μM 2,4-decadienoyl-CoA as a substrate. The substrates and the purified yeast Sps19p protein, as a positive control, were generously provided by J. Kalervo Hiltunen and Kari T. Koivurantac (University of Oulu, Finland).

*6.2.8 **Northern blot analysis***—For identification of the tissue distribution of the reductase transcripts, a Northern blot with mRNA from eight different rat tissues (2 μg each) was purchased from Clontech and prehybridised, hybridised and analysed as described in the Southern blot analysis section above. The rat px-2,4-DCR#1 cDNA probe was comprised of nucleotides -96 to 177 (Fig. 4).

6.3 RESULTS

6.3.1 DnaK-affinity purification of rat px-2,4-DCR#1 and mt-2,4-DCR#1—In Chapters 2 and 5 (Naylor *et al.*, 1995, 1999) it was established that two distinct subsets of mature rat liver organellar proteins were selectively retained on immobilised *E. coli* Hsp70 (DnaK). One subset constitutes the mitochondrial GrpE-like co-chaperones which form a complex with DnaK, that is not disrupted in the presence of 1 M KCl, but readily in the presence of 5 mM ATP (Naylor *et al.*, 1995, 1998, 1999). The other subset most likely represents general Hsp70 protein substrates and these are dissociated from DnaK in the presence of 1 M KCl and the DnaK-bound GrpE-like co-chaperones (Naylor *et al.*, 1999) (Fig. 1*A*, lane 3). Previous studies are consistent with K^+ ions being an indispensable cofactor for the GrpE mediated release of substrate proteins from Hsp70 members (Palleros *et al.*, 1993; Harrison *et al.*, 1997; Motohashi *et al.*, 1997). Amongst the major constituents identified by peptide sequencing from the 1 M KCl eluate of a DnaK column were peroxisomal multifunctional enzyme isoform-1 (px-MFE-1), the mitochondrial alpha subunit of succinyl CoA synthetase (α-SCS) which is known to form an αβ-dimer, an isoform of mitochondrial 2,4-dienoyl CoA reductase (mt-2,4-DCR#1) which forms an 120 kDa homotetramer, and an undescribed protein suspected to represent a peroxisomal isoform of 2,4-dienoyl CoA reductase (Chapter 5, Naylor *et al.*, 1999). In the present study we have undertaken the further characterisation of the putative rat peroxisomal 2,4-dienoyl CoA reductase (hereafter referred to simply as px-2,4-DCR#1).

Recently, a yeast peroxisomal isoform of 2,4-dienoyl CoA reductase (Sps19p) has been reported and shown to be active as an 69 kDa homodimer (Gurvitz *et al.*, 1997). To determine the oligomeric state of rat px-2,4-DCR#1, the protein constituents of the 1M KCl eluate were subjected to gel filtration chromatography and their elution times compared to those of known standards. Figure 1*B* reveals that as expected, px-MFE-1 exists as a ~70 kDa monomer, α-SCS as a ~31 kDa monomer in the absence of the β-subunit, mt-2,4-DCR#1 as a 120 kDa homotetramer, and rat px-2,4-DCR#1 exists as a homodimer with a native molecular mass of ~65 kDa.

Like the rat px-MFE-1 protein (Osumi *et al.*, 1985), isolated px-2,4-DCR#1 yields no N-terminal sequence during Edman protein sequencing (Chapter 5, Naylor *et al.*, 1999). In order to identify the N-terminal modification group, the molecular mass of purified rat liver

px-2,4-DCR#1 was determined by mass spectrometry as 32,344 ± 2 Da. As will be discussed in the following section, I have isolated the full-length cDNA encoding rat liver px-2,4-DCR#1 and determined its sequence. The predicted molecular mass of the primary translation product comprising Met^{1} to Leu^{303} is 32, 432.91 Da. Removal of the N-terminal methionine and acetylation of Gly^{2} would therefore result in a protein with a M_r of 32,345.71 Da in excellent agreement with the experimentally determined M_r of 32,344 ± 2 Da.

*6.3.2 **Cloning of rat px-2,4-DCR#1 cDNA***—Using eight peptide sequences I previously obtained from the putative rat px-2,4-DCR#1 (Chapter 5, Naylor *et al.*, 1999) (Fig. 2) to search various databases, an uncharacterised 269 bp rat EST (GenBank™ accession no. H35326) was identified and later shown to correspond to a segment within the 5'-end of the px-2,4-DCR#1 cDNA (nucleotides -69 to 198, Fig. 2). Based on the sequences of peptide #6 and the EST, degenerate and specific oligonucleotides were designed and used to obtain the first 273 bp of the rat px-2,4-DCR#1 transcript by 5'-RACE PCR (nucleotides -96 to 177, Fig. 2). The PCR product was then used to probe a rat liver cDNA library and a px-2,4-DCR#1 cDNA clone (nucleotides -16 to 1092, Fig. 2) was isolated. An ATG codon (nucleotides 1 to 3) that fulfils the Kozak requirements for initiation of translation in eukaryotes (Kozak, 1987) was evident in both the 5'-RACE PCR product and the corresponding cDNA clone, which are identical in a 193 bp overlap. I am therefore confident that the composite cDNA shown in Fig. 2 corresponds to a single full-length px-2,4-DCR#1 transcript. Furthermore, by utilising this cDNA in genomic Southern blot analysis, it was clear that px-2,4-DCR#1 and mt-2,4-DCR#1 are encoded by distinct genes which probably exist as single copies within the rat genome (Fig. 3).

*6.3.3 **Rat px-2,4-DCR#1 exhibits significant sequence identity with members of the 2,4-dienoyl CoA reductase family at the amino acid level***—Database searches established that rat px-2,4-DCR#1 exhibits significant positional similarity with the human homotetrameric mitochondrial 2,4-DCR 120 kDa isoform (mt-2,4-DCR#1, 45.2 %), the corresponding rat enzyme (mt-2,4-DCR#1, 44.2 %), and the yeast peroxisomal 2,4-DCR (Sps19p or px-2,4-DCR#1, 46.7 %) (Fig. 4). In addition, several previously uncharacterised 2,4-dienoyl CoA reductase-like sequences from mouse, human and *Caenorhabditis elegans* were also identified (Fig. 4). The three *C. elegans* genomic sequences were complete, the mouse cDNA sequence was compiled and edited from several ESTs, while the human cDNA sequence was obtained

by the complete sequencing of two independently isolated ESTs (Fig. 5). The deduced amino acid sequence of rat px-2,4-DCR#1 exhibits 94.7 % positional similarity with the mouse sequence (px-2,4-DCR#1), while the positional similarity with the other 2,4-DCR-like sequences are: 81.5 % for human (px-2,4-DCR#1), 47.9 % for the first *C. elegans* sequence (px-2,4-DCR#1), 44.2 % for the second *C. elegans* sequence (2,4-DCR#1) and 43.9 % for the third *C. elegans* sequence (2,4-DCR#2). As expected from the NADPH-dependence of known 2,4-dienoyl CoA reductases, all the reductase-like sequences identified in this study contain a $\beta_1\alpha\beta_2$ nucleotide-binding motif (Fig. 4) (Wierenga *et al.*, 1983). Comparison of the sequences identified in this study with previously described sequences of both the rat and human mt-2,4-DCR#1 and yeast Sps19p, revealed that while rat, mouse, human and one of the three *C. elegans* sequences lack apparent N-terminal mitochondrial targeting sequences, they instead contain the PTS-1 tripeptide SKL or a variant thereof (Fig. 4). Utilising a specific antiserum to rat px-2,4-DCR#1, preliminary immunoelectron microscopy analysis revealed that px-2,4-DCR#1 is located in the peroxisomal matrix, suggesting that the C-terminal PTS-1 (...ARL, Fig. 2) is indeed functional (immunoelectron microscopy was performed, as part of a collaboration, with J. Kalervo Hiltunen and Kari T. Koivurantac, University of Oulu, Finland).

Interestingly, the deduced amino acid sequence of rat px-2,4-DCR#1 also revealed considerable positional similarity (42.5 %) with the *Synechocystis* sp. PCC6803 3-ketoacyl-acyl carrier protein (ACP) reductase (Fig. 4). The significance of this similarity, if any, is unknown, although it is unlikely that the similarity reflects the function of rat putative px-2,4-DCR#1 as all mammalian ACPs constitute part of a cytosolic multifunctional enzyme residing in a single polypeptide chain known as fatty acid synthase (reviewed by Zubay, 1989).

6.3.4 Characterisation of recombinant rat px-2,4-DCR#1—While rat liver px-2,4-DCR#1 could be easily affinity purified on a DnaK-column (Fig. 1*A*, *lane 3*), I was unable to ascribe an activity to px-2,4-DCR#1 due to the contamination of this preparation with the mt-2,4-DCR#1 protein. I therefore attempted to remove rat liver mt-2,4-DCR#1 by gel filtration (Fig. 1*B*) and anion exchange chromatography (data not shown) but without success. The limitation of this approach was the amount of px-2,4-DCR#1 (~200 µg from 50 rat livers) that could be obtained from the DnaK-column (~100 mg of DnaK). I therefore expressed recombinant rat px-2,4-DCR#1 in *E. coli* for the further investigation of its activity. Most of the recombinant protein accumulated as insoluble aggregates when produced in *E. coli*, however, some of the

protein was obtained in a soluble homodimeric state, suggesting that it is folded and assembled correctly (data not shown). Fig. 6*A* shows the expression of N-terminally hexahistidine tagged rat px-2,4-DCR#1 in *E. coli* (*lanes 2 and 3*), its purification on an IMAC column (*lane 4*), and removal of the hexahistidine tag from the purified protein with thrombin (*lane 5*). Unfortunately, neither the hexahistidine tagged or untagged versions of recombinant rat px-2,4-DCR#1 were capable of catalysing the NADPH-dependent reduction of either trans-2,trans-4-hexadienoyl-CoA or trans-2,trans-4-decadienoyl-CoA *in vitro* (data not shown). In the quest to establish activity for recombinant px-2,4-DCR#1 I tested whether expression at lower temperature (16 °C) or whether the presence of 10 % (w/v) glycerol and 200 mM NaCl during the extraction, purification and enzyme assays could effect activity. Neither of these parameters alone or in combination allowed the generation of active recombinant px-2,4-DCR#1 (data not shown). Furthermore, I also tested the ability of recombinant px-2,4-DCR#1 to function as a 3-ketoacyl-CoA reductase or as a short chain alcohol dehydrogenase, but again without success (data not shown). Unlike the native px-2,4-DCR#1 protein isolated from rat liver, the px-2,4-DCR#1 protein produced recombinantly in *E. coli* was not N-terminally acetylated and was relatively less stable, being prone to aggregation upon freeze/thawing (data not shown). I therefore suggest that N-terminal acetylation may enhance the stability of the px-2,4-DCR#1 and may be important for its function. However this awaits further investigation.

Interestingly, while a short incubation (2 h at 22 °C) of recombinant px-2,4-DCR#1 with thrombin completely removed the N-terminal hexahistidine tag, a prolonged incubation (2 weeks at 4 °C) resulted in the cleavage of the protein into two distinct domains (Fig. 6*A*, *lane 5*). The amino acid residues which comprise each domain were determined by mass spectrometry analysis and results are shown in Fig. 6*A*. This finding may suggest a bilobal structure of the protein where distinct functions such as dimerisation, nucleotide- and substrate-binding might be assigned to separate domains. Of further interest here is the observation that an ~70 kDa and ~28 kDa protein could be eluted from the column with a 5 mM ATP wash, prior to the removal of hexahistine tagged px-2,4-DCR#1 with 100 mM imidazole (Fig. 6*B*, *lane 2*). N-terminal protein sequencing revealed the identities of these proteins as *E. coli* DnaK (GKIIGIDLGTTN...) and GrpE (SSKEQKTPEGQA...). The identities of DnaK and GrpE are consistent with our previous study, where native rat px-2,4-DCR#1 was observed to form a stable complex with immobilised DnaK in the presence of rat mitochondrial GrpE but absence of ATP (see Chapter 5, Naylor *et al.*, 1999).

6.3.5 Expression of px-2,4-DCR#1 and mt-2,4-DCR#1 transcripts—In mammalian cells, long-chain polyunsaturated fatty acids are initially metabolised in peroxisomes (with the assistance of a 2,4-dienoyl CoA reductase) and the shortened polyunsaturated fatty acids are then shuttled into mitochondria for their further oxidative breakdown (with the assistance of mt-2,4-DCR#1) into intermediates which are eventually employed in the generation of ATP (reviewed by Kunau *et al.*, 1995). Consistent with both the location and coordinate functioning of β-oxidation within peroxisomes and mitochondria of mammalian cells, Northern blot analysis revealed that the expression of the px-2,4-DCR#1 and mt-2,4-DCR#1 ~1.2 kilobase transcripts were similar in several tissues, and was most prominent in kidney, liver, and heart (Fig. 7). I rule out cross-hybridisation of the probes with either transcript as the stringency of hybridisation was identical to that used for the Southern Blot in Fig. 4.

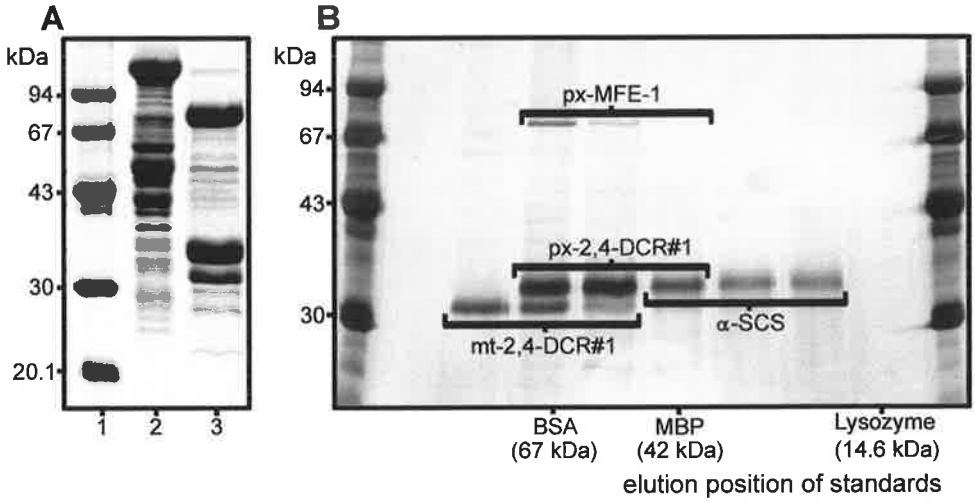


Fig. 1. **The rat liver dimeric px-2,4-DCR#1 and tetrameric mt-2,4-DCR#1 isoforms can be purified by DnaK-affinity chromatography. (*A*)** Organelle preparations were obtained by sucrose gradient centrifugation, lysed and the crude protein mixture loaded onto a DnaK-affinity column. Following extensive washing with equilibration buffer, several bound proteins (including both reductases) were eluted with 1 M KCl. The polypeptide content of all fractions was analysed by SDS-PAGE in a 12.5% gel and stained with Coomassie Brilliant Blue. *Lane 1*, molecular mass standards, *lane 2*, starting material for the DnaK column and *lane 3*, the 1 M KCl eluate. **(*B*)** A portion of the 1M KCl eluate containing predominantly px-2,4-DCR#1, mt-2,4-DCR#1, px-MFE-1, and α-SCS was partially fractionated using a Superose-12 FPLC gel filtration column. The polypeptide content of each fraction was analysed by electrophoresis in a 12.5% Tris-Glycine gel followed by silver staining. The retention time of fractions increase from left to right and the elution position of BSA (69 kDa), maltose binding protein (42 kDa) and lysozyme (14.6 kDa) is shown on the x-axis.

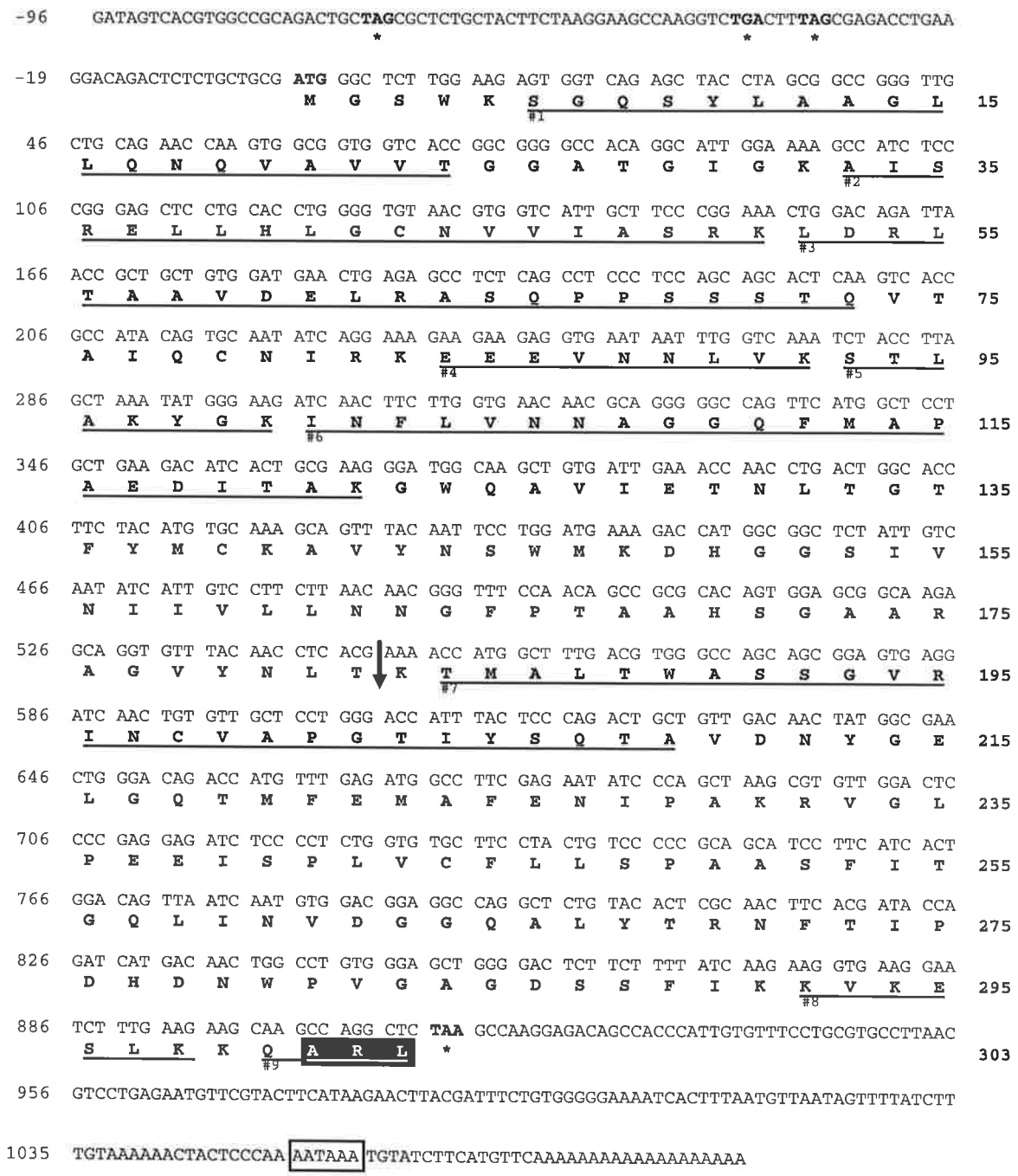


Fig. 2. **The cDNA and deduced amino acid sequence of rat px-2,4-DCR#1.** The numbering of nucleotides is shown on the left-hand side and the first base of the putative initiator codon is numbered +1. Amino acids are given by their single letter code and they are numbered on the right-hand side. Regions of the deduced amino acid sequence that are identical to peptide sequences obtained from purified rat px-2,4-DCR#1 are underlined and labelled #1 to #9. A putative polyadenylation site is boxed and in-frame stop codons, on either side of the open reading frame, are indicated with asterisks. The position at which thrombin appears to cleave the protein into two distinct domains is indicated with a vertical arrow while the residues of a characteristic C-terminal tripeptide PTS-1 are shaded black. This sequence has been submitted to GenBank™ and given the accession number AF021854.

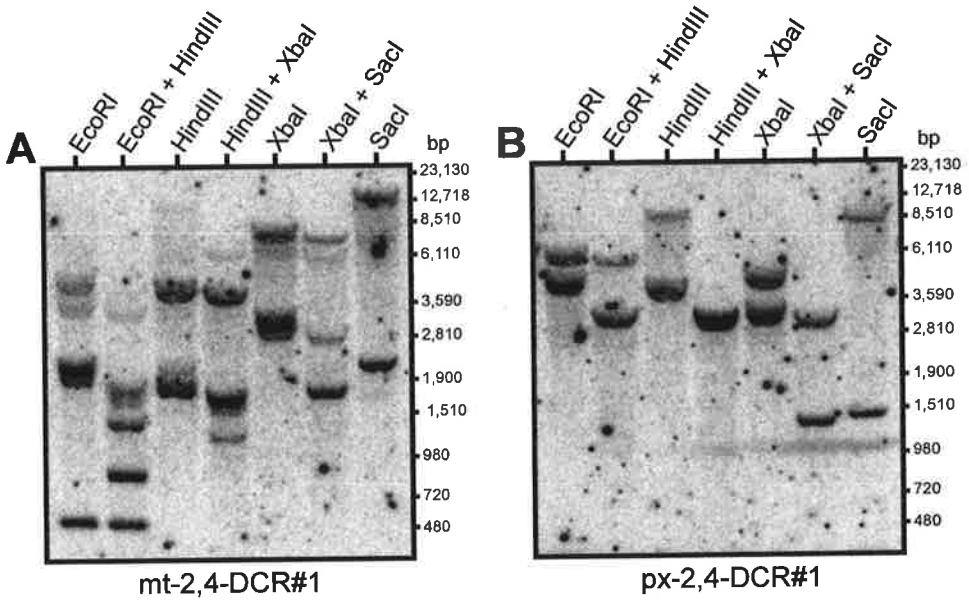
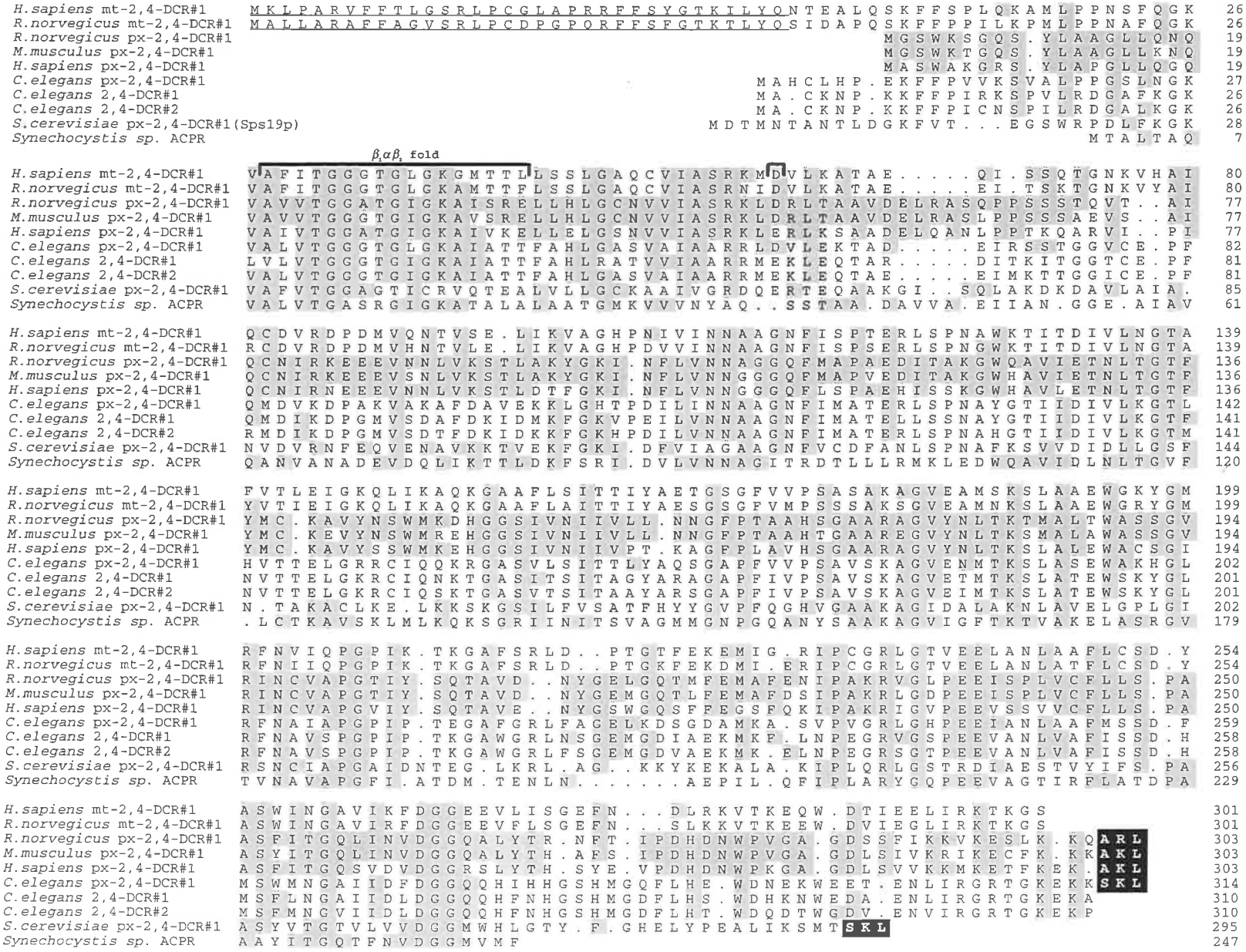


Fig. 3. **Southern blot analysis reveals that px-2,4-DCR#1 and mt-2,4-DCR#1 are encoded by distinct genes within the rat genome.** Each lane contained 10 μg of rat liver genomic DNA digested with the indicated restriction enzymes. **(*A*)** The filter was first probed with the ^{32}P-labelled mt-2,4-DCR#1 cDNA, then stripped and **(*B*)**, reprobed with the ^{32}P-labelled px-2,4-DCR#1 cDNA.

Fig. 4. **Multiple sequence alignment for the predicted amino acid sequences of 2,4-dienoyl-CoA reductase proteins.** The alignment was made using the PILEUP program (Genetic Computer Group, Madison, WI). Residues identical to those in rat px-2,4-DCR#1 are shaded grey while residues of the tripeptide PTS-1 are shaded black. The residues constituting a $\beta_1\alpha\beta_2$ nucleotide-binding motif are indicated above the second sequence block by brackets. Residues denoting the rat mt-2,4-DCR#1 and putative human mt-2,4-DCR#1 mitochondrial targeting sequences are underlined. The sources of the sequences were: *Homo sapiens* (mitochondria, GenBank™ accession no. L26050), *Rattus norvegicus* (mitochondria, PIR proteins accession no. S11021), *Rattus norvegicus* (peroxisomes, this study, GenBank™ accession no. AF021854), *Mus musculus* (peroxisomes, compiled and edited from GenBank™ accession nos. AA241896, AA212886, AA110264 and AA458224 in this study), *Homo sapiens* (peroxisomes, this study, see Fig. 5), *Caenorhabditis elegans* (unknown cellular locations, GenBank™ accession nos. Z79756, Z49969, and Z66500, respectively) and *Saccharomyces cerevisiae* (peroxisomes, GenBank™ accession no. X78898). For comparison, the *Synechocystis* sp. PCC6803 3-ketoacyl-ACP reductase (ACPR) sequence (GenBank™ accession no. D90907) is shown at the bottom of the alignment.


β1αβ2 fold
H.sapiens mt-2,4-DCR#1
R.norvegicus mt-2,4-DCR#1
R.norvegicus px-2,4-DCR#1
M.musculus px-2,4-DCR#1
H.sapiens px-2,4-DCR#1
C.elegans px-2,4-DCR#1
C.elegans 2,4-DCR#1
C.elegans 2,4-DCR#2
S.cerevisiae px-2,4-DCR#1 (Sps19p)
Synechocystis sp. ACPR

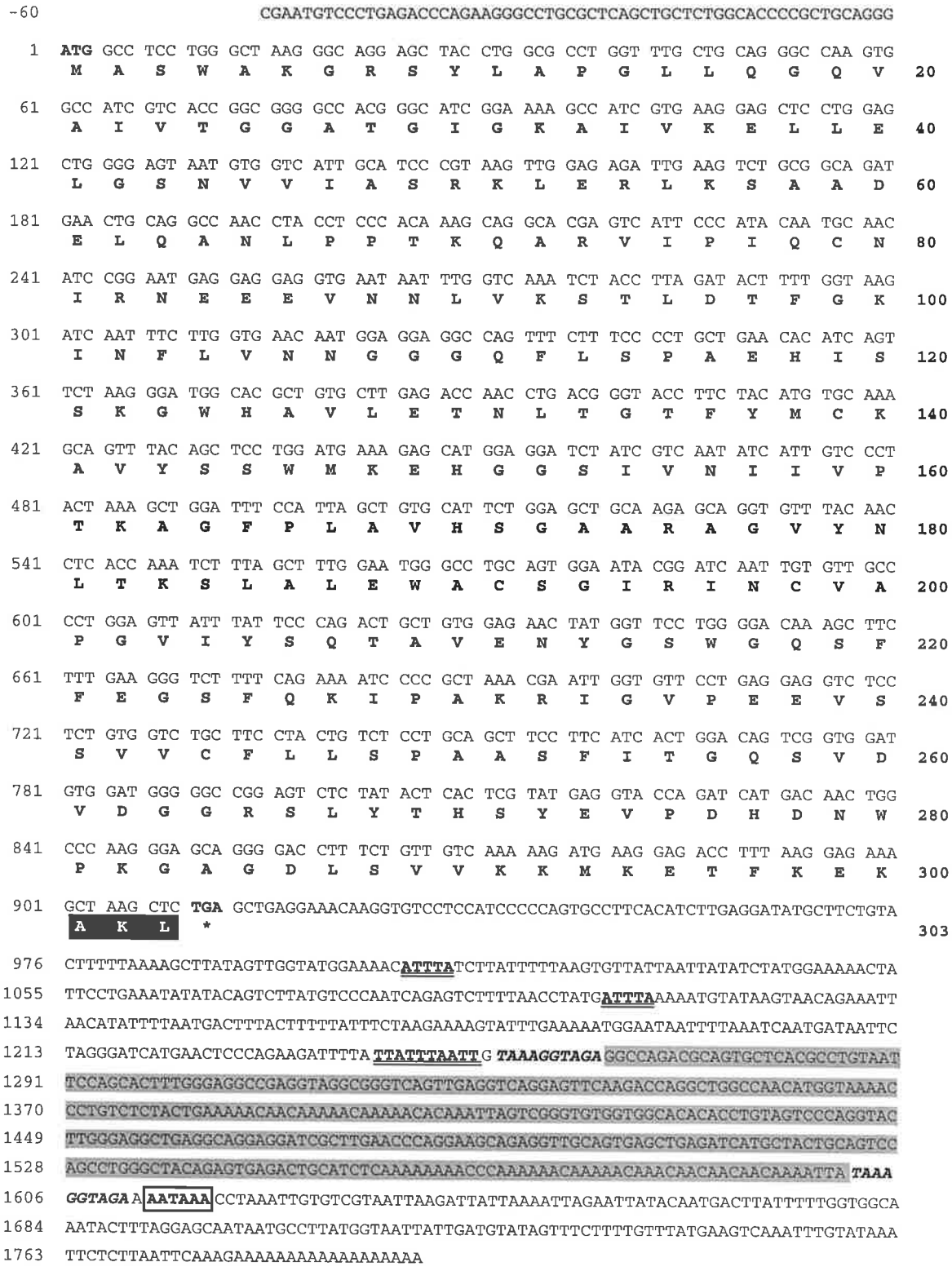


Fig. 5. **The cDNA and deduced amino acid sequence of human px-2,4-DCR#1.** The numbering of nucleotides is shown on the left-hand side and the first base of the putative initiator codon is numbered +1. Amino acids are given by their single letter code and they are numbered on the right-hand side. A putative polyadenylation site is boxed and an in-frame stop codon at the end of the open reading frame is indicated with an asterisk. A characteristic C-terminal tripeptide PST-1 is shaded black. In the 3'-UTR the position of possible RNA destabilisation sequences (double underlined) and an Alu repetitive element (shaded grey) which is flanked by characteristic direct repeats (*TAAAGGTAGA*), are shown. This cDNA sequence was obtained by sequencing both strands of two independently isolated and incompletely characterised ESTs (GenBank™ accession nos. AA232176 and AA232159).

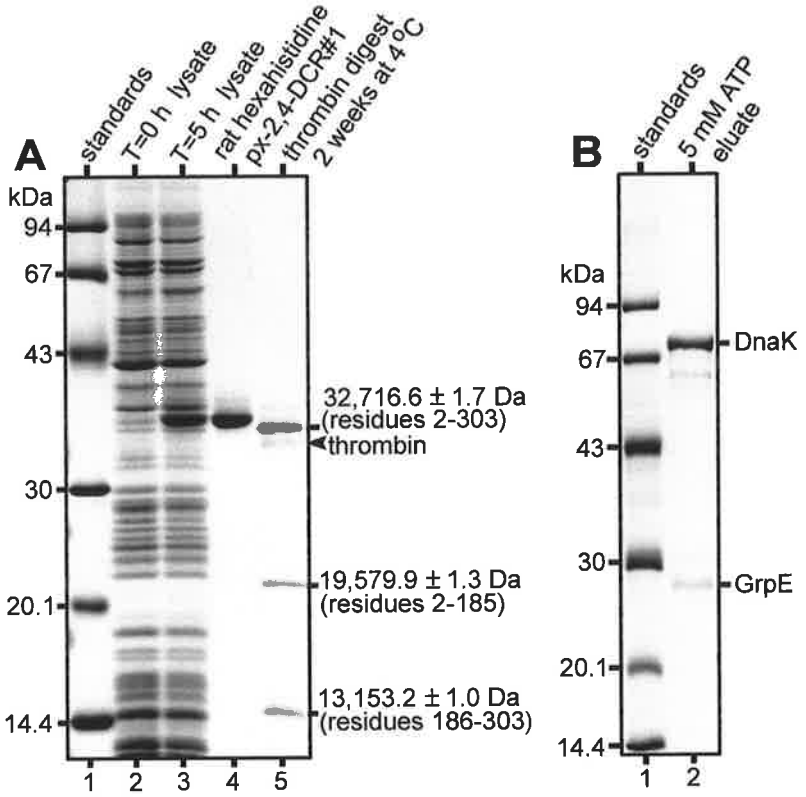


Fig. 6. **Characterisation of rat px-2,4-DCR#1 expressed in *E.coli*.** The synthesis of rat px-2,4-DCR#1, with an N-terminal hexahistidine tag, was induced in *E. coli* and carried out for 5 h (*panel A, lanes 2* and *3*). The recombinant protein was retained on an IMAC column preequilibrated in EB. After extensive washing in EB (200 ml), the column was successively washed with 100 ml of EB containing 10 mM imidazole, 50 ml of EB containing 5 mM ATP (*panel B, lane 2*), 10 mM MgCl and 50 mM KCl, and finally an essentially pure preparation of px-2,4-DCR#1 was eluted with EB containing 100 mM imidazole (*panel A, lane 4*). The N-terminal hexahistidine tag was removed from px-2,4-DCR#1 upon incubation with 2.5 mM $CaCl_2$ and a 1:50 (w/w) ratio of human thrombin: px-2,4-DCR#1 at 22 °C for 2 h. A further incubation at 4 °C for 2 weeks resulted in the cleavage of px-2,4-DCR#1 into two distinct polypeptides (*panel A, lane 5*). The polypeptide content of all fractions was analysed by SDS-PAGE in a 12.5% Tris-Glycine gel followed by staining with Coomassie Brilliant Blue. The molecular mass values of px-2,4-DCR#1 (minus the hexahistine tag) and the two thrombin-derived domains are shown, while the amino acids residues which comprise each entity are given in parentheses. Molecular mass standards were run in *lane 1* of *panels A* and *B*.

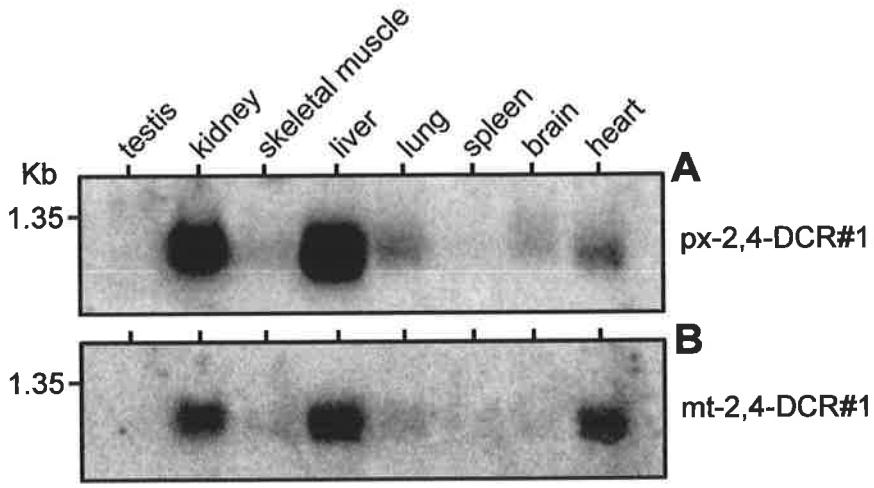


Fig. 7. **Northern blot analysis indicates a similar level of px-2,4-DCR#1 and mt-2,4-DCR#1 mRNA in several rat organs.** Each lane contained approximately 2 μg of mRNA isolated from the indicated tissues and loadings were adjusted to contain equal amounts of β-actin mRNA. **(*A*)** The filter was first probed with the ^{32}P-labelled px-2,4-DCR#1 cDNA, then stripped and **(*B*)**, reprobed with the ^{32}P-labelled mt-2,4-DCR#1 cDNA.

6.4 DISCUSSION

In mammalian cells, β-oxidation of fatty acids is compartmentalised into both mitochondria and peroxisomes (reviewed by Kunau *et al.*, 1995), whilst in yeast cells, it is solely confined to peroxisomes (Kunau *et al.*, 1988). Unsaturated fatty acids with double bonds at even-numbered, and possibly also at odd-numbered positions, require the auxiliary enzyme 2,4-dienoyl CoA reductase for their breakdown (Kunau and Dommes, 1978; Cuebas and Schulz, 1982; Smeland *et al.*, 1992; Chen *et al.*, 1994). The essential requirement of 2,4-dienoyl CoA reductases has been exemplified in both *E. coli* and *S. cerevisiae* mutants grown on petroselineate as the sole carbon source (You *et al.*, 1989; Gurvitz *et al.*, 1997). However, their importance is perhaps best exemplified in humans, where despite dietary therapy, an inborn deficiency in 2,4-dienoyl-CoA reductase was lethal (Roe *et al.*, 1990). At least three isoforms of 2,4-dienoyl CoA reductase exist in mammals, two mitochondrial reductases (native molecular mass 120 kDa and 60 kDa) and a third peroxisomal reductase (Dommes *et al.*, 1981; Hakkola and Hiltunen, 1993). Although a peroxisomal 2,4-dienoyl CoA reductase (Sps19p) has been cloned and studied from *S. cerevisiae* (Gurvitz *et al.*, 1997), the lack of information on a mammalian peroxisomal reductase has undoubtedly obscured investigations into the evolutionary reason for the cellular compartmentalisation of β-oxidation in mammals, and has fueled a debate for either a distinct peroxisomal isoform or the dual targeting of a mitochondrial isoform to both organelles in mammalian cells (Hakkola *et al.*, 1989; Mizugaki *et al.*, 1996).

In the present study, I have undertaken the characterisation of a DnaK-bound ~33 kDa rat liver protein of which several derived peptide sequences displayed significant homology to the rat mitochondrial 120 kDa isoform of 2,4-dienoyl CoA reductase (mt-2,4-DCR#1) (Chapter 5, Naylor *et al.*, 1999). I report that, like the yeast Sps19p protein, the ~33 kDa rat protein is a homodimer with a molecular mass of ~65 kDa and is localised to the peroxisomal matrix, due to the presence a C-terminal PTS-1 (....ARL) (Gurvitz *et al.*, 1997). Isolation of the cDNA encoding the ~33 kDa protein revealed that, at the amino acid level, it shares ~44.2 % positional similarity with rat mt-2,4-DCR#1, ~46.7 % positional similarity with yeast Sps19p and up to ~94.7 % positional similarity with several previously uncharacterised mouse, human and *C. elegans* 2,4-dienoyl CoA reductase-like ESTs. Consistent with this identification, all of the amino acid sequences, including px-2,4-DCR#1, contain an $\beta_1\alpha\beta_2$ nucleotide-binding motif which is expected given the NADPH-dependence of all previously

described 2,4-dienoyl CoA reductases (Wierenga *et al.*, 1983; Gurvitz *et al.*, 1997). Genomic Southern blot analysis showed that the ~33 kDa rat protein and mt-2,4-DCR#1 are encoded by distinct genes within the rat genome, but Northern blot analysis revealed that their transcripts are expressed at almost identical levels within several distinct rat organs. The latter is consistent with the coordinate functioning of β-oxidation in peroxisomes and mitochondria of mammalian cells, and may suggest that the ~33 kDa peroxisomal protein and mt-2,4-DCR#1 fulfil similar and synergistic roles in their respective organelles. For these reasons I suggest that the ~33 kDa protein is most likely a new peroxisomal isoform of 2,4-dienoyl CoA reductase, and it should be designated px-2,4-DCR#1.

While rat liver px-2,4-DCR#1 affinity purified on a DnaK-column is probably active, I could not purify substantial amounts of the protein to homogeneity without the use of RP-HPLC or without contamination by mt-2,4-DCR#1. I were therefore unable to ascribe an activity to the native px-2,4-DCR#1 protein. In a further attempt to ascribe an activity to px-2,4-DCR#1, I expressed the protein in *E. coli*. Most of the recombinant protein accumulated as insoluble aggregates, however, some of the protein was obtained in a soluble homodimeric state. Unfortunately this preparation was inactive when tested on the substrates trans-2,trans-4-hexadienoyl-CoA or trans-2,trans-4-decadienoyl-CoA, but unlike the native px-2,4-DCR#1 protein, the recombinant protein readily precipitated upon freeze/thawing. In our previous studies with the rat molecular chaperone Cpn10 (Hsp10), it was observed that N-terminal acetylation effectively increases the stability of the protein and may augment the ability of the protein to interact with and traverse the mitochondrial membrane, but does not influence the oligomeric structure (Ryan *et al.*, 1995; Jarvis *et al.*, 1995). Interestingly, I determined that native rat px-2,4-DCR#1 is also N-terminally acetylated but, as expected, mass spectrometry revealed that the recombinant form was not acetylated, consistent with the inability of *E. coli* to perform post-translational acetylation. Therefore, the N-terminal acetylation of rat px-2,4-DCR#1 may in fact be essential to both the stability and activity of this protein.

An intriguing question, is how reductases, operating in different cellular organelles, are regulated to function in concert with one another ? One of several possible explanations might be that regulation is brought about at the protein level, through the direct modulation of their activities and perhaps their abundance. Prime candidates that would be capable of imposing such regulation are molecular chaperones, and accordingly, I have in this and the previous Chapter demonstrated that both px-2,4-DCR#1 and mt-2,4-DCR#1 can form a stable interaction with the *E. coli* molecular chaperone DnaK. In *E. coli*, DnaK tightly regulates the

heat shock (stress) response by specifically interacting with, and thereby modulating the activity of, the heat shock promoter-specific σ^{32} subunit of RNA polymerase (Bukau, 1997). In an analogous manner, it could be envisaged that both the mitochondrial and a peroxisomal Hsp70 member may be able to tightly regulate the separate β-oxidation pathways by specifically interacting with, and thereby modulating the activities of the respective reductases. Consistent with this idea, an isoform of the peroxisomal multifunctional enzyme (px-MFE-1), which fulfils several crucial steps in the β-oxidation pathways, has also been observed to interact with DnaK, but most importantly, the presence of mt-Hsp70 and BiP significantly diminished the interaction of both reductases and px-MFE-1 with DnaK (see Chapter 5, Naylor *et al.*, 1999).

The interaction of both px-2,4-DCR#1 and mt-2,4-DCR#1 with members of the Hsp70 family could, alternatively, merely reflect the documented functions of cytosolic Hsp70 in the folding and targeting of both mitochondrial and peroxisomal protein substrates (reviewed in Ryan *et al.*, 1997; Walton *et al.*, 1994; Crookes and Olsen, 1998). However, the enrichment of these reductases and px-MFE-1 within a small subset of fully synthesised organellar proteins, which are selectively retained on DnaK in the absence of mt-Hsp70 and BiP (Chapter 5, Naylor *et al.*, 1999), does give merit to the idea of a regulatory role for organellar Hsp70 members. It should be noted though, that thus far, a Hsp70 member has not been identified in the peroxisomal matrix. Recently, a plastid Hsp70 was shown to be targeted to both the matrix of plastids and glyoxisomes, thus describing the first Hsp70 member to be identified in the matrix of a microbody (Wimmer *et al.*, 1997). The failure to identify a glyoxisomal Hsp70 previously was most likely obscured by the established existence of the protein in plastids. Therefore, an analogous situation may exist for a putative peroxisomal Hsp70. Indeed, in Chinese hamster cells, mt-Hsp70 was observed not only in mitochondria but also in unidentified cytoplasmic vesicles (Singh *et al.*, 1997; Soltys and Gupta, 1999). These unidentified cytoplasmic vesicles could represent peroxisomes.

In conclusion, the findings in this study reveal that in mammalian cells a peroxisomal protein (px-2,4-DCR#1) with a very high degree of sequence similarity and an identical oligomeric structure to the yeast peroxisomal 2,4-dienoyl CoA reductase exists. Furthermore, while px-2,4-DCR#1 also has a very high degree of sequence similarity with mt-2,4-DCR#1, these proteins are clearly encoded by distinct genes and are almost certainly targeted to only one organelle. The tools generated in this study may therefore assist in elucidating the functional and evolutionary reason for the cellular compartmentalisation of β-oxidation in

mammals, and may be invaluable for the future screening and molecular characterisation of patients with inborn defects in the oxidative degradation of unsaturated fatty acids.

Chapter 7

Summary and future directions

Summary and future directions

The attainment of well-defined three dimensional structures and correct subcellular location is a prerequisite for protein function. It is now generally accepted that the efficient targeting of proteins to their correct subcellular destinations and their folding often, if not always, requires the action of so called molecular chaperones. Following the recent completion of the *S. cerevisiae* and *E. coli* genome sequencing projects, scientists have been permitted, for the first time, to analyse a complete set of molecular chaperones involved in protein targeting and folding within an organism. Furthermore, the extensive characterisation of chaperones from bacteria and fungi has proven instrumental to the investigation of their less studied counterparts in higher eukaryotes such as mammals. Acknowledging that there is likely to be unique requirements for mitochondria from cells that experience developmental- and tissue-specific gene expression, our laboratory has focused its efforts on the study of the mammalian molecular chaperone complement that is involved in mitochondrial protein targeting, import and folding. Specifically, as part of this overall effort, the major part of the work described in this thesis was concerned with the identification and subsequent characterisation of mammalian, mitochondrial GrpE-like proteins.

Chapter 2 describes the use of affinity chromatography on DnaK-columns to purify for the first time, a mammalian mitochondrial GrpE-like protein (mt-GrpE#1). This rapid and efficient purification method was adapted and refined from a previously described procedure for the affinity purification of *E. coli* GrpE. The apparent general application of this technique indicates its usefulness in future studies of as yet unidentified GrpE homologues.

Chapter 3 reports the cloning of a cDNA specifying rat mt-GrpE#1 and shows that this homologue exhibits only 20% positional identity with its bacterial and fungal counterparts. In fact, comparison of all known GrpE family members revealed that they exhibit a much lower degree of sequence identity than do the well studied members of the Hsp70, Hsp60 and Hsp10 families. Further studies established that the encoded GrpE homologue is of low abundance, only slightly stress inducible and confined to mitochondria but found in most, if not all, rat organs in accordance with its suspected vital cellular function. The low degree of sequence identity amongst the GrpE homologues of different organisms raises the possibility that proteins

with GrpE-like functions exist throughout the biological kingdom and that a search for these have to be based on functional rather than sequence characteristics.

Chapter 4 describes the identification of a second mammalian mitochondrial GrpE homologue (mt-GrpE#2) which exhibits ~47 % positional identity with mt-GrpE#1. The functional integrity of mt-GrpE#1 and mt-GrpE#2 was verified by their ability to specifically interact with and stimulate the ATPase activity of mammalian mitochondrial Hsp70 (mt-Hsp70). The coexistence of GrpE isoforms in a single subcellular location has not been reported previously for any other species, even when complete genome sequences are known as for yeast and bacteria. The significance of this finding is yet to be established in terms of mitochondrial chaperone activities and functions. The tools generated in chapters 2 and 3 will assist in elucidating the importance, or otherwise, of these components for the biogenesis and maintenance of proteins in higher eukaryotic cells. Although highly speculative, the co-existence of similar chaperones with similar functions may help to ultimately explain how mitochondria from mammalian sources have evolved to attain greater specialisation and diversity than their counterparts from lower eukaryotes.

It is expected that like in the *E. coli* cytosol and yeast mitochondrial matrix, multiple isoforms of mt-Hsp70 will be found in the mammalian mitochondrial matrix. If this is the case, the isoforms of mt-GrpE identified in this study may interact differentially with individual Hsp70 members to fulfil specific tasks. Alternatively, it could be envisaged that different individual mt-GrpEs assist a single mt-Hsp70 differentially during the processes of mitochondrial protein import and subsequent folding. Future studies where the expression of the individual mt-GrpE genes is knocked out in transgenic mice may help establish their respective functions.

Chapter 5 describes how immobilised DnaK was employed to identify putative general Hsp70 substrates in several rat organelles. Amongst a subset of organellar proteins selectively retained on DnaK, in the absence of mt-Hsp70 and BiP, the major constituents represent unstable proteins and subunits of oligomeric proteins. The selective retention of these fully synthesised proteins on Hsp70 most likely reflects the function of this molecular chaperone in protein biogenesis, but additionally, could extend the known functions of Hsp70 to include modulating the activities of certain proteins or enzymes which are important in cellular homeostasis.

Chapter 6 reports the cloning of a rat cDNA specifying a previously undescribed peroxisomal protein which was identified in Chapter 5 as a Hsp70 substrate. Although an activity is yet to be established, the results presented in Chapter 6 are most consistent with this protein

constituting a peroxisomal isoform of 2,4-dienoyl CoA reductase (px-2,4-DCR#1). The tools generated in this study may therefore assist elucidating the functional and evolutionary reason for the cellular compartmentalisation of β-oxidation in mammals, and may be valuable for the future screening and molecular characterisation of patients with inborn defects in the oxidative degradation of unsaturated fatty acids. The unsuccessful attempt to establish activity for recombinant rat px-2,4-DCR#1 may be due to the absence of an N-terminal acetyl group on the protein. Future attempts to establish activity could employ a eukaryotic expression vector, such as *Pichia pastoris*, which would be capable of making the recombinant rat px-2,4-DCR#1 protein with an N-terminal acetyl group. Alternatively, the cDNA encoding rat px-2,4-DCR#1 could be use to try and complement the yeast peroxisomal 2,4-dienoyl CoA reductase deletion mutant. These studies are now being attempted, as part of a collaboration, with J. Kalervo Hiltunen and Kari T. Koivurantac University of Oulu, Finland).

In conclusion, work presented in this thesis has confined many similarities between the chaperone complement of lower eukaryotes and mammals, whilst also uncovering unique new knowledge containing the coexistence of GrpE members in a single cellular compartment. The implications of this finding are not evident but certainly worthy of further investigation.

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