HIGH-THROUGHPUT ASSAYS FOR BIOTIN PROTEIN LIGASE : A NOVEL ANTIBIOTIC TARGET

by

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ABSTRACT

Antibiotics are defined as chemical substances that inhibit or limit the growth of microorganisms. Since the second world war, antibiotics have been widely used to reduce the morbidity and mortality associated with serious bacterial infections caused by organisms such as *Staphylococcus aureus*. However, it has become increasingly difficult to treat bacterial infections due to the emergence of antibiotic resistant strains. The first clinical case of drug resistant bacteria was observed in *S. aureus* in 1947, just four years after the mass production of penicillin. Since then, resistance has been reported to every antibiotic ever employed. According to the Centres for Disease Control and Prevention of the United States, more than 70% of hospital-acquired infections show resistance to at least one commonly used antibiotic. Coupled with the paucity of therapeutic agents in the pipeline, there is now an urgent demand for new antibiotics. One of the strategies employed to combat drug resistant bacteria requires new chemical entities that work through novel drug targets for which there is no pre-existing resistance. This thesis focuses on the essential metabolic enzyme biotin protein ligase (BPL) as one such new drug target.

BPL is the enzyme responsible for covalently attaching the cofactor biotin prosthetic group onto the biotin-dependent enzymes such as the carboxylases, decarboxylases and transcarboxylases. Enzymatic biotinylation proceeds via a two-step reaction whereby biotinyl-5'-AMP is synthesized from biotin and ATP before the biotin moiety is transferred onto the side chain of one specific lysine present in the active site of the biotin-dependent enzyme. One example of an important biotin-dependent enzyme is acetyl CoA carboxylase (ACC). ACC catalyzes the first committed step in fatty acid

biosynthesis. Through genetic studies, it has been demonstrated that BPL activity is essential for bacterial survival.

The aim for this project was to develop a convenient, high-throughput assay to measure BPL activity. This assay would permit 1) quantitative kinetic analysis of ligands and inhibitors and 2) screening of compound libraries for new BPL inhibitors. We propose that BPL inhibitors can be developed into new antibiotic agents. The novel BPL assay was developed employing fluorescence polarization (FP). FP is a light based technique which uses plane polarized light for the detection of tumbling motion of fluorescent molecules in solution. As polarization of the emitted light is relative to the apparent molecular mass of the fluorophore, this technique can be use for quantitation of changes in molecular mass of target molecules. This enabled 1) rapid kinetic analysis, 2) a minimal number of handling steps, 3) no washing steps and 4) automation by robotics.

A first generation assay was developed for *Escherichia coli* BPL using peptide 85-11 that has been shown to be a convenient substrate. Following the BPL reaction, biotinylated peptides will form large molecular mass complexes with avidin. The amount of product could then be quantitated using FP. Here, kinetic analysis of MgATP (K_m 0.25 ± 0.01 mM) and biotin (K_m 1.45 ± 0.15 μM) binding produced results consistent with published data. We validated this assay with inhibition studies with end products of the BPL reaction, AMP and pyrophosphate, and a compound, biotinol-5'-AMP. Statistical analysis, performed upon both intraassay and interassay results (n = 30), showed the coefficient of variance to be <10% across all data sets. Furthermore, the Z' factors between 0.5 and 0.8 demonstrated the utility of this technology in high-throughput applications. However, the use of peptide 85-11, a substrate specific to *E. coli* BPL, does limit the application of this methodology to *E. coli*.

In the second generation FP assay, I adapted this technology for *S. aureus* BPL by employing the biotin domain of *S. aureus* pyruvate carboxylase. Insertion of a fluorescein label was achieved by first engineering a cysteine residue into the domain by site directed mutagenesis then incubation with fluorescein-5'-maleimide. A series of mutants was created to investigate optimal positioning of the label into the substrate. Furthermore, the minimal size of the functional domain was determined. Our data showed that the placement of the fluorescein label is an important aspect of this project. Using this approach, I identified that a 90 amino acid domain with the label at position 1134 was optimal. Kinetic analysis of ligand binding showed *Sa*BPL had a K_m for biotin at 3.29 ± 0.37 µM and K_m for MgATP at 66 ± 16.08 µM. This was in good agreement with data obtained from our previous assay measuring ^{3}H-biotin incorporation. Inhibitor studies with pyrophosphate and analogues of biotin and biotinyl-5'-AMP further validated the assay.

Various studies have shown cross-species biotinylation activities by a diverse range of BPLs. Therefore, using this methodology with a biotin domain as the substrate potentially provides a convenient assay for all BPLs.

STATEMENT OF ORIGINALITY

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material that has been previously published or written by another person, except where due reference has been made in the text.

I consent to this thesis, when deposited in the University library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Belinda Ng Ling Nah

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"Praise God for all His grace and blessings!"

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LIST OF PUBLICATIONS

Refereed journal :

Ng, B., Murchland, I., Abell, A. D., Wilce, M. C., Wallace, J. C., Polyak, S. W. and Booker, G. W. (2008) "Engineered biotin domains as fluorescent substrates for biotin protein ligase," *Analytical Biochemistry* - Submitted

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Ng, B., Pendini N.R., Tieu W., Kuan K., Morona R., Abell A., Wilce M.C.J., Wallace J.C., Polyak S.W. and Booker G.W. (2008) Discovery of biotin protein ligase as a novel class of antibiotic. *University of Adelaide, School of Molecular and Biomedical Sciences Research Symposia* pos 12

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LIST OF ABBREVIATION

ACC	acetyl CoA carboxylase
Amp	ampicillin
AMP	adenosine monophosphate
Amp^R	ampicillin resistant
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCCP	biotin carboxyl carrier protein
BirA	biotin inducible repressor A
BLAST	basic local alignment search tool
BME	β-mercaptoethanol
bp	base pair
BPL	biotin protein ligase
BSA	bovine serum albumin
BtnOH-AMP	biotinol-5'-AMP
°C	degree Celsius
C-	carboxyl-
$CaCl_2$	calcium chloride
Cpd	compound
CTP	cytidine triphosphate
cv	column volume
CV	coefficient of variation
DMSO	dimethyl sulfoxide
DNA	deoxynucleotide triphosphate
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
Fl-	fluorescently labeled
FP	fluorescence polarization
FPLC	fast protein liquid chromatography

GST	glutathione-S-transferase
GTP	guanosine triphosphate
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
hr	hour
HRP	horseradish peroxidase
IC_{50}	inhibition concentration at 50% activity
Int	intensity
IPTG	isopropyl β-D-1-thiogalactopyranoside
ITP	inosine triphosphate
kb	kilobase pair
KCl	potassium chloride
kDa	kilo dalton
K_i	inhibition constant
K_M	Michaelis-Menten constant
KPO_4	potassium phosphate
LB	luria broth
m	metre
µ	micron
M	molar
mA	milliampere
Mg	magnesium
MIC	minimal inhibitory concentration
Min	minute, minutes
Mn	manganese
MOPS	3-morpholinopropanesulfonic acid
MS	mass spectrometry
MW	molecular weight
MWCO	molecular weight cut-off
n	nano
N-	amino-
NMR	nuclear magnetic resonance
OD_x nm	optical density at x nm wavelength
p	pico

P	polarization unit
PBS	phosphate buffered saline
PC	pyruvate carboxylase
PCR	polymerase chain reaction
PDB	protein data bank
*Ph*BPL	*Pyrococcus horikoshii* biotin protein ligase
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinyl difluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
*Sa*BPL	*S. aureus* biotin protein ligase
*Sa*PC	*S. aureus* pyruvate carboxylase biotin domain
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
SEM	standard error of the mean
Std. dev.	standard deviation
TBS	tris buffered saline
TBS-T	tris buffered saline and 0.1% (v/v) Tween-20
TEMED	N,N.N,N'N'-tetramethylethylene-diamine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
TTP	thymidine triphosphate
Tween-20	polyoxyethylene-sorbitan monolaurate
U	units (active)
UTP	uridine triphosphate
UV	ultra violet
Vmax	maximum velocity
WB	Western blot
WT	wild type
*y*BPL	yeast (*S. cerevisiae*) biotin protein ligase
ΔmP	milli polarization unit difference between apo and holo substrate