THE REACTION MECHANISM OF PYRUVATE CARBOXYLASE

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CONTENTS

SUMMARY

1. The 2',3'-dialdehyde derivative of ATP (oATP) was prepared by periodate oxidation, and on the following criteria was considered to be an affinity label of the $MgATP^{2-}$ binding site of sheep liver pyruvate carboxylase. The magnesium complex of this inhibitor ($Mg\text{-}oATP^{2-}$) was shown to be a linear competitive inhibitor with respect to $MgATP^{2-}$ in both the acetyl CoA-dependent and -independent activities of the enzyme but was a non-competitive inhibitor with respect to bicarbonate and an un-competitive inhibitor with respect to pyruvate. $Mg\text{-}oATP^{2-}$ was covalently bound to pyruvate carboxylase by reduction using sodium borohydride with concurrent irreversible inactivation of the enzyme. Although pyruvate, oxaloacetate, and bicarbonate were ineffective, both acetyl CoA and $MgATP^{2-}$ protected the enzyme against this chemical modification. Mg^{2+} enhanced the extent of chemical modification. At 100% inactivation, 1.1 ± 0.1 moles of $Mg\text{-}oATP^{2-}$ were bound to the enzyme per mole of biotin. The presence or absence of acetyl CoA had no affect on this stoichiometry. Chromatography of samples of an enzymic digest of Mg-o[^{14}C]ATP^{2-}-labelled enzyme revealed one major band of radioactivity which co-chromatographed with authentic lys-oATP markers.

2. In order to further characterise the lysyl residue(s) in the biotin carboxylation site, the use of cyanate as a potential affinity label of the bicarbonate binding site was investigated. Cyanate was shown to chemically modify sheep liver pyruvate carboxylase, with concurrent inactivation of the enzyme. The rate of loss of acetyl CoA-independent catalytic activity was not pseudo first-order with respect to time. When the inactivation data were analysed assuming that modified

enzyme retains some residual acetyl CoA-independent activity, it was found that only one kinetically significant residue per active site was modified, but cyanate appeared to form a reversible complex with the enzyme prior to modifying this residue.

Acetyl CoA, pyruvate and oxaloacetate were found to have no affect on the rate of modification of the enzyme. Bicarbonate, MgADP, and $MgATP^{2-}$ all afforded protection against inactivation of the enzyme. $MgATP^{2-}$ afforded almost complete protection against loss of both the acetyl CoA-dependent and -independent catalytic activities. Analysis of the concentration dependence of protection by bicarbonate and $MgATP^{2-}$ suggested that cyanate could not inactivate the enzyme-$MgATP^{2-}$ and enzyme-bicarbonate complexes. The enzyme MgADP was found to be inactivated at about 30% of the rate of free enzyme.

Modification of the enzyme with cyanate was found to inactivate the ATP/P_i isotopic exchange reaction, but to enhance the pyruvate/oxaloacetate isotopic exchange reaction. Inactivation of pyruvate carboxylase with [^{14}C]cyanate was shown to be associated with modification of at least six amino acid residues per mole of biotin. Chromatography of samples of an enzyme digest of [^{14}C]cyanate-labelled enzyme revealed that only amino groups in the enzyme had been modified. A major band of radioactivity was shown to co-chromatograph with authentic homocitrulline.

3. The stoichiometry of the products of the pyruvate carboxylase reaction was shown to vary as the concentration of pyruvate was altered. At high concentrations of pyruvate, the ratio of orthophosphate liberated to oxaloacetate produced approached one, but, as the pyruvate concentration decreased, the ratio increased. On the basis of this evidence, a

model for the reaction pathway was proposed in which the carboxybiotin-enzyme complex could react either with pyruvate to produce oxaloacetate, or water to regenerate enzyme-biotin and bicarbonate. The non-productive breakdown of the enzyme-substrate complex provides an explanation for the non-linear double reciprocal plots obtained for both the overall reaction and the pyruvate/oxaloacetate exchange reaction. Since neither the rate of breakdown of the isolated carboxybiotin-enzyme complex, nor the rate of decarboxylation of oxaloacetate in the absence of pyruvate could account for the difference in the amounts of the two products formed in the overall reaction, it was postulated that the presence of pyruvate was necessary for hydrolysis to occur. Rate equations were derived describing the dependence of the initial velocity release of oxaloacetate in the overall reaction, and the rate of the pyruvate/oxaloacetate exchange reaction, on pyruvate concentration. By assigning appropriate values to the various rate constants, theoretical curves were obtained and fitted to the experimental data.

The reaction pathway of the pyruvate carboxylase catalysed reaction was re-examined using two independent experimental approaches not previously applied to this enzyme. To avoid the variable stoichiometry associated with oxaloacetate formation, the reaction rate was measured by following orthophosphate release. Initial velocities, when plotted as a function of varying concentrations of either $MgATP^{2-}$ or bicarbonate, at fixed levels of pyruvate, gave, in double reciprocal form, families of straight, intersecting lines. Furthermore, when the reaction was determined as a function of varying $MgATP^{2-}$ concentration, using pyruvate, 2-ketobutyrate, and 3-fluoro-pyruvate as alternative keto-acid substrates, the slopes of the double reciprocal plots were significantly different. Both results suggest that the pyruvate

carboxylase reaction has a sequential pathway, at least at high keto-acid substrate concentrations. However, an analysis of the slope replots of the initial velocity orthophosphate release data suggested that the reaction via a non-sequential pathway at low keto-acid substrate concentrations. Rate equations describing the dependence of initial velocity release of oxaloacetate and orthophosphate on the concentrations of substrates were derived and were shown to be consistent with the initial velocity kinetic data presently available. Possible extensions of the concept of hydrolytic breakdown of an intermediate enzyme complex to other enzymes were discussed.

4. The interaction of acetyl CoA with sheep liver pyruvate carboxylase was investigated using two independent experimental approaches. Evidence consistent with lack of co-operativity of acetyl CoA binding under initial velocity assay conditions was obtained from an analysis of the protection afforded by acetyl CoA against TNBS modification of the enzyme. Furthermore, the dependence of the rate of acetyl CoA deacylation on acetyl CoA concentration was found to be consistent with lack of co-operativity of binding of acetyl CoA.

Evidence was obtained which suggested that the observed non-classical initial-velocity acetyl CoA profile arose because of acetyl CoA-dependent changes in the saturation of the enzyme with pyruvate and bicarbonate, and in the rate of dilution inactivation of the enzyme. The n_H value obtained from an analysis of the dependence of initial velocity oxaloacetate synthesis on acetyl CoA concentration using the Hill equation was shown to depend on the fixed concentrations of pyruvate and bicarbonate in the assay solutions. The fixed concentrations of $MgATP^{2-}$ and K^+ were found to have no affect on the n_H value. The time period over which the reaction rate was linear with time was found to

depend on the acetyl CoA concentration in the assay solution. At high concentrations of acetyl CoA, the process of dilution inactivation was prevented, and hence the reaction was linear with time for a longer period than at low concentrations of acetyl CoA. Dilution inactivation was shown not to involve formation of catalytically active pyruvate carboxylase dimers or monomers.

When an experiment was designed in which all substrates and activators were present at saturating levels regardless of the acetyl CoA concentration, and the enzyme concentration was raised to a level where dilution inactivation did not occur, the dependence of the rate of oxaloacetate synthesis on acetyl CoA concentration was found to be consistent with lack of co-operativity of binding of acetyl CoA to pyruvate carboxylase.

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ABBREVIATIONS

In addition to those accepted for use in the Journal of Biological Chemistry, the following abbreviations are used in this thesis

DTE	dithioerythritol
FDNB	1-fluoro-2,4-dinitrobenzene
n_H	Hill n coefficient
oATP	2',3' dialdehyde oxidation product of ATP.
OAA	oxaloacetate
pyr	pyruvate
TNBS	2,4,6-trinitrobenzene sulphonic acid
U	international enzyme unit