PYRUVATE CARBOXYLASE: REACTION MECHANISM AND CHEMICAL STUDIES OF THE ACTIVE CENTRE

A thesis submitted by

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CONTENTS

SUMMARY

1. By the use of the classical kinetic techniques, initial velocity, product inhibition and isotopic exchange studies, the reaction catalysed by pyruvate carboxylase (pyruvate:CO_2 ligase (ADP) EC.6.4.1.1) isolated from sheep kidney cortical mitochondria was shown to follow a non-classical ping-pong-bi-bi-uni-uni mechanism. Isotopic exchange studies showed that the overall reaction

$$MgATP^{2-} + HCO_3^- + \text{pyruvate} \underset{\text{acetyl CoA}}{\overset{Mg^{2+}}{\rightleftharpoons}} MgADP^- + Pi + \text{oxaloacetate}$$

consists of two half reactions.

$$E + MgATP^{2-} + HCO_3^- \underset{\text{acetyl CoA}}{\overset{Mg^{2+}}{\rightleftharpoons}} E \sim CO_2 + MgADP^- + Pi$$

$$E \sim CO_2 + \text{pyruvate} \overset{\text{acetyl CoA}}{\rightleftharpoons} \text{oxaloacetate} + E$$

The mechanism is 'non-classical' because, as indicated by the product inhibition data, the two half reactions are catalysed at separate sites on the enzyme. The results are in accord with those obtained by other workers using pyruvate carboxylase from other sources.

The mechanism of the first partial reaction has been further examined, by the use of analogues of ADP and analogues

of a possible intermediate or transition state, in an effort to determine whether the reaction proceeds by concerted or multistep electron transfer. The results obtained are consistent with a concerted mechanism for CO_2 fixation, or with the transitory formation of an enzyme bound carbonyl phosphate intermediate.

2. The nature of the activation of the enzyme by its allosteric effector acetyl CoA has been investigated. The enzyme has been shown to catalyse the acetyl CoA-independent carboxylation of pyruvate at a rate which, under optimal conditions, is approximately 25% of the rate of the acetyl CoA stimulated reaction. Comparison of the properties of the acetyl CoA-stimulated and independent reactions showed that the activator has multiple effects on the enzyme; (i) it stabilised the enzyme at low enzyme concentrations; (ii) it altered the requirement for inorganic ions; (iii) it substantially decreased the apparent K_m values for HCO_3^- and pyruvate with little effect on the apparent K_m value for $MgATP^{2-}$; (iv) it substantially increased V_{max}. Isotopic exchange studies indicated that the V_{max} effect was primarily due to a stimulation of the first partial reaction. Slight quenching of the fluorescence emission intensity of bound 8-anilino-1-naphthalenesulphonate provided some direct evidence for an acetyl CoA-induced conformational change in the protein.

Sheep kidney pyruvate carboxylase preparations, like those isolated from chicken liver (M.C. Scrutton and M.F.

Utter, J. Biol. Chem., 242, 1723 (1967)), catalyse an acetyl CoA deacylase reaction.

3. Sheep kidney pyruvate carboxylase contains a catalytically important lysine residue which can be selectively modified with trinitrobenzenesulphonic acid (D.B. Keech and R.K. Farrant, Biochim. Biophys. Acta, 151, 493 (1968)). Studies reported here show that although chemical modification of the lysyl residue resulted in almost complete inactivation of the acetyl CoA-stimulated reaction, the acetyl CoA-independent reaction was undiminished. This observation indicated that the modified residue was specifically involved in the acetyl CoA activation process. Consistent with this conclusion was the effect of modification on the isotope exchange reactions. The acetyl CoA deacylase reaction was not inhibited by the modification.

Measurement of the increase in extinction at 367 nm due to formation of the chromophoric trinitrophenyl-lysine derivative during the modification reaction showed that inactivation was accompanied by modification of one amino acid residues per enzyme monomer.

4. Previous work in this laboratory (J.B. Edwards and D.B. Keech, Biochim. Biophys. Acta, 146, 576 (1967)) indicated the usefulness of the determination of apparent K_m and V_{max} values for substrates at various stages during a chemical modification reaction. However, a sound theoretical basis for the assessment of this approach and interpretation of

experimental data has been lacking. In this thesis a theory is developed for interpreting kinetic data obtained from chemically modified enzymes in terms of the function of the affected residue. Limitations of the method are also considered.

STATEMENT

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

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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.

AMPPCP	β,γ-methylene-adenosine 5'-triphosphate
ANS	1-analinonaphthalene-8-sulphonate
AOPCP	0-adenylyl methylene diphosphonate (α,β-methylene adenosine 5'-diphosphate)
APS	adenosine 5'-phosphosulphate
BCCP	biotin carboxyl carrier protein
CoA, CoASH	coenzyme A
DNP-	dinitrophenyl-
DTE	dithioerythritol
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EPR	electron paramagnetic resonance
FDNB	1-fluoro-2,4-dinitrobenzene
FDP	fructose 1,6-diphosphate
NMR	nuclear magnetic resonance
OAA	oxaloacetate
PEI	polyethyleneimine
PEP	phosphoenolpyruvate
Pi	orthophosphate
POPOP	1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene
PPO	2,5-diphenyloxazole
PRR	proton relaxation rate
TNBS	2,4,6-trinitrobenzene sulphonic acid
TNP-	trinitrophenyl-