



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

A thesis submitted by

LEONIE KAY ASHMAN, B.Sc.(Hons.) (Flinders, 1970)

To the University of Adelaide

South Australia,

for the Degree of

Doctor of Philosophy.

DEPARTMENT OF BIOCHEMISTRY  
UNIVERSITY OF ADELAIDE  
SOUTH AUSTRALIA

MARCH, 1973.

## CONTENTS

	<u>page</u>
SUMMARY	i.
STATEMENT	v.
ACKNOWLEDGEMENTS	vi.
ABBREVIATIONS	vii.
<u>CHAPTER ONE: INTRODUCTION</u>	
1.1. GENERAL PROPERTIES OF BIOTIN-CONTAINING ENZYMES	1
1.1.1. Classification	1
1.1.2. The Role of Biotin in the Mechanism of Carboxylase Reactions	3
1.1.3. Structural Features of Biotin Enzymes	8
1.2. THE PYRUVATE CARBOXYLASE REACTION AND ITS METABOLIC SIGNIFICANCE	11
1.2.1. Distribution	11
1.2.2. The Reaction	11
1.2.3. Activation by Acetyl CoA	13
1.2.4. Activation by Monovalent Cations	15
1.2.5. The Occurrence and Function of Bound Divalent Cations	15
1.2.6. Kinetic Studies on the Reaction Mechanism	17
1.2.7. The Quaternary Structure of Pyruvate Carboxylase and its Functional Significance	19
1.2.8. Regulation of Pyruvate Carboxylase in Higher Organisms	23
1.3. APPROACHES TO THE STUDY OF ENZYME ACTIVE CENTRES	26
1.3.1. The Active Centre	26
1.3.2. X-Ray Crystallography	27
1.3.3. Chemical Modification	31
1.3.4. The Use of Analogues	35

	<u>page</u>
1.3.5. Other Physical Methods	36
1.3.6. Conclusions	39
1.4 THE AIMS AND FINDINGS OF THE PROJECT	40
<u>CHAPTER TWO: MATERIALS AND METHODS</u>	
2.1. Materials	44
2.2. Preparation and Purification of Acetyl CoA	45
2.3. Preparation of [ $^{14}\text{C}$ ]- $\text{NaHCO}_3$ from [ $^{14}\text{C}$ ]- $\text{BaCO}_3$	46
2.4. Counting Procedures	47
2.5. Measurement of Protein Concentration	48
2.6. Measurement of Pyruvate Carboxylase Activity	48
2.7. Enzyme Purification	51
2.8. Synthesis of [ $\beta$ - $^{32}\text{P}$ ]-ADP	56
2.9. Separation of Radioactive Nucleotides and Inorganic Phosphate	56
2.10. ATP:ADP Isotopic Exchange Assay	57
2.11. ATP:Pi Isotopic Exchange Assay	57
2.12. $\text{HCO}_3^-$ : Oxaloacetate 'Exchange' Assay	58
2.13. Pyruvate: Oxaloacetate Isotopic Exchange Assay	58
2.14. Calculation of Rates of Isotopic Exchange	60
<u>CHAPTER THREE: KINETIC STUDIES OF THE REACTION MECHANISM</u>	
3.1. INTRODUCTION	62
3.2. METHODS	66
3.2.1. Assay Methods	66
3.2.2. Data Analysis	67
3.3. RESULTS	67
3.3.1. Isotopic Exchange Reactions Catalysed by Sheep Kidney Pyruvate Carboxylase	67

	<u>page</u>
3.3.2. Initial Velocity Studies	77
3.3.3. Product Inhibition Studies	78
3.3.4. Equilibrium Exchange Studies of the First Partial Reaction	80
3.4. DISCUSSION	81
<u>CHAPTER FOUR: ON THE MECHANISM OF COUPLING OF CO<sub>2</sub></u>	
FIXATION AND ATP HYDROLYSIS	
4.1. INTRODUCTION	92
4.2. METHODS	97
4.2.1. Preparation of Carbamyl Phosphate Solutions	97
4.2.2. Assay of Pyruvate Carboxylase Catalysed ATP Formation from Carbamyl Phosphate and ADP	98
4.3. RESULTS	99
4.3.1. Carbamyl Phosphate as a Substrate for ATP Synthesis by Pyruvate Carboxylase	99
4.3.2. Phosphonacetic Acid as an Inhibitor of the Overall Reaction	101
4.3.3. Exchange Reactions Indicative of Carbonyl Phosphate Formation	101
4.3.4. Interaction of the Enzyme with Analogues of ADP	102
4.4. DISCUSSION	105
<u>CHAPTER FIVE: INTERACTIONS OF THE ENZYME WITH ACETYL CoA</u>	
5.1. INTRODUCTION	108
5.2. METHODS	109
5.2.1. Assay of the Acetyl CoA-Independent Pyruvate Carboxylase Activity	109
5.2.2. The DTNB Assay for Deacylase Activity	110
5.2.3. Radio-isotopic Assay of Acetyl CoA Deacylation	110

	<u>page</u>
5.2.4. Preparation and Purification of Acyl CoA Derivatives	111
5.3. RESULTS	111
5.3.1. Evidence for the Acetyl CoA-Independent Reaction	111
5.3.2. Activation of the Enzyme by Monovalent Cations and Sulphate Ions	115
5.3.3. The Effect of Enzyme Concentration on Specific Activity	117
5.3.4. Kinetic Studies on the Acetyl CoA Activation	119
5.3.5. Acetyl CoA-Independent Carboxylation by Pyruvate Carboxylases from Other Sources	121
5.3.6. Time Course of Acetyl CoA Activation of Sheep Kidney Pyruvate Carboxylase	124
5.3.7. Attempts to Demonstrate a Conformational Change Induced by Acetyl CoA	124
5.3.8. Acetyl CoA Deacylase Activity	126
5.4. DISCUSSION	131
5.4.1. Activation of the Pyruvate Carboxylase Reaction by Acetyl CoA	131
5.4.2. The Acetyl-CoA Deacylase Reaction	136
 <u>CHAPTER SIX: CHEMICAL MODIFICATION OF SHEEP KIDNEY PYRUVATE CARBOXYLASE WITH TNBS</u>	
6.1. INTRODUCTION	139
6.1.1. The Reactive Lysine Residue of Sheep Kidney Pyruvate Carboxylase	139
6.1.2. The Kinetic Approach to Determining the Number of Residues Modified	140
6.1.3. The Reaction of FDNB and TNBS with Amino Acids and Proteins	142
6.2. METHODS	144

	<u>page</u>
6.2.1. TNBS Modification Conditions	144
6.2.2. Chromatography Systems for the Separation of TNP Derivatives	145
6.2.3. Synthesis of TNP Derivatives	145
6.2.4. Assay Procedures	147
6.3. RESULTS	147
6.3.1. The Effect of Trinitrophenylation on the Catalytic Activity of Sheep Kidney Pyruvate Carboxylase	147
6.3.2. Identification of the Trinitrophenylated Amino Acid Residue	149
6.3.3. Determination of the Stoichiometry of the Trinitrophenylation Reaction	153
6.3.4. Quaternary Structure of the Trinitrophenylated Enzyme	154
6.4. DISCUSSION	154

CHAPTER SEVEN: A KINETIC APPROACH TO DEFINING THE ROLE  
OF CHEMICALLY MODIFIABLE RESIDUES AT  
THE ACTIVE SITES OF ENZYMES

7.1. INTRODUCTION	158
7.2. THEORY	159
7.2.1. Kinetic Properties of a System Containing Modified and Unmodified Enzyme Molecules	159
7.2.2. Determination of Kinetic Constants for the Modified Enzyme	161
7.2.3. Interpretation of Changes in $appV_{max}$ and $appK_m$	163
7.3. CONCLUSIONS	170

CHAPTER EIGHT: GENERAL DISCUSSION

	<u>page</u>
8.1. The Reaction Mechanism	172
8.2. Activation of the Enzyme by Acetyl CoA	175
8.3. Some General Comments on Chemical Modification of Sheep Kidney Pyruvate Carboxylase	178

REFERENCES

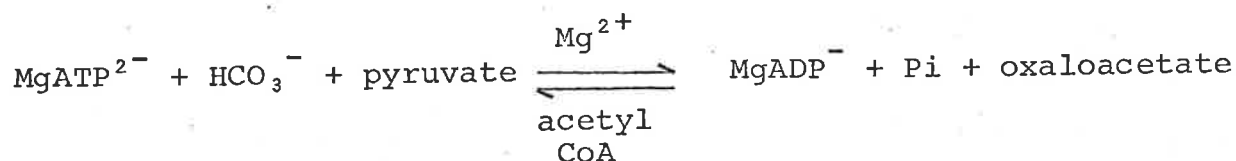
182

APPENDIX A: PRODUCT INHIBITION DATA

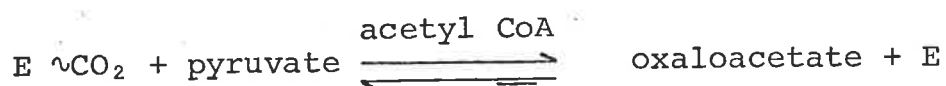
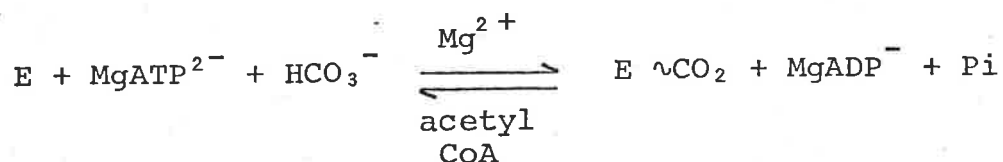
APPENDIX B: PUBLICATIONS

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<sub>2</sub> 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



consists of two half reactions.



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<sub>2</sub> 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<sub>3</sub><sup>-</sup> and pyruvate with little effect on the apparent  $K_m$  value for MgATP<sup>2-</sup>; (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.

LEONIE K. ASHMAN

ACKNOWLEDGEMENTS

I wish to thank Professor W.H. Elliott for permission to undertake this project in the Department of Biochemistry, University of Adelaide.

I am grateful to my supervisor, Dr. D.B. Keech, for his advice, criticisms and encouragement throughout the course of this work, and in the preparation of this thesis. In addition, I wish to thank Dr. J.C. Wallace for his assistance in the analytical ultracentrifugation experiments, and for many helpful discussions, and Dr. R.H. Symons for his guidance in the nucleotide chemistry. I am grateful to Mr. R. Bais and Mr. D.B. Rylatt for allowing me to discuss their unpublished results in this thesis.

I wish to acknowledge the financial support of a Commonwealth Postgraduate Award for the duration of the project.

ABBREVIATIONS

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

AMPPCP	$\beta,\gamma$ -methylene-adenosine 5'-triphosphate
ANS	1-analidonaphthalene-8-sulphonate
AOPCP	0-adenylyl methylene diphosphonate ( $\alpha,\beta$ -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-