STUDIES ON PHOSPHOENOLPYRUVATE CARBOXYKINASE AND CITRATE SYNTHASE IN *ESCHERICHIA COLI*, STRAIN B

by

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To my wife, Linda, for her continuing patience, interest and encouragement.

To my parents, my sister and my brother, who have always shared my interests and for their encouragement, especially in the initial stages of this project.

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ABSTRACT

Phosphoenolpyruvate carboxykinase from Escherichia coli was purified about 400-fold over non-induced levels by ultracentrifugation, protamine sulfate and ammonium sulfate precipitation, Sephadex G-150 elution and DEAE-cellulose chromatography. The enzyme was characterized with respect to optimum hydrogen ion concentration, molecular weight and allosteric inhibition. The enzyme was found to be inhibited in an allosteric manner by NADH. Other coenzymes such as NAD and NADP were completely ineffective and NADPH was only 15% as effective as NADH in the inhibition of the enzyme. Initial velocity and product inhibition patterns show that the free enzyme form possibly binds ADP (or ATP) first. The inhibitor and the remaining substrates possibly bind to the E-ADP or E-ATP complexes. It was found that the sigmoidality of OAA, PEP, and bicarbonate plots could be explained by an isomerization of the enzyme in the presence of NADH or by a kinetic model which postulated the existence of two pathways for the release of products in the presence of NADH.

Citrate synthase from Escherichia coli was highly purified by protamine sulfate and ammonium sulfate precipitation, DEAE-cellulose chromatography, reverse ammonium sulfate elution and Sephadex G-200 elution. The physicochemical properties of the enzyme were investigated by SDS polyacrylamide gel electrophoresis, sucrose gradient

experiments at various pH values, resolution of tryptic peptides by electrophoresis and paper chromatography, sulfhydryl group titrations, ultracentrifugation studies at various pH levels and in the presence of substrate and inhibitors, amino acid analysis, and by polyacrylamide gel electrophoresis at pH 7.0 and pH 8.9. The partial specific volume of the enzyme was calculated and the diffusion coefficients and the molecular weights for two species of the enzyme were found by the sedimentation-diffusion procedure. At pH 11.0 citrate synthase exists almost entirely as identical protomers and each protomer has a molecular weight of 62,000. Below pH 11.0 the protomers are associated in the forms of tetramers and octomers (molecular weights of 248,000 and 496,000). The enzyme was found to be concentration dependent and existed entirely as an octomer at pH 7.0 and in the presence of 2 mM dithiothreitol. Only the enzyme species with a molecular weight of 248,000 shows enzymatic activity, although the heavier species can bind ligands. The data obtained from various initial velocity studies and binding experiments led to the postulation of a reversible isomerization model to explain the allosteric behaviour of citrate synthase. There are 8 binding sites for substrate and inhibitors per molecule of enzyme (molecular weight of 496,000).

It was found that NADH concentrations in cells grown on glucose were 1.5 to 2 times higher than in those grown on succinate. It was concluded that NADH levels may serve as indicators of glycolysis.

Abbreviations

acetyl-CoA	acetyl coenzyme A
acetyl-P	acetyl-phosphate
ADP	adenosine 5'-diphosphate
ADH	alcohol dehydrogenase
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CMP	cytidine 5'-monophosphate
CoA	coenzyme A
DEAE	diethylaminoethane
DTNB	5,5-dithiobis(2-nitrobenzoic acid)
EDTA	ethylenediaminetracetic acid
GDP	guanosine 5'-diphosphate
GSH	reduced glutathionine
GTP	guanosine 5'-triphosphate
Hb	hemoglobin
IDP	inosine 5'-diphosphate
ITP	inosine 5'-triphosphate
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
OAA	oxalacetate
4,4-PDS	4,4'-dithiodipyridine
PEP	phosphoenolpyruvate

Abbreviations Cont'd.

PEP carboxykinase	phosphoenolpyruvate carboxykinase
Pi	inorganic phosphate
SDS	sodium dodecyl sulfate
TCA	tricarboxylic acid
Tris	trihydroxymethylaminomethane
U/V	ultraviolet

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

INTRODUCTION

In various communications (Weitzman, 1967; Sanwal, 1969; Sanwal and Smando, 1969; Wright and Sanwal, 1969) it has been reported that the concentration levels of NADH in Escherichia coli and in other enteric bacteria (Clarke, Wright and Sanwal, 1970) are the central control signals for coordinating the activity of a variety of enzymes which utilize OAA or malate as substrates. Thus, citrate synthase (Weitzman, 1967), malate dehydrogenase (Sanwal, 1969), NADP-specific malic enzyme (Sanwal and Smando, 1969) and PEP carboxykinase (Wright and Sanwal, 1969) are all inhibited in an allosteric manner by NADH.

In the adenylate control hypothesis, as proposed by Krebs (1964) and Atkinson (1965), fluctuations in the levels of ATP-AMP are the central signals controlling glycolysis, gluconeogenesis, lipogenesis, and terminal oxidations. This hypothesis is probably correct, judging from the evidence available from studies of eucaryotic cells. That ATP-AMP levels are also important in the control of glycolytic reactions in E. coli is shown by the fact that ADP (and GDP) activates phosphofructokinase (Atkinson, 1965; Blangy, Buc, and Monod, 1968) and AMP activates pyruvate kinase (Maeba and Sanwal, 1968) and pyruvate dehydrogenase (Shwartz, Old, and Reed, 1968). However, NADH, which is important as a control in the reactions involving OAA and malate as substrates is also the preferred control agent for some reactions of the terminal oxidation pathway (Hansen and Henning, 1966; Weitzman, 1967).

The enzymes controlled by NADH are also regulated by other metabolites (Wright, Maeba, and Sanwal, 1967; Sanwal, Wright, and Smando, 1968) and at least superficially exhibit the same general kinetic features. Unlike the vast majority of other allosteric enzymes (Stadtman, 1966; Atkinson, 1966) they do not show substrate cooperativity, except in the presence of inhibitors. It has been suggested (Sanwal and Smando, 1969; Wright and Sanwal, 1969) that such substrate cooperativity in the presence of the inhibitor may arise either as a result of kinetic interactions (Maeba and Sanwal, 1966) or isomerization of the enzyme (Monod, Wyman and Changeux, 1965; Koshland, Nemethy and Filmer, 1966).

This thesis is an attempt to describe an investigation of two of the enzymes which are allosterically controlled by NADH - PEP carboxykinase and citrate synthase. It presents the results of enzyme purifications, in vivo coenzyme concentration studies, initial velocity studies in the presence and absence of NADH and other effectors, physico-chemical investigations, and some equilibrium binding data. Possible enzyme mechanisms have been postulated and the physiological significance of NADH regulation is discussed.

PHOSPHOENOLPYRUVATE CARBOXYKINASE

The first evidence of CO_2 fixation by chemoorganotrophs was reported by Wood and Werkman (1936, 1938) during an investigation of glycerol fermentation by propionic acid bacteria. For each mole of succinate formed from glycerol, one mole of CO_2 was fixed. The authors postulated that succinate was formed by a $C_1 + C_3$ synthesis. Wood and Werkman (1940) proposed that OAA was synthesized first from pyruvate and CO_2 with a subsequent stepwise conversion to malate, fumarate, and succinate.

$$CO_2 + CH_3.CO.COOH \longrightarrow \underset{\beta}{COOH}.CH_2.\underset{\alpha}{CO}.COOH$$

Krampitz et al (1941, 1943) published the first evidence of the reversibility of the above reaction from their studies of a bacterial enzyme preparation which decarboxylated OAA to pyruvate and CO_2. It was possible to demonstrate an excess of ^{13}C in the β-carboxyl group of the residual OAA when the reaction was carried out in the presence of $NaH^{13}CO_3$.

Many reports soon appeared giving evidence of CO_2 fixation by animal tissues (Wood et al, 1941; Evans and Slotin, 1941) but direct demonstration of the reaction was not accomplished. Evans et al (1943) described an extract from pigeons liver, which fixed $^{13}CO_2$ when fumarate was fermented in the presence of pyruvate and $NaH^{13}CO_2$. Wood, Vennesland, and Evans (1945) later showed that the CO_2 fixed was equally

distributed among the carboxyl groups of pyruvate, lactate, fumarate, and malate. Although this type of distribution could be explained by the Wood-Werkman reaction in conjuction with other well established reactions, an attempt by Wood (1945) to demonstrate an exchange between OAA and $NaHCO_3$ failed. It was later shown by Utter and Wood (1946) that, when ATP is added to the reaction mixture a rapid exchange of isotopic carbon does occur. Their exchange was one of the first indirect suggestions of a PEP carboxykinase reaction, although there were some earlier hints that such a reaction existed. Lohmann and Meyerhof (1934) identified PEP as a phosphate intermediate formed during anaerobic metabolism. Kalckar (1939) reported the accumulation of PEP during the oxidation of malate by a kidney preparation. Leloir and Munoz (1944) were studying a preparation of liver enzymes that would oxidize fatty acids in the presence of adenylic acid, cytochrome c, inorganic phosphate, magnesium ions, and fumarate. In the absence of the fatty acids PEP accumulated.

The importance of CO_2 as an essential metabolite in chemoorganotropic metabolism was shown by Ajl and Werkman (1948). They demonstrated that E. coli and Aerobacter aerogenes did not grow in a mineral glucose medium when incubated in a CO_2-free atmosphere. They were able to show that dicarboxylic acids, α-ketoglutarate, aspartate, or glutamate were equally effective as substitutes for CO_2.

These results supported the reports of Wood and Werkman (1938, 1940) that CO_2 is involved in a synthesis of C_4 compounds.

It was later established that PEP, and not pyruvate was the active C_3 intermediate by Bandurski and Greiner (1953) and Utter and Kurahashi (1953, 1954a, 1954b, 1954c). The former authors had discovered the PEP carboxylase reaction and the latter, the PEP carboxykinase enzyme.

Utter and Kurahashi (1953) proposed the following mechanism:

$$\text{OAA} + \text{ATP (or ITP)} \rightleftharpoons \text{PEP} + HCO_3^- + \text{ADP (or IDP)}.$$

The authors found that the enzyme catalyzed an exchange reaction between $NaH^{14}CO_3$ and OAA in the presence of ATP. The enzyme also produced OAA when it was incubated with PEP, $NaHCO_3$, ADP, and $MnCl_2$. By removing ATP with the hexokinase reaction or by increasing the concentration of the reactants, the amount of OAA produced was raised. The identification of OAA was accomplished by the chromatographic behavior of its 2,4 dinitrophenylhydrozone, and by crystallizing the hydrazone of OAA formed from $NaH^{14}CO_3$ in the presence of a known amount of carrier hydrozone to constant specific activity. PEP was identified by its chromatographic behavior and its reactivity with purified phosphokinase.

Utter and Kurahashi (1954a, 1954b, 1954c) used the chicken liver preparation to show that the exchange reaction occurred with ITP; ATP that had been purified would not

support an exchange reaction. Using a very pure preparation of PEP carboxykinase they confirmed that PEP, and not pyruvate was the acceptor for CO_2 in the exchange reaction.

Graves et al (1956) used deterium as a tracer to establish that the enzymatic addition of CO_2 to PEP gives the keto, and not the enol form of OAA. Kurahashi, Pennington and Utter (1957) reported that guanosine polyphosphates can replace inosine derivatives in the kinase reaction. Adenosine, cytosine, uridine, and xanthine derivatives were inactive when chicken liver was used as the source of enzyme. PEP carboxykinase isolated from lamb liver by Bandurski and Lipmann (1956) was also shown to be inactive with ATP but required ITP or GTP for activity.

Suzuki and Werkman (1958) reported the occurrence of both PEP carboxylase and the PEP carboxykinase reactions in extracts of the chemoautotroph, Thiobacillus thiooxidans.

The intracellular distribution of PEP carboxykinase appears to vary greatly from species to species. Nordlie et al (1963, 1965) reported that in the rat, mouse, and hamster most (more than 90%) of the PEP carboxykinase is localized in the soluble fraction of the cell. In the guinea pig, and rabbit a much greater percentage (35 to 100%) of the total enzyme activity is found in the liver mitochondrial cell fraction. In T. thiooxidans (Suzuki and Werkman, 1958) and E. coli (Theodore and Englesberg, 1964) PEP

carboxykinase is found mainly in the soluble cell fraction.

In animal cells PEP carboxykinase has been implicated in hepatic gluconeogenesis from pyruvate and dicarboxylic acid Krebs cycle intermediates. Shrago et al (1963) found that PEP carboxykinase activity is increased after administration of glucocorticoids, fasting, or the induction of diabetes by pancreatectomy, alloxan, or mannoheptulose. The authors concluded that PEP carboxykinase is closely correlated with carbohydrate formation and is present in amounts sufficient to account for PEP synthesis during glucogenesis. Young et al (1964) also found that the enzyme level in rat liver increases rapidly during fasting but is restored to normal by refeeding diets containing carbohydrate, whereas, refeeding diets free of carbohydrate did not depress the elevated activity level of PEP carboxykinase. It has been shown by Mazumdar et al (1960, 1962) and Heldt et al (1964) that mammalian liver, kidney and heart mitochondria possess an active GTP (or ITP) - generating system in the form of succinic thiokinase. Chang and Lane (1966) suggested that liver mitochondria was particularly well adapted to PEP formation because of the effective coupling of the two enzyme systems.

There is a great deal of evidence connecting the PEP carboxykinase reaction in microorganisms to gluconeogenesis. Theodore and Englesberg (1964) were the first investigators to report that the enzyme is induced 13 to 25-fold when

E. coli is grown in a mineral glycerol-malate medium as compared to a mineral glucose medium. De Torronteque and Palacian (1966) found that growth of two species of yeast, Rhodotorula glutinis and Hansenula anomela, on different carbon sources indicated that the level of PEP carboxykinase was very high when gluconeogenesis is active (acetate, pyruvate, malate and aspartate as carbon sources) and very low when glycolysis is operative (glucose as carbon source). A mutant of E. coli was described by Hsie and Rickenberg (1966) which was isolated on the basis of its inability to grow on succinate as a sole source of carbon. The bacteria were shown to have abnormally low levels of PEP carboxykinase activity. Evidence was presented to show that PEP carboxykinase was essential for growth when TCA cycle intermediates serve as the only source of carbon. Wright and Sanwal (1969) reported the allosteric inhibition of the enzyme in E. coli by NADH. It was also shown that cells grown on glucose contained 1.5 to 2.0-fold higher NADH concentration than cells grown on a succinate carbon source. The authors concluded that PEP carboxykinase in E. coli was important in the mechanism of gluconeogenesis.

During the last few years reports have come from many laboratories describing some of the molecular characteristics of the PEP carboxykinase reaction. Much of the investigation has come from the laboratory of Cannata and Stoppani (1963a,