# PROPIONYL COENZYME A CARBOXYLASE FROM RHODOSPIRILLUM RUBRUM: KINETIC STUDIES AND MECHANISM OF ACTION

by

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MASTER OF SCIENCE



TO MY BROTHER AND MY PARENTS

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# ABSTRACT

The enzyme, propionyl CoA carboxylase (propionyl CoA : carbon dioxide ligase (ADP), EC 6.4.1.3), was extracted and partially purified from a microaerophilic culture of Rhodospirillum rubrum grown in the light. Product formation by propionyl CoA carboxylase from R. rubrum displayed a linear proportionality with time and protein concentration. The pH optimum of the enzyme was determined to be 7.9 - 8.1 with a temperature optimum of $30^{o}$ - $32^{o}$. Propionyl CoA carboxylase was subject to heat denaturation, with the loss of all activity occurring at $50^{o}$. This enzyme was shown to be a biotin-containing enzyme by its inactivation by avidin and protection against such inactivation by excess biotin. The effects of cations and anions on the activity were also examined in this study, using $Na^{+}$, $K^{+}$, $SO_4^{=}$, $PO_4^{=}$, and $C\ell^{-}$ ions.

Apparent and true Michaelis constants were determined for the substrates involved in the reaction; propionyl CoA, $HCO_3^{-}$, ATP, and $Mg^{++}$. As a result of this study, propionyl CoA carboxylase was shown to display a distinct homotropic effect with MgATP. Product in-

hibition studies were carried out with each product, (methylmalonyl CoA, MgADP, and $P_i$), in combination with every substrate, (propionyl CoA, $HCO_3^-$, and MgATP). From the kinetic results, a mechanism was proposed for the action of propionyl CoA carboxylase which involves two separate active sites on the enzyme; one for each partial reaction. The two catalytic sites were assumed to be linked by the biotinyl residue, functioning as a carboxyl carrier between the two sites. Propionyl CoA, the substrate for site II, was assumed to interact in the vicinity of site I at high concentrations. This mechanism satisfactorily explains most of the kinetic data obtained in this study.

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# INTRODUCTION

Biotin carboxylases, in general, have been extensively studied for the past twenty years using acetyl CoA carboxylase and pyruvate carboxylase as model enzymes. However, two carboxylases, β-methylcrotonyl CoA carboxylase and propionyl CoA carboxylase, have not been studied in as much detail as have the other biotin carboxylases. In fact, of the above mentioned enzymes, only those from eukaryotic sources have been used for studying kinetic behavior. An attempt to study the kinetics of such an enzyme from a prokaryotic source would therefore facilitate comparison with previously studied enzymes from eukaryotic sources.

The enzyme, propionyl CoA carboxylase, catalyzes the formation of $D_S$-methylmalonyl CoA by the direct carboxylation of propionyl CoA utilizing energy supplied by the breakdown of ATP to ADP and $P_i$. Propionyl CoA carboxylase is an enzyme which functions as a key anaplerotic distributary for the reactions of the TCA cycle. The kinetic behavior and physiological features of this enzyme have only been studied briefly from microbial sources (1). Therefore, it was of interest to

determine the kinetic behavior of propionyl CoA carboxylase from Rhodospirillum rubrum and to assess both the physical and kinetic properties obtained from such a study.

Furthermore, if possible, a mechanism of action of this enzyme would be proposed; using the nomenclature of Cleland (2, 3, 4); and this would be compared with mechanisms for other biotin carboxylase enzyme systems, already known.

# HISTORICAL

Propionic acid arises from the oxidation of fatty acids with an odd number of carbon atoms, catabolism of branched-chain amino acids, and fermentation by the gastrointestinal flora of ruminants and other animals. Propionate is metabolized by a number of pathways depending on the system studied (5). One such pathway directly involves a biotin-dependent enzyme; propionyl CoA carboxylase, which converts propionyl CoA to $D_S$-methylmalonyl CoA.

The fate of propionate and its metabolic importance have been examined in great detail over the last sixty years. Initially, propionate was thought to be a key glycogenic compound which was eventually converted through acrylate and lactate to pyruvate (6). Later work by Lorber et al (7) suggested that randomization of propionate occurred, resulting in the formation of glycogen, acetyl groups, or lactate. However, with the demonstration of a pathway of propionate metabolism involving carboxylation, in Chlorobium thiosulphatophilum (8),

investigations were initiated to examine this mode of propionate metabolism further.

Lardy and Peanasky (9), using extracts of acetone-dried mitochondria from rats, showed that propionate could be utilized via an ATP-and divalent cation-dependent carboxylation reaction, yielding succinate as the final product. In addition, it was observed that this carboxylating activity was markedly depressed in liver mitochondrial extracts from biotin-deficient rats as compared to mitochondria of normal rats, thereby first implicating biotin involvement in propionate carboxylation in animal tissues. Subsequently, Ochoa and his co-workers (10, 11, 12), working with preparations from animal tissues, showed that the actual substrate of this carboxylation reaction was propionyl CoA and that the product thus formed was $D_S$-methylmalonyl CoA. This latter compound was then converted by the action of a separate enzyme, methylmalonyl CoA isomerase, into succinyl CoA. The carboxylation of propionate to succinate is therefore dependent on propionyl CoA carboxylase, an enzyme associated with biotin in some way.

The discovery of propionyl CoA carboxylase in animal tissues prompted investigations into propionate carboxylation in other systems, and the reaction was looked for in many prokaryotes.

One such prokaryote examined was the photosynthetic bacterium Rhodospirillum rubrum. The studies of Elsden and Ormerod (13) on the effect of monofluoroacetate on the photometabolism of propionate and succinate by R. rubrum, suggested that these compounds were metabolized by a common pathway. Subsequently Ormerod (14), proposed that carbon dioxide played a special role in the photometabolism of propionate. The role of carbon dioxide was further emphasized by the work of Clayton et al (15, 16) who showed that the metabolic pathway of propionate in R. rubrum in both light and dark probably involved a preliminary carboxylation to succinate. The work of Elsden (17), strongly suggested that propionate was metabolized via succinate after propionate assimilation. Further studies carried out by Gibson and Knight (18), and later by Knight (19), using crude extracts of R. rubrum indicated the formation of methylmalonate and succinate from propionyl CoA, ATP, and $CO_2$. The reaction quite closely resembled that found in animal tissues, being inhibited in the presence of avidin, a biotin antagonist.

Further investigation of propionyl CoA carboxylase in R. rubrum was later performed by Olsen and Merrick (1). When R. rubrum was grown on acetate, succinate, glutamate or yeast extract, the microorganism displayed relatively high levels of propionyl CoA carboxylase. Furthermore, photoheterotrophic growth on acetate resulted in an accumulation of propionate which, could be used as a $CO_2$ acceptor during acetate assimilation into cellular material. Results from $^{14}CO_2$- fixation experiments obtained under these growth conditions indicated that a propionyl CoA carboxylase reaction existed in R. rubrum with methylmalonate and succinate clearly being identified as the end products of the reaction. Later, studies by Burton (20), demonstrated that propionate accumulation also resulted when R. rubrum was grown photoheterotrophically, on malate or succinate. Under these conditions, not only did propionate accumulate, but propionyl CoA carboxylase displayed high activity as compared with that in cells grown on acetate. Therefore, the presence of propionyl CoA carboxylase in R. rubrum has been demonstrated. However, questions as to how propionate accumulates, or what role propionate plays in the synthesis of cellular material still remain unsolved.

With the initial discovery by Larsen in 1951, of a new carboxylation pathway for propionate metabolism, as mentioned previously, attention was shifted slightly to examine certain metabolic possibilities offered by this new pathway. However, since specific evidence regarding the enzymes directly involved in this pathway did not exist, the significance of the new route for propionate utilization was unclear. Alternative pathways for propionate metabolism had already been proposed at that time (5), so that studies carried out on this carboxylation pathway were only one of several avenues to be explored. However, more recently, the importance of this carboxylation pathway and correspondingly, that of propionyl CoA carboxylase has become clearer.

Hsia and his co-workers (21, 22), using fibroblasts from propionyl CoA carboxylase-lacking children, have shown that the lack of this enzyme through an inborn metabolic defect, resulted in severe metabolic disorders and early death to patients with this hereditary condition (23, 24). Further tests revealed that a high concentration of propionate developed in the plasma and urine of these patients since propionate could not be oxidized to $CO_2$. Hence, propionyl CoA

carboxylase was essential for proper metabolic function and consequently, life itself.

Lynen and Tada (25) as well as Wawszkiewicz and Lynen (26) have suggested that propionyl CoA carboxylase was also important in erythronolide synthesis, the ground structure of erythromycin A. They observed that propionyl CoA acted as a primer in a condensation decarboxylation reaction between units of methylmalonyl CoA. Further evidence by Kanada and Corcoran (27) and also by Grisebach et al (28) clearly supported this proposal.

The importance of propionyl CoA carboxylase is also quite evident in photosynthetic bacteria. Evans and his co-workers (29) demonstrated an alternative pathway for the assimilation of carbon dioxide in photosynthetic cells and in autotrophic bacteria, besides the already known reductive pentose phosphate cycle. In this cycle, it is suggested that photosynthetic bacteria, like R. rubrum, are able to carry out the reversal of two normally irreversible reactions of carbohydrate metabolism. This is accomplished using reduced ferrodoxin generated photochemically by the organism. One such reaction is the conversion of succinyl CoA to α-ketoglutarate by a ferrodoxin-dependent carboxylation reaction. Propionyl CoA carboxylase, being present in microorganisms like R. rubrum, therefore

could replenish the cycle with succinyl CoA, thereby being a key anaplerotic enzyme in this ferredoxin-dependent carbon reduction cycle. However, the route of formation of the propionate used in the propionyl CoA carboxylase reaction is not known as yet and is still under investigation.

Kinetic examination of the behavior of biotin carboxylases, including propionyl CoA carboxylase, has been a topic of research for many years. Extensive studies have been conducted to determine the molecular and kinetic properties of such enzymes in an effort to establish the mechanism of the carboxylase reaction. Although the majority of the work done on propionyl CoA carboxylase has made use of mitochondrial extracts from pig heart (10, 12, 30-35), propionyl CoA carboxylase has also been isolated and purified from mitochondrial extracts from bovine liver (36-40), and from rat liver (41). In addition, the existence of this enzyme has been established in many microbial sources including extracts of Mycobacterium smegmatis (42), Rhodospirillum rubrum (1, 19), Nocardia corallina (43), Streptomyces erythraeus (26), Pseudomonas citronellolis (44), Mycobacterium phlei (44) and Bacillus cereus (44).

Studies with propionyl CoA carboxylase from pig heart mitochondria (5, 32-34, 45) demonstrated that the enzyme had a molecular weight of 700,000 daltons. It contained one mole of bound biotin per 175,000 grams of enzyme, suggesting the existence of four subunits per enzyme molecule, each having a molecular weight of 175,000 daltons. The enzyme had a sedimentation coefficient of 19.7 S and in the presence of 7 M urea, dissociated into inactive subunits of the 2.5 S variety, of undetermined molecular weight. Similar molecular characteristics were observed with the bovine liver mitochondrial enzyme (36-38), although characterization of the enzyme was not as extensive as was the case using the pig heart source.

Avidin inhibition has been verified for the propionyl CoA carboxylase from all sources thus far studied. This inhibition is almost complete in all cases and can be prevented by preincubation of the avidin with biotin. These results demonstrated that propionyl CoA carboxylase is a biotin-containing enzyme. The fact that this co-factor had a function in $CO_2$ fixation had long been postulated since it had been observed in many systems that $CO_2$ fixation was decreased in biotin deficiencies (45).

Lardy and Peanasky (9) first illustrated biotin involvement in the propionyl CoA carboxylase reaction. Later, work by Kosow and Lane (46) verified these findings and showed that incubation of liver slices from biotin deficient rats with (+)-biotin led to a rapid restoration of enzyme activity. Furthermore, incubation of partially purified preparations obtained from livers of biotin deficient rats with ATP and (+)-[$^{14}$C] biotin yielded propionyl CoA carboxylase labeled with $^{14}$C-biotin (47, 48). This evidence not only suggested biotin involvement in the propionyl CoA carboxylase reaction but indicated the presence of bound biotin in the enzyme itself. Through further investigation, many more carboxylases, besides the propionyl CoA carboxylase system, were shown to have a co-relation with biotin in their reaction schemes. It now seemed possible to generalize all biotin-containing carboxylases into one class. In this respect, at least four other carboxylases appeared similar to propionyl CoA carboxylase; β-methylcrotonyl CoA carboxylase (49), acetyl CoA carboxylase (50), pyruvate carboxylase (51), and the transcarboxylase, methylmalonyl-oxalacetictranscarboxylase (52).

Much of the present knowledge of the mechanism

of action of biotin carboxylases was derived from the work of Lynen and his co-workers (49) using β-methylcrotonyl CoA carboxylase. In 1959, Lynen first demonstrated that the biotin content of carboxylase enzyme preparations was directly proportional to the enzyme activity and was inhibited specifically by avidin which formed a complex with the biotin bound to the carboxylase (49). β-methylcrotonyl CoA carboxylase catalyzed a model reaction with free (+)-biotin; reaction (1) (49, 53):

$$ATP + H^{14}CO_3^- + (+)\text{-biotin} \xrightleftharpoons{Me^{++}} {}^{14}COO^-\text{-biotin} + ADP + P_i \quad (1)$$

This model reaction provided the means for obtaining direct evidence as to the mode of linkage of $HCO_3^-$ to biotin, resulting in the formation of carboxybiotin, an unstable reaction intermediate.

By 1961, a more general reaction mechanism had been established for all biotin-containing carboxylases (45). The general mechanism involved two partial reactions and will be mentioned later.

Further insight into the structure and mechanism of action of biotin-containing carboxylases was obtained principally through investigation of pyruvate and acetyl CoA carboxylases. These findings may be summarized as follows:-

1. Molecular Structure

The bulk of the information concerning molecular structure has been obtained by the groups of P. R. Vagelos and of M. D. Lane, using the E. coli acetyl CoA carboxylase. The system consists of the following types of proteins:

(a) Biotin Carboxyl Carrier Protein: This is a low molecular weight protein, which contains a covalently bound biotinyl group, but which has no catalytic activity (54, 55). The biotin carboxyl carrier protein from E. coli has a molecular weight of 45,000 daltons, and consists of two similar polypeptide chains of molecular weight equal to 22,500 daltons. This "native" form is extremely susceptible to proteolytic modification during isolation (56, 57).

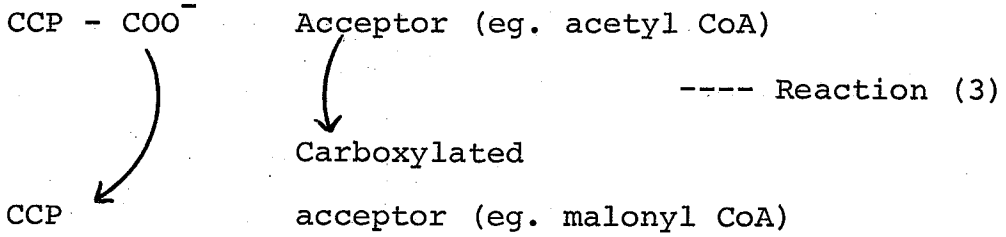
(b) A biotin carboxylase which can carboxylate the biotinyl residue of biotin carboxyl carrier protein, and catalyzes the following reaction:-

(Reaction 2)

$$CCP - Biotin + HCO_3^- + ATP \overset{Me^{++}}{\rightleftharpoons} CCP - Biotin - COO^- + ADP + P_i \quad \text{---- (2)}$$

This enzyme has been crystallized and exists as a dimer composed of apparently identical 51,000 dalton subunits (58, 59, 60).

(c) A carboxyltransferase which is responsible for the transfer of the $COO^-$ function from biotin carboxyl carrier protein to the acceptor molecule.




From E. coli this enzyme has a molecular weight of 130,000 and is composed of non-identical polypeptide chains of 30,000 and 35,000 daltons (58-61).

(d) In some systems a component is present which may be regulatory in function. For example, chicken liver acetyl CoA carboxylase is a polymeric filamentous structure composed of linearly aggregated protomers. The protomeric form consists of a tetrameric array of polypeptide chains having molecular weights equal to 117,000, 129,000, and 139,000 daltons in the ratio 2:1:1. One of these presumably is responsible for the binding of citrate which is an activator of this enzyme, but attempts to assign function to each of the polypeptides in the protomer have met with little success (62).

It is of interest that in E. coli there are apparently four polypeptide chains implicated in

the carboxylation of acetyl CoA. This is similar to the situation in the avian system in which the protomer is tetrameric.

Pyruvate carboxylases from avian liver and from yeast (S. cerevisiae) are also known to have tetrameric structures, and are presumably similar in this respect to acetyl CoA carboxylases (63).

2. Mechanism of Action

Without going into detailed discussion of individual carboxylase systems, the following generalizations can be made concerning the mechanism of action of biotin carboxylases.

The overall reaction catalyzed is the sum of the individual reactions discussed above:

$$\text{CCP - Biotin} + \text{HO-COO}^- + \text{ATP} \underset{\text{Biotin Carboxylase}}{\overset{Me^{++}}{\rightleftharpoons}} \text{CCP - Biotin - COO}^- + \text{ADP} + P_i \quad \text{--(2)}$$

$$\text{CCP - Biotin - COO}^- + \text{Acceptor} \underset{\text{Carboxyltransferase}}{\rightleftharpoons} \text{CCP - Biotin} + \text{Carboxylated Acceptor} \quad \text{--(3)}$$

---

$$\text{Net: HO - COO}^- + \text{ATP} + \text{Acceptor} \rightleftharpoons \text{carboxylated acceptor} + \text{ADP} + P_i \quad \text{--(4)}$$

---

From extensive kinetic analyses, principally using pyruvate carboxylase from a variety of sources, complex