PROPIONYL HOLOCARBOXYLASE SYNTHESIS

by

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List of Abbreviations

CoA	Coenzyme A
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
PPi	Pyrophosphate
Pi	Orthophosphate
GSH	Glutathione
DEAE-cellulose	Diethylaminoethyl-cellulose
TCA	Trichloroacetic acid
PPO	2,5-diphenyloxazole
POPOP	2,2-p-phenylenebis (5-phenyloxazole)

I. Introduction and Literature Review

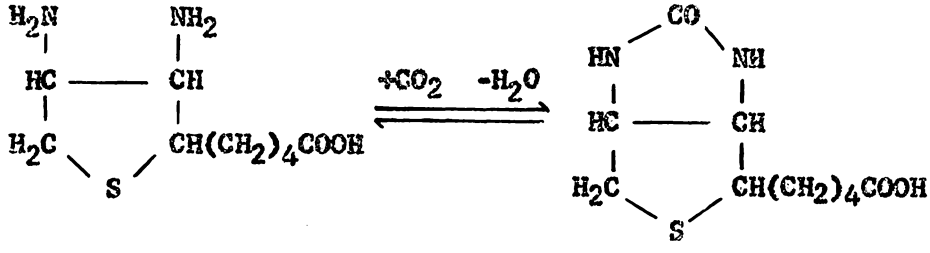
Biotin was first isolated in pure form by Kogl in 1936 (1). Since the identification of this vitamin which is the curative factor for egg white injury (2), many investigators have concerned themselves with the structure, synthesis and the mode of action of this vitamin. By 1943 the structure of the vitamin had been established (3) and its synthesis achieved (4). Early investigators recognized that biotin was required for various carboxylation reactions. The first unequivocal evidence for participation of biotin in an enzymatic reaction was the demonstration that this vitamin is the prosthetic group of acetyl carboxylase (5). Lardy and Peansky first reported (6) that liver mitochondrial extracts prepared from biotin-deficient rats exhibited a greatly reduced ability to carboxylate propionate. It was subsequently shown (7) that the enzymatic lesion involved in biotin-deficient rats was a reduced ability to carboxylate propionyl-CoA. Rapid restoration of the depressed propionyl carboxylase activity due to biotin deficiency has been accomplished by d-biotin administration in vivo and by incubation of liver slices with d-biotin in vitro (7). In 1960, the function of biotin as a prosthetic group of propionyl carboxylase (8,9) as well as β-methylcrotonyl carboxylase (10) and methylmalonyl-oxaloacetic transcarboxylase (11) was well established. Equations 1 and 2 illustrate the manner in which biotin participates as the prosthetic group of propionyl carboxylase (12).

(1) Enzyme-biotin + HCO_3^- + ATP $\overset{Mg^{++}}{\rightleftharpoons}$ Enzyme-biotin-CO_2 + ADP + Pi + H^+

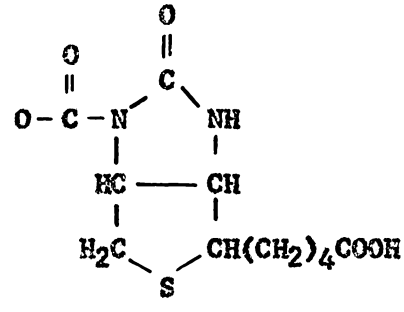
(2) Enzyme-biotin-CO_2 + Propionyl-CoA $\rightleftharpoons$ Enzyme-biotin + Methylmalonyl-CoA

Based on the structure of biotin and its known chemical reactions, it was postulated (13) that the vitamin might enter into biological CO_2 transferring reactions by virtue of an opening and closing of the ureido ring system as shown in equation 3. However, Melville and coworkers (14)

(3)



invalidated this hypothesis by the study of the stability of ureido-C^{14}-labeled biotin during CO_2 fixation by L. arabinosus. This was supported later by Lynen and coworkers (10) who observed that free biotin could be carboxylated by β,β-dimethylacrylyl carboxylase to form N-carboxybiotin. Thus, they assumed the formation of N-carboxybiotin-enzyme



N-carboxybiotin

complex as an intermediate in carboxylation reactions. Lynen's hypothesis received further support by Ochoa and Kaziro (15) and Lane et al. (12,8). Recently Wakil and Waite (16) presented evidence to suggest that the ureido carbon of acetyl-CoA carboxylase-bound biotin is the "active carbon" of biotin and that it is involved in carboxylation reactions. Equations 4 and 5 illustrate the manner in which the ureido carbon of biotin has been postulated to participate in the carboxylation of acetyl-CoA to form malonyl-CoA. Thus, according to these equations,

(4) Biotin-enzyme + Acetyl-CoA $\rightleftharpoons$ Diaminobiotin-enzyme + malonyl-CoA

(5) Diaminobiotin-enzyme + ATP + HCO_3^- $\overset{Mn^{++}}{\rightleftharpoons}$ Biotin-enzyme + ADP + Pi

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Acetyl-CoA + ATP + HCO_3^- $\overset{Mn^{++}}{\rightleftharpoons}$ malonyl-CoA + ADP + Pi

ATP is required only for the conversion of diaminobiotin-enzyme to biotin-enzyme.

Recently (17) a soluble enzyme system which catalyzes the ATP-dependent synthesis of propionyl holocarboxylase from biotin and propionyl apocarboxylase has been isolated from livers of biotin-deficient rats. After $(NH_4)_2SO_4$ fractionation this enzyme has been resolved into two enzyme fractions by gel adsorption (18). One fraction appears to contain the apocarboxylase and the other fraction an activating enzyme which catalyzes the attachment of biotin to propionyl apocarboxylase. Kosow and Lane (19) found that the terminal step of holocarboxylase synthesis is the covalent bonding of biotin to lysyl ϵ-amino groups of propionyl apocarboxylase. The basic requirements for propionyl holo-

carboxylase synthesis have been well studied (20). Concerning the mechanism of holocarboxylase synthesis, Kosow et al. (20) presented evidence to indicate that the involvement of carboxyl-activated biotin derivatives as intermediates in holocarboxylase synthesis seems improbable. Recently, however, Lynen et al. (21) reported that the terminal step in the synthesis of acetyl-CoA carboxylase is: Apocarboxylase + Biotinyl adenylate ——→ Holocarboxylase + AMP.

Wood et al. (11) reported that cell-free extracts of Propionibacterium shermanii do not contain propionyl carboxylase but do contain methylmalonyl-oxalacetic transcarboxylase (22) and probably other biotin enzymes. An enzyme present in cell-free extracts of Propionibacterium shermanii which catalyzes propionyl holocarboxylase synthesis from rat liver apocarboxylase and d-biotin has been recently described (20).

This report describes the further purification of both propionyl apocarboxylase and the activating enzyme. Studies on substrate specificity, reversibility and mechanism of the holocarboxylase synthesis system as well as studies on the structure of enzyme-biotin-CO_2 will be presented.

II. Experimental

Materials and Methods

Coenzyme A and ATP were obtained from Pabst Laboratories; avidin, specific activity 2.5 units per mg (23), from Nutritional Biochemicals Corporation; GSH, alumina C_γ gel, and hexokinase type IV from Sigma Chemical Company; DEAE-cellulose and cellulose phosphate from Carl Schleicher and Schuell Company; methanolic hyamine from Packard Instrument Company, Inc.; Sephadex G-50 from Pharmacia, Uppsala, Sweden, and sodium C^{14}-bicarbonate from New England Nuclear Corporation. d-Biotin-1-C^{14} (specific activity, 23μc per μmole) was kindly supplied by Dr. O. Wiss, Hoffmann-La Roche, Inc., Basle, Switzerland and d- and l-biotin by Dr. W. E. Scott, Hoffmann-La Roche, Inc., Nutley, New Jersey. Propionyl-CoA was prepared as previously described (24). Protein was determined spectrophotometrically (25). Methylmalonyl-CoA was isolated by the method of Halenz and Lane (8). Propionyl carboxylase assays were conducted by the CO_2-fixation method described by Halenz and Lane (8). Weanling male Sprague-Dawley rats were fed the previously described biotin deficient diet (7) from 21-25 days and at this time they were sacrificed by decapitation, the livers removed, rinsed with water and packed in ice. All of the subsequent procedures, including whole liver acetone powder preparation, ammonium sulfate fractionation and alumina C_γ gel fractionation were performed as previously described (20).

Purification of Propionyl Apocarboxylase

A). DEAE-cellulose Chromatographic Fractionation.— The propionyl apocarboxylase containing gel supernatant fraction (about 700 mg of protein) was applied directly to a DEAE-cellulose column (2.5 x 20 cm, packed volume) previously equilibrated (26) with 0.005M potassium phosphate buffer, pH 7.0. Elution was then accomplished by the successive addition of 50 ml quantities of each of the following potassium phosphate buffers, all pH 7.0 0.050M, 0.100M, 0.125M, 0.175M and 0.200M. The column eluate was collected fractionally (10 ml per fraction) and the protein for each fraction was determined by means of a UV recorder. Since the propionyl apo- and holocarboxylase have identical chromatographic properties on DEAE-cellulose (20) and the endogenous carboxylase is always present, propionyl apocarboxylase was located by conducting propionyl carboxylase assays on each fraction. The propionyl apocarboxylase containing fractions were pooled and saturated ammonium sulfate (pH 7.5)[1] was added to bring the solution to 40% saturation with respect to ammonium sulfate. The precipitate was discarded and the supernatant was brought to 60% saturation with saturated ammonium sulfate. The precipitate was then dissolved in a minimal amount of 0.005M sodium phosphate buffer, pH 7.0, containing 2×10^{-4}M neutralized GSH (6 mg of protein per ml) and dialyzed overnight in dialysis tubing (inside diameter, 0.6 cm) with stirring against 500 volumes of the same buffer.

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1 Ammonium sulphate was saturated at room temperature and neutralized with NH_4OH so that when diluted with four volumes of water the pH was 7.5.

B). Hydroxylaptite Chromatographic Fractionation.— The dialyzed ammonium sulfate fractionated enzyme (about 10 mg of protein) from the previous step was placed on a hydroxylapetite column (1 x 10 cm, packed volume) previously equilibrated with 0.005M potassium phosphate buffer, pH 7.0. Elution was then accomplished by the successive addition of 20 ml quantities of each of the same buffers that were used on the DEAE-cellulose column. Location of propionyl apocarboxylase is conducted by propionyl carboxylase assays. The propionyl apocarboxylase containing fractions were pooled and dialyzed overnight against 1 l of 60% saturated ammonium sulfate with stirring. The precipitated enzyme was dissolved in a minimal amount of 0.005M sodium phosphate buffer, pH 7.0, containing 2×10^{-4}M neutralized GSH. Table 1 summarizes the purification of propionyl apocarboxylase.

Purification of the Activating Enzyme

A). Ammonium Sulfate Fractionation.— Saturated ammonium sulfate (pH 7.5)[1] was added to the activating enzyme containing gel eluate fraction to bring the solution to 30% saturation with respect to ammonium sulfate. The precipitate obtained after centrifugation was dissolved in a minimal amount of 0.005M sodium phosphate buffer, pH 7.0, containing 2×10^{-4}M neutralized GSH (20 mg of protein per ml). This enzyme solution was then dialyzed overnight in dialysis tubing (inside diameter, 0.6 cm) with stirring against 200 volumes of the same buffer.

B). Cellulose Phosphate Chromatographic Fractionation.— The dialyzed ammonium sulfate fractionated enzyme (about 150 mg of protein) was

applied directly to a cellulose phosphate column (1 x 20 cm, packed volume) previously equilibrated with 0.050M potassium phosphate buffer, pH 7.0. Elution was then accomplished by the successive addition of 10 ml quantities of each of the following potassium phosphate buffers, all pH 7.0 : 0.100M, 0.125M, 0.150M, 0.175M, 0.200M, 0.250M and 0.300M. The column eluate was collected fractionally (one fraction for each buffer) and the protein for each fraction was determined by means of a UV recorder. The location of the activating enzyme was determined by measurement of propionyl holocarboxylase synthesis. Table 2 summarizes the results of the purification of the activating enzyme.

Measurement of Propionyl Holocarboxylase Synthesis

Propionyl holocarboxylase synthesis was assayed by the method of Kosow et al. (20). Unless otherwise specified, the following system was used: 40 μmoles of sodium phosphate buffer, pH 7.0; 1.25 μmoles of GSH; 1.5 μmoles each of ATP and $MgCl_2$; 0.2 mg (0.5 unit) of avidin; and both the apoenzyme and activating enzyme preparations being tested were preincubated for 10 minutes at 30°C. Holocarboxylase synthesis was then initiated by the addition of 20 μg of d-biotin and the reaction mixture (final volume, 1.0 ml) was incubated for four hours at 30°C. The reaction mixture was then diluted five fold with ice-cold water and a 0.2 ml aliquot was assayed for propionyl carboxylase activity.

Preparation of Cell-Free Extracts of Propionibacterium shermanii

The method of preparation of cell-free extracts of Propionibacterium

shermanii has been reported by this laboratory (20). Propionibacterium shermanii (ATCC 9614) was grown for four to five days at 30°C on the medium described by Delwiche (27), modified to contain a growth rate-limiting quantity of d-biotin (0.2 mg per ml). All subsequent operations were performed at 0-4°C. After cooling to 10°C, the cells were harvested (yield, 13 to 15 g wet weight) with a Sharples centrifuge, resuspended, and washed once with 50 ml of cold 0.9% NaCl. The cell suspension was centrifuged at 32,000 x g for 30 minutes, the pellet was resuspended in 25 ml of 0.3M K_2HPO_4 containing cysteine HCl (0.3 mg per ml), and was then sonically disintegrated at 10 KC. for 20 minutes (28). The temperature of the enzyme was maintained below 5°C during sonic disintegration. After centrifugation at 32,000 x g for 30 minutes, the supernatant solution was retained, and solid ammonium sulfate was added slowly with stirring until the concentration was raised to 80% saturation. The precipitate recovered after centrifugation was redissolved in approximately 25 ml of 0.005M sodium phosphate buffer, pH 7.0, containing 2×10^{-4}M neutralized GSH and was dialyzed against 200 volumes of the same buffer overnight. The dialyzed enzyme was then brought to 45% saturation by the gradual addition of saturated ammonium sulfate. The precipitate after centrifugation was redissolved in 30 ml of 0.005M sodium phosphate buffer, pH 7.0, containing 2×10^{-4}M neutralized GSH. This fraction contained 10 to 11 mg of protein per ml.

Measurement of C^{14}-Biotin Binding to Protein

The method of measurement of C^{14}-biotin binding to protein has been

described by Kosow et al. (20). Unless otherwise specified the following system was used. Sodium phosphate buffer, pH 7.0, 80 μmoles; GSH, 2.5 μmoles; ATP-$MgCl_2$, 3.0 μmoles; d-biotin-1-C^{14} (specific activity, 2.3 x 10^7 cpm per μmole), 0.022 μmole; and the enzyme being tested were incubated for four hours at 30°C in a final volume of 2.0 ml. The tubes were then chilled in an ice bath and the protein precipitated by addition of 6 ml of ice-cold 5% trichloroacetic acid. After centrifugation, the precipitate was retained and washed four times with 8 ml of ice-cold 5% TCA, two times with absolute ethanol, and two times with an ethanol-ether mixture (1:1, V/V). After the final wash the tubes were inverted to drain and 1.5 ml of methanolic Hyamine was added immediately. The mixture was then heated in a water bath at 60°C until the protein was completely dissolved. The volume was adjusted to 2.0 ml with methanolic Hyamine, a 1 ml aliquot added to 15 ml of ppo-popop-toluene (4 g: 0.1 g : 1000 ml) liquid scintillator, and C^{14}-activity bound to protein was measured using a tri-carb liquid scintillation spectrometer.

III. Results

Substrate Specificity of Propionyl Holocarboxylase Synthesized from Propionyl Apocarboxylase and d-Biotin Catalyzed by Activating Enzyme Prepared from Biotin-Deficient Rats

Crude propionyl apocarboxylase (gel supernatant fraction) and crude activating enzyme (gel eluate fraction) were used for the study of the substrate specificity of the synthesized holocarboxylase. Acetyl-CoA, butyryl-CoA and propionyl-CoA were tested as substrates for the synthesized holocarboxylase. The results are shown in Table 3. The relative rates of carboxylation of propionyl-, butyryl- and acetyl-CoA catalyzed by the synthesized holocarboxylase are 100, 8.0 and <1.0. Since highly purified propionyl carboxylase (12,29) exhibits a similar substrate specificity, it is apparant that the holocarboxylase synthesized by the rat liver system is propionyl holocarboxylase.

Activity of d- and l- Biotin for Propionyl Holocarboxylase Synthesis

It has been reported (20) that when either the valeric acid side chain of biotin is altered, as in homo- and nor-biotin, or the sulfur atom is removed or substituted, as in desthio- and hetero-biotin, holocarboxylase synthesis does not occur. In order to complete these studies, the activity of d- and l-biotin for propionyl holocarboxylase synthesis was determined. As shown in Table 4, propionyl holocarboxylase synthesis has an absolute requirement for d-biotin.

Reversibility of Propionyl Holocarboxylase Synthesis

Most enzymatic reactions in biological system are reversible. Reversibility of propionyl holocarboxylase synthesis catalyzed by the activating enzyme from either animal or bacterial origin was investigated. C^{14}-biotin containing propionyl holocarboxylase was prepared from the crude ammonium sulfate fractionated liver acetone powder extracts as described. Although it is now well established (17) that the synthesis of propionyl holocarboxylase from d-biotin and propionyl apocarboxylase is ATP-dependent, the hydrolytic products of ATP (either ADP and Pi or AMP and PPi) have not been reported. Thus the amount of C^{14}-biotin bound to propionyl holocarboxylase was determined after incubation of C^{14}-biotin labelled propionyl carboxylase and the activating enzyme either in the presence of ADP-$MgCl_2$ + Pi or AMP-$MgCl_2$ + PPi. Theoretically, if the activating enzyme also catalyzes the backward reaction of propionyl holocarboxylase synthesis leading to the formation of propionyl apocarboxylase and d-biotin, there should be a loss of radioactivity in propionyl holocarboxylase compared to the control which does not contain ADP-$MgCl_2$ + Pi or AMP-$MgCl_2$ + PPi in the reaction mixture. As shown in Table 5, no significant loss of d-biotin-1-C^{14} bound to protein was observed. This indicates that the synthesis of propionyl holocarboxylase from propionyl apocarboxylase and d-biotin catalyzed by the activating enzyme is an irreversible reaction.

Conversion of Liver Propionyl Apocarboxylase to Propionyl Holocarboxylase Catalyzed by Enzyme Extracts from Propionibacterium shermanii

Cell-free extracts of Propionibacterium shermanii are free of propionyl carboxylase (11) but do contain methylmalonyl-oxalacetic transcarboxylase (22) and probably other biotin enzymes. Preliminary investigations revealed, as shown in Table 6, that the enzyme preparation carried through the dialysis step, in the procedure previously described, catalyzed the binding of C^{14}-biotin to protein. It was subsequently found (see Table 7) that the Propionibacterium shermanii enzyme preparation could catalyze propionyl holocarboxylase synthesis utilizing rat liver apocarboxylase and d-biotin as substrates.

Kinetics of Propionyl Holocarboxylase Synthesis Catalyzed by Cell-Free Extracts of Propionibacterium shermanii

The kinetics of propionyl holocarboxylase synthesis and C^{14}-biotin binding to protein catalyzed by mammalian activating enzyme has been reported (20). The kinetics of propionyl holocarboxylase synthesis catalyzed by the activating enzyme from cell-free extracts of P. shermanii are shown in figure 2. Although the C^{14}-biotin binding to protein was not compared, the same pattern may be expected (20). The reaction is in zero order for the first two hours. Although maximum synthesis is not reached even at the end of a four hour incubation period, only negligable amounts of propionyl holocarboxylase were synthesized after four hours.

Investigations on the Ureido Carbon of Biotin Bound to Enzyme as the "Active Carbon" in Carboxylation Reactions

As stated in the introduction, there are two hypotheses concerning

the mechanism of action of enzyme-bound biotin in carboxylation reactions; (a) the 2' position of biotin serves as the active carboxyl of enzyme-bound biotin and (b) 1'-N-carboxybiotin-enzyme complex is an intermediate in carboxylation reactions. In order to investigate these hypotheses the following experiments were conducted.

Experiment 1: 2'-C^{14}-biotin labelled propionyl holocarboxylase was prepared by use of 2'-C^{14}-biotin[2] in the propionyl holocarboxylase synthsizing system. ATP, free biotin and other low molecular weight compounds were removed from the enzyme by gel filtration on Sephadex G-50. Propionyl-CoA was then incubated with this enzyme preparation for 20 minutes at 37°C. Methylmalonic acid was isolated from the reaction mixture by hydrolysis of its CoA ester and extracted with diethyl ether (8) and chromatographed on Whatman 3MM filter paper strips in the butanol : acetic acid : H_2O (4:1:5) solvent system. The methylmalonic acid was located with bromocresol purple spray indicator and radioactivity was detected by means of a Nuclear-Chicago Actigraph II Chromatogram strip counter. As shown in Table 8, Experiment 1, the ureido carbon of enzyme-bound biotin was not incorporated into methylmalonyl-CoA in the absence of ATP; neither was methylmalonyl-CoA formed nor was radioactivity found in any ether soluble compound under these conditions.

Experiment 2: The ureido C^{14}-biotin enzyme free of ATP prepared as described in Experiment 1, was treated in three different ways; in treat-

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[2] 2'-C^{14}-biotin was kindly supplied by Dr. S. J. Wakil, Duke University, Durham, N. C.

ment 1, enzyme was incubated alone as the negative control; in treatment 2, the enzyme was incubated with propionyl-CoA, ATP and bicarbonate in order to enable the enzyme to act catalytically and in treatment 3, only propionyl-CoA was incubated with enzyme. According to Waite and Wakil (16) the latter treatment should enable the enzyme to turn over once and quantitativily transfer its C^{14} to methylmalonyl-CoA. As shown in Table 8, Experiment 2, only when both ATP and CO_2 together with propionyl-CoA were added to the enzyme was methylmalonyl-CoA formed. However in no case was the ureido carbon of enzyme-bound biotin removed from the biotin as evidenced by the retention of radioactivity in the protein and the lack of incorporation of significant amounts of C^{14} into methylmalonyl-CoA.

Studies on the Mechanism of Propionyl Holocarboxylase Synthesis

As stated in the introduction, the improbability of involvement of carboxyl-activated biotin derivatives as intermediates in holocarboxylase synthesis has already been reported (20). This conclusion was based on the observations that NH_2OH failed to inhibit C^{14}-biotin binding to protein or propionyl holocarboxylase synthesis and biotinyl-AMP could not replace biotin and ATP for the synthesis of propionyl holocarboxylase. However, recently Lynen et al. (21) reported that biotinyl-AMP is an intermediate in the terminal step of the synthesis of acetyl-CoA carboxylase. The following experiments were conducted in order to investigate the mechanism of propionyl holocarboxylase synthesis.

Experiment 1: Hydroxamate formation.— In the presence of activating

enzyme, purified through stage 5, Table 2(0.01 mg protein), 0.011μmoles 1-C^{14}-biotin was incubated with 1,000μmoles neutralized hydroxylamine together with 80μmoles sodium phosphate buffer, pH 7.0; 2.5μmoles GSH and 3μmoles each of ATP and $MgCl_2$ at 30°C for 1 and 4 hours. The hydroxamate formed were extracted from the mixture (28) and chromatographed in the butanol: acetic acid : H_2O (8:1:1) solvent system. The location of biotinyl hydroxamate was detected by means of $FeCl_3$ spray reagent and Co-chromatographed authentic biotinyl hydroxamate. No obvious radioactive peak was observed by means of a Nuclear-Chicage Actigraph II chromatogram strip counter. The radioactivity on the chromatogram was assayed with greater sensitivity by elution of the paper strip with water in the regions of R_f 0.85 (corresponding to biotin) and R_f 0.70 (corresponding to biotinyl hydroxamate) and counting in a Tri-Carb scintillation spectrometer with the ppo-popop-toluene (4 g:2.1 g:1000 ml) liquid scintillator system. The results are shown in Table 9; less than 2% of the biotin added was converted to biotinyl hydroxamate. Also as shown in the table, no marked difference of biotinyl hydroxamate formed was noticed between 1 and 4 hours incubation. Thus, this reveals that the formation of a biotinyl intermediate, if it is an intermediate in the reaction, is not the rate limiting step of holocarboxylase synthesis.

Experiment 2: Attempted isolation of intermediates.— Purified activating enzyme (stage 5, Table 2, 0.01 mg protein) was incubated with 40γ d-biotin in the presence of 4μmoles each of ATP and $MgCl_2$, 80μmoles sodium phosphate buffer, pH 7.0 and 3μmoles GSH at 30°C for 2 hours. The reaction mixture was cooled to 0°C, placed on a sephadex G-50 column

(2 x 25 cm, packed volume) previously equilibrated with 0.005M potassium phosphate buffer, pH 7.0 with 2 x 10^{-4}M neutralized GSH and eluted with the same buffer. The protein containing eluate (first peak) and the low molecular weight compound containing eluate (second peak) were located by means of a UV recorder and were collected separately. Purified propionyl apocarboxylase (stage 6, Table 1) was then incubated with either the first peak (Table 10, Experiment 1) or the second peak (Table 10, Experiment 2) at 30°C for another 2 hours. If enzyme-biotin or any free intermediate such as biotinyl-AMP is an intermediate in the reaction, propionyl holocarboxylase synthesis should occur during incubation of propionyl apocarboxylase with the first peak or the second peak respectively. In Experiment 1, hexokinase and glucose were added to the reaction mixture in order to remove any contaminating ATP completely. As shown in Table 10, no propionyl holocarboxylase was synthesized during the incubation of propionyl apocarboxylase. In Experiment 2, hexokinase and glucose were again added to the reaction mixture in order to remove the ATP which was present in the second peak. The completion of the removal of ATP by the hexokinase and glucose under the condition described in Table 10 are reflected by treatments 3 and 4 of Experiment 2. The results indicate that neither an enzyme-bound nor a free intermediate was formed under the conditions described. Since it is conceivable that the intermediate if it exists, is unstable under the conditions of these experiments, the evidence presented here does not conclusively rule out the involvement of free or enzyme-bound intermediates, although a concerted mechanism is indicated.

IV. Discussion

It is of interest that a bacterium, P. shermanii, which does not possess propionyl carboxylase (11) or propionyl apocarboxylase[3], elaborates an enzyme that catalyzes d-biotin attachment to propionyl apocarboxylase of mammalian origin (rat). This suggests the possibility that other apocarboxylases or apotranscarboxylases (22) common to P. shermanii closely resemble propionyl apocarboxylase from other organisms in the region of the acceptor site. Since the apocarboxylase active site, ultimately becomes the active site of holocarboxylase, possibly all biotin-containing carboxylases have similar active sites.

Attempts to demonstrate apocarboxylase formation from holocarboxylase in the presence of AMP or ADP were unsuccessful. The activating enzyme which catalyzes the binding of C^{14}-biotin to propionyl apocarboxylase does not catalyze the reverse reaction. It is of interest that Ochoa's laboratory has reported (30) that biotinidase, the enzyme which hydrolyzes biocytin and other biotin esters does not release biotin from crystalline pig heart propionyl carboxylase although biotin is bound to propionyl carboxylase via lysyl-ε-amino groups. It seems likely that although, as shown by this laboratory, the covalent bonding of d-biotin to lysyl-ε-amino groups is the terminal step of propionyl holocarboxylase synthesis the release of free biotin from the carboxylase is not the

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3 D. P. Kosow and M. D. Lane, unpublished experiment.

initial step but rather the terminal step in the catabolism of the protein.

2'-C^{14}-biotin bound enzyme, prepared from 2'-C^{14}-biotin and propionyl apocarboxylase catalyzed by the activating enzyme does not transfer its labelled carbon to carboxylate propionyl-CoA. Wood and coworkers have recently reported (31) similar results with ureido-C^{14}-biotin labelled methylmalonyl-oxalacetic transcarboxylase isolated from Propionibacterium shermanii. Although these results do not indicate the nature of the active form of biotin, they strongly suggest that the ureido carbon of biotin is not the source of CO_2 for biotin dependent carboxylation reactions.

The observation that only less than 2% of total biotin added forms biotinyl hydroxamate when incubated with the activating enzyme raises again the question, as to whether a carboxyl-activated biotinyl intermediate is involved in holocarboxylase synthesis. Since attempts to isolate intermediates of the reaction by the techniques described have been unsuccessful, the synthesis of propionyl holocarboxylase appears to occur via a concerted mechanism, however further investigation is indicated.

V. Summary

A soluble enzyme system, isolated from livers of biotin-deficient rats, catalyzes the ATP-dependent synthesis of propionyl holocarboxylase from d-biotin and propionyl apocarboxylase. This system has been resolved by alumina C γ gel fractionation into two essential components; (a) gel supernatant which contains propionyl apocarboxylase and (b) gel eluate which contains an enzyme which catalyzes the covalent bonding of d-biotin to propionyl apocarboxylase. The propionyl holocarboxylase synthesis catalyzed by these enzyme systems is irreversible and d-biotin specific. The gel supernatant has been further purified by hydroxylapatite chromatography and $(NH_4)_2SO_4$ fractionation and the gel eluate by $(NH_4)_2SO_4$ precipitation and cellulose-phosphate chromatography. An enzyme similar to the gel eluate enzyme has been isolated from cell-free extracts of Propionibacterium shermanii. Although cell-free extracts of P. shermanii do not contain propionyl apo- or holocarboxylase, they do catalyze ATP-dependent propionyl holocarboxylase formation from d-biotin and rat liver propionyl apocarboxylase. Biotin-2'-C^{14}-labelled propionyl holocarboxylase, synthesized with these enzyme systems, does not transfer $C^{14}O_2$ to propionyl-CoA indicating that the ureido carbon of enzyme-bound biotin is not the "active carbon" of biotin.

VI. Acknowledgement

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IX. Appendix

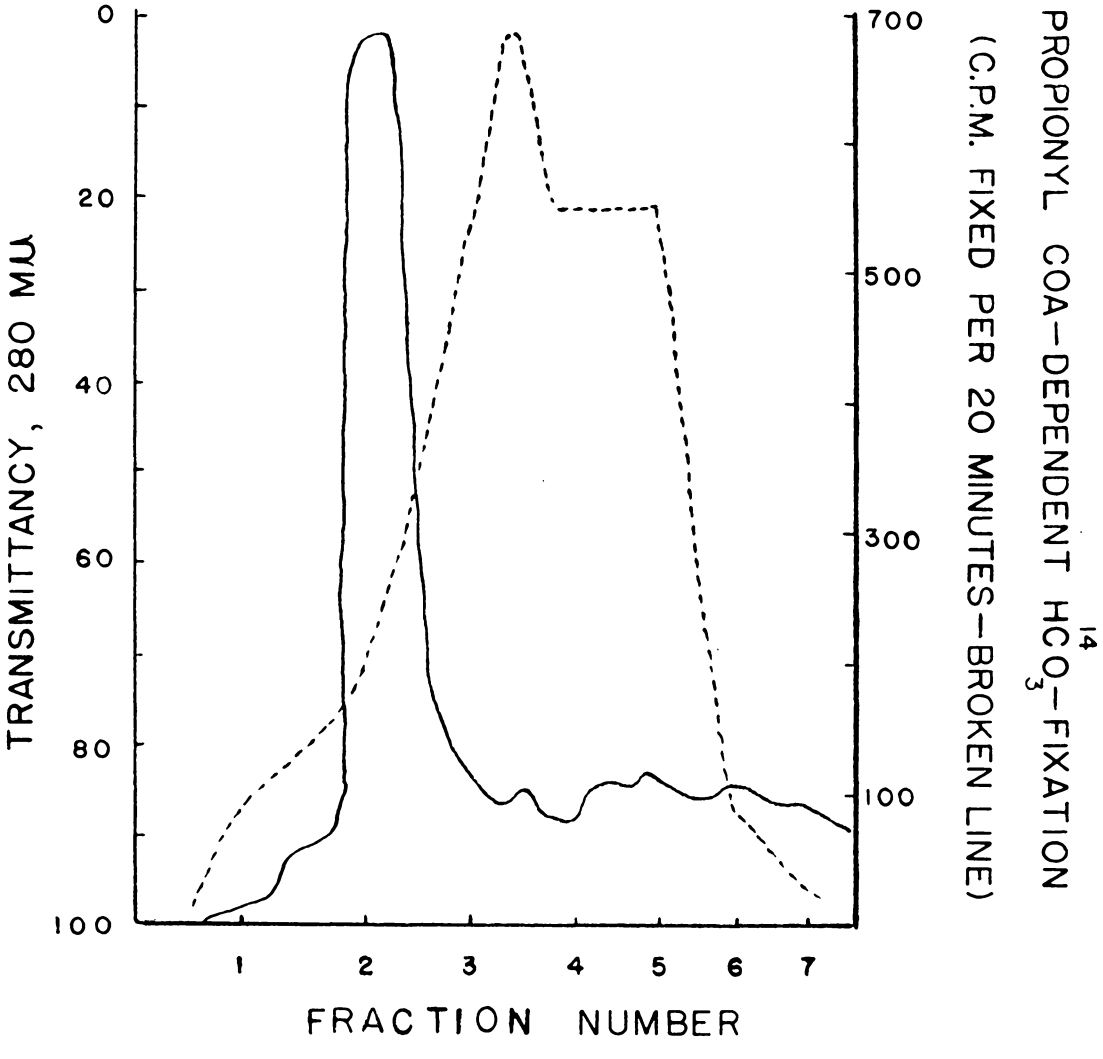

Figure 1. Cellulose Phosphate Chromatography of Activating Enzyme from Biotin-deficient Rats.

The 30% ammonium sulfate saturated gel eluate fraction containing 100 mg of protein was chromatographed as described in the text. Propionyl carboxylase activity was determined on a 0.2 ml aliquot of the 5-fold diluted holocarboxylase synthesis reaction mixture after 2-hour incubation with each column fraction (0.5 ml aliquot) and gel supernatant fraction (10.5 mg of protein).

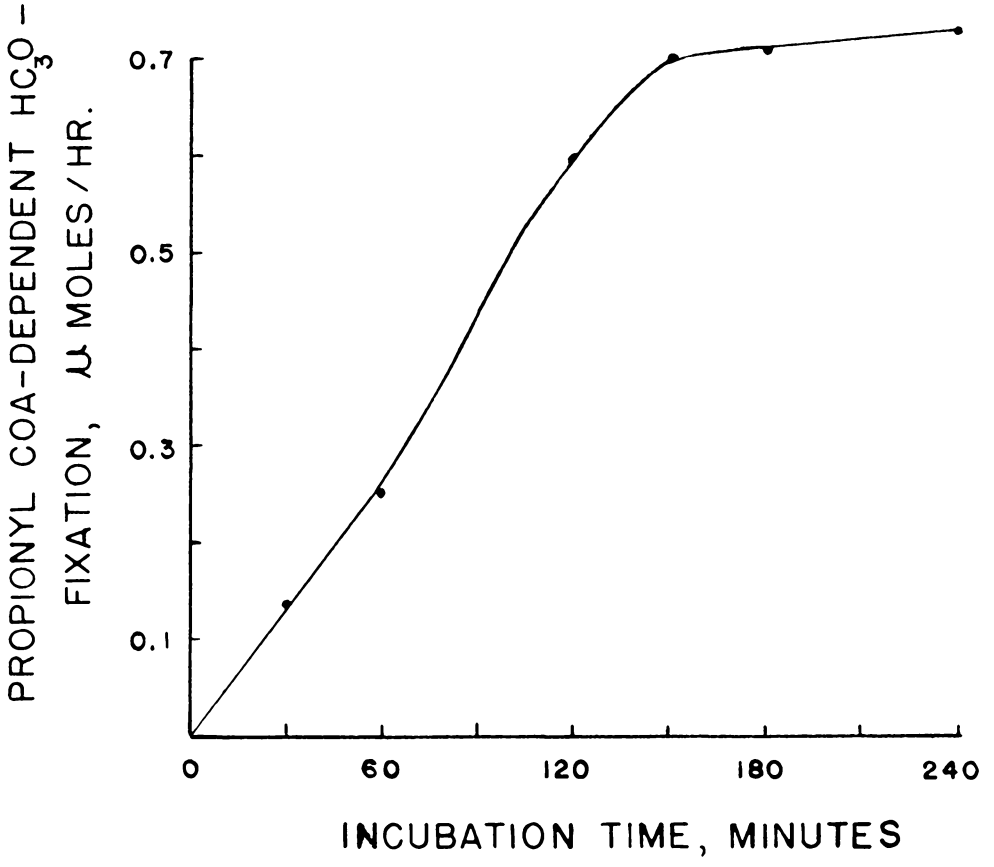

Figure 2. Kinetics of Propionyl Holocarboxylase Synthesis.

Holocarboxylase synthesis was determined as described in the text. Each tube contained 0.1 mg of DEAE-cellulose chromatographed gel supernatant enzyme fraction and 0.4 mg of the 40-45% ammonium sulfate saturated cell-free extracts of P. shermanii.

Table 1. Purification of Propionyl Apocarboxylase from Biotin-Deficient Rats.

Stage of purification	Volume (ml)	Protein (mg)	Enzyme activity*(u)	Specific activity
1. Acetone powder extract	135	1296	51.7	0.039
2. 45% $(NH_4)_2SO_4$ fraction	16.5	743	46.8	0.063
3. Dialyzed $(NH_4)_2SO_4$ fraction	18.7	760	48.3	0.063
4. Gel supernatant	21.5	581	40.0	0.068
5. DEAE-cellulose chromatography	33	15	16.0	1.060
6. 2nd $(NH_4)_2SO_4$ fraction (40-60%)	1.4	8	12.0	1.500
7. Hydroxylapatite chromatography	19.4	1.3	10.0	7.700

* Unit of enzyme activity is expressed as units of endogenous propionyl holocarboxylase.

Table 2. Purification of Activating Enzyme from Biotin-Deficient Rats.

Stage of purification	Volume (ml)	Protein (mg)	Enzyme activity* (μ)	Specific activity
1. Gel eluate fraction	25	225	67	0.297
2. 30% $(NH_4)_2SO_4$ saturated fraction	5	102	60	0.588
3. Dialyzed 30% $(NH_4)_2SO_4$ saturated fraction	5.2	104	61.6	0.592
4. Cellulose phosphate chromatography	28	5.3	47	8.86
5. 2nd $(NH_4)_2SO_4$ fraction (50% saturation)	7	4	45	11.25

* One unit of enzyme activity is defined as the amount of enzyme which catalyzes the synthesis of one unit of propionyl carboxylase per four hours.

Table 3. Substrate Specificity of Propionyl Holocarboxylase Synthesized from Propionyl Apocarboxylase and d-Biotin Catalyzed by Activating Enzyme Prepared from Biotin-Deficient Rats.

Propionyl holocarboxylase synthesis was performed as described in the text. Acetyl-(0.5μm), propionyl-(0.4μm) and butyryl-CoA (0.4μm) were used as substrates in the carboxylation reaction catalyzed by the synthesized holocarboxylase. Each tube contained the gel supernatant fraction (5.5 mg of protein) and the gel eluate fraction (0.8 mg of protein). Enzyme activity is corrected for controls which consist of the complete system of holocarboxylase synthesis minus ATP.

Substrates	Synthesized holocarboxylase
	Carboxylase units
Acetyl-CoA	0.00
Butyryl-CoA	0.085
Propionyl-CoA	1.060

Table 4. Activity of d- and l-Biotin for Propionyl Holocarboxylase Synthesis

Propionyl holocarboxylase synthesis was determined as described in the text. However, d-biotin was not included in the basic reaction mixture. Each tube contained the gel supernatant fraction (7.5 mg of protein) and the gel eluate fraction (1.5 mg of protein).

Addition to basic reaction mixture	Propionyl holocarboxylase synthesis
	Carboxylase units
20 μg l-biotin	0.05
20 μg d-biotin	0.37

Table 5. Reversibility of Propionyl Holocarboxylase Synthesis.

C^{14}-biotin labelled propionyl holocarboxylase was prepared from crude ammonium sulfate fractionated liver acetone powder extracts as described in the text. The amount of C^{14}-biotin bound in propionyl holocarboxylase is determined as described after four hours incubation at 30°C. The basic reaction mixture in each tube contains sodium phosphate buffer, pH 7.0, 120μmoles; GSH 2.5μmoles; C^{14}-biotin labelled propionyl holocarboxylase (12 mg and 14.5 mg of protein in Experiments 1 and 2, respectively) and ammonium sulfate fractionated enzyme (10.8 mg and 12 mg of protein in Experiments 1 and 2, respectively).

Additions to basic reaction mixture	d-Biotin-1-C^{14} bound in protein
	mμg/mg
Experiment 1	
None	7.9
Pi + ADP + $MgCl_2$, 4μmoles	9.0
Pi + ADP + $MgCl_2$, 4μmoles plus 0.5 mg hexokinase in 20μM glucose	9.3
Experiment 2	
None	18.2
PPi + AMP + $MgCl_2$, 4μmoles	18.7
PPi + AMP + $MgCl_2$, 4μmoles plus 0.5 mg hexokinase in 20μM glucose	17.6
*PPi + AMP + $MgCl_2$, 4μmoles plus 0.5 mg hexokinase in 20μM glucose	17.9

* 3 mg of protein of the 0-70% saturated ammonium sulfate fraction of P. shermanii cell-free extracts was used instead of ammonium sulfate fractionated rat liver enzyme.

Table 6. C^{14}-Biotin Binding to Protein Catalyzed by Cell-Free Extracts of Propionibacterium shermanii.

The reaction mixture and procedure for determining C^{14}-biotin binding to protein are as described in the text. Each tube contained 16 mg of protein of the 0-80% saturated ammonium sulfate fraction of Propionibacterium shermanii cell-free extracts.

Duration of incubation	d-Biotin-1-C^{14} bound to protein		
	Minus ATP	Plus ATP	Net due to ATP
Minutes	mμg	mμg	mμg
15	4	187	183
30	6	313	307

* Average of duplicates corrected for zero time controls.

Table 7. Propionyl Holocarboxylase Synthesis from Mammalian Apocarboxylase and d-Biotin Catalyzed by a Propionibacterium shermanii Enzyme.

Propionyl holocarboxylase synthesis was determined as described in the text. The 0-40% saturated ammonium sulfate fraction of Propionibacterium shermanii extracts (1.0 mg of protein) and DEAE-cellulose chromatographed rat liver apocarboxylase (0.4 mg of protein) were added as indicated.

Enzyme addition	Propionyl holocarboxylase synthesis
	Carboxylase unit*
P. shermanii enzyme	0.03
Liver apocarboxylase	0.00
P. shermanii enzyme plus liver apocarboxylase	1.23

* Average of duplicates.

Table 8. Investigation on the Ureido Carbon of Biotin Bound in Enzyme as the "Active Carbon" in Carboxylation Reactions.

The enzyme was prepared as described in the text. The basic reaction mixture of Experiment 1 contained 29 ml of enzyme solution, 75μ moles GSH and 1.2 millimoles sodium phosphate buffer, pH 7.0; of Experiment 2 contained 9 ml of enzyme solution, 25μ moles GSH and 0.4 millimoles sodium phosphate buffer, pH 7.0.

	Amount of C^{14}-biotin bound to enzyme (cpm)	Activity of propionyl carboxylase (units/ml enzyme)	Addition to basic reaction mixture	Methyl-malonyl-CoA formed	Radio-activity in methyl-malonyl-CoA
Experiment 1	2780	5.22	Propionyl-CoA, 5μM	-	0
Experiment 2					
Treatment 1	938	1.44	None	-	0
Treatment 2	948	1.44	ATP, 20μM; propionyl-CoA, 2μM; $KHCO_3$, 20μM	+	46
Treatment 3	907	1.44	Propionyl-CoA, 2μM	-	0

Table 9. Biotinyl Hydroxamate Formation Catalyzed by the Activating Enzyme.

The amount of free C^{14}-biotin left in the reaction mixture and biotinyl hydroxamate formed at the end of incubation period were determined with a Tri-Carb scintillation spectrometer after the elution of the paper chromatograms with H_2O in the region of R_f 0.85 and 0.70 on the chromatogram respectively. All counts per minute were corrected for controls which did not contain ATP in reaction mixture.

Incubation time (hour)	Amount of free C^{14}-biotin left in reaction mixture (cpm)	Amount of biotinyl hydroxamate formed (cmp)	% biotinyl hydroxamate formed
1	9237	143	1.52
4	10156	186	1.79

Table 10. Studies of the Intermediates Involved in Propionyl Holocarboxylase Synthesis.

The basic reaction mixture in Experiment 1 contained 3.5 ml of first peak eluate from Sephadex G-50 column (2 mg of protein); 120 μmoles sodium phosphate buffer, pH 7.0; 3.75 μmoles GSH; 600 units of hexokinase type IV (6 μmoles/min.) and 5 μmoles glucose; in Experiment 2, 6.8 ml of redissolved lyophlized 2nd peak eluate from Sephadex G-50 column containing 40 μmoles sodium phosphate buffer, pH 7.0 and 1.25 μmoles GSH.

Addition to basic reaction mixture	Propionyl holocarboxylase synthesis
	Carboxylase assay
Experiment 1	cpm/0.2 ml reaction mixture
1. None	103
2. Propionyl apocarboxylase	144
Experiment 2	cpm/0.04 ml reaction mixture
1. Propionyl apocarboxylase, hexokinase and glucose	60
2. Propionyl apocarboxylase	100
3. Propionyl apocarboxylase, activating enzyme, hexokinase and glucose	60
4. Propionyl apocarboxylase and activating enzyme	670
5. None	64

Notes:

1. The final volume of reaction mixture was 4 ml and 1 ml in Experiments 1 and 2 respectively.

2. Propionyl apocarboxylase used was purified through stage 6, Table 2 (0.4 mg and 0.31 mg of protein in Experiments 1 and 2 respectively).

3. Hexokinase type IV, 100 units (1 mole/min.); glucose, 1 mole were used in Experiment 2.

4. The activating enzyme used in Experiment 2 was purified through stage 5, Table 2 (0.004 mg of protein).

5. Propionyl holocarboxylase syntheses were corrected for endogenous propionyl carboxylase contained in propionyl apocarboxylase.

Abstract

A soluble enzyme system, isolated from livers of biotin-deficient rats, catalyzes the ATP-dependent synthesis of propionyl holocarboxylase from d-biotin and propionyl apocarboxylase. This system has been resolved by alumina C_γ gel fractionation into two essential components; (a) gel supernatant which contains propionyl apocarboxylase and (b) gel eluate which contains an enzyme which catalyzes the covalent bonding of d-biotin to propionyl apocarboxylase. The propionyl holocarboxylase synthesis catalyzed by these enzyme systems is irreversible and d-biotin specific. The gel supernatant has been further purified by hydroxyl-apatite chromatography and $NH_4)_2SO_4$ fractionation and the gel eluate by $(NH_4)_2SO_4$ precipitation and cellulose-phosphate chromatography. An enzyme similar to the gel eluate enzyme has been isolated from cell-free extracts of Propionibacterium shermanii. Although cell-free extracts of P. shermanii do not contain propionyl apo- or holocarboxylase, they do catalyze ATP-dependent propionyl holocarboxylase formation from d-biotin and rat liver propionyl apocarboxylase. Biotin-2'-C^{14}-labelled propionyl holocarboxylase, synthesized with these enzyme systems, does not transfer $C^{14}O_2$ to propionyl-CoA indicating that the ureido carbon of enzyme-bound biotin is not the "active carbon" of biotin.