Metabolism of Poly-β-hydroxybutyrate. II. Enzymatic Synthesis of D-(−)-β-Hydroxybutyryl Coenzyme A by an Enoyl Hydrase from *Rhodospirillum rubrum**

G. J. Moskowitz† and J. M. Merrick‡

ABSTRACT: Two enoyl coenzyme A hydrases have been separated from *Rhodospirillum rubrum* extracts whose combined activity results in the racemization of D-(−)-β-hydroxybutyryl coenzyme A to L-(+)-β-hydroxybutyryl coenzyme A. Both hydrases catalyze the reversible hydration of crotonyl coenzyme A to β-hydroxybutyryl coenzyme A. One of the hydrases is specific for the synthesis of the D-(−) isomer (enoyl coenzyme A hydrase (D)) while the other catalyzes the synthesis of the L-(+) isomer. The following reactions are proposed for the racemization of the D-(−) isomer to the L-(+) isomer: D-(−)-β-hydroxybutyryl coenzyme A → crotonyl coenzyme A → L-(+)-β-hydroxybutyryl coenzyme A. Enoyl coenzyme A hydrase (D) has been purified 680-fold and its properties were studied. The enzyme also hydrates crotonyl thioesters of pantetheine and acyl carrier protein. The following pathway for poly-β-hydroxybutyrate synthesis from acetate by *R. rubum* is proposed: acetate → acetyl coenzyme A → acetoacetyl coenzyme A → L-(+)-β-hydroxybutyryl coenzyme A → crotonyl coenzyme A → D-(−)-β-hydroxybutyryl coenzyme A → poly-β-hydroxybutyrate.

Poly-β-hydroxybutyrate, the principal lipid reserve of many different types of bacteria, is a polyester composed of the D-(−) stereoisomer of β-hydroxybutyrate. The structure, composition, and properties of purified poly-β-hydroxybutyrate granules has recently been reported (Griebel *et al.*, 1968; Ellar *et al.*, 1968). The synthesis of this stereoregular polymer has been studied with cell-free preparations from *Rhodospirillum rubrum* and *Bacillus megaterium* (Merrick and Doudoroff, 1961; Griebel *et al.*, 1968). In both cases it has been shown that the poly-β-hydroxybutyrate synthetase is associated with isolated granules and is presumably located in the membrane coat which surrounds the granule. Although D-(−)-β-hydroxybutyryl-CoA can readily be shown to be the substrate for the polymerization reaction, the biosynthesis of this thioester by bacteria was unclear. Schindler (1964) and Kominek and Halvorson (1965) have examined crude extracts of *Hydrogenomonas* and *Bacillus cereus*, respectively. In both cases an acetoacetyl-CoA reductase was found to be present and in the case of *B. cereus* this enzyme appears at the time that poly-β-hydroxybutyrate is accumulated by the cells. In neither case, however, was it shown whether the immediate product of the reduction was the D-(−)- or L-(+)-β-hydroxybutyryl-CoA derivative. An acetoacetyl-CoA reductase which catalyzes the synthesis of D-(−)-β-hydroxybutyryl-CoA from acetoacetyl-CoA and NADPH has been isolated from pigeon liver by Wakil and Bressler (1962).

In order to investigate the biosynthesis of D-(−)-β-hydroxybutyryl-CoA by *R. rubrum*, studies were initiated to examine whether a specific D-(−)-β-hydroxybutyryl-CoA dehydrogenase was present in extracts from this organism. Indeed, it was found that crude extracts catalyzed a NAD^+-dependent oxidation of D-(−)-β-hydroxybutyryl-CoA. However, on further purification it was found that the D isomer was converted into L-(+)-β-hydroxybutyryl-CoA prior to oxidation. Stern *et al.* (1955) had previously found an L-(+)-β-hydroxybutyryl-CoA dehydrogenase in *R. rubrum* extracts and also reported that such extracts catalyzed the interconversion of the D-(−) and L-(+) isomers but the mechanism of this racemization was not further studied. Evidence will be presented which demonstrates that the interconversion of the D and L isomers is catalyzed by two specific enoyl-CoA hydrases according to the following reactions:

$$\text{D-(}-\text{)-}\beta\text{-hydroxybutyryl-CoA} \rightleftharpoons \text{crotonyl-CoA} + H_2O \quad (1)$$

$$\text{crotonyl-CoA} + H_2O \rightleftharpoons \text{L-(}+\text{)-}\beta\text{-hydroxybutyryl-CoA} \quad (2)$$

The purification and properties of the enoyl-CoA hydrase which reversibly hydrates crotonyl-CoA to D-(−)-β-hydroxybutyryl-CoA (reaction 1) are reported in this communication. A preliminary report of this work has been presented (Moskowitz and Merrick, 1967).

Experimental Procedure

Materials and Methods. Cultures of *R. rubrum* (strain III, C. B. van Niel) were grown on acetate medium in the light as described by Shuster and Doudoroff (1962). Uniformly labeled D-(−)-β-hydroxybutyric-^{14}C acid was prepared by alkaline hydrolysis of uniformly labeled poly-β-hydroxybutyrate as previously described by Merrick and Doudoroff (1961). DL-β-Hy-

* From the Biological Research Laboratories, Department of Bacteriology and Botany, and Biochemistry Committee, Syracuse University, Syracuse, New York 13210. *Received January 27, 1969.* This investigation was supported by Research Grant AI-07176 from the National Institutes of Health, U. S. Public Health Service.

† Present address: Department of Pharmacology, Baylor University College of Medicine, Houston, Texas 77025. The early studies of this work were carried out in the Department of Biochemistry at the State University of New York at Buffalo. Parts of this work were submitted to that institution in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ To whom inquiries regarding this paper should be sent. Mailing address: Biological Research Laboratories, Syracuse, N. Y. 13210.

droxybutyric acid-3-^{14}C was purchased from Nuclear Science and Engineering Corp.; crotonic-1-^{14}C acid from Nuclear Research Chemicals, Inc.; *trans*-2-hexenoic acid from Sapon Labs; and crystalline pig heart L-(+)-β-hydroxyacyl-CoA dehydrogenase from Calbiochem. Pantethine purchased from Sigma was reduced to pantetheine with borohydride at pH 8.0. D-(−)-β-Hydroxybutyric dehydrogenase was purified from *Pseudomonas lemoignei* as described by Delafield *et al.* (1965). Acyl carrier protein was kindly provided by Dr. P. Roy Vagelos. Native poly-β-hydroxybutyrate granules from *B. megaterium* were prepared as described by Merrick and Doudoroff (1964).

β-Hydroxybutyryl-CoA, crotonyl-CoA, crotonylpantetheine, acrylyl-CoA, crotonylglutathione, and *trans*-2-hexenoyl-CoA were prepared by the method of Wieland and Rueff (1953); crotonyl acyl carrier protein was synthesized by the procedure described by Ailhaud *et al.* (1967) with the modification of Birge *et al.* (1967). L-(+)-β-Hydroxbutyryl-CoA was prepared from DL-β-hydroxybutyryl-CoA by selective removal of the D isomer by its polymerization to poly-β-hydroxybutyrate with poly-β-hydroxybutyrate synthetase. DL-β-Hydroxybutyryl-3-^{14}C-CoA (2.5 μmoles) was added to a reaction mixture containing 50 μmoles of potassium phosphate (pH 7.5), 5 μmoles of $MgCl_2$, and excess poly-β-hydroxybutyrate synthetase (added as native poly-β-hydroxybutyrate granules) in a final volume of 0.6 ml. After incubation at 30° for 15 min the labeled polymer was removed by centrifugation. Native poly-β-hydroxybutyrate granules were again added to the supernatant fluid, and the incubation was repeated by removal of the polymer by centrifugation. This procedure was repeated a third time to ensure complete removal of D-(−)-β-hydroxybutyryl-CoA; 0.84 μmole of L-(+)-β-hydroxybutyryl-CoA remained as assayed with L-(+) crystalline pig heart β-hydroxyacyl-CoA dehydrogenase (Stern, 1957).

β-Hydroxybutyrate thioesters were measured by the hydroxamate method described by Stadtman (1957). α,β-Unsaturated thioesters were assayed as described by Stern and Del Campillo (1956). The hydration of α,β-unsaturated thioesters was followed by measuring the decrease in absorbance at 263 mμ. D-(−)-β-Hydroxybutyric acid was assayed with D-(−)-β-hydroxybutyric dehydrogenase (Shuster and Doudoroff, 1962). Protein was determined by the method of Lowry *et al.* (1951). β-Hydroxybutyrylhydroxamate was identified by paper chromatography in two solvent systems: 1-butanol–water (100:18, v/v) and *sec*-butyl alcohol–formic acid–water (75:13:12, v/v).

Assay of Enoyl-CoA Hydrase. The hydrases were conveniently assayed by following the hydration of crotonyl-CoA by measuring the disappearance of absorbance at 263 mμ due to the crotonyl thioester bond (Stern *et al.*, 1956). Reaction mixtures contained Tris-HCl buffer (pH 8.5, 10 μmoles), crotonyl-CoA (9 mμmoles), and enzyme in a final volume of 0.25 ml. Enzyme preparations were diluted as necessary in 0.05 M Tris-HCl buffer (pH 8.5) containing 0.001 M EDTA and 0.01% egg albumin. The reactions were carried out at 25° and readings were taken at 30-sec intervals in a Zeiss spectrophotometer. Under these conditions the rate of reaction was proportional to enzyme concentrations and linear for at least 3 min.

Conversion of D-(−)-β-Hydroxybutyryl-CoA into L-(+)-β-Hydroxybutyryl-CoA. Early experiments suggested that crude extracts of *R. rubrum* were capable of catalyzing the oxidation of D-(−)-β-hydroxybutyryl-CoA in the presence of NAD^+. However on ammonium sulfate fractionation of the extracts, preliminary evidence was obtained which suggested that more than one step was involved in the oxidation of the D isomer. Thus, two fractions were isolated which on combination gave at least a twofold stimulation in rate over that expected from additive effects. Moreover, the rate of oxidation of the D isomer by one of these fractions could be stimulated over 13-fold when pig heart crystalline L-(+)-specific β-hydroxyacyl-CoA dehydrogenase was added to the reaction mixtures. These studies suggested that the observed oxidation of the D isomer by crude extracts may have been a result of its prior conversion into the L isomer. In addition, it could readily be demonstrated that *R. rubrum* extracts contain L-(+)-β-hydroxybutyryl-CoA dehydrogenase, an observation which has been previously reported by Stern *et al.* (1955). Support for the conclusion that the D isomer was indeed racemized to the L isomer was obtained by the studies reported below. Thus, by subjecting the extracts to further purification two fractions were resolved whose combined activity resulted in the conversion of D-(−)-β-hydroxybutyryl-CoA into L-(+)-β-hydroxybutyryl-CoA.

The conversion of the D isomer into the L isomer was measured by following the oxidation of L-(+)-β-hydroxybutyryl-CoA in the presence of NAD^+ and crystalline L-(+)-β-hydroxyacyl-CoA dehydrogenase. Reaction mixtures contained (in micromoles): Tris-HCl (pH 8.0, 8.5), $MgCl_2$ (3.4), NAD^+ (0.2), D-(−)-β-hydroxybutyryl-CoA (0.016), L-(+)-β-hydroxyacyl-CoA dehydrogenase (1 μg), and enzyme in a final volume of 0.25 ml. The reaction was followed by measuring the increase in absorption at 340 mμ at 30-sec intervals. The following fractionation was carried out. Extracts were prepared by subjecting a 10% (w/v) suspension of cells (8.5 g) in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.01 M 2-mercaptoethanol and 0.001 M EDTA to sonic oscillation with a Bronwill Biosonic probe for 6 min at maximum intensity. All operations were carried out at 0° and centrifugations at 17,000*g* for 10 min unless otherwise indicated. The disrupted cells were centrifuged and the resulting supernatant fluid was further centrifuged at 105,000*g* for 90 min in a Spinco Model L ultracentrifuge. The supernatant fluid obtained after the 105,000*g* centrifugation was brought to 35% ammonium sulfate saturation with solid ammonium sulfate and the resulting precipitate was discarded after centrifugation. The supernatant solution was adjusted to 70% ammonium sulfate saturation with solid ammonium sulfate and the precipitate after centrifugation was dissolved in 5.0 ml of 0.01 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol and 0.001 M EDTA (PME) buffer and desalted by gel filtration over Sephadex G-25 before application to a DEAE-cellulose column (diameter 2.0 cm, bed volume 100 ml). The column was previously equilibrated with the PME buffer. Two fractions were separated by this procedure both of which were required for the oxidation of the D isomer in the presence of the crystalline L-(+)-specific dehydrogenase. Since later studies established that both fractions were enoyl-CoA hydrases, the enzymatic activity eluted from the column could be conveniently followed by measuring the decrease in absorption of crotonyl-CoA at 263 mμ. After application of the ammonium sulfate fraction to the column, the DEAE-cellulose was washed with the PME buffer (460 ml). The column was then washed with the PME buffer containing 0.05 M NaCl. Fractions (11.0 ml) were collected and enzyme possessing enoyl-CoA hydrase activity appeared after 110 ml of eluate had passed through the column. The next 94

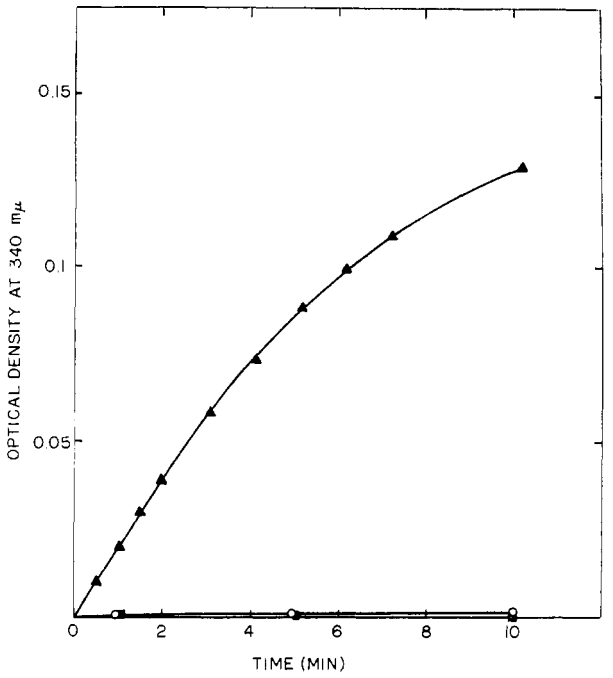

FIGURE 1: Conversion of D-(−)-β-hydroxybutyryl-CoA into L-(+)-β-hydroxybutyryl-CoA. Each reaction mixture contained (in micromoles): D-β-hydroxybutyryl-CoA, 0.016; NAD^+, 0.2; $MgCl_2$, 3.4; and Tris-HCl buffer (pH 8.5), 8.5. Fraction I/(0.006 μg), fraction II (2.6 μg), and L-(+)-β-hydroxyacyl-CoA dehydrogenase (1 μg) were added as indicated. Final volume was 0.25 ml; incubation time was 10 min at 25°. Reactions were followed at 340 mμ. (▲——▲) Fractions I and II plus L-(+)-β-hydroxyacyl-CoA dehydrogenase. (○——○) Fractions I and II minus L-(+)-β-hydroxyacyl-CoA dehydrogenase. (■——■) Fraction I or II, alone or in combination with L-(+)-β-hydroxyacyl-CoA dehydrogenase.

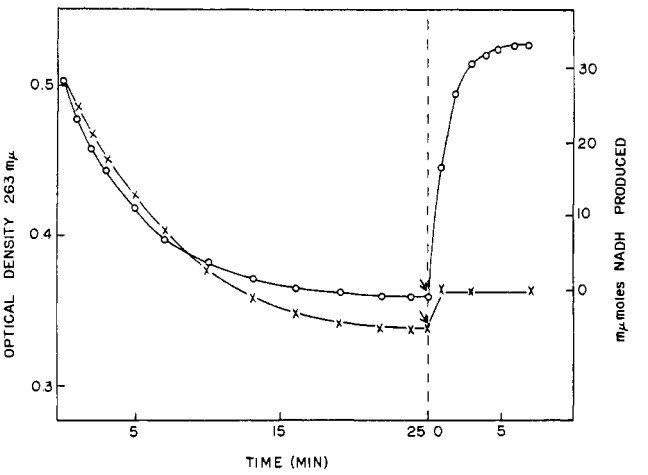

FIGURE 2: Hydration of crotonyl-CoA by fraction I or II. Reaction mixtures contained (in micromoles): Tris-HCl buffer (pH 9.0), 30; crotonyl-CoA, 0.04; and 0.005 μg of fraction I (x——x) or 0.5 μg of fraction II (○——○) as indicated. Final volume was 0.8 ml. The reactions were carried out at 25° in cuvets of 0.5-cm light path. Reactions were followed by measuring change in optical density at 263 mμ. After the reaction had reached equilibrium, $MgCl_2$ (10 μmoles), NAD^+ (0.3 μmole), and L-(+)-β-hydroxyacyl-CoA dehydrogenase (0.5 μg) were added as indicated by the arrow. Final volume was 0.9 ml. NAD^+ reduction was followed at 340 mμ.

ml was collected and the pooled eluate was made 90% ammonium sulfate saturated. The precipitate was collected by centrifugation and dissolved in a minimal volume of the PME buffer (fraction I). The column was further washed with PME buffer containing 0.1 M NaCl until no further 280-mμ-absorbing material was detected (627 ml) and the eluting buffer was changed to the PME buffer containing 0.2 M NaCl. Enzyme possessing enoyl-CoA hydrase activity was eluted after 209 ml of buffer had passed through the column. The next 143 ml was collected and the pooled eluate was made 90% saturated with ammonium sulfate. The precipitate was collected by centrifugation and dissolved in a minimal volume of the PME buffer (fraction II).

Figure 1 demonstrates that neither fraction I nor fraction II alone or in combination could effect the oxidation of D-(−)-β-hydroxybutyryl-CoA. However in the presence of added L-(+)-hydroxyacyl-CoA dehydrogenase, oxidation of the D isomer was obtained and was dependent upon the presence of both fractions. These results therefore suggested that fraction I and fraction II catalyzed the conversion of the D isomer into the L isomer. Both fractions I and II are capable of catalyzing the hydration of crotonyl-CoA as assayed by loss in absorption at 263 mμ (Figure 2). However, only the product of the reaction catalyzed by fraction II was oxidized by L-(+)-specific β-hydroxyacyl-CoA dehydrogenase (Figure 2). After incubation with neutralized hydroxylamine the products of both reactions gave identical R_F values with authenic β-hydroxybutyrylhydroxamic acid in the butanol–water and butanol–formic acid–water solvent systems. These results suggested that both fractions I and II were enoyl-CoA hydrases which catalyzed the formation of β-hydroxybutyryl-CoA from crotonyl-CoA and H_2O. Fraction II catalyzed the formation of the L isomer and fraction I presumably gave rise to the D isomer. Additional evidence which supports this conclusion will be presented later. The oxidation of D-(−)-β-hydroxybutyryl-CoA through the following series of reactions is thus suggested.

$$\text{D-(}-\text{)-}\beta\text{-hydroxybutyryl-CoA} \rightleftharpoons \text{crotonyl-CoA} + H_2O \quad (1)$$

$$\text{crotonyl-CoA} + H_2O \rightleftharpoons \text{L-(}+\text{)-hydroxybutyryl-CoA} \quad (2)$$

$$\text{L-(}+\text{)-}\beta\text{-hydroxybutyryl-CoA} + NAD^+ \rightleftharpoons \text{acetoacetyl-CoA} + NADH^+ + H^+ \quad (3)$$

The enzyme which catalyzes the formation of the L and D isomers from crotonyl-CoA will be referred to as enoyl-CoA hydrase (L) and enoyl-CoA hydrase (D), respectively. The properties of enoyl-CoA hydrase (D) was further studied. A more extensive purification of this enzyme was carried out by the procedures described below.

Purification and Properties of Enoyl-CoA Hydrase (D). ENZYME ASSAY. Enoyl-CoA hydrase (D) activity was measured by coupling the enoyl hydrase (D) catalyzed reaction (reaction I) with the reactions catalyzed by enoyl-CoA hydrase (L) and L-(+)-specific β-hydroxyacyl-CoA dehydrogenase (reactions 2 and 3). Assay mixtures contained: Tris-HCl (pH 8.0, 8.5 μmoles), $MgCl_2$ (3.4 μmoles), NAD^+ (0.2 μmole), D-(−)-β-hydroxybutyryl-CoA (0.016 μmole), enoyl-CoA hydrase (L) (2.6 μg), and L-(+)-β-hydroxyacyl-CoA dehydrogenase (1 μg). The final volume was 0.25 ml and incubations were carried out at 25°. Reactions were initiated by addition of enzyme and changes in absorbance at 340 mμ were recorded every 30 sec.

TABLE I: Purification of Enoyl-CoA Hydrase (D).

Step	Vol (ml)	Units (ml)	Total Units	Protein (mg/ml)	Sp Act. (units/mg)	% Recov
17,000*g* supernatant fluid	74	8.4	621	30.4	0.28	100
105,000*g* supernatant fluid	67	8.8	590	15.0	0.59	95
Ammonium sulfate precipitate (35–70%)	11	42.0	462	21.0	2.0	75
Heated fraction	37	7.5	277	0.5	15.0	45
DEAE-cellulose column eluate	235	0.78	183	0.014	55.7	30
Calcium phosphate gel eluate	4.9	25.0	122	0.13	192	20

Under these conditions the assay was linear for several minutes and proportional to enzyme concentration. An enzyme unit is defined as that amount of enzyme which is required to catalyze the reduction of 1 μmole of NAD^+/min under the experimental conditions.

Purification of Enoyl-CoA Hydrase (D). Crude extracts of *R. rubrum* (starting material, 12.8 g of cell paste, wet weight) were prepared as previously described except that 20% (w/v) suspension were subjected to sonic oscillation. The supernatant fluid obtained after centrifugation in the ultracentrifuge was partially freed of nucleic acids by precipitation with 0.22 volume of 10% streptomycin. After 15 min the mixture was centrifuged and the precipitate was discarded. The supernatant fluid was adjusted to 40% of ammonium sulfate saturation and the precipitate was removed by centrifugation. The supernatant solution was brought to 75% ammonium sulfate saturation and the precipitate was collected by centrifugation and dissolved in a minimal volume of 0.01 M sodium–potassium phosphate buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol and 0.001 M EDTA. After dialysis for 1 hr against the same buffer, the solution (11.0 ml) was heated to 90° for 5 min in a water bath. The suspension was cooled to 0° and the precipitate was removed by centrifugation. The supernatant fluid was desalted by gel filtration over Sephadex G-25 before application to a DEAE-cellulose column (diameter 2.0 cm; bed-volume 113 ml) which had been previously equilibrated with the 0.01 M sodium–potassium phosphate buffer (pH 7.0). The column was washed with 0.01 M sodium–potassium phosphate (pH 7.0) containing 0.004 M 2-mercaptoethanol and 0.001 M EDTA until no further protein was detected as measured at 280 mμ (350 ml). The column was then washed with 0.01 M sodium–potassium phosphate buffer (pH 7.0) containing 0.004 M 2-mercaptoethanol, 0.001 M EDTA, and 0.05 M NaCl. Fractions (4.0 ml) were collected and activity of the enoyl-CoA hydrase (D) was conveniently assayed by measuring the hydration of crotonyl-CoA according to the assay previously described. Enzyme activity was eluted after 130 ml of the buffer had passed through the column and the next 235 ml was col-

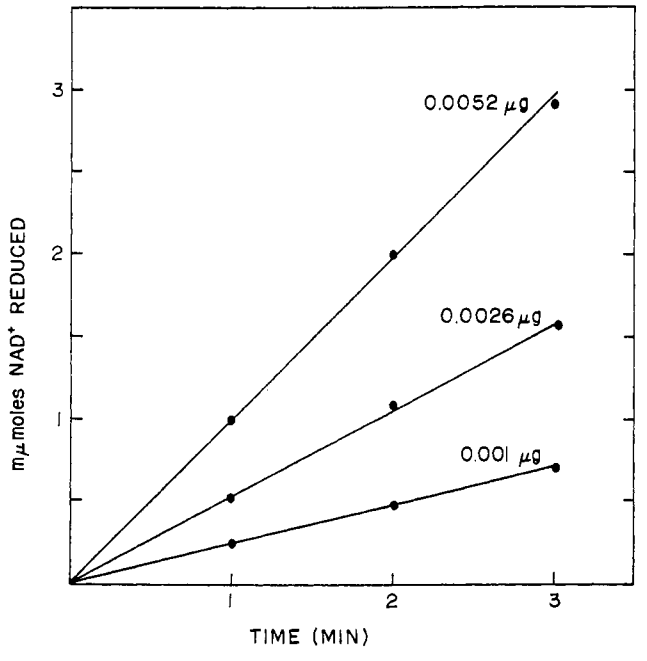

FIGURE 3: Effect of enoyl-CoA hydrase (D) protein concentration and incubation time on D-(−)-β-hydroxybutyryl oxidation. Assays were conducted as described in the Experimental Section.

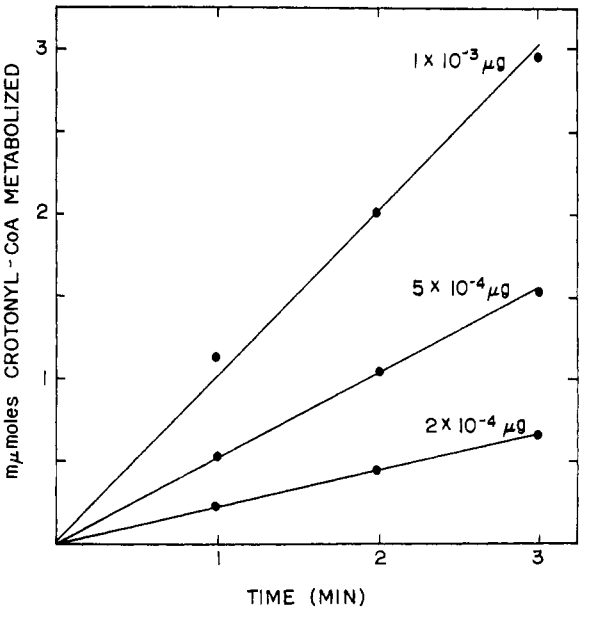

FIGURE 4: Effect of enoyl-CoA hydrase (D) concentration and incubation time on crotonyl-CoA hydration. Assays were conducted as described in the Experimental Section.

TABLE II: Enoyl-CoA Hydrase Stereospecificity and Stoichiometry.[a]

Expt	Crotonyl-CoA Added (mμ-moles)	Crotonyl-CoA Converted into β-Hydroxy-butyryl-CoA (mμ-moles)	NAD^+ Reduced with D-(−)-β-Hydroxy-butyric Dehydro-genase (mμ-moles)	NAD^+ Reduced with L-(+)-β-Hydroxy-acyl-CoA Dehydro-genase (mμ-moles)
1	40	40	37	0
2	40	31	0	33

[a] The reaction mixtures contained 30 μmoles of Tris-HCl buffer (pH 8.5), 0.005 μg of enoyl-CoA hydrase (D) in expt 1 or 0.52 μg of enoyl-CoA hydrase (L) in expt 2, and crotonyl-CoA in a volume of 0.8 ml. The reactions were allowed to proceed to completion as noted by the decrease in absorbance at 263 mμ. For the determination of D-(−)-β-hydroxybutyrate 0.1 ml of 4 N NaOH was added to the reaction mixture and further incubation was carried out at 30° for 1 hr. The mixture was then acidified with Dowex 50 (H^+) and neutralized. D-(−)-β-Hydroxybutyrate was measured as described by Shuster and Doudoroff (1962). For the determination of L-(+)-β-hydroxybutyryl-CoA, 10 μmoles of $MgCl_2$, 0.3 μmole of NAD^+, and 0.5 μg of L-(+)-β-hydroxyacyl-CoA dehydrogenase were added to the cuvet (final volume 0.9 ml) and the reactions were followed at 340 mμ.

TABLE III: Substrate Specificity of Enoyl Hydrase (D).[a]

Thioester Added	α,β-Unsaturated Thioester Metabolized (mμmoles/min per ml)
Crotonyl-CoA	2.4
Crotonylpantetheine	1.3
Crotonyl acyl carrier protein	1.2
Crotonylglutathione	0.0
Acrylyl-CoA	0.0
2-Hexenoyl-CoA	0.9

[a] The reaction mixtures contained 10 μmoles of Tris-HCl buffer (pH 8.5), 9 mμmoles of indicated substrate, and 5×10^{-4} μg of enoyl-CoA hydrase (D). Reactions were run at 25° and final volume was 0.25 ml. The reactions were followed by measuring the decrease in absorption at 263 mμ.

lected, pooled, and the concentration of 2-mercaptoethanol in the pooled eluate was increased to 0.01 M. Calcium phosphate gel (260 mg) was added to the eluate and the suspension was stirred for 30 min. The gel was removed by centrifugation and the supernatant fluid was discarded. The gel was suspended in 5.0 ml of 0.1 M sodium–potassium phosphate buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol and 0.001 M EDTA and stirred for 30 min. The gel was removed by centrifugation and the supernatant fluid was discarded. The procedure was repeated twice more. The gel was then washed twice with 0.15 M sodium–potassium phosphate buffer containing 0.01 M 2-mercaptoethanol and 0.001 M EDTA. Finally the enzyme was eluted from the gel with 0.25 M sodium–potassium phosphate buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol and 0.001 M EDTA. Final volume was 5.0 ml. 2-Mercaptoethanol was added to the eluted enzyme to bring the final concentration to 0.15 M. Under these conditions the enzyme is stable for several months at 0°. At lower concentrations of 2-mercaptoethanol (0.01 M) approximately 43% of the activity is lost in 10 days. The over-all purification was usually approximately 650–680-fold with 20% recovery of total enzyme units. A summary of a typical purification is shown in Table I.

The effect of enoyl-CoA hydrase (D) protein concentration and incubation time on the oxidation of D-(−)-β-hydroxybutyryl-CoA is shown in Figure 3. For further studies of the enoyl-CoA hydrase (D), use was made of the more convenient assay which measures the disappearance of absorbance at 263 mμ due to the hydration of crotonyl-CoA. The relationship of enoyl-CoA hydrase (D) concentration and incubation time on the hydration reaction is shown in Figure 4. The pH optimum for the reaction was between 8.4 and 8.6 and the K_m calculated according to Lineweaver and Burk (1934) was 9.26×10^{-6} M.

Product of Enoyl-CoA Hydrase (D). To determine the product of the enoyl-CoA hydrase (D) reaction, crotonyl-CoA, 0.9 μmole was incubated with 0.1 μg of enoyl-CoA hydrase (D) in a reaction mixture containing 50 μmoles of Tris-HCl (pH 8.5) in a final volume of 0.5 ml. Incubation was carried out for 1 hr at 25°. Neutral hydroxylamine (400 μmoles) was then added to the product and the mixture was further incubated at 25 for 1 hr. The reaction mixture was desalted by repeated precipitation with absolute alcohol and chromatographed in the butanol–water and butanol–formic acid–water solvent systems. Authenic crotonyl-, butyryl-, and β-hydroxybutyrylhydroxamates were used as standards. The hydroxamates were stained with ferric chloride spray. The hydroxamate obtained from the product of the enoyl-CoA hydrase (D) reaction migrated with R_F values identical with β-hydroxybutyryl-hydroxamate in both systems. This experiment suggests that enoyl-CoA hydrase (D) catalyzes the hydration of crotonyl-CoA to β-hydroxybutyryl-CoA. Other experiments to be presented below support this conclusion and also identify the product as the D-(−) isomer.

Stereospecificity and Stoichiometry. To determine which optical isomer was formed, the products of the reactions catalyzed by the enoyl-CoA hydrase (D) and enoyl-CoA hydrase (L), respectively, were examined with a D-(−)-specific β-hydroxybutyrate dehydrogenase (after alkaline hydrolysis of the thioester) and with pig heart crystalline L-(+)-specific β-hydroxyacyl-CoA dehydrogenase. As shown in Table II only the product obtained with enoyl-CoA hydrase (L) is oxidized in the presence of the L-(+)-specific dehydrogenase and NAD^+. Also after alkaline hydrolysis only the product of the enoyl-CoA hydrase (D) catalyzed reaction is oxidized with the D-(−)-specific dehydrogenase. Both enoyl-CoA hydrases catalyzed a

TABLE IV: Enzymatic Synthesis of Poly-β-hydroxybutyrate.[a]

Substrate	Addn to Reaction Mixture	Poly-β-hydroxybutyrate Synthesized (mμmole)
	Experiment 1	
D-(−)-β-Hydroxybutyryl-CoA	None	60.1
Crotonyl-CoA	None	0.0
Crotonyl-CoA	Enoyl hydrase (L)	0.0
Crotonyl-CoA	Enoyl hydrase (D)	60.3
	Experiment 2	
D-(−)-β-Hydroxybutyryl-CoA	None	47.8
L-(+)-β-Hydroxybutyryl-CoA	None	0.6
L-(+)-β-Hydroxybutyryl-CoA	Enoyl hydrase (L)	0.5
L-(+)-β-Hydroxybutyryl-CoA	Enoyl hydrase (D)	0.5
L-(+)-β-Hydroxybutyryl-CoA	Enoyl hydrase (L) plus (D)	18.0
Crotonyl acyl carrier protein	None	0.13
Crotonyl acyl carrier protein	Enoyl hydrase (D)	0.15
Crotonyl acyl carrier protein	Enoyl hydrase (L)	0.11

[a] The reaction mixtures contained 50 μmoles of potassium phosphate buffer (pH 7.5), 5 μmoles of $MgCl_2$, and native poly-β-hydroxybutyrate granules (240 μg of poly-β-hydroxybutyrate and 10.9 μg of protein), and as indicated, 0.13 μmole of D-(−)-β-hydroxybutyryl-CoA (1.73×10^4 cmp/μmole), 0.15 μmole of L-(+)-β-hydroxybutyryl-CoA (4×10^4 cpm/μmole), 0.2 μmole of crotonyl-CoA (1.2×10^4 cpm/μmole) of 0.1 μmole of crotonyl acyl carrier protein (3.1×10^5 cpm/μmole). Enoyl hydrase (D) or enoyl hydrase (L) was added at 5×10^{-3} μg or 0.25 μg, respectively, were indicated. Final volume was 0.5 ml; incubation time was 15 min at 30°.

stoichiometric disappearance of crotonyl-CoA with the formation of β-hydroxybutryl-CoA. These experiments indicate that crotonyl-CoA is converted into D-(−)-β-hydroxybutyryl-CoA by the enoyl-CoA hydrase (D).

Substrate Specificity. Various crotonyl thioesters were examined as possible substrates for the enoyl-CoA hydrase (D) reaction (Table III). Crotonylpantetheine and crotonyl acyl carrier protein were hydrated but at approximately 50% of the rate of crotonyl-CoA. Crotonylglutathione and acrylyl-CoA do not act as substrates. *trans*-2-Hexenoyl-CoA is also hydrated but at a lower rate than crotonyl-CoA. The K_m's for crotonylpantetheine, crotonyl acyl carrier protein, and *trans*-2-hexenoyl-CoA are 1.18×10^{-4}, 8×10^{-5}, and 2.63×10^{-5} M, respectively.

Role of Enoyl-CoA Hydrase (D). It has been previously demonstrated that the substrate for poly-β-hydroxybutyrate synthesis is D-(−)-β-hydroxybutyryl-CoA and that the polymerization enzyme is associated with isolated poly-β-hydroxybutyrate granules (Merrick and Doudoroff, 1961; Griebel *et al.*, 1968). It can readily be shown that the product of hydration of crotonyl-CoA by enoyl-CoA hydrase (D) is polymerized to poly-β-hydroxybutyrate (Table IV), thus providing further evidence that the product is D-(−)-β-hydroxybutyryl-CoA. As expected, the product of the enoyl-CoA hydrase (L) catalyzed reaction does not act as a substrate. Although it has been shown that the D-(−) isomer is converted into the L-(+) isomer, presumably the enoyl-CoA hydrases functions in poly-β-hydroxybutyrate synthesis in the reverse direction. Thus the D-(−) isomer is derived from the L-(+) isomer by the following series of reactions:

L-(+)-β-hydroxybutyryl-CoA ⟶ crotonyl-CoA ⟶
D-(−)-β-hydroxybutyryl-CoA ⟶ poly-β-hydroxybutyrate

L-(+)-β-Hydroxybutyryl-CoA is synthesized from acetoacetyl CoA and NAD^+ by an L-(+)-specific β-hydroxybutyryl-CoA dehydrogenase previously reported to be present in *R. rubrum* extracts by Stern *et al.* (1955) and also confirmed in this laboratory (unpublished observations). As shown in Table IV L-(+)-β-hydroxybutyryl-CoA can act as a substrate for the polymerizing enzyme when incubated in the presence of both enoyl-CoA hydrase (D) and enoyl-CoA hydrase (L). In the absence of either hydrase negligible incorporation was obtained. These results therefore provide evidence for the pathway of poly-β-hydroxybutyrate synthesis as indicated above.

The role of the thioesters of acyl carrier protein in the biosynthesis of poly-β-hydroxybutyrate has not been previously investigated. Since enoyl-CoA hydrase (D) also catalyzed the hydration of crotonyl acyl carrier protein, it was possible to examine the role of D-(−)-β-hydroxybutyryl acyl carrier protein in poly-β-hydroxybutyrate synthesis. As seen in Table IV, crotonyl-1-^{14}C acyl carrier protein when incubated with enoyl-CoA hydrase (D) and poly-β-hydroxybutyrate synthetase does not result in poly-β-hydroxybutyrate synthesis, thus suggesting that acyl carrier protein thioesters may not play a significant role in poly-β-hydroxybutyrate synthesis.

Reversibility of Enoyl Hydrase (D) *and* (L) *Reactions.* To study the reversibility of both enoyl-CoA hydrases, reactions were carried out with D-(−)- and L-(+)-β-hydroxybutyryl-CoA as substrates as indicated in Table V. As shown, both enoyl-CoA hydrases are capable of converting their respective substrates into crotonyl-CoA. The stereospecificity of the reactions is further confirmed since only enoyl-CoA hydrase (D) is capable of metabolizing D-(−)-β-hydroxybutyryl-CoA and similarly only the enoyl-CoA hydrase (L) converts L-(+)-β-hydroxybutyryl-CoA into crotonyl-CoA.

TABLE V: Reversibility of Enoyl Hydrase.[a]

Substrate (mμmoles/ml)	Enzyme	Crotonyl-CoA Formed (mμmoles/ml)
D-(−)-β-Hydroxybutyryl-CoA (65.6)	Enoyl hydrase (D)	6.4
L-(+)-β-Hydroxybutyryl-CoA (67.2)	Enoyl hydrase (D)	0.0
L-(+)-β-Hydroxybutyryl-CoA (67.2)	Enoyl hydrase (L)	6.4
D-(−)-β-Hydroxybutyryl-CoA (65.6)	Enoyl hydrase (L)	0.0

[a] The reaction mixtures contained (in micromoles): Tris-HCl (pH 8.5, 10), indicated amounts of substrate, and 2.5×10^{-4} μg of enoyl hydrase (D) or 0.52 μg of enoyl hydrase (L). The increase in absorption at 263 mμ, due to the production of α,β-unsaturated thioester, was followed to equilibrium. Final volume was 0.25 ml and incubation temperature was 25°.

Inhibitors. No clear-cut experimental evidence was obtained which demonstrates that enoyl-CoA hydrase (D) is a sulfhydryl enzyme similar to other enoyl-CoA hydrases. *p*-Mercuribenzoate was the most effective inhibitor studied and at 3×10^{-3} M, 65% inhibition was obtained. However, *N*-ethylmaleimide, iodoacetamide, and iodoacetic acid at 10^{-2} M inhibited only 23, 30, and 12%, respectively. Extensive dialysis of the enzyme under N_2 did not result in increased sensitivity of the enzyme to sulfhydryl reagents. Alberts *et al.* (1965) have demonstrated that some of the enzymes from *Escherichia coli* involved in fatty acid biosynthesis are somewhat resistant to sulfhydryl reagents unless they are reduced by prior incubation with sulfhydryl compounds. Prior reduction of enoyl-CoA hydrase (D) was carried out as described by Alberts *et al.* (1965) and gave similar results to those reported above.

The enzyme was not stimulated or inhibited by the following cations, Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, K^+, and Na^+. Addition of EDTA at relatively high concentration (10^{-2} M) was without effect.

Discussion

In an earlier study Stern *et al.* (1955) has reported that *R. rubrum* extracts catalyze a racemization of the D-(−) and L-(+) isomers of β-hydroxybutyryl-CoA. The present investigation describes the resolution of the intermediate steps in this pathway and demonstrates that the interconversion of the two isomers is catalyzed by specific enoyl-CoA hydrases. Thus, two enoyl-CoA hydrases have been isolated whose action results in the hydration of crotonyl-CoA to β-hydroxybutyryl-CoA. Enoyl-CoA hydrase (D) gives rise to the D-(−) isomer while the action of enoyl-CoA hydrase (L) results in the formation of L-(+)-β-hydroxybutyryl-CoA. The latter enzyme is thus similar in this regard to crotonase previously crystallized by Stern *et al.* (1956). It is now possible to account for the over-all pathway which leads to poly-β-hydroxybutyrate synthesis from acetate by *R. rubrum* by the following reactions:

acetate ⟶ acetyl-CoA ⟶ acetoacetyl-CoA ⟶
L-(+)-β-hydroxybutyryl-CoA ⟶ crotonyl-CoA ⟶
D-(+)-β-hydroxybutyryl-CoA ⟶ poly-β-hydroxybutyrate

The enzymes in the early steps of this pathway have been reported to be present in *R. rubrum* by Eisenberg (1955) (acetyl-CoA kinase) and Stern *et al.* (1956) (thiolase, L-(+)-hydroxybutyryl-CoA dehydrogenase).

It is clear from studies reported here that the enoyl-CoA hydrase (D) catalyzes the enzymatic synthesis of the D-(−) isomer of β-hydroxybutyryl-CoA from crotonyl-CoA and water. It has a relative broad specificity with respect to various crotonyl thioesters since it hydrates crotonylpantetheine and crotonyl acyl carrier protein and thus differs from the enzyme isolated from *E. coli* by Majerus *et al.* (1965). The latter enzyme is specific for acyl acyl carrier protein thioesters and does not metabolize other compounds. Although *R. rubrum* enoyl-CoA hydrase (D) is capable of hydrating crotonyl acyl carrier protein, the product of the reaction does not serve as a substrate for the poly-β-hydroxybutyrate synthetase thus suggesting that acyl carrier protein thioesters may not play a significant role in poly-β-hydroxybutyrate synthesis.

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The Amino Acid Sequence of Bovine Carboxypeptidase A. I. Preparation and Properties of the Fragments Obtained by Cyanogen Bromide Cleavage*

Masao Nomoto,† N. G. Srinivasan,‡ Ralph A. Bradshaw,§ Roger D. Wade, and Hans Neurath

ABSTRACT: Bovine carboxypeptidase A_γ has been subjected to cleavage by cyanogen bromide and the fragments were produced isolated by gel filtration on Sephadex G-75 equilibrated in 0.1 M propionic acid. In addition to the amino- and carboxyl-terminal peptides previously isolated and characterized, two large fragments, designated F_I and F_{III}, have been obtained. The first of these, F_I, occurs mainly as a high molecular weight aggregate in 0.1 M propionic acid while F_{III} shows little or no aggregation properties but exhibits nonideal behavior. Sedimentation equilibrium analyses in 6 M guanidine hydrochloride show F_I to possess a molecular weight about 23,000 and F_{III} a molecular weight of about 9000. Amino acid analyses of each fragment indicate that F_I and F_{III} are composed of 198 and 81 amino acid residues, respectively. Each fragment contains a single aspartyl residue as amino terminus. The relationship of the four cyanogen bromide fragments of bovine carboxypeptidase A_γ to the primary structure of the enzyme is discussed.

Bovine pancreatic carboxypeptidase A_γ is composed of a single polypeptide chain containing 300 amino acids. Extensive chemical and physical studies recently reviewed by Neurath *et al.* (1968, 1969), and Vallee and Riordan (1968) have disclosed many of the structural and mechanistic features of this enzyme. In addition, X-ray analysis of the crystal structure has been undertaken (Lipscomb *et al.*, 1968). In view of this concerted approach to the structure and function of this enzyme, it was deemed of importance to determine the complete amino acid sequence of carboxypeptidase A by chemical methods.

Earlier studies from this laboratory (Bargetzi *et al.*, 1964) suggested that a promising route to the solution of this structure was cleavage of the polypeptide chain at each of three methionyl residues by the CNBr method of Gross and Witkop 1961). This method has already been successfully applied to a number of sequence problems (Hofmann, 1964; Edmundson, 1963; Delaney and Hill, 1968), and indeed both the amino- (Sampath Kumar *et al.*, 1964) and carboxyl-terminal peptides (F_N and F_C)[1] of carboxypeptidase A composed of 15 and 6 amino acids, respectively, have been isolated (Bargetzi *et al.*, 1964). Complete amino acid sequence analysis of these fragments has supplied the first primary structural data for the protein.

In continuation of these studies, the preparation and characterization of the other two major fragments has been carried out. One of these fragments contains 81 residues and the other one 198. Amino acid analyses of these two fragments yield compositions which, together with the amino acid content of the N- and C-terminal peptides, account satisfactorily for the composition of carboxypeptidase A_γ determined from acid hydrolysates.

Experimental Procedure

Materials. Carboxypeptidase A_γ was obtained from Worthington Biochemical Corp. as an aqueous suspension containing 40 mg/ml of twice-crystallized protein. The specific activity of these preparations toward the ester substrate hippuryl-DL-β-phenyllactate was of the magnitude described by Bargetzi *et al.* (1963).

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† Present address: Seikagaku Kogyo Co., Ltd., 9, 2-Chome, Nihombashi-Honcho, Chuo-Ku, Tokyo, Japan.
‡ Present address: Cancer Research Institute, Madras, India.
§ Portions of this work were completed during the tenure of U. S. Public Health Service Postdoctoral Fellowship 1-F2-GM-23, 968-02. Present address: Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo. 63110.

[1] F_C, F_N, F_{III}, and F_I, CNBr fragments of carboxypeptidase A.