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Purification, New Assay, and Properties of Coenzyme A Transferase from *Peptostreptococcus elsdenii*[1]

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Coenzyme A (CoA) transferase from *Peptostreptococcus elsdenii* has been purified and crystallized, and some of its properties have been established. The work was facilitated by a newly developed coupled and continuous spectrophotometric assay in which the disappearance of added acrylate could be followed at 245 nm. The rate-limiting conversion of acetyl- and β-hydroxypropionyl CoA to acrylyl CoA by CoA transferase was followed by the non-rate-limiting conversion to β-hydroxypropionyl CoA by excess crotonase. Thus, a small priming quantity of acetyl CoA served to generate acrylyl CoA, which, by hydration, generated β-hydroxypropionyl CoA. This product then served to generate more acrylyl CoA in cyclic fashion. The net result was the CoA transferase-limited conversion of acrylate to β-hydroxypropionate. The purified transferase has a molecular weight of 125,000 and is composed of two subunits of 63,000 each, as determined by disc gel electrophoresis. Short-chain-length monocarboxylic acids are substrates, whereas dicarboxylic or β-ketocarboxylic acids are not. The reaction kinetics are typical of a ping-pong *bi bi* mechanism composed of two half reactions linked by a covalent enzyme intermediate. Incubation of the transferase with acetyl CoA in the absence of a fatty acid acceptor yielded a stable intermediate which, by absorption spectrophotometry, radioactivity measurements, reduction with borohydride, reactivity with hydroxylamine, and catalytic activity, was identified as an enzyme-CoA compound. Kinetic constants for CoA transferase are: final specific activity, 110 U/mg of protein corresponding to 1.38×10^4 μmol of acrylate activated per μmol of transferase; K_m for acrylate, 1.2×10^{-3} M; K_m for acetyl CoA (β-hydroxypropionyl CoA), 2.4×10^{-5} M.

The enzyme that catalyzes the transfer of coenzyme A (CoA) from short-chain acyl CoA derivatives to short-chain monocarboxylic acids was first discovered in *Clostridium kluyveri* by Stadtman (26); a similar enzyme has also been partially purified from *Peptostreptococcus elsdenii* (2). This CoA transferase is distinct from the 3-keto acid CoA transferase from mammals (29) and the CoA transferase from *Propionibacterium shermanii* (1) in that it will not utilize succinic acid or 3-keto acids as substrates.

Extensive studies have not been reported for the short-chain CoA transferase, presumably due to the inconvenience of the assay. In this paper, we report the crystallization and characterization of the CoA transferase from *P. elsdenii*, as well as a new assay procedure which greatly facilitated the work. (A preliminary report of this work has been published [K. K. Tung and W. A. Wood, Fed. Proc. **30**:1266, 1971].)

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MATERIALS AND METHODS

Bacteriological. *P. elsdenii* strain B-159 was grown on a medium of corn steep liquor and sodium D,L-lactate as described by Ladd and Walker (19). Cells grown in deep culture at 37 C were collected in a Sharples centrifuge. Approximately 700 g (wet weight) of cells was harvested from a 200-liter culture grown in a 55-gallon (ca. 209-liter) drum. These were stored at −14 C.

Enzymes. A new assay, based on the hydration of acrylic acid in the presence of a catalytic amount of acetyl CoA, CoA transferase, and excess crotonase, was developed. The theoretical background will be detailed in Results. The standard enzyme assay mixture contained 2 μmol of sodium acrylate, 5 nmol of acetyl CoA, 10 μmol of potassium phosphate buffer (pH 7.0), limiting amounts of CoA transferase, and 1 unit of crotonase in a total volume of 0.2 ml. Incubation was conducted at room temperature, and the hydration reaction was followed continuously at 245 nm with a Gilford spectrophotometer. One unit of

CoA transferase will convert 1 μmol of acrylate to β-hydroxypropionate per min under the described conditions. The hydration of 1 μmol of acrylate will cause a decrease of 0.8 absorbance units at 245 nm (A_{245}). The CoA transferase assay of Stadtman (26) involving phosphotransacetylase was used to confirm the newly developed assay. Crotonase and phosphotransacetylase were assayed according to Stern and del Campillo (30) and Stadtman and Barker (28), respectively. D-Lactate dehydrogenase, acyl CoA dehydrogenase, and an electron transfer flavoprotein used in identification of acrylyl CoA by reduction to propionyl CoA were purified fractions from *P. elsdenii* supplied by H. L. Brockman (submitted for publication).

Chromatography. For analysis of volatile carboxylic acids, a sample containing the acids was acidified to pH 2.0 with a few drops of 1 N sulfuric acid and centrifuged to remove any insoluble material present. Suitable aliquots of the supernatant solution were subjected to gas-liquid chromatography using a precoated 10% free fatty acid, aqueous phase column at a running temperature of 120 C using N_2 as a carrier gas at a flow rate of 20 ml/min.

β-Hydroxypropionic acid was isolated and identified on a silicic acid column according to Marvel and Rands (21). It was eluted from the column by 100 ml of 25% butanol in chloroform. The acyl CoA in the reaction mixture was converted to its hydroxamate as described by Sokatch et al. (24), and the hydroxamates were then identified by ascending thin-layer chromatography on cellulose using 2-butanol-HCOOH-H_2O (75:13:12) and water-saturated butanol as solvents. Acid hydroxamates were detected by spraying with an $FeCl_3$ reagent.

Gel electrophoresis. Polyacrylamide gel electrophoresis was carried out at pH 8.3 according to the method of Davis (5) and at pH 7.0 by the method of Williams and Reisfeld (34). Protein was visualized with Coomassie blue according to Chrambach et al. (4). For relating the position of the protein band to CoA transferase activity, the gel was scanned at 280 nm immediately after electrophoresis. Each 0.25-cm section was then treated with 0.1 ml of 0.1 M phosphate buffer, pH 6.0, and the eluates were assayed for CoA transferase as described above.

The molecular weight of the CoA transferase subunit was determined by the sodium dodecyl sulfate-polyacrylamide gel procedure of Weber and Osborn (33), and the molecular weight of intact CoA transferase was determined by the variable cross-linked gel electrophoresis procedure of Hedrick and Smith (11).

Protein determination. Protein concentrations were determined by the A_{280}/A_{260} method (17) or by the modified biuret procedure of Gornall et al. (9), using pure CoA transferase standardized by dry weight measurement. One milligram (dry weight) of CoA transferase per ml gave an absorbance of 0.5 at 280 nm, and $A_{280}/A_{260} = 1.50$.

Amino acid analysis. Amino acid content was determined after acid hydrolysis by standard automated chromatographic methods. Threonine and serine values were obtained by extrapolation to zero-time hydrolysis. Half-cysteine residues were determined as cysteic acid (22). Tryptophan was analyzed by the method of Edelhoch (7).

Other methods. Radioactivity was determined in a liquid scintillation counter using the counting solution of Bray (3). The determination of difference spectra of CoA transferase and the enzyme-CoA intermediate was made on a recording spectrophotometer (Cary model 14) equipped with double-compartmented microcuvettes for reagent compensation.

Materials. Acrylic acid was obtained from B. F. Goodrich Co. and was redistilled over copper wire before use. Acrylic anhydride was purchased from City Chemical Corp. and used without further purification. Crystalline crotonase was a gift from M. J. Coon. 2-Keto-3-deoxy-6-phosphogluconic aldolase was prepared in this laboratory (10). All other proteins and chemicals were commercial products and were used without further purification.

β-Hydroxypropionyl CoA was synthesized from propionolactone according to Vagelos and Earl (32). All other acyl CoA compounds were prepared from their acid anhydrides according to the method of Simon and Shemin (23).

RESULTS

Characteristics of the new CoA transferase assay. A system containing CoA transferase, crotonase, and catalytic amounts of acetyl CoA will quantitatively hydrate acrylate to form β-hydroxypropionate, utilizing the following reactions:

$$\text{acrylyl CoA} + H_2O \xrightleftharpoons{\text{crotonase}} \beta\text{-hydroxypropionyl CoA} \quad (1)$$

$$\beta\text{-hydroxypropionyl CoA} + \text{acrylate} \xrightleftharpoons{\text{CoA transferase}} \beta\text{-hydroxypropionate} + \text{acrylyl CoA} \quad (2)$$

Net reaction:

$$\text{acrylate} + H_2O \rightleftarrows \beta\text{-hydroxypropionate} \quad (3)$$

The initial level of acrylyl CoA is produced by the addition of small amounts of acetyl CoA through the action of CoA transferase. Since acrylate absorbs in the ultraviolet region, the reaction can be followed quantitatively between 230 and 260 nm in a Gilford spectrophotometer. When crotonase and acrylate are in excess, the A_{245} decrease is linear with time and the velocity is proportional to the amount of CoA transferase present (Fig. 1). The omission of CoA transferase, acrylate, or acetyl CoA results in

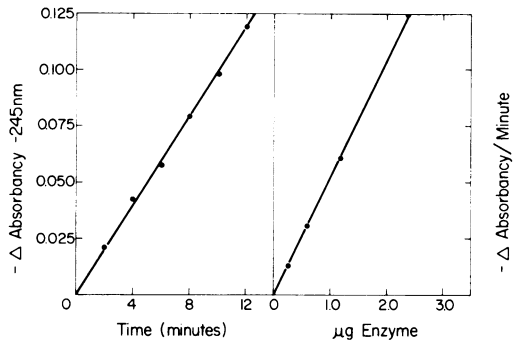


FIG. 1. *Reaction rate as a function of time and the amount of column-purified CoA transferase. The reaction conditions were as described in Methods and Materials.*

complete loss of activity, and with extensively purified CoA transferase exogenous crotonase also must be added. Butyryl CoA or other suitable acyl CoA's could substitute for acetyl CoA, whereas free CoA or phosphoryl donors such as adenosine 5'-triphosphate, phosphoenolpyruvate, and acetyl phosphate did not function in generation of the initial level of acrylyl CoA.

Disappearance of acrylate. The ultraviolet absorption spectrum of sodium acrylate was independent of pH between pH 5.0 and 9.0, and the absorbance between 230 and 260 nm is proportional to the acrylate concentration. Analysis by silicic acid column chromatography of a reaction mixture before enzyme action showed only acrylic acid (Fig. 2). After extensive enzyme reaction, the acrylic acid peak was reduced to less than 5% of the original size, indicating that acrylate was modified in the combined enzyme system. Further, the disappearance of acrylate was also established by gas-liquid chromatography. Another acid peak representing 80% of the total acids appeared in a position corresponding to that of authentic β-hydroxypropionic acid.

Isolation and identification of β-hydroxypropionate. Twenty millimoles of acrylic acid, in 20 ml of water and titrated to pH 7.0 with NaOH, was incubated with CoA transferase and crotonase at room temperature for 3 days. The reaction was stopped by acidifying with HCl, and the excess acrylic acid was removed by evaporation. The reaction product was crystallized as a Zn-Ca salt as described by Den et al. (6). One hundred and eighty milligrams of crystals was collected and dried over P_2O_5 overnight. The infrared spectrum shown in Fig. 3 clearly indicates that the product is identical

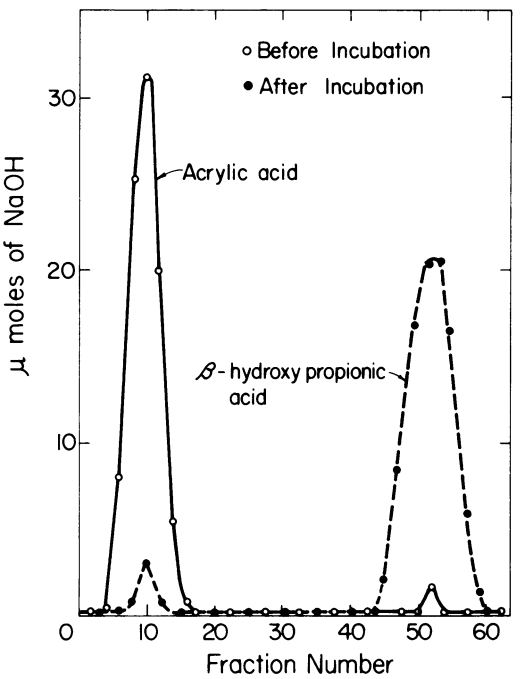


FIG. 2. *Elution pattern of carboxylic acids from silicic column. The reaction mixture contained 0.5 mmol of sodium acrylate, 1 μmol of acetyl CoA, and 0.1 mg of column-purified CoA transferase. Either before (O) or after (●) incubation at room temperature for 2 h, the mixture was acidified with 1 M H_2SO_4 and layered on a silicic column (12 by 1.5 cm), according to Marvel and Rands (21). The column was eluted sequentially with 100 ml each of chloroform-butanol, 95:5, 85:15, and 75:25. The effluent was collected in 5-ml fractions and titrated with 1.0 N NaOH in the presence of phenol red.*

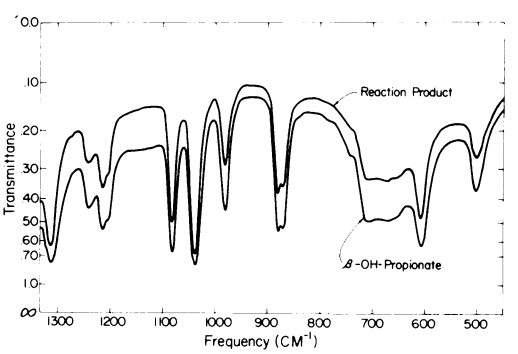


FIG. 3. *Infrared spectra of authentic Zn-Ca-β-hydroxypropionate and the isolated reaction product.*

to authentic Ca-Zn β-hydroxypropionate.

Acrylyl CoA and β-hydroxypropionyl CoA as the reaction intermediates. To demonstrate the involvement of acrylyl CoA and β-hydroxy-

propionyl CoA as reaction intermediates, 1 μmol of acetyl CoA was incubated with acrylate or β-hydroxypropionate and the enzymes at 37 C for 30 min. The reaction was stopped by boiling, the acyl CoA's in the reaction mixture were converted to their hydroxamates, and these were separated and identified by thin-layer chromatography. Duplicate experiments showed that β-hydroxypropionyl hydroxamate was the major component and that acrylyl hydroxamate was the minor component present on the plates. This indicates that the steady-state level of acrylyl CoA in reaction mixtures is low, as would be expected if the equilibrium between acrylyl CoA and β-hydroxypropionyl CoA favors β-hydroxypropionyl CoA.

The continuous production of acrylyl CoA and its direct involvement was also established by enzymatic reduction to propionyl CoA. The reducing system composed of acyl CoA dehydrogenase, D-lactate dehydrogenase, and an electron transfer flavoprotein isolated from *P. elsdenii* (Brockman, submitted for publication) will quantitatively reduce acrylyl CoA to propionyl CoA as follows:

$$\begin{array}{c} \text{D-lactate} \\ + \\ \text{acrylyl CoA} \end{array} \xrightarrow[\text{electron transfer flavoprotein, acyl CoA dehydrogenase}]{\text{D-lactate dehydrogenase}} \begin{array}{c} \text{pyruvate} \\ + \\ \text{propionyl CoA} \end{array} \quad (4)$$

The data presented in Table 1 show that the combination of CoA transferase, crotonase, and this acrylyl CoA dehydrogenase system could convert more than 70% of either acrylate or β-hydroxypropionate into propionic acid. Omission of acetyl CoA, acrylate, or β-hydroxypropionate caused a complete loss of activity. Since the acyl CoA dehydrogenase fraction contained contaminating CoA transferase, the omission of CoA transferase from the system did not completely abolish propionate formation. This experiment suggested that the continuous production of acrylyl CoA is due to the action of CoA transferase and crotonase acting on either acrylate or β-hydroxypropionate.

The involvement of acrylyl CoA in the CoA transferase assay was also shown by the effects of adding a thiol compound. It has been reported (27) that thiols such as mercaptoethanol and dithiothreitol easily add across the double bond of acrylyl CoA, but not across that of acrylate. In the normal spectrophotometric assay system for the disappearance of acrylate, either dithiothreitol or glutathione stopped the reaction in a few minutes. The reaction mixture contained 10 μg of partially purified CoA transferase, 5 nmol of acetyl CoA, 20 μmol of phosphate buffer, pH 7.0, 2 μmol of acrylate, and 0.1 μmol of dithiothreitol or reduced glutathione. This inhibition could be reversed by simply adding in excess acetyl CoA or another acyl CoA donor. Since preincubation of thiol compound with CoA transferase had no effect on the enzyme activity, the effect must be due to the formation of unreactive thiol-substituted acrylyl CoA. This experiment also pointed out that acrylyl CoA is the obligatory intermediate for the CoA transferase assay.

Tritium incorporation into acrylate and β-hydroxypropionate. To determine whether the hydration and dehydration reactions are stereospecific, acrylate, enzymes, and acetyl CoA were incubated in T_2O at room temperature for 4 h. Since the reaction reaches equilibrium within 0.5 h, the prolonged incubation would insure the exchange of tritium among tritiated water, acrylate, and β-hydroxypropionate. The reaction was stopped by adjusting the pH to 10 with sodium hydroxide, and the excess tritiated water was removed by repeated lyophilization and addition of H_2O. The acidified reaction products were then separated and characterized by silica gel column chromatography as described in Materials and Methods. Theoretically, repeated non-stereospecific hydration and dehydration would result in the incorporation of one equivalent of tritium at the α-carbon of acrylate and two equivalents of tritium at the α-carbon of β-hydroxypropionate. On the other hand, a stereospecific reaction should leave no tritium in acrylate and one equivalent of tritium in β-hydroxypropionate. The data in Table 2 indicate that only 0.7 equivalent of tritium was incorporated per mol of β-hydroxypropionate and less than 0.015 equivalent into acrylate. Therefore, our data suggest a stereospecific hydration-dehydration process, as reported previously for crotonase (30).

Purification of CoA transferase. The frozen cells of *P. elsdenii* were mixed with an equal weight of distilled water and treated in a Raytheon sonic oscillator for 20 min at 10 kHz. The suspension of disrupted cells was centrifuged at 30,000 × *g* for 20 min to remove cellular debris. About 170 ml of clear, dark brown solution was obtained from 100 g of cell paste. The crude extract can be stored at −15 C for months without loss of activity.

First heat treatment. The crude extract, containing 40 to 60 mg of protein per ml, was brought to 50 C by incubating in a 55 C water

TABLE 1. *Production of propionic acid from acrylate or β-hydroxypropionate with the combined enzyme system*[a]

Enzyme	Substrate	Propionic acid (μmol)
Complete	Acrylate and acetyl CoA	14.2
Complete	β-Hydroxypropionate and acetyl CoA	17.5
Complete	Without acetyl CoA	0.2
Complete	Acetyl CoA only	0.4
Without CoA transferase	β-Hydroxypropionate and acetyl CoA	3.0
CoA transferase only	β-Hydroxypropionate and acetyl CoA	0.0

[a] The reaction mixture included 0.5 mg of acyl CoA dehydrogenase, 0.5 mg of a D-lactate dehydrogenase fraction (Brockman, submitted for publication), 0.1 mg of electron transfer flavoprotein fraction, 10 μg of partially purified CoA transferase, 40 μmol of D-lactate, 50 nmol of acetyl CoA, 20 μmol of phosphate buffer, pH 7.0, and 20 μmol of acrylate or β-hydroxypropionate, in a total volume of 0.25 ml. The mixture was incubated under N_2 at 25 C for 4 h. Suitable aliquots were removed from the reaction mixture, acidified within H_2SO_4, and examined for propionic acid by gas-liquid chromatography.

TABLE 2. *Tritium incorporation into acrylate and β-hydroxypropionate*[a]

Enzyme	Sp act (counts/min per μmol)			Tritium incorporation (mol/mol)	
	Tritiated H_2O	Acrylate	β-Hydroxypropionate	Acrylate	β-Hydroxypropionate
Active	4.4×10^4	340	1.55×10^4	0.015	0.706
Boiled	4.4×10^4	~0	~0	~0	~0

[a] Ten micromoles of sodium acrylate, 0.02 μmol of acetyl CoA, 5 μmol of phosphate buffer (pH 7.4), and 200 μg of partially purified CoA transferase were incubated with tritiated H_2O for 4 h at 25 C. The reaction products were analyzed as described in the text.

bath for no more than 10 min, and then it was heated to 58 C for 5 min in a 70 C water bath. The denatured proteins were removed by centrifuging at 30,000 × *g* for 10 min.

Ammonium sulfate fractionation. Over a period of 20 min, 31.3 g of ammonium sulfate was added to 100 ml of dark green supernatant from the heat treatment. This 50% saturated solution was centrifuged at 30,000 × *g* for 30 min, and the pellet was discarded. Similarly, 15.7 g of ammonium sulfate was added to produce a 70% saturated solution, which was allowed to stand in an ice bath for 1 h before centrifugation (30,000 × *g*, 30 min). The yellow precipitate was collected and re-dissolved in 20 ml of 0.05 M phosphate buffer (pH 6.0).

Second heat treatment. The ammonium sulfate fraction was diluted to 20 mg of protein per ml with 0.05 M phosphate buffer (pH 6.0) and transferred to a round-bottom flask. The diluted solution was brought to 55 C in a 70 C water bath within 10 min. The flask was immersed in water at 65 C for 7 min with constant stirring and then placed in an ice-water bath for 1 h before centrifugation. The precipitate was discarded.

Alcohol fractionation. To each 100 ml of the heated fraction, 80 ml of 90% ethyl alcohol at −20 C was slowly added while the mixture was maintained near its freezing point. The resulting turbid solution was equilibrated at −20 C for 20 min and then centrifuged at 30,000 × *g* at −20 C for 10 min. An additional 120 ml of cold alcohol for each 100 ml of heated fraction was added to the supernatant solution; the temperature was kept around −20 C. The mixture was centrifuged at −20 C, and the supernatant solution was discarded. The precipitate was re-dissolved in 0.02 M phosphate buffer (pH 6.0). The insoluble residue was removed by centrifugation, and the clear, light yellow solution was dialyzed overnight against 0.02 M phosphate buffer (pH 6.0).

Chromatography on SE-Sephadex. About 50 ml of the dialyzed enzyme preparation was applied to an SE-Sephadex C-50 column (2.0 by 20 cm) equilibrated with 0.02 M phosphate buffer, pH 6.0, and was carefully washed into the bed. The column was treated with the same buffer at a flow rate of 24 ml/h, and the effluent was collected in 5-ml fractions. The enzyme emerges in a peak at the void volume with only a threefold dilution. The pooled fractions were used directly for further purification.

Chromatography on CM-Sephadex. A CM-50 Sephadex column (2.0 by 20 cm) was equilibrated with 0.02 M phosphate buffer, pH 6.0. About 150 ml of the eluate from SE-Sephadex was applied to the column, which was then washed with 50 ml of the equilibration buffer. Elution was carried out with a linear gradient consisting of 150 ml of 0.03 M phosphate buffer, pH 6.0, and 150 ml of 0.5 M phosphate buffer, pH 6.0. Fractions of 5 ml were collected every 15 min. CoA transferase eluted at a salt concentration between 0.05 to 0.1 M. The fractions with specific activity higher than 30 units/mg were pooled and concentrated by ammonium sulfate precipitation.

The final preparation is greater than 90%

pure, as judged by polyacrylamide gel electrophoresis. Trace amounts of very active crotonase still contaminated the enzyme preparation at this stage. Only repeated crystallization can remove the crotonase. A summary of the purification procedure for CoA transferase is given in Table 3.

Crystallization. The ammonium sulfate-precipitated enzyme was dissolved in a minimum amount of 40% saturated ammonium sulfate solution in 0.1 M phosphate, pH 6.0. Saturated ammonium sulfate solution was then added dropwise; crystallization will start after a few hours. If there is no sign of crystallization after 48 h, the procedure of Jakoby (16) can be used to help start crystallization. After 2 to 3 weeks, some of the crystals grow to 0.1 mm in the longest dimension. The crystals are flat plates of triangular shape (Fig. 4). The gel electrophoresis data of the twice-crystallized CoA transferases are also shown in Fig. 4. Figure 5 indicates the correspondence of the enzyme activity with the protein band on acrylamide gel electrophoresis.

Characteristics of CoA transferase. Molecular weight. The molecular weight of native CoA transferase was determined by gel electrophoresis, as described by Hedrick and Smith (11). Five different gel concentrations (4, 5, 8, 10, and 12%) were used to calculate the slope characteristic of each marker enzyme and CoA transferase. CoA transferase fell on the line well above hexokinase and below lactic dehydrogenase, giving a value of 125,000.

Electrophoresis in the presence of sodium dodecyl sulfate yielded a single band, which migrated between L-amino acid oxidase and pyruvate kinase at a point corresponding to a molecular weight of 62,000. This result indicates that CoA transferase is composed of either two identical subunits or two different subunits with similar molecular weight.

Amino acid content. The amino acid analysis of 27 μg of CoA transferase is shown in Table 4. The values in the table were derived from the results after 25 h of hydrolysis, except for those of threonine and serine, which were obtained by extrapolation to zero-time hydrolysis. The weight of each amino acid was also calculated; the recovery was 24.5 μg from 27 μg hydrolyzed, or 91%. The number of residues is based on a molecular weight of 125,000, as determined by polyacrylamide gel electrophoresis. The eight half-cystines per 125,000 were obtained as cysteic acid (22). Five tryptophan residues were found by Edelhoch's procedure (7). The low content of tryptophan agrees with the observed high ratio (2:1) of dry weight in milligrams to A_{280}. The moles of asparagine and glutamine were assumed to be 10 for the purpose of these calculations.

Specificity of CoA transferase. The new assay based on the spectrophotometric measurement of acrylate hydration could not be applied to specificity studies for other carboxylic acid acceptors. An indication of analogue reactivity was obtained from the relative ability to inhibit the hydration of acrylate (Table 5). Further, their ability to form acyl CoA derivatives was confirmed by thin-layer chromatography of their hydroxamates.

Propionyl CoA, butyryl CoA, hexanoyl CoA, crotonyl CoA, and isobutyryl CoA could substitute for acetyl CoA as the CoA donor in the enzyme assay. Succinyl CoA and acetyl pantetheine are inactive as substrates for the enzyme.

Other properties of CoA transferase. The pH profile indicates a pH optimum of 7.0 and 90% of the maximum activity at pH 6.0 and 8.0. Beyond these points, activity decreases rapidly with change in pH. The denaturation of CoA transferase at low pH may contribute to the sharp drop in activity at pH 5.0. Since the reaction remains linear at pH 9.5, the decrease in activity at higher pH is probably not due to the decomposition of the thiol ester bond of acyl CoA.

TABLE 3. *Purification of CoA transferase from P. elsdenii*

Step	Vol (ml)	Total protein (mg)	Total activity (units)	Yield	Sp act (units/mg)	Purification (fold)
Cell-free extract	170	9,600	6,850	100	0.71	
First heat treatment	150	5,300	7,500	110	1.42	2.0
Ammonium sulfate fractionation	50	2,980	6,250	91	2.10	3.0
Second heat treatment	150	1,900	5,600	82	2.94	3.8
Alcohol fractionation	45	950	4,400	65	4.63	6.5
Column chromatography						
SE-50 Sephadex	70	325	4,100	59	12.6	17.7
CM-50 Sephadex	60	53	2,700	39	51.0	75.0

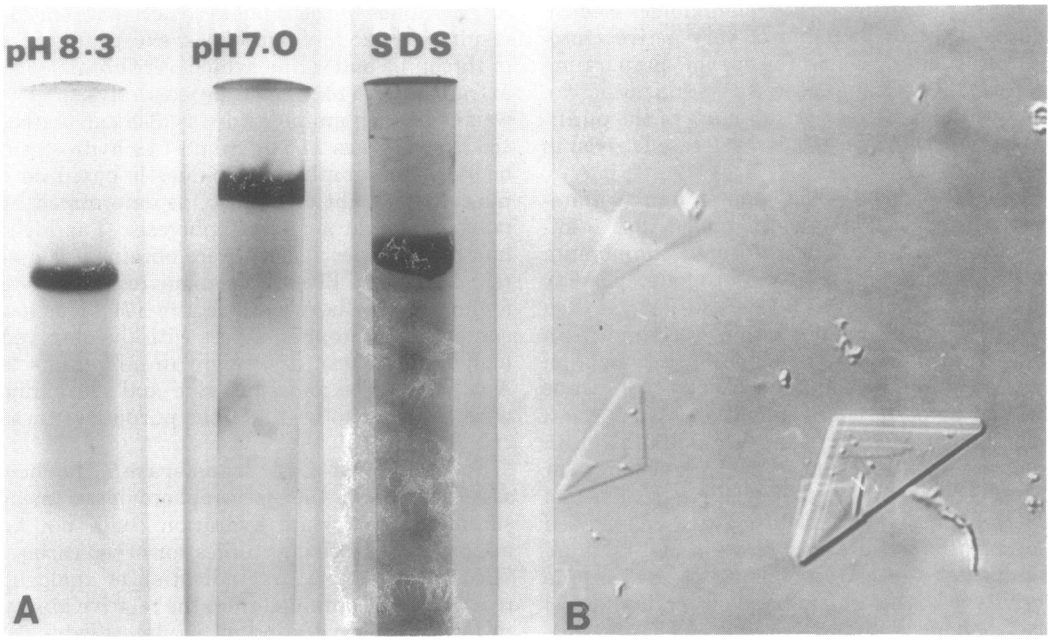


FIG. 4. *(A) Polyacrylamide disc gel electrophoresis of 50 μg of twice-crystallized CoA transferase. Runs were made at pH 8.3 (33), pH 7.0 (Brockman, submitted for publication), and in sodium dodecyl sulfate (11). (B) CoA transferase was crystallized from ammonium sulfate. The total magnification was 500-fold.*

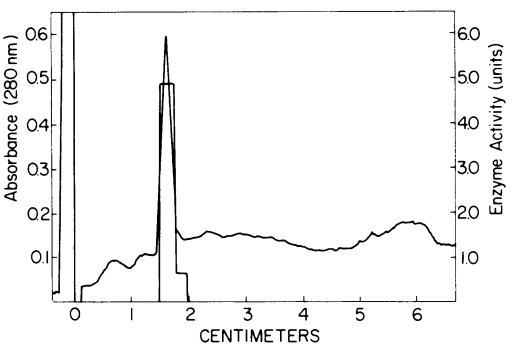


FIG. 5. *Electrophoretogram of twice-crystallized CoA transferase. Gels run at pH 8.3 by the method of Davis (5) were scanned at 280 nm. The same gel was then cut laterally into 0.25-cm sections, extracted with buffer, and assayed for CoA transferase activity.*

Pretreatment of the enzyme with 10^{-3} M *N*-ethylmaleimide had no effect on the activity. However, preincubating the enzyme with 2×10^{-4} M *p*-mercuribenzoate caused an 80% inactivation, which could not be reversed by dithiothreitol or β-mercaptoethanol.

Initial velocity characteristics. Since there are two substrates for the CoA transferase, the initial velocity was measured by fixing the concentration of one substrate and varying the concentration of the other. Under all conditions

TABLE 4. *Amino acid analysis of CoA transferase*

Amino acid	Amt (nmol) per 27 μg of protein	Mol/mol[a]	Residues/mole	Wt (μg)
Cys[b]	1.84	8.52	9	0.221
Asp	24.19	111.99	112	3.22
Thr[c]	12.42	57.50	58	1.48
Ser[c]	7.80	36.11	36	0.82
Glu	23.34	108.05	108	3.43
Pro	13.33	61.71	62	1.53
Gly	25.15	116.44	116	1.89
Ala	21.92	101.48	101	1.95
Val	17.02	78.80	79	2.00
Met	3.51	16.25	16	0.52
Ile	3.44	62.22	62	1.76
Leu	17.01	78.75	79	2.23
Tyr	7.46	34.54	35	1.35
Phe	9.61	44.49	44	1.56
Lys	13.65	63.19	63	1.99
His	5.46	25.28	25	0.85
Arg	8.25	38.19	38	1.44
Try[d]	1.22	5.65	6	0.25
NH_3[e]		10.0		

[a] Based on molecular weight of 125,000 (see text).
[b] Determined as cysteic acid.
[c] From hydrolyses at three time intervals; extrapolated to zero-time hydrolysis.
[d] Determined spectrophotometrically.
[e] Ten amide residues assumed.

TABLE 5. *Inhibition of CoA transferase by carboxylic acids*[a]

Group	Carboxylic acid	Concn (M)	Inhibition (%)
1	Formate	10^{-2}	10
	Acetate	10^{-2}	70
	Propionate	10^{-2}	65
	Butyrate	10^{-2}	75
	Valerate	10^{-2}	28
	Caproate	10^{-2}	5
	Capryate	10^{-2}	0
2	Glycolate	10^{-2}	35
	D,L-Lactate	10^{-2}	60
	α-Hydroxybutyrate	10^{-2}	30
	α-Hydroxyvalerate	10^{-2}	15
	α-Hydroxycaproate	10^{-2}	0
3	β-Hydroxypropionate	10^{-2}	45
	β-Hydroxybutyrate	10^{-2}	20
4	Malonate	2×10^{-2}	0
	Succinate	2×10^{-2}	0

[a] Two micromoles of sodium acrylate, 5 nmol of acetyl CoA, 2 μg of partially purified CoA transferase, and 10 μmol of phosphate (pH 7.0) were incubated in a total volume of 0.2 ml at room temperature. Suitable amounts of various carboxylic acids were added to the system as inhibitor.

used in these studies, reaction 3, the transfer of CoA between β-hydroxypropionyl CoA and acrylate, is rate limiting for the following reasons. (i) Since initial rates are measured, the back reaction can be considered negligible; (ii) the reverse conversions involving acrylyl CoA and acetate are negligible because the ratio of acetyl CoA (3×10^{-5} M) to acrylate (10^{-3} M) is very low; (iii) under the most extensive conditions used in kinetic studies, crotonase was present at five times the activity of CoA transferase; and (iv) crotonase has a very low K_m for acrylate ($\sim 2 \times 10^{-5}$ M) and a broad pH versus velocity curve (pH 5.5 to 9.0). Also, there is little competition between the two reactions of CoA transferase, i.e., that involving acetyl CoA and that involving β-hydroxybutyryl CoA. In the extreme case with 0.2 units of CoA transferase and 3.0×10^{-5} M acetyl CoA, all of the acetyl CoA will be converted to acrylyl CoA in less than 5 s, which is before rate measurements start. Thus, in the forward direction, it is reasonable to assume that both acetyl CoA and acrylyl CoA concentrations are negligible and that β-hydroxypropionyl CoA and acrylate concentrations determine the rates observed.

Care was taken to insure the reproducibility of these results. Reciprocal plots of the resulting data in Fig. 6 show a series of parallel lines characteristic of a ping-pong *bi bi* mechanism, in which one product is released before the second substrate combined with the enzyme. This type of kinetics suggests that the CoA transferase from *P. elsdenii* catalyzes the reaction through the two half reactions (see Discussion, equation 5).

Sodium acetate was used in inhibition studies as a product or substrate analogue. Figure 7 indicates a linear competitive inhibition of acrylate utilization by acetate when the concentration of acetyl CoA (β-hydroxypropionyl CoA) was varied and a linear noncompetitive inhibition of β-hydroxypropionyl CoA utilization when the concentration of acrylate was varied. These data fit the description of a ping-pong *bi bi* mechanism according to Garces and Cleland (8) (see Discussion).

Kinetic constants of CoA transferase are as follows: final specific activity, 110 units/mg of protein (corresponds to 1.38×10^4 μmol of acrylate hydrated per μmol of CoA transferase; molecular weight, 125,000); K_m for acrylate, 1.2×10^{-3} M; and the K_m for acetyl CoA, 2.4×10^{-5} M. (Although acrylyl CoA can be synthesized, it is relatively unstable and difficult to purify. For this reason, acrylyl CoA was generated in situ by adding a small amount of acetyl CoA and excess acrylate. In the presence of excess acrylate and excess crotonase, virtual quantitative conversion to propionyl CoA occurs. For kinetic studies involving β-hydroxypropionyl CoA binding, graded levels of acetyl CoA were added.)

Isolation of the enzyme-CoA intermediate. The ping-pong *bi bi* mechanism indicated by kinetic studies requires that an intermediate be produced during the reaction. An experimental demonstration of the intermediate was accomplished by preincubating CoA transferase with the acyl CoA donor in the absence of acceptor, followed by chromatographic separation. Specifically, 1 mg of column-purified CoA transferase was incubated with 1 μmol of acetyl CoA at pH 7 for 30 min. The reaction mixture was chromatographed on Sephadex G-50, which completely separated the transferase from both CoA and acetyl CoA. The ultraviolet difference absorption spectrum between untreated CoA transferase and that incubated with acetyl CoA and separated chromatographically is shown in Fig. 8. The difference absorption peak at 260 nm indicated the presence of CoA in the transferase, which had been preincubated with acetyl

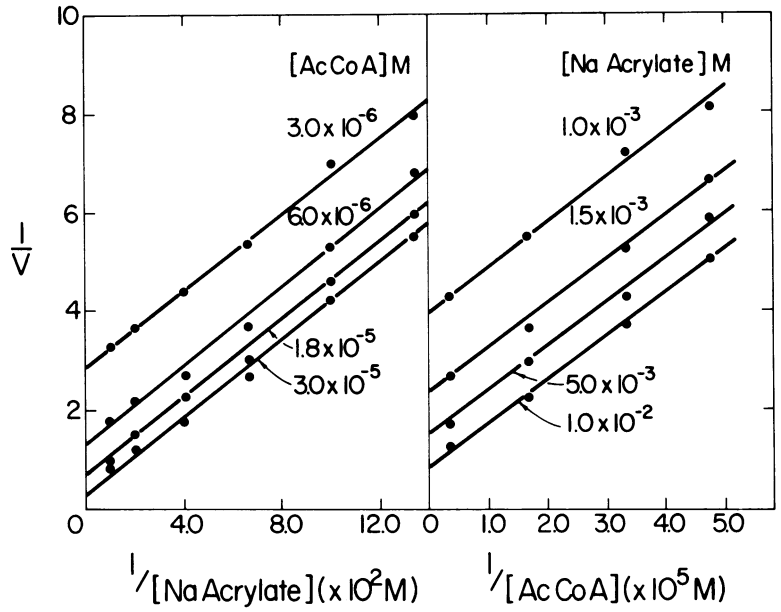


FIG. 6. *Initial velocity pattern for the hydration of acrylate with acrylate (A) or acetyl CoA (B) as the variable substrate. As described in the text, the variable amounts of acetyl CoA used may be considered to very rapidly yield similar amounts of β-hydroxypropionyl CoA. The reaction was conducted by premaking 10 ml of reaction mixture components which contained 200 μg of column-purified CoA transferase, 1 mmol of potassium phosphate, pH 7.0, and a suitable amount of stationary substrate (acetyl CoA for [A] and acrylate for [B]). After equilibration to room temperature, 0.2 ml of the mixture was added to the cuvette, and the reaction was then started by injecting the variable substrate with stirring from a Hamilton syringe.*

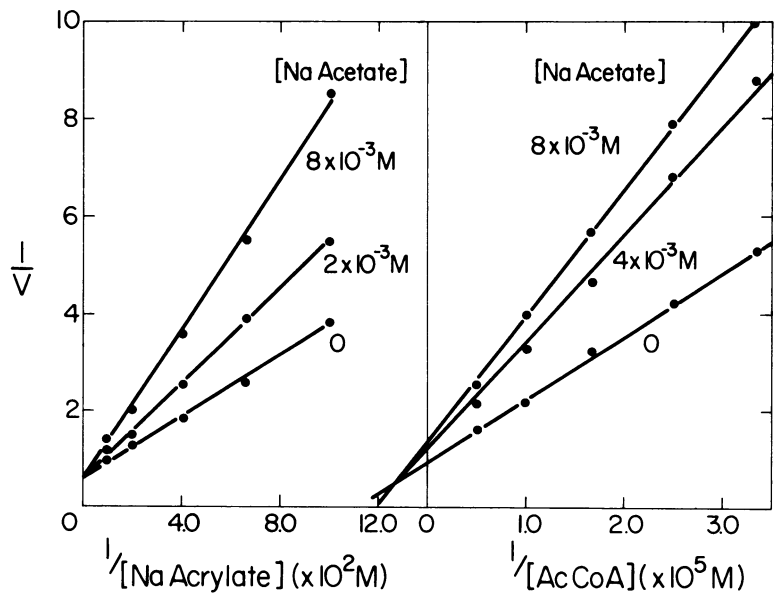


FIG. 7. *Inhibition by acetate. The experimental conditions were similar to those in Fig. 6 except for the addition of various amounts of acetate into the reaction mixture.*

CoA. A value of 1.60 mol of CoA per mol of CoA transferase was calculated based on the E_{260} for free CoA (16.4×10^3 liters/mol per cm) and a molecular weight of 125,000 for CoA transferase. Nonspecific association of acetyl CoA with CoA transferase was eliminated by preincubating CoA transferase with acetyl CoA containing ^{14}C in the acetyl group. Based upon radioactivity, less than 0.1 mol of acetate was present per mol of the isolated transferase-CoA intermediate. This indicated that more than 94% of the CoA associated with CoA transferase must have existed in some form other than one containing acetate carbon.

General properties of enzyme-CoA intermediate. Table 6 summarizes the general properties of the isolated enzyme-CoA intermediate. As shown in line 5 of the table, the enzyme-CoA intermediate displays a considerable amount of activity, even in the absence of acetyl CoA. The free CoA transferase, however, requires acetyl CoA for activity (lines 1 and 2). Acetate or coenzyme A will not substitute for acetyl CoA. No inactivation of the free enzyme occurs upon treatment with sodium borohydride or neutral hydroxamine (lines 3 and 4). However, these same treatments of the putative intermediate caused 80 and 60% inactivation, respectively (lines 7 and 8). The intermediate retained all of the properties described above after storage at −20 for 7 days at pH 6.0 (0.1 M phosphate). No differences in the specific activities of the free CoA transferase or the enzyme-CoA intermediate were observed using acetyl CoA as the substrate.

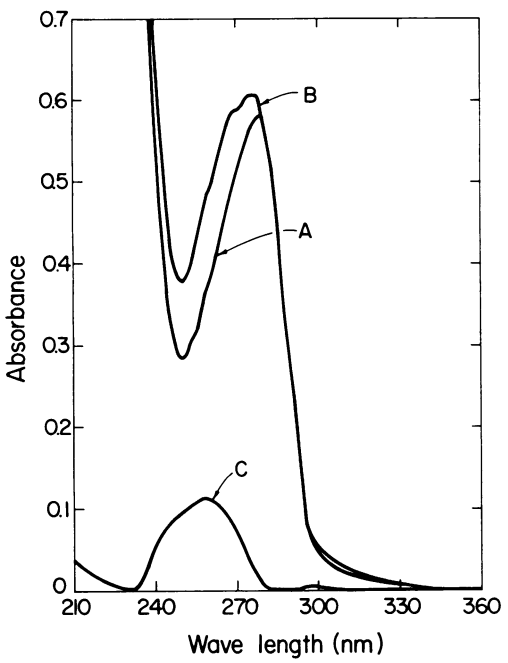


FIG. 8. *Comparison of absorption spectrum of CoA transferase (A) and the putative enzyme-CoA intermediate (B). Line C is the absorption difference spectrum between them. The experiment was conducted by incubating 10 mg of column-purified CoA transferase, 1 μmol of acetyl CoA, and 50 μmol of phosphate buffer, pH 7.0, in a 0.5-ml reaction volume at room temperature for 30 min. The reaction mixture was then chromatographed at room temperature on a Sephadex G-50 column (0.8 by 20 cm) with 0.05 M phosphate, pH 7.0. The protein was collected in one 3-ml fraction. For the control, 1 μmol of CoA was substituted for acetyl CoA. Since identical procedures were used, the protein concentration is assumed to be the same in (A) and (B). The ultraviolet spectra were then produced on a Cary model 14 recording spectrophotometer.*

TABLE 6. *Evidence for enzyme-CoA intermediate*

Treatment	Assay condition	Sp act
Free enzyme		
None	−AcCoA	0.1
None	+AcCoA	20.0
$NaBH_4$	+AcCoA	19.0
NH_2OH	+AcCoA	19.5
Enzyme-CoA intermediate		
None	−AcCoA	4.0
None	+AcCoA	15.0
$NaBH_4$	+AcCoA	2.5
NH_2OH	+AcCoA	4.5

[a] The $NaBH_4$ treatment was conducted by preincubating 0.2 mg of CoA transferase or the enzyme CoA intermediate with 1.5 μmol of $NaBH_4$ and 5 μmol of potassium phosphate, pH 7.5, in a final volume of 0.15 ml at room temperature for 30 min. After the treatment, 2 μl of incubation mixture was assayed for remaining enzyme activity under standard conditions. The hydroxamine treatment was identical to the $NaBH_4$ treatment except 150 μmol of neutral hydroxamine was used instead of 1.5 μmol of $NaBH_4$.

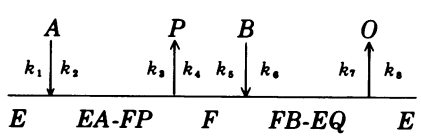
DISCUSSION

Comparison of CoA transferase from P. elsdenii and 3-keto acid CoA transferase from pig heart. Thus far, only the 3-keto acid CoA transferase from pig heart (13) and, in this report, the CoA transferase from *P. elsdenii* have been purified to homogeneity and extensively studied. Although both enzymes catalyze similar reactions, possibly through identical mechanisms, the enzymes were clearly distinguished from each other by their distribution, substrate specificity, and general role in metabolism.

The 3-keto acid CoA transferase is widely distributed in mammalian heart, skin, kidney, and muscle (15). The enzyme is believed to initiate the metabolism of acetoacetate. The aliphatic acid type of CoA transferase has only been detected in anaerobically grown bacteria such as *C. kluyveri* and *P. elsdenii*. Since both organisms are known for their ability to utilize or produce short-chain carboxylic acids, it is likely that CoA transferase has a major role in the metabolism of these acids.

Table 7 showed that both enzymes have clearly defined and non-overlapping substrate specificities. The 3-keto acid CoA transferase reacts only with succinic acid and β-keto acids, with the highest activity toward acetoacetate. On the other hand, the CoA transferase from *P. elsdenii* reacts with straight-chain, branched-chain, α,β-unsaturated, and hydroxy-substituted short-chain carboxylic acids, with highest activity toward carboxylic acids containing three carbon atoms. Similar substrate specificities were reported by Stadtman for the CoA transferase from *C. kluyveri* (26).

Kinetics and the back reaction. The catalytic amount of acetyl CoA used in the system for hydration of acrylate will immediately donate its CoA moiety to acrylate or to β-hydroxypropionate. Since there is a great excess of crotonase, the data we obtained in kinetic studies reflect the behavior of CoA transferase with β-hydroxypropionyl CoA and acrylate as substrates. Its mechanism may be represented by equation 5. *E* is free enzyme, *F* is the enzyme-CoA intermediate. In the present discussion, *A* and *P* are β-hydroxypropionyl CoA and β-hydroxypropionate, respectively, and *B* and *Q* are acrylate and acrylyl CoA, respectively. *K* is the equilibrium constant for the hydration of acrylyl CoA to β-hydroxypropionyl CoA (equation 3).



(5)

The kinetic expression (equation 6) for the reaction was derived by the King-Altman method (18):

$$v = \frac{V_1V_2(AB - PQ/K_{eq})}{K_bV_2A + K_aV_2B + V_2AB + \frac{K_pV_1}{K_{eq}}Q + \frac{K_qV_1}{K_{eq}}(P) + V_1\frac{PQ}{K_{eq}} + \frac{K_qV_1}{K_aK_{eq}}AP + \frac{K_aV_2}{K_{eq}}BQ} \quad (6)$$

TABLE 7. *Comparison of CoA transferases from pig heart and P. elsdenii*

Determination	Pig heart	*P. elsdenii*
Substrate specificity		
Saturated carboxylic acids (C_2-C_6)	−	+
α-Hydroxy carboxylic acids (C_2-C_6)	−	+
β-Hydroxy carboxylic acids (C_3-C_4)	−	+
Dicarboxylic acids (C_3-C_4)	+	−
β-Keto carboxylic acids (C_4-C_6)	+	−
Molecular weight	78,000	125,000
pH optimum	~8.5	7.0

When measuring the initial velocities, the concentration of *P* is approximately zero. The concentration of *Q* is determined by the equilibrium for equation 7,

$$A/Q = K \cdot [H_2O] = 1.2 \times 10^2 \quad (7)$$

and can be calculated from equations 8 and 9:

$$[A] + [Q] = [\text{acetyl CoA}]_{\text{initial}} \quad (8)$$

$$[Q] = \frac{[\text{acetyl CoA}]_{\text{initial}}}{1 + K[H_2O]} \quad (9)$$

We determined a $K[H_2O]$ value of 1.2×10^2 using freshly prepared acrylyl CoA and crotonase from *P. elsdenii*. Since $Q = [\text{AcCoA}]/1 + K[H_2O]$, the large value of $K[H_2O]$ indicates that the concentration of *Q* is negligible. Setting both *P* and *Q* equal to zero, the initial rate can be obtained (equation 10).

$$v = \frac{V_1AB}{K_bA + K_aB + AB} \quad (10)$$

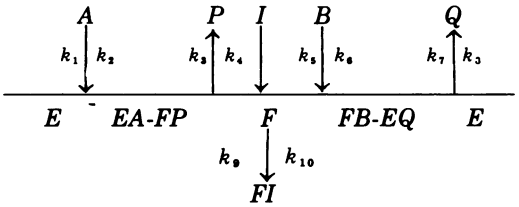
Equation 10 predicts a series of parallel lines for the double reciprocal plots. We observed such a pattern in our kinetic studies (Fig. 6).

For the reverse reaction (i.e., the dehydration of β-hydroxypropionate), our data suggested a slow rate and a complicated mechanism. This can be explained by examining the rate equation 6.

In the present case, *Q* (acrylyl CoA) is one of the substrates and *A* (β-hydroxypropionyl CoA) is one of the products. The low concentration of acrylyl CoA obviously leads to a slow reaction rate. The high concentration of β-hydroxypropionyl CoA will produce competitive inhibition for acrylyl CoA and a noncompetitive inhibition

for β-hydroxypropionate. Therefore, the double reciprocal plots will not yield simple parallel lines in this case. No further studies have been done on the reverse reaction.

Product analogue inhibition. When acetate (*I*) is used as an inhibitor of the overall reaction measured as the hydration of acrylate, again the velocity data obtained are for CoA transferase because crotonase is present in excess. The kinetic mechanism can be written as follows:



The initial rate equation and velocity expression were derived by the King-Altman method (equation 11).

$$v = \frac{VAB}{K_bA + K_aB + AB + K_b\frac{AI}{K_iP}} \quad (11)$$

Equation 11 is consistent with the observed competitive inhibition when acrylate is varied and noncompetitive inhibition when acetyl CoA is varied (Fig. 7).

Enzyme-CoA intermediate. A covalent intermediate between CoA and CoA transferase was isolated and identified for the 3-keto acid transferase from pig heart by Hersh and Jencks (12). It was demonstrated that the intermediate can donate CoA to either succinate or acetoacetate and that it can be reduced by $NaBH_4$. We described the isolation of a similar enzyme-CoA complex by preincubating acetyl CoA and CoA transferase from *P. elsdenii*.

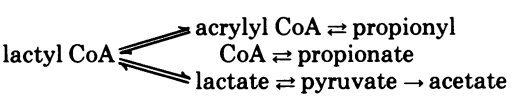
The complete separation of the CoA transferase intermediate from acetyl CoA, from CoA transferase plus radioactive acetyl CoA, and from free CoA eliminated the possibility of noncovalent association of CoA or acetyl CoA with the enzyme. This suggests that CoA is covalently linked to the enzyme to form the isolated intermediate. Table 6 shows that the isolated enzyme-CoA complex, but not free CoA transferase, can catalyze the hydration of acrylate in the absence of acetyl CoA. This observation indicates that CoA is linked to the enzyme such that it can serve as a CoA donor during the reaction with an added carboxylic acid.

The pretreatment of the putative enzyme-CoA intermediate with $NaBH_4$ at pH 7.5 resulted in an 80% inactivation of the enzyme. Since no inactivation occurred in a contol with untreated CoA transferase, and since borohydride is known to reductively cleave thiol esters to the corresponding thiol and alcohol (25), there is reason to believe that a thiol ester bond between CoA and enzyme was reduced with resulting inactivation. Inactivation of an enzyme-CoA intermediate, but not the free enzyme by neutral hydroxylamine, also indicated a reaction of the thiol ester bond, resulting in enzyme-hydroxamate formation.

Reduction with borotritide and acid hydrolysis of the enzyme-CoA intermediate of the 3-keto acid CoA transferase from pig heart by Solomon and Jencks yielded a tritium-labeled α-amino-δ-hydroxyvaleric acid (25). Therefore, it was concluded that CoA is bound to the γ-carboxyl group of glutamic acid residue on the protein.

For the enzyme-CoA intermediate for the CoA transferase of *P. elsdenii*, our results strongly suggest that a similar thiol linkage exists between the enzyme and CoA. However, there is a distinct difference between these two enzyme-CoA intermediates. For instance, Hersh and Jencks (14) reported a half-life of 42 min for the pork heart enzyme-CoA intermediate at pH 6.5. Under similar conditions, the enzyme-CoA intermediate from *P. elsdenii* can be prepared at room temperature and stored at −20 C for several days without deterioration. Whether this stability difference simply reflects, in the case of the *P. elsdenii* enzyme, that CoA is bound to an amino acid other than glutamic acid, or is the result of microenvironmental difference between these two proteins, is not known.

Metabolism of acrylate in P. elsdenii. The metabolism of acrylate by *P. elsdenii* was studied by Lewis and Elsden and Ladd and Walker, both in vivo (20) and in vitro (19). These authors proposed that at pH 6.0 acrylate was metabolized as follows:



Although most enzymes in this scheme have been detected in *P. elsdenii*, the identity of the key enzyme which converts acrylyl CoA to lactyl CoA still remains unresolved. Furthermore, their reports indicated that there was no accumulation of either lactate or pyruvate. Schneider (D. L. Schneider, Ph.D. thesis, Michigan State Univ., East Lansing, 1969) reported that, even in the presence of excess L-lactate

dehydrogenase, formation of lactate from acrylate by the crude extract of *P. elsdenii* could not be detected. Our data strongly suggest that the presence of excess crotonase and CoA transferase will quantitatively convert acrylate to β-hydroxypropionate. It may be that some of the acrylate is metabolized via β-hydroxypropionyl CoA, semi-malonyl CoA, and malonyl CoA to acetate as described by Vagelos and his colleagues in *C. kluyveri* (31, 32).

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