Methylmalonyl Isomerase

IV. PURIFICATION AND PROPERTIES OF THE ENZYME FROM PROPIONIBACTERIA*

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Methylmalonyl isomerase catalyzes the interconversion of methylmalonyl coenzyme A (b) and succinyl coenzyme A (3, 4), a reaction which plays an important role in both the conversion of pyruvate to propionate in propionibacteria (5-7), and of propionate to succinate in animal tissue (3, 8). The enzyme has been the object of considerable interest since it is one of the few purified enzymic reactions requiring cobamide coenzymes for catalytic activity (5, 7, 9, 10). Barker, Weissbach, and Smyth (11) initially discovered a D12-coenzyme as an essential reactant in the isomerization of glutamate and β-methyl aspartate. Stjernholm and Wood (7) obtained methylmalonyl isomerase in a purified form from propionibacteria, but subsequently found that this enzyme preparation contained methylmalonyl racemase (12), an enzyme which catalyzes the interconversion of the two isomeric forms (a and b) of methylmalonyl-CoA (4). Methylmalonyl isomerase also has been obtained in a purified form from propionibacteria by Overath, Stadtman, Kellerman, and Lynen (13) and from animal tissue by Mazumder, Sasaki, and Ochoa (14).

There is considerable information pertaining to the mechanism of the isomerase reaction; yet the role of the cobamide coenzymes is not clear. By degradation of the succinyl-CoA formed from specifically 14C-labeled methylmalonyl-CoA, it was determined that the isomerization occurred by a shift of the carbonyl thioester group and not the free carboxyl group (15-17). It was proposed by Hegre, Miller, and Lane (17) and Marston, Mills, and Smith (18) that the transfer of the carbonyl thioester group of methylmalonyl-CoA occurs by an intramolecular mechanism involving the formation of a cyclic dimer, followed by cleavage to form two molecules of succinyl-CoA. Eggerer, Stadtman, Overath, and Lynen (15), on the other hand, proposed an intramolecular shift resulting from a one electron oxidation-reduction through action of the cobalt, with formation of a methylmalonyl-CoA free radical which rearranges to form succinyl-CoA. Kellermeyer and Wood (1) and Phares, Long, and Carson (19) have now shown by mass analysis of the succinyl-CoA, formed from specifically 14C-labeled methylmalonyl-CoA that the conversion occurs by an intramolecular shift. Current studies, however, indicate there is no proton exchange with the medium (20) as was predicted for the free radical mechanism (see Wood, Kellermeyer, Stjernholm, and Allen (21) for more complete discussion).

This report results from an extension of the studies of Stjernholm and Wood (7) on the purification and characterization of methylmalonyl isomerase. Data are presented on the following: determination of the equilibrium constant in the absence of methylmalonyl racemase, the effect of sulfhydryl inhibitors, ultracentrifugal and electrophoretic mobilities, a calculation of molecular weight, and a method for purification of a preparation with a specific activity greater than previously reported. This preparation was colorless and was found to contain little cobamide coenzyme as determined by spectral analysis.

EXPERIMENTAL PROCEDURE

Materials and Methods

All enzymes used in this study were prepared from Propionibacterium shermanii, 52W; the bacteria were grown on a glucose yeast extract medium and the cell extract was prepared as described by Wood et al. (22). The dimethylbenzimidazolyl-cobamide (DBC) was a gift of Karl Folkers, Merck, Sharp and Dohme Company; a fresh aqueous solution (10⁻⁴ M) was prepared from a 7 x 10⁻⁴ M stock solution at weekly intervals, both were stored at -20° and protected from the light. Methylmalonyl-oxaloacetate transcarboxylase (22), malate dehydrogenase (23), methylmalonyl racemase (12), and CoA transferase (23) were also prepared from propionibacteria. None of these preparations contained methylmalonyl isomerase. Coenzyme A esters were prepared as described by Swick and Wood (6), using modifications of the methods by Simon and Shemin (24) or by Beck, Flavin, and Ochoa (25). The DPNH (Sigma), pyruvate (Sigma), glutathione (Boehringer), and Coenzyme A (Cutolo Calosi, S.P.A., Naples, Italy) were commercial preparations. The protein concentration in the crude extract was measured by the biuret method (26) and subsequently by the spectrophotometric method of Warburg and Christian as described by Layne (27).

Purification of Methylmalonyl Isomerase—The initial steps in the purification of the methylmalonyl isomerase were the same as those reported by Stjernholm and Wood (7) for the preparation obtained from propionibacteria. The DBC was added to the crude extract and the mixture was incubated with shaking at 30° for 2 hours, after which time the DBC was removed by gel filtration (27) and the enzyme preparation was dialyzed against Tris buffer, pH 7.8, for 24 hours. The isomerase was then purified as described by Stjernholm and Wood (7) on the purification and characterization of methylmalonyl isomerase. Data are presented on the following: determination of the equilibrium constant in the absence of methylmalonyl racemase, the effect of sulfhydryl inhibitors, ultracentrifugal and electrophoretic mobilities, a calculation of molecular weight, and a method for purification of a preparation with a specific activity greater than previously reported. This preparation was colorless and was found to contain little cobamide coenzyme as determined by spectral analysis.

* The abbreviations used are: DBC, dimethylbenzimidazolyl-cobamide; TEAE-cellulose, triethylaminoethyl cellulose; HMB, p-hydroxymercuribenzoate; BC, benzimidazolylcobamide; AC, adenylcobamide.
as those described by Wood et al. (22) for the purification of oxaloacetic transcarboxylase. The crude extract, which contained 115.4 g of protein obtained from 1.22 kg of propioni-bacteria, was absorbed on DEAE-cellulose and eluted batchwise with potassium phosphate buffer, pH 6.9, at 4°; the isomerase activity was recovered in the 0.3 M buffer eluate with more than a 3-fold increase in the specific activity (Table I). The isomerase was precipitated with ammonium sulfate (90% saturation) and sedimented by centrifugation at 25,000 × g. The precipitate was dissolved in 0.05 M phosphate buffer, pH 6.9, and was then diluted with H₂O to make an ammonium sulfate concentration of 0.05 M. The enzyme preparation was then passed through a cellulose phosphate column as described previously (22). The protein passing through the column, plus that eluted with 0.05 M phosphate buffer, pH 6.9, contained nearly all of the isomerase with more than a 2-fold increase in specific activity. This latter step removed the transcarboxylase activity and most of the racemase.

The 0.05 M eluate was divided into two equal parts and processed separately. The treatment of the half resulting in the highest specific activity will be described first. For this preparation the eluted material was stored at −20° for 3 months and then absorbed on a TEAE-cellulose column (Brown Company, 0.8 meq per g, 4.5 × 21 cm). The TEAE-cellulose had been washed with 0.1 M NaOH, 0.1 M HCl, water, 0.1 M, and then 0.05 M phosphate buffers, pH 6.9, and before use the column was equilibrated with 0.05 M potassium phosphate buffer at 4°. The protein was eluted successively with approximately 600 ml each of 0.1, 0.15, 0.2, and 0.25 M potassium phosphate buffer, pH 6.9; the isomerase was eluted with the 0.15 M phosphate buffer. The fractions containing isomerase were brought to 90% saturation with ammonium sulfate, the precipitate was taken up in 0.05 M phosphate buffer, pH 6.9, and the solution was dialyzed against 0.05 M phosphate buffer, pH 6.9. The dialyzed product was placed on a second TEAE-cellulose column, prepared as before, and eluted with a gradient phosphate buffer (500 ml of 0.0/5 M phosphate buffer, pH 6.9, in the mixing bottle with addition of 0.3 M phosphate buffer, pH 6.9). The isomerase in a protein peak obtained from this column, was precipitated with ammonium sulfate (90% saturation) and was redissolved in 0.05 M phosphate buffer, pH 6.9. The solution (20.2 mg of protein per ml), was fractionated by addition of saturated ammonium sulfate; 71% of the activity was precipitated between 60 and 90% ammonium sulfate saturation. A second ammonium sulfate fractionation of this precipitate resulted in a slight increase in specific activity of the fraction obtained between 67 to 90% saturation. The preparation had a specific activity of 14.4 and was colorless. The recovery was 51% of the total activity in the crude extract.

Analytical ultracentrifugal studies of this preparation showed a 5 to 10% slowly sedimenting impurity (by comparison of the areas on the schlieren photograph). An aliquot of this preparation was subjected to a final purification step consisting of centrifugation in a 10 to 37% sucrose gradient (28) and the sucrose column was fractionated according to the method of Martin and Ames (29). The protein was prepared for this step by dialysis against 0.05 M phosphate buffer, pH 6.9; 0.5 ml of this dialyzed preparation containing 15 mg of protein was placed on top of the sucrose column in a 2- × 0.5-inch tube and spun for 15 hours at 0° and 30,000 r.p.m. in a SW 39 rotor with a Spinco model L ultracentrifuge. This purification removed the slowly sedimenting and faintly yellow protein but did not significantly alter the specific activity. The isomerase protein was now homogeneous in the ultracentrifuge.

The other half of the cellulose phosphate eluate (Table I, Step 4a) was processed immediately; the final preparation was pink and had a specific activity of 9.7. Analytical ultracentrifugal studies showed that this preparation contained small amounts (less than 5%) of a low molecular weight impurity. An aliquot of this preparation was also subjected to a final purification step consisting of centrifugation in a 10 to 37% sucrose gradient. The isomerase was easily identified as a pink band after centrifugation and was found in Fractions 15 to 20 of 30 fractions collected from the bottom of the tube. This purification likewise removed a yellow protein but did not significantly alter the specific activity of the isomerase. The protein was now homogeneous in the ultracentrifuge.

To determine the equilibrium constant for the isomerase reaction, it was necessary to remove all remaining traces of methylmalonyl racemase from an isomerase preparation. A preparation corresponding to that of Table I, Step 6a was further purified by a method described by Overath et al. (13), who reported that racemase could be separated from the isomerase on a Sephadex G-75 F column. A tightly packed Sephadex G-75 M column (1 × 85 cm) was equilibrated at 4° with 0.05 M potassium phosphate, pH 7.3. Approximately 50 units of an isomerase preparation (specific activity, 8.3) which contained 9 units of methylmalonyl racemase (specific activity, 0.85) was placed on the column. The column was washed with 0.05 M phosphate buffer, pH 7.3, and 1-nl fractions were collected. Little or no protein was present in the first 14 fractions. Isomerase, with a specific activity of 9.0, was eluted in Fractions 15 to 17. This enzyme preparation, which contained no detectable racemase, was concentrated with ammonium sulfate (90% saturation) and stored at −10°. When the activity was assayed 2 days later, the specific activity had inexplicably decreased to 4.1. However, because the preparation was free of racemase, it was used for the equilibrium studies.

**Table I**

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bacterial extract</td>
<td>3450</td>
<td>0.03</td>
</tr>
<tr>
<td>2. DEAE-cellulose, 0.3 M buffer</td>
<td>3480</td>
<td>0.09</td>
</tr>
<tr>
<td>3. Cellulose-phosphate, 0.05 M buffer*</td>
<td>3720</td>
<td>0.21</td>
</tr>
<tr>
<td>4. TEAE-cellulose, 0.15 M buffer</td>
<td>2100</td>
<td>2.5</td>
</tr>
<tr>
<td>5. TEAE-cellulose, gradient buffer</td>
<td>1290</td>
<td>3.8</td>
</tr>
<tr>
<td>6. (NH₄)₂SO₄, 0.60-0.90 saturated</td>
<td>1800</td>
<td>9.7</td>
</tr>
<tr>
<td>7. (NH₄)₂SO₄, 0.67-0.90 saturated</td>
<td>872</td>
<td>14.4</td>
</tr>
</tbody>
</table>

* After this step the eluate was divided in two equal parts; one part (a) was processed immediately by Steps 4 and 6 above and resulted in a pink preparation with a specific activity of 9.7; the other half (b) was frozen at −20° for 3 months and then processed by Steps 4 through 7.
modified from that described by Stjernholm and Wood (7). The coupled reactions are as follows.

\[ \text{Acetyl-CoA} + \text{succinate} \xrightarrow{\text{transferase}} \text{acetate} + \text{succinyl-CoA} \]  
\[ \text{Succinyl-CoA} \xrightarrow{\text{isomerase}} \text{methylmalonyl-CoA} \]  
\[ \text{Methylmalonyl-CoA} \xrightarrow{\text{racemase}} \text{methylmalonyl-CoA} \]  
\[ \text{Methylmalonyl-CoA} + \text{pyruvate} \xrightarrow{\text{transcarboxylase}} \text{propionyl-CoA} + \text{oxaloacetate} \]  
\[ \text{Oxaloacetate} + \text{DPNH} \xrightarrow{\text{malate dehydrogenase}} \text{DPN} + \text{malate} \]

Because synthetic succinyl-CoA is unstable relative to acetyl-CoA on storage at 0°, succinyl-CoA usually was generated in the reaction mixture using CoA transferase. All enzymes used in this assay were free of interfering amounts of isomerase. The reaction was carried out in a final volume of 0.3 ml in a 0.5-ml microcuvette with a 1-cm light path. The rate of DPNH oxidation was directly proportional to the amount of isomerase added (Fig. 1).

When the extracts contained DPNH oxidase or lactate dehydrogenase, the assay was carried out in two steps similar to that described by Stjernholm and Wood (7). Reactions 1, 2, 3, and 4 were allowed to proceed for 5 minutes at 30° and the reaction mixture contained the following (in micromoles): pyruvate, 10; Tris-HCl, pH 7.3, 75; reduced glutathione, 2.5; DBC, 0.02; succinyl-CoA, 0.6; and in units: transcarboxylase, 0.5. The reaction was started by addition of the isomerase sample, giving a final volume of 0.6 ml, and it was stopped by the addition of 0.4 ml of 10% trichloroacetic acid. After 5 minutes at 0° the precipitate was removed by centrifugation. The solution was neutralized with 0.13 ml of 0.4 M Tris base, and an aliquot of the resultant mixture (0.2 ml) was assayed immediately for oxaloacetate, in a final volume of 0.51 ml containing Tris-HCl, pH 7.3, 100 μmoles; DPNH, 0.05 μmole; and malate dehydrogenase, 0.25 unit.

Variations of this two-step assay procedure were used for the determination of the pH optimum, the effect of sulphydryl-binding agents, for equilibrium studies and for the determination of Michaelis constants.

**Determination of Methylmalonyl-CoA and Succinyl-CoA by Enzymic Methods**—The approximate concentration of CoA esters was determined with the hydroxamate method (30) and this value was used for routine work. However, for the more precise measurement used in determining \( K_m \) values and the equilibrium constant, the concentration of the CoA ester was assayed enzymatically.

The quantitative determination of methylmalonyl-CoA (a and b) involves Reactions 3, 4, and 5. The CoA ester was assayed spectrophotometrically in a microcuvette with a 1-cm light path at room temperature or 30°. The reaction mixture contained the sample of the CoA ester and the following in units: methylmalonyl racemase, 0.1; transcarboxylase, 0.1; malic dehydrogenase, 0.25; and in micromoles: pyruvate, 1.7; reduced glutathione, 0.8; Tris-HCl, pH 7.4, 8.3; DPNH, 0.043. The final volume was 0.31 ml and the reaction was started by the addition of transcarboxylase. For quantitative determination of each member of the racemase pair the racemase is omitted and the reaction is allowed to proceed until the (a) form is spent and then racemase is added for the determination of the (b) form.

Succinyl-CoA can be determined enzymatically by coupling Reactions 2, 3, 4, and 5. The assay system is similar to that for methylmalonyl-CoA except the succinyl-CoA sample is added in place of the methylmalonyl-CoA and in addition 0.1 unit of isomerase and 0.01 μmole of DBC. The control lacking either isomerase or acetyl-CoA gave a reading equivalent to approximately 0.1 mpmole per minute which has been subtracted from the value of the complete assay.

**Properties of Methylmalonyl Isomerase**

**pH Optimum**—The highest methylmalonyl isomerase activity occurred at pH 7.4 and diminished only slightly at pH 6.0 and 8.0 (Fig. 2). The pH optimum was measured in a two-step assay to insure that the effect of the pH was only on the isomerase. The first step was the conversion of succinyl-CoA to methylmalonyl-CoA(b) at different pH values. The second step assayed the quantity of methylmalonyl-CoA(b) and was carried out at a neutral pH (see Fig. 2 for details). These results are comparable to those obtained by Stjernholm and Wood (7).

**Michaelis Constants**—The Michaelis constants for succinyl-CoA, methylmalonyl-CoA, and benzimidazolylcobamide are 3.45 × 10⁻⁶ M, 8.0 × 10⁻⁶ M, and 3.45 × 10⁻⁷ M, respectively (Fig. 3). The \( K_m \) values for the cobamide coenzymes have been reported previously (7, 9, 13). The \( K_m \) value for cobinamide conjugate (Co₅'-deoxyadenosylcobamide) has been reported to be 1.7 × 10⁻⁶ M (13) with isomerase from propionibacteria. However, this compound did not activate any of the isomerase preparations discussed in this report. The cobinamide conjugate
was inactive at a final concentration of $3.8 \times 10^{-4}$ m. This compound was a generous gift from Dr. Otto Müller, Stuttgart, Germany.

Equilibrium of the Methylmalonyl Isomerase Reaction— Previously it was reported by Stjernholm and Wood (7) that the equilibrium constant of the methylmalonyl isomerase reaction was 10.5 favoring succinyl-CoA. This determination was done prior to the discovery of methylmalonyl racemase and the enzyme preparation used undoubtedly contained racemase. Since the equilibrium constant of the racemase reaction is 1 (12), twice as much methylmalonyl-CoA should accumulate in the presence of racemase as in its absence. The measurements have been repeated with an isomerase preparation containing no racemase and the equilibrium constant ([succinyl-CoA]/[methylmalonyl-CoA]) was found to be 23.1 (Table II), a value, as expected, that is about twice that obtained by Stjernholm and Wood. The $K_{eq}$ was calculated from this equilibrium constant to be $-1.86 \times 10^{3}$ calories.

The amount of succinyl-CoA used for the calculation of the equilibrium constant was that determined by the difference between the amount of succinyl-CoA added and the methylmalonyl-CoA formed. This calculation was necessary since the succinyl-CoA determined after deproteinization was always underestimated (Table II). The low recovery is evident since only 87% of the added succinyl-CoA could be recovered at 0 time when the perchloric acid was added prior to the enzyme. Stjernholm and Wood (7) encountered the same difficulty in recovering succinyl-CoA from the deproteinized solutions.

Spectrum of Purified Enzyme—The absorption spectra of two highly purified preparations of methylmalonyl isomerase processed as described previously (Table I), one pink (specific activity, 9.7) and one colorless (specific activity, 14.4), were determined in a Zeiss PMQ II spectrophotometer (Fig. 4). Both preparations were inactive without added cobamide coenzyme. The pink preparation had absorbance peaks at 352, 407, 505, and 535 mμ. This spectrum is nearly identical to the light inactive benzimidazolyl-, dimethylbenzimidazolyl-, and adenylcobamide coenzymes. These, as reported by Overrath et al. (18) and Webesch et al. (31, 32), exhibit absorbance peaks at 352, 407, 495, and 522. The colorless preparation had only small peaks at 352 and 407 indicating that little inactive coenzyme was still attached to the enzyme. It is postulated (see "Discussion") that the higher specific activity of this colorless preparation is due to the relatively greater abundance of free attachment sites for the added active coenzyme.

In view of the spectral changes observed by Abeles and Lee (33) with diol dehydrase and cobamide coenzyme, it was considered important to look for similar spectral shifts caused by methylmalonyl isomerase. When $3.5 \times 10^{-5}$ μoles of DBC were incubated with 2.4 $\times 10^{-5}$ μoles of colorless isomerase and 26 μoles of potassium phosphate buffer, pH 7.5, in a total volume of 0.25 ml, no increase in absorbance at either 352 or 362 mμ occurred during 10 minutes at 25°. Furthermore, no decrease in specific activity of the isomerase was detected at the end of the incubation. Likewise, addition of methylmalonyl-CoA to the incubation mixture did not alter the absorbance at 352 or 362 mμ.

Effect of p-Hydroxymercuribenzoate and N-Ethylmaleimide— Aliquots of isomerase (0.075 unit) were incubated with HMB, in a final concentration of $10^{-3}$ and $10^{-2}$ m, or N-ethylmaleimide, in a final concentration of $10^{-2}$ m, in 0.02 m phosphate buffer, pH 7.6, for 20 minutes at 0° in a final volume of 0.5 ml. A 0.1-m1 aliquot of each of the above mixtures was added to 10 μoles of phosphate buffer, pH 7.6, 0.022 μole of DBC and 1 μole of succinyl-CoA, in a final volume of 0.5 ml. This mixture was incubated for 4 minutes at 32° and the reaction was stopped by adding 0.25 ml of 10% trichloroacetic acid. After 5 minutes at 0°, the methylmalonyl-CoA in 0.2 ml of the mixture was determined using the following in micromoles: Tris base, 40; Tris-HCl, pH 7.4, 14; DPNH, 0.03; and in units: lactate dehydrogenase, 0.1; methylmalonyl racemase, 0.13; and oxaloacetate transcarboxylase, 0.1. The final volume was 0.45 ml and the reaction was started by the addition of the transcarboxylase.

Stability of Enzyme—The isomerase was incubated in 0.05 m...
I.5

Methylmalonyl Isomerase. IV

\[ \text{ Succinyl-CoA } \quad \text{Km} = 3.45 \times 10^{-5} \]

\[ \text{ methylmalonyl-CoA } \quad \text{Km} = 8 \times 10^{-5} \]

Fig. 3. The \( K_m \) values of substrates of the methyl isomerase reaction. For succinyl-CoA, the reaction mixture contained in micromoles: pyruvate, 2.3; reduced glutathione, 1.2; DPNH, 0.043; potassium phosphate, pH 7.4, 11.5; DBC, 0.001; and in units: racemase, 0.13; transcarboxylase, 0.25; and isomerase (specific activity, 14.4), 0.0034. Variable amounts of succinyl-CoA were added in a final volume of 0.32 ml. The reaction was started by adding the substrate after initial incubation at 30° for 5 minutes for temperature equilibration. A similar assay system was used for the determination of \( K_m \) value for the benzimidazolylcobamide except succinyl-CoA was generated in the cuvette from 0.1 umole of acetvl-CoA and 0.8 umole of succinate using a purified CoA-transferase (0.25 unit). The following series of reactions were used for determination of \( K_m \) of methylmalonyl-CoA.

Methylmalonyl-CoA (b) \[ \xrightarrow{\text{isomerase}} \] succinyl-CoA

Succinyl-CoA + propionate \[ \xrightarrow{\text{CoA transferase}} \] propionyl-CoA + succinate

The reaction mixture contained in micromoles: reduced glutathione, 1.5; potassium phosphate buffer, pH 7.4, 15; MgCl₂, 1.5; ATP, 1.5; potassium bicarbonate, 3.0; P-enolpyruvate, 0.6; DPNH, 0.025; propionate, 2; DBC, 0.001; and in units: pyruvate kinase, 0.25; lactate dehydrogenase, 0.25; propionyl carboxylase, 0.25; CoA transferase, 0.25; and isomerase, 0.0034 in a final volume of 0.34 ml. The methylmalonyl-CoA contained equal amounts of the two isomeric forms (a and b) and all \( K_m \) calculations are based on the concentration of one isomer (b). The method of least squares was used to determine the slopes.

Effect of Ionic Composition of Medium on Methylmalonyl Isomerase Activity—Although no metal requirement has been noted for methylmalonyl isomerase, the diol dehydrase reported by Abeles and Lee (33) is stimulated by K⁺ and NH₄⁺ ions. Reaction rates with the purified isomerase reported here showed no stimulation by K⁺ ions and NH₄⁺ ions up to a final concentration of 0.33 M. Further when EDTA was incubated with the enzyme at concentrations of \( 4 \times 10^{-4} \) to \( 8 \times 10^{-2} \) M for 10 minutes no alteration in the specific activity of the isomerase was noted.

Sedimentation Coefficient, Electrophoretic Mobility, and Molecular Weight—The isomerase gave a single peak in the ultracentrifuge with an \( s_{20, w} = 7.0 \) (Fig. 5A). It had an electrophoretic
mobility of $14.6 \times 10^{-5}$ cm$^2$ per sec per volt (Fig. 5B). The small peak trailing the major isomerase peak in the electrophoretic pattern represents a maximum contamination of 8.3%.

The molecular weight of the pure isomerase was determined using the sedimentation equilibrium method of Archibald (34) described by Schachman (35). An aliquot of the isomerase preparation with a specific activity of 9.7 (Table I, Step 6a) was dialyzed against 0.1 M phosphate buffer, pH 7.5, and then placed in a standard 12-mm cell at a protein concentration of 12.8 mg per ml. The centrifugation was done in a Beckman model E ultracentrifuge at a temperature of 6.5°. The partial specific volume of the isomerase was assumed to be 0.75 and the molecular weight was determined to be 56,000 ± 3,000. The molecular weights of crystalline papain, muscle aldolase, bovine serum albumin, and purified bovine hemoglobin were found to be 30,000, 176,000, and 59,000, respectively, using the same method. These values agree within 12% with those reported for these proteins.

**Table II**

Equilibrium of methylmalonyl isomerase reaction

The reaction mixture contained in micromoles: Tris-HCl, pH 7.4, 20; DBC, 0.02; and succinyl-CoA, 1.35 in a volume of 1.52 ml. At 0 time, 0.19 ml was removed before addition of isomerase and added to 0.05 ml of 2 N HClO$_4$ plus 0.14 unit (0.01 ml) of isomerase free of racemase. To the remaining 1.33 ml, 0.98 unit of isomerase (0.07 ml) was added to start the reaction. The temperature was 25°. At 1, 3, 6, 9, and 12 minutes, 0.2 ml was removed and added to 0.05 ml of 2 N HClO$_4$, at 0°. Tris buffer (0.1 ml, 0.5 M), pH 7.4, and KOH (0.04 ml, 2 N), was added and the potassium perchlorate was removed by centrifugation. The amount of methylmalonyl-CoA formed was determined enzymatically using 0.05 ml of supernatant solution.

<table>
<thead>
<tr>
<th>Time</th>
<th>Methylmalonyl-CoA</th>
<th>Succinyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Experimental]</td>
<td>[By difference]</td>
</tr>
<tr>
<td>0</td>
<td>1.18</td>
<td>1.18</td>
</tr>
<tr>
<td>1</td>
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<td>1.14</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>9</td>
<td>0.066</td>
<td>1.114</td>
</tr>
<tr>
<td>12</td>
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<td>1.126</td>
</tr>
<tr>
<td>Average</td>
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</tr>
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</table>

* By difference refers to the difference between the original amount of succinyl-CoA added (determined enzymatically) and the amount of methylmalonyl-CoA determined at each time interval.

**Fig. 4.** Spectrum of two highly purified preparations of methylmalonyl isomerase (Table I), one pink with a specific activity of 9.7 (broken line) and the other colorless with a specific activity of 14.4 (solid line).

**Fig. 5.** Sedimentation and electrophoretic patterns of purified methylmalonyl isomerase. A, sedimentation patterns: the isomerase preparation with a specific activity of 9.7 (Table I, Step 6a) was further purified by sucrose gradient centrifugation. This protein (8.7 mg per ml) was dialyzed 16 hours against 0.05 M potassium phosphate buffer, pH 7.4. Sedimentation in the direction of the arrows in a Spinco model E ultracentrifuge at 60,000 r.p.m. The schlieren photographs were taken at 16 and 72 minutes. The temperature was set for 5°. The sedimentation coefficient was corrected for temperature as well as for viscosity of the buffer. $s_20,w = 7.0$. B, electrophoretic pattern: electrophoresis in a Spinco model H apparatus for 195 minutes at 2.5 volts per cm. The isomerase preparation was the same one as in A. Electrophoretic mobility = $14.6 \times 10^{-5}$ cm$^2$ per sec per volt.

**DISCUSSION**

Considerable data has now been collected for the characterization of methylmalonyl isomerase derived from mammalian and bacterial sources. Although both catalyze the identical reaction there are some intriguing differences between the enzymes from the two sources. Mazumder, Sasakawa, and Ochoa (14) have purified a preparation from sheep liver and have found that the holoenzyme is not readily separated into apoenzyme and coenzyme nor is the attached coenzyme inactivated by light. The
specific activity of their purified preparation (estimated to be 71% pure by ultracentrifuge analysis) was 5.5 without added cobamide and 6.43 with added coenzyme, an increase in activity of 17%. The two isomerase preparations described in this report and prepared from the same crude bacterial extract, one colorless (specific activity, 14.4) and the other pink (specific activity, 9.7), were inactive without added B12 coenzyme. The absorption spectra (240 to 600 m\(\mu\)) of these two preparations indicate that the pink color, when present, was attributable to light-inactivated cobamide and that the colorless preparation contained very little cobamide. Thus, it would appear that the bacterial apoenzyme is separated from the coenzyme to varying degrees and that the residual attached coenzyme has been inactivated by light. The coenzyme requirements for the mammalian and bacterial enzymes differ; the mammalian enzyme is active only with DBC and BC coenzymes while the bacterial enzyme is active with with DBC, BC, and AC coenzymes. The reasons for the differences between the two enzymes is not known.

It is postulated that the difference in specific activity between the pink and colorless enzyme preparations described in this report can be attributed to inactive coenzyme still attached to some of the apoenzyme molecules, which blocks the attachment of the added active coenzyme. This postulation is in accord with the calculated amount of inactive coenzyme present in the pink and colorless preparations. The molar extinction coefficient at 352 r\(\mu\) was estimated to be 2.1 \(\times\) 10\(^6\) cm\(^2\) per mole from pink and colorless preparations. The molar extinction coefficient of the added active coenzyme. This postulation is in accord with some of the apoenzyme molecules, which blocks the attachment of the active coenzyme. This data also confirms the molar ratio of the added active coenzyme. If the remaining 1.9% of inactive coenzyme were removed from the colorless preparation, it is calculated that the specific activity of the colorless preparation would be increased from 14.4 to 14.6. The specific activity of the pink preparation is 34% less than the calculated maximum specific activity of the colorless one. Thus, this difference in specific activity is comparable to the calculated percentage of molecules in the pink preparation still attached to inactive coenzyme (38%) and the difference in specific activity between the pink and colorless enzymes appears to be due to blockage of active sites by tightly bound light-inactivated coenzyme. This data also confirms the molar ratio of 1.0 for the cobamide coenzyme and the isomerase apoenzyme (14).

The highest specific activity of methylmalonyl isomerase which was previously reported was 7.3 by Overath et al. (13) and this preparation from propionibacteria moved as a homogeneous peak in the ultracentrifuge (\(s_{20,w} = 7.30\)). The highest specific activity reported by Mazumder, Sasakawa, and Ochoa (14) was 6.4 and this sheep liver preparation was considered 71% pure on the basis of an ultracentrifuge pattern (\(s_{20} = 6.21\)). The difference in specific activity between these preparations may be attributable to the source of the protein or to the different methods of assay, however, it also might be due to inactive coenzyme attached to the enzyme thus preventing the combination of active cobamide with the enzyme.

It is also of interest to note that the molecular weight of 56,000 as determined in this report is in agreement with the molecular weight (54,000) for the mammalian derived enzyme as determined by an indirect calculation based on coenzyme content and an assumption of a 1:1 ratio of apoenzyme and coenzyme (14). This would indicate that the mammalian and bacterial enzymes have comparable molecular weights, however, a preliminary estimation gave a value of 88,200 for the mammalian enzyme from the sedimentation constant (14).

Cobamide coenzymes are essential for the activity of at least three purified enzymes; methylmalonyl isomerase, glutamate isomerase (36), and diol dehydrase (37). The investigation of Toohey, Perlman, and Barker (38) with glutamate dehydrase from Clostridium tetanomorphum, and of Overath et al. (13) with methylmalonyl isomerase from propionibacteria indicate, that in contrast to the nucleoside portion, the nucleotide portion of the coenzyme can be modified extensively without complete loss of the activity as a coenzyme. In fact, Overath et al. (13) reported that cobinamide conjugate (Co-5'-deoxyadenosylcobinamide) which does not contain the nucleotide portion, was active with the enzyme from propionic acid bacteria and had a \(K_m\) of 1.7 \(\times\) 10\(^{-2}\). Similar incomplete coenzymes were not active in the glutamate isomerase reaction (36). We have had an opportunity to reexamine this problem and find that cobinamide conjugate is not active at 3.8 \(\times\) 10\(^{-5}\) M. The cause of the discrepancy is not known.

Each of the three enzymes known to require cobamide coenzymes is concerned with the shift of a hydrogen without incorporation of a proton from the medium. Lee and Abeles (39) working with diol dehydrase demonstrated that incubation of DBC with the purified dehydrase, in the absence of the substrate resulted in the inactivation of both the enzyme and coenzyme. This was attributed to the hydration of the coenzyme by the enzyme resulting in the formation of an inactive hydroxycofactor-enzyme complex. This reaction could be followed by the development of an absorbance peak at 362 m\(\mu\), which was attributed to the hydroxycofactor. On the basis of these changes, Abeles and Lee proposed a mechanism, for the enzymic conversion of diols to aldehydes, which requires a cleavage of the carbon to cobalt bond of the cobamide. The colorless methylmalonyl isomerase afforded a useful model to study shifts in cobamide absorption spectra, without the interfering absorption by inactive cobamide coenzyme on the enzyme. When equal quantities of the colorless methylmalonyl isomerase and DBC were combined, there was no development of a 362 peak nor was there a disappearance of the absorbance pattern of the active.
cobamide. This was true either in the presence or absence of methylenaloyl-CoA, and the enzyme was not inactivated. These differences may indicate that the role of cobamide coenzyme is not the same in the various types of reactions.

**SUMMARY**

Methylenaloyl isomerases from propionibacteria have been purified by column chromatography and ammonium sulfate fractionation. A colorless enzyme has been obtained with a specific activity of 14.4. A pink preparation with lower activity has also been obtained, and the lower activity is believed to result because part of the active sites were blocked by bound inactivated coenzyme. Comparison of the spectra of cobamide in the presence or absence of methylmalonyl-CoA are $3.45 \times 10^{-5}$ and $8 \times 10^{-5}$ M, respectively. No spectral shifts were noted during interaction of the enzyme with methylmalonyl-CoA, and the enzyme was not inactivated.

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