The conversion of propionyl coenzyme A to succinyl-CoA involves carboxylation to methylmalonyl-CoA (a), isomerization of methylmalonyl-CoA to its enantiomorph methylpropionyl-CoA (b), and isomerization of the latter to succinyl-CoA (3). Since methylmalonyl-CoA (a) has recently been shown to have the $\beta$ configuration (4, 5), the reversible reaction catalyzed by methylmalonyl-CoA racemase will henceforth be written as $\beta$-methylmalonyl-CoA $\Leftrightarrow$ $\alpha$-methylmalonyl-CoA and that catalyzed by methylmalonyl-CoA mutase as $\alpha$-methylmalonyl-CoA $\Leftrightarrow$ succinyl-CoA.

Methylmalonyl-CoA mutase is present in animal tissues and in propionic acid bacteria, and its activity is dependent on the presence of cobamide coenzyme which is firmly attached to the mutase of animal origin. A 5000-fold purification of the sheep liver holoenzyme was reported previously (3). However, this preparation was only about 70% pure, as judged by sedimentation in the ultracentrifuge, and the amounts then obtained were insufficient for study of the properties of the enzyme. An ultracentrifugally homogeneous preparation of the sheep liver holoenzyme has now been obtained, and the apoenzyme has been prepared by resolution with acid in the presence of ammonium sulfate (6). A report of the properties of the enzyme, including molecular weight, coenzyme content and kinetics, the equilibrium constant of the reaction, and the effect of inhibitors, is the subject of this paper. The apoenzyme has been found to be highly sensitive to $-$SH-binding reagents.

EXPERIMENTAL PROCEDURE

Methods

Enzyme Assay—The standard enzyme assay, the measurement of the rate of conversion of methylmalonyl-CoA to succinyl-CoA, as well as the definition of units and specific activity were as previously described (3). Frequently a modified assay was used in which, instead of generating methylmalonyl-CoA from propionyl-CoA directly in the assay samples, $\alpha$-methylmalonyl-CoA-3-14C was added as such. This was prepared as follows. A 5.0-ml reaction mixture containing (in micromoles unless otherwise stated) Tris-HCl buffer, pH 7.5, 250; MgCl2, 30; ATP, 9; phosphoenolpyruvate, 10; ECL, 500; Na214CO3 (0.16 $\mu$Ci per $\mu$ mole), 29; propionyl-CoA, 5.8; crystalline pyruvate kinase (EC 2.7.1.40), 0.1 mg, and propionyl-CoA carboxylase (EC 6.4.1.3), specific activity 3 to 4 (7), 24 units, was incubated for 30 min at 30°, and the reaction was stopped by heating for 25 min at 100°. $\alpha$-Methylmalonyl-CoA is converted by heating to the $\alpha$-racemic mixture (8). The precipitate of denatured protein was removed by centrifugation, and aliquots of the supernatant were used for the assay. The modified assay samples contained, per 0.5 ml, Tris-HCl buffer, pH 7.5, 50 $\mu$ moles; $\alpha$-methylmalonyl-CoA-3-14C, 0.15 $\mu$ mole, and mutase. The incubation was for 10 min at 30°. For assay of the apoenzyme, DDC coenzyme was added to the samples containing the apoenzyme and all other additions except substrate. After incubation for 5 min at 30°, the reaction was started by addition of $\alpha$-methylmalonyl-CoA.

For the modified assay either (a) the formation of succinyl-CoA was determined as in previous work by estimation of the permanganate-stable radioactivity (9), or (b) the disappearance of methylmalonyl-CoA was measured enzymatically as described below under "Determination of Methylmalonyl-CoA and Succinyl-CoA." Method b was used unless otherwise specified. Assay of the mutase in the direction succinyl-CoA $\rightarrow$ methylmalonyl-CoA was carried out by direct optical assay according to Wood et al. (10). The reactions are as follows.

Succinyl-CoA $\Leftrightarrow$ $\alpha$-methylmalonyl-CoA

mutase, EC 5.4.99.2

1 Methylmalonyl-CoA mutase is the trivial name recommended by the Enzyme Commission of the International Union of Biochemistry (1) for 2-methylmalonyl-CoA CoA-carbonyl mutase (EC 5.4.99.2). For the preceding paper of this series see Kaziro, Grossman, and Ochoa (2).

The abbreviations used are: DBC, dimethylbenzimidazolyl-cobamide; HMB, $p$-hydroxymercuroibenzoate.

3249
HCl buffer, pH 7.5, 50; sodium pyruvate (brought to pH 6.5 to 7.0), 10; GSH, 10; ammonium sulfate, 30; DPNH, 0.32; methylmalonyl-CoA transcarboxylase, 7.7 μg; bacterial (Propionibacterium shermanii), methylmalonyl-CoA racemase, 4.3 μg; crystalline malate dehydrogenase, 23 μg; and succinyl-CoA, 0.02 to 0.9. After a zero time reading of the absorbance at 340 μm, the reaction was started by the addition of mutase, 2.8 μg, and followed at 15 sec intervals for a few minutes.

**Determination of Methylmalonyl-CoA and Succinyl-CoA**—For determination of D,L-methylmalonyl-CoA (Reactions 2 through 4 above) the samples contained, per ml, (in micromoles unless otherwise specified), Tris-HCl buffer, pH 7.5, 50; sodium pyruvate, 10, GSH, 10; ammonium sulfate, 30; DPNH, 0.32; methylmalonyl-CoA transcarboxylase, 7.7 μg; methylmalonyl-CoA racemase, 4.3 μg; malate dehydrogenase, 23 μg; and sample with 0.02 to 0.1 μmole of total methylmalonyl-CoA. The reaction in 1.0 ml of Corex cells (l = 1.0 cm) was started by addition of transcarboxylase and readings were taken at λ = 340 μm in the Beckman DU spectrophotometer until the oxidation of DPNH had ceased.

In an experiment with D,L-methylmalonyl-3-14C the concentration of methylmalonyl-CoA and succinyl-CoA at equilibrium was determined by measuring the radioactivity of the free acids separated by electrophoresis following deacylation of the thioesters. Aliquots of the reaction mixture, 0.1 ml, were treated with 0.03 ml of 1.0 M KOH, and 2 μmoles each of methylmalonic and succinic acid were added as carrier. The mixture was kept at 30° for 15 min and then heated for 30 sec at 100°. After adding 0.01 ml of 4.0 N formic acid, the sample was spotted on Whatman No. 3MM filter paper and subjected to electrophoresis (31 volts per cm) in pyridine, acetic acid, water (1:10:89, v/v)., pH 3.5, for 3 hours. The acid spots were located by spraying with 0.04% bromoresol green in 95% ethanol (adjusted to pH 8.0), and the radioactivity was detected with use of a strip, gas flow counter (Actigraph, Nuclear-Chicago) equipped with an automatic recorder. The spots were eluted with water, and the radioactivity was measured on aliquots of the eluates with a window gas flow counter. When succinyl-CoA-1,4-14C was processed in this manner, it gave rise to two radioactive spots. The major one migrated in the position of authentic succinic acid (9 cm from the origin). The minor spot, migrating about 2 cm from the origin, has not been identified. Authentic D,L-methylmalonyl-CoA-3-14C gave by this procedure only one spot that moved in the position of methylmalonic acid (30 cm from the origin). Experimental samples yielded, besides the spots corresponding to free methylmalonic and succinic acids, the above additional spot. For this reason, the aggregate radioactivity from the two spots given by succinyl-CoA was used in calculating the amount of this compound present experimentally. Under these conditions the recovery of methylmalonyl-CoA and succinyl-CoA was 78.5 and 93%, respectively. The experimental values were corrected for these recoveries.

**Determination of Molecular Weight**—The molecular weights were determined by sedimentation equilibrium in short columns of solution as described by Yphantis (11) for the schlieren optical system. In later work longer columns were used, and calculations were made by the method of La Bar and Baldwin (12) from Raleigh interference patterns. The partial specific volume of the holoenzyme, assumed to be equal to the apparent specific volume, was calculated from the density of solutions measured in a density gradient column by the method of Lindstrom-

Lang and Lanz (13). Kerocene and o-dichlorobenzene were used in making the gradients. The protein concentration was based on refractometric determination in a synthetic boundary cell with 1.862 × 10⁻⁴ as the specific refractive index increment per mg per ml. This value is the average for several proteins determined by Perimann and Longsworth (14) corrected to a wave length of 546 μm and 20°. The partial specific volume of the apoenzyme was assumed to be the same as that of the holoenzyme.

**Preparations**

Methylmalonyl-CoA—D,L-Methylmalonyl-CoA-3-14C was prepared enzymatically as described under “Enzyme Assay.” For the kinetics and equilibrium experiments the methylmalonyl-CoA was purified chromatographically as described by Kazior and Ochoa (15). This material was used for the preparation of L-methylmalonyl-CoA-3-14C through removal of the D enantiomorph by conversion to propionyl-CoA. This is accomplished by the reversal of the propionyl-CoA carboxylase reaction in the presence of hexokinase (EC 2.7.1.1) and glucose (Reactions 5 through 7).

\[
\text{ADP} + P_i + \text{D,L-methylmalonyl-CoA-3-14C} \rightarrow \text{Mg}^{2+} \rightarrow \text{ATP} + H^+ \text{CO}_3 + \text{propionyl-CoA} + L-\text{methylmalonyl-CoA-3-14C}
\]

Glucose + ATP → glucose 6-phosphate + ADP

\[
\text{Sum: } P_i + \text{D,L-methylmalonyl-CoA-3-14C} + \text{glucose} \rightarrow H^+ \text{CO}_3 + \text{propionyl-CoA} + \text{L-methylmalonyl-CoA-3-14C} + \text{glucose 6-phosphate}
\]

The reaction mixture contained (in micromoles unless otherwise stated): Tris-HCl buffer, pH 7.5, 100; potassium phosphate buffer, pH 7.5, 125; ADP, 30; MgCl₂, 80; glucose, 120; chromatographically purified D,L-methylmalonyl-CoA-3-14C, 3.9; crystalline hexokinase, 0.04 mg; and three times crystallized pig heart propionyl-CoA carboxylase (specific activity, 10 (7)), 1.9 mg; final volume, 1.6 ml. After incubation for 10 min at 30°, the reaction was stopped by chilling to 0°. Aliquots of this mixture were used immediately to avoid nonenzymatic racemization of the L-methylmalonyl-CoA. It was found that reversal of the propionyl-CoA carboxylase reaction removed 57%, rather than 50%, of the methylmalonyl-CoA originally present. This indicates that there was some spontaneous racemization during the incubation.

**Holoenzyme**—Ultraspeed centrifugally homogeneous holoenzyme (290, 77, 78) was obtained from sheep liver by a slight modification of the procedure previously described (3). Based on the refractometric determination of protein, 90 Kg of mince, worked up over a period of 8 months, yielded 24 mg of enzyme of specific activity 7.2 (micromoles per min per mg at 30°). Since the specific activity of the initial extract was of the order of 0.001, the over-all purification was about 7000-fold. Based on the spectrophotometric determination of protein (9) the yield of enzyme was 32 mg. However, because of the high absorption of the cobamide coenzyme at 260 μm, the spectrophotometric method gives erroneously high protein values for highly purified preparations of the holoenzyme. For this reason, concentrations of the purified enzyme will be given in terms of refractometric
protein values. The factor for conversion of spectrophotometric to refractometric values, as determined with homogeneous holoenzyme, is 0.73.

In what follows only the main deviations from the previously published purification procedure (3) will be indicated. At the end of each step, from Step 5 on, the enzyme was always concentrated by precipitation with solid ammonium sulfate (80 to 85% saturation) and solution in a small volume of potassium phosphate buffer, and kept frozen, if required, until the next step was carried out. The protein values given in connection with the purification procedure are spectrophotometric values.

Steps 1 through 4 were the same as in previous work (3). However, the average specific activity of the Step 4 enzyme was around 0.03, or about one-fourth of the previous value (3), probably because of long frozen storage periods (up to 6 months) of Step 4 fractions, and other fractions at earlier steps of purification, necessitated by the very large scale at which the purification was undertaken.

**Step 5. Adsorption on Calcium Phosphate Gel and Elution—** The dialyzed enzyme from Step 4 was diluted to 10 mg of protein per ml, and the pH was 7.3 rather than 6.5. The gel was added in successive amounts of 43, 20, 41, and 81 mg of Ca(PO₄)₂ per 100 mg of protein in the original solution, and the enzyme was eluted from the third and fourth gels.

**Step 6. Chromatography on Triethylaminoethyl Cellulose—** Triethylaminoethyl cellulose was substituted for DEAE-cellulose. The resin was successively washed with 1.0 N NaOH, water, 1.0 N HCl, water, and 0.5 M, 0.1 M, and 0.01 M potassium phosphate buffer, pH 7.3, and then packed into a column (4.5 \( \times \) 30 cm) without applying pressure. The enzyme solution from Step 5 was dialyzed against 0.01 M potassium phosphate buffer, pH 7.3, and adjusted to a protein concentration of 30 mg per ml. The enzyme was eluted in a way similar to that described previously.

**Step 7. Chromatography on Hydroxylapatite—** The enzyme solution from Step 6 (about 30 mg of protein per ml) was dialyzed for 22 hours against 4 liters of 0.01 M potassium phosphate buffer, pH 7.3, with a change of buffer after 12 hours. A hydroxylapatite column (3 \( \times \) 30 cm) was used. Elution was carried out in a similar way as previously described. Chromatography disclosed two distinct, well separated colored bands, a pink band corresponding to the mutase. The former was eluted with 0.1 to 0.15 M \( \text{KHPO}_4 \) with 0.2 ml of 0.2 M Tris-HCl buffer, pH 7.3, followed by precipitation with ammonium sulfate and centrifugation. This washing was repeated once more, and the precipitate was finally dissolved in 0.5 ml of 0.2 M potassium phosphate buffer, pH 7.3. A typical preparation was as follows (Table I). Holoenzyme, 0.2 ml, with 27.8 mg of protein per ml was diluted with 1.8 ml of 0.01 M Tris-HCl buffer, pH 7.5. To the diluted solution were added 2.2 ml of saturated ammonium sulfate, with vigorous stirring, followed by 0.5 ml of 0.004 M HCl. The pH was then adjusted to 3.5 (measured with the glass electrode) with a 1.0 N HCl. The mixture was immediately centrifuged in the Servall centrifuge at 6000 rpm for 7 min. The protein precipitate was taken up at once in 2.0 ml of 75% saturated ammonium sulfate (adjusted with NH₄OH to pH 9.4), thoroughly dispersed with a glass rod, and centrifuged as above. This washing was repeated once more, and the precipitate was finally dissolved in 0.5 ml of 0.5 M potassium phosphate buffer, pH 7.5. A small amount of insoluble material was removed by centrifugation. Ultra-centrifugation of the resolved enzyme showed two components, a fast (at 85% saturation) and a slow (at 41%) sedimenting component. The fast component could be removed by chromatography on hydroxylapatite, as in Step 7 of the holoenzyme purification procedure, with buffers containing 0.01 M GSH to prevent inactivation of the apoenzyme. Elution with 0.18 M potassium phosphate buffer, pH 7.3, containing 0.01 M GSH, followed by precipitation with ammonium sulfate and solution in 0.2 ml of 0.2 M potassium phosphate buffer-0.01 M GSH, pH 7.3, yielded an ultracentrifugally homogeneous preparation of the apoenzyme (at 85%, 7.8 S). Since the specific activity of the apoenzyme (assayed in the presence of an excess of cobamide coenzyme) rose by 32%, following chromatography (Table I), the eliminated fast component probably was denatured enzyme.

As previously noted (3), all holoenzyme preparations contain a small amount of apoenzyme as judged by a slight increase in specific activity when assayed in the presence of added cobamide coenzyme. From the specific activity columns of Table I the holoenzyme preparation consisted of 88% holoenzyme and 12% apoenzyme. After resolution the holoenzyme dropped to 25% and the apoenzyme rose to 75%, i.e. the enzyme was resolved to an extent of 85%.

A higher degree of resolution can be obtained by decreasing the pH to slightly below 3.5 during the acid treatment of the holoenzyme, but this significantly
decreases the yield of apoenzyme. It should be pointed out that the holoenzyme used for the apoenzyme preparation of Table I had been stored for about 10 months. Loss of activity during storage may be responsible for the drop in specific activity from 7.2, the value obtained for the freshly prepared holoenzyme, to 5.05.

Other Preparations—Propionyl-CoA and succinyl-CoA were prepared by the method of Simon and Shemin (16). Racemase-free P. shermanii methylmalonyl-CoA transcarboxylase (specific activity, 7 (17)) and P. shermanii methylmalonyl-CoA racemase (specific activity, 33 (18)) were kindly provided by Dr. H. G. Wood, Western Reserve University. DBC coenzyme was a gift from Dr. Karl Folkers, Merek Institute for Therapeutic Research, Rahway, New Jersey. Crystalline hexokinase, pyruvate kinase (type II), and phosphoenolpyruvate (sodium salt) were from the Sigma Chemical Company; crystalline malate dehydrogenase and GSH from C. F. Boehringer and Sons, Mannheim, Germany. Pronase (a proteolytic enzyme from Streptomyces griseus) grade B was purchased through Calbiochem. Triethyl aminoethyl cellulose (Serva) was purchased through Gallard-Schlessinger Manufacturing Corporation, Garden City, New York. Hydroxylapatite was prepared as described by Tiselius, HjerMn, and Levine (19). We are indebted to Mr. H. Lozina for this preparation. Other preparations including sodium pyruvate and N-ethylmaleimide (Calbiochem), H. Lozina for this preparation. Other preparations including sodium pyruvate and N-ethylmaleimide (Calbiochem), DPNH (Sigma), CoA (Fabst and Cutoio-Calosi, S.P.A., Naples, Italy), p-hydroxymercuribenzoate (Sigma), crystalline bovine serum albumin and iodononicamide (Nutritional Biochemicals) were obtained commercially.

RESULTS

Properties of Holoenzyme

It was earlier reported (6) that the activity of crude preparations of methylmalonyl-CoA mutase of sheep kidney cortex is not affected by treatment with charcoal or illumination. Thus, not only is the cobamide coenzyme firmly bound to the mammalian mutase protein, but this binding protects it from photolytic cleavage. These results were confirmed with the homogeneous holoenzyme from sheep liver. Treatment of a solution (0.6 mg of protein per ml in 0.2 M potassium phosphate buffer, pH 7.3) with 1.0 mg of Nuchar C per mg of protein for 5 min at 0°, or illumination of a more dilute solution (0.25 mg of protein per ml) with two 100-watt tungsten lamps at a distance of 12 cm for 1 hour at 0° did not change the specific activity of the holoenzyme.

Sedimentation, Molecular Weight, and Coenzyme Content—The sedimentation pattern of the holoenzyme in 0.175 M potassium phosphate buffer, pH 7.3, is shown in Fig. 1A. The sedimentation coefficient $s_{20,w} = 7.78$. A value of 7.68 was found in another preparation in 0.5 M phosphate buffer. The molecular weight averaged from two short column equilibrium runs was found to be 165,000 ± 3,000.

The coenzyme content was determined spectrophotometrically as done previously (3). Based on the refractometric determination of protein and a content of 88% holoenzyme and 12% apoenzyme, the holoenzyme was found to contain approximately 1 mole of cobamide coenzyme per 75,000 g of protein. This assumes that the coenzyme is dimethylbenimidazoylcobamide coenzyme and that there is no change in absorbance due to binding by the protein. This value is much smaller than the one (1 mole per 54,000 g of protein) determined previously (3), but since the present value was obtained with a higher concentration of holoenzyme, it is probably more accurate than the earlier one.

pH Optimum—The pH optimum of the holomutase was at 7.0, in good agreement with earlier results with crude enzyme (20). The activity dropped sharply below pH 7.0, less so between pH 7.0 and 8.6, and again sharply above pH 8.6 (Fig. 2).

Michaelis Constants—The effect of the concentration of substrate on reaction velocity is shown in Fig. 3A for methylmalonyl-CoA and Fig. 3B for succinyl-CoA. Since the inactive $\beta$ enantiomer might be inhibitory, separate experiments were run with $\beta$- and $\alpha$-methylmalonyl-CoA. The latter was prepared from the racemate as described under "Preparations." It may be seen from Fig. 3A that the $\beta$ isomer was indeed inhibitory. Lineweaver-Burk (21) plots of the data for $\beta$-methylmalonyl-CoA and succinyl-CoA gave apparent $K_m$ values of 2.4 × 10$^{-4}$ M and 6.2 × 10$^{-4}$ M, respectively.

Equilibrium—The attainment of equilibrium of the methylmalonyl-CoA mutase reaction starting from either side, i.e. from methylmalonyl-CoA or succinyl-CoA, is shown in Fig. 4. The substrate for Curve A was $\alpha$-methylmalonyl-CoA, and the reaction was allowed to proceed in the presence of excess methylmalonyl-CoA racemase. The substrate for Curve B was succinyl-CoA, and no racemase was present. The concentration of total methylmalonyl-CoA determined enzymatically at various times, is expressed as the percentage of the initial concentration of methylmalonyl-CoA (Curve A) or succinyl-CoA (Curve B). As may be seen, the relative proportion of reactants at equilibrium was 10% (BL) methylmalonyl-CoA and 90% succinyl-CoA.
for Curve A (racemase present) and 5% (L) methylmalonyl-CoA and 95% succinyl-CoA for Curve B (racemase absent). The fact that the proportion of methylmalonyl-CoA was twice as high in the experiment with as in that without racemase is in agreement with an equilibrium constant of 1 for the racemase reaction (18). From Curve B the equilibrium constant of the mutase reaction is 85:5 = 19. Values for the apparent equilibrium constant, \( K' = \frac{\text{succinyl-CoA}}{(\text{L-methylmalonyl-CoA})} \), from data of the experiments of Fig. 4 and one experiment with \( \text{D}-\text{methylmalonyl-CoA}-3-\text{C} \) with separate determination of methylmalonyl-CoA and succinyl-CoA, are given in Table II. The average value was 18.6. Kellermeyer et al. (22) reported \( K' = 23.1 \) with homogeneous apomutase of \( P. \ shermanii \); for \( K' = 19.0 \) and \( t = 30^\circ \), \( \Delta F^\circ_{30^\circ} = -1760 \) cal.

**Properties of Apoenzyme**

Resolution of the enzyme did not result in release of the coenzyme from the protein; the ammonium sulfate supernatant solution after resolution was colorless whereas the precipitate and solutions thereof had a strong pink color. The absorption spectrum of apomutase solutions was similar to that of the pink \( P. \ shermanii \) apomutase preparations (22) and photoinactivated cobamide coenzymes (23) with peaks at 354, 405, 505, and 535 mp. A colorless bacterial apomutase obtained by Kellermeyer et al. (22) had higher specific activity than their pink preparation, a fact interpreted by the authors as due to blocking of coenzyme attachment sites by inactive coenzyme. There would seem to be no such blocking in the case of the sheep liver apomutase, for its specific activity was the same as that of the original holoenzyme when assayed with excess DBC coenzyme. Furthermore, incubation of apomutase and DBC coenzyme with or without a 20-fold excess of photoinactivated coenzyme showed only moderate inhibition (24%) by the latter. Cyanocobalaminamenta

**Fig. 2.** Activity as a function of pH. Modified assay with \( \text{D}-\text{methylmalonyl-CoA-3-C} \) and determination of permanganate-stable radioactivity. Samples contained, per ml, buffer, 50 \( \mu \)moles; \( \text{D}-\text{methylmalonyl-CoA}, 0.108 \) \( \mu \)mole; and enzyme, 0.43 \( \mu \)g. The pH was determined with the glass electrode before adding the enzyme. ●, citrate; ○, Tris-HCl; ◊, phosphate; ▲, glycine-NaOH.

**Fig. 3.** Reaction velocity as a function of the concentration of substrate. A, substrate, methylmalonyl-CoA (chromatographically purified). The samples contained, per 0.5 ml, Tris-HCl buffer, pH 7.5, 25 \( \mu \)moles; methylmalonyl-CoA as indicated; and enzyme, 0.42 \( \mu \)g. Incubation, 10 min at 30\(^\circ\). Determination of permanganate-stable radioactivity. Upper curve, \( \text{L}-\text{methylmalonyl-CoA}; \) lower curve, \( \text{D}-\text{methylmalonyl-CoA}. \) B, substrate, succinyl-CoA. Optical assay (pH, 7.5; enzyme, 2.8 \( \mu \)g) as described under "Methods." Temperature, about 25\(^\circ\).
and cobamide coenzyme photolytic products can form complexes with a number of proteins.

**Stability**—Contrary to the holoenzyme, the apomutase was very unstable. Whereas the apoenzyme solution obtained by dissolving the acid ammonium sulfate precipitate in a small amount of buffer could be kept at -20°C for several weeks without appreciable loss of activity, dialysis at 5°C overnight against dilute (0.01 M) phosphate buffer, pH 7.5, or gel filtration through a column of Sephadex G-100 led to 80 to 90% loss of activity. When similarly dialyzed, but in the presence of 0.01 M GSH, the enzyme was fully protected. However, once it occurred, inactivation appeared to be irreversible, for activity could not be restored by addition of GSH. Moreover, even in the presence of GSH, dialyzed solutions lose activity gradually (about 80% in a month) on storage at -20°C. Incubation of solutions of apomutase in dilute buffer (5 to 20 min at 30°C, 60 min at 0°C) led to 50 to 80% loss of activity which was largely prevented by the presence of either GSH or DBC coenzyme.

The reconstituted holoenzyme, i.e. the active enzyme formed on incubation of apomutase with an excess of DBC coenzyme, showed considerable resistance to illumination (Table III) approaching that of the native holoenzyme. These results, somewhat at variance with previous observations with crude sheep kidney apomutase (6), suggest that the native type of binding between apoenzyme and coenzyme is largely restored on reconstitution of the holoenzyme.

**Sedimentation and Molecular Weight**—The sedimentation pattern of the apomutase purified by chromatography on hydroxylapatite (Table I, last line) is shown in Fig. 1B. Its sedimentation coefficient, $s_{20,w} = 7.85$, was close to that of the holoenzyme (7.75). The molecular weight was found to be 144,000. This value, however, is based on an initial concentration determined spectrophotometrically and calculated on the refractometric basis because of failure of the synthetic boundary

<table>
<thead>
<tr>
<th>TABLE II</th>
<th>Equilibrium constant</th>
</tr>
</thead>
</table>
| Experiments 1 and 3 correspond to the 5- and 4-min samples of Curves A and B, respectively, of Fig. 4. In Experiment 2, the reaction mixture contained, per 0.5 ml: Tris-HCl buffer, pH 7.5, 25 μmoles; Dl-methylmalonyl-CoA-3-14C (chromatographically purified, specific radioactivity, 85,000 cpm per pmole), 0.765 μmole; P. shermanii methylmalonyl-CoA racemase, 45 μg; and enzyme, 82 μg. Incubation was for 4 min at 30°C. In this experiment methylmalonyl-CoA-3-14C and succinyl-CoA-14C were determined on 0.1-ml aliquots following electrophoretic separation of the free acids as described under "Methods." In Experiments 1 and 2 (with added racemase) L-methylmalonyl-CoA is taken to be one-half of the total for Dl-methylmalonyl-CoA.

| Experiment | Concentration of reagents | Initial | Final | $K$
<table>
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<tr>
<td></td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>1</td>
<td>1.242</td>
<td>0.621</td>
<td>0</td>
<td>0.122</td>
</tr>
<tr>
<td>2</td>
<td>1.330</td>
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</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2.85</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Difference between initial and final Dl-methylmalonyl-CoA.

<sup>b</sup> Difference between initial succinyl-CoA and final L-methylmalonyl-CoA.

and adenosine at much higher concentration produced little or no inhibition.

When resolution of the enzyme was carried out in dim light, to prevent or decrease photoinactivation of the coenzyme, the pink apoenzyme had the usual low activity in the absence of added coenzyme and was activated by this addition to the usual extent. The absorption spectrum of this apoenzyme preparation was not determined as only very small amounts were available. Thus we cannot definitely rule out the occurrence of coenzyme cleavage, through photolysis or otherwise, when the holoenzyme was resolved in dim light. In any case, attempts to detect the presence of biologically active coenzyme after resolution, by preparing boiled extracts of the pink apomutase and testing them with active apomutase, gave negative results irrespective of whether resolution had been carried out under usual illumination conditions or in dim light. Digestion in the dark with Pronase (0.15 mg per mg of mutase), suggested that the native type of binding between apoenzyme and coenzyme is largely restored on reconstitution of the holoenzyme.

**Sedimentation and Molecular Weight**—The sedimentation pattern of the apomutase purified by chromatography on hydroxylapatite (Table I, last line) is shown in Fig. 1B. Its sedimentation coefficient, $s_{20,w} = 7.85$, was close to that of the holoenzyme (7.75). The molecular weight was found to be 144,000. This value, however, is based on an initial concentration determined spectrophotometrically and calculated on the refractometric basis because of failure of the synthetic boundary

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Stability of reconstituted holoenzyme to illumination</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td>0.21</td>
</tr>
<tr>
<td>B. Incubation with coenzyme followed by dark period</td>
<td>4.15</td>
</tr>
<tr>
<td>C. Incubation with coenzyme followed by illumination period</td>
<td>3.05</td>
</tr>
<tr>
<td>D. Incubation with light-inactivated coenzyme</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Table IV

Effect of -SH-binding reagents on mutase

Enzyme preincubated at 30° with coenzyme or inhibitors or both in the order indicated, each time for 5 min, and the reaction then started by addition of methylmalonyl-CoA. Modified assay. The amount of holoenzyme per ml reaction mixture was 0.47, 0.58, and 1.1 μg, respectively, in the experiments with HMB, iodoacetamide, and N-ethylmaleimide. The amount of apo-enzyme per ml of reaction mixture was 0.94 μg throughout. DBC coenzyme, 1.38 × 10⁻⁴ M.

<table>
<thead>
<tr>
<th>Enzyme and additions</th>
<th>Relative activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holoenzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ HMB (10⁻⁴ M)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ Iodoacetamide (10⁻⁴ M)</td>
<td>59</td>
<td>41</td>
</tr>
<tr>
<td>+ N-Ethylmaleimide (10⁻⁴ M)</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>+ DBC coenzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ HMB (10⁻⁴ M) + DBC coenzyme</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>+ Iodoacetamide (10⁻⁴ M) + DBC coenzyme</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>+ DBC coenzyme + iodoacetamide</td>
<td>80</td>
<td>11</td>
</tr>
<tr>
<td>+ N-Ethylmaleimide (10⁻⁴ M) + DBC coenzyme</td>
<td>41</td>
<td>59</td>
</tr>
<tr>
<td>+ DBC coenzyme + N-ethylmaleimide (10⁻⁴ M)</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

The facts that (a) inhibition by HMB is reversed by GSH and (b) the mutase-bound coenzyme is resistant to illumination, made it possible to ascertain whether blocking of -SH groups by HMB interferes with binding of the coenzyme. The experiments of Table V (cf. especially Samples C and D) showed that cell run. Sufficient material was not available to repeat the determination. The sedimentation coefficient of the irreversibly inactivated apomutase (7.4 S) was essentially the same as that of the active apoenzyme.

Sensitivity to -SH-binding Reagents—Whereas the holoenzyme was moderately sensitive to -SH-binding reagents, the apo-enzyme was very sensitive when incubated with these compounds before adding the coenzyme. Prior addition of the coenzyme had a marked protective effect. Typical results are shown in Table IV. Moreover, it is apparent from Fig. 5 that reaction of the holoenzyme with N-ethylmaleimide was very sluggish whereas that of the apoenzyme was fairly rapid. The activity of apomutase inhibited by HMB was extensively restored by subsequent incubation with excess GSH. These observations suggest that sheep liver methylmalonyl-CoA mutase possesses essential -SH groups which are masked or protected in the holoenzyme but become exposed upon resolution. Exposure of such groups may also be responsible for the intrinsic instability of the apomutase for, as previously pointed out, the presence of either GSH or DBC coenzyme protected the apomutase from spontaneous inactivation.

Fig. 5. Kinetics of inhibition of holo- and apomutase by N-ethylmaleimide. Enzymes, dissolved in 0.05 M Tris-HCl buffer, pH 7.5, at a final concentration of 40 μg per ml (holoenzyme) and 3.14 μg per ml (apoenzyme), were incubated at 0° with 10⁻⁴ M N-ethylmaleimide. At various times, 0.02-ml samples were withdrawn and the activity was measured by the modified assay with addition of DBC coenzyme (1.38 × 10⁻⁴ M) for the apoenzyme assay. Controls without N-ethylmaleimide were run simultaneously. Curve 1 (○—○), holoenzyme alone; Curve 2 (●—●), apoenzyme alone; Curve 3 (●—●), holoenzyme and N-ethylmaleimide; Curve 4 (■—■), apoenzyme and N-ethylmaleimide; Curve 5 (□—□), activity of apoenzyme in the absence of added dimethylbenzimidazolylcobamide coenzyme.

Table V

Reconstitution of holoenzyme in the presence of -SH-binding reagents

A, apoenzyme assayed in the absence (Assay a) or presence (Assay b) of added DBC coenzyme (1.38 × 10⁻⁴ M). B, apoenzyme assayed as in A, following incubation with HMB (final concentration, 2 × 10⁻⁴ M) for 3 min at 30°. C, apoenzyme (60 μg per ml) incubated with HMB (final concentration, 2 × 10⁻⁴ M) for 3 min at 30°, the mixture was then incubated with GSH (final concentration, 8 × 10⁻⁴ M) for 5 min at 30°. DBC coenzyme (final concentration, 1.6 × 10⁻⁴ M) was then added, and the mixture was incubated for 10 min at 30° in the dark followed by illumination (in a Beckman cell, l = 1.0 cm, 100-watt tungsten lamp at a distance of 10 cm) for 60 min at 0°. Activity was then assayed without further addition of DBC coenzyme (Assay a); D, as C, except that the order of incubations was HMB, DBC coenzyme, illumination, and GSH. E, as C, but without HMB treatment (Assays a and b). Activity measured in 0.02-ml portions of reaction mixture (1.2 μg of mutase) by the modified assay. Experiment 1, in 0.05 M Tris-HCl buffer, pH 7.5; Experiment 2, in 0.1 M potassium phosphate buffer, pH 6.8.
this was not the case for the activity of HMB-blocked apomutase was restored by incubation with DBC coenzyme followed by photoactivation of unbound coenzyme and removal of the blocking HMB with GSH (Table V, Samples D).

**DISCUSSION**

The coenzyme content of the holoenzyme, about 1 mole per 75,000 g of protein, with a measured molecular weight of approximately 165,000 suggests that mammalian methylmalonyl-CoA holomutase may be a polymer of two subunits of molecular weight around 80,000 each containing 1 molecule of cobamide coenzyme. The sedimentation coefficient of the apoenzyme is close to that of the holoenzyme, and our single molecular weight determination is only slightly lower indicating that resolution does not lead to dissociation of the protein.

The experiments reported in the preceding section suggest that resolution of the enzyme leads to the exposure of essential —SH groups. These —SH groups may participate (a) in the methylmalonyl-CoA = succinyl-CoA isomerization reaction itself or (b) in binding of the coenzyme. The fact that the methylmalonyl-CoA apomutase of *P. shermanii* has low sensitivity to —SH groups, explains high sensitivity of the apomutase to, inactivation by —SH-binding reagents (RX in diagram). Resolution, with ensuing detachment of the coenzyme and exposure of the enzyme in mammalian methylmalonyl-CoA holoenzyme.

Involvement of —SH groups at one site (Site b) explains sluggish inactivation by —SH-binding reagents (RX in diagram). Resolution, with ensuing detachment of the coenzyme and exposure of the —SH groups, explains high sensitivity of the apomutase to, and rapid inactivation by, —SH reagents. Blocking of —SH at Site b still permits binding of the coenzyme at Site a, in a form resistant to photolysis, but reconstitution of active holoenzyme through binding at second binding site (Site b) cannot take place until the blocking agent is removed by a thiol. Site a might be a multiple rather than a single binding site.

**Fig. 6.** Hypothetical two-point attachment of cobamide coenzyme in mammalian methylmalonyl-CoA mutase holoenzyme. Involvement of —SH groups at one site (Site b) explains sluggish inactivation by —SH-binding reagents (RX in diagram). Resolution, with ensuing detachment of the coenzyme and exposure of the —SH groups, explains high sensitivity of the apomutase to, and rapid inactivation by, —SH reagents. Blocking of —SH at Site b still permits binding of the coenzyme at Site a, in a form resistant to photolysis, but reconstitution of active holoenzyme through binding at second binding site (Site b) cannot take place until the blocking agent is removed by a thiol. Site a might be a multiple rather than a single binding site.

### TABLE VI

**Comparison of properties of mammalian and bacterial mutase**

The *V*ₘₐₓ values for the mammalian holoenzyme were calculated from Lineweaver-Burk plots of the data of Fig. 3. *V*ₘₐₓ for L-methylmalonyl-CoA was 19.9 mmoles per min per 0.42 μg of enzyme (at pH 7.5 and 30°), and *V*ₘₐₓ for succinyl-CoA was 7.7 mmoles per min per 2.8 μg of enzyme (at pH 7.5 and 25°). These values were multiplied by the factor 100/88 = 1.14, to correct for 12% apoenzyme content, and the factor 100/90 = 1.11 to correct for 10% higher activity at pH 7.0, the optimal pH. The holoenzyme used for the experiments of Fig. 3 was the same used for the preparation of apoenzyme in Table I; there is therefore a further correction, for the decrease of specific activity on storage from the initial value of 7.2 to 0.18, through multiplication by the factor 7.2/0.18 = 4.3. The *V*ₘₐₓ for L-methylmalonyl-CoA values for the bacterial apoenzyme represent an approximate estimate from the Lineweaver-Burk plots (Fig. 3) of Kellermeyer et al. (22) with a correction (through multiplication by the factor 13/5 = 2.6) for inhibition by the n-methylmalonyl-CoA in their samples and correction from 25° to 30° by assuming a Q₁₀ = 2 for the reaction. The *V*ₘₐₓ for succinyl-CoA of 14.4 amoles per min per mg corresponds to the maximal specific activity of the bacterial enzyme in the standard assay of Kellermeyer et al.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mammalian</th>
<th>Apoenzyme</th>
<th>Bacterial apoenzyme³</th>
</tr>
</thead>
<tbody>
<tr>
<td>$s_{20, w}$</td>
<td>7.78</td>
<td>7.88</td>
<td>7.05</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>165,000</td>
<td>(144,000)</td>
<td>56,000</td>
</tr>
<tr>
<td>Cobamide coenzyme content (moles/mole)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>pH optimum</td>
<td>7.0</td>
<td>7.0</td>
<td>7.2</td>
</tr>
<tr>
<td>$V_{\max}$ (30°), L-methylmalonyl-CoA (μmoles/min/mg)</td>
<td>85.3</td>
<td>85.3</td>
<td>127.0</td>
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<tr>
<td>$V_{\max}$ (25°), succinyl-CoA (μmoles/min/mg)</td>
<td>5.0</td>
<td></td>
<td>14.4</td>
</tr>
<tr>
<td>$V_{\max}$ (30°), L-methylmalonyl-CoA (moles/min/mole)</td>
<td>14,000</td>
<td>(12,300)</td>
<td>7,100</td>
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<tr>
<td>$K_{m}$, L-methylmalonyl-CoA (μ)</td>
<td>825</td>
<td></td>
<td>810</td>
</tr>
<tr>
<td>$K_{m}$, succinyl-CoA (μ)</td>
<td>2.4 x 10⁻⁴</td>
<td>0.85 x 10⁻⁴</td>
<td>3.45 x 10⁻⁴</td>
</tr>
<tr>
<td>$K_{m}$, cobamide coenzyme (μ)</td>
<td>6.2 x 10⁻⁴</td>
<td>3.45 x 10⁻⁴</td>
<td>3.45 x 10⁻⁴</td>
</tr>
<tr>
<td>Sensitivity to —SH-binding reagents²</td>
<td>Low</td>
<td>Very high</td>
<td>Low</td>
</tr>
</tbody>
</table>

* Assay with excess DBC coenzyme added.
* Value for DBC coenzyme.
* Value for benzimidazolylcobamide coenzyme.
* In the absence of added DBC coenzyme.
from this paper; those for the bacterial enzyme from the recent paper of Kellermeyer et al. (22). The mammalian mutase is a slightly asymmetrical molecule as judged by the value of $f_\alpha$ of 1.3 that can be calculated from the molecular weight and sedimentation coefficient given in Table VI. The molecular weight of the bacterial enzyme is much lower. Since the sedimentation coefficient is inconsistently high for a protein of the reported molecular weight no further comparison seems warranted. It may be noted that both enzymes have similar "molecular activity" (1), although the specific activity of the bacterial mutase is higher than that of the animal enzyme. Moreover, the affinity of the \textit{P. shermanii} enzyme for the substrates, \textit{L}-methylmalonyl-CoA and succinyl-CoA, is somewhat higher than that of the enzyme from sheep liver. Since animals do not synthesize vitamin B\textsubscript{12}, it would seem to have been advantageous from an evolutionary point of view to develop a cobamide enzyme which binds its prosthetic group more tightly than the corresponding enzyme in microorganisms capable of synthesizing the vitamin, even at the expense of substantial loss of intrinsic catalytic activity.

\textbf{SUMMARY}

An ultracentrifugally homogeneous preparation of sheep liver methylmalonyl coenzyme A mutase holoenzyme was obtained representing a 7000-fold purification of the enzyme in the initial extract. The enzyme had a sedimentation coefficient of 7.7S, a molecular weight of 165,000, and contained 1 mole of cobamide coenzyme per 75,000 g or roughly 2 moles per mole of enzyme. Substrate affinity and other catalytic constants as well as the equilibrium constant of the reaction were determined. The average value of $K'$ ([succinyl-CoA]/[L-methylmalonyl-CoA]) was 18.6. \textit{D}-Methylmalonyl-CoA, which is not a substrate for the mutase, inhibited the isomerization of \textit{L}-methylmalonyl-CoA.

Ultracentrifugally homogeneous apoenzyme, prepared by acidification in the presence of ammonium sulfate followed by chromatography on hydroxylapatite, was similar in sedimentation coefficient and molecular weight to the native holoenzyme. The apoenzyme was essentially inactive, but full activity could be restored by the addition of DBC coenzyme. Resolution did not result in release of the coenzyme from the protein (which remained intensely pink), but no active coenzyme could be recovered from apoenzyme preparations by boiling or other procedures even if the resolution had been carried out in dim light to avoid or minimize coenzyme photolysis.

Whereas the holoenzyme was relatively stable, the apoenzyme was very unstable, particularly in solutions of low ionic strength; it was stabilized in the presence of glutathione or cobamide coenzyme. Likewise, the holoenzyme was relatively resistant whereas the apoenzyme was very sensitive to \textit{--SH}-binding reagents. The sensitivity of the reconstituted holoenzyme (apoenzyme + cobamide coenzyme) to \textit{--SH} inhibitors was similar to that of the native holoenzyme. These results suggest that resolution of the holoenzyme leads to exposure of essential \textit{--SH} groups. The possibility that these groups are involved in coenzyme binding is discussed.

\textbf{Acknowledgments}—We are indebted to Mr. Horace Lozina for help with the preparation of the enzyme and to Mr. Frank Zaboretsky for help with the ultracentrifuge runs.

\textbf{REFERENCES}

Metabolism of Propionic Acid in Animal Tissues: XII. PROPERTIES OF MAMMALIAN METHYLMALONYL COENZYM E A MUTASE
J. J. B. Cannata, Aldo Focesi, Jr., Rajarshi Mazumder, Robert C. Warner and Severo Ochoa


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