Purification and characterization of methylmalonyl-CoA epimerase from *Propionibacterium shermanii*

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Methylmalonyl-CoA epimerase, which specifically interconverts the (2*R*)- and (2*S*)-epimers of methylmalonyl-CoA, was purified 95-fold from *Propionibacterium shermanii* by a new method that affords apparently homogeneous enzyme, in 80–100 mg quantities, in yields representing about 40% of the activity in cell-free extracts. The specific activity of the purified enzyme, 10.1 μ kat/mg, is much greater than previously reported. Native methylmalonyl-CoA epimerase has M_r about 33 000, and apparently consists of two identical subunits. The purified enzyme is stable indefinitely when stored at -20°C and pH 8.5, but contrary to previous reports it is not unusually acid-stable. The activity of methylmalonyl-CoA epimerase is increased by Co^{2+} , and to a smaller extent by Ni^{2+} , Mn^{2+} and Zn^{2+} .

Methylmalonyl-CoA epimerase (classified as a racemase, EC 5.1.99.1) catalyses the interconversion of (2*R*)- and (2*S*)-methylmalonyl-CoA, an essential step in the interconversion of propionyl-CoA and succinyl-CoA (carboxypropionyl-CoA) both in mammalian tissues (Mazumder *et al.*, 1962) and in the propionate-producing bacteria (Overath *et al.*, 1962*a*). The enzyme has been purified from *Propionibacterium shermanii* (Allen *et al.*, 1963) as a heat- and acid-stable protein of M_r about 30 000, and from sheep liver (Mazumder *et al.*, 1962). Methylmalonyl-CoA epimerase catalyses extensive exchange of the C-2 hydrogen atoms in methylmalonyl-CoA with protons in the medium (Overath *et al.*, 1962*a*; Mazumder *et al.*, 1962; Allen *et al.*, 1963), so mechanistic proposals for the epimerization that involve proton transfers are obviously attractive (see, e.g., Overath *et al.*, 1962*a*).

The present paper describes the purification and detailed characterization of methylmalonyl-CoA epimerase from *P. shermanii* for use in studies of the mechanism of proton transfer. By using a simplified spectrophotometric assay, an efficient purification procedure was developed that leads to apparently homogeneous enzyme, with a specific activity much greater than previously obtained.

Materials and methods

Materials

Deionized distilled water and A.R.-grade reagents were used except where otherwise stated.

(*RS*)-Methylmalonyl-CoA, malate dehydrogenase (pigeon muscle), lysozyme (egg-white), myoglobin (horse), ovalbumin and carbonic anhydrase (bovine erythrocytes) were obtained from Sigma Chemical Co. Poly(ethylene glycol) 6000 and polyamide t.l.c. sheets were from BDH Chemicals. Sephadex G-75 and Blue Dextran were bought from Pharmacia, and Bio-Gel HT and Bio-Gel A-1.5m from Bio-Rad Laboratories. Guanidinium chloride and urea (both from BDH) were twice recrystallized from ethanol, and iodoacetic acid (BDH) was recrystallized from *n*-heptane. *N*-Ethylmorpholine (BDH) was redistilled before use.

Dimethyl suberimidate dihydrochloride was kindly provided by Dr. J. R. Coggins (University of Glasgow). Glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle) and a sample of dansyl-histidine were kindly given by Dr. R. N. Perham (University of Cambridge). (*RS*)-Methylmalonyl-CoA was also synthesized by the method of Overath *et al.* (1962*b*).

Enzyme assays

Transcarboxylase activity was assayed by the method of Wood *et al.* (1969). Methylmalonyl-CoA epimerase activity was assayed by a modification of the method of Allen *et al.* (1963).

Assays were performed at 30°C in reduced-volume 1 cm-path-length cells. Reaction mixtures contained, in a final volume of 1.185 ml; 5 mM-sodium pyruvate, 0.16 mM-NADH, 0.2 M-potassium phosphate buffer, pH 6.9, 0.08 mM-(*RS*)-methyl-

malonyl-CoA, 0.1 M-(NH₄)₂SO₄ and malate dehydrogenase (0.56 µg; 120 nkat/µg). Purified transcarboxylase (0.5 µg; 670 nkat/mg) was added, and the decrease in *A*₃₄₀ [due to consumption of (2*S*)-methylmalonyl-CoA] was observed. After 2–3 min, the rate of decrease in *A*₃₄₀ reaches a low constant value (approx. 0.005/min). The epimerase-catalysed reaction was then started by the addition of 10 µl of enzyme (1 µg/ml or less), and the rate of decrease of *A*₃₄₀ was monitored. The rate was usually linear for about 3 min, and proportional to the concentration of added epimerase under these conditions. Further additions of coupling enzymes confirmed that their turnover was not rate-limiting.

Growth of P. Shermanii

P. shermanii strain N.C.I.B. 9885 was obtained as a freeze-dried culture from the National Collection of Industrial Bacteria, Aberdeen, Scotland, U.K. It was maintained in stab culture on solid medium (Difco tomato agar) and grown at 30°C in a defined glucose/salts/yeast-extract medium (Brown *et al.*, 1973). An inoculum of 0.5 litre was added to 9 litres of medium. At intervals, the pH of the culture was raised to pH 6.5–7.0 (pH indicator paper) by careful addition of aq. NH₃ (sp.gr. 0.880). After 65 h, the bacteria were collected by centrifugation at 6000 *g* for 10 min at 4°C, and washed twice with distilled water. The yield (wet weight) was approx. 35–40 g/litre.

Purification of methylmalonyl-CoA epimerase

Except where otherwise stated, operations were performed at 4°C, and all buffers contained 0.1 mM-phenylmethanesulphonyl fluoride, unless otherwise stated.

Step 1: initial extraction. Bacterial cell paste (350 g) was suspended in 0.7 litre of 0.2 M-potassium phosphate buffer, pH 6.9, containing 0.2 mM-phenylmethanesulphonyl fluoride and 1 mM-EDTA. The cells were ruptured by continuous passage through a Manton-Gaulin laboratory homogenizer operated at 60 MPa (type 15M8; Manton-Gaulin Manufacturing Co., Everett, MA, U.S.A.) with intermittent cooling in a methoxyethanol/solid CO₂ mixture. The extract was then centrifuged at 35 000 *g* for 30 min.

Step 2: salt fractionation. Solid (NH₄)₂SO₄ (32.6 g/100 ml) was added to the red supernatant from step 1, to give 55% saturation, and the precipitate was removed by centrifugation at 25 000 *g* for 30 min. To the supernatant was added solid (NH₄)₂SO₄ (23.5 g/100 ml) to give 90% saturation, and the protein precipitate was collected by centrifugation as above. The pellets were resuspended and combined by using a total of 40 ml of ice-cold 50 mM-Tris/HCl buffer, pH 7.5, and the

suspension was dialysed against 2 litres of this buffer overnight, with several changes.

Step 3: streptomycin treatment. The protein solution was treated with 0.2 vol. of a 10% (w/v) solution of streptomycin sulphate in 50 mM-Tris/HCl buffer, pH 7.5. After 15 min, the precipitate was removed by centrifugation at 35 000 *g* for 15 min and the pellet was discarded. The dark-red supernatant was dialysed for 4 h, with at least one change, against 2 litres of 50 mM-Tris/HCl buffer, pH 7.5.

Step 4: fractionation with poly(ethylene glycol). The non-diffusible material from the previous step was gently stirred during the rapid addition of small amounts (approx. 0.04 vol.) of 50% poly(ethylene glycol). In a typical preparation, methylmalonyl-CoA epimerase activity was first lost from the supernatant after a total of 0.11 vol. of 50% poly(ethylene glycol) had been added. When the poly(ethylene glycol) concentration had been adjusted to the point where more than 95% of the epimerase remained in solution, the mixture was centrifuged at 35 000 *g* for 15 min and the pellet was discarded. Methylmalonyl-CoA epimerase was then precipitated by addition of more 50% poly(ethylene glycol), to give a final concentration of 15% (w/v) poly(ethylene glycol). The precipitate was collected at 35 000 *g* for 15 min and the pellet was resuspended in 100–250 ml of 10 mM-sodium phosphate buffer, pH 6.5, with gentle stirring for at least 1 h.

Step 5: hydroxyapatite chromatography. The enzyme solution was applied to a column (2.3 cm high × 4.5 cm diam.) of hydroxyapatite equilibrated with 10 mM-sodium phosphate buffer, pH 6.5, at a flow rate of 60 ml/h. After loading of the sample, the column was washed with 50 ml of buffer, followed by a linear gradient (1 litre) of 10–200 mM-sodium phosphate buffer, pH 6.5, at a flow rate of 100 ml/h (Fig. 1a). Fractions (10 ml) were collected and assayed for epimerase activity, protein concentration and electroconductivity. Fractions containing epimerase activity were pooled (approx. 450 ml) and the solution was concentrated by ultrafiltration.

Step 6: gel filtration on Sephadex G-75. The concentrated solution from the previous step (15 ml) was gel-filtered on a column (80 cm × 5 cm) of Sephadex G-75 in 50 mM-Tris/HCl buffer, containing 0.1 mM-EDTA. Fractions (17 ml) were collected at a flow rate of 80 ml/h (Fig. 1b). Fractions containing epimerase activity were pooled, and the solution was concentrated by ultrafiltration, to a final protein concentration of 6–8 mg/ml, and stored frozen at –20°C.

Measurement of protein concentration

Protein concentration in crude extracts was measured by the micro biuret method (Itzhaki & Gill, 1964). A calibration curve was constructed by using crystalline bovine serum albumin as the

Fig. 1. *Chromatography of methylmalonyl-CoA epimerase*

(a) Chromatography on hydroxyapatite (step 5). The column was eluted with a linear gradient of sodium phosphate buffer, pH 6.5 (----). A_{280} (—) was monitored, and fractions (10 ml) were collected and assayed for methylmalonyl-CoA epimerase activity (●). (b) Chromatography on Sephadex G-75 (step 6). Fractions (20 ml) were collected and assayed for protein (—) and enzymic activity (●).

standard. For rapid measurement of the protein concentration during the enzyme purification, the spectrophotometric method of Warburg & Christian (1941) was used. The A_{280} of solutions (0.5–1 mg/ml) of pure methylmalonyl-CoA epimerase was measured after thorough dialysis against 10 mM-*N*-ethylmorpholine/acetic acid buffer, pH 7.5, and samples of these solutions were taken for absolute amino acid analysis. The average value of $A_{1\text{cm}}^{1\%}$ at 280 nm was determined to be 11.5 ± 0.2 .

Amino acid analysis

Dry salt-free samples (50–75 μg) of methylmalonyl-CoA epimerase were hydrolysed in 0.25 ml of AristaR-grade 6 M-HCl in sealed evacuated tubes at 105°C. 2-Mercaptoethanol (0.05%, v/v) was added to protect methionine and tyrosine (Houston, 1971). Duplicate samples were hydrolysed for each of 24, 48, 72 and 96 h. The amino acids were analysed on a Rank-Hilger Chromaspek analyser coupled with a Digico micro 16V computer. Valine and isoleucine values were taken from the 96 h hydrolysates only. *S*-Carboxymethylcysteine values were taken from 24 h hydrolysates of *S*-carboxymethylated methylmalonyl-CoA epimerase. *S*-Carboxymethylation was performed in 0.5 M-Tris/HCl buffer, pH 8.5, in the presence of 6 M-guanidinium chloride, essentially as described by Perham (1978). Serine and threonine values were extrapolated to zero time of hydrolysis. Tryptophan was determined by hydrolysing the protein in 6 M-HCl containing thioglycolic acid (2%,

v/v) (Matsubara & Sasaki, 1969). Norleucine was added as an internal standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of methylmalonyl-CoA epimerase was performed at pH 8.4 by the method of Davis (1964), with omission of the large-pore gel. After electrophoresis, gels were stained with Coomassie Blue by using standard procedures. Alternatively, gels were sliced manually into 4.5 mm sections, and each gel slice was macerated with 100 μl of Tris/HCl buffer, pH 7.4, containing 0.1 mM-EDTA. After 48 h at 4°C, each mixture was centrifuged and samples of the supernatant were taken for assay of epimerase activity.

Methylmalonyl-CoA epimerase was also examined by electrophoresis in 10% (w/v) polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulphate (Weber & Osborn, 1969). The subunit molecular weight was estimated by comparing its mobility with those of bovine serum albumin (M_r 68 000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (M_r 36 000), soya-bean trypsin inhibitor (M_r 21 500) and hen egg-white lysozyme (M_r 14 300).

Ultracentrifugation

Sedimentation-velocity studies of methylmalonyl-CoA epimerase were conducted at 20°C in a Beckman model E analytical ultracentrifuge (Schachman, 1957). Before use, protein solutions were dialysed at 4°C against a buffer, pH 7.0 (*I* 0.1),

containing 12.8 mM- Na_2HPO_4 , 11.5 mM- KH_2PO_4 and 50 mM- NaCl . Sedimentation coefficients ($s_{20,w}$) were calculated as described by Schachman (1957). A partial specific volume of 0.74 ml/g for methylmalonyl-CoA epimerase was calculated from its amino acid composition (Cohn & Edsall, 1943). Sedimentation-equilibrium centrifugation of methylmalonyl-CoA epimerase was performed at 20°C in a Beckman model E analytical centrifuge equipped with Rayleigh interference optics. The procedures of Nureddin & Johnson (1977) were followed. After attainment of equilibrium, a full-length interference photograph was taken, and apparent weight-average molecular weights were calculated from the slopes of $\ln c$ -versus- x^2 plots (where c is the protein concentration and x is the radial distance).

Gel filtration

Sephadex G-75 was used to obtain an estimate of the relative molecular mass of native methylmalonyl-CoA epimerase by the method of Andrews (1964). Blue Dextran (M_r 2000000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 29000) and myoglobin (M_r 18000) were used to calibrate the column.

N-Terminal analysis

S-Carboxymethylated methylmalonyl-CoA epimerase was examined for free N-terminal residues by the dansyl procedure in 8 M-urea (Gray & Hartley, 1963). Dansyl-amino acids were separated and identified by chromatography on polyamide thin-layer sheets (Gray, 1972).

Cross-linking

Dimethyl suberimidate was made up just before use as a solution (70 mg/ml) in 0.5 M-triethanolamine/HCl buffer, pH 8.7, containing 0.5 M-NaOH. Cross-linking was performed at 20°C in 90 mM-triethanolamine/HCl buffer, pH 8.5, with an epimerase concentration of 0.3 mg/ml. The reaction was stopped by addition of neutral 1 M-

hydroxylamine, and, after dialysis, samples of protein were examined by electrophoresis in 10% (w/v) polyacrylamide gels containing sodium dodecyl sulphate (0.1%, w/v) (Figs. 2b and 2c).

Peptide 'mapping'

Samples of salt-free S-carboxymethylated methylmalonyl-CoA epimerase (10 mg/ml), dissolved in 0.5% (w/v) NH_4HCO_3 solution, were digested for 4 h at 37°C with trypsin (10 µg/mg of epimerase). Peptide mixtures were analysed by the methods of Bates *et al.* (1975).

Atomic absorption

The presence of bivalent metal ions in purified methylmalonyl-CoA epimerase was investigated by atomic-absorption spectrometry, by using a Pye-Unicam SP.90B instrument with a conventional nebulizer. Enzyme samples (2.0 ml; 0.8–1.6 mg/ml) were first dialysed overnight at 4°C, against metal-ion-free 10 mM-Tris/HCl buffer, pH 7.4. In each case the diffusate served as a blank for the subsequent atomic-absorption measurements. Calibration curves were constructed by using A.R.-grade reagents. Buffers were made metal-ion-free by extraction with dithizone solutions (0.02%, w/v) in CCl_4 .

Results

Purification of methylmalonyl-CoA epimerase

The purified methylmalonyl-CoA epimerase could be stored frozen at -20°C for several months without loss of activity. An overall yield of about 40% gave approx. 90 mg of enzyme (from 350 kg of cell paste), with a specific activity of approx. 10 µkat/mg under the conditions of the coupled assay. A summary of the purification is given in Table 1.

Characterization of methylmalonyl-CoA epimerase

Polyacrylamide-gel electrophoresis. Methylmalonyl-CoA epimerase, when examined by electro-

Table 1. Purification of methylmalonyl-CoA epimerase from *P. shermanii*
For full experimental details see the text.

Fraction	Volume (ml)	Total activity (µkat)	Total protein (mg)	Specific activity (nkat/mg)	Activity yield (%)
1. Extract	750	2310	21 700	106	100
2. 55–90%-satn.-(NH_4) ₂ SO ₄ pellet	51	1710	*	—	74.0
3. Streptomycin supernatant	63	1590	2270	700	69.0
4. 5–15%-(w/v)-poly(ethylene glycol) pellet	250	1425	890	1600	61.6
5. Hydroxyapatite pool	450	1050	207	5072	45.4
6. Sephadex G-75 pool after centrifugation	11.5	885	87.4	10 125	38.0

* Not determined in this preparation. In similar preparations the corresponding value was 2.5–3.5 g.

phoresis at pH 8.4 in the system of Davis (1964), in the presence of 2-mercaptoethanol, migrated essentially as a single band of protein, coincident with epimerase activity (Fig. 2*a*). Electrophoresis in the presence of sodium dodecyl sulphate (0.1%, w/v) and 2-mercaptoethanol also showed the methylmalonyl-CoA epimerase to be essentially homogeneous (Fig. 2*b*). A plot of $\log M_r$ against mobility was constructed for standard proteins and was found to be linear. The mobility of the methylmalonyl-CoA epimerase band corresponded to a subunit M_r of 17000 ± 1000 (S.E.M. for four determinations).

Relative molecular mass of methylmalonyl-CoA epimerase. Gel filtration on a column of Sephadex G-75, calibrated with suitable marker proteins, showed that native methylmalonyl-CoA epimerase has M_r approx. 35000 (average of three deter-

Fig. 2. Gel electrophoresis of methylmalonyl-CoA epimerase

The position of the marker dye is shown by a horizontal arrow. Approx. 50 μ l of protein solution was applied to each gel (a) in the absence of sodium dodecyl sulphate (Davis, 1964). Analysis of an identical gel showed a single peak of enzymic activity coincident with the position of the protein band. (b) Electrophoresis in the presence of sodium dodecyl sulphate, before cross-linking, showing bands at (1) M_r 34000 (trace) and (2) M_r 17000. (c) As (b), but after cross-linking with dimethyl suberimidate, showing bands at (3) M_r 34000, (4) M_r 30500 and (5) M_r 17300.

minations). The size of methylmalonyl-CoA epimerase was also investigated by means of sedimentation-equilibration studies. Centrifugation was performed at several protein concentrations, and in each case a plot of $\ln c$ versus x^2 (where c is the protein concentration and x is the radial distance) was linear. The apparent weight-average molecular weight of methylmalonyl-CoA epimerase, calculated from the slope of the plots, was estimated to be 33000 ± 2000 . Comparison of these data with the results of gel filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, indicates that methylmalonyl-CoA epimerase is composed of two similar or identical subunits. The M_r of each subunit was taken to be 16500.

Sedimentation-velocity studies. Methylmalonyl-CoA epimerase sediments as a single, apparently symmetrical, boundary. From a plot of $s_{20,w}$ against protein concentration, it was found that $s_{20,w}^0 = 3.05S$, in good agreement with the previously published value of $s_{20,w} = 2.9-3.0S$ (Allen *et al.*, 1963) for a single protein concentration.

Subunit structure. The subunit structure of native methylmalonyl-CoA epimerase was also examined by cross-linking studies with dimethyl suberimidate. Cross-linking was almost complete after 22 min under the conditions used, as judged by the appearance of a 'doublet' of protein bands on 10% sodium dodecyl sulphate/polyacrylamide gels with apparent M_r values of 34000 and 30500 respectively, and the presence of a relatively minor band with an apparent M_r of 17300 (Fig. 2*c*). No evidence was obtained for the formation of any aggregates larger than dimers. The appearance of two 'sub-bands' at the dimer position after cross-linking may be caused by the anomalous migration of polypeptide chains that contain intrachain cross-links and therefore cannot unfold completely in sodium dodecyl sulphate, as suggested previously for catalase by Hucho *et al.* (1975).

Histidine was the only free *N*-terminal residue identified when *S*-carboxymethylated methylmalonyl-CoA epimerase was subjected to the dansyl procedure. This is consistent with the two subunits being identical. The results of peptide-'mapping' experiments supported this conclusion, since a maximum of 14-15 tryptic peptides could be resolved, close to the number predicted from amino acid analysis of methylmalonyl-CoA epimerase. The amino acid composition of methylmalonyl-CoA epimerase is given in Table 2, and is based on a subunit M_r of 16500.

Effects of bivalent metal ions

It was found that several metal-ion-chelating reagents, at high concentrations, progressively inactivate methylmalonyl-CoA epimerase, although these effects could subsequently be reversed by

Table 2. *Amino acid composition of methylmalonyl-CoA epimerase from P. shermanii*

The amino acid composition of methylmalonyl-CoA epimerase was determined as described in the text. Results are expressed as means for the numbers of values given in parentheses. The subunit M_r was taken as 16 500.

Amino acid	Composition (mol/mol of subunit)
Lys	7.7 (8)
His	11.2 (8)
Arg	7.8 (8)
Cys	2.0 (2)
Asx	8.8 (8)
Thr	7.8 (2)
Ser	5.9 (2)
Glx	17.1 (8)
Pro	8.8 (8)
Gly	11.3 (8)
Ala	14.1 (8)
Val	12.4 (2)
Met	5.0 (8)
Ile	6.0 (2)
Leu	12.4 (8)
Tyr	2.9 (8)
Phe	3.1 (8)
Trp	3.3 (8)

dialysis (results not shown). Therefore a number of bivalent metal ions were tested for their effects on the activity of the epimerase. After incubation of the enzyme ($5\mu\text{M}$ in subunits) with each metal ion ($20\mu\text{M}$, added as the chloride salt) in 10mM-Tris/HCl buffer, pH 7.4, for 20min, the enzyme possessed the activity shown in parentheses (expressed as a percentage of the activity of a control sample without added metal ions) for the following bivalent metal ions: Cu^{2+} (53); Cd^{2+} (64); Zn^{2+} (102); Mg^{2+} (106); Ni^{2+} (156); Mn^{2+} (212); Co^{2+} (283). The stimulation observed with Co^{2+} , Mn^{2+} and Ni^{2+} ions was only marginally greater when preincubation was for 1h, or when the concentration of these ions in the incubation mixtures was increased from $10\mu\text{M}$ to $100\mu\text{M}$. Samples withdrawn from these incubation mixtures were diluted 50-fold into metal-ion-free buffer, and then another 120-fold into the assay mixture, so the added metal ions evidently became very tightly bound to methylmalonyl-CoA epimerase.

Metal content of purified methylmalonyl-CoA epimerase

Atomic-absorption spectroscopy was used to analyse samples of freshly purified methylmalonyl-CoA epimerase for the presence of tightly bound zinc, manganese, nickel and cobalt. Results obtained with a single preparation of methylmalonyl-CoA

epimerase were as follows (expressed in g-atoms/mol of enzyme subunits): zinc, 0.22; manganese, 0.24; nickel, 0.61; cobalt, <0.025 .

In an attempt to find conditions where tightly bound metal ions might be released from methylmalonyl-CoA epimerase, samples of the enzyme were dialysed at 4°C , under N_2 , against 50mM-glycine/HCl buffer, pH 2.4 at 20°C , either in the absence of added bivalent metal ions or in the presence of Co^{2+} , Ni^{2+} , Mn^{2+} or Zn^{2+} ions (all at 4mM). After 24h, the enzyme activity in all samples had fallen to approx. 1% of its original value. The activity of the sample without metal ions was not restored by dilution into metal-ion-free 10mM-Tris/HCl buffer, pH 7.4 at 20°C , even when the buffer contained (each at $20\mu\text{M}$) either Co^{2+} , Ni^{2+} , Mn^{2+} or Zn^{2+} ions. However, at least 60–70% of the original activity was restored simply by dialysis for 24h at 4°C , under N_2 , against metal-ion-free 10mM-Tris/HCl buffer, pH 7.4 at 20°C . When the enzyme samples containing metal ions were dialysed in the same way, various amounts of precipitate appeared. The supernatants did possess some epimerase activity, but in each case had a lower specific activity than the control sample. Clearly, if methylmalonyl-CoA epimerase does require tightly bound metal ions for activity, they are not released even after extensive dialysis at pH 2.4.

Discussion

The purification procedure described in the present paper allows the rapid preparation of relatively large (100mg) amounts of methylmalonyl-CoA epimerase from *P. shermanii*, in good yield. The purified methylmalonyl-CoA epimerase has a specific activity, when assayed at 30°C with (2R)-methylmalonyl-CoA as substrate, apparently 10–15-fold higher than obtained previously for an apparently homogeneous preparation (Wood *et al.*, 1969).

The results obtained in the present work show that methylmalonyl-CoA epimerase from *P. shermanii* contains two identical subunits, each of M_r approx. 16 500. The number of active sites per dimer has not been firmly established, but the stoichiometry of inactivation of the enzyme with thiol-blocking reagents (P. F. Leadlay, unpublished work) suggests that there are two.

The stimulation of the activity of methylmalonyl-CoA epimerase, caused by preincubation with certain bivalent metal ions, is worthy of note because, as pointed out by Roeder & Kohlhaw (1980), the involvement of metal ions has been demonstrated, or is at least suspected, in several other enzyme-catalysed reactions where thioester-stabilized carbanions are thought to be enzyme-bound intermediates. It may also be relevant that

mandelate racemase from *Pseudomonas putida*, where there is evidence for the formation of a carbanion intermediate (Kenyon & Hegeman, 1970), has an absolute requirement for a bivalent metal ion (Fee *et al.*, 1974). By analogy, a mechanistic role can be envisaged for a bivalent metal ion in methylmalonyl-CoA epimerase, but the mechanism of activation remains to be established.

Atomic-absorption spectroscopy clearly shows that methylmalonyl-CoA epimerase contains tightly bound metals, in stoichiometric amounts, but a correlation between metal content and enzymic activity has not yet been demonstrated, and further work is required to establish whether these metals play any direct part in catalysis.

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