The subunit structure of methylmalonyl-CoA mutase from *Propionibacterium shermanii*

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5'-Deoxyadenosylcobalamin-dependent methylmalonyl-CoA mutase was purified to homogeneity from *Propionibacterium shermanii* by a simplified procedure. The native enzyme has an apparent M_r of 165000, similar to the enzyme from other sources but larger than previously reported. It consists of two non-identical subunits, of M_r 79000 and 67000 respectively. The smaller subunit is apparently not a proteolytic fragment of the larger one. The final preparation usually contained some inactive mutase, bearing a tenaciously bound cobalamin species. This protein proved to be readily separable from apoenzyme by fast protein liquid chromatography on anion-exchange columns.

INTRODUCTION

5'-Deoxyadenosylcobalamin acts as a cofactor for several enzymes which catalyse unusual rearrangements of carbon skeletons (Barker *et al.*, 1964; Kellermeyer & Wood, 1969; Kung *et al.*, 1971). Prominent among these is the interconversion of (2*R*)-methylmalonyl-CoA and succinyl-CoA, catalysed by methylmalonyl-CoA mutase (EC 5.4.99.2). The enzyme has previously been isolated from sheep liver (Cannata *et al.*, 1965) and human liver (Fenton *et al.*, 1981), from the intestinal worm *Ascaris* (Han *et al.*, 1984) and from the Gram-positive bacterium *Propionibacterium shermanii* (syn. *freudenreichii*) (Kellermeyer & Wood, 1969; Zagalak *et al.*, 1974). As part of a structural and mechanistic characterization of this enzyme, we have purified the mutase from *P. shermanii* by a simplified method. The properties of the enzyme isolated in this way are significantly different from those previously reported (Zagalak *et al.*, 1974). A convenient method is also presented for the rapid separation of apoenzyme from other, inactive, forms of the enzyme.

EXPERIMENTAL

Materials

DEAE-cellulose (type DE-52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. Bio-Gel A1.5m was from Bio-Rad Laboratories, Richmond, CA, U.S.A. The Mono Q anion-exchange column was from Pharmacia, and the TSK 3000 SWG gel-filtration column was supplied by Anachem, Luton, Beds., U.K. 5'-Deoxyadenosylcobalamin was from Sigma Chemical Co., Poole, Dorset, U.K. Procion Navy MXR, Coomassie Brilliant Blue R250 and activated charcoal were supplied by BDH Chemicals, Poole, Dorset, U.K. The Bradford (1976) reagent for protein assays was from Pierce Chemical Co., Rockford, IL, U.S.A. The purification of methylmalonyl-CoA epimerase (EC 5.1.99.1) and methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1) from *P. shermanii*, and the sources of other reagents and enzymes, have been given previously (Leadlay, 1981; Leadlay & Fuller, 1983).

Protein assays

Protein was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Enzyme assay

Methylmalonyl-CoA mutase was assayed, with succinyl-CoA as substrate, by coupling the formation of (2*R*)-methylmalonyl-CoA to the action of methylmalonyl-CoA epimerase, carboxyl transferase and malate dehydrogenase, and monitoring the fall in NADH concentration by the change in A_{340} . This was carried out essentially as described by Zagalak *et al.* (1974), except that assays were conducted at 30 °C rather than 37 °C. All operations with 5'-deoxyadenosylcobalamin were performed in dim red light.

Purification of the enzyme

Initial extraction. *Propionibacterium shermanii* (N.C.I.B. 9885) was grown as described by Brown *et al.* (1973). Bacterial cell paste (100 g) was suspended in 200 ml of 0.2 M-potassium phosphate buffer, pH 7.0, containing 0.1 mM-phenylmethanesulphonyl fluoride, 1 mM-EDTA, 1 mM-benzamidine hydrochloride, 1 mM-dithiothreitol and 5 g of charcoal. The cells were ruptured by two passes through a KDL Dyno-Mill bead mill (W. Bachofen, Basle, Switzerland) operated at 2 °C. The mixture was centrifuged (12000 *g* for 30 min), and the pelleted beads and cell debris was resuspended with 100 ml more of buffer, and centrifuged as above. The supernatants were combined to obtain the crude extract.

Batch-elution from DEAE-cellulose. This step was carried out essentially as described by Kellermeyer & Wood (1969), by using 0.5 kg of moist DEAE-cellulose.

(NH₄)₂SO₄ fractionation. Solid (NH₄)₂SO₄ (32.6 g/100 ml) was added to give 55% saturation at 0–2 °C, and the precipitate was removed by centrifugation at 12000 *g* for 20 min. More (NH₄)₂SO₄ (19.7 g/100 ml) was added to the supernatant to give 85% saturation, and the protein precipitate was collected by centrifugation as

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Fig. 1. Purification of mutase from *P. shermanii*

(a) Gel filtration on Bio-Gel A 1.5 m. For experimental details see the text. Fractions (10 ml) were pooled as indicated by the bar. (b) DEAE-cellulose chromatography. For experimental details see the text. Fractions (12.5 ml) were pooled as indicated by the bar.

above. The pellet was resuspended in about 15 ml of buffer M (50 mM-Tris/HCl, pH 7.5 at 20 °C, containing 2 mM-EDTA, 1 mM-benzamidine and 1 mM-dithiothreitol), and dialysed for 2 h against the same buffer.

Bio-Gel A1.5m chromatography. The concentrated solution from the previous step (29 ml) was clarified by centrifugation (25000 *g* for 10 min) and gel-filtered on a column (5 cm × 90 cm) of Bio-Gel A1.5 m, equilibrated with buffer M at 2 °C. The active material was pooled (Fig. 1a).

DEAE-cellulose-chromatography. The active material from the previous step was loaded directly on to a DEAE-cellulose column (2.5 cm × 20 cm) previously equilibrated with buffer M. After washing with the equilibration buffer, the enzyme was eluted by a linear 0–0.25 M-KCl gradient formed from 500 ml volumes in buffer M, at a flow rate of 200 ml/h. Active fractions were eluted near the beginning of the gradient (Fig. 1b), and were pooled and concentrated by ultrafiltration.

Anion-exchange fast protein liquid chromatography of purified mutase

A Pharmacia Mono Q column and Pharmacia fast-protein-liquid-chromatography apparatus (GP-250 gradient programme, and P-500 pumps) were used in

anion-exchange chromatography. The running buffers were 20 mM-Tris/HCl, pH 8.0, containing 1 mM-benzamidine (buffer A) and 0.5 M-Tris/HCl, pH 8.0, containing 1 mM-benzamidine (buffer B). Proteins were eluted at 1 ml/min, with the following gradient programme: 0–10 min, 0–80% buffer B; 10–30 min, 80–100% buffer B. Fractions (1 ml) were collected, and samples of each protein peak were taken for SDS/polyacrylamide-gel electrophoresis (Fig. 3).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of methylmalonyl-CoA was performed in the system of Laemmli (1970). After electrophoresis, gels were stained with Coomassie Brilliant Blue 250R by standard procedures. Alternatively, gels were stained with Procion Navy MX-R as described by Smith (1984). Stained protein was quantified by repeated scanning of each gel track with a Transidyne 2955 densitometer, linked to an Apple IIe microcomputer. Silver staining was by the method of Morrissey (1981). Individual subunits of the mutase were isolated by preparative SDS/polyacrylamide-gel electrophoresis of enzyme which had been previously reduced and carboxy-methylated (Perham, 1978). Protein bands were made visible by brief immersion of the gel in 1 M-KCl at 4 °C. Protein was recovered from excised gel slices by electro-elution.

Amino acid analysis

Amino acid analysis of isolated methylmalonyl-CoA mutase subunits was carried out as described by Leadlay (1981).

Ultracentrifugation

Sedimentation-velocity analysis of methylmalonyl-CoA mutase was conducted at 18 °C in a Beckman model E analytical ultracentrifuge (Schachman, 1957). Before use, the protein solution (3 mg/ml) was dialysed against a buffer, pH 7.8 (*I* 0.1), containing 16.2 mM- Na_2HPO_4 , 1.45 mM- KH_2PO_4 and 50 mM-NaCl. Sedimentation coefficients were calculated as described by Schachman (1957). A partial specific volume of 0.71 ml/g was calculated (Cohn & Edsall, 1943) from its amino acid composition (see Table 2). The procedures for sedimentation-equilibrium centrifugation were as described by Leadlay (1981).

Gel filtration

This was carried out with a TSK 3000SWG column (21.5 mm × 300 mm) and h.p.l.c. (Varian 5000 liquid chromatograph) to obtain an estimate of the M_r of native mutase. The running buffer was 50 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA, 1 mM-benzamidine and 0.4 M- $(\text{NH}_4)_2\text{SO}_4$, at a flow rate of 0.4 ml/min. The standard proteins used were rabbit pyruvate kinase (M_r 237000), yeast alcohol dehydrogenase (M_r 150000), muscle glyceraldehyde-phosphate dehydrogenase (M_r 144000), bovine serum albumin (M_r 68000) and ovalbumin (M_r 43000).

Peptide mapping

Samples of purified salt-free *S*-carboxymethylated mutase subunits were digested with trypsin, chymotrypsin, papain or *Staphylococcus aureus* V8 proteinase, by the method of Cleveland *et al.* (1977): gel slices containing individual mutase subunits (approx. 20 µg of protein)

Table 1. Purification of methylmalonyl-CoA mutase from *P. shermanii*

For experimental details see the text. Enzyme activity cannot be assayed accurately in crude extracts by the coupled assay. Values in parentheses represent activity in the absence of added cofactor.

Fraction	Volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)
Batch DEAE-cellulose eluate	1780	2530	7200 (7200)	2.8
(NH ₄) ₂ SO ₄ fractionation pellet	29	940	6100 (970)	6.5
Bio-Gel chromatography	114	239	4200 (190)	17.6
DEAE-cellulose chromatography	74	66	5900 (200)	89

were subjected to proteolysis *in situ* for 50 min, after electro-elution into the stacker gel of an SDS/20%-polyacrylamide slab gel, in the presence of increasing concentrations of proteinase. The resulting peptides were separated and silver-stained (Fig. 4).

RESULTS AND DISCUSSION

Purification of methylmalonyl-CoA mutase

A summary of the purification procedure is given in Table 1. One preparation was conducted throughout in dim red light, and the charcoal treatment was omitted, but the results were not significantly different from those reported here. An apparent increase in total activity during the purification has been previously noted (Kellermeyer & Wood, 1969) and has been ascribed to the cleavage of light-inactivated coenzyme from the apoenzyme. The overall yield is comparable with that previously achieved (Zagalak *et al.*, 1974), and the procedure may be scaled up 5-fold without major changes.

After DEAE-cellulose column chromatography, methylmalonyl-CoA mutase purified by the present method is essentially homogeneous as a protein. It exhibits a single band in non-denaturing polyacrylamide-gel electrophoresis, and two bands of apparently equal intensity in SDS/polyacrylamide-gel electrophoresis (Fig. 2). However, from the mobility of the two bands observed in the presence of SDS, the M_r of these polypeptides may be estimated to be 79000 and 67000 respectively, rather greater than the values of 66000 and 61000 reported previously (Zagalak *et al.*, 1974). The purified enzyme was usually pink, and, in different preparations, had a specific activity of 90–120 nkat/mg. A final specific activity of approx. 200 nkat/mg (at 37 °C, rather than 30 °C) was reported previously (Zagalak *et al.*, 1974), but in our hands the assay was insufficiently linear at 37 °C to allow a direct comparison to be made. The enzyme activity was stable when the enzyme was stored as a suspension in (NH₄)₂SO₄. In solution at 4 °C, activity was slowly lost, and SDS/polyacrylamide-gel electrophoresis showed the appearance of some material of lower M_r . It was subsequently found that anion-exchange fast protein liquid chromatography could resolve such 'aged' mutase into several protein fractions (Fig. 3), one of which (peak II) was both colourless and highly active, having a specific activity of 120–150 nkat/mg. Material in peak I was inactive and contained material of apparent subunit M_r 67000 and 60000, whereas material in peak III was almost inactive

Fig. 2. Gel electrophoresis of purified mutase from *P. shermanii*

(a) Electrophoresis of mutase (25 µg) on an SDS/15%-polyacrylamide slab gel (Laemmli, 1970), and staining with Coomassie Blue. (b) Electrophoresis of mutase (approx. 10 µg) on a 10%-polyacrylamide slab gel in the absence of SDS (Laemmli, 1970), and staining with Coomassie Blue.

and had a u.v./visible absorption spectrum characteristic of photoinactivated 5'-deoxyadenosylcobalamin (results not shown). The ability to resolve apoenzyme from closely related forms of the mutase should be extremely valuable in further studies with this enzyme.

Activity of mutase in the absence of 5'-deoxyadenosylcobalamin

Purified methylmalonyl-CoA mutase from *P. shermanii* has previously been reported to possess low but

Fig. 3. Fast protein liquid chromatography of mutase from *P. shermanii* on Mono Q

A sample of purified mutase (1.5 mg), after storage in solution at 4 °C for 3 weeks, was purified by anion-exchange fast protein liquid chromatography. For experimental details see the text. Insert: SDS/polyacrylamide gel electrophoresis of samples of the material applied to the column (L), and of the pooled fractions represented by the bars (I–III).

detectable activity (1–2% of total activity) in the absence of added cofactor (Overath *et al.*, 1962). Other workers have found this activity (Kellermeyer & Wood, 1969) or have not tested for it (Zagalak *et al.*, 1974). Such a holoenzyme activity was routinely observed in our preparations of mutase (3–10% of total activity) even after DEAE-cellulose column chromatography. Before precipitation with $(\text{NH}_4)_2\text{SO}_4$, essentially all of the detectable enzyme was active in the absence of added cofactor, but after this step the proportion of holoenzyme was greatly decreased. In future it may prove possible to isolate the holoenzyme form of the mutase by avoiding $(\text{NH}_4)_2\text{SO}_4$ fractionation.

M_r of the native mutase

In sedimentation-velocity experiments, mutase purified by DEAE-cellulose column chromatography sedimented as a single symmetrical boundary, with a sedimentation coefficient ($s_{20,w}$) of 7.7 S, as against 7.0 S previously found at the same enzyme concentration (Zagalak *et al.*, 1974). Sedimentation equilibrium analysis of the native mutase gave an estimate of the weight-average M_r of 165 000, again greater than the value of 124 000 reported previously (Zagalak *et al.*, 1974). Interestingly, the native mutase from sheep liver also has a sedimentation coefficient $s_{20,w}$ of 7.7 S, and an estimated M_r of 165 000 (Cannata *et al.*, 1965). Gel filtration on a TSK 3000SWG h.p.l.c. column was also used to provide an independent estimate of the M_r . A good linear correlation between $\log M_r$ and K_{av} was obtained at high ionic strength ($I \sim 1.3$).

Under these conditions, freshly purified mutase was eluted in a single major peak (> 95%) with an apparent M_r of 163 000 (average of six determinations). The lower values reported by Zagalak *et al.* (1974) may perhaps result from proteolytic degradation during purification. Suitable inhibitors were routinely present in all buffers used in the present work.

Subunit stoichiometry

Since the sum of the M_r values of the two mutase subunits falls short of the estimated native M_r of the enzyme, it is possible that the observed subunits are related and that the smaller (M_r 67 000) subunit is derived from the larger by limited proteolysis, despite the inclusion of proteolytic inhibitors in all buffers. The molar ratio of the subunits was measured after separation by SDS/polyacrylamide-gel electrophoresis. Densitometric scanning of the bands after staining with Coomassie Brilliant Blue R250 showed that the polypeptide-chain ratio was reasonably constant in all freshly-purified mutase samples, including material from peaks II and III in Fig. 3. In all cases it was 1:1, within experimental error, or 0.85:1 when allowance was made for the expected proportionately greater binding of dye to the larger polypeptide.

Table 2 shows that the isolated polypeptides have a remarkably similar amino acid composition, and should therefore differ little in intrinsic dye-binding ability. Also, densitometric scanning of gels stained with Procion Navy MXR, a covalently bound dye with an absorption

Table 2. Amino acid composition of individual polypeptide chains of methylmalonyl-CoA mutase from *P. shermanii*

The results are given as mol% [$\pm$ S.E.M. ($n = 6$) where appropriate] obtained from duplicate analyses of 24, 48 and 72 h hydrolysates. The values for threonine and serine were obtained by linear extrapolation to zero hydrolysis time. Isoleucine and valine were taken from 72 h hydrolysates. Half-cystine was determined as CM-cysteine. N.D., not determined.

Residue	Subunit...	Content (mol %)	
		α (M_r 79000)	β (M_r 67000)
Asx		8.1 $\pm$ 0.2	8.6 $\pm$ 0.3
Thr		4.8	4.9
Ser		13.1	11.7
Glx		11.4 $\pm$ 0.4	11.9 $\pm$ 0.3
Pro		3.5 $\pm$ 0.3	3.6 $\pm$ 0.5
Gly		16.7 $\pm$ 1.4	16.2 $\pm$ 0.9
Ala		8.6 $\pm$ 0.3	8.9 $\pm$ 0.2
Cys		0.8 $\pm$ 0.3	0.6 $\pm$ 0.3
Val		5.2	5.7
Met		1.5 $\pm$ 0.3	1.4 $\pm$ 0.4
Ile		3.9	4.0
Leu		6.6 $\pm$ 0.7	6.9 $\pm$ 0.2
Tyr		2.5 $\pm$ 0.2	2.4 $\pm$ 0.3
Phe		2.7 $\pm$ 0.2	2.9 $\pm$ 0.2
His		2.8 $\pm$ 0.4	2.6 $\pm$ 0.5
Trp		N.D.	N.D.
Lys		4.4 $\pm$ 0.2	4.2 $\pm$ 0.3
Arg		4.2 $\pm$ 0.2	4.3 $\pm$ 0.2

Fig. 4. Peptide mapping of individual subunits of mutase from *P. shermanii*

Digestion of electrophoretically purified mutase subunits (M_r 79000 and 67000) was for 30 min, with increasing concentrations of *Staph. aureus* V8 proteinase. The peptides obtained were separated on an SDS/20%-polyacrylamide slab gel and detected by silver staining.

spectrum indifferent to the amino acid composition of a protein (Smith, 1984), gave the same ratio. Whatever the explanation for the small apparent deficit of the larger subunit, the observed constancy of the ratio argues strongly against adventitious proteolysis as a mechanism for producing the M_r -67000 polypeptide. The M_r of the

native protein suggests that the mutase may exist as an asymmetric $\alpha\beta$ dimer, with limited contacts between the two subunits. Consistent with this, cross-linking experiments using dimethyl suberimidate (Packman & Perham, 1982) have failed to detect intramolecular cross-linking of the mutase subunits (S. Fowell & P. F. Leadlay, unpublished work).

Peptide mapping

Confirmation was sought for the non-identity of the two polypeptides by peptide-mapping experiments. Fig. 4 shows the results of one-dimensional peptide mapping by the method of Cleveland *et al.* (1977), in which reduced *S*-carboxymethylated M_r -79000 and -67000 polypeptides were separately digested with *Staph. aureus* V8 proteinase. The two polypeptides yielded many peptides that were different, and very few that could plausibly have been identical. A wide range of proteinase concentrations was used, to ensure that similarities were not masked by differences in the rate of digestion. After similar digestion with chymotrypsin, papain or trypsin, the two polypeptides also gave substantially different peptide patterns (results not shown). These results support the idea that methylmalonyl-CoA mutase from *P. shermanii* contains two different types of subunit, in contrast with the mutase from other sources, which exists as a dimer of apparently identical subunits (Han *et al.*, 1984). However, in view of their very similar amino acid compositions, there may well be substantial sequence homology between the two subunits of *P. shermanii* methylmalonyl-CoA mutase. A detailed comparison of the respective amino acid sequences will be required to establish this point.

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