Methylmalonyl-CoA mutase from Propionibacterium shermanii: characterization of the cobalamin-inhibited form and subunit–cofactor interactions studied by analytical ultracentrifugation

E. Neil Marsh†‡ and Stephen E. Harding†

*Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K., and †Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington, LE12 5RD, U.K.

A large proportion of adenosylcobalamin-dependent methylmalonyl-CoA mutase from Propionibacterium shermanii is isolated in an inactive form which contains a tightly bound cobalamin. Even when the enzyme was denatured in 5.0 M guanidine hydrochloride the cobalamin remained associated with the protein. However, when dithiothreitol was added to the denatured protein, the pink inhibitor was rapidly converted into a yellow-brown compound which could be removed by dialysis. Enzyme activity could be recovered after removal of the denaturant, although surprisingly this did not depend on prior treatment with dithiothreitol. The interaction between the protein and inhibitor was investigated by using analytical ultracentrifugation under denaturing conditions. The sedimentation coefficient $s_{20,w}$ was measured in various concentrations of guanidine hydrochloride. A complicated picture emerged in which at low denaturant concentrations subunit dissociation, partial unfolding and aggregation occur, whereas at high concentration the protein behaves as a monodisperse species. No major differences in sedimentation were observed between the enzyme–cobalamin complex and the cobalamin-free enzyme, suggesting that the inhibitor does not significantly stabilize higher-order structure within the protein.

INTRODUCTION

Methylmalonyl-CoA mutase (EC 5.4.99.2) from Propionibacterium shermanii is one of a group of adenosylcobalamin-requiring enzymes that catalyse unusual rearrangements which involve the interchange of a hydrogen atom on one carbon atom with an electron-withdrawing group on an adjacent carbon atom (Babiou and Krouwer, 1979; Retey, 1982; Halpern, 1985; Golding and Rao, 1986). The enzyme in its native form is an $\alpha/\beta$ dimer of $M_r \sim 150000$ (Francalanci et al., 1986). The $\alpha$-subunit, which is the product of the mutB gene, has $M_r$ 80174, and the $\beta$-subunit, product of the mutA gene, has $M_r$ 69465 (Marsh et al., 1989a). The two subunits show clear sequence homology, with 22% of amino acid residues being identical between the two. Previously we have investigated the dissociation of the two subunits as the ionic strength of the solvent is increased and have shown that this leads to a progressive decrease in enzymic activity (Marsh et al., 1989b).

A substantial proportion (varying from 60 to 80% between different preparations) of the mutase as isolated from P. shermanii is in an inactive 'pink' form that contains a tightly bound cobalamin species. This form of the enzyme, which can be separated from the apo-enzyme by ion-exchange chromatography on a Mono Q column, appears otherwise identical in its hydrodynamic properties and $M_r$ as judged by gel filtration and SDS/PAGE (Francalanci et al., 1986; Marsh et al., 1989b). It was hoped that characterization of this 'pink' enzyme might provide information on the mechanism by which the enzyme is inactivated. In this study we have used velocity and equilibrium analytical ultracentrifugation under denaturing conditions to investigate interactions between the protein subunits and the cobalamin inhibitor. We have also been able to reconstitute the methylmalonyl-CoA mutase apoenzyme by treatment of protein-bound cobalamin with dithiothreitol (DTT), followed by gradual removal of denaturant from the enzyme in dilute solution.

EXPERIMENTAL

Materials

Guanidine hydrochloride (GuHCl) (Aristar grade) was purchased from B.D.H. Chemicals, Poole, Dorset, U.K.; hydroxocobalamin and DTT were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The purification of methylmalonyl-CoA mutase and the sources of all other reagents and chemicals are as described previously (Leadlay, 1981; Leadlay and Fuller, 1983; Francalanci et al., 1986).

Preparation of samples for analytical ultracentrifugation and reaction with DTT

Methylmalonyl-CoA mutase (approx. 10 mg/ml in a volume of 20–100 $\mu$l) was dialysed overnight at 4°C against a solution of 1.0 M, 2.0 M, 3.0 M, 4.0 M or 5.0 M GuHCl in 50 mM Tris/HCl, pH 7.5, containing 5 mM EDTA. Before ultracentrifugation the enzyme was diluted to the appropriate concentration with more of the same GuHCl-containing buffer. The enzyme was allowed to react with DTT either by including 10 mM DTT in the dialysis buffer or by addition of 1.0 M DTT solution to a final concentration of 10 mM to the enzyme after denaturation in GuHCl-containing buffer. Samples treated with DTT in either way exhibited identical behaviour in the ultracentrifuge: there was no evidence that the presence of modified cobalamin influenced the sedimentation of the protein.

Abbreviations used: DTT, dithiothreitol; GuHCl, guanidine hydrochloride.
† To whom correspondence should be addressed.
Renaturation studies on mutase

A solution of the cobalamin-binding form of the mutase in buffer A (50 mM Tris/HCl buffer, pH 7.5, 5 mM EDTA, 1.0 mM benzamidine) was added to 6.5 M GuHCl solution dissolved in buffer A. The final protein concentration was 3.5 mg/ml and the final GuHCl concentration was 5.0 M. A small sample was removed and the protein was diluted to 0.01 mg/ml with buffer A containing 5.0 M GuHCl; this was then dialysed overnight at 4°C against 500 ml of buffer A, which had been degassed. The rest of the protein solution was incubated with DTT (10 mM) at 30°C for 30 min. A serial dilution into buffer A containing 5.0 M GuHCl and 2 mM DTT was then made to give the final protein concentrations of 1.0, 0.1, 0.01 and 0.001 mg/ml. Each of these was dialysed overnight at 4°C against buffer A containing 2 mM DTT, to allow gradual renaturation of the protein.

The protein concentration of the samples was assayed with Bradford's (1976) reagent, and the specific activity of the samples was measured with the assay described by Zagalak et al. (1974). The most dilute sample (0.001 mg/ml) had to be concentrated approx. 10-fold by ultrafiltration, to bring the protein to a suitable concentration for assay.

Sedimentation analysis

Details of these procedures, using an MSE Centrisec 75 analytical ultracentrifuge for sedimentation-velocity analysis and a Beckman model E centrifuge for sedimentation-equilibrium analysis, are essentially as described previously (Marsh et al., 1989b). For sedimentation velocity, both scanning u.v. absorption and scanning schlieren optics were employed. Due to scarcity of material, 'single point' concentration measurements were made for a given set of solvent conditions (Marsh et al., 1989b). These were between 0.5 and 0.7 mg/ml, except when schlieren optics were used (3.0 mg/ml). Density and viscosity data for GuHCl solutions for the correction of sedimentation coefficients to standard solvent conditions, s20,w, were taken from Kawahawa and Tanford (1966).

For sedimentation-equilibrium analysis, Rayleigh interference optics were employed. The 'low'-or 'intermediate'-speed method was used (Creeth and Harding, 1982); 12 mm-path-length cells were used at cell loading concentrations of ~1 mg/ml. As previously (Marsh et al., 1989b), M, determinations were done in triplicate with the use of an appropriate combination of cells with wedge windows.

RESULTS AND DISCUSSION

Chemical reactivity of the bound cobalamin

The u.v.-visible absorption spectrum of the enzyme-bound cobalamin appears 'typical' in that an intense γ-band is seen at 364 nm, with weaker, broader, α and β bands at 538 and 510 nm (Figure 1). The cobalamin remained associated with the protein even after extensive dialysis against buffer containing 5.0 M GuHCl, demonstrating it to be very tightly bound. Addition of DTT to 10 mM final concentration caused the cobalamin to be converted into a yellow-brown compound in a rapid reaction which was complete within 10 min at room temperature. The u.v.-visible spectrum of this compound was characteristic of cob(II)alamin (B12), with maxima at 408, 430 and 470 nm (Figure 1). This yellow-brown compound was easily removed from the protein by dialysis. Denaturation of the protein was essential for the reaction to occur: after 30 h at room temperature no reaction of the native enzyme with DTT was observed in buffer alone, nor when 2.0 M (NH4)2SO4 [which dissociates the subunits (Marsh et al., 1989b)] was also present.

Previously it has been shown that thiols, including cysteine and β-mercaptoethanol, can reduce various cobalamins to cob(II)alamin (Peal, 1963; Cavallini et al., 1968). However, reduction was shown to be dependent on catalytic quantities of bivalent metal ion such as Cu2+. We found no evidence that bivalent metal ions are required for the reduction of cobalamin by DTT. DTT readily reduced aquocobalamin in 50 mM Tris/HCl, pH 7.5, containing 5.0 M GuHCl and 5 mM EDTA, and, more slowly, reduced aquocobalamin in distilled water containing 5 mM EDTA. Removal of DTT by gel filtration resulted in rapid oxidation of cob(II)alamin to cobalamin.

The co-ordination chemistry of cobamides has been of great interest to inorganic and theoretical chemists (Pratt, 1972). As a result, a large body of work has accumulated on the electronic spectra of differently co-ordinated cobalamins. The u.v.-visible spectra of cobalamins have been found to be diagnostic for the nature of the axial ligand co-ordinated to cobalt, with the position and intensity of the γ-band being particularly informative (Pratt, 1972), 'Hydroxycobalamin', which at physiological pH is more likely to be aquocobalamin (water, as opposed to hydroxide co-ordinated to cobalt), has been reported as a tightly bound inhibitor of several B12 enzymes (Baker et al., 1973; Taylor and Weissbach, 1967). The γ-band of aquocobalamin has λmax at 350 nm, which is significantly lower than that for the mutase-associated cobalamin, which absorbs at 364 nm. Thus we propose that the cobalamin in the 'pink' mutase is co-ordinated by a protein side chain which acts as a ligand to cobalt. This would explain its continued binding to the protein, even after denaturation in GuHCl. The cob(II)alamin formed by reduction with DTT would not be expected to remain bound to the protein, as under the conditions of the reaction it is stable as the 5-co-ordinate species (De Tacconi et al., 1979), and would diffuse away.

It is not possible to determine from the u.v.-visible spectrum the exact nature of the amino acid side chain which is proposed to co-ordinate to cobalamin. Alkyl, cysteinyl and tyrosyl complexes are ruled out, since they have 'atypical' spectra in which the γ-band is shifted to longer wavelengths and is greatly decreased in intensity relative to the αβ-bands (Pratt, 1972; Hill et al., 1970). Co-ordination by oxygen, as carboxylate, is also unlikely, as the γ-band for the cobalamin-acetate complex occurs at 352 nm (Firth et al., 1969). Cobalamin complexes with simple alcohols have not been reported. Most likely the cobalamin is co-
Table 1  Recovery of enzymic activity from the 'pink' mutase form

The inactive 'pink' enzyme was denatured in 5.0 M guanidinium hydrochloride and allowed to renature as described in the text. (a) Samples which were treated with DTT before renaturation in DTT-containing buffer. (b) A sample which was not treated with DTT at any stage. The recovery of activity is expressed as a percentage of the specific activity of highly purified mutase, which had a specific activity of 12.0 \( \mu \text{mol/min per mg} \).

<table>
<thead>
<tr>
<th>Protein concn. (mg/ml)</th>
<th>Sp. activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.047 ± 0.0005</td>
<td>0.4</td>
</tr>
<tr>
<td>0.1</td>
<td>0.73 ± 0.05</td>
<td>6.1</td>
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<tr>
<td>0.01</td>
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<td>28.3</td>
</tr>
<tr>
<td>0.001</td>
<td>0.009 ± 0.001</td>
<td>0.08</td>
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<tr>
<td>B</td>
<td>1.47 ± 0.05</td>
<td>12.2</td>
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</table>

Figure 2  Schlieren sedimentation-velocity diagram of mutase

Measurements were made in the presence of 5.0 M GuHCl and monitored by schlieren optics at 546 nm as described in the text. The cell was scanned at 9 min intervals.

Table 2  Sedimentation-velocity and sedimentation-equilibrium data for mutase at various concentrations of GuHCl

<table>
<thead>
<tr>
<th>[GuHCl] (M)</th>
<th>10 mM DTT</th>
<th>( s_{20,W} ) (S)</th>
<th>( M^0e_{w} ) (± 5000)</th>
<th>( M^0e_{w}(f_{\text{ave}}) ) (± 5000)</th>
<th>( M^0e_{w}(f_{\text{ave}})(\pm 10000) )</th>
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<tr>
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<td>65000</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>4.0</td>
<td>No</td>
<td>2.24*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>No</td>
<td>1.57**</td>
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* Determined using scanning u.v. optics, initial protein concn. 0.5–0.7 mg/ml.
† Determined using schlieren optics, initial protein concn. 2.0–3.0 mg/ml.

Recovery of activity from the 'pink' form of mutase

The cobalamin-binding form of the mutase is completely inactive. However, after DTT treatment in the presence of GuHCl, followed by dialysis to remove the denaturant, a proportion of the mutase activity could be recovered. The activity was totally dependent on the addition of adenosylcobalamin to the assay solution. The specific activity of the reconstituted enzyme was highly dependent on the concentration of the protein during renaturation. The optimal protein concentration for renaturation appeared to be about 10 \( \mu \text{g/ml} \), when the specific activity of the reconstituted enzyme was about one-quarter that of highly purified preparations of mutase (Table 1). This observation is consistent with cobalamin being attached at the enzyme active site. It also demonstrates that attachment of the cobalamin inhibitor to the enzyme does not cause irreversible modification of the protein.

A surprising finding was that, at the optimum protein concentration for the renaturation, about 10% of the mutase activity could be recovered by dialysis of the GuHCl-denatured protein without prior DTT treatment. Again, this activity was totally dependent on adenosylcobalamin added in the assay. Such a result could be obtained if at some step in the refolding pathway water is able to displace the protein as a ligand to cobalamin. The higher specific activity of the enzyme refolded in the presence of DTT is most likely attributable to the thiol preventing adventitious oxidation of the protein during renaturation. Alternatively, the protein could refold in a conformation in which cobalamin, although still attached to the enzyme, is excluded from the active site. Scarcity of material and losses during concentration prevented our determining whether, at dilute protein concentrations, cobalamin remained bound after removal of GuHCl. Interpretation of this result is further complicated by the fact that only a small percentage of the enzyme activity is recovered (most of the protein presumably does not refold correctly). To distinguish unambiguously between the two possibilities, it will be necessary to separate the active enzyme from incorrectly folded protein to allow the cobalamin content to be determined accurately.

Analytical ultracentrifugation

Sedimentation-velocity analysis was used to investigate the binding of the cobalamin to the enzyme. Both scanning u.v. and schlieren optics were used to record the data; an example of the schlieren data in 5.0 M GuHCl is reproduced in Figure 2. The sedimentation coefficient \( s_{20,W} \) was measured for the protein in increasing concentrations of GuHCl in the presence and in the absence of 10 mM DTT. Since \( s_{20,W} \) depends not only on \( M_e \) but also on shape, it can be used to compare the degree of unfolding in different concentrations of GuHCl. We wished to examine whether the very tight binding of cobalamin resulted in protection from denaturation by GuHCl, implying substantial organization of structure around the coenzyme, or whether binding was localized within the protein, which would not affect denaturation.

In initial experiments the denaturant was first removed by dialysis before centrifugation. It was hoped that it would be possible to tell if the enzyme was now in the monomer or dimer form: in fact the protein sedimented as very-high-\( M_e \) aggregates.
whose sedimentation coefficients could not be measured under the conditions used. This may explain why the enzyme failed to refold at high protein concentrations, as described in the previous section. In subsequent experiments, therefore, the enzyme was centrifuged in the presence of denaturant to prevent aggregation.

The effects of increase in GuHCl concentration in the presence and absence of 10 mM DTT on the $M_r$ and sedimentation-velocity behaviour are summarised in Table 2. Even in the presence of only 2.0 M GuHCl the native mutase had fully dissociated into its constituent subunits with a whole-cell weight-averaged $M_r$ (i.e. averaged over all macromolecular species in solution in the ultracentrifuge cell), $M_r^{0.0}$, as measured by low-speed sedimentation-equilibrium ultracentrifugation, of 68000 ± 5000: a similar result was found for the protein in the presence of 2.0 M GuHCl + 10 mM DTT.

The sedimentation-velocity data show there to be little difference in the $s_{20,w}^0$ of samples in which the cobalamin inhibitor had been released by DTT treatment compared with those in which it had not. However, DTT treatment always resulted in the protein sedimenting with a slightly lower $s_{20,w}^0$ value. Most likely this is due to the reduction of non-specific inter- and intra-subunit disulphide bonds by DTT [the native enzyme contains no disulphide bonds (Marsh and Leadlay, 1989)]. This is borne out by the sedimentation-equilibrium measurements which show a significant fall in $M_r^{0.0}$ at the cell base when DTT is present in the cell, indicating a more homogeneous sample.

The sedimentation-velocity traces recorded with scanning u.v. optics, reproduced in Figure 3, illustrate the complex behaviour of the protein as the concentration of denaturant is increased. In 1.0 M GuHCl very broad sedimenting boundaries were observed, so that it was impossible to measure the sedimentation coefficient. The highly polydisperse nature of the sample was probably due to a mixture of dissociated, partly denatured and aggregated subunits. The relatively low concentration of denaturant may cause partial unfolding of the protein to reveal hydrophobic portions of the chain leading to the formation of aggregates. In 2.0 M GuHCl broad boundaries were again observed, although it was possible to measure the $s_{20,w}^0$ for the major sedimenting component as 2.85 S. Clearly visible, however, was a fast-moving component sedimenting at about 4.3 S. It is possible to predict an approximate $s_{20,w}^0$ for the monomeric mutase subunits based on that measured for the native dimer by using the Mark–Houwink–Kuhn–Sakurada relationship (see, e.g., Harding et al., 1991) for spheres:

$$s = M_r^{0.1}$$

This assumes that the protein subunits adopt a globular conformation after dissociation. Using the value of 7.67 S (Marsh et al., 1989b) for the mutase dimer, it is possible to estimate that $s_{20,w}^0$ for the pure monomer should be approx. 4.8 S. We speculate that in 2.0 M GuHCl a mixture of dissociated, but folded, subunits (4.3 S species) and partially unfolded subunits (2.85 S

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**Figure 3** Sedimentation-velocity diagrams recorded with scanning absorption optics for mutase in increasing concentrations of GuHCl

The diagrams were recorded in the absence of DTT and have been offset on the ordinate. The direction of sedimentation in all cases is from left to right. Abbreviation: m, meniscus. [GuHCl]: (a) 0; (b) 1.0 M; (c) 2.0 M; (d) 3.0 M; (e) 4.0 M. Rotor speed, 47 000 rev./min; scan interval, 12 min [except for (a): 44 000 rev./min, scan interval 9 min]. Temperature, 20.0 °C.

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**Figure 4** Co-sedimentation of mutase with cobalamin, illustrated with scanning u.v. optics

The scans were recorded in the presence of 5.0 M GuHCl and in the absence of DTT. Bottom scan: protein sedimenting boundary (295 nm). Top scan: cobalamin sedimenting boundary (550 nm), signal expanded 4-fold. The two scans were taken within 2 min of each other. The point of inflexion of the two sedimenting boundaries is essentially the same. Rotor speed, 44 000 rev./min; temperature, 20 °C; protein loading concentration, 0.6 mg/ml.
species) may exist. In higher concentrations of denaturant the protein sedimented as a well-defined monodisperse species. A progressive decrease in $s_{20,w}$ was observed as GuHCl concentration was increased, indicating that the protein adopts an increasingly extended conformation.

Under all concentrations of GuHCl examined, but in conditions where the DTT is absent, the cobalamin co-sedimented with the protein, suggesting that it was indeed tightly bound (Figure 4). When the enzyme in 5.0 M GuHCl was treated with DTT before centrifugation, $s_{20,w}$ values of 1.45±0.01 S were obtained by using schlieren optics or scanning u.v. optics. The modified cobalamin was now no longer associated with the protein, confirming the spectroscopic observations referred to above.

Conclusions

The experiments described above demonstrate cobalamin to be very tightly associated with the protein in the pink, inactive, form of methylmalonyl-CoA mutase. The ultracentrifuge data reveal a complicated unfolding pathway with many differently sedimenting species present at lower concentrations of denaturant. However, no major differences are seen between the cobalamin-binding protein and apoenzyme generated by reduction with DTT. It therefore seems unlikely that cobalamin significantly stabilizes protein structure, and in the presence of 5.0 M GuHCl the continued association of cobalamin with the protein indicates a covalent attachment. We propose that this occurs through a protein side chain acting as the sixth axial ligand to cobalt. The u.v.–visible spectrum of the protein–cobalamin complex suggests that co-ordination is most probably through nitrogen. Reduction of the cobalamin by DTT results in the release of cofactor to give apoenzyme and cob(II)alamin. This is in accord with known reactions of cobalamin with thiols and the physicochemical properties of cob(II)alamin. That some mutase activity can be recovered after removal of denaturant suggests, but does not prove, that cobalamin becomes co-ordinated by a residue at or close to the active site.

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