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Subunit interactions in *Propionibacterium shermanii* methylmalonyl-CoA mutase studied by analytical ultracentrifugation

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The effect of increasing ionic strength on adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii* was studied by using analytical ultracentrifugation. Both sedimentation-velocity and low-speed sedimentation-equilibration measurements show that the enzyme dissociates progressively into its two dissimilar subunits with increasing ionic strength. Equilibrium between the $\alpha\beta$-dimer and the separated subunits is rapidly established under these conditions. Dissociation is accompanied by loss of enzymic activity, but the position of the equilibrium is unaffected by the presence of either substrate or adenosylcobalamin cofactor.

INTRODUCTION

Methylmalonyl-CoA mutase is one of several adenosylcobalamin-dependent enzymes that catalyse unusual rearrangements involving the carbon skeleton of the substrates (Barker *et al.*, 1964; Kellermeyer & Wood, 1969; Kung *et al.*, 1971). The first step in these rearrangements is the homolytic cleavage of the carbon–cobalt bond of adenosylcobalamin at the active site of the enzyme to produce a 5′-deoxyadenosyl radical. This then abstracts hydrogen from the substrate to give 5′-deoxyadenosine and a carbon-based substrate radical (Abeles, 1979; Golding & Rao, 1986). The substrate radical is then presumed to rearrange to give a product radical, followed by replacement of hydrogen to give product and regenerate the cofactor. Despite numerous chemical studies on models of these reactions (Babior & Krouwer, 1979; Dolphin, 1982; Tada *et al.*, 1988), the exact nature of the rearranging species and the manner in which the enzyme controls the course of the reaction remain obscure. This is due in large part to the lack of structural information for any of these enzymes.

Methylmalonyl-CoA mutase catalyses the interconversion of succinyl-CoA (3-carboxypropionyl-CoA) and (2*R*)-methylmalonyl-CoA both in animal tissues and in propionate-producing micro-organisms (Rétey, 1982). The enzyme from *Propionibacterium shermanii* is an $\alpha\beta$-dimer in solution (Zagalak *et al.*, 1974; Francalanci *et al.*, 1986) with an apparent M_r of about 150000 (Francalanci *et al.*, 1986), and is one of the smallest and simplest of adenosylcobalamin-dependent enzymes studied so far.

An improved purification of the enzyme (Francalanci *et al.*, 1986) has provided homogeneous enzyme in sufficient quantities for detailed structural studies to be undertaken. The structural genes for both subunits of the enzyme have been cloned and sequenced (Marsh *et al.*, 1989). Preliminary X-ray-diffraction data have been obtained for crystals of a pink cobalamin-containing form of the enzyme (Marsh *et al.*, 1988). Here we report the results of studies, using sedimentation velocity and low-speed equilibrium ultracentrifugation, on both this pink form and the apoenzyme, which show that subunit dissociation is brought about by an increase in the ionic strength. The degree of dissociation, though, is unaffected by the presence of substrate or cofactor.

MATERIALS AND METHODS

Materials

The purification of methylmalonyl-CoA mutase (EC 5.4.99.2), methylmalonyl-CoA epimerase (EC 5.1.99.1) and methylmalonyl-CoA carboxytransferase (EC 2.1.3.1) from *P. shermanii* and the sources of all other reagents and chemicals have been given previously (Leadlay, 1981; Leadlay & Fuller, 1983; Francalanci *et al.*, 1986.)

Enzyme assay

Methylmalonyl-CoA mutase was assayed with succinyl-CoA as a substrate, by coupling the formation of (2*R*)-methylmalonyl-CoA to the action of methylmalonyl-CoA epimerase, transcarboxylase 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 the assay mixtures contained various concentrations of $(NH_4)_2SO_4$. Even at the highest concentration of $(NH_4)_2SO_4$ used (2.0 M), the rate of the coupled enzyme-catalysed reactions was fast enough to ensure that the mutase remained clearly rate-limiting. Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as standard.

Sedimentation velocity

Sedimentation-velocity experiments were performed in an MSE Centriscan 75 analytical ultracentrifuge

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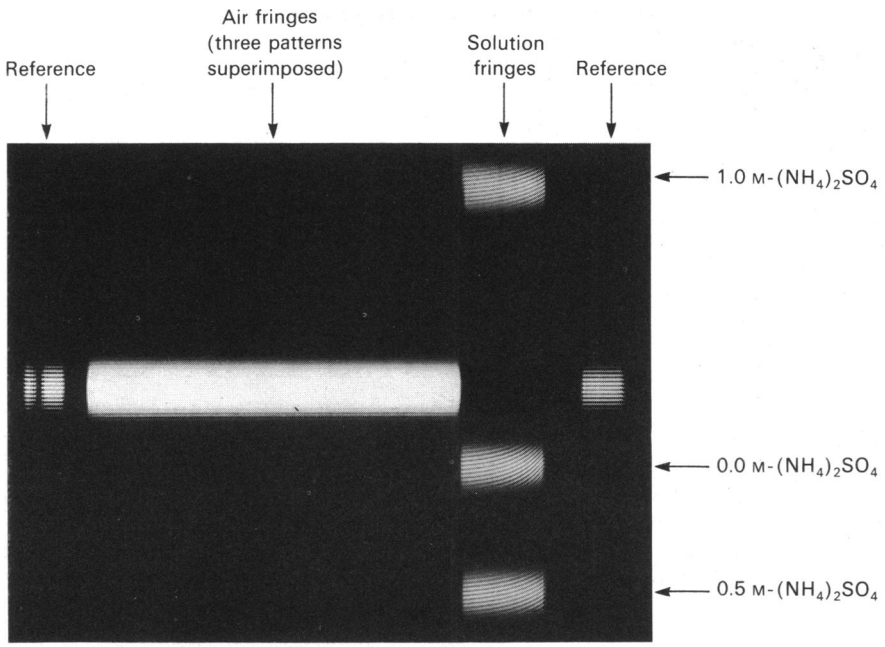

Fig. 1. Rayleigh interference low-speed sedimentation-equilibrium fringes from methylmalonyl-CoA mutase

Cells of 12 mm path length were used, two with wedge windows. The rotor speed was 9332.8 rev./min and the temperature was 20 °C. C_0, the initial loading concentration, was approx. 0.8 mg/ml. Middle: 50 mM-Tris/HCl buffer, pH 5, containing 5 mM-EDTA. Top: Tris/HCl buffer containing 1 M-$(NH_4)_2SO_4$. Bottom: Tris/HCl buffer containing 0.5 M-$(NH_4)_2SO_4$.

equipped with scanning schlieren and scanning absorption optics, and a monochromator. All experiments on the effects of electrolyte concentration were performed at 20.0 °C at rotor speeds of 44000–47000 rev./min. The apparent sedimentation coefficient at a given concentration was evaluated by using a computer digitizing tablet.

Sedimentation-velocity experiments were performed under a variety of electrolyte concentrations but with a fixed protein concentration of 0.5–0.7 mg/ml, before correction for radial dilution effects. A partial specific volume of 0.696 mg/g was used (calculated from the amino acid composition; Cohn & Edsall, 1943), and this partial specific volume was assumed to be constant for all the conditions used in this work. Correction of measured sedimentation coefficients for solvent viscosity and density was done in the usual way, to convert them into values at standard conditions of water at 20.0 °C (see, e.g., Creeth & Pain, 1967).

Sedimentation equilibrium

A Beckman model E analytical ultracentrifuge was used for sedimentation-equilibrium studies, employing Rayleigh interference optics and an RTIC temperature-measurement system. The low- or 'intermediate'-speed method was used (Creeth & Harding, 1982), where the speed is sufficiently low to allow adequate resolution of the fringes near the base of the cell (see, e.g., Fig. 1). In this method, the concentration at the air/solution meniscus remains finite, and can be calculated from the fringe data (Creeth & Harding, 1982).

Before use, all samples were dialysed for at least 24 h against the appropriate buffers. Determinations were made at 9330 rev./min at 20.0 °C in cells of 12 mm optical pathlength. The lowest possible loading concentration was used, in order to minimize possible effects of thermodynamic non-ideality. M_r determinations were done in triplicate with the use of an appropriate combination of 12 mm-pathlength cells with wedge windows. An example of the fringe patterns obtained is give in Fig. 1.

Weight-average M_r values were obtained over the whole solute distribution in a cell. The 'whole-cell' weight-average M_r values ($M^0_{r,w}$) were extracted by using the limiting value at the cell base of a directly determinable point average (the 'star' average, M^*; Creeth & Harding, 1982). An independent estimate for the initial concentration is not required. Point weight-average M_r values ($M_{r,w}$) were obtained by using sliding-strip quadratic fits to the observed fringe data (see, e.g., Harding, 1984).

RESULTS AND DISCUSSION

Extent of dissociation of methylmalonyl-CoA mutase

The dissociation behaviour of methylmalonyl-CoA mutase, in the presence of high concentrations of added salt, was analysed in two ways. First, we used sedimentation velocity, which provides a rapid and sensitive measure of changes in sedimentation coefficient, reflecting changes in either shape or mass. We also used low-speed sedimentation equilibrium, which gives an absolute measure of change in M_r without the need for assumptions concerning shape or hydration. The results are summarized in Table 1.

In sedimentation-velocity experiments, the mutase sedimented as a single boundary under all conditions used (Figs. 2*a* and 2*b*), confirming the absence of any higher-order association products. The single boundary is either evidence for a monodisperse protein preparation or for a rapid monomer–dimer equilibrium, in which case

Table 1. Ultracentrifugation data for methylmalonyl-CoA mutase

Column 1, salt concentration (M); column 2, ionic strength (I); column 3, sedimentation coefficient corrected to standard conditions ($s_{20,w}$) (S); column 4, 'whole-cell' weight-average M_r ($M^0_{r,w}$); column 5, point average-weight M_r at the meniscus [$M_{r,w}(a)$]; column 6, point weight-average M_r at the cell-base [$M_{r,w}(b)$]; column 7, point weight-average M_r extrapolated to zero fringe concentration [$M_{r,w}(J \rightarrow 0)$]. Quoted errors for $M^0_{r,w}$ are estimated errors obtained from the graphical extrapolation of M^* values to the cell base (see Creeth & Harding, 1982). Similarly, the error estimates for $M_{r,w}(a)$, $M_{r,w}(b)$ and $M_{r,w}(J \rightarrow 0)$ were obtained from graphical extrapolations of the point weight-average M_r to the cell meniscus, to the cell base and to zero fringe concentration respectively.

Salt concentration (M)	Ionic strength (I)	$s_{20,w}$ (S)	$M^0_{r,w}$ ($\pm$5000)	$M_{r,w}(a)$ ($\pm$5000)	$M_{r,w}(b)$ ($\pm$5000)	$M_{r,w}(J \rightarrow 0)$ ($\pm$8000)
$(NH_4)_2SO_4$						
0.0	0.0	7.35$\pm$0.04	116000	100000	130000	85000
0.5	1.5	6.69$\pm$0.04	100000	90000	125000	85000
1.0	3.0	6.41$\pm$0.05	99000	90000	105000	85000
1.5	4.5	5.77$\pm$0.04	94000	90000	100000	85000
2.0	6.0	5.18$\pm$0.05	85000	85000	87000	82000
$MgSO_4$						
1.0	4.0	6.33$\pm$0.04				
NH_4Cl						
4.0	4.0	6.25$\pm$0.04				

the measured sedimentation coefficient will be a weighted average of the two states. From Figs. 2(*a*) and 2(*b*) it seems clear that the protein is sedimenting more slowly at high $(NH_4)_2SO_4$ concentrations. Even after allowing for changes in the viscosity and density of the solvent, by correction to standard conditions ($s_{20,w}$), there is still a significant decrease in the $s_{20,w}$ value with increase in $(NH_4)_2SO_4$ concentration (Table 1 and Fig. 3*a*). The $s_{20,w}$ value of the 'native' material in 50 mM-Tris/HCl buffer is 7.35$\pm$0.04 S, which decreases to 5.18$\pm$0.05 S in 2.0 M-$(NH_4)_2SO_4$. This effect is not specific for $(NH_4)_2SO_4$, since experiments in 1.0 M-$MgSO_4$ (I 4.0) and in 4.0 M-NH_4Cl (I 4.0) gave similar values of $s_{20,w}$ (Table 1).

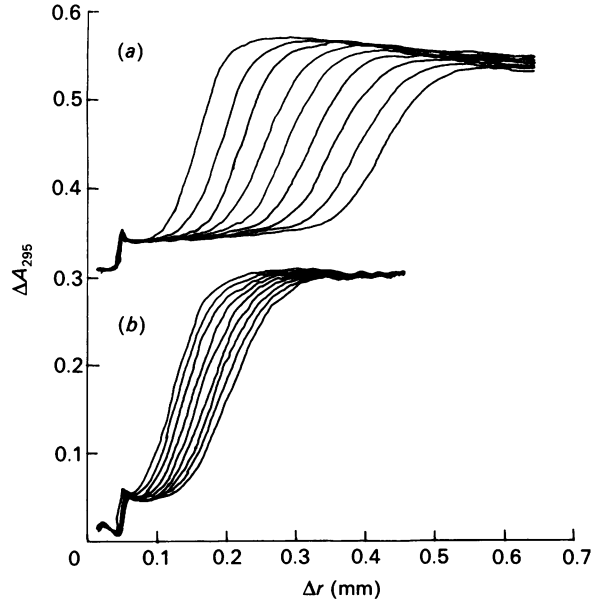

Fig. 2. Sedimentation-velocity traces from methylmalonyl-CoA mutase

The rate of sedimentation under various conditions of ionic strength was monitored by using scanning u.v. optics at 295 nm. (*a*) Sedimentation in 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA. (*b*) Sedimentation in Tris/HCl buffer containing 2.0 M-$(NH_4)_2SO_4$. Data obtained from these traces were corrected to standard conditions ($s_{20,w}$) as described in the text.

Changes in hydration, or a change to a more extended conformation, could account for these data. Because of the high rotor speeds required (47000 rev./min), effects of hydrostatic pressure on the monomer–dimer equilibrium cannot be excluded (Fujita, 1975), although this difficulty does not apply to the case of low-speed sedimentation equilibrium (Fujita, 1975). However, low-speed sedimentation-equilibrium measurements show that dissociation does occur, and so the data are best analysed in terms of the displacement of a rapidly established equilibrium between monomer and dimer forms (Table 1). Of course, changes in hydration or shape may well accompany this gross change. Two types of weight average are presented here: the 'whole-cell' weight-average M_r values ($M^0_{r,w}$) and the point weight-average M_r values at respectively the cell meniscus, the cell base and extrapolated-to-zero absolute fringe concentration ($J = 0$). In buffer alone the weight average over the whole solute distribution $M^0_{r,w} = 116000 \pm 5000$, with point weight-average M_r values ($M_{r,w}$) ranging from 100000 at the cell meniscus to 130000 at the cell base, where a larger proportion of dimer should be present. When the salt concentration was increased, the $M^0_{r,w}$ progressively decreased, reaching 85000$\pm$5000 at 2.0 M-$(NH_4)_2SO_4$ (Fig. 3*b*), with a corresponding decrease in the difference between $M_{r,w}$ values at the meniscus and base (approx. 85000 and approx. 87000 respectively) as shown in Fig. 4. These observations correlate well with the observed changes in sedimentation coefficient (Fig. 3*a*).

Effect of increasing salt concentration on methylmalonyl-CoA mutase activity

The variation in methylmalonyl-CoA mutase activity ($V_{max.}$) with increasing $(NH_4)_2SO_4$ concentration is shown

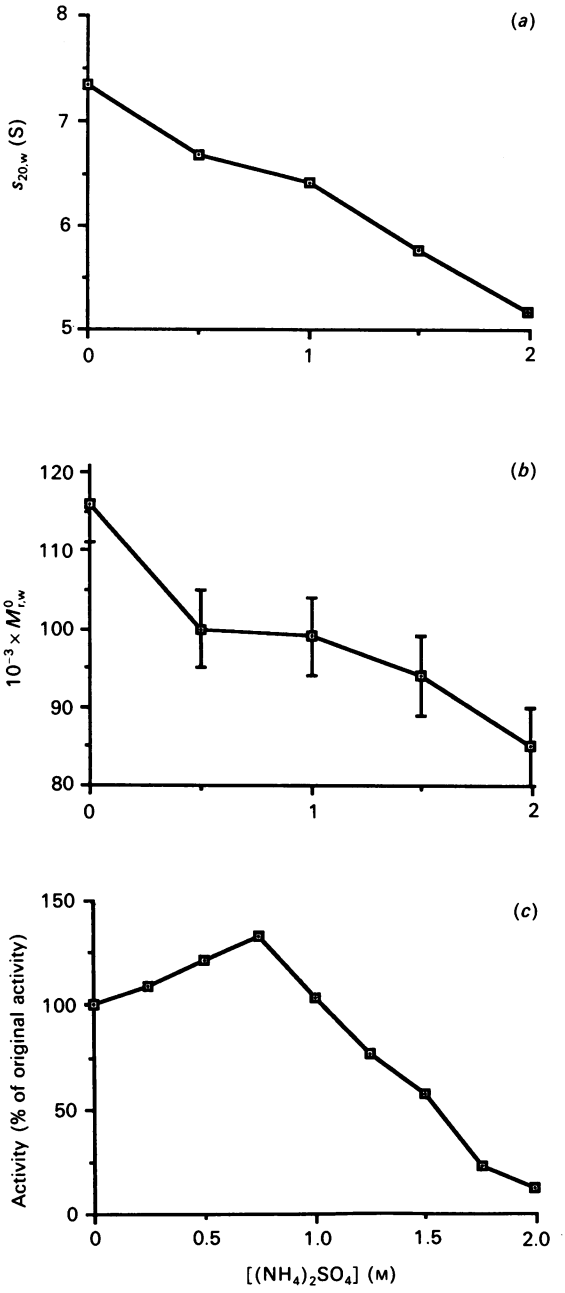

Fig. 3. Variation in $s_{20,w}$, $M^0_{r,w}$ and specific activity of methylmalonyl-CoA mutase with increasing $(NH_4)_2SO_4$ concentration

(*a*) Variation of sedimentation coefficient ($s_{20,w}$) with $(NH_4)_2SO_4$ concentration. (*b*) Variation of 'whole-cell' weight-average M_r ($M^0_{r,w}$) with $(NH_4)_2SO_4$ concentration. (*c*) Variation of enzyme activity (expressed as a percentage of the activity with no salt present) with $(NH_4)_2SO_4$ concentration. For experimental details and comment see the text.

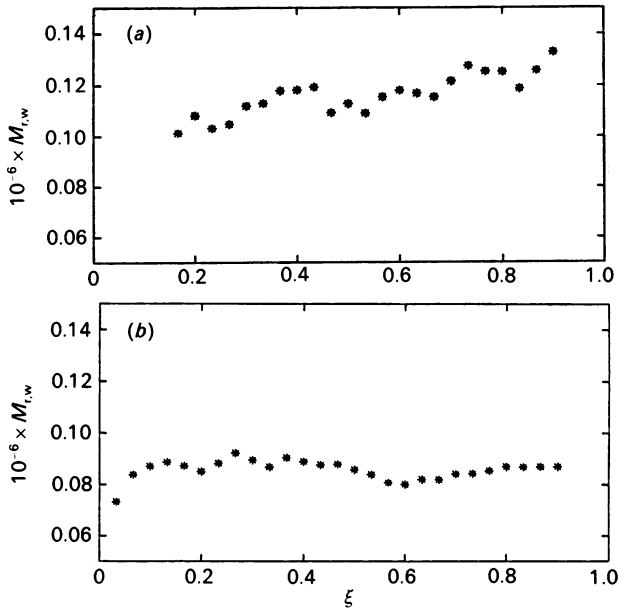

Fig. 4. Effect of increasing $(NH_4)_2SO_4$ concentration on the point weight-average M_r ($M_{r,w}$) data for methylmalonyl-CoA mutase

$M_{r,w}$ values are plotted versus ξ, where $\xi = (r^2-a^2)/(b^2-a^2)$, r being the radial displacement of a given point in the cell and a and b the corresponding values of r for the meniscus and cell-base respectively. $M_{r,w}$ values were obtained by applying quadratic sliding-strip fit procedures to the observed fringe data. (*a*) 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA. (*b*) Tris/HCl buffer containing 2.0 M-$(NH_4)_2SO_4$.

in Fig. 3(*c*). There is an initial small increase and at 0.75 M-$(NH_4)_2SO_4$ the specific activity reaches a maximum and then declines sharply. In 2.0 M-$(NH_4)_2SO_4$ the enzyme retains only 12% of the activity in buffer alone. The K_m for both substrate and coenzyme remained unchanged over this range (results not shown). The initial increase in activity seen at lower salt concentrations may be due to activation of the enzyme by NH_4^+ ions, which has been noted for some other adenosylcobalamin-dependent enzymes, albeit at much lower concentrations (Morley & Stadtman, 1970; Toraya *et al.*, 1971). Alternatively, increased ionic strength may induce a conformational change in the enzyme to give a more active form. This was not investigated further. At higher $(NH_4)_2SO_4$ concentrations, comparison of Figs. 3(*a*), 3(*b*) and 3(*c*) makes it clear that dissociation of the enzyme is accompanied by loss of activity, and the simplest interpretation is that the individual subunits are inactive. Of couse, the data do not exclude alternative explanations in which the ionic strength affects dissociation and activity by unrelated mechanisms.

Effect of added substrate on sedimentation coefficient of methylmalonyl-CoA mutase

All the experiments described above were conducted with a mixture of enzyme species comprising about 20% apoenzyme and 80% inactive enzyme containing tightly bound cobalamin. Under all conditions tested, the pink cobalamin-containing species remained associated with the sedimenting protein peak. No evidence was obtained that the two species behaved differently in sedimentation velocity. To investigate this further, the two enzyme forms were separated by f.p.l.c. on a Mono Q column as described by Francalanci *et al.* (1986) and measurements were conducted on the pure apoenzyme. The $s_{20,w}$ values determined for purified apoenzyme, either in buffer alone or in buffer containing 1.0 M-$(NH_4)_2SO_4$, were essentially

Table 2. Effect of substrate and cofactor on the sedimentation of methylmalonyl-CoA mutase

$s_{20,w}$ values were measured for methylmalonyl-CoA mutase in the presence and in the absence of substrate (methylmalonyl-CoA) and/or coenzyme. Measurements were made with no salt present (column 2) and in 1.0 M-$(NH_4)_2SO_4$ (column 3). Quoted errors are standard deviations from the mean. Abbreviation: N.D., not determined.

	$s_{20,w}$ (S)	
$[(NH_4)_2SO_4]$ ...	0.0 M	1.0 M
Enzyme only	7.30±0.04	6.49±0.05
Enzyme + substrate (200 μM)	7.18±0.04	6.51±0.05
Enzyme + coenzyme (1 μM)	7.29±0.04	N.D.
Enzyme + substrate (200 μM) + coenzyme (1 μM)	7.21±0.04	6.33±0.06

identical with those for the mixture (Table 2). Measurements were also made on apoenzyme with adenosylcobalamin (1 μM) or methylmalonyl-CoA (200 μM) or both present. Surprisingly, the presence of either substrate or both substrate and cofactor, at concentrations above their reported K_m values (Kellermeyer & Wood, 1969), has no significant effect on the observed value of $s_{20,w}$ even at high ionic strength. This implies that the binding of coenzyme and substrate by the enzyme has no effect on the monomer–dimer equilibrium and is independent of the state of dissociation of the subunits. This is in marked contrast with results obtained with adenosylcobalamin-dependent diol dehydrase from *Klebsiella aerogenes*, where the association of the two dissimilar components F and S is strongly promoted by propane-1,2-diol substrate (Toraya & Fukui, 1982) and where both F and S components are required for strong binding of cobalamin.

M_r values of methylmalonyl-CoA mutase monomer and dimer

It is apparent that, even in 50 mM-Tris/HCl buffer, the enzyme is not totally in dimeric form: the weight-average M_r over the whole solute distribution is approx. 118000. Both the measured $s_{20,w}$ and $M^0_{r,w}$ values for the enzyme in 50 mM-Tris/HCl buffer are substantially lower than the previously reported values (Francalanci *et al.*, 1986) of 7.7 S (at 3.0 mg/ml) and 165000 respectively in NaCl/sodium phosphate buffer, pH 7.0 and I 0.1 (Leadlay, 1981). The value of 165000 for $M^0_{r,w}$ was also obtained by these workers using low-speed equilibrium. Nucleotide sequence analysis of the structural genes (Marsh *et al.*, 1989) has indicated that the total M_r of the dimer is 149602. We have re-measured and confirmed the higher $s_{20,w}$ value of the enzyme in the above phosphate buffer: by using the methods in this paper, a value of 7.67±0.05 S was obtained at a protein concentration of 0.6 mg/ml and 7.72±0.15 S at 1.5 mg/ml. These results strongly support the idea that in 50 mM-Tris/HCl buffer there is already partial dissociation of the enzyme.

In Table 1, we have also given estimates of the point weight-average M_r extrapolated to zero (fringe) concentration. For the range of salt concentrations analysed, a value of approx. 85000±8000 is obtained. This is in reasonable agreement with the weighted average (74800) of the M_r values of the two dissimilar subunits, assuming that they are in rapid equilibrium with the dimer form. Lower values for the sedimentation coefficient (7.1 S) and $M^0_{r,w}$ (125000) have been obtained by Zagalak *et al.* (1974) in phosphate buffer, perhaps because of differences in the enzyme preparations (Francalanci *et al.*, 1986).

The results in this paper explain at least in part why, during the fractionation of methylmalonyl-CoA mutase from *Propionibacterium shermanii* with $(NH_4)_2SO_4$, the bulk of the holoenzyme activity is lost. The dissociation of the enzyme into α- and β-subunits probably exposes the bound cofactor to attack by O_2 or water, and generates the inactive pink form of the mutase. As yet, it has not been possible to separate the (highly homologous) subunits in a native form, to allow more detailed examination of the roles of individual subunits in binding either substrate or cofactor. Expression of individual subunits from the cloned genes may open the way to such studies, as a useful complement to the X-ray-crystallographic analysis (Marsh *et al.*, 1988).

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