The synthetic substrate succinyl(carbadethia)-CoA generates cob(II)alamin on adenosylcobalamin-dependent methylmalonyl-CoA mutase

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Succinyl(carbadethia)-coenzyme A, a synthetic substrate for adenosylcobalamin-dependent methylmalonyl-CoA mutase, has been prepared by a simplified procedure. When recombinant mutase was mixed with the synthetic substrate, the u.v./visible absorption spectrum of the bound cofactor changed rapidly to resemble that of cob(II)alamin, with an absorption maximum at 467 nm. Addition of the natural substrates, in contrast, produced only minor changes in the u.v./visible spectrum. The recent report of the generation of a complex e.p.r. spectrum on addition of substrate to the holo-methylmalonyl-CoA mutase was confirmed with the recombinant enzyme. The signals observed were stronger when the succinyl(carbadethia) analogue was used. Cobalt K-edge X-ray absorption spectroscopy confirmed that the addition of this analogue to holoenzyme leads to the generation of a cob(II)alamin-like species. These results strongly support the generation of cob(II)alamin during the 1,2-skeletal rearrangement catalysed by methylmalonyl-CoA mutase, as required if this enzyme follows the reaction pathway involving radical intermediates previously proposed for other adenosylcobalamin-dependent enzymes.

INTRODUCTION

Adenosylcobalamin-dependent enzymes, with the exception of ribonucleotide reductase, catalyse unusual 1,2-rearrangements in which a hydrogen atom is exchanged with a group on an adjacent carbon (for reviews see Dolphin, 1982; Golding and Rao, 1986). For methylmalonyl-CoA mutase, which catalyses the interconversion of succinyl-CoA and (2*R*)-methylmalonyl-CoA, it is the COSCoA group that migrates (Eggerer et al., 1960). A minimal scheme for the mechanism is given in Figure 1 (Halpern, 1985). Homolysis of the cobalt–carbon bond generates cob(II)alamin and the 5′-deoxyadenosyl radical. The protein apparently promotes this cleavage by a factor of 10^{11} or more (Pratt, 1993), affecting both the rate of homolysis and the equilibrium constant. The 5′-deoxyadenosyl radical then abstracts a specific hydrogen atom from the substrate, producing a substrate radical which rearranges by an unknown pathway. Hydrogen transfer back from the cofactor radical generates the product, and the reaction cycle is completed by re-formation of the carbon–cobalt bond. The presence of cob(II)alamin as an enzyme-bound intermediate is supported by e.p.r. spectra produced on the addition of substrate to diol dehydrase (Finlay et al., 1973), glycerol dehydrase (Cockle et al., 1972) and ethanolamine ammonia-lyase (Babior et al., 1974). For ethanolamine ammonia-lyase, the organic radical was identified as derived from the substrate.

Glutamate mutase, 2-methyleneglutarate mutase and methylmalonyl-CoA mutase all catalyse the rearrangement of carbon skeletons, and there has been particular interest in whether or not they share mechanistic features with the other, better-studied adenosylcobalamin-dependent enzymes. Very recently, using e.p.r., the presence of cob(II)alamin [or a cob(II)inamide] has been reported on addition of substrates to, respectively, glutamate mutase (Leutbecher et al., 1992), 2-methyleneglutarate mutase (Michel et al., 1992) and methylmalonyl-CoA mutase from *Propionibacterium shermanii* (Zhao et al., 1992). The intermediate species on glutamate mutase was formed within 15 ms (Leutbecher et al., 1992). We present here further evidence for the generation of cob(II)alamin-like species during catalysis by methylmalonyl-CoA mutase, using a combination of u.v./visible spectroscopy, e.p.r. and analysis of cobalt K-edge X-ray absorption spectra of the enzyme with the substrate analogue succinyl(carbadethia)-CoA.

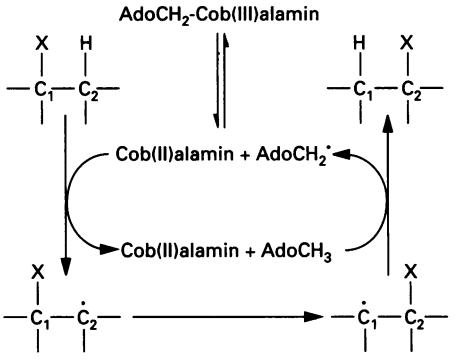

Figure 1 Minimal scheme for the mechanism of adenosylcobalamin-dependent reactions

Substrate-promoted homolysis of the cobalt–carbon bond in adenosylcobalamin initially generates cob(II)alamin and the 5′-deoxyadenosyl radical ($AdoCH_2^{\bullet}$). The abstraction of hydrogen from C-1, generating a substrate radical, is followed by the 1,2-intramolecular migration of the electronegative group X (-COSCoA in the case of methylmalonyl-CoA mutase), and then transfer of a hydrogen from deoxyadenosine ($AdoCH_3$) to C-2 to yield the product. The same scheme is proposed for the reaction of the carbadethia- analogue of succinyl-CoA with methylmalonyl-CoA mutase, although the relative rates of individual steps are clearly altered (see the Discussion for details).

Abbreviations used: e.x.a.f.s., extended X-ray absorption fine structure; x.a.n.e.s., X-ray near-edge spectrum; Co(TPP), 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine cobalt(II).

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EXPERIMENTAL

Materials and enzyme assays

Recombinant methylmalonyl-CoA mutase was prepared from an overexpressing clone of *Escherichia coli* as described previously (McKie et al., 1990). The activity of the enzyme was determined at 30 °C essentially by the method of Zagalak et al. (1974). One unit of activity transformed 1 μmol of succinyl-CoA into product per min under these conditions.

Synthesis of propionyl(carbadethia)pantetheine 4′-phosphate

The synthesis of propionyl(carbadethia)pantetheine 4′-phosphate was carried out in six steps, as previously described in outline by Michenfelder and Rétey (1986), and as described in more detail for the analogous synthesis of isobutanoyl(carbadethia)-pantetheine 4′-phosphate (Brendelberger and Rétey, 1989). Some important modifications were made, as noted below. All reactions were monitored by silica t.l.c. using various mixtures of either ethyl acetate in toluene or methanol in chloroform, and using chloroform/methanol/water/ammonia (18.1 M) (50:35:6:2, by vol.) for the phosphate derivatives. Detection was by iodine staining or charring with ammonium sulphate solution, and by u.v. absorption where appropriate. Benzene was in all cases replaced by toluene.

1-Nitrohexan-4-one was prepared as for 5-methyl-1-nitrohexanone (Brendelberger and Rétey, 1989), except that the isopropyl vinyl ketone was replaced with ethyl vinyl ketone and the product (95%) was distilled at 87 °C/0.5 mm; i.r. (film) 3000 (s), 2940 (s), 1715 (s), 1570 (s), 1440 (m), 1380 (s), 1125 (m) cm^{-1}.

2-(3-Nitropropyl)-2-ethyl-1,3-dioxolane was prepared as for the isopropyl analogue (Brendelberger and Rétey, 1989), but increasing the amount of ethylene glycol 3-fold. Distillation at 90 °C/0.5 mm gave the product (99%). I.r.: 3000 (s), 2960 (s), 2900 (s), 1570 (s), 1470 (m), 1440 (m), 1380 (s), 1200 (m), 1150 (m), 1080 (s), 930 (m) cm^{-1}; δ (p.p.m.) (60 MHz, C^2HCl_3) 0.9 (3 H, t, *J* 7Hz), 1.55 (2 H, t, *J* 9 Hz), 1.759 (2 H, q, *J* 7 Hz), 2.1 (2 H, m), 4.0 (4 H, s), 4.45 (2 H, t, *J* 8 Hz).

2-(3-Aminopropyl)-2-ethyl-1,3-dioxolane was prepared as for the isopropyl analogue (Brendelberger and Rétey, 1989), except for replacing the Raney nickel with 10% palladium on charcoal catalyst and carrying out the reduction, which was complete overnight, at atmospheric temperature and pressure, rather than under 100 atmospheres (1.013×10^7 Pa) of H_2 at 50 °C. The product (99%) had b.p. 60 °C/0.5 mm, δ (p.p.m.) (60 mHz, C^2HCl_3) 0.9 (3 H, t, *J* 7 Hz), 1.6 (6 H, m), 2.9 (2 H, m), 3.95 (4 H, s), 4.8 (2 H, d).

2-{3-[(*N*-Benzyloxycarbonyl-β-alanine)-amino]propyl}-2-ethyl-1,3-dioxolane (99%) was prepared as for the isopropyl analogue (Brendelberger and Rétey, 1989).

Propionyl(carbadethia)pantetheine was prepared essentially as for the isobutanoyl analogue (Brendelberger and Rétey, 1989). The product was initially obtained quantitatively as a pale yellow glass which contained some ethylene glycol (δ 3.36 p.p.m.) that was not removed by vacuum drying. The product was obtained pure by crystallization from diethyl ether in 81% yield, m.p. 33–35 °C. ^1H n.m.r. δ (p.p.m.) (400 MHz, ^2H_2O): 0.885 (3 H, s), 0.922 (3 H, s), 1.007 (3 H, t, *J* 7 Hz), 1.74 (2 H, quin, *J* 7 Hz), 2.485 (2 H, t, *J* 6.7 Hz), 2.54 (2 H, t, *J* 7 Hz), 2.58 (2 H, t, *J* 6.7 Hz), 3.17 (2 H, t, *J* 6.7 Hz), 3.39 (1 H, d, *J* 14 Hz), 3.5 (3 H, m), 3.67 (2 H, s), 3.98 (1 H, s). ^{13}C n.m.r. δ (p.p.m.) (104 MHz, ^2H_2O): 10.05, 21.87, 23.29, 25.61, 38.10, 38.27, 38.55, 41.41, 41.49, 41.85, 65.33, 71.16, 78.52, 176.52, 177.89, 220.95.

For propionyl(carbadethia)pantetheine 4′-phosphate, the phosphorylation was carried out as for the isobutanoyl analogue (Brendelberger and Rétey, 1989). The acetone was omitted during the precipitation of the barium salt of the product.

Synthesis of propionyl(carbadethia)-CoA

Adenosine-2′,3′-cyclic phosphate 5′-phosphoromorpholidate (Moffatt and Khorana, 1961) was treated with 1.5 molar equivalents of propionyl(carbadethia)pantetheine 4′-phosphate, by a method described previously for the analogous coupling of pantothenonitrile 4′-phosphate (Shimizu, 1970). When the pyrophosphate coupling was complete, as judged by t.l.c., the pyridine was removed by multiple methanol and water evaporation *in vacuo*, and the residue was dissolved in 100 mM ammonium acetate buffer containing 5 mM EDTA at pH 6.3 and treated with ribonuclease T_2 (Sigma) at 30 °C. This pH is much more favourable for the ring-opening reaction than the originally chosen pH of 4.6 (Shimizu, 1970). When the reaction was complete, the protein was denatured by brief heating at 80 °C and removed by centrifugation, and the clarified solution was freeze-dried. After column chromatography on DEAE-Sephacel, eluted with LiCl gradient (Shimizu, 1970) the product was isolated by acetone precipitation as the lithium salt, in greater than 90% yield. Reverse-phase h.p.l.c. over octadecylsilica using 10% (v/v) acetonitrile in triethylammonium acetate solution (10 mM) showed the presence of only one component. In contrast, a sample of the product prepared by hydrolysis with 0.1 M HCl of the 2′,3′-cyclic phosphate (Brendelberger and Rétey, 1989) showed the presence of a second component in equal amount, corresponding to the iso-CoA analogue.

Synthesis of succinyl(carbadethia)-CoA

Propionyl(carbadethia)-CoA (50 mg) was incubated with 100 mg of oxaloacetic acid, 0.5 unit of methylmalonyl-CoA:pyruvate transcarboxylase (Wood et al., 1977), 0.5 unit of methylmalonyl-CoA epimerase (Leadlay, 1981) and 0.5 unit of methylmalonyl-CoA mutase (McKie et al., 1990) in a final volume of 10 ml of 0.1 M Tris/HCl buffer, pH 7.5, at 37 °C for 3 h. Protein was removed by centrifuging the solution through a Centricon 10 filtration unit (Amicon). The product was purified by anion-exchange chromatography on a Mono-Q (Pharmacia) column using a 0–0.2 M KCl gradient in 50 mM Tris/HCl buffer, pH 7.5. The pooled fractions containing succinyl(carbadethia)-CoA were freeze-dried, desalted on a column (1 cm × 50 cm) of Sephadex G10 (Whatman) and freeze-dried once again, to yield 10 mg of product. The purity of the succinyl(carbadethia)-CoA (Figure 2) was checked by enzymic assay (Zagalak et al., 1974).

U.v./visible absorption spectroscopy

Absorption spectra were recorded on a Shimadzu UV2100 spectrophotometer in a 1 cm path-length cuvette, of 0.4 ml volume, using a Peltier temperature cell to maintain the sample at 30 °C. The concentration of the enzyme was estimated assuming the following molar absorption coefficients: ϵ_{528} 8000; ϵ_{376} 11000; ϵ_{336} 12800 $M^{-1} \cdot cm^{-1}$ (Dawson et al., 1986). It seems that 1 mol of adenosylcobalamin is bound per heterodimer, as judged by comparing the enzyme concentration determined by this method with the value determined from assays of protein concentration (N. H. Keep, unpublished work).

X-ray absorbance spectra

Extended X-ray absorption fine structure (e.x.a.f.s.) spectra were

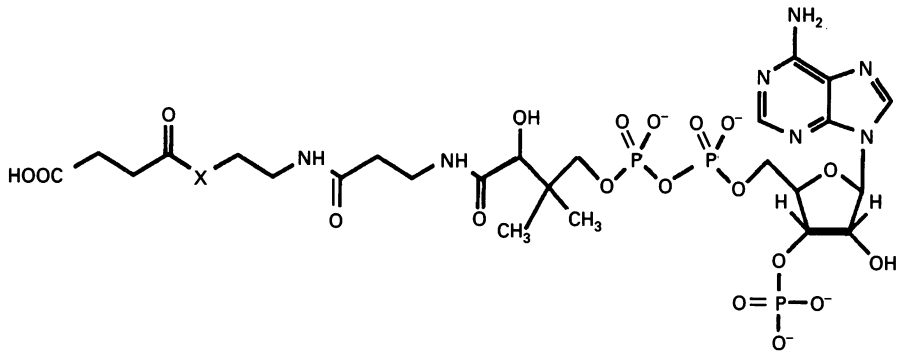

Figure 2 Structure of succinyl(carbadethia)-CoA

The structure of succinyl(carbadethia)-CoA (X = CH_2) and of the natural substrate for the methylmalonyl-CoA mutase-catalysed reaction, succinyl-CoA (X = S), is shown.

recorded on Station 8.1 using a Si (2,2,0) monochromator (van der Hoek et al., 1986) at the Daresbury synchrotron radiation source, operating at an energy of 2 GeV with an average current of 180 mA. Enzyme samples were approx. 2 mM, as determined by the u.v./visible absorbance of bound cobalamin. The spectra of holoenzyme, and of holoenzyme with 10 mM succinyl-(carbadethia)-CoA, were recorded at 25 °C. Spectra of adenosylcobalamin (8 mM in water) were recorded at 25 °C. Solutions of cob(II)alamin and cob(I)alamin were obtained by reduction, using sodium borohydride, of hydroxycobalamin (12 mM) under nitrogen in a glove box. The extent of reduction was assessed by u.v./visible spectrometry. All samples were frozen in liquid nitrogen and the spectra were recorded with the sample on a liquid nitrogen cold-finger in a vacuum to prevent re-oxidation by air. Multiple scans were taken between 7450 and 8600 eV and the absorption of incident X-rays was detected by the ensuing fluorescence emission, using a solid-state detector array (Cramer et al., 1988) Each spectrum was checked and the output from every individual detector element was inspected for discontinuities before adding the data. The summed scans were normalized so that the absorbance-edge step was the same size, and the absorbance edge was displayed using the PLOTEK program from the SRS program library.

E.p.r. spectroscopy

E.p.r. spectra were recorded using a JEOL RE-1X fitted with an Oxford Instruments ESR9 liquid helium cryostat. Spectra were recorded and baseline subtraction carried out using a microcomputer with custom-written software. Samples were prepared in standard 3 mm silica e.p.r. tubes. Samples were prepared in 0.1 M potassium phosphate, pH 6.8, which had been previously passed through a 1 cm × 30 cm column of Chelex-100 resin (Sigma) to remove bivalent metals. All protein concentrations were 0.2 mM, as determined by the u.v./visible absorbance spectra of the bound cobalamin. Nitrogen was passed gently over each solution before freezing the solution in liquid nitrogen. Where applicable, the substrates were added to a final concentration of 10 mM and the samples were allowed to warm up to 37 °C, before being re-frozen.

RESULTS

U.v./visible absorption spectroscopy

Figure 3(a) shows the absorption spectrum of the holoenzyme form of methylmalonyl-CoA mutase before and after the addition of 10 mM methylmalonyl-CoA (succinyl-CoA shows the same effect). Addition of substrate caused only a slight decrease in absorbance at 528 nm. In contrast, the addition of succinyl(carbadethia)-CoA (the structure of which is shown in Figure 2) caused an obvious change in the colour of the enzyme, from red-pink to orange-brown. The u.v./visible spectrum (Figure 3b) shows the disappearance of the peak at 528 nm, with concomitant formation of a peak at 467 nm. The new spectrum strongly resembles that of cob(II)alamin, which has an absorbance maximum at 474 nm in the base-on form, and at about 460 nm in the base-off form (Foster et al., 1970). The term 'base-off' refers to the coenzyme form with dimethylbenzimidazole not attached to cobalt. There was no evidence for the formation of cob(I)alamin, which has a characteristically strong absorbance with a maximum at 386 nm (Pratt, 1972).

Cobalt K-edge X-ray absorbance fine structure

Figure 4(a) shows the very similar cobalt K-edge spectra obtained from free adenosylcobalamin and from adenosylcobalamin bound to methylmalonyl-CoA mutase (holoenzyme). In contrast, the edge spectrum of holoenzyme to which succinyl(carbadethia)-CoA had been added showed a significant change (Figure 4b). This spectrum is very similar to that shown by free cob(II)alamin (Figure 4c). The edge spectrum of cob(I)alamin shown in Figure 4(d) is very different, making it unlikely that cob(I)alamin is formed on the enzyme under these conditions. X-ray edge spectra from enzyme-bound cobalamin species have not previously been reported, but the spectra of the free cobalamins resemble those reported by Chance and co-workers (Wirt et al., 1991). The spectra presented here are somewhat sharper, because of the narrower bandwidth afforded by the experimental arrangement used at Daresbury. If the enzyme was frozen in the presence of added succinyl(carbadethia)-CoA sample, even under the conditions used by Zhao et al. (1992) for their e.p.r. experiments, the colour and the observed edge spectrum partially reverted to that of the holo-enzyme.

E.p.r. spectroscopy

Figure 5 shows the e.p.r. spectra of the enzyme. The native enzyme (spectrum 1) has no significant signal in the $g = 2.00$ region. Addition of either the natural substrate, methyl-

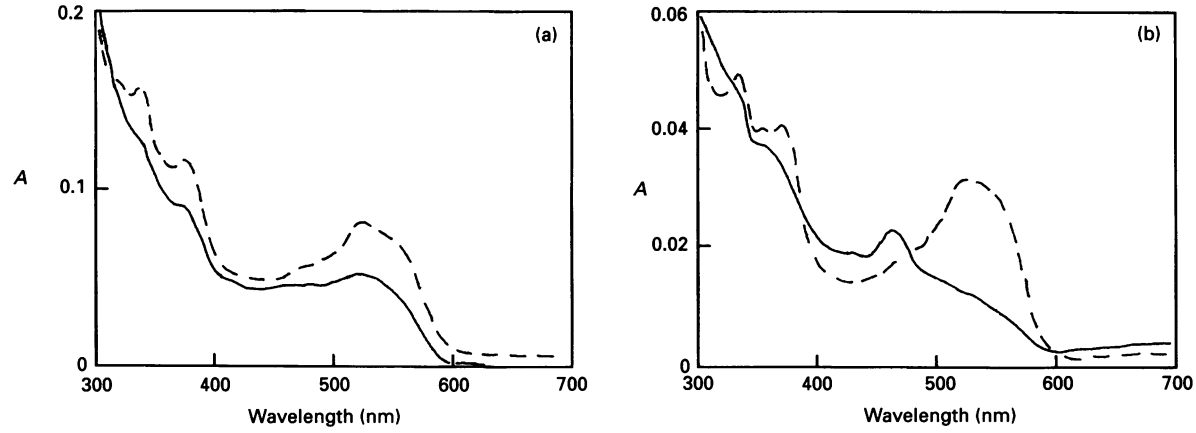

Figure 3 Absorption spectra of mutase after addition of substrates

(**a**) Methylmalonyl-CoA (10 mM) was reacted with methylmalonyl-CoA mutase (10 μM) at 30 °C in Tris/HCl buffer, pH 7.5. Spectra were recorded immediately before (-----) and after (—) addition of methylmalonyl-CoA. (**b**) Succinyl(carbadethia)-CoA (10 mM) was reacted with methylmalonyl-CoA mutase (4 μM) as in (**a**). Spectra were recorded immediately before (-----) and after (—) addition of succinyl(carbadethia)-CoA.

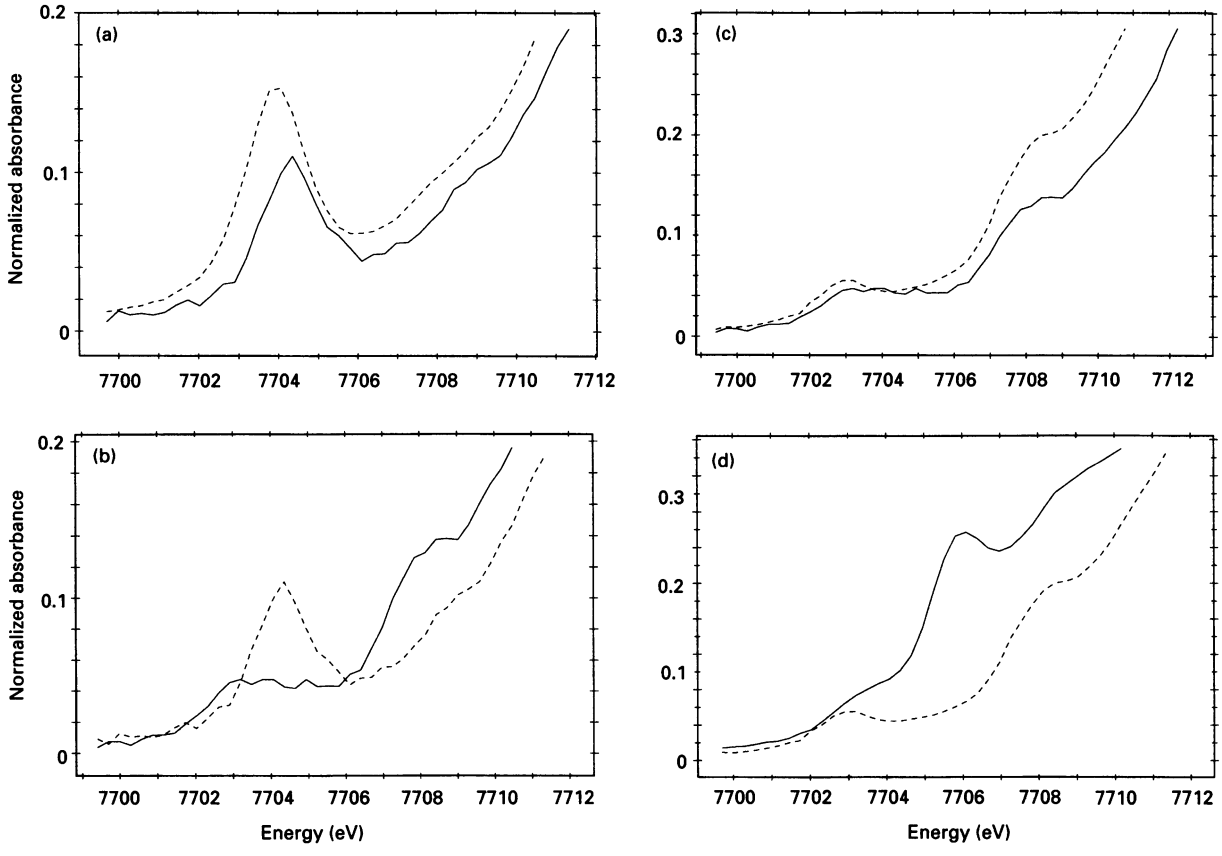

Figure 4 X-ray absorption spectra of methylmalonyl-CoA mutase in the region of the cobalt K-edge

Absorption spectra obtained from the 1s–3d and 1s–4pπ plus 'shakedown' region of the cobalt K-edge absorption spectra are shown, normalized to give the same step at the absorption edge. (**a**) Methylmalonyl-CoA mutase (—) and free adenosylcobalamin (---); (**b**) methylmalonyl-CoA mutase (---) and methylmalonyl-CoA mutase after addition of succinyl(carbadethia)-CoA (10 mM) (—); (**c**) methylmalonyl-CoA mutase after addition of succinyl(carbadethia)-CoA (10 mM) (---) and free cob(II)alamin (—); (**d**) free cob(II)alamin (---) and free cob(I)alamin (—).

malonyl-CoA (spectrum 3), or the artificial substrate, succinyl(carbadethia)-CoA (spectrum 2), results in the appearance of a signal around $g = 2.00$. The signal intensity was considerably greater with the artificial substrate. However, the signal intensity varied between samples. The signal was only seen when the enzyme was in phosphate buffer, pH 6.8. When the

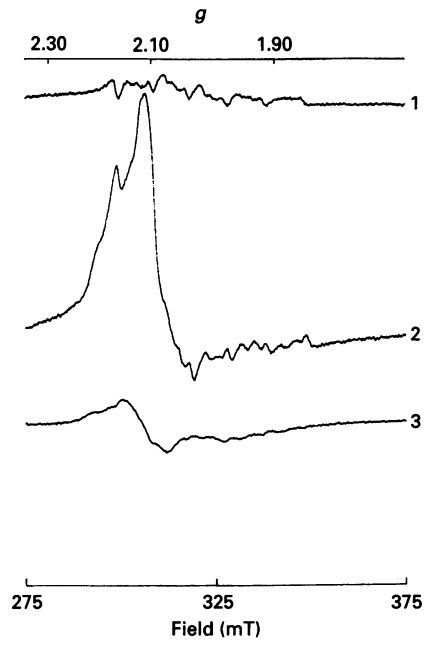

Figure 5 E.p.r. spectra of methylmalonyl-CoA mutase

E.p.r. spectra were obtained at a recording temperature of 15 K, at microwave power 5 mW, modulation width 1 mT, and frequency 9.06 GHz, from methylmalonyl-CoA mutase (0.2 mM) (spectrum 1) in 0.1 M potassium phosphate buffer, pH 6.8, and from mutase in the presence of either succinyl(carbadethia)-CoA (10 mM) (spectrum 2) or methylmalonyl-CoA (10 mM) (spectrum 3).

enzyme was in 50 mM Tris/HCl buffer, pH 7.5, the colour change on substrate addition at room temperature was clearly seen in the sample. On freezing, the colour change was reversed, suggesting changes in the enzyme–substrate complex on freezing under these conditions. Further investigation may allow the preparation of more stable samples for low-temperature measurements. The observed spectra are very similar to those reported previously for the mutase purified from *P. shermanii* (Zhao et al., 1992). Spectra were recorded at 15 K and 80 K, but did not show any change of line shape with temperature.

DISCUSSION

Evidence from u.v./visible, near-edge X-ray absorption and e.p.r. spectroscopy is presented here in support of the view that a cob(II)alamin species is produced during turnover of succinyl(carbadethia)-CoA on recombinant methylmalonyl-CoA mutase. Taken together with the recent reports of e.p.r. signals resembling cob(II)alamin for methylmalonyl-CoA mutase (Zhao et al., 1992), methyleneglutarate mutase (Michel et al., 1992) and glutamate mutase (Leutbecher et al. 1992) (all with natural substrates), this means that these carbon-skeleton rearrangements are likely to have mechanisms similar to that proposed for enzymes such as diol dehydrase and ethanolamine ammonia-lyase. Earlier failures to observe a potential cob(II)alamin intermediate (Brodie et al., 1972) had led to speculation that these enzymes might follow a different mechanistic pathway, possibly including heterolytic cleavage of the cobalt–carbon bond.

Our observation that use of non-natural substrate effectively increases the conversion of the enzyme to a form with a cob(II)alamin-like u.v./visible spectrum has a clear precedent in studies with ethanolamine ammonia-lyase (Holloway et al., 1978). Rapid and complete conversion to a kinetically competent cob(II)alamin intermediate was seen with the non-physiological substrate L-2-aminopropanol, while in the steady state with the natural substrate 2-aminoethanol only about half of the cobalamin was in this form. The e.p.r. signal for enzyme with L-2-aminopropanol was also much stronger than with the natural substrate (Wallis et al., 1982). Presumably there is a change in the rates of some of the steps of the catalytic cycle during turnover with the natural and synthetic substrates. The $k_{cat.}$ for L-2-aminopropanol is about one-hundredth of the $k_{cat.}$ for the natural substrate, mainly due to slower product formation from the cob(II)alamin intermediate. Succinyl(carbadethia)-CoA, in contrast, is an excellent alternative substrate, with a K_m (13.6×10^{-5} M) for the enzyme methylmalonyl-CoA mutase only five times greater than that of methylmalonyl-CoA (2.5×10^{-5} M) and the same $k_{cat.}$ (Michenfelder and Rétey, 1986), and it remains to be determined at what point in the mechanistic pathway the energetics of catalysis are altered. Another important difference is that after a longer (15 min) incubation of L-2-aminopropanol with ethanolamine ammonia-lyase, all the cobalamin is present as hydroxocobalamin, the product of attack by water on cob(II)alamin (Holloway et al., 1978). In contrast, the cob(II)alamin species on methylmalonyl-CoA mutase is stable. A very recent report shows the generation of a similar cob(II)alamin-like u.v./visible spectrum on the enzyme 2-methyleneglutarate mutase by the competitive inhibitor methylenesuccinate (Zelder and Buckel, 1993). For diol dehydrase, the presence of a cob(II)alamin-like u.v./visible absorbance spectrum has also been reported in the steady state with the natural substrates (Foster *et al.*, 1970).

The absorption of X-rays by a metal atom is affected by the environment of that atom. This is normally split into three regions: the pre-edge structure, the x.a.n.e.s. (X-ray near-edge spectrum), and the e.x.a.f.s.. The pre-edge spectrum arises from transition of electrons into vacant orbitals, the relative strength of these transitions being governed by the symmetry (and therefore co-ordination) of the ligands that determine which transitions are allowed (Bart, 1986).

The x.a.n.e.s. and e.x.a.f.s. arise from ionization interference between the photoelectron produced by ionization and photoelectrons back-scattered from nearby atoms. The x.a.n.e.s. arise mainly from multiple scattering and are harder to analyse than the e.x.a.f.s., which are mainly from single backscattering events. E.x.a.f.s. (Sagi et al., 1990) and edge-structure (Wirt et al., 1991) have been recorded and analysed for cobalamins, but this is the first report of such data from enzyme-bound cobalamins. Although a single scan gives all three types of data, only the edge structure is discussed here.

An analysis of the edge spectra of cobalamins has recently been presented (Wirt et al., 1991). These workers studied the size of the 1s–3d peak and the size of the next peak up, which they ascribed to a 1s–4p plus 'shakedown' transition (Kosugi et al., 1986). They measured the shift in energy of the maximum absorbance (which they assigned to 1s–4p) and of the absorption edge. They observed fairly strong 1s–3d transitions for adenosylcobalamin and methycobalamin, which they ascribed to the strong asymmetry introduced by the cobalt–carbon bond, since all the other ligands are nitrogen. In contrast with other studies on iron complexes (Roe et al., 1984) and their results with Co(II)dimethylglyoxime pyridine, this peak was smaller for the five-co-ordinate cob(II)alamin than for adenosylcobalamin. It was suggested that the distorted geometry of the corrin, and the fact that the loss of the cobalt–carbon bond caused all the ligands

to be nitrogen, outweighed the expected effect of the change to five-co-ordinate geometry. A strong 1s–4p plus 'shakedown' peak was observed for the square planar cob(I)alamin and for the model compound Co(TPP) [5,10,15,20-tetraphenyl-21*H*,23*H*-porphine cobalt(II)]. The shifts in the energies of the peaks were small (about 0.5 eV per unit change in oxidation state) for the cobalamins, but agreed with the expected pattern.

The spectra presented here for adenosylcobalamin, either in solution or on the enzyme, contain a strong peak for the 1s–3d transition, in agreement with the results of Wirt et al. (1991). Cob(I)alamin has a small peak in the 1s–3d region but a strong peak at slightly higher energy due to a 1s–4pπ plus 'shakedown' transition, which is strong for square planar complexes. Free cob(II)alamin, and the holoenzyme with added succinyl-(carbadethia)-CoA, both have a much weaker 1s–3d peak than adenosylcobalamin. It is also shifted to slightly lower energy, as expected given the lower charge on the cobalt. There is also a small but clear peak in both cases that is 5 eV higher than the 1s–3d peak and 2 eV higher than the 1s–4pπ plus 'shakedown' peak for cob(I)alamin. This is also probably due to a 1s–4pπ transition. This feature was not resolved by Wirt *et al.* (1991) due to the higher bandwidth they used. There is no study of the 1s–4pπ peak in pentavalent complexes, so it is not possible to say whether this is expected. It is at a different energy from any cob(I)alamin peak and of much lower intensity than the peaks for the square planar complexes of cob(I)alamin and Co(TPP). These results indicate that this weaker peak is due to a square-pyramidal base-on complex. Only small changes in the energy of the 1s–4p peaks can be seen, but the small differences show the same pattern of lower energy for lower oxidation state. There is an almost invariant bump on the shoulder of this peak, which is not discussed by Wirt et al. (1991). This is much larger for cyanocobalamin (Wirt et al., 1991; N. H. Keep, unpublished work). It can be tentatively assigned to a 1s–5pπ plus 'shake-down' transition (Kosugi et al., 1986) but does not contain much geometric information, as it does not vary with co-ordination. It may well be sensitive to π-donor ligands. It is clear that the enzyme–succinyl(carbadethia)-CoA complex most resembles cob(II)alamin in its X-ray absorbance spectrum.

The e.p.r. spectrum for either substrate with the recombinant mutase is similar to that previously reported for this enzyme (Zhao et al., 1992) and for other adenosylcobalamin-dependent enzymes (see the Introduction section). The spectrum for diol dehydrase has been analysed as a cob(II)alamin radical coupled to an unidentified organic radical at either 0.5 nm (Schepler et al., 1975) or 0.99 nm (Büttner and Coffman, 1977). The apparently more complete conversion to the cob(II)alamin form when succinyl(carbadethia)-CoA is used as substrate makes this compound (Michenfelder and Rétey, 1986) a valuable tool in further kinetic and structural studies on this enzyme, one aim of which will be to define more precisely the nature of the yellow-brown cob(II)alamin form. Crystals of the succinyl(carbadethia)-CoA complex with the mutase have now been obtained (N. H. Keep, unpublished work). Determination of the X-ray crystal structure of methylmalonyl-CoA mutase from *P. shermanii* is in progress (Marsh et al., 1988; McKie et al., 1990; N. H. Keep, F. Mancia and P. R. Evans, unpublished work) and, once this structure is solved, the form containing the bound analogue should be a particularly interesting structure to study.

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