Protein–coenzyme interactions in adenosylcobalamin-dependent glutamate mutase

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Glutamate mutase catalyses an unusual isomerization involving free-radical intermediates that are generated by homolysis of the cobalt–carbon bond of the coenzyme adenosylcobalamin (coenzyme B_{12}). A variety of techniques have been used to examine the interaction between the protein and adenosylcobalamin, and between the protein and the products of coenzyme homolysis, cob(II)alamin and 5′-deoxyadenosine. These include equilibrium gel filtration, isothermal titration calorimetry, and resonance Raman, UV-visible and EPR spectroscopies. The thermodynamics of adenosylcobalamin binding to the protein have been examined and appear to be entirely entropy-driven, with $\Delta S = 109\ J \cdot mol^{-1} \cdot K^{-1}$. The cobalt–carbon bond stretching frequency is unchanged upon coenzyme binding to the protein, arguing against a ground-state destabilization of the cobalt–carbon bond of adenosylcobalamin by the protein. However, reconstitution of the enzyme with cob(II)alamin and 5′-deoxyadenosine, the two stable intermediates formed subsequent to homolysis, results in the blue-shifting of two of the bands comprising the UV-visible spectrum of the corrin ring. The most plausible interpretation of this result is that an interaction between the protein, 5′-deoxyadenosine and cob(II)alamin introduces a distortion into the ring corrin that perturbs its electronic properties.

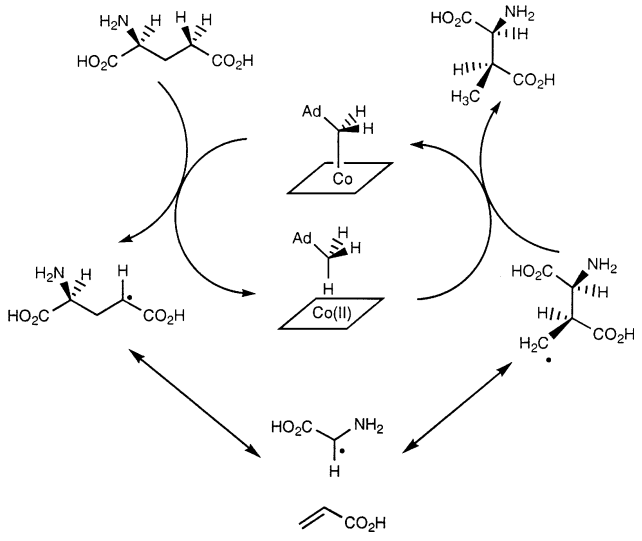
Key words: enzyme, resonance Raman spectroscopy, vitamin B_{12}.

INTRODUCTION

Glutamate mutase (EC 5.4.99.1) catalyses the reversible interconversion of L-glutamate into L-*threo*-3-methylaspartate as the first step in the fermentation of L-glutamate by various species of Clostridia [1]. It is one of a group of enzymes that catalyse unusual isomerizations in which a hydrogen atom on one carbon atom is interchanged with an electron-withdrawing group on an adjacent carbon [2–6]. These enzymes require adenosylcobalamin (AdoCbl, coenzyme B_{12}) for activity, which serves as source of carbon-based free radicals that are required in the mechanism of the rearrangements.

The first step in these rearrangements is generally considered to be the reversible homolysis of AdoCbl to form a 5′-deoxyadenosyl radical and cob(II)alamin [Cbl(II)] [7]. The adenosyl radical abstracts the migrating hydrogen to generate a carbon-based radical on the substrate, which then, in a poorly understood step, undergoes a 1,2-rearrangement to give a product radical. In the final step, a hydrogen atom is replaced from the 5′-carbon of deoxyadenosine to give the product and regenerate the adenosyl radical, which is then 'stored' by recombination with Cbl(II) to reform the cobalt–carbon bond. This mechanism is illustrated for glutamate mutase in Scheme 1.

In free solution the AdoCbl cobalt–carbon bond is quite inert: the bond-dissociation energy is about 30 kcal/mol (1 cal ≡ 4.184 J) [8] and the half-life with respect to thermolysis is estimated to be around 6 months at room temperature. For cobalt–carbon bond cleavage to be a catalytically competent step, AdoCbl-dependent enzymes must accelerate the rate of homolysis by at least 10^{12}-fold [8,9]. The mechanism by which the enzyme accelerates homolysis of AdoCbl is one of the least-well-understood aspects of B_{12} chemistry. Several enzymes, including glutamate mutase, exhibit deuterium isotope effects on the pre-steady-state rate of cobalt–carbon bond homolysis when they are reacted with substrates deuterated at the abstractable hydrogen [9–11]. This kinetic coupling of AdoCbl homolysis and hydrogen abstraction demonstrates that the adenosyl radical can only be present as a transient, high-energy intermediate.



Scheme 1 Mechanistic scheme for the rearrangement of L-glutamate to L-*threo*-methylaspartate catalysed by AdoCbl-dependent glutamate mutase

The interconversion of glutamyl and methylaspartyl radicals is thought to occur by a fragmentation and recombination mechanism with glycyl radical and acrylate as intermediates [5]. Ad, adenosyl group.

Abbreviations used: AdoCbl, adenosylcobalamin; Cbl(II), cob(II)alamin.

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The crystal structures of glutamate mutase complexed with methylcobalamin and cyanocobalamin, and with tartrate bound at the active site, have recently been solved [12]. The structure reveals that the substrate and coenzyme are probably too far apart for a concerted mechanism for cobalt–carbon bond cleavage and hydrogen abstraction, which has been discussed previously [9–11], to be operating in this enzyme. Unfortunately, since these structures lack the adenosyl moiety of the coenzyme, it remains unclear as to how the protein activates the coenzyme towards homolysis.

There are two general mechanisms by which an enzyme could potentially catalyse the homolysis of AdoCbl. The first, which involves a protein-induced ground-state destabilization of the cobalt–carbon bond, is based on studies of model organocobalt compounds [13–17] and has been termed the 'mechanochemical' hypothesis [7]. Two factors are envisaged to contribute to bond weakening: first, an enzyme-induced upward flexing of the corrin ring would apply steric strain to the adenosyl moiety, thereby weakening the Co–C bond; secondly, changes to the donor strength of the axial ligand to cobalt have also been shown to affect Co–C bond strength, although the magnitude and nature of this *trans* effect appear to depend on the system being studied. The alternative mechanism may be termed a 'transition-state stabilization' mechanism. It recognizes that because radical recombination has a very low activation energy, the rate of homolysis could be increased if the enzyme stabilizes the products of homolysis, Cbl(II), 5′-deoxyadenosine and substrate radical, making recombination less favourable. Support for this type of mechanism has been obtained from studies on AdoCbl-dependent ribonucleotide reductase and methylmalonyl-CoA mutase [18,19].

Here we describe experiments designed to probe the interactions between the protein and the coenzyme that may be responsible for the remarkable rate acceleration for Co–C bond homolysis. Experiments employing microcalorimetry indicate that binding of AdoCbl by the protein is almost entirely entropy-driven. Low-temperature resonance Raman spectroscopy has been used to examine possible protein-induced distortion of the corrin ring when glutamate mutase binds AdoCbl. UV-visible and EPR spectroscopies have also been used to examine interactions between protein-bound Cbl(II), 5′-deoxyadenosine and substrates, in complexes that mimic the intermediates formed after homolysis of the coenzyme. Whereas our results do not support ground-state weakening of the AdoCbl Co–C bond by the enzyme as a mechanism for catalysis, they do provide some of the first evidence for a change in the conformation of the corrin ring in the enzyme–Cbl(II)–5′-deoxyadenosine complex that may stabilize radical intermediates.

EXPERIMENTAL

Materials

Plasmid pOZ5 [20], expressing the *glmE* gene, was the kind gift of Professor W. Buckel and Dr O. Zelder (University of Marburg, Marburg, Germany). GlmE (the glutamate mutase E subunit from *Clostridium cochlearium*) was over-expressed in *Escherichia coli* and purified as described by Zelder et al. [20]. MutS, the small subunit of glutamate mutase from *Clostridium tetanomorphum*, was over-expressed in *E. coli* and purified as described by Holloway and Marsh [21]. The engineered single-subunit form of glutamate mutase, GlmES, was purified as described by Chen and Marsh [22]. AdoCbl, hydroxycobalamin and 5′-deoxyadenosine were purchased from Sigma. The sources of other materials have been described previously [21], or the materials were purchased from commercial suppliers.

Preparation of Cbl(II)

Cbl(II) was generated *in situ* by stoichiometric reduction of hydroxycobalamin with dithiothreitol under anaerobic conditions. All solutions were degassed on a water pump for 2 h, and purged with argon for another 10 min. A 300 μl solution of the desired concentration of hydroxycobalamin and 300 μl of 50 μM dithiothreitol were injected into a septum-sealed 2 ml anaerobic cuvette that had been flushed with argon for 5 min prior to use. The reduction of hydroxycobalamin to Cbl(II) was monitored by the changes in the UV-visible spectrum and usually took about 6 min. Anaerobic solutions containing the enzyme and/or 5′-deoxyadenosine or substrates were then introduced into the cuvette by syringe and the spectra recorded.

Resonance Raman spectra

Two samples, one containing 1.5 mM AdoCbl, the other containing 1.5 mM MutS, 300 μM GlmE and 300 μM AdoCbl, were prepared in 50 mM potassium phosphate buffer, pH 8.0, with 1 mM EDTA. Then 40 μl of each sample was pipetted into separate sample wells machined into the cold plate of a cryostat. A third sample of vacuum-distilled neat indene served for spectral calibration. To minimize frost accumulation, the samples were loaded in a glove bag purged with dry air and cooled to 200 K prior to applying the vacuum. The cryostat was maintained at 30 K using a closed-cycle helium refrigerator.

Samples were excited at 514.5 nm using a coherent Innova 307 argon ion laser operated in single line mode and filtered through a Pellin Broca prism stage to remove extraneous light prior to sample illumination. Spectra were collected in a 130°-scattering geometry from samples frozen to the cold plate. Rayleigh scattering was attenuated using a holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI, U.S.A.) that was angle tuned for maximum spectra coverage. Back-scattered photons were analysed using a Chromex 500IS single-stage 0.5 m imaging spectrograph, equipped with a 1200 groove/mm holographic grating, coupled to a Princeton Instruments (Trenton, NJ, U.S.A.) LN/CCD-1024TKB detector. Baseline corrections were computed using polynomial or cubic spline fits to a user-defined baseline. The program PEAKFIT (Jandel Scientific) was used to determine Raman band centre frequencies and linewidths.

Isothermal titration calorimetry

Titrations were performed using a Calorimetry Sciences Corp. (Spanish Fork, UT, U.S.A.) SC Model 4200 ITC microcalorimeter. The calorimetry cell contained 1.25 ml of a solution of GlmES in either 10 mM potassium phosphate buffer, pH 7.0, containing 10 % glycerol, or 10 mM Tris/HCl buffer, pH 8.3, containing 10 % glycerol. The concentration of protein ranged from 40 to 130 μM. The protein solution was titrated with 10 μl injections of a solution containing 0.56 mM AdoCbl in the same buffers, made at 900 s intervals. The coenzyme concentration ranged from 0.3 to 0.55 mM depending upon the experiment. To correct for heats of dilution, a series of blank injections was made under identical conditions except that the protein was omitted. All experiments were conducted at 25 °C. Calorimetry traces were integrated using software supplied by the instrument's manufacturer.

EPR spectroscopy

Cbl(II) was first prepared by *in situ* reduction of hydroxycobalamin with dithiothreitol in argon-flushed EPR tubes. Deoxygenated solutions (100 μl each) of 400 μM hydroxy-

cobalamin and 1 mM dithiothreitol were introduced into the tube and the reduction was allowed to proceed to completion (10 min). Then 200 μl of a deoxygenated solution of glutamate mutase containing 400 μM GlmE and 1 mM MutS in 50 mM Tris/HCl buffer, pH 8.3, was introduced into the tube and the samples were mixed thoroughly. The final concentration of protein (200 μM) was 2-fold greater than that of Cbl(II) (100 μM) to ensure that essentially all the coenzyme was bound. The protein and Cbl(II) were incubated at room temperature for 5 min and then frozen in liquid nitrogen.

EPR measurements were made using a Varian Century line X-band (9 GHz) EPR spectrometer equipped with a cryogenic Dewar system. The conditions for the detection of the Co(II) ions were: microwave power, 10 mW; microwave frequency, 9.176 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; temperature, 125 K. For each spectrum, the analogue output was digitized on the computer using a data-acquisition board made by ComputerBoard (Mansfield, MA, U.S.A.).

RESULTS AND DISCUSSION

Thermodynamics of AdoCbl binding to glutamate mutase

Two techniques, equilibrium gel filtration and isothermal titration microcalorimetry, were used to examine the thermodynamics of AdoCbl binding to glutamate mutase. To avoid complications arising from the protein-concentration-dependent association of the E and S subunits that has been examined previously [22], the engineered single subunit for of glutamate mutase, GlmES protein, was used in these experiments. Equilibrium gel filtration may be used to determine binding isotherms for ligands to macromolecules, from which equilibrium constants and thence free energies of binding can be calculated. For AdoCbl binding to glutamate mutase at 25 °C and neutral pH, the $K_d = 2.0 \pm 0.2\ \mu$M, which corresponds to $\Delta G = -32\ \text{kJ}\cdot\text{mol}^{-1}$. These values are similar to those measured previously for the enzyme under slightly different conditions [22].

Isothermal titration calorimetry can in principle be used to determine both the enthalpy and entropy changes associated with ligand binding. However, experiments with glutamate mutase failed to detect any significant heat changes when solutions of the GlmES protein ranging from 40 to 130 μM were titrated with successive injections AdoCbl solutions (0.3–0.55 mM) in either 10 mM potassium phosphate, pH 7.0, or 10 mM Tris/HCl, pH 8.3. The amount of heat evolved was very small and probably reflects the heat of dilution since control experiments in which protein was omitted from the calorimetry cell gave similar traces (Figure 1). Measurements of enzyme activity in the same buffers before and after calorimetry established that the enzyme retained at least 90 % of its activity under the conditions of the experiment and the concentrations of protein and coenzyme used were sufficient to have completely saturated the protein with ligand. It is therefore highly unlikely that the lack of an observable heat change during the titration was the result of the protein adopting an inactive conformation and failing to bind AdoCbl.

Normally, a negative result such as this would be difficult to interpret. However, because ΔG was determined independently by gel filtration, in the same buffer and at the same temperature and pH (10 mM potassium phosphate, pH 7.0, at 25 °C), it is reasonable to conclude that the absence of any discernible enthalpic component to binding must mean that the binding of AdoCbl to glutamate mutase is almost entirely entropy-driven.

From the data we estimate that greater than 90 % of the ΔG associated with AdoCbl binding to glutamate mutase is accounted for by a favourable change in entropy, with $\Delta S = 109\ \text{J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$. To our knowledge, this is the first time that the enthalpic and entropic components of B_{12} binding to a protein have been determined. One possibility is that the entropy change derives from a conformational change in the protein that expels bound water molecules from the active site. In support of this idea, we have recently obtained evidence from NMR studies for a conformational change in the S subunit of glutamate mutase upon binding to AdoCbl [23].

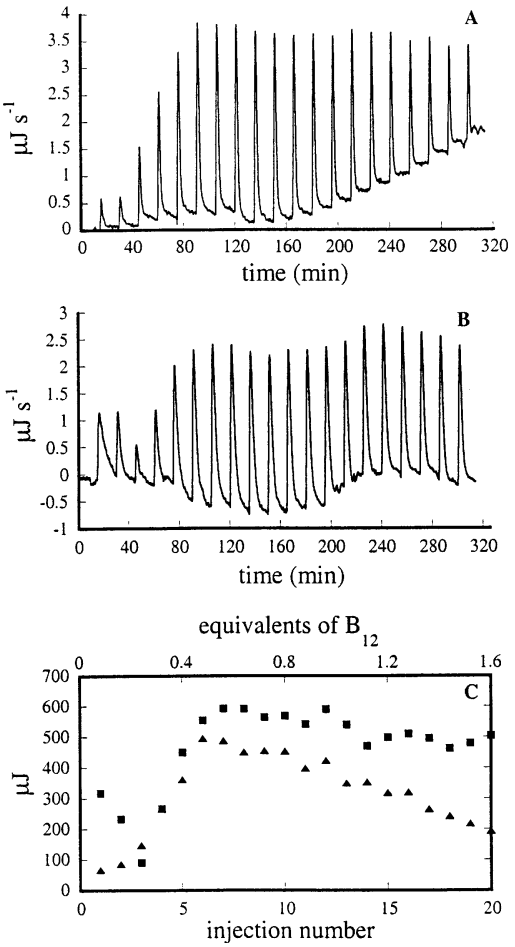

Figure 1 Isothermal titration microcalorimetry of AdoCbl binding to glutamate mutase

(**A**) Trace obtained when a 60 mM solution of glutamate mutase apoenzyme was titrated with 10 ml injections of 0.56 mM AdoCbl (10 mM potassium phosphate buffer, pH 7.0/10 % glycerol, 25 °C). (**B**) Control titration without protein, conditions identical with (**A**). (**C**) Integration of heats of injection for traces shown in (**A**) and (**B**). ▲, Enzyme titration; ■, blank titration. The differences in heat observed lie within the limits of experimental error.

Resonance Raman spectrum of AdoCbl bound to glutamate mutase

There has been much speculation that upon binding AdoCbl the protein may activate the Co–C bond towards homolysis, either through strain or electronically through a *trans* effect mediated by the axial ligand [7,13–17]. We have used resonance Raman spectroscopy to seek evidence for enzyme-mediated distortion of the coenzyme when bound to the protein, as the vibrational modes of the corrin ring should be sensitive to changes in conformation. The absorption maximum of the coenzyme at

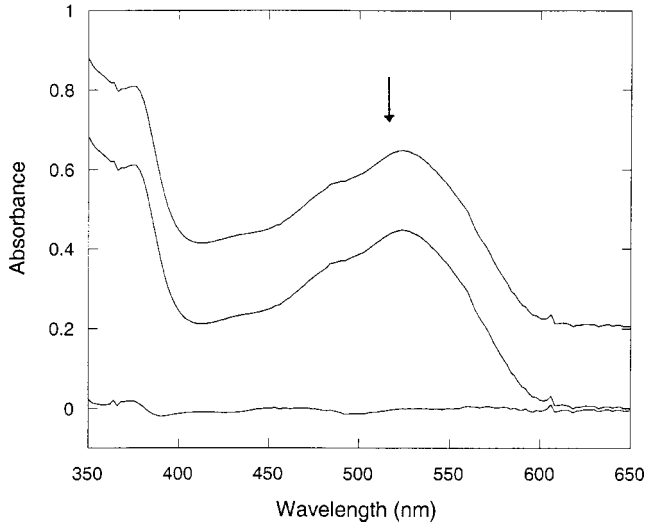

Figure 2 UV-visible spectra of AdoCbl and AdoCbl–glutamate mutase complex

Top curve: enzyme–AdoCbl complex (50 μM enzyme/40 μM AdoCbl). Middle curve: 40 μM AdoCbl in free solution. Bottom curve: difference spectrum obtained from subtracting the middle from the top curve. The spectra were recorded in 50 mM potassium phosphate buffer, pH 8.0. The arrow marks the wavelength of the Raman laser (514.5 nm).

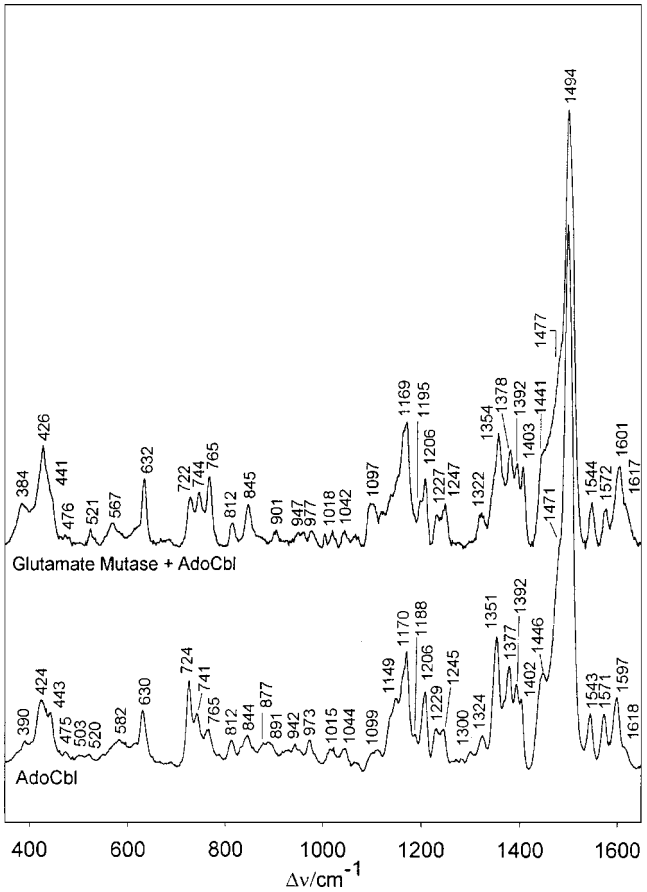

Figure 3 Resonance Raman spectra of AdoCbl and AdoCbl–glutamate mutase complex

Upper spectrum: glutamate mutase–AdoCbl complex (holoenzyme concentration 300 μM); lower spectrum: 1.5 mM AdoCbl. The spectra have been scaled so that they are of similar amplitudes.

522 nm provides a convenient chromophore with which to excite vibrational transitions in the corrin ring.

In these experiments the excitation wavelength was 514.5 nm, which is close to the absorption maximum at 522 nm. It is noteworthy that the UV-visible absorption spectrum of AdoCbl is changed very little upon binding to the protein (Figure 2). It appears that there are no major changes in the electronic transitions associated with the corrin chromophore, as might have been expected had extensive changes in either the conformation of the corrin ring or the co-ordination state of cobalt occurred.

Initial attempts to obtain spectra at room temperature were unsuccessful due to homolysis of the coenzyme caused by the excitation laser. However, at 30 K no degradation of the coenzyme was detected during the course of the experiment and the enzyme retained activity after the experiment. The concentrations of protein and AdoCbl (300 μM each) were sufficiently high that almost all the coenzyme was bound to the protein under the conditions of these experiments [21].

The resonance Raman spectra of free AdoCbl and AdoCbl bound to glutamate mutase are shown in Figure 3. The spectrum of free AdoCbl (1.5 mM, excitation wavelength 514.5 nm) is similar to that obtained previously by Spiro, Banerjee and coworkers [24,25], who have assigned vibrational bands at 424 cm^{-1} and 443 cm^{-1} to the cobalt–carbon bond stretching mode. The presence of two stretching frequencies has been attributed to the adenosyl moiety occupying two different conformations with respect to the corrin that interconvert by rotation about the Co–C bond [24]. This interpretation is supported by NMR studies that show the adenosyl portion of the coenzyme to populate two conformations with respect to the corrin ring in free solution [26].

The resonance Raman spectrum of AdoCbl bound to glutamate mutase (effective holoenzyme concentration $\approx$ 300 μM, excitation wavelength 514.5 nm) exhibits a number of changes in the relative intensities of many of the absorption bands. These changes are most evident at the low-frequency end of the spectrum. Remarkably, however, there are only very minor changes seen in the frequencies of the Raman-active bands of the coenzyme upon binding. In most cases these shifts are in the order of 2 cm^{-1} and may not be experimentally significant. In particular, the bands assigned to the Co–C stretch at 443 and 426 cm^{-1} appear little altered in frequency, although a significant increase in the relative intensity of the band at 426 cm^{-1} is observed. This may be explained if the enzyme prefers to bind the coenzyme in one conformation over another, as might be expected, and a similar phenomenon has been observed in resonance Raman studies of AdoCbl bound to methylmalonyl-CoA mutase [27]. Alternatively, the change in relative band intensities could be due to selective resonance enhancement of one or another vibrational mode of the corrin ring.

Overall, it appears that the ground-state stretching modes of the corrin ring chromophore are little perturbed by binding to the protein. This argues against a major distortion of the corrin ring in the ground state that would be expected on the basis of the mechanochemical hypothesis. It should be noted, however, that resonance Raman spectroscopy is most sensitive to changes in the curvature of the bottom of the electronic potential well that describes the covalent bond, i.e. the ground state. Whereas both our own studies reported here and those on methylmalonyl-

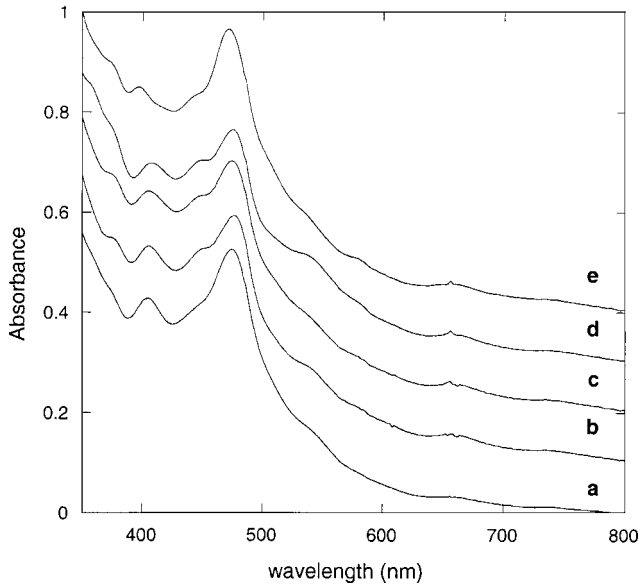

Figure 4 UV-visible spectra of Cbl(II)–glutamate mutase complexes

(**a**) Cbl(II) in free solution; (**b**) enzyme–Cbl(II) complex; (**c**) enzyme–Cbl(II) complex in the presence of 10 mM L-glutamate; (**d**) enzyme–Cbl(II) complex in the presence of 2.5 mM L-*threo*-3-methylaspartate; (**e**) enzyme–Cbl(II) complex in the presence of 5 mM 5′-deoxyadenosine. The spectra were recorded in 50 mM potassium phosphate buffer, pH 8.0 (50 μM enzyme/40 μM AdoCbl).

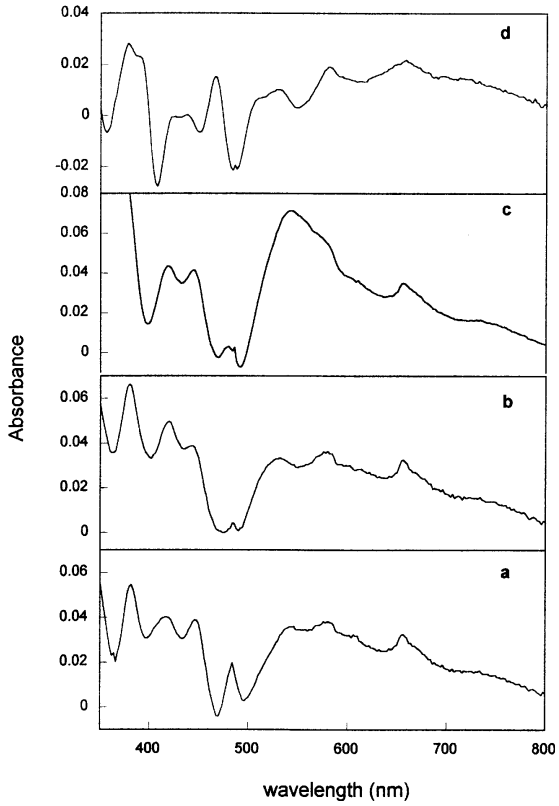

Figure 5 Difference spectra obtained after subtracting the spectrum of free Cbl(II) from that of the Cbl(II)–enzyme complexes

To correct for possible small differences in concentrations between the samples, the spectra were first normalized with respect to the most intense band at 470 nm. (**a**) Enzyme–Cbl(II) complex; (**b**) enzyme–Cbl(II) complex in the presence of 10 mM L-glutamate; (**c**) enzyme–Cbl(II) complex in the presence of 2.5 mM L-*threo*-3-methylaspartate; (**d**) enzyme–Cbl(II) complex in the presence of 5 mM 5′-deoxyadenosine.

CoA mutase [24,27] point to the ground-state Co–C stretch being very little perturbed by the protein, this does not rule out weakening of this bond by lowering the free energy of the transition state. Since radical recombination reactions typically have very low activation energies, the transition state for AdoCbl homolysis is expected to resemble the product radicals, and therefore could be stabilized by lowering the free energy of the enzyme–Cbl(II)–organic radical complex.

UV-visible spectra of glutamate mutase–Cbl(II) complexes

Various studies have examined the UV-visible absorption spectra of AdoCbl-dependent enzymes in the presence of substrates. For glutamate mutase such spectra show features expected for a mixture of AdoCbl and Cbl(II) enzyme forms [9]. This is consistent with EPR spectra that exhibit features which have been interpreted as an unpaired electron on cobalt interacting with the C-4 radical of glutamate, in accord with mechanistic hypotheses [28]. The UV-visible spectra obtained during turnover reflect a mixture of the various enzyme intermediates formed during catalysis. The relative concentrations of these intermediates are hard to determine, and therefore it is very difficult to reliably deconvolute the spectra to obtain pure Cbl(II) spectra.

We have used UV-visible and EPR spectroscopy to examine inactive complexes of the enzyme with Cbl(II) bound in the presence of 5′-deoxyadenosine and/or substrate. [We were unable to study the glutamate mutase–Cbl(II) complex by resonance Raman spectroscopy because the specialized apparatus for performing resonance Raman spectroscopy at cryogenic temperatures did not allow us to analyse samples under anaerobic conditions necessary to keep Cbl(II) reduced.] The non-productive complex of glutamate mutase with Cbl(II), 5′-deoxyadenosine and substrate differs from the true intermediate by only a single hydrogen atom and so, sterically, should mimic the intermediate formed by homolysis reasonably well. If the protein interacts strongly with the Cbl(II) form of the enzyme, this should be reflected in a change in the spectral properties of Cbl(II) upon binding to the protein.

The UV-visible absorption spectrum was recorded for Cbl(II) complexed with glutamate mutase at relatively high concentrations [50 μM enzyme/40 μM Cbl(II)], where the equilibrium favours formation of the enzyme–coenzyme complex. The absorption spectra are shown in Figure 4. To facilitate comparison, the spectra were normalized relative to the most intense band at 470 nm and the difference spectra resulting from subtraction of the spectrum of Cbl(II) in the absence of protein and/or ligands were calculated (shown in Figure 5). Binding of Cbl(II) by the protein resulted in small but significant changes to the spectrum of Cbl(II). In particular, the bands in the 600–800 nm region of the spectrum, which probably represent d–d transitions, increase in intensity relative to the main band at 470 nm, as do the shoulders at 380 and 450 nm. The presence of saturating concentrations (10 mM) of L-glutamate causes very little further change to the spectrum. However, in the presence of L-*threo*-3-methylaspartate (2.5 mM) significant increases in the intensity of bands at 375 and 535 nm in the spectrum of the enzyme–Cbl(II) complex are apparent. These bands are characteristic of cob(III)alamin species, suggesting that the cobalt may have undergone oxidation. However, this sample was prepared under

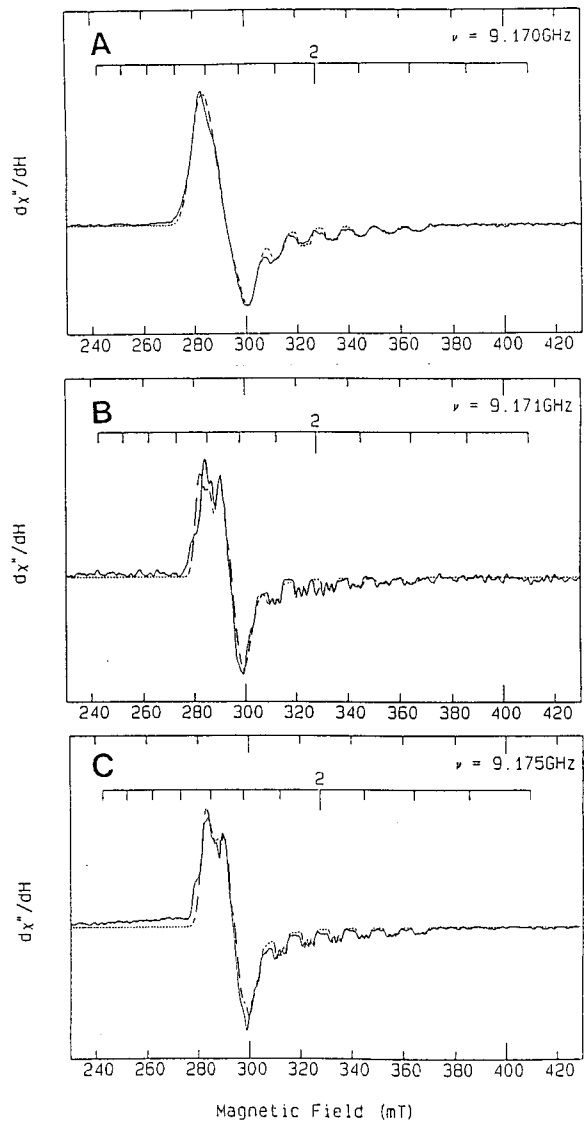

Figure 6 EPR spectra of Cbl(II)–glutamate mutase complexes

(**A**) Cbl(II) in free solution; (**B**) enzyme–Cbl(II) complex; (**C**) enzyme–Cbl(II)–5′-deoxyadenosine complex. Fits to the experimental data are indicated by dashed lines.

the same anaerobic conditions as the others and the spectral changes were reproducible, so therefore we consider it unlikely that adventitious oxidation of the sample is responsible for these changes.

In contrast, when 5 mM 5′-deoxyadenosine, the intermediate formed through Co–C-bond homolysis and hydrogen abstraction, was present, quite extensive further changes in the UV-visible spectrum of the enzyme–coenzyme complex were observed (Figure 4, spectrum e). The difference spectrum (Figure 5, spectrum d) clearly shows the blue shifts of the 470 and 404 nm absorption bands that move by about 6 nm. Control experiments established that the spectral changes were specific to the ternary complex of protein, Cbl(II) and 5′-deoxyadenosine: in the absence of protein, 5′-deoxyadenosine caused no changes in the spectrum of Cbl(II) between 350 and 800 nm. Addition of the substrates to the enzyme–Cbl(II)–5′-deoxyadenosine complex did not result in further changes to the UV-visible spectrum (results not shown).

EPR spectra of glutamate mutase–Cbl(II) complexes

EPR spectroscopy was used to examine the possibility that the changes in the UV-visible spectra of the enzyme–Cbl(II) complexes result from changes in the co-ordination of the axial histidine ligand that co-ordinates cobalt when the protein binds the coenzyme. Indeed, changes in the co-ordination strength of the axial base have been postulated as a potential mechanism for controlling the reactivity of AdoCbl. EPR spectroscopy of Cbl(II) provides a useful probe to examine the co-ordination of ligands to the paramagnetic Co(II) atom. The axial nitrogen ligand to cobalt imposes a superhyperfine coupling that results in a characteristic triplet splitting of the hyperfine octet structure [29,30].

The EPR spectra of free Cbl(II), the glutamate mutase–Cbl(II) complex and the glutamate mutase–Cbl(II)–5′-deoxyadenosine complex are shown in Figure 6. It is evident that binding of Cbl(II) results in some sharpening of the superhyperfine structure, suggesting that the histidine ligand of the protein (MutS His-16) may co-ordinate cobalt more strongly than the intrinsic dimethylbenzimidazole ligand of the coenzyme. However, the inclusion of 5′-deoxyadenosine in the sample results in an EPR spectrum that is virtually identical with that of the glutamate mutase–Cbl(II) complex. We may conclude, therefore, that 5′-deoxyadenosine does not perturb the electronic structure of the cobalt atom to any measurable extent. The addition of substrates to the glutamate mutase–Cbl(II) complex similarly did not noticeably change the superhyperfine coupling pattern (results not shown).

The EPR data suggest that it is unlikely that a change in the co-ordination strength of the axial cobalt ligand is responsible for the changes observed in the UV-visible spectra of the Cbl(II) complexes. An alternative explanation is that the UV-visible spectral changes arise from a distortion of the corrin ring that in turn results in changes in π–π^* transitions of the chromophore. As discussed below, this phenomenon is well documented for the more extensively studied haem-containing proteins.

The electronic spectra of corrin tetrapyroles have not been studied extensively and as a result the electronic transitions associated with their UV-visible spectra have not been assigned. There is, however, a very large body of literature dealing with the electronic properties of porphyrins that possess a 4-fold symmetric π system that results in a simplified electronic spectrum. Porphyrins, of course, play a wide variety of roles in Nature and there has been much interest in how proteins modulate their reactivity (see, for example, [31–33]). A well-documented example of a protein-enforced distortion of the porphyrin ring is the ruffling twist of the iron porphyrin cofactor in cytochrome *c* [34]. Shelnutt and co-workers [35] have used a series of model porphyrins to demonstrate that the Soret and Q bands of the porphyrin spectrum undergo a red shift dependent on the degree of ruffling distortion.

The electronic spectra of cobalamins are more complex than the spectra of porphyrins, but arise from similar π–π^* electronic transitions. The magnitude of the observed blue shifts of the 404 and 470 nm bands in the spectrum of Cbl(II) are perfectly consistent with a distortion of the corrin ring, although they do not reveal the nature of the deformation. Certainly, it seems reasonable, given that adenosine is initially covalently linked to cobalamin, that steric interactions between 5′-deoxyadenosine and Cbl(II) bound at the active site could result in a deformation of the conformationally flexible corrin ring. The mechanistic significance of this interaction is presently unclear. One attractive

idea is that such a distortion of the corrin ring might disfavour the recombination of adenosyl radical and Cbl(II) and thereby stabilize the free-radical species that participate in the subsequent steps of the mechanism.

Conclusions

The binding of AdoCbl to glutamate mutase appears to be entirely entropically driven. We find no evidence that, in the absence of substrate, binding of AdoCbl to the protein weakens the Co–C bond. Indeed, such destabilization would be disadvantageous because homolysis would be likely to occur in the absence of substrate and lead to destruction of the coenzyme and inactivation of the enzyme. If homolysis of AdoCbl is triggered as envisioned in the mechanochemical hypothesis, this event must be linked to substrate binding. Activation of the coenzyme by a mechanism that involves stabilizing the products of homolysis appears to be a more likely explanation for the remarkable ability of AdoCbl-dependent enzymes to catalyse the formation of free radicals. The ligand-induced changes in the UV-visible spectrum of the enzyme–Cbl(II) complex provide the first spectroscopic evidence for an enzyme-induced distortion of the Cbl(II) form of the coenzyme. At present the mechanistic significance of this distortion is unclear and the details will have to await high-resolution crystallographic studies that are currently in progress.

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