Evidence that Cobalt−Carbon Bond Homolysis is Coupled to Hydrogen Atom Abstraction from Substrate in Methylmalonyl-CoA Mutase†

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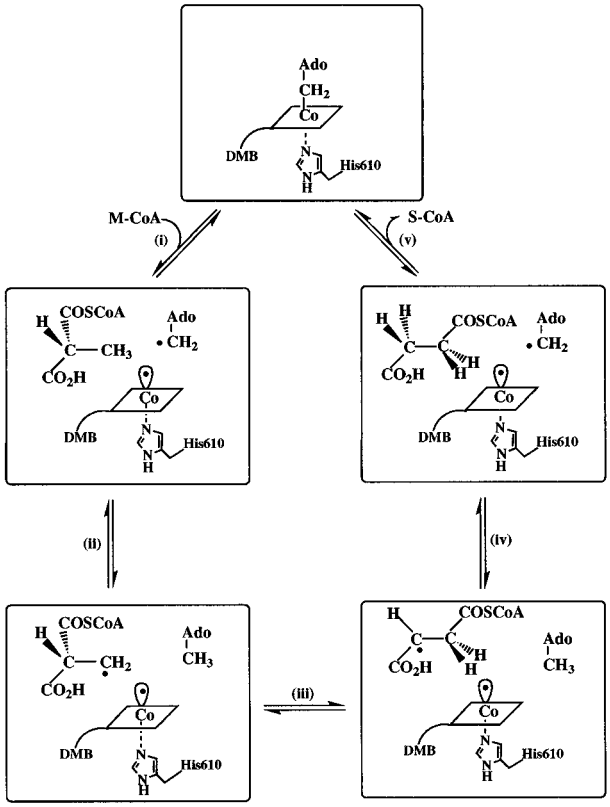
ABSTRACT: Methylmalonyl-CoA mutase catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA. It is dependent on the cofactor, coenzyme B_{12} or adenosylcobalamin, for activity. The first step in this, and other coenzyme B_{12}-dependent reactions, is postulated to be homolysis of the Co−C bond of the cofactor. Methylmalonyl-CoA mutase accelerates the rate of Co−C bond homolysis by a factor of $\sim 10^{12}$. The strategy employed by the enzyme for the remarkable labilization of this bond is not known. Using UV−visible stopped-flow spectrophotometry, we demonstrate that the Co−C homolysis rate in the presence of protiated substrate has a rate constant of >600 s^{-1} at 25 °C. In the presence of [CD_3]methylmalonyl-CoA, this rate decreases to 28 ± 2 s^{-1}. These results suggest that Co−C bond homolysis is coupled to hydrogen atom abstraction from the substrate and that the intrinsic binding energy of substrate may be a significant contributor to catalysis by methylmalonyl-CoA mutase.

Methylmalonyl-CoA mutase (EC 5.4.99.2), a member of the family of AdoCbl-dependent isomerases, catalyzes the 1,2 rearrangement of methylmalonyl-CoA to succinyl-CoA. In this group of enzymes, the cofactor, AdoCbl, functions as a latent source of radicals (Halpern, 1985). The first common step is postulated to be homolysis of the Co−C bond of the cofactor to generate a pair of radicals, cob(II)alamin and dAdo•[1] (Scheme 1, step i). This sets the stage for the subsequent radical chain propagation reaction which, in the simplest mechanism, involves hydrogen atom abstraction from the substrate (step ii), rearrangement to a product radical (step iii), and reabstraction of a hydrogen atom from deoxyadenosine (step iv). Then, the cofactor-derived radicals, in a reversal of the first step, recombine to complete a catalytic turnover cycle (step v).

A step that is central to this mechanism, and indeed one over which the enzyme must exert substantial control, is the first homolytic cleavage reaction. The reactive Co−C bond is fairly weak, with a bond dissociation energy (BDE) that is estimated to be $\sim 31.4 \pm 1.5$ kcal/mol (Finke & Hay, 1984; Hay & Finke, 1987). Comparison of the homolysis rate for AdoCbl in solution (4×10^{-10} sec^{-1} at 25 °C, (Waddington & Finke, 1993)) with the k_{cat} for methylmalonyl-CoA mutase (~ 60 sec^{-1} for the *Propionibacterium shermanii* enzyme at 25 °C) leads to a specific estimate of a 1.5×10^{11}-fold rate enhancement by the enzyme. This represents a lower limit for the mutase, since the rate constants for the individual steps in the catalytic cycle have not been reported. This mutase-specific acceleration is in the range ($10^{12\pm1}$) reported previously for AdoCbl-dependent enzymes (Finke & Hay, 1984). While the BDE for the cofactor bound to the enzyme has not been determi ned, the rate enhancement data predicts an ~15.5 kcal/mol destabilization of the Co−C bond during the reaction (Garr *et al.*, 1996). This could, in principle, be expressed in the ground-state, in the transition-state, or in a combination of the two [for reviews, see Halpern (1985), Pratt (1985), Finke (1990), Marzilli (1993)]. A sizable ground state destablization, i.e., in the absence of substrate, would be disadvantageous, potentially allowing escape of adenosyl radicals generated in the absence of substrate and leading to inactive enzyme. Direct evidence for substrate-induced labilization of the Co−C bond has been lacking.

Scheme 1: Postulated Reaction Mechanism for the Rearrangement of Methylmalonyl-CoA to Succinyl-CoA Catalyzed by Methylmalonyl-CoA Mutase[a]



[a] M-CoA and S-CoA refer to methylmalonyl-CoA and succinyl-CoA, respectively.

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[1] Abbreviations used: AdoCbl, adenosylcobalamin; Co−C, cobalt−carbon; KPi, potassium phosphate; dAdo•, deoxyadenosyl radical.

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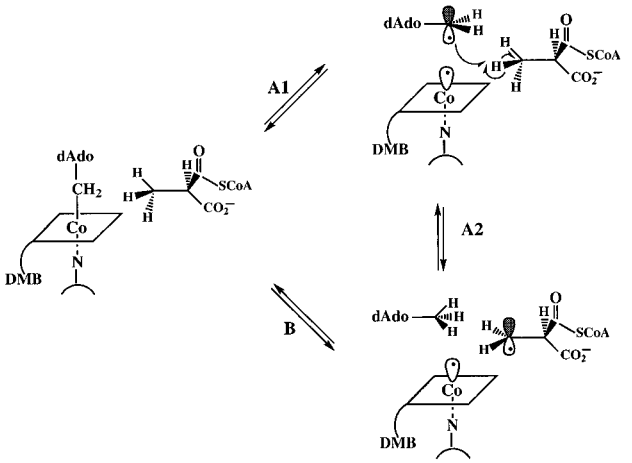
Scheme 2: Alternative Pathways for Coupling Co−C Bond Homolysis to Generation of Substrate Radical[a]



[a] A and B represent stepwise and concerted routes, respectively.

Indirect evidence however implicates a role for the substrate in weakening of the Co−C bond, since in its absence, cob(II)alamin is not formed to a significant extent in AdoCbl-dependent enzymes (Tamao & Blakeley, 1973; Orme-Johnson *et al.*, 1974; Hollaway *et al.*, 1978; Zhao *et al.*, 1992).

In this study, we have employed stopped-flow UV−visible spectroscopy to directly measure the Co−C homolysis rate for AdoCbl bound to methylmalonyl-CoA mutase. Unexpectedly, we find that the rate of Co−C bond homolysis is sensitive to isotopic substitution on the substrate, decreasing ~20-fold when CD_3-methylmalonyl-CoA is employed. Our results provide the first evidence that, on methylmalonyl-CoA mutase, Co−C bond homolysis may be coupled to hydrogen atom abstraction from the substrate (Scheme 2). This indicates that the intrinsic binding energy of the substrate is utilized by methylmalonyl-CoA mutase to labilize the Co−C bond. The strategy of employing a substrate trigger would provide the enzyme with a modus operandi for controlling cofactor reactivity and minimizing dissipation of its free-radical reservoir.

MATERIALS AND METHODS

Materials. Protocatechuic acid (PCA) and AdoCbl were purchased from Sigma. Protocatechuate dioxygenase (PCD) was a gift from Prof. John Lipscomb (University of Minnesota). (*R,S*)-Methylmalonyl-CoA and [CD_3]methylmalonyl-CoA were synthesized as described previously (Padmakumar *et al.*, 1993, 1997). The protiated and deuterated substrates were purified using identical procedures as described previously (Padmakumar *et al.*, 1993, 1997).

Bacterial Strains and Enzyme Preparation. The recombinant expression vector (pMEX2/pGP1-2) harboring the *P. shermanii* genes in *Escherichia coli* strain K 38 (McKie *et al.*, 1990) was a gift from Dr. Peter Leadlay (Cambridge University). The enzyme was purified, reconstituted with AdoCbl, and separated from unbound cofactor by FPLC as described before (Padmakumar & Banerjee, 1995). The specific activity of the enzyme was 28 μmol/min/mg at 37 °C in the radiolabel assay that has been described previously (Padmakumar & Banerjee, 1995).

Pre-Steady-State Kinetic Experiments. Pre-steady-state kinetic experiments were performed at 25 ± 1 °C on a DX.17MV sequential stopped-flow ASVD spectrofluorimeter from Applied Photophysics (U.K.) The temperature of the mixing chamber was controlled by a circulating water bath, and both were continually bubbled with N_2 gas. The syringes, tubing, and mixing chamber of the stopped-flow apparatus were deoxygenated by filling the drive syringes with a solution containing 400 μM PCA and 0.2 units of PCD in 50 mM KPi buffer (pH 7.4), mixing the solutions and waiting for at least 3 h before initiating the experiments. The enzyme solution containing methylmalonyl-CoA mutase (100 μM in bound AdoCbl) in 50 mM KPi buffer, pH 7.5, in a tonometer was made anaerobic by taking it through several cycles of alternately evacuating and flushing with purified nitrogen followed by equilibration with nitrogen at 4 °C over a 60 min period. A syringe containing 12 mM (*R,S*)-methylmalonyl-CoA or 10 mM (*R,S*)-[CD_3]methylmalonyl-CoA was made anaerobic by bubbling with purified nitrogen for 15 min. The concentrations of enzyme and substrate refer to those before mixing; both solutions are diluted 2-fold after mixing. Typically, the results from three shots, each containing 1000 data points, were individually fitted to eq 1, where C is the amplitude, and k is the rate constant.

$$A = Ce^{-kt} \quad (1)$$

Enzyme-monitored turnover was followed at two wavelengths: decrease in absorbance at 525 nm and increase in absorbance at 475 nm which accompanies homolysis of the Co−C bond of AdoCbl.

To obtain spectra of the enzyme at different time points, separate stopped-flow experiments were performed and the wavelength was stepped up from 320 to 620 nm at 20 nm intervals. The data were then collated and smoothed by a cubic spline fit by the "point-by-point" method (Applied Photophysics-supplied software) to give plots of absorbance *versus* wavelength.

RESULTS

Mutase Reaction with [CH_3]Methylmalonyl-CoA. Catalytic events associated with the pre-steady-state phase of the reaction were studied by monitoring spectral changes associated with the mutase-bound AdoCbl following mixing with substrate. A time course for the absorbance changes at 525 nm reveals a very rapid pre-steady-state disappearance of AdoCbl with an estimated $t_{1/2}$ of ~1-1.5 ms and a rate that is too fast to measure (Figure 1). The instrument dead time is ~2 ms, so only a small fraction of the total absorption change is observed in the pre-steady-state phase of the reaction. We estimate k_{obsd} is >600 s^{-1}; however, this should be viewed *strictly* as an approximation.

The pre-steady-state change is followed by the steady-state phase of the reaction, followed by a third phase. We tentatively assign the observed approach out of steady-state to an equilibrium state (data not shown). The reaction catalyzed by methylmalonyl-CoA mutase is reversible, with the equilibrium favoring product by a factor of 23 (Kellermeyer *et al.*, 1964). On the basis of a $\Delta\epsilon = 4.8$ mM^{-1} cm^{-1} (Licht *et al.*, 1996), the change in absorbance at 525 nm between $t = 0$ and $t = 10$ ms indicates that ~78% of the enzyme exists as AdoCbl during steady-state.

Mutase Reaction with [CD_3]Methylmalonyl-CoA. The effect of deuterated methylmalonyl-CoA on the time course

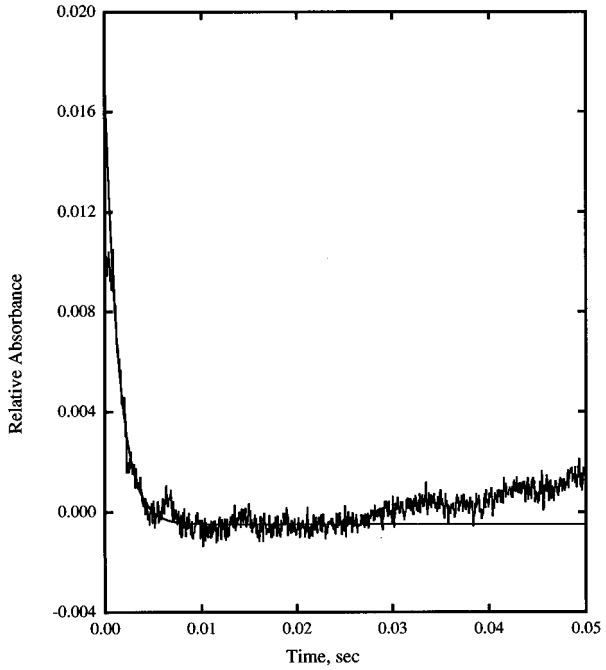


FIGURE 1: Change in absorbance at 525 nm following mixing of methylmalonyl-CoA mutase (50 μM before mixing) with (*R,S*)-[CH_3]methylmalonyl-CoA (5 mM before mixing). The kinetic trace was fitted to a single exponential equation (see Materials and Methods).

of the reaction was examined next. Unexpectedly, isotopic substitution of the substrate affected the pre-steady state phase of the reaction (Figure 2A). The pre-steady-state rate was decreased to 28 ± 2 s^{-1}. The *P. shermanii* methylmalonyl-CoA mutase exhibits a primary deuterium kinetic isotope effect of 6.2 (Michenfelder *et al.*, 1987). This is consistent with the ~5−10-fold longer transit time in the second, steady-state phase of the reaction in the presence of deuterated substrate (Figure 2B). On the basis of the change in absorbance, between $t = 0$ and $t = 1$ s, ~75% of the enzyme is estimated to be present as AdoCbl during steady-state. An isotope effect on the third phase (i.e., approach out of steady-state) is also observed. We do not know what the isotope effect on this phase represents.

Spectrum of Methylmalonyl-CoA Mutase During Steady-State Turnover. To verify whether the data obtained by single-wavelength monitoring truly reflected the proportion of enzyme-bound intermediates, the spectral morphology of the enzyme during turnover was established. As can be seen from the spectra in Figure 3, two species with isosbestic points at 330, 394, 484 nm for their interconversion are discernable. The isosbestic points are comparable to those seen in the steady-state spectrum of ethanolamine ammonia lyase (at 393 nm and 486 nm, (Hollaway *et al.*, 1978)) where a significantly higher proportion of cob(II)alamin (58%) is present, making it easier to observe.

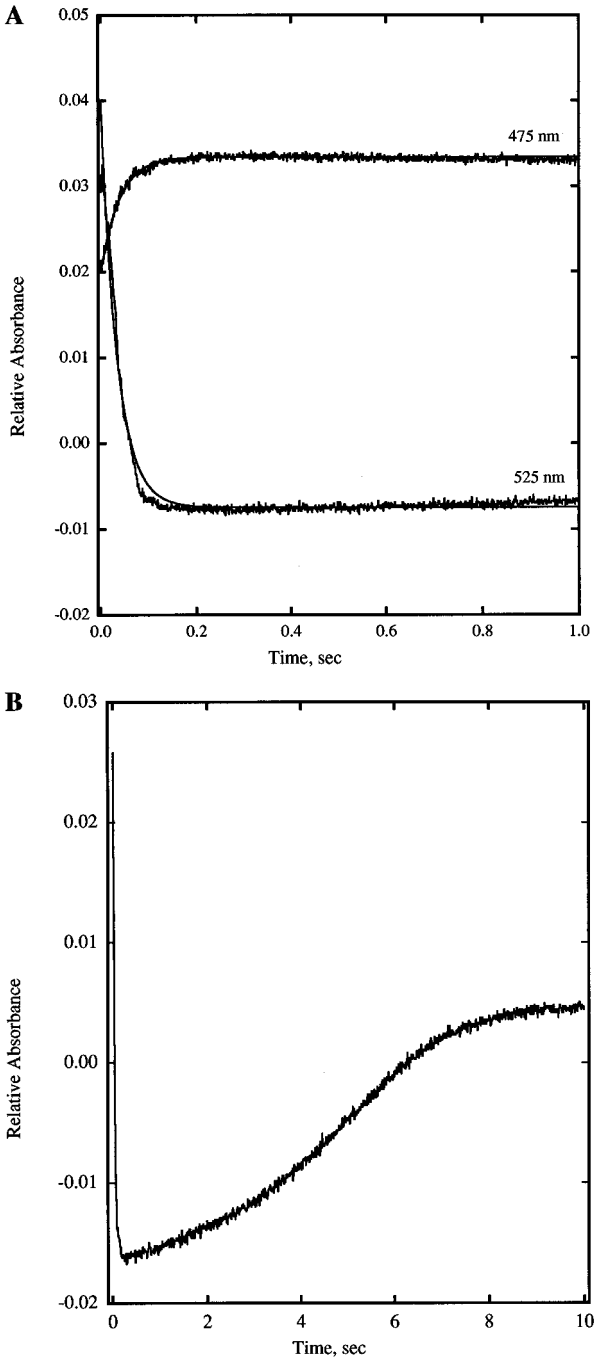


FIGURE 2: Absorbance changes accompanying mixing of methylmalonyl-CoA mutase with [CD_3]methylmalonyl-CoA. (A) Rate of Co−C homolysis in the presence of [CD_3]methylmalonyl-CoA. Holoenzyme was shot against substrate as described under Materials and Methods. The absorbance changes were detected at 475 and 525 nm. The traces were fitted to a single exponential equation (see Materials and Methods). (B) Time course for reaction in the presence of deuterated substrate. Three phases are distinguished: pre-steady state leading to the steady-state phase, and the approach out of the latter to what is tentatively identified as equilibrium. The calculated rate constants were $k_{475} = 27$ and $k_{525} = 29$ s^{-1}.

DISCUSSION

A question that is central to the mechanism of AdoCbl-mediated rearrangement reactions is the strategy and source of energy employed to effect the approximately trillion-fold destabilization of the Co−C bond that is estimated to occur during these reactions (Finke & Hay, 1984; Hay & Finke, 1987). In the debate over this issue, considerable attention has been focused on the properties of the cofactor itself. The mechanochemical destabilization hypothesis argues that the flexibility of the corrin ring is harnessed to cause puckering, butterfly fashion, to create a crowded upper axial face (Grate & Schrauzer, 1979; Marzilli *et al.*, 1979; Toraya *et al.*, 1979; Chemaly & Pratt, 1980; Krouwer *et al.*, 1980; Glusker, 1982; Halpern *et al.*, 1984; Bresciani-Pahor *et al.*, 1985; Brown & Brooks, 1991). Alternatively, trans electronic effects, mediated by the lower axial nitrogenous ligand (which is DMB

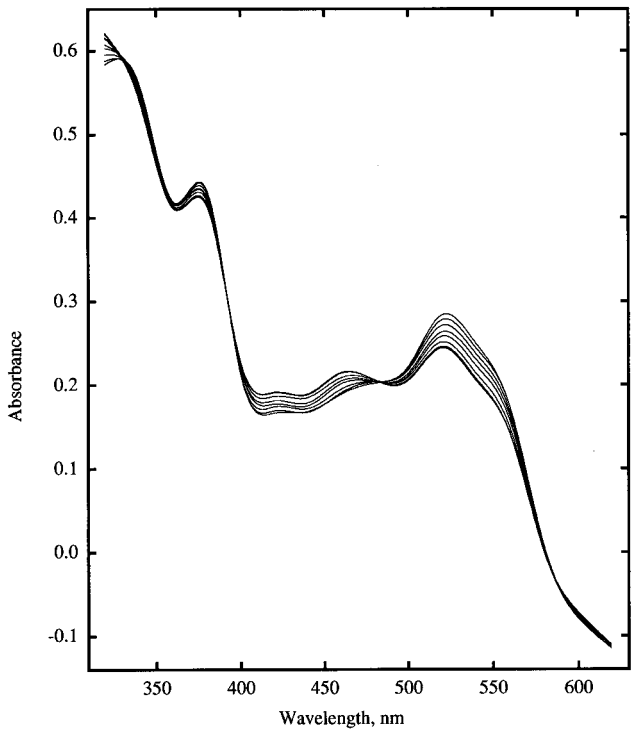


FIGURE 3: Spectra of methylmalonyl-CoA mutase during catalytic turnover in the presence of [CD_3]methylmalonyl-CoA. The spectra were generated from individual shots as described under Materials and Methods. The traces from top to bottom at 525 nm represent spectra at 4, 8, 14, 20 30, 50, 70, and 100 ms, respectively. The last two traces overlay on each other.

in solution and histidine in a subclass of AdoCbl-dependent enzymes) has been invoked (Ng & Rempel, 1982; Geno & Halpern, 1987). The role of the substrate and the utilization of its intrinsic binding energy in activating the Co−C bond have also been discussed, although it is more difficult to evaluate (Kräutler, 1987, Kräutler *et al.*, 1989, 1994; Garr & Finke, 1993; Waddington & Finke, 1993; Garr *et al.*, 1996).

The observed rate constant for Co−C bond cleavage in the presence of protiated substrate is estimated to be >600 s^{-1} at 25°C. Thus, methylmalonyl-CoA mutase accelerates the homolysis reaction by an estimated 1.5×10^{12}-fold, as predicted by earlier studies (Finke & Hay, 1984; Hay & Finke, 1987).[2] The homolysis rate is 10-fold higher than the k_{cat} for the enzyme (∼60 s^{-1} at 25 °C) and indicates that Co−C bond cleavage is not rate determining.[3] Under steady-state conditions, the enzyme exists predominantly as AdoCbl, with approximately 22% being present as cob(II)alamin. This agrees with the estimate obtained by EPR spectroscopic characterization of mutase reaction intermediates (Padmakumar & Banerjee, 1995). When enzyme is mixed with protiated substrate and frozen rapidly under steady-state conditions, spin quantitation of the EPR-active intermediates reveals the presence of 0.4 spin/mol of enzyme (Padmakumar & Banerjee, 1995). Since the intermediate on methylmalonyl-CoA mutase represents a radical pair, this corresponds to cob(II)alamin and a substrate-derived radical, each harboring 0.2 spins, respectively. The remaining 0.8 mol (or 80%) of cofactor is EPR-silent, consistent with it being in the AdoCbl form. This analysis is based on the assumption of a singlet ground state and has been employed previously with ribonucleotide reductase (Orme-Johnson *et al.*, 1974; Gerfen *et al.*, 1996; Licht *et al.*, 1996) and glutamate mutase (Zelder *et al.*, 1994) where similar EPR spectra are observed.

The rate constant for cob(II)alamin formation has been measured in other AdoCbl-dependent enzymes as well. In ethanolamine ammonia lyase it is ≥ 300 s^{-1} at 25 °C (Hollaway *et al.*, 1978; Harkins & Grissom, 1995), whereas in ribonucleotide reductase, it has been reported to be ∼40 s^{-1} (Tamao & Blakeley, 1973) and ∼250 s^{-1} (Licht *et al.*, 1996).[4] In both these enzymes, as in methylmalonyl-CoA mutase, catalytic turnover is not limited by homolysis, despite the ΔG for the homolysis reaction being unfavorable (Stubbe, 1988).

In the presence of [CD_3]methylmalonyl-CoA, a significant retardation of the Co−C bond cleavage rate is observed. The rate constant for this reaction is reduced to 28 s^{-1}, and the isotope effect on this isolated step is substantially larger than the overall deuterium isotope effect of 6.2 reported for this reaction (Michenfelder *et al.*, 1987). Under these conditions, hydrogen atom transfer becomes partially rate limiting. If Co−C bond cleavage (Scheme 1, step i) is independent of substrate radical generation (step ii), a substrate isotope effect on this step would not be expected.

A step that is believed to be common to all AdoCbl-dependent isomerases is the initial Co−C cleavage reaction. Yet, spectroscopic studies have not revealed the presence of an EPR-active radical pair intermediate in which dAdo· is coupled to cob(II)alamin (Orme-Johnson *et al.*, 1974; Licht, *et al*., 1996). In methylmalonyl-CoA mutase (Zhao *et al.*, 1994; Padmakumar & Banerjee, 1995), glutamate mutase (Zelder *et al.*, 1994), and ethanolamine ammonia lyase (Babior *et al*., 1974), the paramagnetic intermediates involve two interacting spins resident on cobalt and on an organic, substrate-derived radical. In ribonucleotide reductase, the EPR spectrum of the intermediate is similar to those of methylmalonyl-CoA mutase and glutamate mutase. The interacting spins are a protein-derived cysteinyl radical and cob(II)alamin (Gerfen *et al.*, 1996; Licht *et al.*, 1996).

A plausible explanation for why the dAdo· has not been detected in these reactions is that its formation is coupled to generation of the next radical in the catalytic cycle, which is either protein-derived (ribonucleotide reductase) or substrate-

[2] Although the overall enzymatic reaction achieves steady-state, the Co−C cleavage is much faster and so behaves like a presteady state rapid equilibrium. Hence, the observed rate constant for Co−C bond homolysis is the sum of those for the forward and reverse steps, and the catalytic efficiency of the enzyme in cleaving the Co−C bond may actually be lower than this estimate. We thank Prof. Kenneth Brown at Ohio University for pointing this out to us. The lower limit for the rate enhancement by methylmalonyl-CoA mutase obtained from k_{uncat}/k_{cat} is 1.5×10^{11} as described in this study.

[3] When apoenzyme is shot against a mixture of [CH_3]-methylmalonyl-CoA and AdoCbl, a significantly slower homolysis rate ($k = 17$ s^{-1} *versus* >600 s^{-1}) is observed. This could indicate that either conversion of apoenzyme to holoenzyme is slow (the base-off form of the cofactor binds to the enzyme although the base-on form predominates in solution at pH 7.5 (Padmakumar *et al.*, 1995; Mancia *et al*., 1996) or that a slow conformational change of the enzyme to a catalytically active form follows cofactor binding. A similar decrease in the rate constant (300 s^{-1} to 5 s^{-1}) for Co−C bond homolysis has been reported for ethanolamine ammonia lyase when apoenzyme rather than holoenzyme is mixed with substrate (Holloway *et al*., 1978).

[4] The discrepancy between the two estimates may arise from the difference in the reduction system employed. Tamao and Blakeley (1973) used dihydrolipoate, whereas Licht *et al.* (1996) employed reduced thioredoxin. We thank Prof. JoAnne Stubbe at MIT for pointing this out to us.

derived (methylmalonyl-CoA mutase) (Scheme 2). In principle, this could occur *via* either a stepwise (Scheme 2, A) or a concerted (Scheme 2, B) pathway. The difference between the two is in the existence of dAdo· as a discrete species in the former but not in the latter. Either pathway would afford the enzyme control over its latent radical reservoir (i.e., bound AdoCbl) by using substrate binding to trigger controlled mobilization of radicals from the cofactor. Recent studies on ribonucleotide reductase indicate that Co−C bond homolysis in this enzyme is coupled to the generation of a thiyl radical (Booker *et al.*, 1994; Licht *et al.*, 1996). Interestingly, when the working thiyl radical (Cys408) is mutated to serine, homolysis is not observed (Booker *et al.*, 1994). In contrast, in ethanolamine ammonia lyase, a substrate isotope effect on Co−C bond cleavage is not observed (Harkins & Grissom, 1995). Large tritium isotope effects and other lines of evidence have suggested the participation of a protein radical on this enzyme (Weisblat & Babior, 1971; O'Brien *et al.*, 1985; Cleland, 1982; Tan *et al.*, 1986). However, there is no evidence from freeze−quench EPR studies (Babior *et al.*, 1974) for a protein-based thiyl radical, which is expected to be more stable than a substrate-derived organic radical (Wu *et al.*, 1995). These results do not exclude the possibility of some other amino acid such as a glycine or tyrosine being the working radical. Alternatively, a discrete dAdo· radical could be formed in ethanolamine ammonia lyase, which is spin correlated with the cobalamin radical, and thus confers magnetic field sensitivity to this reaction (Harkins & Grissom, 1994, 1995). This is in contrast to the insensitivity of the methylmalonyl-CoA mutase-catalyzed reaction to magnetic field effects (Taoka *et al.*, 1997).

The spectrum of methylmalonyl-CoA mutase under steady-state turnover conditions is dominated by AdoCbl. Since we now know that Co−C bond homolysis is rapid, the predominance of AdoCbl suggests that a step following AdoCbl reformation, *viz*., product dissociation, is slow. The crystal structure of *P. shermanii* methylmalonyl-CoA mutase with a substrate fragment, dethia-CoA, bound to it has recently been reported (Mancia *et al.*, 1996). It reveals the presence of a buried active site accessible *via* a long, narrow tunnel that is occupied by the CoA-tail in the substrate-bound complex. The CoA moiety provides an extended binding surface, making multiple contacts with the protein. It is thus not unreasonable to assume that the reaction rate could be limited by the off-rate for product. Recent kinetic studies on methylmalonyl-CoA mutase indicate that the barrier to interconversion of substrate and product is low and the possibility of a rate limiting product dissociation has been raised (Meier *et al.*, 1996).

In summary, our results provide the first evidence that the intrinsic binding energy of substrate may be a significant contributor to the catalytic prowess of methylmalonyl-CoA mutase. This utilization of a substrate trigger would serve the important role of controlling reaction specificity, by ensuring that inherently reactive radical intermediates are generated only within the confines of an active site closed off to solvent by the substrate CoA-tail. Examples of substrate-induced destabilization mechanisms are numerous in the literature (Jencks, 1975). For instance, the binding energies that can be made available from the interaction of a hexose with hexokinase or hexose phosphate with phosphoglucomutase can be as large as −11 and −20 kcal/mol, respectively (Long & Ray, 1972; Jencks, 1975). A comparable rate enhancement on the cleavage of a single bond is displayed by β-galactosidase, in which a net $\Delta\Delta G^{\ddagger}$ lowering of ∼13.5 kcal/mol at 25 °C is achieved (Jones *et al.*, 1977; Garr *et al.*, 1996). These values fall within the realm of destabilization of the Co−C bond (∼15.5 kcal/mol) that is predicted to occur during the mutase-catalyzed reaction. The development of destabilization energy could occur *via* a conformational change in the enzyme resulting from substrate binding. Strong experimental evidence for large conformational changes in enzymes accompanying substrate binding exists; a classic example being hexokinase (Anderson *et al.*, 1979). With methylmalonyl-CoA mutase, the existence of conformational changes can be evaluated by structural and spectroscopic characterization of the enzyme and its bound cofactor in the presence and absence of substrate analogs or inhibitors.

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