Rearrangement of L-2-Hydroxyglutarate to L-*threo*-3-Methylmalate Catalyzed by Adenosylcobalamin-Dependent Glutamate Mutase†

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Received January 20, 2000; Revised Manuscript Received May 15, 2000

ABSTRACT: Adenosylcobalamin-dependent enzymes catalyze a variety of chemically difficult isomerizations in which a nonacidic hydrogen on one carbon is interchanged with an electron-withdrawing group on an adjacent carbon. We describe a new isomerization, that of L-2-hydroxyglutarate to L-*threo*-3-methylmalate, involving the migration of the carbinol carbon. This reaction is catalyzed by glutamate mutase, but $k_{cat} = 0.05\ s^{-1}$ is much lower than that for the natural substrate, L-glutamate ($k_{cat} = 5.6\ s^{-1}$). EPR spectroscopy confirms that the major organic radical that accumulates on the enzyme is the C-4 radical of L-2-hydroxyglutarate. Pre-steady-state kinetic measurements revealed that L-2-hydroxyglutarate-induced homolysis of AdoCbl occurs very rapidly, with a rate constant approaching those measured previously with glutamate and methylaspartate as substrates. These observations are consistent with the rearrangement of the 2-hydroxyglutaryl radical being the rate-determining step in the reaction. The slow rearrangement of the 2-hydroxyglutaryl radical can be attributed to the poor stabilization by the hydroxyl group of the migrating glycolyl moiety of the radical transiently formed on the migrating carbon. In contrast, with the normal substrate the migrating carbon atom bears a nitrogen substituent that better stabilizes the analogous glycyl moiety. These studies point to the importance of the functional groups attached to the migrating carbon in facilitating the carbon skeleton rearrangement.

Adenosylcobalamin (AdoCbl),[1] a biologically active form of vitamin B_{12}, is the cofactor for a group of enzymes that catalyze chemically difficult reactions involving the cleavage of C−C, C−O, or C−N bonds (*1*, *2*). In the 40 years since the coenzyme was discovered only about 12 AdoCbl-dependent reactions have been described (*3*−*6*). These reactions have attracted attention because, in general, they lack obvious counterparts in conventional organic chemistry. They proceed through highly reactive free radical intermediates that are produced by homolysis of the cobalt−carbon bond in the coenzyme. Most AdoCbl-dependent reactions involve an isomerization in which a nonacidic hydrogen on one carbon is interchanged with an electron-withdrawing group on an adjacent carbon (*3*−*6*).

The first B_{12}-dependent reaction to be discovered was the rearrangement of L-glutamate to L-*threo*-3-methylaspartate (*7*), catalyzed by glutamate mutase (*8*−*11*). This reaction constitutes the first step in the anaerobic fermentation of glutamate by various *Clostridium* species (*12*). The enzyme comprises two subunits, E and S, and is active as an E_2S_2 tetramer that binds AdoCbl at the interface of the E and S subunits (*11*, *13*). Recently, crystal structures of the enzyme have been solved with methylcobalamin and cyanocobalamin bound to the enzyme (*14*), and the solution structure of the small S subunit has been determined by NMR methods (*15*).

Various mechanistic studies have investigated the kinetic and chemical competence of postulated intermediates in the enzymic reaction. EPR spectroscopy employing isotopically labeled glutamates has been used to examine the relative steady-state concentrations of various organic radicals on the enzyme (*16*). Rapid-quench experiments have demonstrated that, in both directions, isomerization of the substrate, rather than product release, is rate determining in the reaction and have shown that the formation of 5′-deoxyadenosine is kinetically competent for catalysis (*17*). Stopped-flow spectroscopy has shown that homolysis of AdoCbl and hydrogen abstraction from the substrate are coupled events, implying that the 5′-deoxyadenosyl radical does not accumulate on the enzyme (*18*). Furthermore, the intrinsic isotope effects for hydrogen transfer between substrate and coenzyme were found to be unusually large, $^{D}V \sim 30$, suggesting that quantum tunneling may play an important role in this reaction (*18*).

The mechanism by which substrate and product radicals interconvert is one of the more poorly understood aspects of B_{12}-dependent isomerizations. The carbon skeleton rearrangements catalyzed by methylmalonyl-CoA mutase (*19*−*21*), isobutyryl-CoA mutase (*22*), and 2-methyleneglutarate mutase (*23*) involve the migration of an sp^2 hybridized carbon. The substrate radicals formed in these reactions can rearrange through a low-energy pathway involving a cyclopropyl intermediate, as shown in Figure 1, and this mech-

†This research has been supported by NIH Research Grants GM59227 to E.N.G.M. and GM20877 to D.P.B.

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[1] Abbreviations: AdoCbl, adenosylcobalamin; Cbl(II), cob(II)alamin.

10.1021/bi000121b CCC: $19.00 © 2000 American Chemical Society
Published on Web 07/26/2000

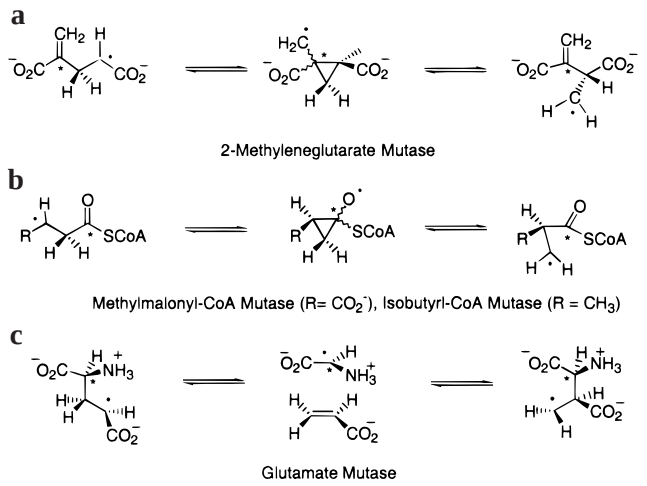

FIGURE 1: AdoCbl-dependent carbon skeleton isomerizations. The rearrangement of substrate radicals is presumed to occur either through a cyclopropyl intermediate (a, b) or, in the case of glutamate mutase, by fragmentation and recombination (c). The migrating carbon is marked by an asterisk.

anism is well supported by model studies (*24*, *25*). However, the interconversion of glutamyl and methylaspartyl radicals catalyzed by glutamate mutase is unique in that it involves the 1,2-migration of an sp^3 hybridized carbon (Figure 1), a reaction that is generally unfavorable. A plausible mechanism (Figure 1) involves fragmentation of the glutamyl radical to give a glycyl radical and acrylate, followed by addition of the glycyl radical to the other end of the acrylate double bond to form the 3-methylaspartyl radical (*26*).

Glutamate mutase exhibits very high substrate specificity, a feature common to most AdoCbl-dependent isomerases, and previously no other substrates have been demonstrated. Here we show that glutamate mutase can catalyze the rearrangement of L-2-hydroxyglutarate to L-*threo*-3-methylmalate. The 2-hydroxyglutaryl radical appears to be formed rapidly in the reaction, but turnover is much slower than with glutamate. Pre-steady-state experiments point to the rearrangement of the 2-hydroxyglutaryl radical as being rate determining.

MATERIALS AND METHODS

Materials. The purification of the glutamate mutase fusion protein, GlmES, from a recombinant *Escherichia coli* strain has been described previously (*27*). AdoCbl, L-2-hydroxyglutaric acid, and D-2-hydroxyglutaric acid were purchased from Sigma Chemical Co. The enzymatic synthesis of 5′-tritiated AdoCbl has been described previously (*28*). The sources of other materials have been described previously (*18*) or were purchased from commercial suppliers.

Synthesis of L-*[4,4-$^2H_2$]-2-Hydroxyglutarate.* Unlabeled L-2-hydroxyglutarate was refluxed in 6 M DCl for 3 days in a modification of the procedure used to synthesize L-[4,4-$^2H_2$]glutamate (*16*). This resulted in material that was greater than 90% dideuterated at the C-4 position as judged by proton NMR.

Assay of Enzyme Activity with L*-2-Hydroxyglutarate.* A solution containing 5 μM GlmES and 25 μM AdoCbl in 10 mM potassium phosphate buffer, pH 7.0, was made anaerobic by repeatedly evacuating and flushing with argon. Using a gastight syringe, 0.35 mL of the solution was transferred to an Eppendorf tube fitted with a septum and purged with argon. A concentrated anaerobic solution of L-2-hydroxyglutarate in the same buffer was introduced into the Eppendorf tubes to initiate the reaction. Fifty microliter aliquots were withdrawn from the reaction at various times and quenched with 2 μL of 2 N hydrochloric acid.

Derivatization of Products as the p-Nitrobenzyl Diester. The pH of the solution was raised by adding 80 μL of 1 M potassium phosphate buffer, pH 6.6. Next 40 μL of 0.5 M *p*-nitrobenzyl bromide in CH_3CN was added. The tubes were sealed, and the derivatization reaction was allowed to proceed overnight at 78 °C. The *p*-nitrobenzyl diester derivatives of L-2-hydroxyglutarate and L-*threo*-3-methylmalate were separated by HPLC on a 15 cm × 4.7 mm C_{18} reverse-phase column. The column was equilibrated in 42:58 acetonitrile:water, and compounds were eluted isocratically at 1 mL/min and detected by their absorbance at 274 nm. L-*threo*-3-Methylmalate *p*-nitrobenzyl diester eluted after 16.3 min. The amount of product formed was determined from standard curves constructed by chromatography of authentic samples of DL-*threo*-3-methylmalate *p*-nitrobenzyl diester of known concentration.

EPR Spectroscopy. Samples containing 450 μL of 235 μM glutamate mutase protein and 235 μM AdoCbl in 149 mM potassium phosphate buffer, pH 8.0, and 30% glycerol were degassed and flushed with argon. The solutions were then introduced by syringe into argon-flushed EPR tubes. Fifty microliters of an anaerobic solution containing either 500 mM L-2-hydroxyglutarate or 500 mM L-[4,4-$^2H_2$]-2-hydroxyglutarate was then added to the EPR tubes to give a total sample volume of 500 μL and a final holoenzyme concentration of 210 μM. The reaction was allowed to proceed for 15−30 s before being frozen in liquid nitrogen. All manipulations were performed in dim light to minimize photolysis. EPR measurements were made in the dark 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 the following: microwave power, 10 mW; microwave frequency, 9.176 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; temperature, 115 K. For each spectrum, the analogue output was digitized on the computer using a data acquisition board made by ComputerBoard Inc. (Mansfield, MA).

Pre-Steady-State Kinetic Experiments. Pre-steady-state kinetic experiments were performed at 10 °C with a Kinetic Instruments stopped-flow apparatus. The temperature of the mixing chamber was controlled by a circulating water bath. The enzyme solution contained 125 μM glutamate mutase in 50 mM potassium phosphate buffer containing 1 mM EDTA and 10% glycerol. Immediately before the experiment, AdoCbl was added to a final concentration of 100 μM so that the effective concentration of holoenzyme was 100 μM. Solutions containing AdoCbl were shielded from bright light. The solution was placed in a glass tonometer and made anaerobic by repeated cycles of evacuation and flushing with purified argon. Substrates were dissolved in the same buffer as the enzyme, placed in glass syringes, and made anaerobic by bubbling purified argon through them for at least 10 min before use. Mixing in the stopped-flow apparatus diluted both substrate and enzyme 2-fold, so that the concentration of holoenzyme in the measured reaction mixture was 50 μM.

The reaction was monitored by following the change in absorbance at 530 nm that accompanies cobalt−carbon bond homolysis. For each concentration of substrate used, the data from at least three shots were averaged and were fitted to either single or multiple parallel exponential functions to obtain rate constants using Program A developed in the Ballou laboratory. Secondary plots of data and fits to binding curves were performed using the Kaleidagraph program (Abelbeck Software).

RESULTS

Although several glutamate analogues have been reported to act as competitive inhibitors of glutamate mutase, none appear to undergo turnover (*29*). Recently though, we discovered that 2-ketoglutarate is able to undergo a partial reaction with the enzyme that results in exchange of tritium between the coenzyme and 2-ketoglutarate, although the enzyme does not appear to catalyze the rearrangement of 2-ketoglutarate (*30*). Prompted by this observation, we investigated whether analogues of glutamate and methylaspartate in which the amino group is replaced by other functional groups could also undergo tritium exchange with AdoCbl.

These experiments used the engineered fusion protein of glutamate mutase, GlmES, in which the S-subunit is fused to the C-terminus of the E-subunit by an 11 amino acid flexible linker (*27*). This protein is better suited to kinetic analysis because complications due to concentration-dependent dissociation of the subunits are avoided (*18*, *27*).

Tritium Exchange between AdoCbl and Substrate Molecules. The tritium exchange assay provides a sensitive test for the ability of substrate analogues to initiate homolysis of AdoCbl and undergo reversible formation of radicals. Therefore, preliminary experiments were undertaken in which glutamate mutase was incubated with 5′-tritiated AdoCbl and the following substrate analogues, chosen to be isosteric with the true substrates: DL-2-methylglutarate, *meso*-2,3-dimethylsuccinate, DL-2-hydroxyglutarate, and DL-*threo*-3-methylmalate. Incubation with DL-2-hydroxyglutarate and DL-*threo*-3-methylmalate resulted in almost all the radioactivity being lost from the coenzyme within 1 h, whereas DL-2-methylglutarate and *meso*-2,3-dimethylsuccinate showed no reaction. When the experiment was repeated with D-2-hydroxyglutarate and L-2-hydroxyglutarate, only the L-isomer elicited tritium exchange.

The results of the tritium exchange experiments suggested that glutamate mutase may interconvert L-2-hydroxyglutarate and L-*threo*-3-methylmalate. This was confirmed by NMR experiments in D_2O. When glutamate mutase (20 μM) was incubated with 10 mM DL-*threo*-3-methylmalate in 50 mM potassium phosphate buffer, pD 7.0, slightly less than half of the racemic material was converted to hydroxyglutarate within 30 min at 25 °C (Figure 2). It is reasonable to assume that the L-isomer of 3-methylmalate is the substrate for the enzyme, in accord with the stereospecificity of the enzyme for L-*threo*-3-methylaspartate (*8*). However, when the enzyme was incubated with 10 mM L-2-hydroxyglutarate under similar conditions, less than 5% of the substrate was converted to 3-methylmalate, as judged by NMR. These results demonstrate that 2-hydroxyglutarate and 3-methylmalate are true substrates for the enzyme and suggest that the equilibrium favors 2-hydroxyglutarate by at least 20:1.

Steady-State Kinetic Properties with L-*2-Hydroxyglutarate.* A discontinuous assay was developed to investigate the kinetic behavior of glutamate mutase with L-2-hydroxyglutarate as the substrate. The holoenzyme was incubated at 25 °C with various concentrations of L-2-hydroxyglutarate for times ranging between 1 and 20 min, and the reaction was quenched by addition of HCl. The substrate and product were subsequently derivatized by treatment with *p*-nitrobenzyl bromide, pH 6.6, to form their di-*p*-nitrobenzyl esters. These were separated and quantified by reverse-phase HPLC as described in the Materials and Methods section.

With L-2-hydroxyglutarate as substrate, the rate of 3-methylmalate formation was constant for the first 10 min of the reaction, allowing reliable kinetic data to be obtained. (No turnover of D-2-hydroxyglutarate was detected in the assay, consistent with the lack of tritium exchange with AdoCbl.) By allowing the reaction to proceed to equilibrium, K_{eq} was determined as 36 ± 3 in favor of hydroxyglutarate, consistent with NMR data. The apparent k_{cat} and K_m for the conversion of 2-hydroxyglutarate to 3-methylmalate were 0.05 ± 0.003 s^{-1} and 1.2 ± 0.3 mM, respectively. For comparison, k_{cat} and K_m for the natural substrate, L-glutamate, are 5.6 s^{-1} and 0.6 mM, respectively (*27*).

EPR Spectroscopy. To establish that the 2-hydroxyglutaryl radical was formed during the reaction, the EPR spectrum of hologlutamate mutase was recorded in the presence of L-2-hydroxyglutarate at 115 K. The spectrum shown in Figure 3 is similar to the spectrum previously recorded for the enzyme when it was reacted with L-glutamate; the g values calculated from the spectrum were $g_{xy} = 2.09$ and $g_z = 1.968$, $A = 4.5$ mT, which compare with $g_{xy} = 2.09$ and $g_z = 1.968$, $A = 5$ mT, reported for the enzyme reacting with glutamate (*16*). The spectrum exhibits the expected 8-fold hyperfine splitting in the g_z region due to the interaction of the unpaired electron ($S = {}^1/_2$) with the nuclear spin of Co ($I = {}^7/_2$). The superhyperfine coupling, resulting from the interaction of the nitrogen ligands with Co(II), is not resolved, most likely due to the influence of the organic radical partner (*16*).

The complex fine structure of EPR spectra of AdoCbl-dependent enzymes arises in part from the coupling of the unpaired electron on cobalt and an organic radical; for glutamate mutase this organic radical has been identified as the C-4 radical of glutamate which is estimated to reside approximately 6.5 Å from the cobalt (*16*). To examine the structure of the organic radical formed by reaction with 2-hydroxyglutarate, the enzyme was reacted with L-[4-D_2]-2-hydroxyglutarate. The EPR spectrum (Figure 3) shows a sharpening of the hyperfine structure in the g_z region, and the $g = 2.14$ signal is resolved into two peaks. These changes to the spectrum are consistent with the C-4 radical of 2-hydroxyglutarate being the major organic radical that accumulates on the enzyme.

Pre-Steady-State Kinetic Studies of Substrate-Induced AdoCbl Homolysis. We examined the ability of L-2-hydroxyglutarate to initiate cleavage of the coenzyme by using UV−visible stopped-flow spectroscopy to measure the rate at which the Co−C bond of AdoCbl is homolyzed. The holoenzyme was reacted with various concentrations of L-2-hydroxyglutarate, and the time course for homolysis was followed by monitoring the decrease in absorbance at 530 nm due to the disappearance of AdoCbl. The reactions were

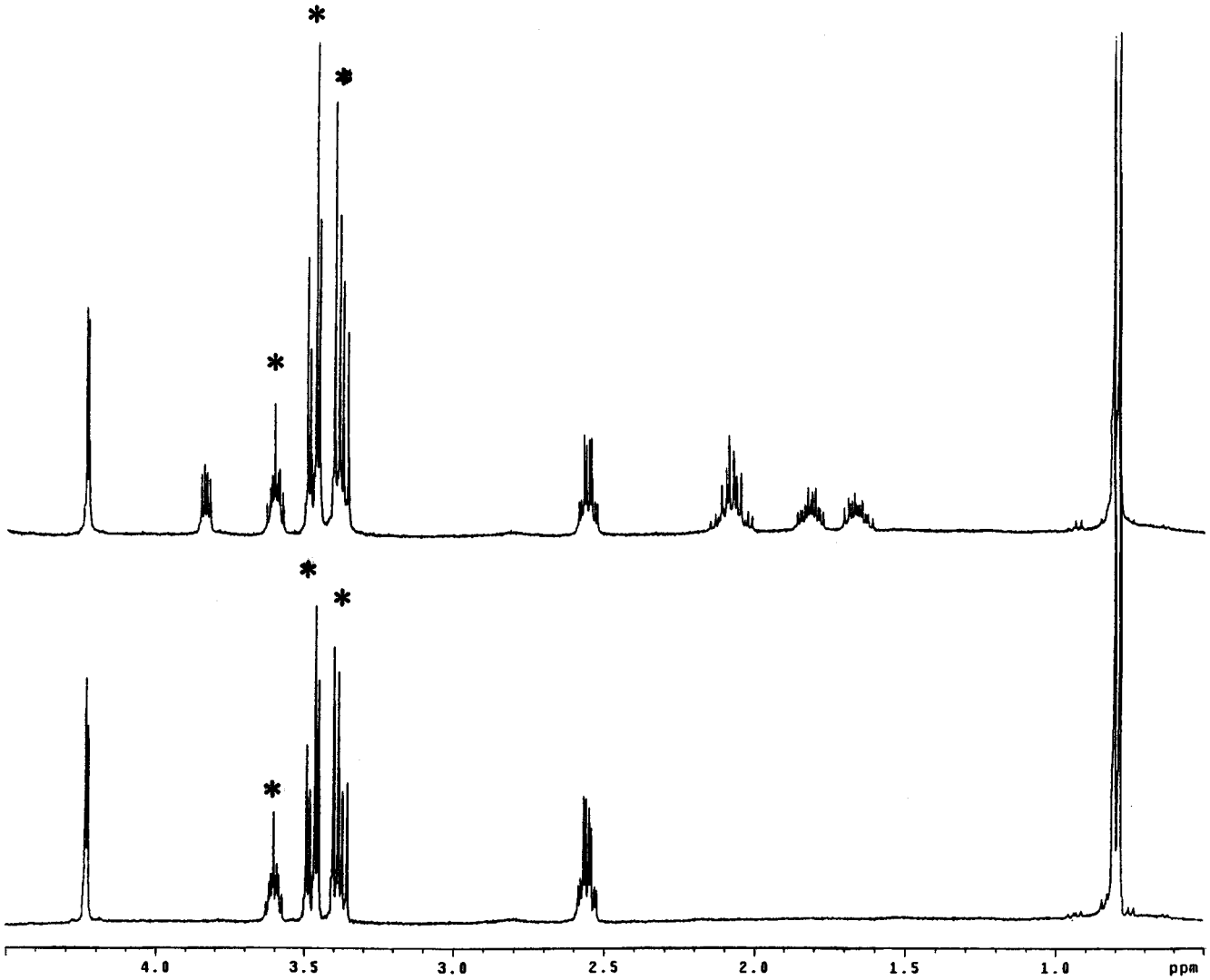

FIGURE 2: Conversion of 3-methylmalate to 2-hydroxyglutarate by glutamate mutase. Lower NMR spectrum: 10 mM DL-*threo*-3-methylmalate and 20 μM apoglutamate mutase in 50 mM potassium phosphate buffer, pD 7.0. Upper NMR spectrum: the same sample after addition of 20 μM AdoCbl and incubation at 25 °C for 30 min; the new peaks at $\delta = 3.85$, 2.1, 1.8, and 1.65 ppm correspond to the α, γ, β, and β' protons of 2-hydroxyglutarate, respectively. Peaks marked by an asterisk arise from residual glycerol present in the enzyme preparation.

typically monitored for 40 s, after which time no further changes in absorbance were evident. The kinetic behavior of the enzyme was studied with concentrations of 2-hydroxyglutarate that ranged from 50 μM (1 $\times$ enzyme concentration) to 100 mM (2000 $\times$ enzyme concentration). The UV−visible spectrum of the holoenzyme−substrate complex, recorded in the stopped-flow spectrophotometer at the end of each reaction, confirmed the formation of Cbl(II) (data not shown).

At concentrations of 2-hydroxyglutarate up to about 10 mM, the major absorbance change was well fitted by a single-exponential function, as shown in Figure 4A. Both the observed rate constant, k_{obs}, and the amplitude of the absorbance change increased as the substrate concentration was increased. At higher concentrations of substrate a much faster phase of the reaction became apparent (Figure 4A) that required a double-exponential function to satisfactorily fit the data. This behavior is reminiscent of that observed with the natural substrates, L-glutamate and L-*threo*-methylaspartate, where biphasic reactions have been attributed to negative cooperativity or half-of-the-sites behavior arising from the dimeric nature of the enzyme (*18*).

Plots of observed rate constants, k_{obs}, against substrate concentration for the slower phase of the reaction can be described by a simple binding isotherm as shown in Figure 4B. At saturating substrate concentrations the slower reaction constitutes about half of the total absorbance change. The reaction at this active site is characterized by a relaxation time, τ, $= 45 \pm 1.6$ ms ($k_{obs} = 22 \pm 1$ s^{-1}) and an apparent K_d for 2-hydroxyglutarate of 3.0 ± 0.6 mM. Because a significant proportion of the faster phase of the reaction occurred within the dead time of the spectrometer, k_{obs} and K_d associated with this active site could not be reliably determined, but k_{obs} is certainly greater than 100 s^{-1}and K_d is above 20 mM.

At all substrate concentrations studied a much slower, very small change in absorbance (less than 5% of the total absorbance change) was just discernible from the kinetic traces (data not shown). The observed rate constant, k_{obs}, for this slow phase was $\sim$0.5 s^{-1} and was independent of substrate concentration. A similar slow phase has also been observed with glutamate as substrate (*18*), but its physical significance is unclear.

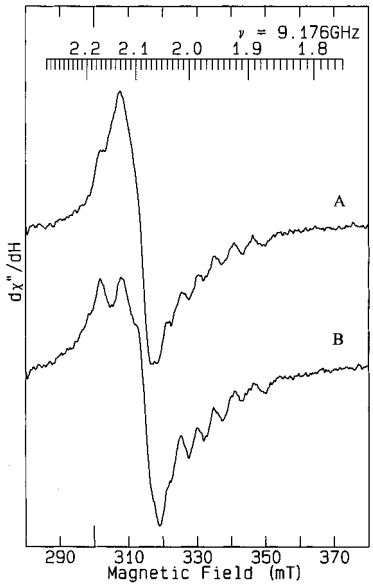

FIGURE 3: EPR spectra of glutamate mutase reacting with L-2-hydroxyglutarate: (A) spectrum obtained with protiated substrate; (B) spectrum obtained with L-[4,4-$^2H_2$]-2-hydroxyglutarate. For discussion see the text.

DISCUSSION

Glutamate mutase exhibits very strict substrate specificity; although various molecules have been tested as potential mechanism-based inhibitors and/or substrates, until now none have been shown to undergo rearrangement. The crystal structure of the enzyme−methylcobalamin−tartrate complex, solved recently (*14*), suggests that substrate specificity results from numerous hydrogen-bonding interactions between active site residues and the substrate. This is borne out by the fact that although 2-methylglutarate is isosteric with glutamate, it lacks hydrogen-bonding interactions at the 2-substituent and does not appear to bind to the enzyme. However, the intrinsic reactivity of the substrate may also be an important factor in determining whether a substrate molecule will turn over. Thus, although 2-ketoglutarate was previously shown to readily form radical species on the enzyme, no rearrangement products could be detected (*30*).

2-Hydroxyglutarate is converted to 3-methylmalate by the enzyme over 100 times more slowly than glutamate is converted to 3-methylaspartate. This is most likely because the rearrangement of the 2-hydroxyglutaryl radical to the 3-methylmalyl radical is very slow. As discussed below, several lines of evidence support this idea. First, 2-hydroxyglutarate reacts with AdoCbl to generate Cbl(II) much faster than it is converted to methylmalate, at a rate that approaches those observed with glutamate and methylasparate (*18*). We have shown previously that homolysis of AdoCbl and the hydrogen abstraction from the substrate are coupled events (*18*), so that the rate at which AdoCbl is cleaved corresponds to the rate at which substrate radicals are generated. This phenomenon has also been demonstrated for several other AdoCbl-dependent enzymes (*20*, *31*). Second, the changes in the EPR spectrum of the holoenzyme−hydroxyglutarate complex that occur upon deuteration at C-4 of 2-hydroxyglutarate are consistent with the C-4 radical of 2-hydroxyglutarate being the major organic radical that accumulates on the enzyme, although low concentrations of other radicals cannot be ruled out. Finally, the mechanism for the rearrangement of the 2-hydroxyglutaryl radical to the 3-methylmalyl radical likely involves a glycolate radical intermediate that is of high energy.

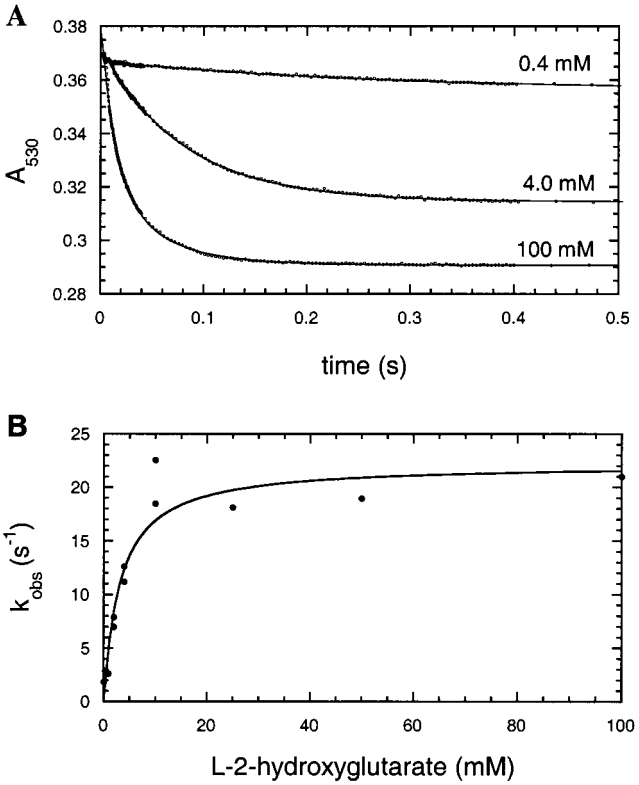

FIGURE 4: (A) Representative stopped-flow traces showing changes in absorbance at 530 nm, indicative of homolysis of AdoCbl, following the rapid mixing of hologlutamate mutase (50 μM final concentration) with various concentrations of L-2-hydroxyglutarate. The traces obtained with 0.4 and 4 mM 2-hydroxyglutarate are fitted to a single-exponential function, whereas the trace obtained with 100 mM 2-hydroxyglutarate is fitted to a double-exponential function. Data points are represented by circles; the fits, by solid lines. (B) Plot of the observed rate constant for homolysis of AdoCbl against 2-hydroxyglutarate concentration for the tighter binding active site of glutamate mutase. The lowest substrate concentration used to construct the plot was 10-fold higher than the enzyme concentration.

Kinetics of Cbl(II) Formation and Substrate Radical Rearrangement. The biphasic reaction kinetics observed in the reaction of the enzyme with 2-hydroxyglutarate most likely arises from negative cooperativity or half-of-the-sites reactivity between the two identical subunits of the enzyme. Such effects arise when substrate binding at one active site on the enzyme induces a conformational change in the protein that alters the affinity of the other active site for the substrate. Similar kinetic phenomena, attributable to negative cooperativity, have been observed previously with the natural substrates (*18*). In this case it appears that the tighter binding active site (apparent $K_d = 3.0 \pm 0.6$ mM, measured kinetically) reacts more slowly, $k_{obs} = 22 \pm 1$ s^{-1}, with 2-hydroxyglutarate to initiate homolysis of AdoCbl than does the weaker binding site ($K_d > 20$ mM) for which k_{obs} is significantly faster than 100 s^{-1}. Interestingly, the crystal structure of the enzyme reveals that the two active sites of the enzyme are separated by about 45 Å and are well away from the protein dimer interface (*14*). This suggests that the active site of the enzyme is very sensitive to what are most likely subtle perturbations in protein structure mediated through the dimer interface.

Scheme 1

$$\text{E:AdoCbl + S} \underset{k_{-1}}{\overset{k_1[S]}{\rightleftharpoons}} \text{E:AdoCbl:S} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E:Cbl(II):S}^{\bullet} \xrightarrow{k_3\ (\text{slow})} \text{E:AdoCbl + P}$$

Negative cooperative effects were not apparent from steady-state kinetic measurements. The K_m for 2-hydroxyglutarate is 1.2 mM, but even when the substrate concentration was raised to 100 mM, there was no evidence that a weak binding site was contributing to catalysis. It seems reasonable that at lower concentrations of substrate, at least, turnover is dominated by the flux of substrate through the tighter binding active site; in which case the true k_{cat} for this active site is 0.1 s^{-1}. (However, one cannot exclude the possibility that very rapid turnover by the weak-affinity active site might contribute significantly to the total flux of substrate, even at low concentrations.)

Assuming a single active site is contributing to catalysis, the reaction of 2-hydroxyglutarate with glutamate mutase may be described by kinetic Scheme 1.The first step is the reversible binding of the substrate to form the Michaelis complex (described by k_1 and k_{-1}); this is followed by the rapid and reversible formation of the 2-hydroxyglutaryl radical and Cbl(II), described by k_2 and k_{-2}. The final step, described by k_3, encompasses the rearrangement of the substrate radical, which we hypothesize to be the slow step, and the subsequent formation of product, which under initial velocity conditions may be considered irreversible.

It may be shown that k_{cat} and K_m for 2-hydroxyglutarate reacting with the enzyme according to Scheme 1 are given by eqs 1 and 2, respectively (*32*). Furthermore, when the

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_{-2} + k_3} \tag{1}$$

$$K_m = \frac{k_{-1}k_{-2} + k_{-1}k_3 + k_2 k_3}{k_1(k_2 + k_{-2} + k_3)} \tag{2}$$

substrate is at saturating concentrations, so that $k_1[S]$ is large, the fraction of the total enzyme in the Cbl(II) form is described by eq 3. When the slower reacting site on the

$$\frac{[\text{Cbl(II)}]}{[E_0]} = \frac{k_2}{k_2 + k_{-2} + k_3} \tag{3}$$

enzyme was reacted with saturating concentrations of 2-hydroxyglutarate, the stopped-flow measurements indicated that approximately 50% of the coenzyme molecules at this active site had undergone homolysis to form Cbl(II) by the time the steady state was reached. In other words the equilibrium constant for the formation of free radicals on the enzyme is ~1.

The observed rate constant for the formation of Cbl(II) is $k_{obs} = k_2 + k_{-2} + k_3$. Therefore, when this expression is substituted into eqs 1 and 3, the forward and reverse rate constants for the formation of Cbl(II) and the 2-hydroxyglutaryl radical, k_2 and k_{-2}, and the rate constant for the conversion of the 2-hydroxyglutaryl radical to product, k_3, may be calculated. k_2 and k_{-2} are both about 11 s^{-1} whereas $k_3 = 0.2$ s^{-1}. From this analysis it is evident that the formation of the 2-hydroxyglutaryl radical on the enzyme is freely reversible and occurs much faster than the conversion of this radical to product.

Previously, we measured the forward and reverse rates for the homolysis of AdoCbl reacting with L-glutamate at the slower active site of glutamate mutase as 19 and 78 s^{-1}, respectively (*18*). However, with this substrate the rate at which the substrate radical is formed and the rate at which it rearranges on the enzyme are similar (*17*), which complicates the interpretation of these data. For example, a significant component of the rate constant for recombination of Cbl(II) is likely to arise from the substrate radical partitioning forward to generate methylaspartate and AdoCbl, i.e., $k_3 \sim k_2$ for L-glutamate. Consistent with this, the steady-state concentration of Cbl(II) formed by reaction of the enzyme with glutamate is significantly lower ([Cbl(II)] = 25% of active sites) than that observed with 2-hydroxyglutarate. 2-Hydroxyglutarate may therefore prove to be a useful substrate with which to examine the kinetic and thermodynamic aspects of Co−C bond homolysis on the enzyme.

In principle, it is also possible to calculate values for k_1 and k_{-1} using eq 2 since the K_m and K_d for 2-hydroxyglutarate are known, but the data are not sufficiently accurate to permit meaningful values for these rate constants to be obtained. However, if k_3 is small relative to k_2 and k_{-2}, then eq 2 simplifies and K_m is approximated by eq 4, from which it is evident that K_m will be smaller than K_d. Using the values

$$K_m = \frac{k_{-1}}{k_1}\frac{k_{-2}}{(k_2 + k_{-2})} \tag{4}$$

obtained for K_d, k_2, and k_{-2} obtained from pre-steady-state measurements, K_m is calculated as 1.5 ± 0.4 mM, which is in qualitative agreement with the value of $K_m = 1.2 \pm 0.3$ mM obtained from steady-state measurements.

Overall, the kinetic properties of the enzyme reacting with 2-hydroxyglutarate are consistent with the rate-determining step occurring after the formation of the 2-hydroxyglutaryl radical.

Mechanism of Substrate Radical Rearrangement. Glutamate mutase is the only AdoCbl-dependent enzyme known that catalyzes the 1,2-migration of an sp^3-hybridized carbon, and chemically this is a much harder reaction than the migrations of sp^2-hybridized carbons catalyzed by acyl-CoA mutases and 2-methyleneglutarate mutase. For the natural substrate, glutamate, a chemically plausible mechanism for the carbon skeleton rearrangement involves fragmentation of the glutamyl radical to give acrylate and the glycyl radical, followed by recombination to give the methylaspartyl radical (*26*) (Figure 1). Tentative support for the intermediacy of the glycyl radical has recently been obtained by EPR spectroscopy of the enzyme reacting with isotopically labeled substrates (*16*) and by rapid-quench experiments aimed at trapping the glycyl radical (H.-W. Chih and E. N. G. Marsh, unpublished data). The analogous rearrangement of hydroxyglutarate would require the formation of a glycolate radical and acrylate, as shown in Figure 5. A precedent for an oxygen-stabilized radical comes from studies on ribonucleotide reductases where the 3′-radical of ribose is implicated in the mechanism of ribonucleotide reduction (*33*). However, a glycolate radical is expected to be less stable than a glycyl radical because the unpaired electron is much less effectively stabilized by

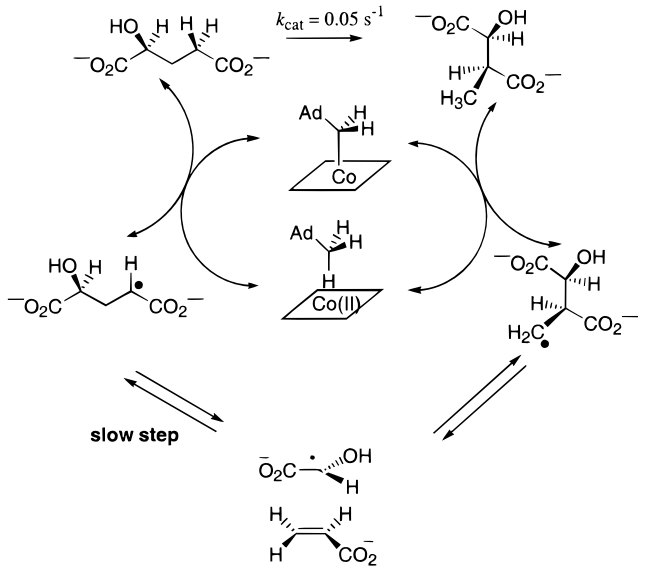

FIGURE 5: Proposed mechanism for the isomerization of L-2-hydroxyglutarate to L-*threo*-3-methylmalate by glutamate mutase.

a hydroxyl group than by an amino group (*34*). This would explain why k_{cat} for hydroxyglutarate is much lower than that for glutamate.

In summary, our results suggest that the rate-limiting step in the glutamate mutase-catalyzed conversion of 2-hydroxyglutarate to 2-methylmalate is most likely the rearrangement of the 2-hydroxyglutaryl radical to the 3-methylmalyl radical. This contrasts with the enzyme-catalyzed rearrangement of glutamate to methylaspartate where the rate constants for the formation of substrate radicals and the rate constants for their rearrangement are similar (*17*). The slow rearrangement of the 2-hydroxyglutaryl radical is in accord with the expected thermodynamically unfavorable formation of the required glycolyl radical. Our results demonstrate the sensitivity of the carbon skeleton rearrangement to changes in functionality at the migrating carbon atom. The need for functional groups in the substrate that stabilize the migrating carbon radical may be one reason AdoCbl-dependent carbon skeleton rearrangements appear to be very rare in nature.

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BI000121B