A Novel Reaction between Adenosylcobalamin and 2-Methyleneglutarate Catalyzed by Glutamate Mutase†

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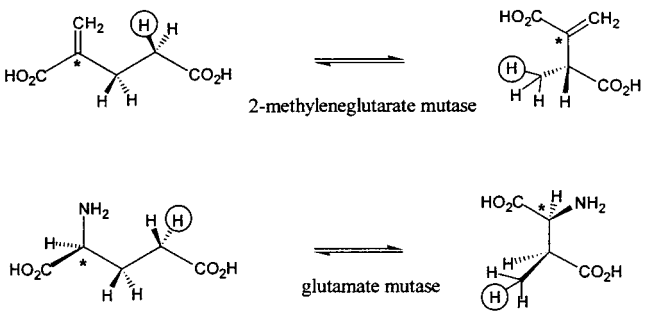
ABSTRACT: We describe a novel reaction of adenosylcobalamin that occurs when adenosylcobalamin-dependent glutamate mutase is reacted with the substrate analogue 2-methyleneglutarate. Although 2-methyleneglutarate is a substrate for the closely related adenosylcobalamin-dependent enzyme 2-methyleneglutarate mutase, it reacts with glutamate mutase to cause time-dependent inhibition of the enzyme. Binding of 2-methyleneglutarate to glutamate mutase initiates homolysis of adenosylcobalamin. However, instead of the adenosyl radical proceeding to abstract a hydrogen from the substrate, which is the next step in all adenosylcobalamin-dependent enzymes, the adenosyl radical undergoes addition to the exo-methylene group to generate a tertiary radical at C-2 of methyleneglutarate. This radical has been characterized by EPR spectroscopy with regiospecifically ^{13}C-labeled methyleneglutarates. Irreversible inhibition of the enzyme appears to be a complicated process, and the detailed chemical and kinetic mechanism remains to be elucidated. The kinetics of this process suggest that cob(II)alamin may reduce the enzyme-bound organic radical so that stable adducts between the adenosyl moiety of the coenzyme and 2-methyleneglutarate are formed.

Glutamate mutase and 2-methyleneglutarate mutase (MGM)[1] are two members of the class of adenosylcobalamin (AdoCbl, coenzyme B_{12})-dependent isomerases that catalyze apparently very similar reactions (*1*−*4*). Glutamate mutase catalyzes the rearrangement of L-glutamate to L-*threo*-3-methylasparate whereas MGM catalyzes the rearrangement of 2-methyleneglutarate to (*R*)-3-methylitaconate (Scheme 1).

Like all B_{12}-dependent isomerases, the initial step of the reaction involves homolysis of the coenzyme and abstraction of the migrating hydrogen atom from the substrate to form a substrate radical (*4*−*9*); in the cases of both glutamate mutase and MGM, this hydrogen is abstracted from C-4 of the substrate (*10, 11*). The next step is the rearrangement of the substrate radical to give a product radical. For the isomerization of glutamate to methylaspartate, this has been shown to occur through the fragmentation of glutamyl radical, to give acrylate and glycyl radical as intermediates, followed by recombination to generate methylaspartyl radical (*12*). An analogous fragmentation−recombination mechanism for the interconversion of 2-methyleneglutaryl and 3-methylitaconyl radicals in the MGM reaction may occur, although in this case an associative mechanism for the

Scheme 1



rearrangement involving a cyclopropyl−carbinyl radical intermediate is also possible (*4, 13*).

The reactions of glutamate mutase and MGM differ in their stereochemical course. The *pro-S* hydrogen at C-4 of glutamate is abstracted in the glutamate mutase-catalyzed reaction (*14*), whereas it is the *pro-R* hydrogen at C-4 of 2-methyleneglutarate that is removed in the MGM-catalyzed reaction (*11*). Since for both enzymes the migration of the carbon-containing fragment occurs with inversion of configuration, this leads to the products of the reaction, methylaspartate and methylitaconate, having the opposite configuration at C-3.

The two enzymes also share structural similarities in that both contain a conserved B_{12}-binding domain characterized by the 'D-X-H-X-X-G' sequence motif (*15, 16*). This domain, which is a variation of the canonical α/β nucleoside-binding Rossman fold, binds the 'lower', α-face of the cobalamin cofactor with the conserved histidine residue displacing the dimethylbenzimidazole moiety of the coenzyme to ligate the central cobalt atom. The crystal structure

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[1] Abbreviations: AdoCbl, adenosylcobalamin; Cbl(II), cob(II)alamin; MGM, 2-methyleneglutarate mutase; 2-MG, 2-methyleneglutarate; 3-MI, 3-methylitaconate; PFL, pyruvate formate-lyase.

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of glutamate mutase has been solved to 1.6 Å (*17*). The structure shows the reactive β face of AdoCbl to be capped by a TIM barrel domain, with the substrate binding in the lumen of the barrel, some 6 Å from the cobalt atom. The global fold of glutamate mutase is shared by the two other B_{12} isomerases, methylmalonyl-CoA mutase and dioldehydrase, whose crystal structures have been determined (*18, 19*). Thus, although the crystal structure of MGM has not been solved, it seems likely that it too will adopt the same basic structure as glutamate mutase.

Recently, it has been shown that glutamate mutase catalyzes the isomerization of L-2-hydroxyglutarate to L-*threo*-3-methylmalate, and can also catalyze the exchange of tritium between the 5′-carbon of the coenzyme and 2-ketoglutarate (*20, 21*). Buckel and co-workers characterized methyleneglutarate as a competitive inhibitor of glutamate mutase, but also reported that when bound to the enzyme this molecule elicited an EPR spectrum, suggesting that homolysis of the coenzyme had occurred (*22, 23*). These findings led us to investigate the reaction of glutamate mutase with 2-methyleneglutarate, since it seemed plausible that the enzyme might be able to catalyze the isomerization of this substrate to methylitaconate, albeit less efficiently than MGM.

Here we describe experiments that show 2-methyleneglutarate not to be a substrate for glutamate mutase. Instead, the enzyme catalyzes a novel reaction of 2-methyleneglutarate with AdoCbl that involves attack of the 5′-deoxyadenosyl radical on the double bond of the 2-methyleneglutarate. The eventual product of this reaction is most likely a stable adduct of 2-methyleneglutarate with 5′-deoxyadenosine. The reaction bears a striking similarity to that described recently for the reaction of the *S*-adenosylmethionine-dependent pyruvate formate-lyase activating enzyme with a peptide substrate containing a dehydroalanine residue (*24*).

MATERIALS AND METHODS

Materials. GlmES, the glutamate mutase fusion protein, was purified as described previously (*25*). AdoCbl, HOCbl, and Sigmacote were purchased from Sigma Chemical Co. [8-^{14}C]-Adenosine (51 mCi/mmol) was purchased from Amersham. [5′-^{3}H]-AdoCbl was synthesized enzymatically as described previously (*26*). [8-^{14}C]-Adenosylcobalamin was synthesized using established protocols described by Brown et al. (*27*) from [8-^{14}C]-adenosine that was purchased from Amersham; the radiolabeled material was first converted to 5′-chloroadenosine that was subsequently used to alkylate Cob(I)alamin generated by in situ reduction of HOCbl with zinc. 2-Methyleneglutarate (2-MG) and 3-methylitaconate (3-MI) were synthesized as described by Hartrampf and Buckel (*11*). Details of the synthesis of the ^{13}C-labeled samples of 2-methyleneglutarate used in this study will be published elsewhere.

Kinetics of Enzyme Inactivation by 2-MG. A solution containing 50 μM GlmES, 50 μM AdoCbl, and 500 μM 2-MG in 50 mM potassium phosphate, pH 7.0, 10 mM KCl, and 1 mM $MgCl_2$ was allowed to incubate in the dark at 4 °C. Then 10 μL aliquots were withdrawn at various times and assayed for activity at 240 nm using the coupled spectrophotometric assay described by Barker (*28*). The assay solution contained 1 unit of β-methylaspartase, 10 mM glutamate, 25 μM AdoCbl in 50 mM potassium phosphate buffer, pH 7.0, 10 mM KCl, and 1 mM $MgCl_2$, in a total volume of 1 mL.

Incorporation of Radiolabeled Coenzyme into GlmES. Reactions were set up containing 10 μM GlmES, 7.5 μM [5′-^{3}H]-AdoCbl (6500 dpm/nmol), and 500 μM 2-MG in 50 mM potassium phosphate, pH 7.0, in the dark at 4 °C. Then 100 μL aliquots were withdrawn at various times and quenched with 100 μL of cold 5% trichloroacetic acid (TCA), and an additional 900 μL of cold 5% TCA was added to the quenched solution, making the final volume 1.1 mL. The solution was then incubated on ice for 2 h. The quenched reactions were filtered by vacuum filtration using a Buchner funnel fitted with glass fiber filters that were pretreated with 5% TCA. The filters and the eluent were analyzed for ^{3}H content by scintillation counting.

UV−Visible Spectra of Enzyme−Substrate Complexes. A solution containing 50 μM GlmES and 50 μM AdoCbl in 50 mM potassium phosphate, pH 7.0, was made anaerobic by repeated evacuation and flushing with argon. The solution was introduced into an evacuated cuvette fitted with a septum. The spectrum of the resting holoenzyme was recorded, and then 10 mM anaerobic 2-MG was added via an airtight syringe to initiate the reaction. Spectra were recorded at various times after the addition of 2-MG.

EPR Spectroscopy. Samples containing 275 μL of 215 μM GlmES and 1 mM AdoCbl in 100 mM potassium phosphate, pH 8.0, and 10% glycerol were made anaerobic by repeated evacuation and flushing with argon. The mixture was transferred via an airtight syringe to argon-purged EPR tubes. Then 25 μL of anaerobic 300 mM 2-MG was added to the solution to bring the final volume to 300 μL, and the holoenzyme concentration to 197 μM. The reaction was frozen with liquid nitrogen within 20−30 s of mixing. The samples were protected from light during all experiments. EPR spectra were recorded in the dark using a Bruker EMX EPR spectrometer equipped with a liquid nitrogen Dewar system. The conditions for obtaining spectra were as follows: temperature, 115 K; microwave power, 16 mW; microwave frequency, 9.2 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.1 mT. The data were analyzed using the Bruker Win-EPR data manipulation program.

Reaction of Glutamate Mutase with [5′-^{3}H]-AdoCbl and 2-MG or 3-MI. Reactions were set up in lightproof Eppendorf tubes at 4 °C. Reactions contained 10 μM GlmES and 7.5 μM [5′-^{3}H]-AdoCbl (13 000 dpm/nmol) in 50 mM potassium phosphate, pH 7.0, and 1 mM DTT. Either 2-MG or 2-MI (500 μM final concentration) was added to initiate the reaction. Then 200 μL aliquots were withdrawn at various times and quenched with 100 μL of 0.5 M HCl, 1 mM DTT. The samples were divided into two portions for analysis. One portion was subjected to reverse-phase HPLC to quantify the amount of AdoCbl remaining and to determine the specific activity of the recovered material by scintillation counting, as described previously (*26*). The other portion was subjected to reverse-phase HPLC on a Spherisorb 5μ ODS2 column (Waters 25 × 4.6 mm) to examine the formation of 5′-deoxyadenosine (5′-dA), 2-MG, and 3-MI. The compounds were eluted isocratically with 15% methanol containing 0.1% TFA at a flow rate of 1 mL/min, and detected by their absorbance at 260 nm. The retention times were as follows: 5′-dA, 5.2 min; 2-MG, 7.2 min; 3-MI, 8.0 min.

The amount of each product formed was calculated from standard curves determined from chromatography of known concentrations of each compound. The tritium content of each of the recovered products was determined by scintillation counting.

Reaction of Glutamate Mutase with [8-^{14}C]AdoCbl and 2-MG. Reactions were set up in light-proofed Eppendorf tubes at 4 °C. Reactions contained 50 μM GlmES and 40 μM [8-^{14}C]-AdoCbl (5600 dpm/nmol) in 50 mM potassium phosphate, pH 7.0, and 1 mM DTT. 500 μM 2-MG was added to initiate the reaction, and 100 μL aliquots were withdrawn at various times and quenched with 50 μL of 0.5 M hydrochloric acid, 1 mM DTT. The samples were analyzed by reverse-phase HPLC as described above for the separation of 5′-dA, 2-MG, and 3-MI. Modifications were made to the separation protocol in order to elute AdoCbl off the same column. The compounds were eluted at 1 mL/min and detected at 260 nm, using the following gradient: 0−26 min, 15% methanol/0.1% TFA; 26−30 min, 15−100% methanol/0.1% TFA; 30−31 min, 100−15% methanol/0.1% TFA; 31−41 min, 15% methanol/0.1% TFA. The retention times were as follows: 5′-dA, 5.2 min; 2-MG, 7.2 min; 3-MI, 8.0 min; compound X, 9 min; compound Y, 15 min; AdoCbl, 30 min. The recovered products were analyzed for ^{14}C content by scintillation counting. The amount of product formed was calculated from the specific activity of the starting material.

Data Analysis. The data were plotted and curves fitted using the Kaleidagraph Program (Abelbeck Software).

RESULTS

Previously, it was reported that 2-methyleneglutarate is a competitive inhibitor of glutamate mutase, with a $K_I \sim 400$ μM (*22*). Recent studies have shown that two other substrate analogues are able to undergo partial reactions or the complete catalytic cycle with glutamate mutase (*20, 21*). These results prompted us to reinvestigate the interaction of glutamate mutase with 2-MG to determine whether it was simply a competitive inhibitor or whether it might in fact be a substrate, a "partial" substrate (i.e., able to reversibly undergo part of the catalytic cycle), or a mechanism-based inhibitor. These experiments used the single-subunit form of glutamate mutase, GlmES, which comprises both subunits of glutamate mutase genetically fused together by an 11 amino acid linker (*25*). This enzyme has a catalytic efficiency similar to wild type, but is not complicated by the concentration-dependent dissociation of subunits (*1, 25*). Except where otherwise noted, no special precautions were taken to exclude oxygen, as the enzyme is not especially sensitive to air in the presence of the natural substrates.

Attempts To Detect the Interconversion of 2-Methyleneglutarate and 3-Methylitaconate by Glutamate Mutase. 2-MG and 3-MI absorb at 240 nm and can readily be separated by reverse-phase HPLC. We first examined whether incubation of holo-glutamate mutase with either 2-MG or 3-MI resulted in the interconversion of these two isomers. However, even after prolonged incubation (up to 20 h) of the holo-enzyme with either 2-MG or 3-MI, we were unable to detect any interconversion of the two isomers by reverse-phase HPLC. The limits of detection were such that under the conditions of the experiment we should have been able to detect a single turnover in 5% of enzyme active sites.

Attempts To Detect Glutamate Mutase-Catalyzed Exchange of Tritium between AdoCbl and 2-Methyleneglutarate or 3-Methylitaconate. The experiments above indicated that neither 2-MG nor 3-MI were substrates for glutamate mutase; however, molecules that undergo only part of the catalytic cycle may be mechanistically informative. Therefore, we next examined the ability of the enzyme to catalyze the transfer of tritium from [5′-^{3}H]-AdoCbl to either 2-MG or 3-MI. This serves as a sensitive test for hydrogen atom transfer between coenzyme and substrate, which is the second common step in all B_{12}-dependent rearrangements. Experiments in which glutamate mutase (10 μM) was incubated with [5′-^{3}H]-AdoCbl (7.5 μM, specific activity 9×10^4 dpm/nmol) and either 1 mM 2-MG or 1 mM 3-MI for 10 min failed to detect any tritium transfer from the coenzyme to either of the potential substrates. For comparison, incubation of the enzyme with [5′-^{3}H]-AdoCbl and L-glutamate under similar conditions results in over 80% of the tritium being transferred from the coenzyme (*26*). Similarly, 2-ketoglutarate, which is not a true substrate for the enzyme, rapidly undergoes enzyme-catalyzed tritium exchange with AdoCbl (*21*). These findings rule out the reversible transfer of hydrogen between the coenzyme and 2-MG or 3-MI.

UV−Visible Spectrum of the Glutamate Mutase/2-Methyleneglutarate Complex. We used UV−visible spectroscopy to examine whether binding of 2-MG to the holo-enzyme resulted in the rapid (i.e., within 30 s) formation of Cbl(II). Addition of 10 mM 2-MG to holo-glutamate mutase (50 μM) resulted in significant changes to the UV−visible spectrum of the holo-enzyme. In particular, a decrease of absorbance at 530 nm and an increase at 470 nm, indicative of formation of Cbl(II) on the enzyme (Figure 1), were evident. The spectral changes were almost identical to those that occurred when the holo-enzyme was incubated with 10 mM L-glutamate. Assuming $\Delta\epsilon_{530} = 4000$ M^{-1} cm^{-1} for the homolysis of AdoCbl, the fraction of enzyme in Cbl(II) form after 30 s incubation with 2-MG was 54%, whereas with L-glutamate it was 43%. This result was in some respects surprising, because, in all the cases that we are aware of, the ability of AdoCbl-dependent enzymes to effect homolytic cleavage of the coenzyme is coupled to either the reversible hydrogen abstraction from the substrate (*29−33*) or, in the case of ribonucleotide reductase, the abstraction of hydrogen from a protein thiol (*34*).

On prolonged incubation of the enzyme with 2-MG, an intense band at 350 nm was observed, together with a recovery of absorbance at 530 nm, both of which are characteristic of the formation of hydroxocobalamin (Figure 1). The oxidation of Cbl(II) to hydroxocobalamin occurs over a period of about 30 min and appears to be a first-order process with an apparent rate constant of 0.1 min^{-1}. The source of the oxidant in the reaction is not clear. The reaction was set up under anaerobic conditions using argon-purged solutions, so oxygen should not have been present. Although the possibility that trace amounts of oxygen remain in solution cannot be ruled out, we think it unlikely that it would be present in sufficient concentrations to explain the observed formation of hydroxocobalamin. Furthermore, control experiments in which the holoenzyme was incubated with L-glutamate resulted in the characteristic formation of a Cbl(II)

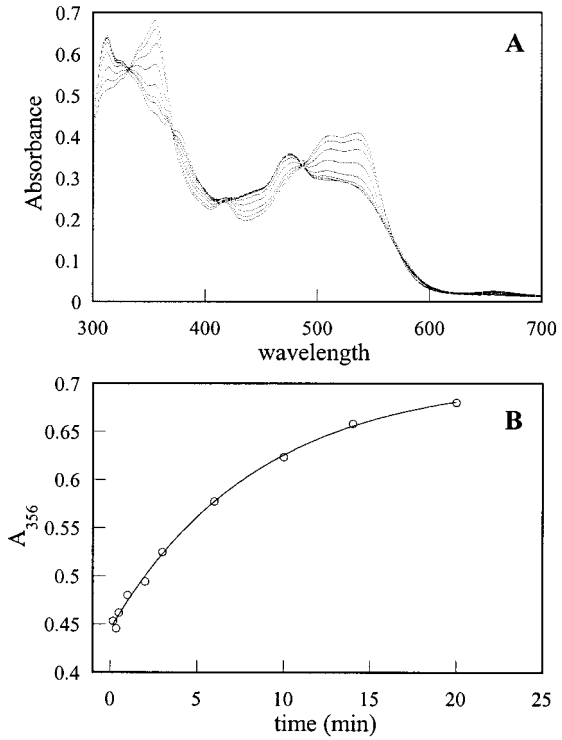

FIGURE 1: (A) UV−visible spectral changes induced by the reaction of 2-MG with holo-glutamate mutase, showing the conversion of the initially produced Cbl(II) species, characterized absorption maximum at 470 nm, to hydroxocobalamin (absorption maxima at 356 and 536 nm). Spectra shown were acquired immediately after mixing 2-MG and holo-enzyme, and at 1, 2, 3, 6, 10, 14, and 20 min after mixing. (B) Time course of formation of hydroxocobalamin after addition of 2-MG, monitored by the increase in absorbance at 356 nm.

spectrum, but even after 30 min, no oxidation of the initially formed Cbl(II) to hydroxocobalamin was evident.

EPR Spectroscopy. Further evidence that binding of 2-MG initiates the formation of free radicals on the enzyme comes from EPR spectroscopy of the holo-enzyme. The spectra (Figure 2) were recorded in the presence of 25 mM 2-MG, at 115 K under anaerobic conditions, with samples that were frozen within 20−30 s of mixing the holoenzyme and 2-MG. The spectra shown in Figure 2 may be ascribed to the coupling of the unpaired electron of Cbl(II) with a carbon-centered radical (*35*, *36*), and are similar to those obtained for other AdoCbl-dependent enzymes. The spectrum of the enzyme reacted with unlabeled 2-MG exhibits features centered at $g = 2.09$, with $A = 5$ mT. The spectrum displays well-resolved hyperfine coupling with the typical 8-fold hyperfine splitting in the g_z region due to the interaction of an unpaired electron ($S = 1/2$) with the nuclear spin of Co ($I = 7/2$). The superhyperfine coupling that usually results from the interaction of the nitrogen ligands with Co(II) is most likely masked by the interaction of Co(II) with an organic radical partner. This phenomenon was also observed when the enzyme was reacted with glutamate (*36*), and 2-hydroxyglutarate (*20*).

Regiospecifically-^{13}C-labeled samples of 2-MG were used to further characterize the nature of the radical species obtained when holo-glutamate mutase was reacted with 2-MG. The EPR spectra (Figure 2) of the holo-enzyme reacted with 2-MG ^{13}C-labeled at either C-4 (the normal site

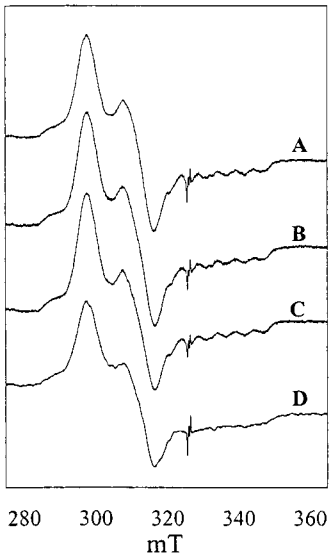

FIGURE 2: EPR spectra of the holo-glutamate mutase reacted 2-MG. (A) Holo-enzyme reacted with unlabeled 2-MG; (B) holo-enzyme reacted with [4-^{13}C]-2MG; (C) holo-enzyme reacted with [*methylene*-^{13}C]-2MG; (D) holo-enzyme reacted with [2-^{13}C]-2MG. A small signal is seen at $g = 2.01$ due to an unidentified organic radical free in solution.

of hydrogen abstraction) or the methylene carbon are indistinguishable from that of the holo-enzyme reacted with unlabeled material. However, the EPR spectrum of the holo-enzyme reacted with [2-^{13}C]-2-MG shows a significant broadening of the hyperfine splitting in the g_z region, which is very similar to that seen when the enzyme is reacted with [4-^{13}C]-L-glutamate (*36*). This result is consistent with an unpaired electron of the organic radical residing on C-2 of 2-MG. Such a radical would be formed if the adenosyl radical, instead of reacting with the substrate to remove a hydrogen atom, underwent addition to the double bond of 2-MG to form a stable tertiary radical.

In all the spectra a very small signal is seen at $g = 2.01$ that may be ascribed to a carbon-based radical that is not coupled to the cobalt. The origin of this signal is unclear, and in any case, it represents at most a few percent of the total spins present.

Inactivation of Glutamate Mutase by 2-Methyleneglutarate. We investigated the effect of 2-MG on the activity of glutamate mutase. Glutamate mutase, 50 μM, AdoCbl, 50 μM, and 2-MG, 500 μM, were incubated together, and at various times, a small portion of the mixture was diluted 100-fold into an assay cuvette containing 10 mM L-glutamate, 25 μM AdoCbl, and 1 unit of β-methylaspartase. 2-MG appears to cause time-dependent irreversible inactivation of glutamate mutase; however, the time course of inactivation is unusual. As shown in Figure 3, there is an initial rapid decrease in glutamate mutase activity, which is followed by a much slower rate of inactivation. The data fit reasonably well to two first-order inactivation reactions, each with similar amplitudes; the faster of these occurs with $k = 1 \pm 0.1$ min^{-1} and the slower with $k = 0.01 \pm 0.001$ min^{-1}. Similar inactivation kinetics were observed when the experiment was repeated under anaerobic conditions. This observation rules out the reaction of radical species with oxygen as the cause of inactivation. Control experiments established that in the absence of AdoCbl,incubation of the enzyme with 2-MG did not result in inactivation.

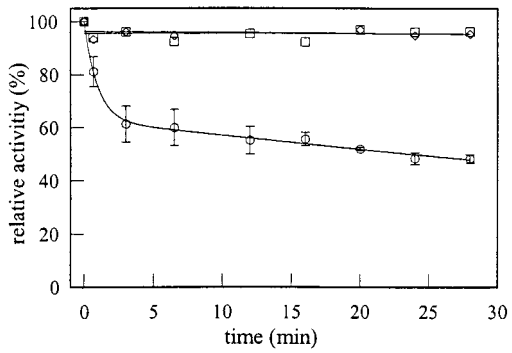

FIGURE 3: Time course of enzyme inactivation by 2-MG; for discussion, see the text. (○) Enzyme activity as a function of time in the presence of saturating concentrations of AdoCbl and 2-MG; (□) control experiment in which AdoCbl was omitted; (◇) control experiment in which 2-MG was omitted.

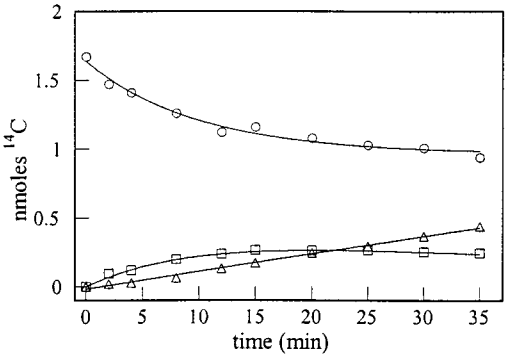

FIGURE 4: Time course for the reaction of [8-^{14}C]-AdoCbl with 2-MG. (○) Degradation of AdoCbl; (□) formation of product X; (△) formation of product Y.

The biphasic nature of inactivation may be the result of negative cooperativity (so-called 'half-of-sites' reactivity) that arises from inhibitor-induced conformational changes that are communicated between the two active sites of the dimeric enzyme. Indeed, we have observed a similar phenomenon in previous pre-steady-state kinetic studies of this enzyme (*20, 31*). We were unable to investigate the effect of 3-MI on glutamate mutase activity, because this compound is a potent competitive inhibitor of 3-methylaspartase, the coupling enzyme used in the assay.

Mechanism of Inactivation. We first examined whether enzyme inactivation resulted from the covalent modification of the protein by 5′-deoxyadenosyl radical. GlmES, [5′-^{3}H]-AdoCbl (6500 dpm/nmol), and 2-MG were incubated together for 10 min in the dark, as described under Materials and Methods. However, no radioactivity was found to be associated with the protein, suggesting that covalent attachment of the adenosyl moiety to the protein is not the cause of inactivation.

Next, we sought to characterize the products of the reaction between AdoCbl and 2-MG. These experiments employed AdoCbl ^{14}C-labeled in the adenine moiety to avoid complications that might arise either if tritium label was lost from compounds during their isolation and characterization or if kinetic isotope effects were to alter the distribution of the products. It was found that AdoCbl was degraded upon incubation of the enzyme with 2-MG and a new ^{14}C-labeled product, X, formed. This product eluted from the HPLC column after 9 min under the conditions described under Materials and Methods. It is further apparent that less than half of the coenzyme is degraded, in accord with the "half of the sites" inhibition noted above. At longer times, a second radiolabeled product, Y, was observed with a retention time of 15 min, which increased in concentration at the expense of product X.

The normal product of AdoCbl homolysis, 5′-deoxyadenosine, elutes after 5 min under the same chromatography conditions, but significantly, no radioactivity was detected in 5′-deoxyadenosine when it was added as a carrier in the HPLC separation. This result suggests that 5′-deoxyadenosine is not formed in the reaction with 2-MG, and is consistent with the lack of tritium exchange between AdoCbl and 2-MG. The time course of formation of X and Y (Figure 4) was well fitted by a model in which AdoCbl and 2-MG react to form X, which subsequently decays to form Y. The data were fitted to the standard eqs 1 and 2 below:

$$[\text{AdoCbl}] = A_o \exp(-k_1 t) \quad (1)$$

$$[\text{X}] = (A_o k_1)/(k_2 - k_1)[\exp(-k_1 t) - \exp(-k_2 t)] \quad (2)$$

The apparent rate of decomposition of AdoCbl, calculated from eq 1, was 0.103 ± 0.017 min^{-1}, which is in good agreement with the apparent rate of formation of X $= 0.106 \pm 0.028$ min^{-1}, calculated from eq 2. The decomposition of X to Y occurs with a rate constant $k_2 = 0.016 \pm 0.008$ min^{-1}. Interestingly, product X is formed at the same rate and with the approximately the same stoichiometry as hydroxocobalamin, as described above, suggesting these reactions are linked.

Attempts to obtain larger quantities of the compounds X and Y that would allow more extensive characterization by NMR were unsuccessful. Increasing the loading of the HPLC column resulted in loss of resolution, so that X and Y could not be separated. Attempts to scale-up the separation on a larger, semipreparative column were also unsuccessful as, again, the products could not be resolved. LC/ES-TOF mass spectrometry of products X and Y was also attempted using ~0.5 nmol samples of these products which could be isolated by analytical HPLC. However, these experiments failed to yield reliable mass spectral data for these products.

DISCUSSION

The experiments described above suggest that 2-MG reacts with AdoCbl at the active site of glutamate mutase in a novel manner. All previous studies on AdoCbl-dependent enzymes have found that 5′-deoxyadenosyl radical, once formed by homolysis of the cobalt−carbon bond of the coenzyme, reacts very rapidly to abstract hydrogen, either from the substrate (*30*−*33*) or, in the case of ribonucleotide reductase, from a cysteine residue on the protein (*34*). In this case, it appears, however, that the exo-methylene group of 2-MG reacts with adenosyl radical as a Michael acceptor to form an adduct between adenosine and 2-MG. This reaction results in the formation of a σ-bond at the expense of a π-bond and the transfer of the radical from the primary carbon of 5′-deoxyadenosine to the tertiary carbon of 2-MG, both of which are expected to be energetically favorable.

The clearest evidence in support of this initial step in the reaction of glutamate mutase with 2-MG comes from the EPR studies. The EPR spectrum of the holo-enzyme/2-MG

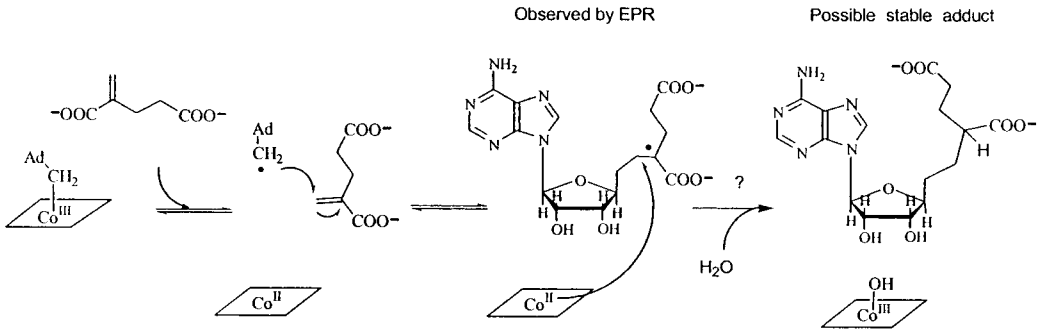

FIGURE 5: Proposed mechanism for the glutamate mutase-catalyzed reaction of 2-MG with AdoCbl.

complex is characteristic of cob(II)alamin coupled to a carbon-based radical partner (*35*, *36*). Furthermore, the ^{13}C-labeling studies clearly point to the organic radical being located at C-2 of 2-MG. Reaction of the holo-enzyme with 2-MG results in characteristic changes to the UV−visible spectrum of the coenzyme indicating the formation of cob(II)alamin. Also significant is the fact that the enzyme does *not* appear to catalyze hydrogen transfer between 2-MG and AdoCbl. Thus, neither the exchange of tritium between the coenzyme and 2-MG, nor the formation of 5′-deoxyadenosine can be detected. A mechanism for the reaction of 2-MG with glutamate mutase is outlined in Figure 5.

The fate of the radical adduct formed between 5′-deoxyadenosine and 2-MG and the precise details by which 2-MG inactivates glutamate mutase remain unclear. It does not appear that either adenosine or 2-MG becomes covalently attached to the protein, to cause its inactivation. The initial formation of the Michael adduct between adenosyl radical and 2-MG is probably rapid and reversible, as the UV−visible spectral changes occur as soon as the holo-enzyme and 2-MG are mixed in the cuvette. In contrast, inactivation appears to be characterized by two rates and takes place over several minutes (Figure 3). It is noteworthy that the rate at which product X, the stable covalent adduct between adenosine and 2-MG (Figure 4), and the rate at which Cbl(II) is oxidized to hydroxocobalamin are the same (0.1 min^{-1}). It seems plausible that X may result from reduction of the radical by Cbl(II) to yield a carbanion, that would be readily protonated, and hydroxocobalamin. If this were the case, then X would be expected to possess the structure shown in Figure 5, although it may well be that the presumed carbanion may react further before being quenched. Unfortunately, because this product was only formed in very small amounts, there was insufficient material for more detailed characterization by, for example, NMR.

A strikingly similar example of mechanism-based inactivation has recently been described by Frey and co-workers for the substrate-dependent inactivation of AdoCbl-dependent lysine 5,6-aminotransferase (*37*). In this case, the rate of enzyme inactivation coincides with the formation of hydroxocobalamin and 5′-deoxyadenosine. Furthermore, inactivation is accompanied by the incorporation of a proton from the solvent into the substrate. These observations are consistent with the transient formation of a carbanion arising from electron transfer from Cbl(II) to the substrate radical that is quenched either by the solvent or by a solvent-exchangeable proton on the protein.

The fact that only about 50% of the enzyme activity appears to be susceptible to inactivation by 2-MG was initially puzzling. However, we have encountered evidence for the asymmetric reactivity of the two active sites of the dimeric protein in various pre-steady-state kinetic experiments on this enzyme. Thus, when the enzyme is reacted with glutamate or methylaspartate, the kinetics of AdoCbl homolysis appear biphasic and are well described by a model in which the two active sites exhibit negative cooperativity, leading to a 'fast' and a 'slow' reacting site (*31*). This biphasic behavior is also observed when the enzyme is reacted with the slow substrate L-2-hydroxyglutarate (*20*). The biphasic kinetics of inactivation by 2-MG may be plausibly described by a model in which reaction of 2-MG with adenosyl radical at one active site locks the enzyme into a conformation in which the other active site is unable to react with the inhibitor or can only react very slowly.

A precedent for the addition of 5′-deoxyadenosyl radical to a double bond is provided by a recent study of the reaction of pyruvate-formate-lyase (PFL) activating enzyme with a peptide containing dehydroalanine (*24*). The activating enzyme is responsible for generating the catalytic glycyl radical in PFL. In this case, 5′-deoxyadenosyl radical is generated by a one-electron reduction of *S*-adenosylmethionine, and this radical is used to abstract a hydrogen atom from the glycine residue of PFL. By substituting PFL with an 8-residue peptide corresponding to the sequence of PFL in which the catalytic glycine was replaced with dehydroalanine, the adenosyl radical could be trapped as a Michael adduct between 5′-deoxyadenosine and the peptide, which was subsequently reduced. Because the enzyme catalyzed multiple turnovers, it was possible to isolate sufficient quantities of the peptide/adenosine adduct to confirm its structure by NMR.

A further noteworthy example of an enzyme-catalyzed radical addition to a double bond is provided by benzylsuccinate synthase (*38*, *39*). This recently discovered enzyme catalyzes the first step in the anaerobic degradation of toluene; the enzyme is mechanistically related to the glycyl radical-containing enzymes PFL and anaerobic ribonucleotide reductase. The glycyl radical is believed to abstract a hydrogen from the methyl group of toluene to generate a benzyl radical. The benzyl radical is then proposed to undergo addition to fumarate to generate a benzylsuccinyl radical that is finally quenched by return of the abstracted hydrogen from the glycine residue.

Given the mechanistic similarities between glutamate mutase and MGM and the structural similarities between their respective substrates, L-glutamate and 2-MG, it is quite striking that glutamate mutase should react so differently with 2-MG. That the adenosyl radical appears to undergo addition

to the exo-methylene group of 2-MG, rather than abstracting hydrogen from C-4, suggests that the exo-methylene group occupies the equivalent position of the *pro-S* hydrogen on C-4 of glutamate. This could occur if 2-MG were bound in the opposite orientation to glutamate, so that instead of the α-carboxylate being hydrogen-bonded to Arg66 and Arg149, and the γ-carboxylate being hydrogen-bonded to Arg100, as occurs with glutamate, the α-carboxylate is recognized by Arg100 and the γ-carboxylate by Arg66 and Arg149. This provides a further example of the exquisite sensitivity of the reactions catalyzed by this class of enzymes to small changes in the structure of either the substrate or the enzyme active site.

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