How Enzymes Control the Reactivity of Adenosylcobalamin: Effect on Coenzyme Binding and Catalysis of Mutations in the Conserved Histidine-Aspartate Pair of Glutamate Mutase

Hao-Ping Chen and E. Neil G. Marsh*

Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055

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ABSTRACT: Glutamate mutase is one of a group of adenosylcobalamin-dependent enzymes that catalyze unusual isomerizations that proceed through the formation of radical intermediates. It shares a structurally similar cobalamin-binding domain with methylcobalamin-dependent methionine synthase. In particular, both proteins contain the "DXHXXG" cobalamin-binding motif, in which the histidine provides the axial ligand to cobalt. The effects of mutating the conserved histidine and aspartate residues in methionine synthase have recently been described [Jarrett, J. T., Amaratunga, M., Drennan, C. L., Scholten, J. D., Sands, R. H., Ludwig, M. L., & Matthews, R. G. (1996) *Biochemistry 35*, 2464−2475]. Here, we describe how similar mutations in the "DXHXXG" motif of glutamate mutase affect coenzyme binding and catalysis in an adenosylcobalamin-dependent reaction. The mutations made in the MutS subunit of glutamate mutase were His16Gly, His16Gln, Asp14Asn, Asp14Glu, and Asp14Ala. All the mutations affect, in varying degrees, the rate of catalysis, the affinity of the protein for the coenzyme, and the coordination of cobalt. Mutations of either Asp14 or His16 decrease k_{cat} by 1000-fold, and whereas cob(II)alamin accumulates as an intermediate in the wild-type enzyme, it does not accumulate in the mutants, suggesting the rate-determining step is altered. The apparent K_d for adenosylcobalamin is raised by about 50-fold when His16 is mutated and by 5−10-fold when Asp16 is mutated. There are extensive differences between the UV−visible spectra of wild-type and mutant holoenzymes, indicating that the mutant enzymes coordinate cobalt less well. Overall, the properties of these mutants differ quite markedly from those observed when similar mutations were introduced into methionine synthase.

The corrinoid coenzymes exemplified by adenosylcobalamin (AdoCbl)[1] and methylcobalamin (MeCbl) are unique among biological cofactors in containing stable carbon−metal bonds (Ludwig & Matthews, 1997; Pratt, 1993; Finke, 1990). They catalyze two quite different types of reactions that hinge upon the manner in which the cobalt−carbon bond is broken during enzymic catalysis. In methylcobalamin, which serves as an intermediate methyl donor in some methyl-transfer reactions (Banerjee & Matthews, 1990), the cobalt−carbon bond is broken heterolytically during catalysis to form Cbl(I). In contrast, AdoCbl serves as an initiator of free radicals that are "unmasked" by homolytic cleavage of the cobalt−carbon bond to generate Cbl(II) and 5′-deoxyadenosyl radical (Buckel & Golding, 1996; Marsh, 1995a; Stubbe, 1989). Both cofactors are quite inert in the absence of their respective enzymes, implying that the cobalt−carbon bond is substantially weakened when the coenzyme is bound by the protein. Measurements of the cobalt−carbon bond dissociation energy of free AdoCbl suggest that, to achieve the rates of catalysis observed, enzymes must accelerate homolysis by about 10^{12}-fold (Hay & Finke, 1986).

Although AdoCbl and MeCbl function quite differently, they appear to be bound in a very similar way; this was first suggested by sequence similarities between AdoCbl-dependent glutamate mutase, methylmalonyl-CoA mutase (MMCM), and MeCbl-dependent methionine synthase (MetH) (Marsh & Holloway, 1992). This has subsequently been confirmed with the solution of the crystal structures of MMCM and MetH (Mancia et al., 1996; Drennan et al., 1994). They show that the common cobalamin-binding domains are superimposable with a root mean square deviation of only 1.7 Å. Most interestingly, the coenzymes are bound in an extended conformation in which the dimethylbenzimidazole "tail" of cobalamin is displaced by a histidine residue that coordinates cobalt through the ϵ-nitrogen of the imidazole ring (Figure 1). The histidine is, in turn, hydrogen bonded to an aspartate residue. These two residues form part of an invarient "DXHXXG" motif that is found in a number of B_{12}-dependent enzymes (Marsh & Holloway, 1992). Although this B_{12}-binding motif and the conserved domain that encompasses it are not universal in B_{12} enzymes, the coordination of histidine to cobalt may be a more widespread feature of B_{12}-mediated catalysis. Thus, the coordination of histidine was first demonstrated by EPR experiments on whole cells of the cobamide-requiring *o*-methyltransferase from *Sporomusa ovata* (Stupperich et al., 1990, 1993), an enzyme that does not contain the conserved B_{12}-binding domain.

The role of the axial base in modulating the reactivity of alkylcobalamins remains controversial. Studies employing alkylcobaloximes as putative model compounds have shown that increasing the nucleophilicity of the transaxial ligand to cobalt results in a stronger carbon−cobalt bond (Ng et al., 1982). However, recent investigations of the thermolysis of adenosylcobinamide in the presence of various *para*-

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[1] Abbreviations: AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin; Cbl(II), cob(II)alamin; Cbl(I), cob(I)alamin; MMCM, methylmalonyl-CoA mutase; MetH, methionine synthase.

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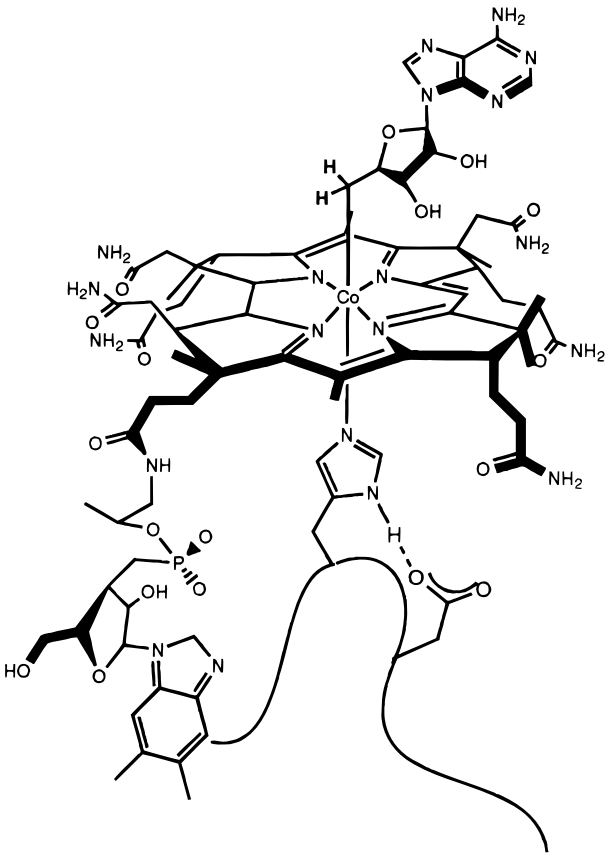

FIGURE 1: Cobalt-histidine-aspartate hydrogen-bonded "triad" formed by the residues of the DXHXXG motif found in the AdoCbl-dependent carbon skeleton isomerases and MeCbl-dependent methionine synthase. For glutamate mutase, these residues are MutS-His16 and MutS-Asp14 The histidine-aspartate pair displaces the axial dimethylbenzimidazole ligand of the coenzyme when it is bound by the protein.

substituted pyridines have identified both heterolytic and homolytic cleavage reactions and indicate that the nucleophilicity of the axial ligand only signifcantly affects the abiological heterolytic cleavage pathway (Garr et al., 1996). In light of the structures of MetH and MMCM, one might expect the histidine-aspartate pair to play an important role in both binding cobalamin and controlling the reactivity of the cobalt−carbon bond. But whether the protein ligand serves to stabilize or labilize the cobalt−carbon bond and the relative importance of any such effect in catalysis remain unclear.

We have used site-directed mutagenesis to investigate the role of the conserved histidine-aspartate pair in both coenzyme binding and catalysis by AdoCbl-dependent glutamate mutase from *Clostridium tetanomorphum*. This enzyme is one of a group of AdoCbl-dependent enzymes that use free radicals to catalyze a variety of unusual isomerizations, in this case the carbon skeleton rearrangement of L-glutamate to L-*threo*-3-methylaspartate, the first step in the fermentation of L-glutamate by *C. tetanomorphum* (Barker et al., 1958). Glutamate mutase is comprised of two weakly associating subunits, MutE and MutS, which combine with AdoCbl to form the active holoenzyme (Holloway & Marsh, 1994). MutE is a dimer with an M_r of 53 703 (Holloway & Marsh, 1993), whereas MutS is a monomer with an M_r of 14 748 that comprises the conserved cobalamin-binding domain and contains the "DXHXXG" motif (Marsh & Holloway, 1992).

The results presented here show that both of these residues are important in both catalysis and coenzyme binding. Further, the results indicate that the histidine-aspartate pair in glutamate mutase modulates the reactivity of AdoCbl in a manner quite different from that seen in MeCbl-dependent MetH, consistent with the different modes of cobalt−carbon bond cleavage in these two coenzymes.

MATERIALS AND METHODS

Materials. The purification of MutE and MutS proteins from recombinant *Escherichia coli* strains has been described previously (Holloway & Marsh, 1994). 3-Methylaspartase was purified from *C. tetanomorphum* as described by Hsiang and Bright (1967). AdoCbl was supplied by Fluka Chemical Co.; restriction enzymes and DNA-modifying enzymes were purchased from Boeringer Mannheim. The sources of other materials have been described previously (Holloway & Marsh, 1994) or were purchased from commercial suppliers.

Construction of MutS Mutant Proteins. Standard techniques, as described by Sambrook et al. (1989), were used to prepare plasmid and single-stranded DNA and to subclone DNA fragments. Site-directed mutagenesis was performed using the Altered Sites in vitro mutagenesis system (Promega) according to the manufacturer's protocol. The *mutS* gene was excised from the pUC119-based plasmid pmutS (Holloway & Marsh, 1994) as an *Eco*RI−*Pst*I fragment and subcloned into the commercial vector pALTER-1 to give pALmutS, which was maintained in *E. coli* JM109. Single-stranded DNA template for mutagenesis was obtained by transfection with helper phage R408. Mutations were introduced into the *mutS* gene using the following oligonucleotides as primers for second-strand synthesis: *mutS*-H16G, GGTTCAGACTGTGGTGCAGTTGGTAA; *mutS*-H16Q, CAGACTGTGCTGCAGTTGGTAA; *mutS*-D14N, TATTGGTTCAAACTGTCATGC; *mutS*-D14A, ATTGGTTCAGCCTGTCATGCA; *mutS*-D14E, TTGGTTCAGAATGTCATGCAG. Mutations were confirmed by DNA sequencing, and the mutant *mutS* genes were then excised from pALmutS as *Nde*I−*Sal*I fragments and subcloned into pT7-7 (Tabor & Richardson, 1985) to facilitate overexpression of the mutant proteins in *E. coli* BL12(DE3) cells as described previously (Holloway & Marsh, 1994).

Enzyme Assay. Wild-type glutamate mutase activity was assayed using the spectrophotometric assay described by Barker et al. (1964). For the mutant proteins, glutamate mutase activity was assayed using uniformly labeled L-[^{14}C]glutamate with a specific activity of 2000 dpm/nmol (Amersham). Typically, reactions were set up in 50 μL of 50 mM potassium phosphate buffer (pH 8.0) containing 5 μM MutE and 12 μM MutS mutant. For routine determination of enzyme activity, 10 mM L-glutamate and 150 μM AdoCbl were present. The reaction was made irreversible by coupling the formation of 3-methylaspartate to the production of mesaconate formed through the action of 3-methylaspartase. At various times, 15 μL aliquots were withdrawn and the reaction was stopped by the addition of 5 μL of 5% trifluoroacetic acid; 80 μL of 1 mM unlabeled mesaconate was then added as carrier. Mesaconate was isolated by reverse phase HPLC on a C_{18} column as described previously (Marsh, 1995b) and the radioactivity determined by scintillation counting. Typically, to measure the rate of reaction, the amount of radioactivity incorporated into

mesaconate was determined at three time points spaced over 2 h; the reaction rate was linear for up to 5 h. The concentrations of MutE and MutS were determined by measuring their absorbance at 280 nm and using the following values for their absorption coefficients: MutE ϵ_{280} = 56 300 M^{-1} cm^{-1} and MutS ϵ_{280} = 9380 M^{-} cm^{-1} (Holloway & Marsh, 1994). MutS mutant proteins were assumed to have the same extinction coefficients as wild-type protein.

Measurement of AdoCbl Binding. Equilibrium gel filtration (Hummel & Dreyer, 1962) was used to measure the binding of AdoCbl to glutamate mutase. Measurements were made using a 5 × 200 mm column of Bio Gel P-6 (fine) equilibrated in 50 mM potassium phosphate buffer (pH 7.0) and various concentrations of AdoCbl at room temperature. The column and all solutions containing AdoCbl were shielded from light. The column was attached to a Beckman HPLC system controlled by "System Gold" software. The column was pumped at 0.1 mL/min and the AdoCbl concentration of the eluate monitored at 522 nm. Samples were prepared in 50 mM potassium phosphate buffer (pH 7.0) in a final volume of 80 μL. In each case, the final concentration of MutE was 12 μM and the final concentration of MutS (or MutS mutant) was 60 μM. AdoCbl was added from a 1 mM stock solution to give a final concentration which was the same as that in which the column was equilibrated. Samples were incubated in the dark at room temperature for 10 min, and then 40 μL was injected onto the column. The integrated areas of the peaks were used directly to construct binding curves.

UV−Visible Spectra of Holoenzymes. Samples containing 20 μM MutE, 100 μM MutS (wild-type or mutant), and 40 μM AdoCbl in 50 mM potassium phosphate buffer (pH 7.0) were dialyzed at 4 °C, in the dark, against 50 mM potassium phosphate buffer (pH 7.0) containing 40 μM AdoCbl for 24 h, by which time equilibrium had been reached. Spectra were recorded using a Hewlett-Packard diode array spectrophotometer; a sample of the dialysis buffer was used to subtract out the contribution of unbound AdoCbl from the spectra of the holoenzymes.

RESULTS

Introduction of Mutations at MutS-His16 and MutS-Asp14 of Glutamate Mutase. Mutations were made in MutS with the intention of examining the effects of both conservative changes and deletion of amino acid functionality at the conserved positions. Therefore, the following mutations of the conserved histidine and aspartate residues were introduced into MutS: H16G, H16Q, D14A, D14N, and D14E. The mutations were confirmed by DNA sequencing. All the mutant proteins were overexpressed as soluble proteins in *E. coli*, at levels similar to that of recombinant wild-type MutS. The molecular weights of purified mutant proteins were determined by electrospray mass spectrometry. In all cases, the experimentally determined molecular weight agreed, within experimental error, with that calculated from the amino acid sequence of the mutant protein, confirming that they had been correctly expressed.

Kinetic Properties of the MutS Mutants. All the MutS mutants were profoundly impared in catalysis. Even at the highest concentrations of protein and AdoCbl feasible, none of the mutant MutS proteins exhibited any detectable glutamate mutase activity when the holoenzyme was reconstituted in the usual spectroscopic assay (Barker et al., 1964). We therefore developed a radiolabeled assay using ^{14}C-labeled glutamate as the substrate and following the incorporation of radioactivity into mesaconate. Using this much more sensitive assay, we were able to observe turnover with all of the mutants and determine their steady-state kinetic properties. Control experiments in which either MutE, AdoCbl, or the mutant MutS protein was omitted from the assay resulted in only background levels of radioactivity being associated with recovered carrier mesaconate.

Table 1: Coenzyme Binding and Kinetic Parameters for Wild-Type and Mutant Glutamate Mutase Enzymes[a]

glutamate mutase	apparent K_d for AdoCbl (μM)	k_{cat} (s^{-1})	apparent K_m for AdoCbl (μM)	apparent K_m for L-Glu (mM)
wild type[b]	1.8 ± 0.2	18.1	5.8 ± 0.2	1.1 ± 0.1
H16G	≈ 100	0.016	16 ± 3	2.5 ± 0.4
H16Q	≈ 100	0.023	29 ± 4	4.3 ± 1.0
D14N	34 ± 4	0.021	7.4 ± 0.6	4.5 ± 0.5
D14E	13 ± 4	0.014	34 ± 5	1.3 ± 0.4
D14A	20 ± 2	0.043	20 ± 2	2.0 ± 0.3

[a] The assay conditions are described in the text. [b] Data taken from Holloway and Marsh (1994).

The radioassay allowed us to probe further the absolute requirement for MutS protein in the glutamate mutase reaction. Even after overnight incubation with 150 μM AdoCbl and 10 mM glutamate, there was no detectable turnover in the absence of MutS. Similarly, no incorporation of tritium into AdoCbl was observed when tritiated glutamate was used as a substrate. We estimate the limits for detection of activity to be $\approx 10^{-6}$ of that of wild-type enzyme; MutS, therefore, appears to be absolutely required for turnover.

The mutants exhibited rather similar kinetic properties, regardless of the position or nature of the mutation (Table 1). In each case, k_{cat} was decreased by about 1000-fold when compared with that of wild-type enzyme, although unexpectedly, MutS-D14A was 2−3-fold more active than the other mutants. The apparent K_m for glutamate of the mutants was raised by only 2−4-fold, indicating that glutamate binding is little affected by mutations to the histidine-aspartate pair. The apparent K_m for AdoCbl varied more widely among the mutants. The *mutS*-D14N mutation, the most conservative change to the Co-His-Asp triad, appears to cause the least change to the K_m for AdoCbl; the value is only slightly higher than that measured previously for wild-type enzyme (Holloway & Marsh, 1994). The other mutants have K_ms that are 3−5-fold higher than that of wild type. Interpretation of these data is complicated by the fact that the K_m for AdoCbl is dependent upon the relative concentrations of MutS and MutE (Holloway & Marsh, 1994; Switzer & Barker, 1967). The protein in the radiolabeled assay was about 50 times more concentrated than in the spectroscopic assay used to determine the properties of the wild-type enzyme. This implies that the apparent K_ms reported here are probably lower than they would be if it were possible to measure them under conditions identical to wild-type conditions.

UV−Visible Spectra of Wild-Type and Mutant Holoenzymes. The UV−visible spectrum of the wild-type holoenzyme is shown in Figure 2. The wild-type holoenzyme has a spectrum very similar to that of free AdoCbl; in particular, the absorbance band at 530 nm is characteristic of the "base-

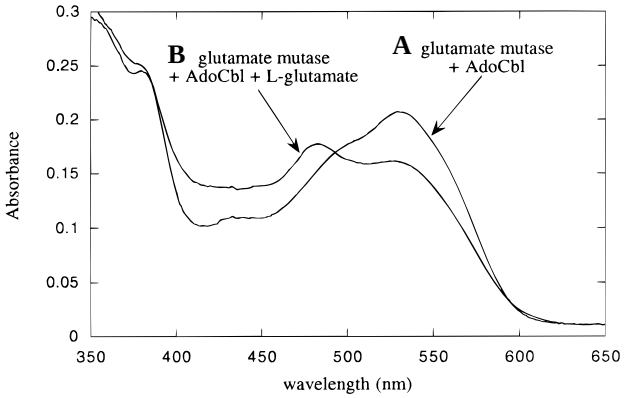

FIGURE 2: UV−visible spectrum of wild-type hologlutamate mutase. (A) The spectrum of the resting holoenzyme. The peak at 520 nm is characteristic of base-on AdoCbl. (B) The spectrum of the holoenzyme after addition of L-glutamate (final concentration of 10 mM). The increase in absorbance at 470 nm indicates that Cbl(II) is accumulating during turnover.

on" form. This implies that MutS-His16 is coordinated to cobalt, in accord with previous EPR studies (Zelder et al., 1995). The addition of L-glutamate (10 mM) caused a change in the spectrum consistent with the formation of Cbl(II) on the enzyme. Assuming $\Delta\epsilon_{530} \approx 4000\ M^{-1}\ cm^{-1}$, calculated from the extinction coefficients of free AdoCbl and Cbl(II), we estimate that 15−20% of the enzyme exists in the Cbl(II) form during steady-state turnover. This is in accord with estimates of Cbl(II) obtained from spin quantification of EPR spectra (Zelder et al., 1994).

The spectra of the mutant holoenzymes (Figure 3A) differ according to the nature of the mutation. MutS-D14N appears to bind AdoCbl predominantly in the base-on form, implying His16 is coordinated to cobalt, whereas the increased absorption at 450 nm in the spectra of the MutS-D14A and MutS-D14E mutants indicates that a substantial proportion of the holoenzyme is in the base-off form. The spectra of the MutS-H16G and MutS-H16Q mutants, although much less intense because these mutants bind AdoCbl very weakly, indicate that, as expected, the coenzyme is bound in the base-off form. No spectral changes were observed with any of the mutant enzymes when L-glutamate was added. The steady-state concentration of Cbl(II) during turnover must, therefore, be very small.

MeCbl, the other biologically active form of coenzyme B_{12}, is a competitive inhibitor of glutamate mutase (E. N. G. Marsh, unpublished results). Therefore, we thought it might be informative to compare the spectra of the wild-type and mutant enzymes with MeCbl bound. The spectra were obtained under the same conditions as those for AdoCbl. Both the wild-type and mutant enzymes give spectra that are very similar to those obtained with AdoCbl (Figure 3B). Thus, it appears that glutamate mutase binds both AdoCbl and MeCbl with similar affinity, and the nature of the alkyl group makes little difference with respect to coordination of cobalt by His16.

Effect of Mutations on AdoCbl Binding. To quantify the effect of mutations to the cobalt-His-Asp triad on AdoCbl binding, the apparent dissociation constants of the mutants for the coenzyme were measured by equilibrium gel filtration (Table 1), as described previously (Holloway & Marsh, 1994). Although the K_d for AdoCbl is also dependent upon the relative concentrations of MutE and MutS, in this case, it was possible to perform the measurements under experimental conditions the same as wild-type conditions, allowing for a direct comparison. Mutations of MutS-H16 considerably weaken the binding of AdoCbl by glutamate mutase. The apparent K_ds for AdoCbl of both MutS-H16G and MutS-H16Q were too high to be measured accurately, but were estimated to be $\approx 100\ \mu M$. The histidine ligand to cobalt

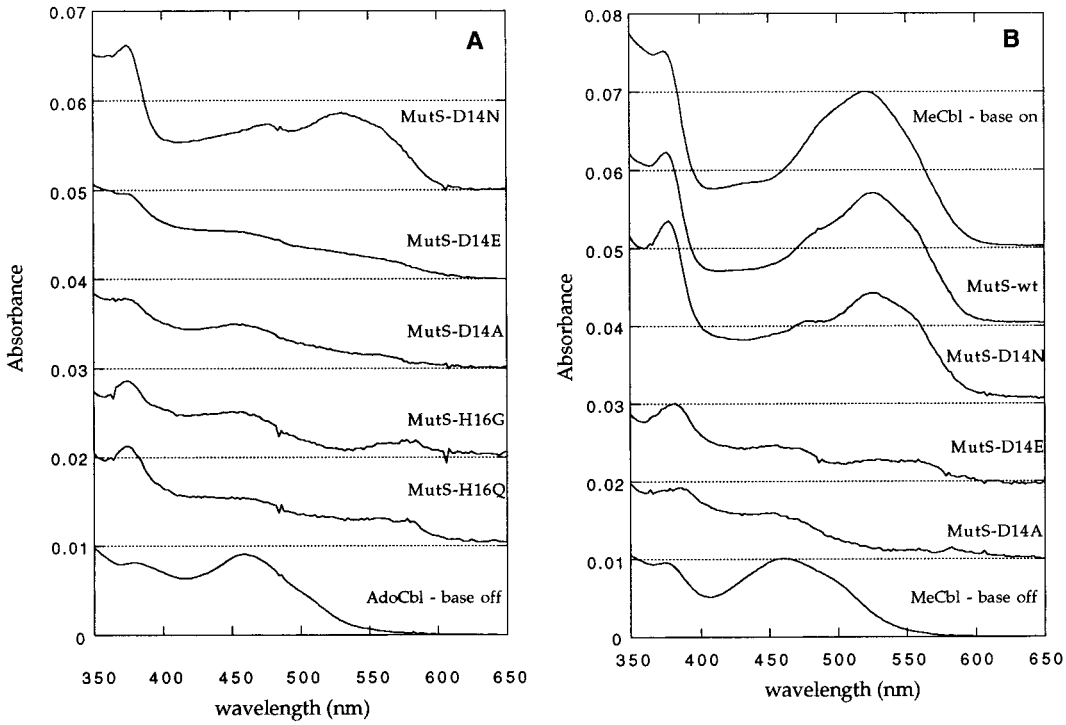

FIGURE 3: UV−visible spectra of glutamate mutase mutants with (A) AdoCbl bound and (B) MeCbl bound. The spectra of base-off AdoCbl and MeCbl are included for comparison. The spectra indicate that coordination of cobalt by MutS-His16 is sensitive to mutations introduced at MutS-Asp14. Mutants lacking His16 appear to bind cobalamin, as expected, in the base-off form. The traces for MutS-H16G and MutS-H16Q are shown with the absorbance scale expanded 2-fold.

appears, therefore, to be important for binding AdoCbl in glutamate mutase. Mutations of MutS-Asp14 also weaken AdoCbl binding, although to a lesser extent. The apparent K_ds for AdoCbl of the MutS-D14 mutants were increased between 6- and 18-fold in comparison with the apparent K_d for AdoCbl of wild-type glutamate mutase determined under the same conditions.

DISCUSSION

Numerous model studies have been directed toward understanding the influence of steric and electronic effects on the strength of the cobalt−carbon bond in alkylcobalamins. Despite this, the relative importance of these effects in the enzyme-catalyzed homolysis of AdoCbl has remained unclear. Our study, in which we have investigated the effect of changing or perturbing the axial ligand to cobalt by site-directed mutagenesis, is the first to begin to address these questions in an adenosylcobalamin-dependent enzyme; a similar study has recently been reported for methylcobalamin-dependent methionine synthase (Jarrett et al., 1996). Our results indicate that, in the glutamate mutase-catalyzed reaction, the axial base to cobalt probably contributes no more than 10^3 of the estimated 10^{12}-fold increase in the rate of AdoCbl homolysis required to explain the observed rate of turnover.

Nature of the Rate-Determining Step in the Mutated Enzymes. The proximity of the His-Asp pair to the cobalt−carbon bond of AdoCbl, revealed by the structures of MetH and MMCM, immediately suggested that these residues may be involved in promoting homolysis of the coenzyme. However, the DXHXXG motif is not present in all AdoCbl-dependent enzymes, and there is evidence that in some cases the coenzyme is bound with the pendant dimethylbenzimidazole moiety still coordinated to cobalt (Toraya, 1997). Our experiments have shown the importance of the His-Asp pair in catalysis but do not demonstrate directly that these residues serve to weaken the cobalt−carbon bond. Several lines of evidence support this idea, though. Firstly, mutation of either His16 or Asp14 greatly reduces k_{cat}, by 1000-fold, consistent with a chemical step in the mechanism being slowed. Secondly, in contrast to wild-type glutamate mutase, Cbl(II) does not appear to accumulate during catalysis in the mutant enzymes. This suggests that the rate-limiting step is changed and that it must occur either before or at homolysis of AdoCbl. Finally, Hay and Finke (1987) have measured the non-enzymatic rate of homolysis of adenosylcobinamide, the derivative of the coenzyme lacking the nucleotide tail, and found it to be 200-fold slower than the homolysis of AdoCbl where a dimethylbenzimidazole is coordinated to cobalt. Again, this is consistent with the imidazole ring of His16 serving to labilize the carbon−cobalt bond.

Relationship between the Apparent K_m and the Apparent K_d. That mutations of MutS-His16 raised the apparent K_d for AdoCbl much more than they raised the apparent K_m was unexpected and deserves some comment. We note that, whereas K_d is a simple equilibrium constant, the apparent K_m reflects binding of both AdoCbl and L-glutamate together with subsequent chemical steps. Consider the minimal three-step kinetic scheme below in which AdoCbl binding is followed first by a reversible step, k_2, that represents events such as substrate binding, a conformational change or reversible chemical steps, and finally an irreversible step, k_3, leading to product formation.

$$\text{E + AdoCbl} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E:AdoCbl + S} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{S:E':AdoCbl} \xrightarrow{k_3} \text{E:AdoCbl + P}$$

In this scheme, K_d is simply k_{-1}/k_1, whereas K_m is given by:

$$K_m = \frac{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3}{k_1(k_2 + k_{-2} + k_3)} \quad (1)$$

This can be rearranged to give:

$$K_m = \frac{k_{-1}}{k_1}\left[\frac{k_{-2} + k_3}{k_2 + k_{-2} + k_3} + \frac{k_2k_3}{k_{-1}(k_2 + k_{-2} + k_3)}\right] \quad (2)$$

It is evident from eq 2 that the relationship between K_m and K_d is complicated, and K_m may in principle be larger or smaller than K_d. In particular, mutations that increase K_d by making k_{-1} larger will increase K_m by a smaller amount because of the inverse dependence of the second term in eq 2 on k_{-1}. Further, if the second term is much larger than first term, i.e. k_2 dominates, then K_m becomes independent of K_d. Mutations of MutS-His16 do indeed result in a substantially increased K_d for AdoCbl, as well as causing a 1000-fold decrease in k_{cat}. It appears plausible then that changes in the elementary rate constants may easily result in a change in the rate-limiting step that could be reflected in the apparent K_ms for these mutants.

Comparison with the Effects of Mutating the DXHXXG Motif in Methionine Synthase. It is informative to compare our results with those obtained by Matthews and co-workers, who have used mutagenesis to dissect the His-Asp-Ser triad of methionine synthase (Amaratunga et al., 1996; Jarrett et al., 1996). The mutation MetH-D757N results in an enzyme that retains 6% of the activity under steady-state turnover conditions, whereas the rate constant for the chemical step, methyl transfer, is only reduced 2-fold. Since product release has been shown to be rate-limiting in MetH (Banerjee et al., 1990), it is this step that is most likely being effected by the MetH-D757N mutation. Given the relatively minor perturbation of the catalytic properties of MetH caused by this mutation, it is quite remarkable that mutation of MutS-Asp14 causes such a large decrease in k_{cat} for glutamate mutase. However, the MetH-H759G mutant, in which the cobalt-coordinating histidine is deleted, is essentially inactive; the enzyme is unable to undergo full turnover, and the rate of demethylation of MeCbl is slower by 10^5. This contrasts with only a 10^3-fold decrease in k_{cat} when MutS-His16 is mutated in glutamate mutase. Clearly, similar mutations when introduced into the DXHXXG motif of methionine synthase and glutamate mutase have quite different effects on the enzymes' kinetic properties. This is presumably a consequence of the fact that the homolytic cleavage of AdoCbl catalyzed by glutamate mutase and heterolytic cleavage of MeCbl catalyzed by methionine synthase proceed through different transition states.

MetH binds MeCbl very tightly so that mutant enzymes, even those lacking His759, were isolated with cobalamin bound to them (Amaratunga et al., 1996). His759 in MetH appears to coordinate cobalt much more tightly than does

MutS-His16 in glutamate mutase. Whereas the mutation MetH-D757E has no discernible effect on the coordination state of MeCbl bound to MetH, the mutation MutS-D14E is sufficient to switch the coordination state of cobalt from being base-on (coordinated by MutS-His16) to being predominantly base-off. This effect is observed with either AdoCbl or MeCbl bound to glutamate mutase and implies that it is an intrinsic property of the enzyme and is not caused by differences in the σ-donating properties of the alkyl group bonded to cobalt. Whether the coordination strength of the axial ligand is responsible for determining the different modes of cobalt−carbon bond cleavage observed with enzymes that use MeCbl and AdoCbl, as suggested by recent studies (Garr et al., 1996), is an interesting question that requires further study. Intriguingly, the structure of MMCM revealed an unusually long (2.5 Å) cobalt−nitrogen bond between the coenzyme and the axial histidine (Mancia et al., 1996), suggesting that the ligand is weakly coordinated even though the coenzyme is bound very tightly. In comparison, the cobalt−nitrogen bond in MetH is much shorter and very similar to that observed in the free coenzyme (Drennan et al., 1994).

In conclusion, we have shown that mutants of glutamate mutase in which MutS-His16 or MutS-Asp14 are changed turn over at only $^1/_{1000}$ of the rate of wild-type enzyme. Kinetic and spectral data point to a change in the rate-limiting step, consistent with AdoCbl homolysis being impaired. This suggests that the cobalt-His-Asp triad plays an important role in modulating the reactivity of the coenzyme but that other factors must also contribute to weakening the AdoCbl cobalt−carbon bond.

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