Adenosylcobalamin-Dependent Glutamate Mutase: Examination of Substrate and Coenzyme Binding in an Engineered Fusion Protein Possessing Simplified Subunit Structure and Kinetic Properties†

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ABSTRACT: Glutamate mutase is comprised of two weakly associating subunits, E and S, that combine to form the coenzyme binding site. The active holoenzyme assembles in a kinetically complex process in which both the stoichiometry and apparent K_d for adenosylcobalamin (AdoCbl) are dependent upon the relative concentrations of the two subunits, as is the enzyme's specific activity. To facilitate mechanistic and structural studies on this enzyme we have genetically fused the S subunit to the C-terminus of the E subunit through an 11 amino acid $(Gly\text{-}Gln)_5$-Gly linker segment. This protein, GlmES, binds AdoCbl stoichiometrically and neither the affinity for AdoCbl nor the turnover number depends upon protein concentration. The k_{cat} and K_m for both substrate and coenzyme, together with the deuterium isotope effects on V_{max} and V_{max}/K_m, have been determined for the GlmES-catalyzed reaction proceeding in both directions. Compared with wild type, the affinity for AdoCbl is unchanged, but for the conversion of L-glutamate to (2S,3S)-3-methylaspartate both k_{cat} and K_m for L-glutamate are decreased by about a third and the isotope effects are reduced, suggesting product release to be more rate-limiting. To test hypotheses concerning the activation of the coenzyme, we examined the binding of adenosylcobalamin, methylcobalamin, and cob(II)alamin to the enzyme. Each of these is bound with essentially the same affinity (2 μM), suggesting that, contrary to expectations, interactions between the protein and the adenosyl moiety do not serve to weaken the cobalt−carbon bond in the ground state.

Adenosylcobalamin- (AdoCbl,[1] coenzyme B_{12}) dependent glutamate mutase (EC 5.4.99.1) catalyzes the reversible interconversion of L-glutamate to (2*S*,3*S*)-3-methylaspartate as the first step in the fermentation of L-glutamate (*1*) by various species of clostridia. The enzyme has been cloned from *Clostridium tetanomorphum* (*2*) and *Clostridium cochlearium* (*3*). It is one of a group of AdoCbl-dependent isomerases that catalyze unusual isomerizations in which a hydrogen atom on one carbon atom is interchanged with an electron-withdrawing group on an adjacent carbon (*4*−*7*). These rearrangements proceed through a mechanism involving free radicals that are generated by homolysis of AdoCbl. The B_{12}-dependent isomerases are one group in an emerging class of enzymes that use carbon-based free radicals to catalyze a variety of chemical transformations on otherwise unreactive substrates (*8, 9*). In general, the radical, generated on the enzyme, is used to remove a nonacidic hydrogen atom from the substrate. The substrate radical is thereby activated to undergo reactions such as carbon−carbon, carbon−oxygen, or carbon−nitrogen bond cleavage that would otherwise be difficult to achieve.

Glutamate mutase shares a conserved cobalamin-binding domain with AdoCbl-dependent methylmalonyl-CoA mutase (MMCM) and 2-methyleneglutarate mutase (MGM); this domain is also found in methylcobalamin-dependent methionine synthase (MetH) (*10*). The crystal structures of MMCM and a methylcobalamin-binding fragment of MetH have been solved (*11*−*13*). In both cases the conserved B_{12}-binding domain is revealed to possess an α/β structure—a variant of the canonical nucleotide-binding (Rossmann) fold—that recognizes the lower α-face and dimethylbenzimidazole ribofuranosyl "tail" of the coenzyme. A surprising and important feature is that the cofactor is bound in an extended conformation in which the nucleotide tail is displaced by a conserved histidine residue that is, in turn, hydrogen-bonded to an aspartate residue in a manner reminiscent of the catalytic triad of the serine proteases. Both these residues have been shown to play a significant role in catalysis and coenzyme binding in glutamate mutase (*14*).

The upper, reactive face of AdoCbl is recognized by a catalytic domain that also contains the substrate-binding site, for which there are no apparent sequence similarities evident between enzymes. In MMCM and MGM the catalytic and conserved B_{12}-binding domains are present as the N- and C-terminal portions, respectively, of a single protein subunit (Figure 1). However, glutamate mutase is unusual in that the catalytic portion and the B_{12}-binding domain comprise two separately encoded subunits, designated MutE and MutS respectively for *C. tetanomorphum* enzyme and GlmE and GlmS for the enzyme from *C. cochlearium*. The E subunit is a dimeric protein of subunit M_r 54 000, and the S subunit is a monomer of M_r 14 800 (*2, 3, 15*).

We have shown that the interaction between MutE and MutS is relatively weak and this results in complex kinetic behavior (*15*): MutS binds to MutE in a cooperative manner

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[1] Abbreviations: AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin; HOCbl, hydroxocobalamin; Cbl(II), cob(II)alamin; MMCM, methylmalonyl-CoA mutase; MGM, 2-methyleneglutarate mutase; MetH, methionine synthase.

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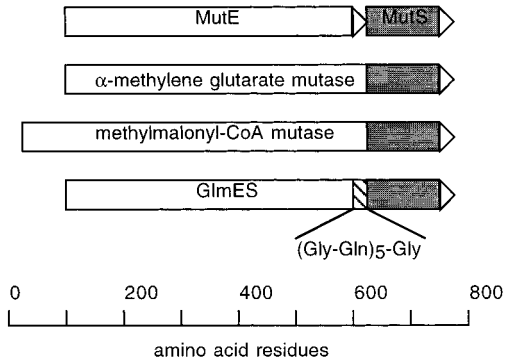

FIGURE 1: Distribution of the conserved MutS-like B_{12}-binding domain (shaded region) in AdoCbl-dependent isomerases. Top to bottom: E and S subunits of glutamate mutase (GM), 2-methyleneglutarate mutase (MGM); methylmalonyl-CoA mutase (MMCM), and GlmES. GlmES was created by fusing the *glmE* gene from *C. cochlearium* to the *mutS* gene from *C. tetanomorphum* using the linker sequence indicated.

(Hill coefficient 1.3), and under normal assay conditions a severalfold molar excess of MutS is required for maximal enzyme activity. The association of both subunits is required for the enzyme to bind AdoCbl, consistent with the coenzyme-binding site being formed at the interface between the MutE and MutS subunits, and this results in the apparent K_m and K_d for AdoCbl being dependent upon the relative concentrations of the two subunits (*15*). Even measurements of the stoichiometry with which AdoCbl is bound are complicated by this phenomenon; values of between 1 and 2 molecules of AdoCbl/glutamate mutase dimer have been reported, depending upon the conditions under which the measurements are made (*3, 15*). In contrast, MMCM and MGM bind AdoCbl far more tightly (nanomolar as opposed to micromolar affinities) and do not exhibit cooperative behavior (*16, 17*).

The fact that both k_{cat} and the apparent K_m and K_d for AdoCbl are dependent on protein concentration makes it difficult to interpret the results from experiments (such as pre-steady-state stopped-flow measurements or kinetic characterization of highly impaired mutants) where the concentration of enzyme used must necessarily be very different from that typically employed in the spectroscopic assay. To simplify the kinetics and to facilitate mechanistic and physical studies, we have engineered a fusion protein of glutamate mutase in which the B_{12}-binding subunit is linked to the C-terminus of the catalytic subunit by an artificial linker sequence. Here we describe the construction and purification of this protein together with the characterization of its physical and kinetic properties.

EXPERIMENTAL PROCEDURES

Materials. 2-Hydroxyglutarate dehydrogenase and plasmid pOZ5 (*3*), expressing the *glmE* gene, were the kind gifts of Professor W. Buckel and Dr. O. Zelder. The construction of the plasmid pmutS has been described previously (*15*). 3-Methylaspartase was purified as described by Hsiang and Bright (*18*). Restriction endonucleases and DNA modifying enzymes were purchased from Promega. AdoCbl, MeCbl and HOCbl were purchased from the Sigma Chemical Co. The sources of other materials have been described previously (*14, 15, 19*) or were purchased from commercial suppliers

PCR Assembly of glmES Gene. The linker sequence encoding the peptide repeat -(Gly-Gln)$_5$-Gly- was introduced between the *glmE* and *mutS* genes using "recombinant PCR" (*20*). Four oligonucleotide primers were synthesized. Oligo-1 was designed to prime toward the 3′-terminus of the *glmE* gene, upstream of a unique internal *Bsm*I site. Two overlapping, complementary oligonucleotide primers, oligo-2 and oligo-3, were designed to introduce the linker sequence at the 3′-terminus of the *glmE* gene and the 5′-terminus of the *mutS* gene, respectively. Oligo-4, was designed to prime from the 3′-terminus of the *mutS* gene. The sequences of the primers were as follows: oligo-1, ATTGGTATACCAACAAAAGAAGC; oligo-2, (TCCTTG)$_5$TCCTTCTGGTCTTCCAATTAATCTTCC; oligo-3, (GGACAA)$_5$GGAGAGAAAAAGACTATTGTTCTTGGA; oligo-4, CCCCGTCGACTTATTCTACTCCTAA.

A 400 base pair 3′-region of the *glmE* gene was amplified using oligo-1 and oligo-3 as primers and 25 ng of plasmid pOZ5 as template, following a protocol described previously (*19*); the PCR product was designated *GlmE′*. To amplify *mutS*, oligo-3 and oligo-4 were used as primers and 25 ng of plasmid pmutS as template; this PCR product was designated *mutS′*. The fragments *glmE′* (30 ng) and *mutS′* (10 ng) were assembled in a third PCR reaction using oligo-1 and oligo-4 as primers and as cotemplates; this PCR product was designated *glmES′*. *GlmES′* was restricted with *Bsm*I and *Sal*I and the resulting *Bsm*I−*Sal*I fragment purified by electrophoresis through a 0.7% agarose gel. This fragment was subcloned into pOZ5 that had been restricted with *Bsm*I and *Sal*I and the liberated *Bsm*I−*Sal*I fragment was purified away by electrophoresis. The resulting plasmid, designated pglmES, was maintained in *Escherichia coli* TG1.

Expression and Purification of GlmES. Expression and purification of the GlmES protein (*15*) followed that devised by Zelder et al. (*3*). Cells were grown in LB medium in the presence of ampicillin (100 mg/L) at 37 °C until they reached an A_{600} of 1.0−1.5. Synthesis of GlmES was induced by the addition of IPTG (200 mg/L) and the cultures were allowed to grow overnight.

In a typical purification 13 g of cells (damp weight) from 6 L of culture were resuspended in 20 mL of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT and 1 mM EDTA. The cells were ruptured by sonication and cell debris was removed by centrifugation at 25000*g* for 15 min. The supernatant (30 mL) was brought to 30% saturation in ammonium sulfate by slow addition of the solid. The precipitate was removed by centrifugation at 25000*g* for 15 min and the supernatant (28 mL) was brought to 80% saturation in ammonium sulfate. The precipitated proteins were recovered by centrifugation at 25000*g* for 15 min and redissolved in 20 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. The protein solution was cleared by centrifugation at 25000*g* for 15 min and applied to a 2.6 × 20 cm column of phenyl-Sepharose CL-4B equilibrated in 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. Contaminating proteins were removed by washing the column with 500 mL of 5 mM potassium phosphate buffer, pH 7.0, at a flow rate of 1 mL min^{-1}. GlmES protein was then eluted isocratically with H_2O containing 1 mM DTT, and 4.5 mL fractions were collected. Fractions containing GlmES protein were pooled and concentrated by ultrafiltration in a stirred cell fitted with a PM 30 membrane (exclusion limit 30 kDa).

Final purification was achieved by FPLC using a phenyl-Superose HR 5/5 hydrophobic interaction column (Pharma-

cia). The concentrated protein solution was adjusted to contain 50 mM potassium phosphate, pH 7.0, and 1 M ammonium sulfate by addition of 1 M potassium phosphate buffer, pH 7.0, and solid ammonium sulfate. The sample was cleared by centrifugation at 10000*g* for 10 min and approximately 5 mg was applied to the column equilibrated in 1 M ammonium sulfate and 50 mM potassium phosphate, pH 7.0. Proteins were eluted with a linear, descending gradient of ammonium sulfate. GlmES eluted in a well-resolved, broad peak. The protein was stored at −20 °C in the presence of 50% glycerol.

Enzyme Assay. To assay glutamate mutase in the direction glutamate to 3-methylaspartate, activity was measured spectroscopically by coupling the formation of 3-methylaspartate to mesaconate through the action of methylaspartase, as described by Barker et al. (*1*), except that the buffer was 100 mM potassium phosphate containing 1 mM $MgCl_2$ at pH 7.0. To assay in the direction 3-methylaspartate to glutamate, glutamate formation was coupled to the reduction of NADH through the action of glutamate−oxalacetic transaminase and 2-hydroxyglutarate dehydrogenase (*21*). The assay contained 1 mM oxalacetate, 0.15 mM NADH, 0.5 μg of glutamate−oxalacetic transaminase, and 0.3 unit of 2-hydroxyglutarate dehydrogenase in 100 mM potassium phosphate, pH 7.0, together with required concentrations of substrate, coenzyme, and GlmES in a total volume of 0.5 mL. After an initial lag phase the rate was steady for several minutes. Both assays were initiated by addition of substrate and carried out at 22 °C.

Preparation of Cob(II)alamin and Anaerobic Assay Procedure. In experiments to investigate the inhibition of GlmES by Cbl(II), Cbl(II) was generated *in situ* by stoichiometric reduction of HOCbl with DTT under anaerobic conditions. All solutions were degassed on a water pump for 2 h and purged with argon for another 10 min. A 300 μL solution of the desired concentration of HOCbl and 300 μL of 50 μM DTT were injected into a septum-sealed 2 mL anaerobic cuvette that had been flushed with argon for 5 min prior to use. The reduction of HOCbl to Cbl(II) was monitored by the changes in the UV−visible spectrum and usually took about 6 min. The solutions of Cbl(II) were stable for next 10 min. Once reduction was complete, 300 μL of a deoxygenated solution of adenosylcobalamin and 500 μL of a deoxygenated solution containing 150 pmol of glutamate mutase and 0.1 unit of methylaspartase were injected into the cuvette immediately. After mixing and incubation in the dark for a further 2 min, the reaction was initiated by injection of 100 μL of a deoxygenated solution of glutamate.

Curve Fitting. Modified computer programs of Duggleby (*22*) were used to fit kinetic, inhibition, and binding data to eqs 1, 2, or 3 as appropriate:

$$v = VA(V/K_a)/[V + (V/K_a)A] \quad (1)$$

$$v = VAB/(K_{ia}K_b + K_bA + K_aB + AB) \quad (2)$$

$$v = VA/[K_a(1 + I/K_i) + A] \quad (3)$$

where V is maximal velocity or extent of binding; A, B, and I are substrate, cofactor, and inhibitor concentrations; K_a and K_b, Michaelis or dissociation constants for A and B; K_{ia} is the dissociation constant for A in the absence of B; and K_i is the inhibition constant for I. Plots of kinetic and binding data were generated using the KaleidaGraph program (Abelbeck Software).

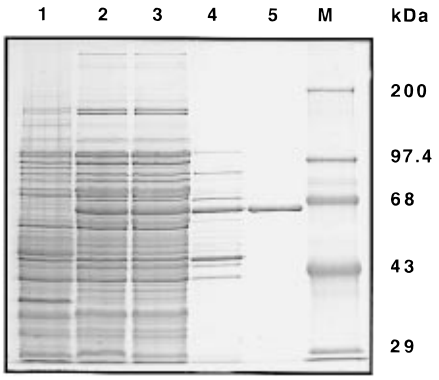

FIGURE 2: Expression and purification of GlmES. Coomassie-stained 12% polyacrylamide gel showing (lane 1) crude cell extract before induction, (lane 2) crude cell extract after induction, (lane 3) ammonium sulfate fractionation, (lane 4) chromatography on phenyl-Sepharose CL4B column, and (lane 5) FPLC on phenyl-Superose HR column. The molecular masses of marker proteins (lane M) are indicated at the side.

Determination of Protein Concentration. Samples of GlmES for amino acid analysis were hydrolyzed in 6 N HCl for 24 h at 110 °C and their amino acid composition was determined using a commercial analyzer. The amino acid composition deduced from the gene sequence was then used to calculate the molar concentration of the protein samples.

RESULTS

Construction and Purification of GlmES. In the construction of the single-subunit glutamate mutase we used the homologue of *mutE* from *C. cochlearium*, *glmE*, which has previously been cloned and over-expressed in *E. coli* (*3*), rather than the *mutE* gene. In initial experiments, constructs encoding a MutE−MutS fusion protein expressed the protein as inclusion bodies that could not be efficiently refolded into active apoenzyme. This may be related to the previously observed tendency of MutE to aggregate irreversibly at high concentrations (*15*).

Complementary oligonucleotides were synthesized to encode an 11-residue linker sequence, $(Gly\text{-}Gln)_5$-Gly, that was inserted between the last residue of GlmE and the first residue of MutS (Figure 1) using standard PCR techniques as described in the Experimental Procedures section. The final construct, derived from pOZ5 and designated pglmES, encoded an open reading frame encoding a protein of 633 amino acid residues comprising the *glmE* sequence, the linker sequence, and finally the *mutS* sequence. The *glmES* gene was placed under the control of the *lacUV5* promoter, and induction of exponentially growing cultures with IPTG resulted in reasonable levels of protein expression.

GlmES protein was purified by hydrophobic interaction chromatography, following a scheme similar to that used by Zelder and co-workers for the purification of GlmE (*3*). To obtain highly pure protein it was necessary, however, to perform a final FPLC purification step, using a phenyl-Sepharose HR column (Figure 2). The purified protein was stable and could be stored for several months in the presence of 50% glycerol at −20 °C without loss of activity. The molar extinction coefficient of GlmES at 280 nm, ϵ_{280}, was determined by amino acid analysis as $\epsilon_{280} = 108\,000\ M^{-1}$

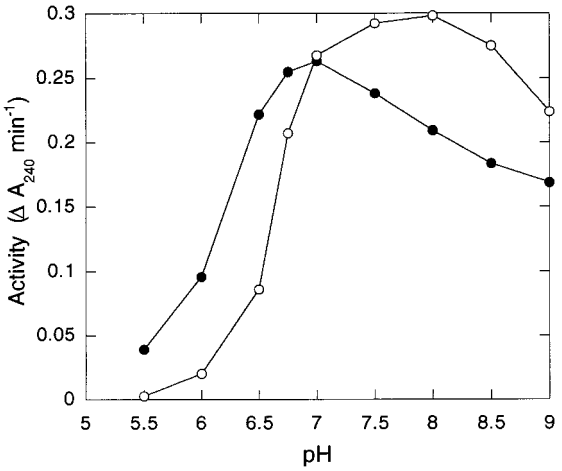

FIGURE 3: pH dependence of glutamate mutase activity for GlmES protein (●) and wild-type GlmE:MutS system (○). Assays were performed in 0.05 M phosphate buffer between pH 5.5 and 7.5 and in 0.05 M Tris-H/Cl buffer between pH 8.0 and 9.0, in the presence of 10 mM L-glutamate and 25 μM AdoCbl.

cm^{-1}. The apparent M_r for GlmES, as estimated by gel filtration on a calibrated Superose-12 FPLC column, was 150 000, suggesting that, as expected, it retains a dimeric subunit structure.

pH Dependence of GlmES Activity. Preliminary experiments revealed GlmES to be active in the glutamate mutase assay, although k_{cat} was somewhat lower than for wild type. Unexpectedly, it was found that whereas MutS requires prior reduction with thiol-reducing reagents such as dithiothreitol for activity (*15*), GlmES was active without reduction and dithiothreitol had no effect on activity. Presumably the GlmE domain protects the MutS domain from adventitious oxidation and disulfide formation. To determine the optimal pH at which to conduct kinetic measurements, the pH of the assay solution was varied between 5.5 and 9.0. The concentrations of glutamate (10 mM) and AdoCbl (25 μM) were kept constant and remained close to saturating over the pH range examined. Maximal rates were observed at about pH 7.0 (Figure 3), a value significantly lower than for wild-type enzyme, which is maximally active between pH 8.0 and 8.5. All further kinetic and binding experiments were therefore conducted at pH 7.0.

Concentration Dependence of GlmES Activity. Our primary objective in constructing a single-subunit glutamate mutase was to create an enzyme in which k_{cat} and the apparent K_ms for substrates and coenzyme were not dependent upon protein concentration. We therefore compared the variation of GlmES activity over a 30-fold concentration range with that of the wild-type enzyme reconstituted from GlmE and MutS. GlmE and MutS were present in an equimolar ratio and their concentrations varied over the same concentration range. The data plotted in Figure 4 demonstrate that GlmES activity increases linearly with protein concentration, implying that the specific activity is independent of concentration. In contrast, the activity of wild-type glutamate mutase increases significantly with protein concentration leading to an upwardly curved plot; this implies that the concentration-dependent association of GlmE and MutS is altering the proportion of enzyme in the active form.

We have previously shown that AdoCbl binding by wild-type glutamate mutase is also dependent on the relative concentrations of the E and S subunits (*15*). To investigate

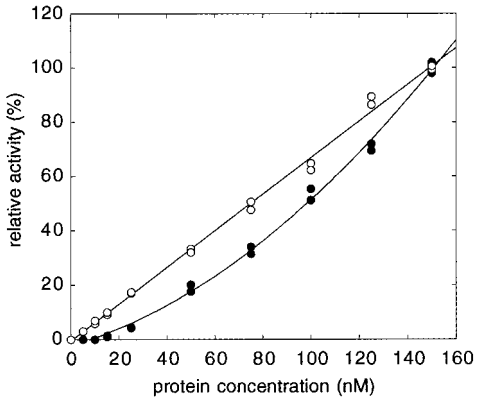

FIGURE 4: Concentration dependence of GlmES activity (○) compared with that of equimolar concentrations of GlmE and MutS (●). In the comparison, the activity measured at 150 nM protein concentration has been normalized to 100% for each enzyme.

Table 1: Variation of the Apparent K_m for AdoCbl with Protein Concentration for GlmES and for Equimolar Concentrations of GlmE and MutS

protein concn (nM)	GlmES appK_m (AdoCbl) (μM)	GlmE + MutS appK_m(AdoCbl) (μM)
50	8.4 ± 0.9	> 25
100	8.6 ± 1.0	16.7 ± 3.5
150	8.5 ± 0.9	12.0 ± 2.2

whether coenzyme binding by GlmES was dependent upon protein concentration, we determined the apparent K_m for AdoCbl at three different concentrations of GlmES. For comparison, the apparent K_m for AdoCbl was also determined at the same concentrations for equimolar ratios of GlmE and MutS (Table 1). Whereas for the wild-type enzyme the apparent K_m for AdoCbl increased significantly as the protein concentration was lowered, the apparent K_m for AdoCbl of GlmES was, within experimental error, the same at each concentration of protein used.

Steady-State Kinetic Properties. Having established that GlmES behaved as intended, the steady-state kinetic properties of this enzyme were investigated for the reaction proceeding in both directions.[2] In particular, the kinetic constants for the rearrangement of 3-methylaspartate to glutamate catalyzed by glutamate mutase have not, to our knowledge, previously been reported. Therefore, we used a spectroscopic assay (*21*) to measure the conversion of (2*S*,3*S*)-3-methylaspartate to L-glutamate, which was achieved by coupling the formation of glutamate to the oxidation of NADH through the successive action of glutamate:aspartate transaminase and α-hydroxyglutarate dehydrogenase. Although the assay suffers from an initial lag phase of about 1 min, using the concentrations of coupling enzymes and cosubstrates described in the Experimental Procedures section, the assay was subsequently linear and reliable kinetic data could be obtained.

The velocity of the GlmES-catalyzed reaction was measured in both directions as the concentrations of both substrates and AdoCbl were varied. The data were analyzed graphically using the Hanes plot and by computer fitting to

[2] We apply the term forward to refer to the reaction proceeding in the direction L-glutamate to (2*S*,3*S*)-3-methylaspartate and reverse to refer to the reaction proceeding in the direction (2*S*,3*S*)-3-methylaspartate to L-glutamate.

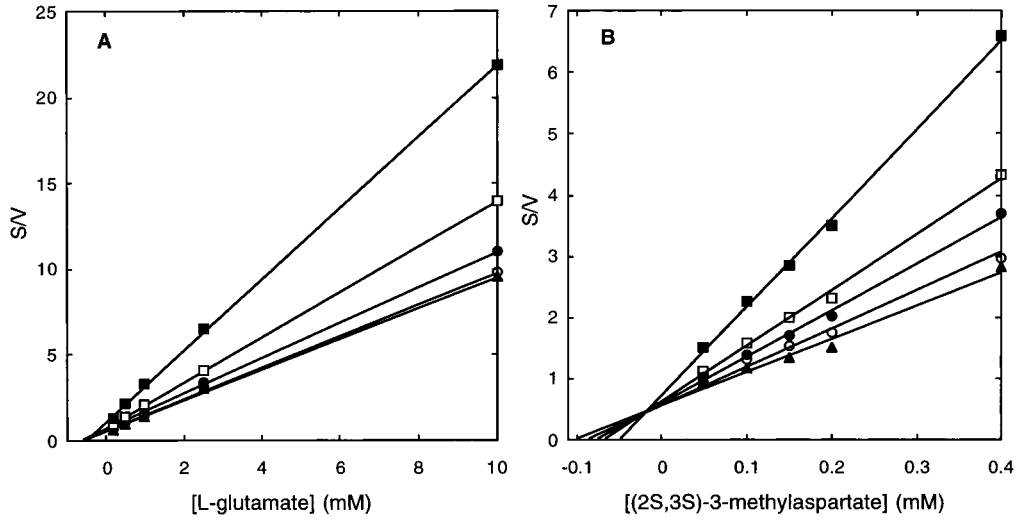

FIGURE 5: Hanes plots of kinetic data for GlmES. (A) Glutamate mutase activity measured in the forward direction. The concentrations of AdoCbl were 25 (▲), 15 (○), 10 (●), 6 (□), and 3 (■) μM. The pattern of lines intersecting on the *x*-axis indicates that binding of L-glutamate does not alter the affinity of the enzyme for AdoCbl. (B) Glutamate mutase activity measured in the reverse direction. The concentrations of AdoCbl were 15 (▲), 10 (○), 5 (●), 2.5 (□), and 1.25 (■) μM. The intersecting pattern of lines indicates that the affinity of the enzyme for AdoCbl is lowered when (2*S*,3*S*)-3-methylaspartate is bound.

eq 2. In both cases the Hanes plots show an intersecting pattern of lines expected for the formation of a ternary complex by either a random or ordered sequential mechanism (*23*). In the forward direction [L-glutamate to (2*S*,3*S*)-3-methylaspartate] the lines intersect on the *x*-axis (Figure 5A). This is diagnostic for the formation of a ternary complex in which the affinity of L-glutamate is not influenced by the binding of AdoCbl and *vice versa*. Accordingly, the K_ms for L-glutamate and AdoCbl, which were 0.58 ± 0.08 mM and 5.5 ± 0.7 μM, respectively, are, within experimental error, the same as the K_i values, which were 0.49 ± 0.18 mM and 4.3 ± 0.9 μM, respectively. A similar noncompetitive mode of binding between L-glutamate and AdoCbl has also been observed for wild-type enzyme (*15*). k_{cat} for the conversion of L-glutamate to (2*S*,3*S*)-3-methylaspartate was 5.8 ± 0.3 s^{-1}.

In the reverse direction [(2*S*,3*S*)-3-methylaspartate to L-glutamate], different kinetic behavior was observed. The pattern of lines generated by the Hanes plot (Figure 5B) intersects above the *x*-axis, implying that when 3-methylaspartate is bound to the enzyme, the affinity of the enzyme for AdoCbl is *weakened*, and *vice versa* (*23*). This is reflected in the values of K_m and K_i for 3-methylaspartate, which are 0.14 ± 0.02 mM and 0.015 ± 0.011 mM, respectively, and in K_m and K_i for AdoCbl, which are 3.1 ± 0.2 μM and 0.34 ±0.15 μM, respectively. Interestingly, k_{cat} for the conversion of (2*S*,3*S*)-3-methylaspartate to L-glutamate was 5.8 ± 0.2 s^{-1}, the same as for the forward direction.

Deuterium Isotope Effects. The deuterium isotope effects upon V_{max} (^DV) and V_{max}/K_m ($^DV/K$) were determined for the reaction in both directions in the presence of a fixed concentration (25 μM) of AdoCbl. In the forward reaction commercially available DL-glutamate-2,4,4-d_3 was used. Control experiments established that the D-isomer of glutamate was neither an inhibitor nor a substrate. In the reverse direction (2*S*,3*S*)-3-methylaspartate and (2*S*,3*S*)-3-(methyl-d_3)aspartate, synthesized enzymatically as described by Eagar et al. (*24*), were used. In the forward direction $^DV = 3.9 \pm 0.3$ and $^DV/K = 4.0 \pm 1$, whereas in the reverse direction $^DV = 6.3 \pm 0.5$ and $^DV/K = 3.4 \pm 0.7$. For comparison, the values measured for the wild-type enzyme in the forward direction were $^DV = 7.0 \pm 0.5$ and $^DV/K = 6.4 \pm 0.6$. The smaller isotope effects measured for GlmES, taken together with the lower values for V_{max} and K_m, suggest that product release may be more rate-limiting in GlmES.

Binding of Cobalamins to GlmES. The construction of GlmES enabled us to examine the binding, free from protein concentration effects, of AdoCbl and other cobalamins analogues so that mechanistic hypotheses regarding the enzyme-promoted homolysis of AdoCbl could be tested. Binding constants were measured by equilibrium gel filtration (*15*, *25*) in the same buffer, 100 mM potassium phosphate, pH 7.0, as the kinetic assays. The K_d for AdoCbl binding to GlmES was 2.0 ± 0.2 μM, very similar to the value of 1.8 ± 0.2 μM determined previously for wild-type glutamate mutase under conditions where MutS was present in 5-fold molar excess over MutE. At saturating concentrations of coenzyme, GlmES bound AdoCbl with a stoichiometry of 2 mol of AdoCbl/mol of GlmES dimer, indicating that, as expected, each subunit binds one molecule of coenzyme.

To examine the role played by the adenosyl moiety in the recognition of AdoCbl by GlmES we measured the binding of MeCbl to the enzyme. This analogue retains the carbon−cobalt bond, and therefore a similar electronic environment around the cobalt atom, whereas the methyl group presents minimal steric bulk. The K_d for MeCbl was 1.9 ± 0.2 μM—within error the same as that for AdoCbl. The binding of MeCbl to GlmES was also studied kinetically: MeCbl behaved as a competitive inhibitor of GlmES with a K_i of 3.7 ± 0.7 μM. These results suggest that, at least in the resting enzyme, the adenosyl group does not contribute significantly to the binding or recognition of AdoCbl.

Finally, we investigated the binding of the posthomolysis reaction intermediates cob(II)alamin and 5′-deoxyadenosine to the enzyme. Attempts to measure the K_d for Cbl(II) directly were unsuccessful because Cbl(II) undergoes slow decomposition during the course of the binding experiment and therefore reliable data could not be obtained. The decomposition may be caused by disproportionation of

Cbl(II) to Cob(I) and Cob(III) that is subsequently re-reduced to Cbl(II) by DTT. This was not a problem in the much shorter time scale of the kinetic assay, and it was possible to measure the inhibition of GlmES by Cbl(II). Cbl(II) behaved as a competitive inhibitor of GlmES with a K_i of 2.3 ± 0.3 μM.

To study the binding of 5′-deoxyadenosine, the reverse assay was employed because 5′-deoxyadenosine has too strong an absorption at the wavelength used to assay the enzyme in the forward direction (240 nm) for reliable measurements to be made. 5′-deoxyadenosine behaved as a competitive inhibitor of the enzyme with respect to AdoCbl, although it was bound relatively weakly; $K_i = 2.2 \pm 0.3$ mM. The K_i for 5′-deoxyadenosine was also determined in the presence of 10 μM Cbl(II) to examine whether synergistic effects between Cbl(II) and 5′-deoxyadenosine might result in tighter binding of these intermediates, as has been observed with AdoCbl-dependent ribonucleotide reductase (*26*). However, the presence of Cbl(II) resulted in no significant change in the enzyme's affinity for 5′-deoxyadenosine, $K_i = 2.7 \pm 0.3$ mM. It appears, therefore, that differential binding of Cbl(II) and 5′-deoxyadenosine provides little stabilization energy to offset the bond dissociation energy of the AdoCbl cobalt−carbon bond.

DISCUSSION

Engineering of GlmES. By joining MutS to the C-terminus of GlmE through an 11 amino acid linker sequence we have successfully engineered the subunit structure from an $\alpha_2\beta_2$ tetramer to an α_2 homodimer. This protein, GlmES, possesses very similar catalytic properties to the wild type, but the kinetics of the assembly of active holoenzyme are greatly simplified. In particular, coenzyme binding and k_{cat} are no longer dependent on protein concentration.

GlmES was constructed without detailed knowledge of the enzyme's structure. It has not been necessary to engineer new contacts between the two subunits; rather we have exploited the existing weak interaction of the subunits by using a flexible tether to provide a high local concentration of one subunit relative to the other and thus promote their association. The length of the linker, 11 amino acids, which theoretically can span a distance of about 40 Å, was necessarily somewhat arbitrary. Sequences of similar length are often found in loops on the surface of proteins and have successfully been employed to link the two subunits of bacterial tryptophan synthase (*27*) and the four subunits comprising the glucose phosphotransferase system of *E. coli* (*28*). The sequence we chose, $(Gly\text{-}Gln)_5$-Gly, was intended to avoid charged and hydrophobic amino acids, those with a high propensity to form secondary structure, and known protease sites. However, we do not believe there is anything special about this sequence and arguably many other linking sequences would have sufficed. This approach should be of general utility for studying weakly interacting proteins, provided the N- and C-termini are close enough to be spanned by a relatively short linker.

Our approach was guided, in part, by the observation that the mechanistically similar MMCM and MGM both incorporate the conserved B_{12}-binding region as the C-terminal domain. The fact that glutamate mutase can be made to adopt a similar topology supports the idea that these enzymes may possess a similar global fold, despite the lack of sequence similarity between their N-terminal regions. In MMCM the N-terminal domain adopts a β-barrel structure: notably, this most common of folds is shared among many globular proteins that show no sequence similarity to each other.

Kinetics and Substrate Binding. Knowledge of the steady-state kinetic parameters of GlmES is a prerequisite for further pre-steady-state and physical studies aimed at elucidating the energetics of chemical steps in which free radicals are generated. GlmES possesses comparable kinetic properties to wild-type glutamate mutase, indicating the mechanism to be little perturbed by the alteration to its structure. GlmES binds AdoCbl with the same affinity as wild-type glutamate mutase when MutS is present in saturating concentrations. Thus, the introduction of the linker sequence does not seem to have at all perturbed the structure of the coenzyme-binding site. k_{cat} (in the forward direction) is about 3-fold slower than for wild type, whereas k_{cat}/K_m is unaltered. Since the primary deuterium kinetic isotope effects on V_{max} and V_{max}/K_m are also diminished relative to wild type, this suggests that product dissociation is most likely the step that is slowed in GlmES.

In any examination of the role of substrate binding energy in catalysis, it is important to know to what extent the Michaelis constants measured for the substrates and coenzyme represent the true dissociation constants for these molecules bound to the enzyme. Klinman and Matthews (*29*) have shown that measurements of ^{D}V and $^{D}V/K$ kinetic isotope effects may be used to calculate dissociation constants from K_ms. Although the calculation is only strictly applicable when there is only one isotopically sensitive step in the mechanism (which excludes the present case), the general conclusions are probably still qualitatively valid; specifically, that where $^{D}V = {}^{D}V/K$, then $K_m = K_d$, whereas if $^{D}V > {}^{D}V/K$, then $K_m > K_d$, and conversely if $^{D}V < {}^{D}V/K$, then $K_m < K_d$.

In the forward direction, a random sequential mechanism is indicated for the binding of AdoCbl and L-glutamate in which the affinity of one ligand is unchanged by the presence of the other. Furthermore, when deuterated glutamate is used the ^{D}V and $^{D}V/K$ isotope effects are roughly equal. This suggests that the K_m of 0.58 mM measured for L-glutamate is close to the intrinsic K_d for this substrate. However, in the reverse direction ^{D}V is significantly larger than $^{D}V/K$. This indicates that the intrinsic K_d for 3-methylaspartate will be significantly lower than the value of 0.14 mM measured for the K_m. This is supported by the kinetic analysis which indicates that the K_i for 3-methylaspartate binding in the absence of AdoCbl is only about 10 μM. These results suggest that the enzyme exploits differential substrate binding to catalyze the reaction. That is, in order to shift the internal equilibrium constants toward unity, as required for optimal catalysis, 3-methylaspartate, which is thermodynamically less stable than glutamate by about 1.5 kcal/mol (*1*), is bound more tightly by the enzyme.

Mechanism of AdoCbl Homolysis. The mechanism by which the enzyme accelerates homolysis of AdoCbl is one of the most speculated upon and least well understood aspects of B_{12} chemistry. Much attention has been focused on the idea that the enzyme may exploit binding energy to force the coenzyme into an unfavorable conformation leading to a weakening of the cobalt−carbon bond: the mechanochemical hypothesis (*4, 7, 30*). An alternative hypothesis is that

dissociation is promoted by binding the reaction intermediates, Cbl(II), 5′-deoxyadenosine, and substrate radical, more tightly than AdoCbl and substrate. To try and obtain experimental evidence to support or refute either of these hypotheses we investigated the binding of AdoCbl, MeCbl, Cbl(II), and 5′-deoxyadenosine to GlmES.

Our results tend not support the idea that the enzyme uses steric compression to destabilize the coenzyme. If strain were to play a major role in weakening the Co−C bond, one might expect MeCbl and Cbl(II) to bind much more tightly than AdoCbl, whereas we observe AdoCbl, MeCbl, and Cbl(II) all to bind with essentially the same affinity. Although we were not able to measure the K_d for Cbl(II) directly, the fact that the K_m and K_i values for AdoCbl and MeCbl are very similar to their K_ds suggests that the K_i we measured for Cbl(II) is a good measure of binding. We cannot rule out the explanation that favorable interactions between the adenosyl moiety and the protein exactly counterbalance unfavorable steric interactions—so that there is no net energetic gain in binding the adenosyl moiety—but this seems less likely.

Our results provide some support for the alternative hypothesis, that stabilization of intermediates promotes homolysis, although the stabilization appears to be relatively modest. 5′-Deoxyadenosine binds with a $K_i \approx 2$ mM. If all this binding energy were realized in the posthomolytic state it would offset the unfavorable carbon−cobalt bond dissociation energy by about 4 kcal mol^{-1}. As discussed above, no additional stabilization appears to be achieved by binding Cbl(II). This is perhaps not surprising, as the X-ray structure of Cbl(II) is very similar to that of the cobalamin portion of AdoCbl (*31*). Kräutler et al. (*31*) have argued that the conformational change in the cobalamin portion in going from AdoCbl to Cbl(II) and 5′-deoxyadenosine is too small for the protein to exploit in stabilizing radical intermediates. Set against this is the example of AdoCbl-dependent ribonucleotide reductase, in which Cbl(II) is bound approximately 10 times more tightly than AdoCbl, but even so, this only results in a $K_d \approx 10$ μM for Cbl(II) (*26*). Also, tight binding of Cbl(II) only occurs when both the allosteric activator, dGTP, and 5′-deoxyadenosine are present. 5′-deoxyadenosine itself is bound by ribonucleotide reductase with $K_i \approx 10$ μM. Thus, in this more complicated case it still seems that binding of 5′-deoxyadenosine is the more important stabilizing factor.

In conclusion, we have successfully reengineered the subunit structure of glutamate mutase to one analogous to MMCM and MGM, thereby simplifying the enzyme's kinetic properties. The GlmES protein will be useful for future physical and kinetic studies aimed at understanding coenzyme B_{12}-dependent catalysis. The steady-state kinetic constants for the GlmES-catalyzed reaction have been determined; these reveal differences in AdoCbl-binding dependent upon whether the enzyme is combining with glutamate or methylaspartate. The enzyme does not appear to destabilize the cobalt−carbon bond by differential binding of AdoCbl and Cbl(II); rather, binding of the intermediate 5′-deoxyadenosine may offset some of the unfavorable bond dissociation energy. However, this explanation does not appear sufficient to fully explain the remarkable rate accelerations for AdoCbl homolysis seen in these enzymes, and further investigations are in progress to elucidate the mechanism in more detail.

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