Adenosylcobalamin-dependent glutamate mutase: properties of a fusion protein in which the cobalamin-binding subunit is linked to the catalytic subunit

Daniel E. HOLLOWAY*§, Stephen E. HARDING† and E. Neil G. MARSH*‡‖

*Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K., †Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington, LE12 5RD, U.K., and ‡Department of Chemistry, University of Michigan, 930 N. University, Ann Arbor, MI 48109-1055, U.S.A.

Adenosylcobalamin-dependent glutamate mutase (EC 5.4.99.1) from *Clostridium tetanomorphum* comprises two protein components, MutE and MutS. The formation of the holoenzyme is a kinetically complex process that involves the co-operative association of MutS, MutE and adenosylcobalamin. The MutS portion of the cobalamin-binding site is conserved within a group of adenosylcobalamin-dependent enzymes that catalyse similar isomerizations. However, in contrast with glutamate mutase, in these other enzymes the cobalamin-binding region represented by MutS is present as a C-terminal domain. We have investigated the effect on the structural and kinetic properties of glutamate mutase of linking MutS to the C-terminus of MutE. Kinetic analysis of this protein, MutES, showed, unexpectedly, that enzyme activity was still co-operatively dependent on protein concentration. The K_m for L-glutamate was unchanged from the wild type, whereas V_{max} was decreased to approx. one-thirtieth and the K_m for coenzyme increased approx. 10-fold. Investigation of the quaternary structure of MutES by equilibrium ultracentrifugation indicated that the protein existed in equilibrium between monomeric and dimeric forms. Thus linking MutE and MutS together seems to substantially weaken the contacts that are responsible for the dimerization of MutE. The two domains of the MutES monomer seem unable to communicate, so that active enzyme is formed by the intermolecular association of two MutES subunits in a co-operative manner.

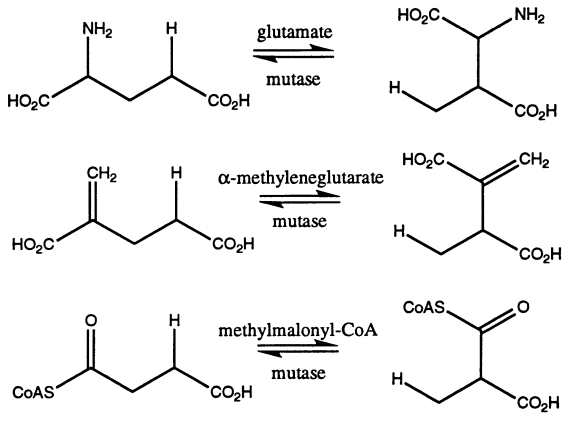
INTRODUCTION

Glutamate mutase (EC 5.4.99.1) catalyses the reversible interconversion of L-glutamate to L-*threo*-3-methylaspartate as the first step in the fermentation of L-glutamate by *Clostridium tetanomorphum* [1]. The enzyme is one of a group of adenosylcobalamin (AdoCbl, coenzyme B_{12})-dependent enzymes that catalyse unusual isomerizations in which a carbon-containing fragment on one carbon atom is interchanged with a hydrogen atom on an adjacent carbon to effect a skeletal rearrangement [2,3] (Scheme 1).

AdoCbl functions as a source of 5′-deoxyadenosyl radical, which is 'unmasked' by homolysis of the cofactor's unique cobalt–carbon bond [4]. In the glutamate mutase reaction this adenosyl radical abstracts the migrating hydrogen from the substrate [5,6], which is thus activated to undergo rearrangement in a poorly understood step of the mechanism.

Glutamate mutase from *C. tetanomorphum* comprises two readily separable subunits: MutE, a dimeric protein of subunit M_r 54000, and MutS, a monomer of M_r 14800 [7,8]. The kinetic properties of the enzyme are quite complex. MutS binds to MutE in a co-operative manner (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, and consistently with this the apparent K_m and K_d for AdoCbl are dependent on the relative concentrations of the two subunits [9]. These properties are consistent with the coenzyme-binding site's being formed at the interface between the MutE and MutS subunits. Glutamate mutase has also been purified [10] and the genes cloned [11] from *C. cochlearium*: this enzyme exhibits very similar properties to the *C. tetanomorphum* enzyme.

MutS shows sequence similarity to several cobalamin-dependent enzymes [7,12] including AdoCbl-dependent methylmalonyl-CoA mutase (EC 5.4.99.2; MMCM) from *Propionibacterium shermanii* and methylcobalamin-dependent meth-



Scheme 1 Carbon skeleton rearrangements catalysed by adenosylcobalamin-dependent enzymes

The migrating hydrogen atom is shown explicitly.

Abbreviations used: AdoCbl, adenosylcobalamin; (Bza)AdoCba, (benzimidazolribofuranosyl)-adenosylcobinamide; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; MetH, methionine synthase; MMCM, methylmalonyl-CoA mutase; MGM, 2-methyleneglutarate mutase.

§ Present address: Division of Biochemistry, School of Biological Sciences, Royal Holloway College, Egham, Surrey, TW20 0EX, U.K.

‖ To whom correspondence should be addressed.

ionine synthase (EC 2.1.1.13; MetH) from *Escherichia coli*. The crystal structures of MMCM and the methylcobalamin-binding region of MetH were solved during the course of this work [13,14]. In both cases the MutS-like domain was shown to possess an α/β structure that is a varient of the canonical nucleotide-binding (Rossmann) fold and binds 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 and a histidine residue co-ordinates the central cobalt atom of the coenzyme from below. EPR studies of glutamate mutase [15] suggest that cobalt is similarly co-ordinated by a conserved histidine residue.

Glutamate mutase is unusual in that the cobalamin-binding domain represented by MutS is a discrete protein, encoded by a separate gene, whereas in MMCM and 2-methyleneglutarate mutase (EC 5.4.99.4; MGM) from *C. barkeri*, which catalyse very similar isomerizations, this conserved region is present as the C-terminal domain of a single subunit [12,16]. In contrast with glutamate mutase, these enzymes bind AdoCbl essentially irreversibly and do not exhibit co-operative behaviour [17,18]. Apart from this domain, there is no apparent sequence similarity between any of the AdoCbl-dependent isomerases; however, the similarity of the carbon skeleton rearrangements that they catalyse makes the idea that these proteins might also share a common global fold attractive. To investigate the structural relationship between glutamate mutase, MMCM and MGM we have engineered a fusion protein of glutamate mutase in which MutS is linked to the C-terminus of MutE by a linker sequence derived from MGM. Here we describe the construction and purification of this protein together with the characterization of its physical and kinetic properties.

EXPERIMENTAL

Materials

The construction of the plasmids pmutS, pmutE, pmutEX and pmutSX has been described previously [9]. 3-Methylaspartase was purified as described by Hsiang and Bright [19]. Restriction endonucleases and DNA-modifying enzymes were purchased from Promega. AdoCbl was purchased from the Fluka Chemical Company. The sources of other materials have been described previously [9] or were purchased from commercial suppliers.

PCR

The PCR was performed in a volume of 100 μl with 5 units of *Taq* polymerase in the manufacturer's buffer; nucleoside triphosphates were present at 250 μM each and oligonucleotide primers at 10 μM each. Amplification was achieved with 25 cycles at the following temperatures: 95 °C, 1 min; 40 °C, 1 min; 72 °C, 2 min. Finally, the reaction was maintained at 72 °C for 10 min. PCR products were purified with the Wizard® PCR Preps DNA Purification System.

PCR assembly of *mutES* gene

The *mutE* and *mutS* genes were fused by using 'recombinant PCR' [20] to introduce the desired linking sequence. Four oligonucleotide primers were synthesized. The first, oligo-1, was designed to introduce *Bam*HI and *Nde*I restriction sites at the 5′-terminus of the *mutE* gene. Two overlapping, complementary, oligonucleotide primers, oligo-2 and oligo-3, were designed to introduce the linker sequence at the 3′-terminus of the *mutE* gene and the 5′-terminus of the *mutS* gene respectively. The last, oligo-4, was designed to prime from the 3′-terminus of the *mutS* gene. The sequences of the primers were as follows: oligo-1, CCCCGGATCCATATGGAACTTAAGAATAAAAA; oligo-2, CCAAGAACAATTTTTTCTGGTCTTCCGATAAG; oligo-3, AAGACCAGAAAAAATTGTTCTTGGAGTTATTG; oligo-4, TAGTTAAATTATTCTAC.

mutE was amplified by using oligo-1 and oligo-2 as primers and 25 ng of plasmid pmutEX as template; the PCR product was designated *mutE′*. To amplify *mutS*, oligo-3 and oligo-4 were used as primers with 25 ng of plasmid pmutS as template; this PCR product was designated *mutS′*. To assemble the *mutES* gene, oligo-1 and oligo-4 were used as primers and 30 ng of *mutE′* and 10 ng of *mutS′* as co-templates in a single PCR reaction (see Figure 2).

Subcloning and expression of *mutES*

The *mutES* PCR product was end-repaired and phosphorylated by standard methods [21]. The PCR product was then ligated with *Sma*I-restricted and dephosphorylated pUC119, and the ligation mixture was used to transform *E. coli* TG1 *recO*. Plasmids were isolated from recombinant bacteria, and clones with correctly sized inserts were identified by restriction analysis. The DNA sequence of their inserts was determined by sequencing plasmid DNA directly with specifically synthesized oligonucleotides as primers. No recombinant plasmids were found that contained completely error-free copies of *mutES*. Therefore an error-free 550 bp portion of *mutES* that was downstream from a unique internal *Eco*RI site and included the linker sequence was excised from one plasmid by restriction with *Eco*RI. This fragment was then ligated into *Eco*RI-restricted and dephosphorylated pmutE (pmutE contains a complete copy of the *mutE* gene) from which the 200 bp fragment liberated by restriction had been purified away. The ligation mixture was used to transform *E. coli* TG1 *recO*. Plasmids were isolated from recombinant bacteria and restriction analysis identified a plasmid containing the reconstituted *mutES* gene. This plasmid was designated pmutES.

To facilitate the expression of *mutES*, the gene was excised from pmutES by digestion with *Nde*I and *Sac*I and the mixture treated with calf intestine alkaline phosphatase to prevent religation with the vector. After purification, the fragments were ligated with *Nde*I- and *Sac*I-restricted pT7-18, and the ligation mixture was used to transform *E. coli* TG1 *recO*. Plasmids were isolated from recombinant bacteria, and restriction analysis identified a plasmid containing the *mutES* gene cloned into pT7-18 in the correct orientation. This plasmid was designated pmutESX. To express *mutES*, pmutESX was used to transform *E. coli* BL21 (DE3) pLysS, which contains phage-T7 RNA polymerase under the control *lac*UV5 promoter. Cultures were grown at 37 °C by inoculating 5 ml of an overnight culture into 1 litre of 2TY containing 100 mg/l ampicillin. After they had reached an attenuance at 600 nm of 1.0–1.5, expression was induced by addition of 200 mg/l isopropyl β-D-thiogalactoside (IPTG) and the culture was grown for a further 3 h.

Purification of inclusion bodies containing MutES

The purification was based on the guidelines of Marston [22]. All steps were performed on ice or at 4 °C. Typically, 12 g of cells (wet weight) were resuspended in 30 ml of 50 mM potassium phosphate buffer, pH 6.9, containing 100 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and 0.1 mM PMSF. The cells were ruptured by sonication, the insoluble material was collected by centrifugation at 12000 ***g*** for 15 min and the supernatant was discarded. The pellet, creamy-white in colour

and topped by a thin dark brown layer, was washed twice to remove lipophilic contaminants. Each wash involved resuspension of the pellet in 25 ml of the same buffer supplemented with 0.5 % (v/v) Triton X-100, followed by centrifugation at 25000 ***g*** for 15 min. After this step the dark brown layer had been removed, and the purity of the MutES inclusion bodies was more than 90 % as judged by SDS/PAGE (results not shown). No steps were taken to purify MutES further before refolding.

Refolding of MutES

The inclusion body preparation was solubilized in 25 ml of 0.1 M potassium phosphate buffer, pH 6.9, containing 6 M guanidine hydrochloride, 10 mM DTT and 1 mM EDTA. The solution was stirred at room temperature for 2 h and then insoluble material was removed by centrifugation at 25000 ***g*** for 15 min. A small dark pellet was discarded. Solublized protein was added dropwise to 100 ml of 0.1 M potassium phosphate buffer, pH 7.6, containing 1 M guanidine hydrochloride, 10 % (v/v) glycerol and 1 mM DTT, with gentle stirring. The final protein concentration was approx. 50 μg/ml. The solution was stirred for a further 20 min and then dialysed overnight against 4 litres of 0.1 M potassium phosphate buffer, pH 7.6, containing 10 % (v/v) glycerol and 1 mM DTT. The dialysate was then cleared by centrifugation at 25000 ***g*** for 15 min and concentrated to a volume of 2 ml by ultrafiltration in a stirred cell fitted with YM30 membrane (M_r exclusion limit 30000). The concentrated solution was again cleared by centrifugation at 25000 ***g*** for 15 min.

Enzyme assay

Glutamate mutase activity was assayed by monitoring at 240 nm the producton of mesaconate formed by the deamination of 3-methylaspartate by methylaspartase [1]. MutES was reduced with 5 mM DTT for at least 2 h on ice before all assays. Assays were performed at 25 °C.

Protein concentration

The molar extinction coefficients of MutE and MutS [9] were summed to provide an estimate of the MutES extinction coefficient. This gave an ϵ_{280} of 65700 $M^{-1} \cdot cm^{-1}$ and an $\epsilon_{280}^{0.1\%}$ of 0.97 cm^{-1}.

Analytical ultracentrifugation

Sedimentation equilibrium measurements were performed on a Beckman XLA machine, by methods described previously [23]. Measurements were made at 4 °C with a rotor speed of 10000 ***g***. Sedimentation was monitored at 280 nm by scanning absorption optics. Protein samples were made up at concentrations between 0.4 and 1.0 mg/ml in 0.1 M potassium phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT and 20 % (v/v) glycerol. The 'whole-cell' weight-average M_r values, $M^0_{r,w}$, and point weight-average M_r, as a function of either radial position, $M_{r,w}(r)$ or the corresponding protein concentration, $M_{r,w}(c)$, were obtained by the M* procedure of Creeth and Harding [24]. The dissociation constant for the MutES dimer was evaluated by direct fitting to the equilibrium concentration distribution [25].

RESULTS

Design of MutES fusion protein

In the absence of a three-dimensional structure for glutamate mutase, and before the structure of MMCM was available, the sequence of MGM from *C. barkeri* was used to guide the design of the glutamate mutase fusion protein (MutES). MGM catalyses a very similar isomerization to glutamate mutase and contains the conserved cobalamin-binding domain (Figure 1) [12]. Interestingly, the combined length of MutE and MutS is almost identical with that of MGM. We were therefore optimistic that if the similarities between these two proteins extended to a common global fold, an appropriate sequence from MGM would function to join MutE and MutS as an active fusion protein.

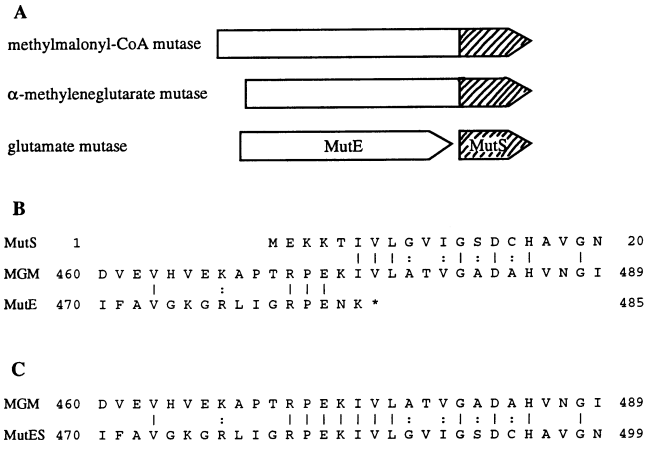


Figure 1 Design of MutES fusion protein

(**A**) The primary structures of the AdoCbl-dependent isomerases are represented with the conserved cobalamin-binding domain hatched. (**B**) The alignment of the N-terminal of MutS and the C-terminal of MutE with MGM. (**C**) The alignment of MutES with MGM. The region of fusion consists of seven contiguous residues in common with MGM. In (**B**) and (**C**) identical residues are indicated by vertical lines, conservatively substituted residues by dotted lines.

To identify a suitable sequence within MGM, MutS was first aligned with the C-terminal domain of MGM by using the University of Wisconsin Genetics Computing Group program GAP [26]. MutE and MGM have no substantial regions of sequence similarity and so computer alignment of these proteins was unreliable; however, both proteins have similar predictions for secondary structure and isolated regions of sequence similarity are discernible. The C-terminal region of MutE was therefore aligned manually with the region of MGM immediately upstream of the cobalamin-binding domain (Figure 1). Interestingly, the C-terminus of MutE contains a three-residue identity, Arg-Pro-Glu, with a sequence just upstream from the MGM cobalamin-binding domain. To test the validity of this alignment the C-terminal 50 residues of MutE were aligned with a 50-residue segment of MGM from Asn-431 to Val-480 with the GAP program. The computer-generated alignment confirmed that for these two short regions of sequence the manual alignment was optimal. In the alignment shown in Figure 1 the C-terminus of MutE and the N-terminus of MutS overlap by six residues. However, these residues are conserved in neither MGM nor MMCM, which suggested that these residues might be removed and the truncated proteins linked by a seven-residue segment of MGM. This fusion protein was called MutES.

Construction of *mutES* gene

mutES was assembled by 'recombinant' PCR [20] by the strategy outlined in Figure 2. Primers were synthesized to introduce the linker sequence at the 3′-terminus of *mutE* and the 5′-terminus of *mutS*. These primers were used in separate PCR processes to amplify the *mutE* and *mutS* genes, so that the PRC products

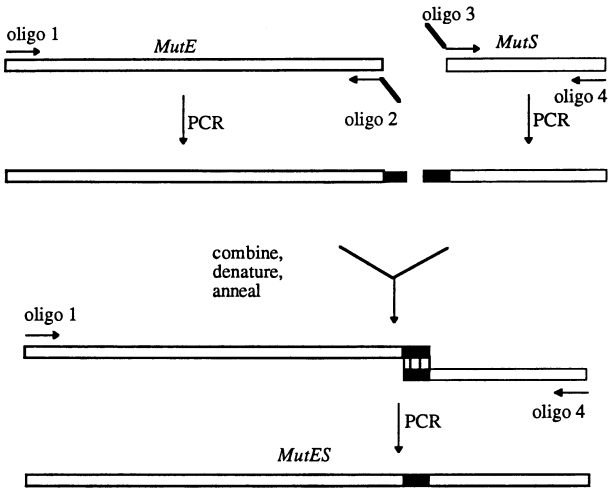


Figure 2 PCR strategy used to construct the *mutES* gene

Two separate PCR processes were used introduce complementary ends encoding the linker sequence on the 3′-terminus of *mutE* and the 5′-terminus of *mutS*. In a third PCR the full-length gene was then assembled. Details are given in the text.

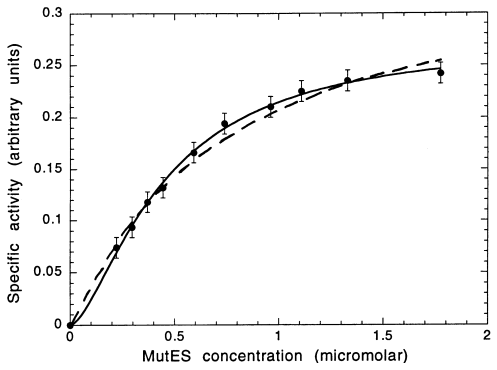


Figure 3 The specific activity of MutES as a function of protein concentration

The data were fitted to the Hill equation (solid line) by using the Kaleidagraph® program. For comparison the fit to a simple ($n = 1$, non-co-operative) binding curve is also shown (broken line). Each point represents the average of three measurements; the error bars are estimates of S.E.M. based on these measurements.

mutE′ and *mutS′* carried a 20 bp overlapping sequence. *mutE′* and *mutS′* were combined and used co-templates with primers to the 5′- and 3′-termini of *mutE* and *mutS* respectively in a further PCR to assemble the full-length *mutES* gene. Initially the PCR was attempted with proof-reading polymerases such as *Vent* and *Pfu* to minimize errors. However, these enzymes gave very poor yields of PCR products and so the experiment was performed using the less accurate *Taq* polymerase. *mutES* was thus successfully assembled and the PCR product subcloned by standard methods into pUC119.

DNA sequencing of five subcloned PCR products failed to identify a *mutES* gene free from unintended point mutations. This was attributed to the low fidelity of *Taq* polymerase and the relatively great length of the PCR product (1851 bp). To overcome this a subclone was identified that contained no errors within a 550 bp *Eco*RI fragment that encompassed the 3′ portion of the gene. This fragment was subcloned into pmutE, which contains an error-free copy of the *mutE* gene [9], to give the plasmid pmutES. To express *mutES*, the gene was subcloned as an *Nde*I–*Sac*I fragment into the vector pT7-18 to facilitate expression from the bacteriophage T7 promoter [27]. This plasmid, designated pmutESX, was used to transform *E. coli* BL21(DE3) pLysS so that expression could be induced with IPTG.

Purification of MutES from inclusion bodies

MutES was expressed predominantly as inclusion bodies in *E. coli*. Attempts to express the protein in soluble form by reducing the concentration of IPTG and/or growing the cultures at lower temperatures were unsuccessful. The protein was therefore purified as inclusion bodies that were then solubilized in 6 M guanidine hydrochloride. The solubilized protein was refolded by dilution into buffer containing 1 M guanidine hydrochloride, 1 mM DTT and 10% (v/v) glycerol; the residual guanidine hydrochloride was removed by dialysis against buffer containing only DTT and glycerol. Precipitation of the protein during refolding and subsequent concentration proved to be a significant problem. Excessive precipitation occurred if the protein was refolded at concentrations higher than approx. 0.05 mg/ml or if the refolded protein was concentrated much above 1 mg/ml. Under these conditions the yield of refolded MutES was approx. 30%, on the basis of solubilized protein.

Kinetic properties of MutES

Refolded MutES was active in the glutamate mutase assay. The activity was dependent on the proteins being reduced with 5 mM DTT before assay. The kinetic properties of the enzyme were investigated with (Bza)AdoCba (in which the dimethylbenzimidazole moiety of AdoCbl is replaced by benzimidazole), as this cofactor binds more tightly to the enzyme than AdoCbl [28]. At 25 °C, with 4 μM (Bza)AdoCba and 2.1 μM MutES present in the assay, the K_m(app) for L-glutamate was 1.2 ± 0.1 mM (S.D., $n = 3$), a value unchanged from the wild-type enzyme [9]. The K_m(app) for (Bza)AdoCba, measured under similar conditions in the presence of 10 mM L-glutamate, was 4.8 ± 0.3 μM. V_{max}, calculated from these results, was 0.58 ± 0.02 units/mg, which, assuming the enzyme has one active site per monomer, corresponds to a k_{cat} of 0.65 ± 0.03 s^{-1}. These values contrast with those obtained for the wild-type enzyme in which K_m(app) for (Bza)AdoCba is much lower [K_m(app) = 0.5 ± 0.1 μM when measured under similar conditions with equimolar (80 nM) MutE and MutS], whereas k_{cat} is substantially higher at 20 s^{-1}. However, because of the instability of the protein it is not clear whether all of the sample was catalytically active or whether only a subpopulation of molecules are able to catalyse the reaction, in which case the true activity of MutES might be higher.

Co-operative behaviour of MutES

During the purification of MutES it was noticed that the protein seemed to be active only when concentrated. Therefore the variation of glutamate mutase activity with MutES concentration was investigated. Assays were conducted in which the concentrations of L-glutamate and (Bza)AdoCba were fixed at 10 mM and 4 μM respectively, and MutES concentration was varied from 0 to 1.8 μM. The variation in enzyme specific activity is plotted against MutES concentration in Figure 3. The curve indicates that the specific activity of MutES is not constant over this concentration range but instead approaches a maximum value at higher concentrations. At the lowest concentrations the

Table 1 The effect on glutamate mutase activity of adding exogenous MutE or MutS to MutES

MutE or MutS was added to MutES (0.33 μM) in the presence of either a high (15 μM) or a low (1 μM) concentration of (Bza)AdoCba coenzyme. Assays were performed at 25 °C with 10 mM L-glutamate as substrate. See text for discussion. Glutamate mutase activities are shown as means ± S.E.M. for three or more measurements.

[MutS] (μM)	[MutE] (μM)	[(Bza)AdoCba] (μM)	Glutamate mutase activity (nmol/min)
–	–	1	1.3 ± 0.7
–	–	15	4.8 ± 0.5
1.00	–	1	11.7 ± 0.6
1.00	–	15	14.3 ± 0.7
–	0.11	1	11.0 ± 0.5
–	0.11	15	19.6 ± 0.6

specific activity was close to zero. The concentration dependence of MutES activity was not well described by simple binding kinetics but instead exhibited co-operative behaviour. The Hill coefficient, n, calculated from the results was 1.6 ± 0.1, and K, the apparent dissociation constant, was 0.44 ± 0.02 μM. This co-operative behaviour is similar to that observed when MutS binds to MutE and, importantly, implies that in the active form of MutES there is more than one active site.

One explanation for this behaviour is that the MutS domain is not able to form an active complex with the MutE segment to which it is covalently linked, and that the activity is the result of association of domains from different subunits. To test this, the effect of exogenously added MutS or MutE on the activity of MutES was measured in the presence of high (15 μM) or low (1 μM) concentrations of (Bza)AdoCba (Table 1). The rate was stimulated considerably by the addition of either MutE or MutS to the assay mixture. In the presence of 1 μM (Bza)AdoCbl the addition of a 3-fold molar excess of MutS resulted in a 9-fold increase in the specific activity of MutES, whereas in the presence of 15 μM (Bza)AdoCba addition of MutS increased the rate by only 3-fold. A similar phenomenon was observed when MutES was present in 3-fold molar excess over MutE. This could be explained if added MutS or MutE bound to MutES to form a functional coenzyme-binding site and so increased the enzyme's apparent affinity for coenzyme, as is observed with the wild-type enzyme. Taken together the results indicate that MutES possesses both functional MutE and MutS segments that are accessible to exogenous MutE and MutS subunits.

Analytical equilibrium ultracentrifugation studies

The experiments described above suggested a model in which active glutamate mutase is formed by the intermolecular association of MutES subunits. Wild-type MutE protein is dimeric; active enzyme is formed by its association with MutS to give an $\alpha_2\beta_2$ tetramer. However, it was not clear whether MutES would retain the dimeric structure of MutE so that active enzyme would be formed by the association of two dimers into a tetramer, or whether inactive MutES is a monomer that would associate to give an active dimer. To clarify this, the subunit structure of MutES was investigated by equilibrium analytical ultracentrifugation.

Centrifugation was performed at 4 °C with an initial protein concentration of 0.8 mg/ml. Despite the presence of 20 % (v/v) glycerol to stabilize the protein, by the time the samples had reached equilibrium (48 h) some precipitation of the sample was evident. The weight-averaged relative molecular mass over the whole solute distribution, $M^{\circ 0}_{r,w}$, was 98000 ± 5000, a value consistent with MutES (subunit M_r 67000) existing in an equilibrium between monomer and dimer forms. The point weight-averaged relative molecular mass, $M_{r,w}(c)$, plotted as a function of protein concentration is shown in Figure 4. As expected for a system in true equilibrium between monomer and dimer, $M_{r,w}(c)$ tends towards the monomer value as the concentration tends towards zero. At the highest concentrations $M_{r,w}(c)$ exceeds the value expected for the dimer, which suggests that high-molecular-mass aggregates are also present.

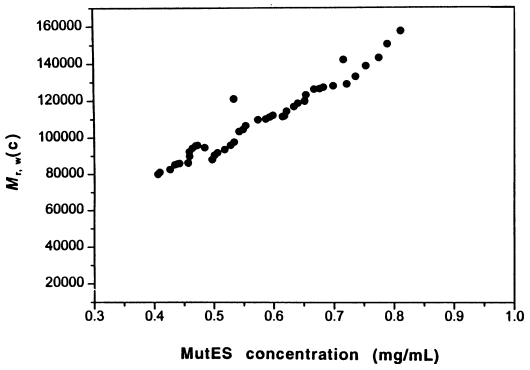


Figure 4 Concentration-dependent association of MutES

The point weight-averaged M_r of sedimenting species in the ultracentrifuge cell is plotted against protein concentration as measured by its absorbance at 280 nm. The initial concentration of protein in the cell was 0.8 mg/ml.

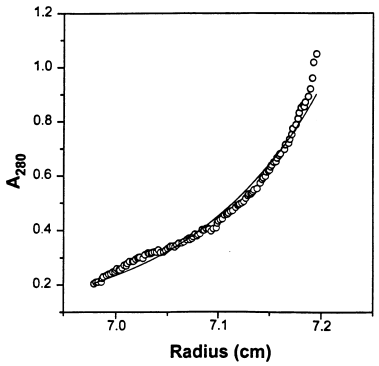


Figure 5 The equilibrium distribution of MutES in the ultracentrifuge cell

Protein absorbance is plotted against cell radius. The superimposed curve is fitted for a monomer–dimer equilibrium of the protein assuming a subunit M_r of 67000. Some systematic deviation of the data from the curve is evident: see text for discussion.

The variation of protein concentration as a function of radial displacement (Figure 5) is modelled reasonably well by the theoretical curve calculated for a monomer–dimer equilibrium. These results show some systematic deviation from the fitted curve. In part this is likely to have been caused by minor variations in absorbance properties of the cells along their radius. However, species of higher molecular masses are clearly present towards the cell base and it is possible that a small proportion of MutES exists as trimers or tetramers. The apparent K_d for the MutES dimer, calculated from these results, was 13 ± 1.5 μM, a value substantially higher than the apparent K_d estimated from the kinetic experiments. This discrepancy might in part be an artifact of the different techniques used to make

these measurements. One important difference is that the sedimentation experiments had to be performed in the absence of (Bza)AdoCba because its absorbance masked that of the protein. Evidence from this and previous studies indicates that the coenzyme binds between the MutE and MutS domains and would therefore be expected to promote the association of MutES. This might explain why the subunits seem to associate more tightly in the presence of coenzyme. An interesting but currently unanswered question is whether coenzyme binding is also responsible for inducing co-operativity.

DISCUSSION

The strategy used to link MutE and MutS relied on sequence alignments with MGM. Using this unsophisticated approach we did not expect that the linker sequence chosen would be optimal, but in the absence of structural information it provided a best guess for the construction of a 'single-subunit' glutamate mutase. The fact that MutES was active, albeit at lower levels than the wild type, supports the idea that MutS represents a transferable cobalamin-binding domain [29]. Topologically, MutES resembles MGM and MMCM, which lends weight to the suggestion that the AdoCbl-dependent skeletal isomerases, in addition to catalysing rather similar rearrangements, might share a similar global protein fold.

An unexpected effect of linking MutE and MutS seems to be that the contacts responsible for the dimerization of MutE are substantially weakened so that at low concentrations MutES is predominantly monomeric. This would explain the poor solubility of MutES, as dissociation would expose the MutE:MutE interface, which is likely to be mainly hydrophobic, to the solvent. Despite the instability of MutES, the overall integrity of the MutE and MutS segments seems to have been maintained. Thus both exogenous MutS and MutE stimulate MutES activity to levels approaching that of wild-type glutamate mutase. The affinity for L-glutamate, which is thought to bind to MutE, also seems unchanged as judged by the K_m for this substrate.

The linker sequence seems to constrain the MutE and MutS domains of MutES so that they are unable to interact directly with each other. Kinetic analysis indicates that active enzyme is formed by the association of two (or possibly more) MutES molecules in a co-operative process. In this respect MutES resembles the wild-type enzyme in which the binding of MutS to MutE is also co-operative [9]. Interestingly, although subunit contacts are much weaker in the MutES dimer than in MutE, dimerization of MutES is a more co-operative process ($n = 1.6$) than the binding of MutS to MutE ($n = 1.3$).

The simplest explanation, consistent with the kinetic and physical results, is that the active site is formed between the two subunits by the dimerization of MutES in a symmetrical 'head-to-tail' manner. Alternatively, dimerization might be required to force the protein subunits into an active conformation. Either process could give rise to the observed co-operativity. Support for the latter hypothesis is provided by the structure of the MMCM from *P. shermanni*, an $\alpha\beta$ dimer, that was solved during the course of the present work [13]. This enzyme contains only one active site that is entirely formed within the α subunit. The β subunit, although having a similar tertiary structure, contains no substrate- or coenzyme-binding sites and its function is therefore unclear. It might be important for stabilizing the α subunit in the correct conformation, because the α subunit, when expressed on its own, is inactive [30].

The strategy used to create MutES caused MutE and MutS to be slightly truncated in the fusion protein. An alternative strategy would be to link the subunits with a longer 'artificial' sequence. This might result in a glutamate mutase with quite different physical properties. One might imagine that a longer, more flexible linker would allow the two domains sufficient freedom for the tightly associated dimeric structure of MutE to be retained. This strategy has been successfully exploited to obtain an active and soluble fusion protein of *E. coli* tryptophan synthase [31]. Such a protein would be expected to be more stable and hence better suited to physical and kinetic characterization.

This work was supported by a University Research Fellowship from The Royal Society (E.N.G.M.) and the BBSRC (E.N.G.M. and D.E.H.).

REFERENCES

1 Barker, H. A., Roove, V., Suzuki, F. and Iodice, A. A. (1964) J. Biol. Chem. **239**, 3260–3266
2 Ochiai, E.-I. (1994) Metal Ions Biol. Syst. **30**, 255–278
3 Abeles, R. H. (1979) in Vitamin B_{12} (Zagalak, B. and Friedrich, W., eds.), pp. 373–388, W. de Gruyter, Berlin
4 Halpern, J. (1985) Science **227**, 869–875
5 Marsh, E. N. G. (1995) Biochemistry **34**, 7542–7547
6 Switzer, R. L., Baltimore, B. G. and Barker, H. A. (1969) J. Biol. Chem. **244**, 5263–5268
7 Marsh, E. N. G. and Holloway, D. E. (1992) FEBS Lett. **310**, 147–149
8 Holloway, D. E. and Marsh, E. N. G. (1993) FEBS Lett. **317**, 44–48
9 Holloway, D. E. and Marsh, E. N. G. (1994) J. Biol Chem. **269**, 20425–20430
10 Leutbecher, U., Böcher, R., Linder, D. and Buckel, W. (1992) Eur. J. Biochem. **205**, 759–765
11 Zelder, O., Beatrix, B., Leutbecher, U. and Buckel, W. (1994) Eur. J. Biochem. **226**, 577–585
12 Beatrix, B., Zelder, O., Linder, D. and Buckel, W. (1994) Eur. J. Biochem. **221**, 101–109
13 Mancia, F., Keep, N. H., Nakagawa, A., Leadlay, P. F., McSweeney, S., Rasmussen, B., Bosecke, P., Diat, O. and Evans, P. R. (1996) Structure **4**, 339–350
14 Drennen, C. L., Huang, S., Drummond, J. T., Matthews, R. G. and Ludwig, M. L. (1994) Science **266**, 1669–1674
15 Zelder, O., Beatrix, B., Kroll, F. and Buckel, W. (1995) FEBS Lett. **369**, 252–254
16 Marsh, E. N., McKie, N., Davis, N. K. and Leadlay, P. F. (1989) Biochem. J. **260**, 345–352
17 Michel, C., Albracht, S. P. J. and Buckel, W. (1992) Eur. J. Biochem. **205**, 767–773
18 Francalanci, F., Davis, N. K., Fuller, J. Q., Murfitt, D. and Leadlay, P. F. (1986) Biochem. J. **236**, 489–494
19 Hsiang, M. W. and Bright, H. J. (1967) J. Biol. Chem. **242**, 5263–5268
20 Higuchi, R. (1990) In PCR Protocols: A Guide to Methods and Applications (Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J., eds.), pp. 177–183, Academic Press, San Diego
21 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22 Marston, F. A. O. (1987) In DNA Cloning: A Practical Approach (Glover, D. M., ed.), vol. 3, pp. 59–88, IRL Press, Oxford
23 Marsh, E. N. and Harding, S. E. (1993) Biochem. J. **290**, 551–555
24 Creeth, J. M. and Harding, S. E. (1982) Biochem J. **205**, 639–641
25 Kim, H., Deonier, R. C. and Williams, J. W. (1977) Chem. Rev. **77**, 659–690
26 Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. **12**, 387–395
27 Tabor, S. and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1074–1078
28 Switzer, R. L. and Barker, H. A. (1967) J. Biol. Chem. **242**, 2658–2674
29 Marsh, E. N. G. (1995) Bioessays **17**, 431–441
30 McKie, N., Keep, N. H., Patchett, M. L. and Leadlay, P. F. (1990) Biochem. J. **269**, 293–298
31 Burns, D. M., Horn, V., Paluh, J. and Yanofsky, C. (1990) J. Biol. Chem. **265**, 2060–2069

Received 28 May 1996/29 July 1996; accepted 6 August 1996