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The mechanism of glutamate mutase: An unusually substrate-specific enzyme

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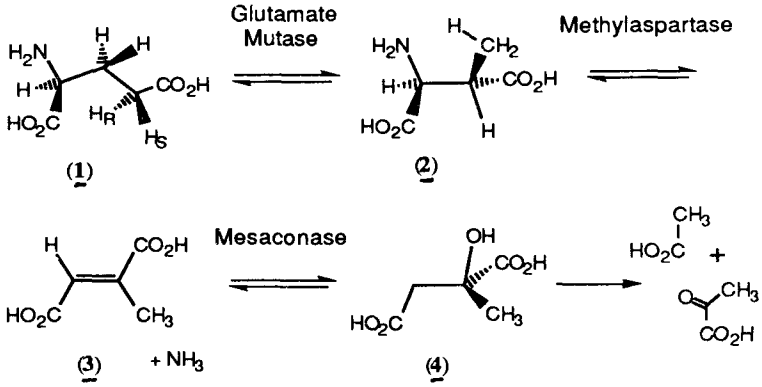
Abstract. Coenzyme B_{12}-dependent glutamate mutase catalyses the interconversion of (2*S*)-glutamic acid and (2*S*, 3*S*)-3-methylaspartic acid. The enzyme is unable to accept alternative substrates for the rearrangement reaction but is inhibited by substrates analogues including (2*S*, 3*R*)- and (2*S*, 3*S*)- and (2*S*, 3*S*)-3-methylglutamic acid, 2-bromo-2, 3-methanosuccinic acid, (2*S*)-homocysteic acid. The primary isotope effect upon V_{max} and V/K for the isomerisation of the (2*S*)-glutamic acid is $3{\cdot}7 \pm 0{\cdot}2$ and $13{\cdot}5 \pm 1{\cdot}0$ respectively. There are two C-H bond breaking steps involved in the isomerization reaction. The relative sizes of ^{D}V and $^{D}(V/K)$ indicate that neither of these steps are cleanly rate limiting.

Keywords. Coenzyme-B_{12}; glutamate mutase; isotope effect; substrate analogues; inhibitors.

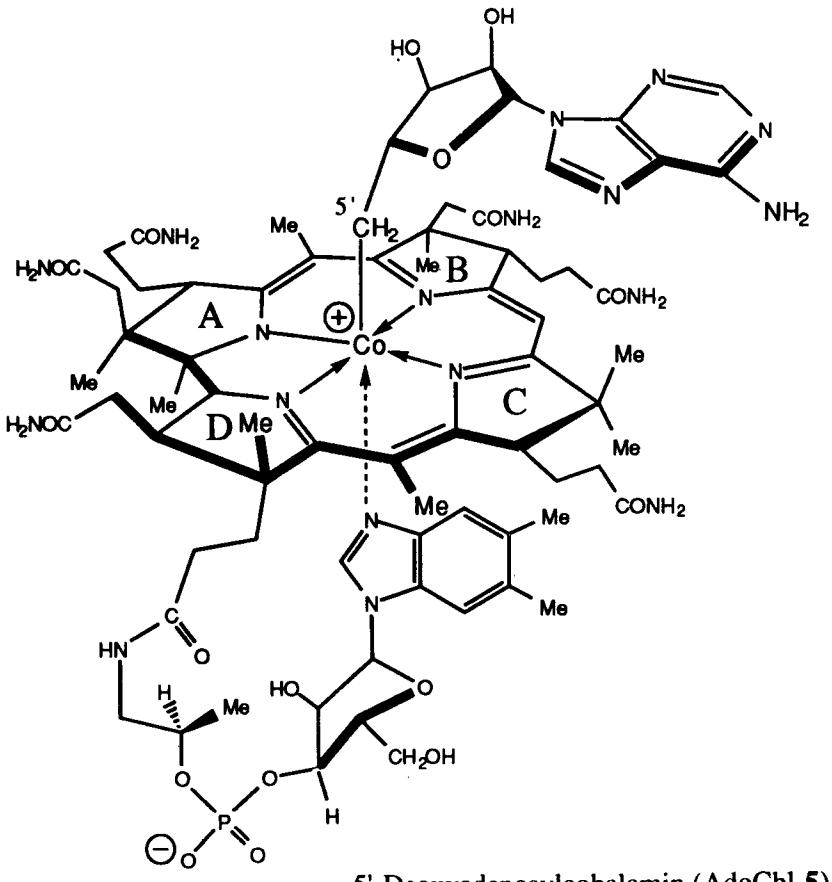
1. Introduction

Many bacteria contain a pathway for the anaerobic degradation of (2*S*)-glutamic acid (1) to acetic and pyruvic acid via the intermediacy of (2*S*, 3*S*)-3-methylaspartic acid (2), mesaconic acid (3) and (2*S*)-citramalic acid (4), (scheme 1). The first enzyme in the pathway, glutamate mutase, uses coenzyme-B_{12} (AdoCbl, 5) (figure 1) as a cofactor and catalyses a reversible 1, 2-carbon skeleton rearrangement. During this rearrangement, the glycyl moiety of (2*S*)-glutamic acid (1) migrates from C-3 to C-4



Scheme 1.

*For correspondence



5'-Deoxyadenosylcobalamin (AdoCbl, **5**)

Figure 1. Structure of 5'-deoxyadenosylcobalamine (AdoCbl, 5), coenzyme-B_{12}.

while concomittantly, a hydrogen atom migrates from C-4 to C-3 of glutamic acid, to give (2*S*, 3*S*)-3-methylaspartic acid (2). The enzyme was first isolated by Barker and coworkers from the soil anaerobe *Clostridium tetanomorphum* (Barker *et al* 1963). The active enzyme was shown to consist of two polypeptide components, a large air-stable subunit designated component E (128 kDa) (Suzuki and Barker 1966) and a small air-sensitive thiol containing subunit designated component S (17·5 kDa) (Switzer and Barker 1967).

Component E was shown to be able to bind to the coenzyme although its affinity was increased in the presence of subunit S. In the absence of component S, the E-AdoCbl complex was not active. In a similar manner it was shown that high concentrations of coenzyme increased the affinity of the two components for each other (Switzer and Barker 1967). Nevertheless, it emerged that in the active enzyme, the two subunits are loosely associated. Stable complexes could not be detected by gel filtration and, indeed, the stoichiometry of the two components in the active enzyme remains unknown. Furthermore, although it was established that the coenzyme binds to component E (Suzuki and Barker 1966), the stoichiometry of AdoCbl and component E in the resulting complex(es) also remains unknown. On this issue our

own analysis of Barker's original initial rate kinetic data (Switzer and Barker 1967) (which allows for the reported overestimate of the molecular mass of subnuit E, *vide infra*) suggests that the molar ratio is 1:1. However, other experiments performed by Barker and coworkers (Switzer *et al* 1969) in which [*adenosyl*-5′-H]-coenzyme was incubated with the enzyme such that the label was transformed to the product amino acids, gave a deduced stoichiometry of 3:1. At present this discrepancy cannot be explained.

The recent completion of the purification to homogeneity of both of the components in our own laboratory (Hartzoulakis 1994) revealed that the relative molecular mass of component E is, in fact, 53 kDa rather than 128 kDa as reported by Barker (Suzuki and Barker 1966). This value is in accord with other recent reports from Hollaway and Marsh (1993) and from Brecht *et al* (1993), for the enzyme from *C. tetanomorphum.* However, we were able to detect a protein band possessing a molecular mass of approximately 120 kDa on SDS-polyacrylamide gel electrophoretograms which possessed glutamate mutase E-subunit activity. This finding may indicate that the interactions between the two components are stronger than was originally thought (Suzuki and Barker 1966). It is now known that the molecular mass of the S component is 14 kDa (Marsh and Holloway 1992), thus, it is likely that this stable complex (possibly oxidised $E_2S = 120$ kDa) corresponds to the species observed by Barker (Suzuki & Barker 1966).

Over the past three years enormous advances have been achieved in establishing how the genes in the pathway for glutamic acid catabolism are organised within *Clostridium tetanomorphum.* In 1992 the gene for 3-methylaspartase, the second enzyme on the pathway, was sucessfully sequenced and expressed in *E. coli* (Goda *et al* 1992). In accord with expectations derived from the determination of the molecular mass of the native homodimeric enzyme by size exclusion chromatography and by SDS-PAGE analysis, the gene was found to encode a polypeptide of 413 amino acid residues of molecular mass 44,539 Da. This work also identified two large but incomplete open reading frames up- and downstream of the *MeAsp* gene (Goda *et al* 1992). These were believed to code for the E-subnuit of glutamate mutase and for mesaconase, respectively. The suggestion that the gene for the E-subunit existed immediately upstream of *MeAsp* was later confirmed when the complete gene was sequenced (Holloway and Marsh 1993). The open reading frame for the E-subunit was shown to code for a polypeptide 485 amino acids of 53,708 Da. Other work identified the gene for the S component from which it was deduced that the S component consisted of a 137-residue polypeptide of 14,748 Da (Marsh and Holloway 1992). Interestingly, these studies revealed that there was a single intervening gene, designated L, coding for a protein of 50,171 Da, between the genes for the two glutamate mutase components and immediately upstream of gene for the E-subunit, see figure 2. No role has yet been ascribed for the product of the intervening gene.

The glutamate mutase reaction is one of ten vicinal interchange reactions mediated by AdoCbl in which a functional group, which may vary widely in structure, swaps places with a hydrogen atom from an adjacent carbon atom, table 1. In four of these rearrangements (table 1, entries 1–4) the migrating group is a substituted carbon atom and of these, only for glutamate mutase is the migrating centre saturated (i.e. sp^3 hybridised). The involvement of competent radical species has been shown for methylmalonyl-CoA mutase (Zhao *et al* 1992) and glutamate mutase (Leutbecher *et al* 1992a). The EPR signal recorded for each holoenzyme complex, after the addition

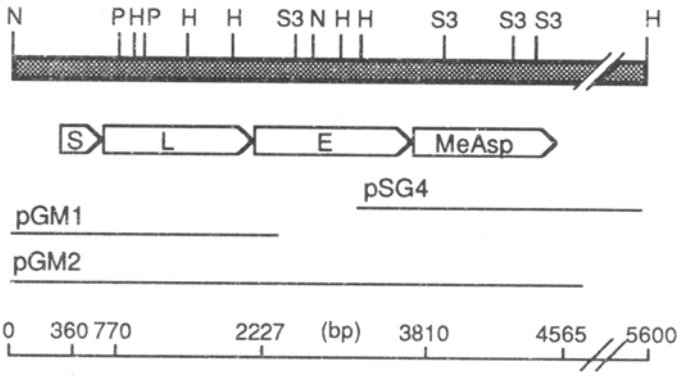


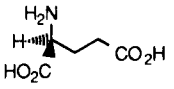
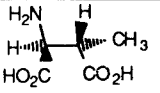
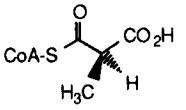
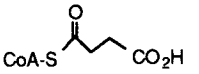
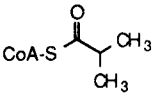
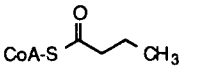
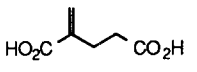
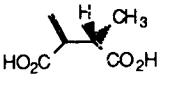
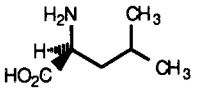
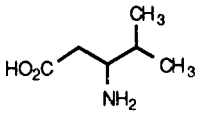
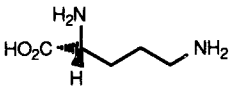
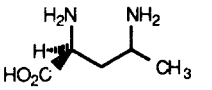
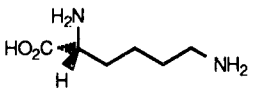
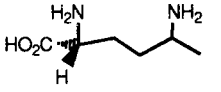
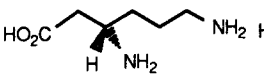
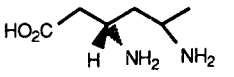
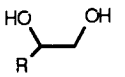
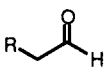
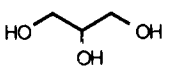
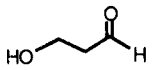
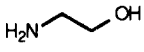
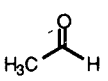
Figure 2. Organisation of the genes for glutamate mutase components S and E, protein L, and 3-methylaspartase in *Clostridium tetanomorphum*. Restriction sites for *Hind*III (H), *N*col (N), *Pst*I (P) and *Sau*3A (S3) are also indicated. Plasmids containing *C. tetanomorphum* genomic fragments are labelled pSG4 (Goda *et al* 1992), pGM1 (Marsh and Hollaway 1992) and pGM2 (Holloway and Marsh 1993).

of the substrate, showed similar characteristics to those obtained for other B_{12}-dependent enzymes including diol dehydrase (Finlay *et al* 1973) and ethanolamine ammonia-lyase (Babior 1981).

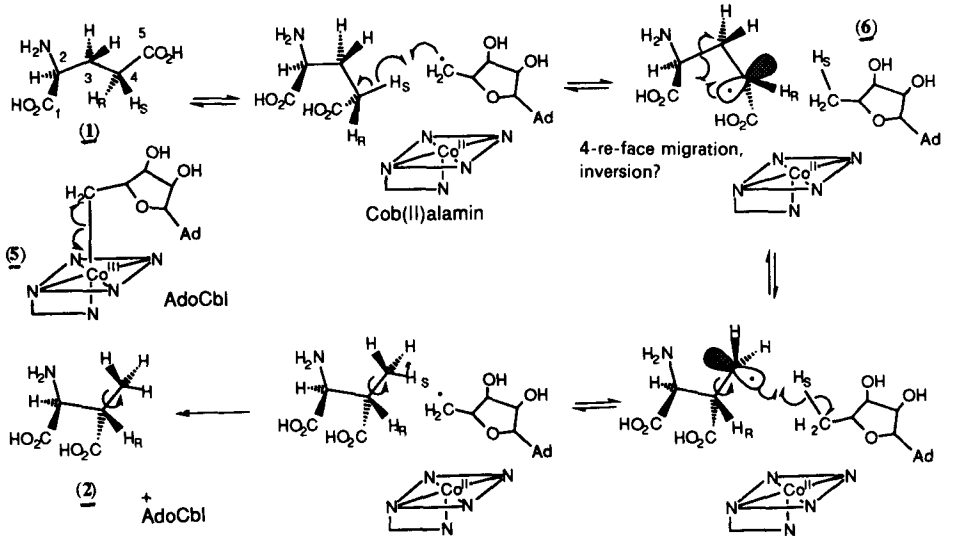
Three carbon centres are involved in the glutamate mutase-catalysed rearrangement reaction and it is evident that to fully understand the mechanism of the enzyme, it is necessary to determine the stereochemical course of the migrations with respect to each carbon centre. Early work by Sprecher *et al* (1966) demonstrated that the 4-*pro*-S hydrogen of glutamic acid (1) migrates to become one of the three hydrogens of the methyl group of the product (2) and is replaced by the migrating glycyl moiety with inversion of configuration at C-4. It is now generally believed that the migrating hydrogen atoms becomes one of the three hydrogen atoms of the methyl group of the 5′-deoxyadenosine portion of the coenzyme during the rearrangement and that one of these hydrogen atoms is transferred back to the product radical when the rearrangement is complete, (scheme 2). For the glutamate mutase system it has been demonstrated that tritium present in the 5′-deoxyadenosyl portion of the coenzyme at C-5′ is transferred to the substrate (Switzer *et al* 1969). Furthermore, in model systems it has been shown that the methyl group of the stable intermediate, 5′-deoxyadenosine (6), is able to serve as a source of hydrogen atoms and in doing so generates a 5′-deoxyadenosly radical (Gani *et al* 1980). The equivalance of the three hydrogens at C-5′ of deoxyadenosine was demonstrated by Richards and coworkers (Eager *et al* 1972). Their data also showed that there was a large deuterium isotope effect of $7{\cdot}5 \pm 1$ for the removal of a hydrogen atom from the methyl group of methylaspartic acid (2).

Given that Barker had determined that both the substrate and the product possess the same absolute configuration at C-2, the glycyl moiety must migrate to C-4 of glutamic acid with retention of configuration at C-2 (Barker *et al* 1964). In order to define the stereochemical course of the rerrangement with respect to the other carbon centre, C-3 of glutamic acid (1), Hartrampf and Buckel (1984) prepared (2*S*)-glutamic acid labelled at C-3 and C-4 with all three isotopes of hydrogen in the expectation that the enzymic product would contain a chiral methyl group. However, analysis

Table 1. Coenzyme-B_{12}-dependent enzymes catalysing rearrangements.

Enzyme	Substrate	Product	E.C. Number
1) (S)-Glutamate Mutase ⇌ *			5.4.99.1
2) (R)-Methylmalonyl-CoA mutase ⇌			5.4.99.2 (Zhao *et al* 1992)
3) Isobutyryl-CoA mutase ⇌			5.4.99.3 (Reynolds *et al* 1986)
4) α-Methyleneglutarate mutase ⇌			5.4.99.4 (Michel & Buckel 1991)
5) (S)-Leucine 2,3-aminomutase ⇌			5.4.3.7 (Baker *et al* 1981)
6) (R)-Ornithine 4,5-aminomutase ⇌			5.4.3.5 (Baker *et al* 1981)
7) (R)- α-Lysine 5,6-aminomutase ⇌			5.3.3.3 (Baker *et al* 1981)
((S)- β-Lysine mutase)			
8) Diol dehydrase →			4.2.1.28 (Finke *et al* 1983)
9) Glycerol dehydrase →			4.2.1.30 Toraya *et al* 1981)
10) Ethanolamine ammonia lyase →			4.3.1.7 (Hollaway *et al* 1980 & Gani *et al* 1983)

* Reaction reversibility under physiological conditions.



Scheme 2.

of the product indicated that the potentially chiral methyl group was, in fact, racemic. This is the expected result for a reversible reaction in which the product contains a torsiosymmetric methyl group, or where the putative methylene radical intermediate is able to rotate about the C-3-C-3′ sigma bond prior to hydrogen atom capture at C-3′ (scheme 2). Thus, while it is tempting to suggest that the rearrangement reaction should proceed with inversion of configuration at C-3 of glutamic acid, because this outcome requires minimal motion (scheme 2), it is evident that the stereochemical course of the reaction with respect to C-3 cannot be determined directly using the natural substrate.

Here we report on the results of experiments using substrate, transition state and product analogues as probes to gain further structural and mechanistic information on the glutamate mutase system.

2. Materials and methods

All chemicals, reagents, solvents and gases were of the highest commercially available purity or were purified further before use where necessary. Substrates and inhibitors were purchased from Sigma (Poole, Dorsèt, UK) or were prepared as outlined below.

Enzyme: Glutamate mutase was prepared from *Clostridium tetanomorphum* H1 (ATCC 15920) using modified literature procedures (Switzer and Barker 1967; Switzer *et al* 1969). Full details will be reported elsewhere. Routinely homogeneous component S was obtained in 10% yield and homogeneous component E was obtained in 5% yield. Typically, 50g of wet *C. tetanomorphum* cell paste gave 17 units of component S possessing a specific activity of 0·87 units mg^{-1} and 16 units of component E possessing a specific activity of 1·27 units mg^{-1}. The activity of the enzyme was determined spectrophotometrically in degassed *tris* buffer solution at *p*H 8·35 according to the method of Barker *et al* (1964) using a coupled assay containing

excess 3-3-methylaspartase activity. Under such conditions the conversion of (2*S*)-glutamic acid to (2*S*, 3*S*)-3-methylaspartic acid can be followed conveniently by measuring the increase in absorbance at 240 nm due to the formation of mesaconic acid.

Enzyme assay: Assay solutions containing 50 mmol dm^{-3} tris HCl buffer, 5 mmol dm^{-3} sodium (2*S*)-glutamate, 5 mmol dm^{-3} potassium chloride, 1 mmol dm^{-3} magnesium chloride, 3 mmol dm^{-3} mercaptoethanol and component S were preincubated for 10 min at 37°C to activate component S and then component E was added. AdoCbl (5 μl of a 3 mmol dm^{-3} solution) was added to initiate the reaction and the change in absorbance at 240 nm was followed for several minutes. One unit of glutamate mutase is defined as the amount of activity that converts 1 μmol of (2*S*)-glutamic acid to (2*S*, 3*S*)-3-methylaspartic acid per minute under the assay conditions.

Kinetic data: Reaction courses were followed using the spectrophotometric assay described above or by ^{1}H-NMR spectroscopy at 200 MHz on a Varian Gemini 200 or 300 MHz on a Bruker AM300 instrument. Data were processed using graphical and standard nonlinear regression analyses (Leatherbarrow 1987).

Substrates and inhibitors: (2*S*, 3*S*)-3-Methylaspartic acid (2) and (2*S*, 3*S*)-3-ethylaspartic acid (6) were prepared through the enzymic amination of the appropriate alkylfumaric acid using 3-methylaspartase as described by Akhtar *et al* (1987). [3′-$^2H_3$]-(2*S*, 3*S*)-3-Methylaspartic acid (2a) was prepared similarly starting from [2′-$^2H_3$]-mesaconic acid. (2*S*, 3*R*)-3-methylaspartic acid (7) was prepared by the method of Archer *et al* (1993). (2*S*, 3*R*)-3-Methylglutamic acid (8) was prepared via Arndt–Eisert homologation of (2*S*, 3*S*)-3-methylaspartic acid (2) or through the reaction of (2*R*)-2-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine with isopentyl-(*Z*)-but-2-enoate followed by appropriate deprotection. (2*S*, 3*S*)-3-methylglutamic acid (9) was prepared using similar methodology and methyl (*E*)-but-2-enoate (Hartzoulakis 1994; Hartzoulakis and Gani 1994). Racemic mixtures of (1*R*, 2*S*)- and (1*S*, 2*R*)-2-bromo-2, 3-methanosuccinic acid (10) and (1*R*, 2*R*) and (1*S*, 2*S*)-2-bromo-2, 3-methanosuccinic acid acid (11) were prepared using a modification of the method of McCoy and Nachtigall (1962). [4-$^2H_2$]-Glutamic acid (12) was prepared as described by Murray and Williams (1972). All compounds and key intermediates were fully characterised and gave the expected spectral and analytical data.

3. Results and discussion

Alternative substrates and inhibitors: The only known substrates for glutamate mutase are (2*S*)-glutamic acid and (2*S*, 3*S*)-methylaspartic acid. Furthermore, very few inhibitors for the enzyme have been described (Barker *et al* 1964; Leutbecher *et al* 1992b) (see table 2). According a systematic study of the active-site space was undertaken by replacing hydrogen atoms in the natural substrate and product by methyl groups. Given that Hartrampf and Buckel (1984) had been unsuccessful in determining the stereochemical course of the reaction with respect to C-3 of the substrate, due to the reversible formation of a torsiosymmetric methyl group in the product, we first wished to test the ability of (2*S*, 3*S*)-3-ethylaspartic acid (6) to serve as a substrate. This compound might be expected to rearrange to give a

Table 2. Compounds studied with glutamate mutase.

Compound	Substrate	Inhibitor	K_m or K_i (mmol dm^{-3})	References
(2*S*)-glutamic acid (1)	+		1·35	Suzuki and Barker (1966); Barker *et al* (1964)
(2*S*, 3*S*)-3-methylaspartic acid (2)	+		0·5	Barker *et al* (1964)
(*R*)-glutamate	–	†	–	Barker *et al* (1964)
(*RS*)-2-methylglutamate	–	†	–	Barker *et al* (1964)
2-Ketoglutarate	–	†	–	Barker *et al* (1964)
(2*R*, 3*R*)-3-methylaspartic acid	–	†	–	Barker *et al* (1964)
(2*S*, 4*S*)-4-fluoroglutamic acid*	–	+	0·07	Leutbecher *et al* (1992b)
2-Methyleneglutaric acid*	–	+	0·4	Leutbecher *et al* (1992b)
(*S*)-3-Methylitaconic acid*	–	+	0·1	Leutbecher *et al* (1992b)
Itaconic acid*	–	+	1·2	Leutbecher *et al* (1992b)

† No additional data available; * compounds were tested with glutamate mutase from *C. cochlearium*

3-methylglutamic acid (8 or 9). Since the ethyl group is not torsiosymmetric and its methylene group can be easily labelled stereospecifically with deuterium, it appeared that (2*S*, 3*S*)-3-ethylaspartic acid might be useful in solving the overall stereochemical course of the reaction. However, (2*S*, 3*S*)-3-ethylaspartic acid did not serve as a substrate for glutamate mutase as determined both by using standard activity assays and by monitoring the incubation solution by 200 MHz ^{1}H-NMR spectroscopy over prolonged periods of up to 36 h. In the presence of increased quantities of enzyme it was determined that ethylaspartic acid is at least 10^5-times slower as a substrate than the natural product, methylaspartic acid. This surprising and disappointing results became even more curious when it was learnt that ethylaspartic acid was not a strong inhibitor for the enzyme. Incubations containing the compound and glutamic acid did not show a reduced rate of methylaspartic acid formation relative to controls when monitored directly by ^{1}H-NMR spectroscopy.

The result was particularly surprising because many other AdoCbl-dependent enzymes have been shown to process methyl homologues of their natural substrates, including diol dehydrase (Baker and Standman 1981), ethanolamine ammonia-lyase (Toraya and Fukui 1981; Finke and Shiraldi 1983) and methylmalonyl-CoA mutase (Zhao *et al* 1992), Given that the equilibrium constant for the formation of methylglutamic acid from ethylaspartic acid could have favoured ethylaspartic acid to the extent that the product might not be formed in sufficient quantity to be detected by NMR spectroscopy, two new experiments were devised to test the ability of ethylaspartic acid to enter the active site.

First, methylglutamic acid was prepared in order to evaluate it as a substrate. As the potential rearrangement of (2*S*, 3*S*)-3-ethylaspartic acid could give either (2*S*, 3*R*) or (2*S*, 3*S*)-3-methylglutamic acid, syntheses of both compounds were developed and the pure individual diastereomers were obtained (Murray and Williams 1972). When

each of the compounds was incubated with glutamate mutase, examination of the incubation solutions by ^{1}H-NMR spectroscopy over prolonged periods of time indicated that neither of the 3-methylglutamic acids were substrates. Nevertheless, both compounds served as competitive inhibitors for the enzyme with K_m values close to the value of K_m for the natural substrate, glutamic acid, $K_i = 1{\cdot}35$ mM (table 3). This result clearly indicates that both methylglutamic acids can bind at the active-site of the mutase, but that neither of the homologues can achieve a conformation suitable for rearrangement. The fact that neither of the methylglutamic acids is a substrate precludes the possibility that 3-ethylaspartic acid is the thermodynamically favoured isomer in an isomerisation reaction catalysed by the enzyme and suggests that the mutase simply cannot bind to ethylaspartic acid. Nevertheless, there were other possible explanations as to why ethylaspartic acid did not rearrange and one of these was that the enzyme might recognise the methyl group of the side-chain rather than the methylene group. Such recognition should lead to the removal of a hydrogen atom for the methyl group and its equilibration with the two hydrogen atoms at the 5′-position of the 5′-deoxyadenosyl moiety of the coenzyme.

In order to investigate this possibility, (2*S*, 3*S*)-3-ethylaspartic acid was prepared in which the ethyl group was either fully deuteriated, deuteriated in the methylene group or deuteriated in the methyl group (Gulzar *et al* 1994). These samples were mixed with appropriately labelled methylaspartic acids (either [*methyl*-$^2H_3$] or unlabelled) which are known to exchange hydrogen with the 5′-position of the 5′-deoxyadenosyl moiety (Sprecher *et al* 1966; Switzer *et al* 1969), see scheme 2, and the mixtures were incubated with the enzyme. Although the methylaspartic acids rearranged to form appropriately labelled glutamic acids, no label was transferred either to or from the ethylaspartic acid, even after prolonged incubation. Thus, it is evident that no hydrogen is transferred from ethylaspartic acid to the coenzyme and together with the other results, *vide supra*, the finding indicates that ethylaspartic acid is too large to fit into the active-site. (2*S*)-Aminoadipic acid (13) showed similar properties to ethylaspartic acid and did not serve as a substrate or as an inhibitor for the enzyme. Likewise, (2*S*)-aspartic acid, (2*RS*)-2-methylglutamic acid, (2*S*, 3*R*)-3-methylaspartic acid and (2*S*, 3*S*)-N-methyl-3-methylaspartic acid (Gulzar *et al* 1994) all failed to serve as substrates or inhibitors, see table 3.

Table 3. Compounds tested in this study.

Compound	Substrate	Inhibitor*	K_m or K_i
(2*S*)-glutamic acid (1)	+		1·35
(2*S*, 3*R*)-3-methylaspartic acid	–	–	–
(2*S*, 3*S*)-3-ethylaspartic acid	–	–	–
N-methyl-(2*S*, 3*S*)-3-methylaspartic acid	–	–	–
(2*S*)-aspartic acid	–	–	–
(2*S*, 3*R*)-3-methylglutamic acid	–	+	1·5 ± 0·1
(2*S*, 3*S*)-3-methylglutamic acid	–	+	1 ± 0·1
(2*S*)-homocysteic acid	–	+	5 ± 0·5
1-Bromo-cyclopropane-*cis*-1, 2-diacid	–	+	2·2 ± 0·2
1-Bromo-cyclopropane-*trans*-1, 2-diacid	±	+	~ 3 mM
(*RS*)-2-methylglutamic acid	–	–	–

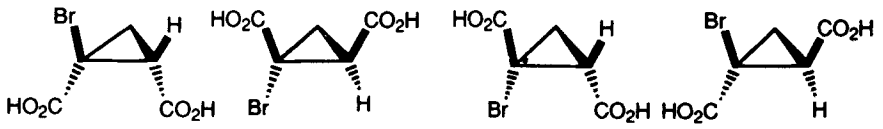
– No activity as substrate or inhibitor; + active as a substrate or inhibitor; ± possible substrate – see text for details

Surprisingly, (2*S*)-homocysteic acid (14), in which the γ-carboxyl group of glutamic acid was replaced by a sulphonyl group, was inactive as a substrate. However, the compound served as a competitive inhibitor for the mutase, $K_i = 5 \pm 0{\cdot}5$ mM. Both the facts that the compound did not serve as a substrate for the enzyme and that the K_i value for (2S)-homocysteic acid was large suggest that either the γ-carboxyl group of glutamic acid is protonated in the enzyme bound complex, or, the active site cannot easily accommodate the extra O-atom of the sulphonate group. In accord with expectations, (2*S*)-phosphohomoserine (15), in which the γ-carboxyl group of glumatic acid (1) is replaced by a phosphate group, did not serve as an inhibitor or as a substrate for glutamate mutase.

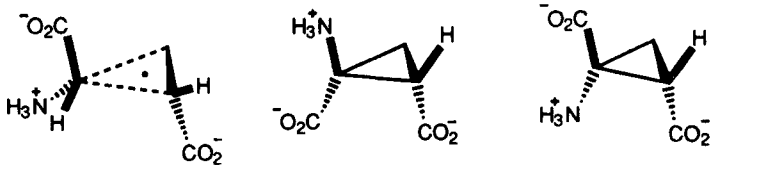
The emerging strict structural requirements for substrates for glutamate mutase prompted us to consider the structure of the transition state for the natural reaction. If the rearrangement occurs with inversion of stereochemistry at C-3 of glutamic acid, as indicated in scheme 2, the transition state might possess a three-membered ring (16) and resemble one of the 2,3-methanoaspartic acids (17) or (18), depending on the C-2-C-3 torsion angle in the active conformation of the substrate.

In order to test this possibility, the mutase was challenged with racemic *cis*-2-bromo-2,3-methanosuccinic acid (10) and *trans*-2-bromo-2,3-methanosuccinic acid (11). The bromides were used in lieu of the amines because it had been reported that the amines (17 and 18) were unstable and spontaneoulsy decomposed to give the 2-ketobutan-1,4-dioate and ammonia *via* the ketimine (Stammer 1990). Furthermore, it was expected that the bromine atom would fit into the site normally occupied by the presumably protonated ammonium group of the natural substrates; the Van der Waals radii of -Br and $-NH_3^+$ are 1·14 Å and 1·00 Å respectively.

Both the cyclopropane diacids (10) and (11) were found to be inhibitors of the enzyme but their modes of inhibition were very different. The *cis*-isomer (18) behaved as a simple competitive inhibitor and displayed a K_i value of 2·2 mmol dm^{-3}. On the other hand, the *trans*-isomer (11) prevented the enzyme from processing any of the substrate for several minutes, as determined using the spectrophotometric assay, after which time the substrate was converted into the enzymic product in the usual way but at a reduced rate. The duration of the lag period was dependent upon the concentration of the inhibitor and increased with increasing concentration. The rate of substrate conversion after the lag period, however, decreased with increasing



(10) (11)



(16) (17) (18)

inhibitor concentration. From the change in rate with inhibitor concentration it was deduced that the minimum K_i value for the *trans*-2-bromo-2, 3-methanosuccine acid (11) was 3 mmol dm^{-3}.

Since the *trans*-2-bromo-2, 3-methanosuccinic acid inhibitor (11) is a racemic mixture of the (2*R*, 3*R*)- and (2*S*, 3*S*)-stereoisomers, it seemed reasonable to believe that only one isomer was responsible for causing the lag period and that this occurred because one isomer was substrate for a rearrangement reaction. In order to test this possibility the reaction of the inhibitor with the enzyme was examined by NMR spectroscopy. While it was confirmed that the inhibitor was consumed, as judged by the time-dependent decrease in the intensity of signals due to the inhibitor, no new signals appeared. The fact that some inhibitor remained unprocessed even after prolonged incubation periods does support the notion that only one stereoisomer is processed. Presumably, it is the other stereoisomer that causes the observed inhibition. From the length of the lag periods measured using the spectrophotometric assay for experiments performed at differing inhibitor concentrations it was calculated that, if half of the inhibitor is processed by the enzyme, k_{cat}/K_M is much larger than for the natural substrate by a factor > 50. It is hoped that on-going work in this area will provide a mechanistic rational for these observations.

Deuterium isotope effects: Given that probing the mechanism of the glutamate mutase system with alternative substrates was proving to be unusually difficult, attention was turned to the use of deuteriated isotopomers of the natural substrate. Although on the basis of isotope partition experiments reported by Richards and coworkers (Eager *et al* 1972) there was a significant deuterium isotope effect of $7{\cdot}5 \pm 1{\cdot}0$ for the cleavage of the methyl-C-H bond in methylaspartic acid, the effects of the isotopic substitution had not been resolved into isotope effects upon V_{max} and V/K. Accordingly the values of the kinetic parameters for unlabelled and [4-$^2H_2$]-labelled (2*S*)-glutamic acid were determined at *p*H 8·4 and the primary isotope effects upon V_{max} [ie. V_H/V_D {or ^DV}] and upon V/K [ie. $(V/K)_H/(V/K)_D$ {or $^D(V/K)$}] were calculated to be $3{\cdot}7 \pm 0{\cdot}2$ and $13{\cdot}5 \pm 1{\cdot}0$ respectively. For a uni-uni reaction the Haldane equation requires that $(V/K)_{forward}/(V/K)_{reverse} = K_{eq}$. Using data from the competition experiments between unlabelled and [*methyl*-$^2H_3$]-3-methylaspartic acid described by Eager *et al* (1972), the deuterium isotope effect for $(V/K)_{reverse}$ was calculated to be at least 5·5. This finding suggests that the isotope effect on the equilibrium constant ($^DK_{eq}$) is small and close to unity. Furthermore, these large values of $^D(V/K)$ indicate that the chemical steps are slow compared to substrate and product debinding steps. The relatively low value for ^DV in the forward reaction direction indicates that a relatively slow internal isomerisation occurs during the catalytic cycle. This step clould be a conformation change in the protein.

In summary, the glutamate mutase system shows remarkable control over its evolved catalytic role. The protein appears to be able to protect highly reactive radical species from traversing reaction pathways which might lead to the destruction of the protein or the coenzyme. Aside from the curious behaviour of the *trans*-2-bromo-2, 3-methanosuccinic acid which appears to indicate that one isomer may be processed, there are no documented alternative substrates for the enzyme. Indeed, even close structural analogues of the substrate and product are unable to participate in partial reactions which involve equilibration of hydrogen on the coenzyme with the structural analogues. Given the difficulty in probing the structure and mechanism of the enzyme,

it may be many years before its chemistry is unravelled. Nevertheless, the recent availability of overexpressed protein will significantly aid progress.

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References

Akhtar M, Botting N P, Cohen M A and Gani D 1987 *Tetrahedron* **43** 5899
Archer C H, Thomas N R and Gani D 1993 *Tetrahedron Asymmetry* **4** 1141
Babior B M 1981 In B_{12} (ed.) D Dolphin (New York: Wiley) vol. 2, ch. 10, p. 263
Baker J J and Standtman T C 1981 In B_{12} (ed.) D Dolphin (New York: Wiley) vol. 2, ch. 8, p. 203
Barker H A, Rooze V, Suzuki F and Iodice A A 1964 *J. Biol. Chem.* **239** 3260
Barker H A, Smyth R D, and Hogenkamp H P C 1963 *Biochem. Preparations* **10** 27
Brecht M, Kellerman J and Pluckthun A 1993 *FEBS Lett.* **319** 84
Eagar R G, Baltimore B G, Herbst M M and Richards J H 1972 *Biochemistry* **11** 253
Finke R G and Shiraldi D A 1983 *J. Am. Chem. Soc.* **105** 7605
Finlay T H, Valinski J, Mildvan A S and Abeles R H 1973 *J. Biol. Chem.* **248** 1285
Gani D, Johnson A W and Lappert M F 1980 *J. Chem. Soc., Chem. Comman* 1244
Gani D, Wallis O C and Young D W 1983 *Eur. J. Biochem.* **136** 303
Goda S, Minton K, Botting N P and Gani D 1992 *Biochemistry* **31** 10747
Gulzar S, Badiani K and Gani D 1994 (unpublished results)
Hartrampf G and Buckel W 1984 *FEBS Lett.* **171** 73
Hartzoulakis B 1994 *Mechanistic studies on glutamate mutase*, Ph D thesis, University of St. Andrews, St. Andrews, Fife
Hartzoulakis B and Gani D 1994 *J. Chem. Soc., Perkin Trans I* (in press)
Hollaway M R, Johnson A W, Lappert M F and Wallis O C 1980 *Eur. J. Bicochem* **111** 177
Holloway D H and Marsh E N G 1993 *FEBS Lett.* **317** 44
Leatherbarrow R J 1987 Enzifitter – A nonlinear regression data analysis program (from the Chemistry Department, Imperial College of Science, Technology and Medicine London SW7 2AY, UK), Biosoft Cambridge CB2 1JP UK
Leutbecher U, Albracht S P J and Buckel W 1992a *FEBS Lett.* **307** 144
Leutbecher U, Bocher R, Linder D and Buckel W 1992b *Eur. J. Biochem.* **205** 759
Marsh E N G and Holloway D H 1992 *FEBS Lett.* **310** 167
McCoy L L and Nachtigall G W 1962 *J. Org. Chem* **27** 4312
Michel C and Buckel W 1991 *FEBS Lett.* **281** 108
Murray A C and Williams D L 1972 In *Organic synthesis with isotopes* (New York: Interscience) vol 4, p. 1302
Reynolds K, Gani D and Robinson J A 1986 *J. Chem. Soc., Chem. Commun.* **17** 1334
Sprecher M, Switzer R L and Sprinson D B 1966 *J. Biol. Chem.* **241** 864
Stammer C H 1990 *Tetrahedron* **46** 2231
Suzuki F and Barker H A 1966 *J. Biol. Chem.* **241** 878
Switzer R L, Baltimore B G and Barker H A 1969 *J. Biol. Chem.* **244** 5263
Switzer R L and Barker H A 1967 *J. Biol. Chem.* **242** 2658
Toraya T and Fukui S 1981 In B_{12} (ed.) D Dolphin (New York: Wiley) vol. 2, ch. 9, p. 233
Zhao Y, Such P and Retey J 1992 *Angew. Chem., Int. Ed. Engl.* **31** 215