

Cloning and sequencing of glutamate mutase component E from *Clostridium tetanomorphum*

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The nucleotide sequence of the large subunit E of glutamate mutase of *Clostridium tetanomorphum* was determined. The protein consists of 483 amino acids and is not made in a precursor form, thus excluding the possibility of subunit E being a pyruvoyl enzyme. It shows no homology to any other protein in the database, and while binding coenzyme B₁₂, a conspicuous B₁₂ binding motif, shared amongst other proteins, is not detectable at the sequence level.

Glutamate mutase; Cobalamin-dependent enzyme; Vitamin B₁₂

1. INTRODUCTION

For the fermentation of glutamate, *Clostridium tetanomorphum* requires the enzyme, glutamate mutase, which catalyzes the rearrangement of L-glutamate to threo- β -methyl-L-aspartate in a vitamin B₁₂-dependent reaction [1].

Several other adenosylcobalamin-dependent enzymes are known, which catalyze similar carbon skeleton rearrangements. Both for methylmalonyl-CoA-mutase and for α -methylglutamate mutase, the reaction may involve a cyclopropane ring as an intermediate in the rearrangement reaction [2,3]. Glutamate mutase has a mechanistic problem, however, in that there is no obvious way to form such an intermediate (Fig. 1).

Therefore several ideas have been considered as to how an analogous mechanism might be operative for glutamate mutase as well. The formation of a Schiff's base at the amino group of glutamate mutase might be such a possibility, and a model substrate was shown to rearrange under such conditions [4,5]. The efficient inhibition of the enzyme by 2-methyleneglutarate and (*S*)-3-methylitaconate [6], but not by (*R,S*)-2-methylglutarate is consistent with an active site accommodating a planar group at the position of the nitrogen. However, no evidence could be obtained for the presence of pyridoxal

phosphate in the enzyme, nor for any effect upon external addition of this coenzyme [7]. A remaining possibility was the presence of a pyruvoyl group as an acceptor of the glutamate amino group in Schiff's base formation.

To test this hypothesis, the enzyme from *Clostridium tetanomorphum* was purified, cloned and sequenced. Pyruvoyl enzymes are generated from precursors [8] and should therefore show a disagreement between the N-terminal sequence determined by Edman degradation and the one deduced from the gene. In these enzymes the protein chain is rearranged at a peptide bond via the formation of anhydro-serine at the new N-terminus, which then hydrolyzes to a pyruvoyl group [8]. A pyruvoyl mechanism would therefore require a continuous gene encoding both E and S subunits, or a precursor form for at least one of them.

The results reported here show that the sequence for component E lies on a gene separate from the sequence of component S, that there is an intervening gene between E and S, and that there is no precursor form of either E or S.

2. MATERIALS AND METHODS

2.1. Protein purification

Glutamate mutase was purified from *Clostridium tetanomorphum* strain H1 (DSM 528), obtained from Deutsche Sammlung für Mikroorganismen. It was grown according to the method of Barker et al., using a semi-synthetic medium [9].

The purification largely followed the work of Barker and co-workers [7]. Both subunits were purified from a single preparation. The step gradients of phosphate buffers in the DEAE column and the hydroxylapatite column [7] were replaced by linear gradients. As a final purification step, we used a chromatofocusing column (Pharmacia, Freiburg). The column was prepared according to the manufacturer's

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The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under accession number X70695

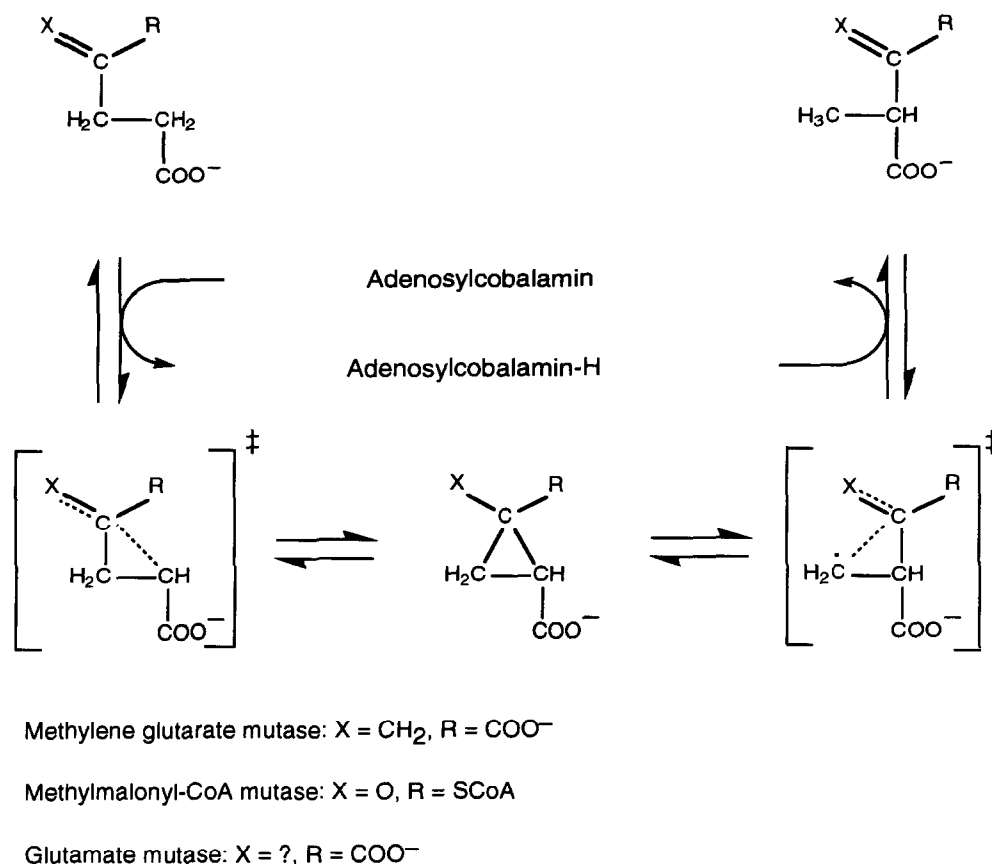


Fig. 1. Suggested reaction mechanism for the three vitamin B₁₂-dependent mutase reactions. The reaction is suggested to involve a cyclopropane ring as intermediate of the rearrangement reactions.

instructions (equilibration buffer: 25 mM histidine/HCl, pH 6.2). Subunits E and S both elute from the column with polybuffer PBE 74 adjusted to pH 4.0.

2.2. Enzymatic assay

The enzymatic activity of glutamate mutase was determined by the aerobic assay system described by Barker et al. [1]. It is based on coupling the conversion of glutamate to methylaspartate with the conversion of the latter to mesaconic acid, catalyzed by methylaspartase, which can be followed by the increase in OD₂₄₀. The assay mixture consisted of 10 mM L-glutamate, 50 mM Tris/HCl, pH 8.2, 3 mM coenzyme B₁₂, 10 mM KCl, 2 mM MgCl₂. In the early stages of the purification, methylaspartase is present in the glutamate mutase fractions; in later stages it was added at about 2 U/assay.

2.3. Tryptic digestion and Edman degradation of the resulting peptides

The protein (10 µg) was dissolved in 80 µl 0.1 M Tris/HCl, pH 8.0, and digested with 0.5 µg of trypsin for 4 h. The reaction was stopped by the addition of 2 µl formic acid and the resulting peptide mixture was separated by reverse-phase HPLC on a Hibar LiChrosphere column (125 × 2 mm) (Merck, Darmstadt) with a flow rate of 0.3 ml/min. A gradient of 0–70% buffer B (0.1% trifluoroacetic acid (TFA) in acetonitrile) was used over a period of 60 min. Buffer A was 0.1% TFA in water.

The tryptic peptides were sequenced, using the Edman degradation method in the sequencer version [10] on a gas-phase sequencer 477A (Applied Biosystems). The phenylthiohydantoin derivatives were identified on a HPLC system 130A (Applied Biosystems) according to the manufacturer's instructions.

2.4. Molecular cloning

Molecular cloning techniques were carried out as described in Sambrook et al. [11]. Genomic DNA of *Clostridium tetanomorphum* (DSM528) was prepared as described in [12]. Total RNA from *Clostridium tetanomorphum* was isolated by the hot-phenol technique [13]. cDNA was prepared, with the You-prime kit (Pharmacia, Freiburg) according to the manufacturer's instructions. RNA-PCR was carried out as described by Fritz et al. [14].

After preliminary attempts to use the sequence information of various peptides for PCR reactions of genomic DNA were not successful, cDNA was prepared and used as starting material. Using a primer corresponding to the N-terminus and a random primer, a 453 bp fragment of the glutamate mutase gene was amplified. This sequence information was then used for Southern blots and inverse PCR [15].

To determine which DNA restriction fragments were of suitable size for inverse PCR (Fig. 2), genomic DNA was digested with various restriction enzymes. For Southern blotting, the direct in-gel hybridization technique was used [16]. A 453 bp fragment corresponding to the N-terminus of subunit E obtained from PCR of cDNA served as the probe. The fragment was labelled with [α-³²P]CTP for 1 h at 37°C, using the Megaprime-labelling kit (Amersham, Braunschweig). The Southern blot showed hybridization of the probe to a *Hind*III (1.2 kb) and an *Aff*III (2.3 kb) fragment, together large enough to contain the whole gene of the E subunit. After ligation at high dilution, the outward primers were used to amplify fragments of 1.1 kb from the *Hind*III digest and 2.2 kb from the *Aff*III digest. Sequencing of these fragments gave information about the 5' and 3' ends of the gene, which was used to design primers for a new inward PCR, starting from genomic DNA.

The final sequence was established from three independent PCR amplifications of clostridial genomic DNA by sequencing both strands.

3. RESULTS AND DISCUSSION

Glutamate mutase consists of two subunits termed component E (mol. wt. 50 kDa) and component S (14 kDa). N-Terminal sequencing of both subunits resulted in the sequence of MELKNKKWTDEEFFKQREE-VLKQWP for the N-terminus of component E and MEKKTIVLGVIGSDDHA VGNKILD for the N-terminus of component S. Since both subunits start with

methionine, as do the homologous subunits from *Clostridium cochlearium* [6], a pyruvoyl group at the N-terminus may appear less likely, although such a group might have been labile. We could rule out the possibility that the N-terminus of the protein might be blocked and that the determined sequence might only be that of a small fraction of precursor by the fact that the yield of the N-terminal methionine in the Edman degradation was about 60%. Additional attempts to obtain chemical evidence for the presence of a pyruvoyl group [8] by the use of NaB^3H_4 and phenylhydrazine were largely negative, even though some reaction was always detectable.

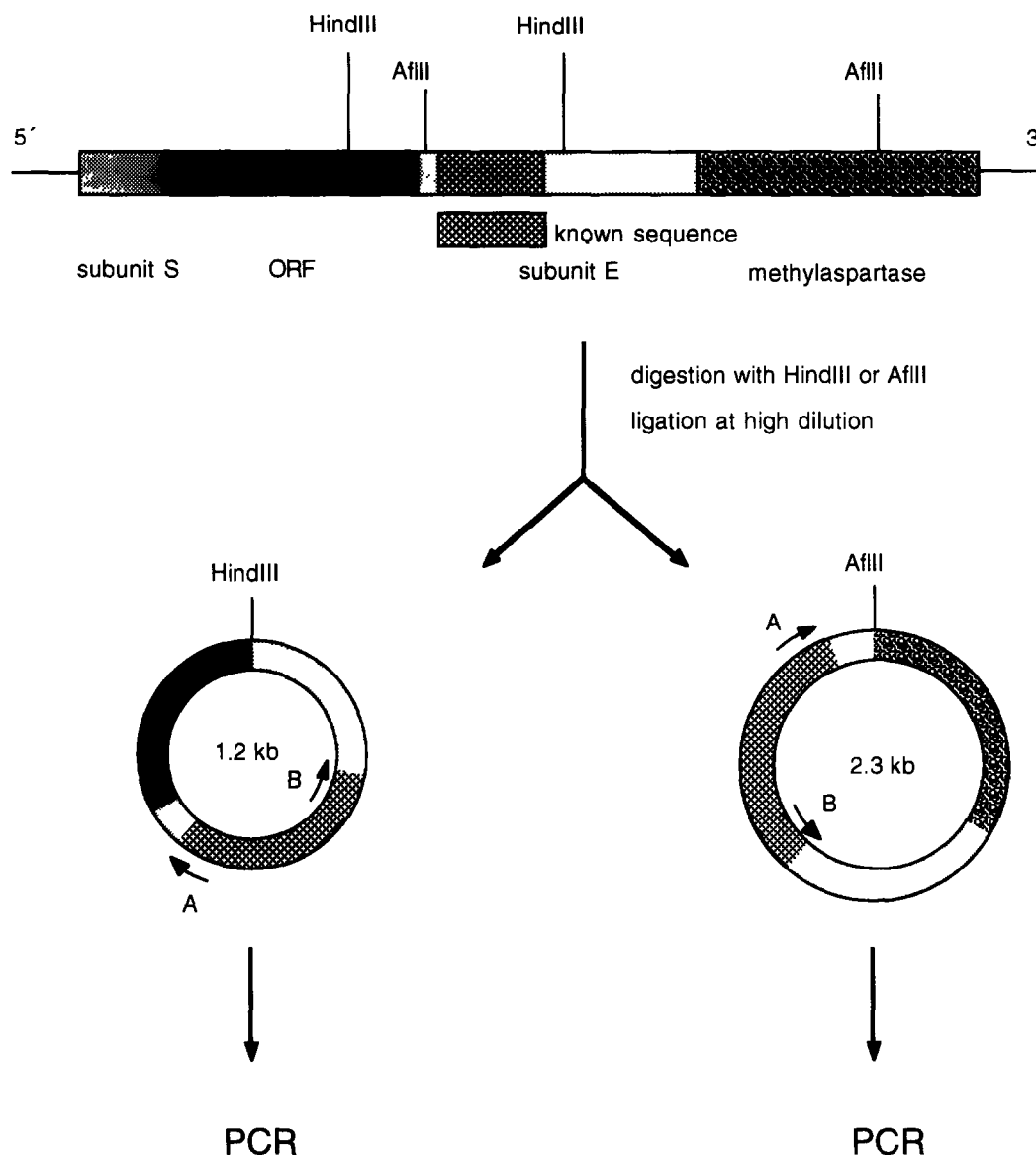
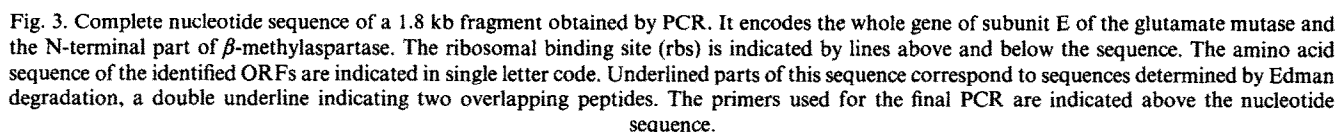


Fig. 2. Cloning and sequencing strategy for glutamate mutase subunit E. On the top, the organization of glutamate mutase and its neighboring genes is shown. *HindIII* or *AflIII* digests of *Clostridium tetanomorphum* were circularized at high dilution and used as starting material for inverse PCR. The primer information was obtained from an initial PCR using an N-terminal and a random primer (top, labeled as 'known sequence'). After sequencing the inverse PCR products, the final sequence was obtained by inward PCR directly from *Clostridium tetanomorphum* DNA, using three independent reactions.



To obtain firm evidence, we decided to clone and sequence the DNA coding for the enzyme, since pyruvoyl-containing enzymes are known to be generated from precursors.

To determine the DNA sequence of the large subunit of glutamate mutase, the products of three independent PCR reactions were cloned and sequenced, and a consensus was determined (Fig. 3). The sequence encoding the N-terminal peptide is found to begin at nucleotide 95, but 5 codons upstream is an in-frame stop codon. The GTG codon is the first reasonable start codon after this stop codon and it is preceded by a ribosomal binding site 8 nucleotides upstream (see below). We conclude therefore that the translated sequence is identical to the sequence determined by Edman degradation, and that there is no precursor form for glutamate mutase subunit E. The protein consists of 486 amino acids and has a calculated molecular weight of 53,706 Da and an isoelectric point of pH 5.85. These data are consistent with the behavior of the protein in SDS-PAGE and on chromatofocusing columns. All peptide sequences were precisely found again in the nucleotide sequence. The distribution of hydrophobic and hydrophilic regions in the protein is inconspicuous.

A homology search of the protein sequence using the FASTA algorithm [17] revealed no significant homology to any known protein in the databases. No significant homology to any other vitamin B₁₂-containing protein was detectable, either in individual or clustered alignments. These sequence alignments with other vitamin B₁₂-containing proteins gave no hints for any common residue which may be involved in a vitamin B₁₂ binding pocket.

There is no homology between components E and S. While spectroscopic data clearly show that vitamin B₁₂ binds to subunit E of glutamate mutase [6], Marsh and Holloway postulated a binding motif in subunit S [18]. Using all known B₁₂-containing proteins, we could not find evidence for a general use of this motif in other proteins nor for any other significant motif in subunit E, shared with other proteins. It appears that B₁₂ binding pockets may have found individual solutions in different proteins, perhaps conserved at the structural level.

On the DNA level the gene exhibits many of the features characteristic of genes from other clostridial species [19]. The gene initiates with the alternative initiator codon, GUG, which was also found for *celA* and *celE* genes of *Clostridium thermocellum*, as well as for the *nifD* gene of *Clostridium pasteurianum*. The gene is preceded by the sequence GGAGG spaced 8 nucleotides from the initiator codon, which is characteristic of clostridial ribosomal binding sites [19]. Approximately 130 bp downstream from the stop codon, the gene coding for methylaspartase starts. Combining our data with those obtained for methylaspartase and glutamate mutase subunit S [18,20], we obtained the genomic organi-

zation of the subunits of glutamate mutase and methylaspartase, shown in Fig. 2.

The mechanism by which glutamate mutase catalyzes the carbon skeleton rearrangement of glutamate to β -methylaspartate still remains unclear. Our data and those obtained by Marsh and Holloway [18] indicate that the genes for the two subunits are separated by an unknown open reading frame of 1.3 kb.

A mechanism, in which an N-terminal pyruvoyl residue is involved in a Schiff's base formation, can be excluded. For such a mechanism it would be necessary that at least one subunit was synthesized as a precursor molecule and was post-translationally modified via a rearrangement involving an anhydro-serine.

Based on the assumption that the reaction mechanism for all three vitamin B₁₂-dependent mutases is the same, there are still plausible solutions to this problem. One possibility is that the glutamate binding pocket in the enzyme facilitates a non-radical rearrangement, using charged amino acids or partial charges from the backbone or other amino acid side chains. The second possibility is the existence of a specific cofactor. We are currently investigating possibilities of other post-translational modifications which may catalyze this reaction.

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