Cloning, Sequencing, and Expression in *Escherichia coli* of the *Clostridium tetanomorphum* Gene Encoding β-Methylaspartase and Characterization of the Recombinant Protein†

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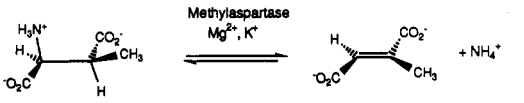
ABSTRACT: The gene encoding methylaspartase (EC 4.3.1.2) from *Clostridium tetranomorphum* has been cloned, sequenced, and expressed in *Escherichia coli*. The open reading frame (ORF) codes for a polypeptide of 413 amino acid residues (M_r 45 539) of which seven are cysteine residues. The size of the ORF indicates that methylaspartase is a homodimer rather than an $(AB)_2$ tetramer. The deduced primary structure of the protein shows no homology to enzymes that catalyze similar reactions or, indeed, any convincing homology with any other characterized protein. The recombinant protein is identical to the enzyme isolated directly from *C. tetanomorphum* as determined by several criteria. The enzyme is obtained in a highly active form (~70% of the activity of the natural enzyme) and migrates as a single band (M_r 49 000) in SDS–polyacrylamide gels. The kinetic parameters for the deamination of (2*S*,3*S*)-3-methylaspartic acid by the natural and recombinant proteins are very similar, and the proteins display identical potassium ion-dependent primary deuterium isotope effects for V and V/K when (2*S*,3*S*)-3-methylaspartic acid is employed as the substrate. In accord with the activity of the natural enzyme, the recombinant protein is able to catalyze the slow formation of (2*S*,3*R*)-3-methylaspartic acid, the L-*erythro*-epimer of the natural substrate, from mesaconic acid and ammonia. Earlier work in which the cysteine residues in the protein were labeled with *N*-ethylmaleimide had indicated that there were eight cysteine residues per protein monomer. One cysteine residue was protected by substrate. Here evidence is forwarded to suggest that the residue that was protected by the substrate is not a cysteine residue but the translation product of a serine codon. Kinetic data indicate that this serine residue may be modified in the active enzyme. The implications of these findings on the mechanism of catalysis are discussed within the context of a new emerging mode of action for methylaspartate ammonia-lyase.

The enzyme β-methylaspartase (3-methylaspartate ammonia-lyase, EC 4.3.1.2) catalyzes the reversible α,β-elimination of ammonia from L-*threo*-(2*S*,3*S*)-3-methylaspartic acid (**1**) to give mesaconic acid (Scheme I) (Barker et al., 1959). The enzyme lies on the main catabolic pathway for glutamate in *Clostridium tetanomorphum* (Barker et al., 1959) and a number of other species (Williams & Traynham, 1962; Ueda et al., 1982). The clostridial enzyme, which is the best studied, was shown to deaminate (2*S*)-aspartic acid and a number of 3-alkyl homologues (Winkler & Williams, 1967) and also the L-*erythro*-(2*S*,3*R*)-diastereomer of methylaspartic acid (Barker et al., 1959). The enzyme was reported to possess an $(AB)_2$ structure, M_r 100 000 (Hsiang & Bright, 1967; Wu & Williams, 1968), and was demonstrated to require monovalent as well as divalent cations for activity.

In early work on the mechanism of deamination, the Clostridial enzyme was shown to catalyze the exchange of the C-3 hydrogen atom of the physiological substrate with hydrogen derived from the solvent, at greater rates than the overall deamination reaction, under some conditions. No primary deuterium isotope effect was detected for the deamination reaction over the pH range 5.5–10.5, and on the basis of these observations, together with the reported ability

Scheme I



of the enzyme to process both diastereomers of L-3-methylaspartic acid, a mechanism involving the intermediacy of a carbanion was proposed (Bright, 1964; Bright et al., 1964a) [see Hansen and Havir (1970) for a review of the early work]. Since Bright's work, the methylaspartase system has been regarded as the archetypal example of an enzyme which operates via a carbanion elimination mechanism, and indeed, studies of the related systems, L-aspartase (Nuiry et al., 1984), phenylalanine ammonia-lyase (Hermes et al., 1985), and argininosuccinate lyase (Kim & Raushel, 1986) have revealed the operation of similar elimination mechanisms.

Research in our own laboratories led us to investigate the kinetics of the methylaspartase system using a range of substrate analogues (Akhtar et al., 1987; Botting et al., 1988a) and, furthermore, prompted an examination of the primary deuterium isotope effects for the deamination of these substrate analogues (Botting et al., 1988b). In contrast to the reported findings (Bright, 1964), we found that (2*S*,3*S*)-3-methylaspartic acid, the physiological substrate, showed a significant isotope effect of ~1.7 on V and V/K over a wide pH range. It was also shown that the C-3 hydrogen-exchange reaction displayed a significant primary deuterium isotope effect (Botting & Gani, 1992). Together with the results of product

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inhibition experiments and $^2H/^1H$–$^{15}N/^{14}N$-double isotope fractionation experiments (Botting et al. 1989), it was suggested that the chemical elimination process was concerted and was followed by a slow step in which ammonia or ammonium ion was relocated within the active site of the enzyme prior to the release of the products (Botting & Gani, 1992). In this paper we describe the cloning, sequencing, and expression of the β-methylaspartase gene from *C. tetanomorphum* in *E. coli* and the characterization of the recombinant enzyme. The sequence indicates that the enzyme is an A_2 homodimer and that an active site *N*-ethylmaleimide-labeled tryptic peptide, previously believed to result from the modification a cysteine residue (Wu & Williams, 1967), is derived from serine rather than cysteine.

MATERIALS AND METHODS

Materials. Tris(hydroxymethyl)aminomethane (Tris),[1] magnesium chloride hexahydrate, and deuterium oxide (99.8 atom %) and all reagents for molecular biology were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. (2*S*,3*S*)-3-Methylaspartic acid and (2*S*,3*S*)-[3-^2H]-3-methylaspartic acid were prepared as previously reported (Botting et al., 1987; Akhtar et al., 1987). All batches of the deuteriated substrate contained >95 atom % deuterium at the 3 position. All other chemicals were of analytical grade or were purified before use.

Enzyme. 3-Methylaspartate ammonia-lyase was purified from *C. tetanomorphum*, strain H1 (ATCC 15920), obtained from the American Typed Culture Collection, grown according to the method of Barker et al. (1959). The purification protocols are modifications of literature procedures (Barker et al., 1959; Hsiang & Bright, 1969) and are identical to those described for the recombinant enzyme, see below.

Enzyme Assay. The enzyme was assayed according to the method of Barker et al. (1959). One unit of enzyme is defined as the amount that catalyzes the formation of 1 μmol of mesaconic acid min^{-1} at pH 9.0 at 30 °C as determined by the increase in OD_{240} in the presence of 4 mM (2*S*,3*S*)-3-methylaspartic acid, 50 mM ethanolamine·HCl (or Tris·HCl), 10 mM KCl, and 1 mM $MgCl_2$ and in the strict absence of other monovalent and divalent metal ions [see Botting and Gani (1992)]. This assay gives the same measured activity as that originally described by Barker et al. (1959), which is performed at pH 9.7 and at 25 °C.

Bacterial Strains, Plasmids, and Culture Conditions. The source of chromosomal DNA was *C. tetanomorphum*, ATCC 15920. The *E. coli* host used as the host for plasmid and M13 recombinants was strain TG1 (K12Δ[*lac-pro*] *supE thi hsdR5*/ F′-*traD36 proA*$^+$*B*$^+$ *lac*]^Q *lacZ*Δ*M15*). *E. coli* strain P2392 (*hsdR514*, *hsdM*, *supE44*, *supF58*, *lacY1*, *galK2*, *galT22*, *metB1*, *trpR55*, P2-lysogen) was employed with the cloning vector λ EMBL3. Cloning vectors used were plasmids pUC8 (Messing & Vieira, 1982), pMTL20/21 (Chambers et al., 1988), and pMTL32 (Whelan et al., 1992), phages M13mp8 (Vieira & Messing, 1982), M13mp18, and M13mp19 (Yanisch-Perron et al., 1985), and the λ EMBL3 (Frischauff et al. 1983). *C. tetanomorphum* was cultivated in a semisynthetic media, essentially as described by Barker (1959). *E. coli* was routinely cultured in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), except when using EMBL3, when NZY broth (0.5% NaCl, 0.2% $MgSO_4·7H_2O$, 0.5% yeast extract, 1.0% NZ amine casein hydrolysate, pH 7.5) was employed. Solidified medium (L-agar) consisted of L-broth with the addition of 2% (w/v) agar (Bacto, Difco, West Holesley, Surrey, U.K.). Antibiotic concentrations used for the maintenance and the selection of transformants were 50 μg/mL ampicillin.

Purification and Manipulation of DNA. Transformation of *E. coli* and large-scale plasmid isolation procedures were as previously described (Minton et al., 1983). Small-scale plasmid isolation was by the method of Holmes and Quigley (1981), while chromosomal DNA from *C. tetanomorphum* was prepared essentially as described by Marmur (1961). Restriction endonucleases and DNA modifying enzymes were purchased from Northumbria Biologicals Limited (Cramlington, Northumberland, U.K.) and were used under the conditions recommended by the supplier. Digests were electrophoresed in 1% agarose slab gels on a standard horizontal system (Bio-Rad Laboratories, Model H4), employing Tris-borate–EDTA (0.09 M Tris-borate, 0.002 M EDTA) buffer. Fragments were isolated from gels using electroelution (Sambrook et al., 1989). M13 template DNA was sequenced by the dideoxynucleotide method of Sanger et al. (1977) using a modified version of T7 polymerase, Sequenase (Tabor & Richardson, 1987). Analysis of generated nucleotide sequence data was undertaken using the computer software of DNASTAR Inc. (Madison, WI).

DNA/DNA Hybridization Experiments. DNA restriction fragments were transferred from agarose gels to "ζ probe" nylon membranes using the procedure of Reed and Mann (1985). After partial depurination with 0.25 M HCl (15 min), DNA was transferred in 0.4 M NaOH by capillary elution for between 4 and 16 h. Bacterial colonies were screened for desired recombinant plasmids by in situ colony hybridization (Grunstein & Hogness, 1975), using nitrocellulose filter disks (Schleicher and Schull, 0.22 μm). DNA fragments were labeled with [α-^{32}P]dATP using a multiprime kit supplied by Amersham International. The oligonucleotide probe was 5′-end labeled by transfer of [γ-^{32}P]rATP with T4 polynucleotide kinase (Maxam & Gilbert, 1977). Hybridizations were carried out in 6× SSC (0.15 M NaCl, 0.15 M Na citrate, pH 7.0) containing 1.0% SDS, 10× Denhardt solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 1 mg/mL of herring sperm DNA, and 10% poly(ethylene glycol) 6000, at a temperature of 53 °C (Sambrook et al., 1989). Following hybridization, membranes were washed for two 15-min periods in 6× SSC containing 0.1% SDS at 50 °C.

Cloning of 5′-End of β-Methylaspartase Gene. To facilitate the detection of the β-methylaspartase structural gene in a recombinant library, the strategy of reverse genetics was applied. The *C. tetanomorphum* enzyme was purified to homogeneity, see below. The enzyme behaved as a homodimer (Cohen, 1989; Botting & Gani, 1989) rather than as a tetramer (Hsiang & Bright, 1967) and was, therefore, subjected to N-terminal amino acid sequence analysis by Edman degradation using a gas-phase sequenator (Applied Biosystems Model 477A). A peptide sequence of 26 amino acids in length was obtained, and the data were used to design a 77′-mer oligonucleotide which could be employed in DNA/DNA hybridization experiments. This oligonucleotide (5′-ATGAAATTTGTTGATGTTTTAAATACTCCAGGATTAACTGGATTTTATTTTGATGATCAAGCTGCTATAAAAAAAGG-3′) corresponded to a sense DNA strand capable of

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; ppm, parts per million (δ); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

encoding the derived N-terminal peptide sequence, MKFVDVLNTPGLTGFYFDDQAAIKKG. The nucleotide bases incorporated in positions of codon degeneracy represented those most frequently utilized in clostridial genes (Young et al., 1989). Southern blot experiments demonstrated that under the conditions employed (see above), appropriately radiolabeled oligonucleotides hybridized strongly to various discrete restriction fragments of *C. tetanomorphum* genomic DNA, including *Bgl*II, *Pvu*II, and *Bam*HI fragments of approximately 14 kb, a large *Sal*I fragment (>23 kb) and *Pst*I and *Sau*3A fragments of 2.3 and 1.5 kb, respectively (data now shown).

To rapidly establish that the probe was specifically hybridizing to the β-methylaspartase gene, the smallest identified fragment was targeted for cloning. Size-fractionated *Sau*3A-cleaved chromosomal DNA, of approximately 1.5 kb in size, was inserted into *Bam*HI-cut pUC8 vector DNA, and positive clones were detected by in situ colony hybridization, using the radiolabeled oligonucleotide as a probe. Approximately 2% of the recombinant clones obtained proved hybridization positive. Purified plasmid DNA from one such clone (designated pSG1) was digested with various restriction endonucleases, and part of its insert was shown to be released as a 1.0-kb *Hin*dIII fragment. Futhermore, the released fragment, following transfer to a nylon membrane, was shown to specifically hybridize to the probe. This fragment was, therefore, subcloned into the *Hin*dIII site of M13mp18, and the templates prepared from the resultant recombinant plaques were sequenced using universal primer. Depending on the orientation of insertion of the cloned fragment, two distinct sequences were obtained. One of these contained a nucleotide sequence exhibiting 79% identity with the oligonucleotide probe, which, upon translation, was found to form part of an open reading frame (ORF).

The encoded polypeptide exhibited amino acid identity with the determined N-terminal sequence of β-methylaspartase in 23 out of 26 positions. The N-terminal sequence of the purified enzyme had assigned the 3rd, 8th, and 21st residues as Phe, Asn, and Ala, respectively. The identified encoded polypeptide had Ile, Cys, and Arg at the equivalent positions.

The origin of the discrepancy in the sequence data was identified upon reexamination of the Edman degradation cleavage products. From this it was apparent that the residue at position 3 had been incorrectly assigned and was, indeed Ile, as predicted from the DNA sequence. Cys residues are not detected during N-terminal amino acid sequencing, unless suitably modified. Because the HPLC peak corresponding to the assigned Asn was unusually small, the Cys predicted to be at position 8 from the nucleotide sequence data was consistent with the Edman sequencing data. In the case of position 21, which was incorrectly assigned as Ala instead of Arg, position 22 is Ala and must have contaminated the products for the 21st sequencing cycle.

Cloning of Entire β-Methylaspartase Gene. Given that the probe hybridized to the desired gene, a λ EMBL3 library was constructed by cloning size-fractionated (approximately 14 kb) *C. tetanomorphum* DNA which had been digested with *Bgl*II (see Figure 1). The resultant recombinant plaques were screened by in situ plaque hybridization using the radiolabeled 1.0-kb *Hin*dIII fragment of plasmid pSG1 as a probe. Of the 2×10^3 plaques screened, 51 positive clones were detected. Cells infected with one of these recombinant λ clones (designated pEM14) were cultivated overnight in NZY medium (Sambrook et al., 1989), and lysate extracts were assayed for methylaspartase activity. Levels of activity

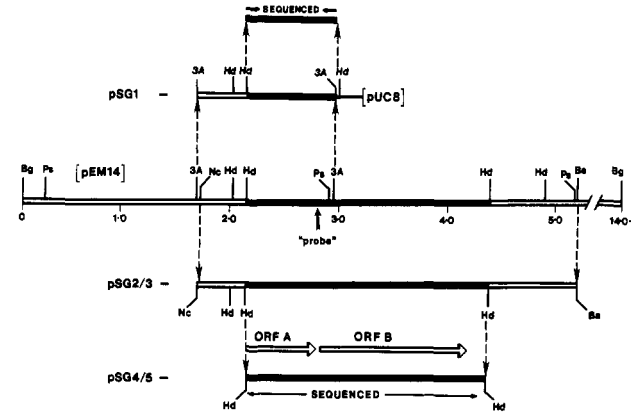


FIGURE 1: Cloning/sequencing strategy employed in the analysis of the β-methylaspartase gene. Restriction enzyme sites are Ba, *Bam*HI; Bg, *Bgl*II; Hd, *Hin*dIII; Nc, *Nco*I; Ps, *Pst*I; and 3A, *Sau*3A. The indicated (vertical dashed arrows) fragments were cloned into pUC8 (pSG1), pMTL21 (pSG2 and pSG4), and pMTL32 (pSG3 and pSG5). The entire 14.0-kb *Bgl*I fragment was cloned into λ EMBL3 (pEM14). The region sequenced is indicated by a bold line, and the position of the identified ORFs is indicated by open arrows.

equating to 3.4 unit/mL of culture were detected. Essentially no activity was seen in cell extracts prepared from a control culture infected with a hybridization negative EMBL3 recombinant.

In order to subclone the gene, the insert of pEM14 was characterized by restriction mapping, and a 3.8-kb *Bam*HI-*Nco*I subfragment was shown to hybridize to both the 1.0-kb *Hin*dIII fragment derived from the insert of pSG1 and the oligonucleotide probe (Figure 1). This fragment was isolated from pEM14 and inserted between the appropriate sites of plasmids pMTL21 (Chambers et al., 1988) and pMTL32 (Whelan et al., 1992), to generate the plasmids pSG2 and pSG3, respectively. That this subcloned fragment still encoded the entire structural gene was confirmed by the demonstration that sonic extracts prepared from cells carrying either recombinant plasmid contained high levels of β-methylaspartase activity.

Determination of Nucleotide Sequence of Gene. Further restriction mapping, combined with Southern blot experiments, indicated that a 2.2-kb *Hin*dIII subfragment of pSG2 (Figure 1) carried the entire β-methylaspartase gene. Accordingly, this gel-isolated fragment was fragmented by sonication. Random fragments of 500–1000 bp were inserted into the unique *Sma*I site of M13mp8, and the nucleotide sequence of the inserts of templates derived from 250 recombinants were determined. The resulting sequence for the *Hin*dIII fragment (Figure 2) was determined in its entirety on both DNA strands (Figure 2), and translation of the nucleotide sequence revealed the presence of two ORFs. The first, ORF A, was apparently incomplete and initiated external to the *Hin*dIII fragment and terminated with a UAA codon at position 627. The second, ORF B, corresponded to that previously identified on the pSG1 insert and coded for β-methylaspartase. ORF B started with an AUG codon at position 756, preceded by a sequence bearing a strong resemblance to the ribosome binding sites of clostridial genes (Young et al., 1989), and terminated with a UAA translational stop codon at position 1995. The deduced primary sequence of the ORF B polypeptide (Figure 2) contains 413 amino acid residues, $M_r = 45\,539$. The codon usage of ORF A and B is shown in Table I.

Overexpression of Gene in E. coli. The polylinker cloning sites of the vector pMTL32 are flanked by tandem copies of *E. coli* transcriptional terminators. Thus, in the case of pSG3,

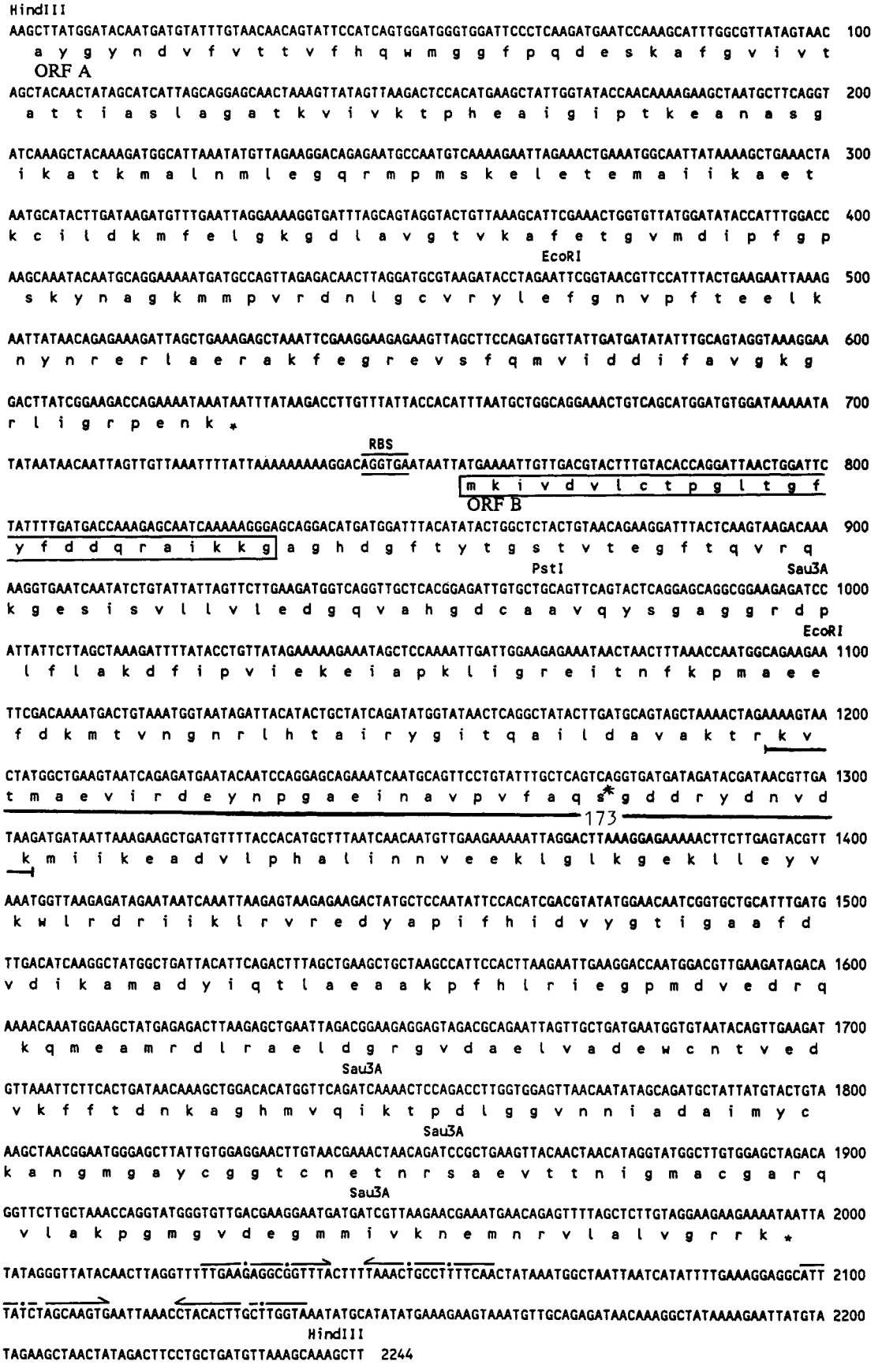


FIGURE 2: Complete nucleotide sequence of the 2.2-kb *Hin*dIII fragment shown to encode the β-methylaspartase gene. The amino acid sequence of the identified ORFs are indicated in the single letter code beneath the second nucleotide of the appropriate codon. The ribosome binding site of ORF B is indicated by lines above and below the sequence. The two regions of dyad symmetry are marked by inward facing arrows above the nucleotide sequence. Gaps in this line indicate mismatch and a dot represents a neutral G·T pairing. ORF B corresponds to the β-methylaspartase gene, and the NH_2-terminal sequence determined by Edman degradation has been boxed.

Table I: Codon Usage of ORF B

amino acid	codon	ORF B	ORF B (%)	*C. acetobutylicum* (%)
Arg	CGU	0	0.0	4.8
	CGC	0	0.0	0.0
	CGA	0	0.0	0.0
	CGG	0	0.0	0.0
	AGA	23	100.0	92.9
	AGG	0	0.0	2.4
Leu	CUU	9	33.3	10.9
	CUC	0	0.0	1.8
	CUA	0	0.0	10.0
	CUG	0	0.0	1.8
	UUA	17	63.0	70.9
	UUG	1	3.7	4.5
Ser	UCU	2	33.3	23.5
	UCC	1	16.7	1.0
	UCA	3	50.0	48.0
	UCG	0	0.0	3.1
	AGU	0	0.0	17.3
	AGC	0	0.0	7.1
Thr	ACU	16	72.7	48.0
	ACC	0	0.0	2.0
	ACA	6	27.3	50.0
	ACG	0	0.0	0.0
Pro	CCU	0	15.4	27.4
	CCC	0	0.0	0.0
	CCA	11	84.6	71.0
	CCG	0	0.0	1.6
Ala	GCU	30	73.2	55.3
	GCC	0	0.0	2.3
	GCA	11	26.8	38.6
	GCG	0	0.0	3.8
Gly	GGU	10	26.3	21.8
	GGC	2	5.3	11.8
	GGA	26	68.4	64.5
	GGG	0	0.0	1.8
Val	GUU	22	62.9	46.2
	GUC	0	0.0	4.3
	GUA	13	37.1	46.2
	GUG	0	0.0	3.2
Ile	AUU	6	41.4	49.5
	AUC	11	37.9	13.8
	AUA	12	20.7	36.7
Lys	AAA	23	82.1	72.0
	AAG	5	17.9	28.0
Asn	AAU	7	38.9	81.8
	AAC	11	61.1	18.2
Gln	CAA	5	41.7	85.7
	CAG	7	58.3	14.3
His	CAU	3	42.9	88.5
	CAC	4	57.1	11.5
Glu	GAA	28	96.4	92.8
	GAG	1	3.6	7.2
Asp	GAU	21	63.6	86.5
	GAC	12	36.4	13.5
Tyr	UAU	6	50.0	80.0
	UAC	6	50.0	20.0
Cys	UGU	7	100.0	70.6
	UGC	0	0.0	29.4
Phe	UUU	7	50.0	79.4
	UUC	7	50.0	20.6
Met	AUG	7	100.0	100.0
Trp	UGG	2	100.0	100.0

expression of the inserted β-methylaspartase gene cannot be attributed to vector-borne transcriptional initiation signals and implies that a sequence residing 5′ to the structural gene is efficiently recognized by an *E. coli* RNA polymerase. To obtain more quantitative data, the completely sequenced 2.24-kb *Hin*dIII fragment was subcloned into both pMTL21 and pMTL32, in the former case, such that transcriptional readthrough of the gene could occur from the vector derived *lac* promoter. Cells carrying the two resultant plasmids (pSG4 and pSG5, respectively) were cultivated in YT media at 37 °C to an optical density of 10 at 600 nm, and the levels of recombinant enzyme produced were assessed in sonic extracts of harvested cells using the standard assay. pSG4 was used to prepare recombinant methylaspartase for characterization and comparison with the authentic enzyme.

Purification and Characterization of Recombinant Enzyme. pSG4 was cultivated in 7 L of YT medium, as described above, to give 95 g of wet cell paste. The cells were frozen in liquid nitrogen and were stored at −80 °C.

Frozen cell paste (10 g) was thawed in 50 mM potassium phosphate buffer (20 mL, pH 7.6) containing mercaptoethanol (1 mM) and the protease inhibitor PMSF (100 μL of a 34-mg mL^{-1} solution in isoPrOH). A further aliquot of PMSF solution (100 μL) was added after thawing was complete, and the solution was sonicated (140 W, 20 kHz) in eight 30-s bursts at 0–5 °C. Acetone (25 mL), precooled to −20 °C, was added with stirring over 5 min, and stirring continued at 4 °C for a further 10 min. The cell debris and precipitated protein were removed by centrifugation at 0 °C (37000*g*, 30 min) to give the crude extract. A further portion of precooled acetone (30 mL) was added, and after being stirred for 10 min, the precipitated protein was collected by centrifugation at 0 °C (37000*g*, 20 min). The protein pellet was resuspended in 50 mM potassium phosphate buffer (2.5 mL, pH 7.6) with swirling at 4 °C, and the insoluble material was removed by centrifugation at 10 °C (37000*g*, 10 min).

The protein solution (2.85 mL) was applied to a column of Sephadex G-150 (1.3 × 170 cm) preequilibrated with 50 mM potassium phosphate buffer (pH 7.6) containing 10 mM mercaptoethanol at 4 °C. The protein was eluted with the same buffer at a rate of 6 mL h^{-1} collecting 6-mL fractions. The active fractions (25–39) were pooled, and the protein solution was concentrated to 3 mL by ultrafiltration (Amicon cell fitted with a YM10 filter) at 4 °C. Distilled water (15 mL) was added to the ultrafiltration cell to dilute the salt, and the protein solution was again concentrated to 3 mL.

A 1-mL aliquot of the protein solution was applied to an LKB-Pharmacia TSK DEAE-5PW FPLC column (2.15 × 15 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.6). The column was washed with the same buffer at a flow rate of 4 mL min^{-1} collecting 4-mL fractions, and a salt gradient of 0–500 mM KCl in the same buffer was applied. The active enzyme eluted in a volume of 24 mL at ~80 mM KCl. The pooled active fractions were concentrated to 1 mL by ultrafiltration as before, and the potassium ion concentration was reduced to ~10 mM by adding distilled water and reconcentrating the protein to ~1 mL, as before. The resulting solution was reapplied to the TSK DEAE-5PW FPLC column preequilibrated as before, and the active enzyme was collected in three 4-mL fractions after application of the salt gradient (see Table II). The protein was obtained in 22% yield, was homogeneous as judged by SDS–PAGE, and was indistinguishable from the authentic protein purified from *C. tetanomorphum*, see below. [This protein purification protocol is identical to the protocol used for preparing the enzyme from *C. tetanomorphum*, except an extra FPLC G3000 gel exclusion chromatography step is required to obtain homogeneous enzyme from *C. tetanomorphum*. The sample used for N-terminal sequencing (see above) was obtained in 8% yield and possessed a specific activity of 233 unit mg^{-1}.]

Protein Electrophoresis. SDS–PAGE was performed on 12% polyacrylamide gels at pH 8.3 according to the method of Laemmli (1970). Buffers contained Tris (25 mM), glycine (200 mM), and SDS (3 mM), and proteins were visualized using Coomassie brilliant blue stain. For molecular weight

Table II: Purification of Recombinant Methylaspartase

fraction	activity					
	vol (mL)	protein (mg)	total (units)	sp. (units mg^{-1})	purifn factor	yield (%)
crude extract[a]	24.0	201.0	(estd 2545 (measd 1556	12.7) 7.8)	1.0	100
resuspended acetone pellet	2.8	80.3	2283	28.4	2.2	90
reduced G-150 fraction	3.0	30.3	1825	60.2	4.7	72
anion-exchange FPLC fraction 1	3.3	10.2	873	85.6	6.7	34
anion-exchange FPLC fraction 2	2.9	4.0	564	141.0	11.1	22

[a] Yields are calculated from the estimated activity present in the crude extract. Assays on this fraction underestimate the actual activity, due to the presence of a methylfumarase activity. Note that the crude extract obtained from unfrozen cells gave 2–3 times more activity and the purified protein possessed higher specific activities, 160–200 unit mg^{-1}, depending on the batch.

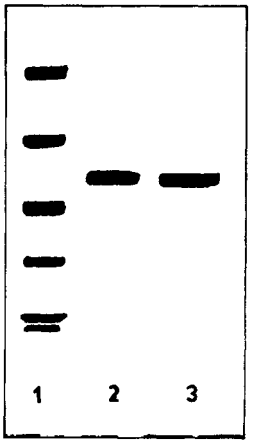


FIGURE 3: SDS–polyacrylamide gel electrophoresis of the natural and recombinant proteins. Lane 1, rabbit muscle phosphorylase *b* (M_r 97 400), BSA (M_r 66 200), hen egg white ovalbumin (M_r 45 000), bovine carbonic anhydrase (M_r 31 000), soybean trypsin (M_r 21 500), and hen egg white lysozyme (M_r 14 400); lane 2, recombinant methylaspartase; lane 3, methylaspartase isolated from *C. tetranomorphum*.

determinations the Bio-Rad SDS–PAGE standards kit was used (Figure 3).

Kinetic Experiments. The kinetic parameters for the recombinant protein in the presence of different potassium ion concentrations were determined as described previously (Botting & Gani, 1992) for the enzyme from *C. tetanomorphum*. The magnitude of primary deuterium isotope effects was also determined as described previously using (2*S*,3*S*)-3-methylaspartic acid and (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid (Table III).

Experiments to measure the formation of the L-*erythro*-diastereomer of methylaspartic acid from mesaconic acid and ammonia contained diammonium mesaconate (0.23 M), NH_4Cl (0.2 M), $MgCl_2$ (0.02 M), and KCl (0.1 M) at pH 9.0 in a total volume of 10 mL. A 1-mL aliquot was removed prior to the addition of enzyme and was lyophilized, reconstituted in deuterium oxide, relyophilized, and redissolved in deuterium oxide (1 mL), and a ^{1}H-NMR spectrum of the sample was recorded. Enzyme (10 units) was added to the remaining 9 mL of the substrate solution, and samples were removed for analysis by NMR spectroscopy at intervals over a period of several days (see Figure 4).

RESULTS AND DISCUSSION

A 77′-mer oligonucleotide, capable of encoding a 26 amino acid peptide sequence (MKFVDVLNTPGLTGFYFDDQ-AAIKKG, derived from N-terminal sequence analysis of the purified β-methylaspartase), was used as a probe to identify and clone a gene in the *C. tetanomorphum* genome encoding β-methylaspartase. The sequenced gene exhibited many of the features characteristic of genes from other clostridial species (Young et al., 1989). The gene initiates with AUG, terminates with an ochre UAA translational stop codon, and is preceded by a sequence characteristic of clostridial ribosome binding sites (Young et al., 1989). There is a general bias for codons ending in U or A, which is especially apparent with those codons encoding Arg, Thr, Pro, Ala, Val, and Cys, where no codon ending in C or G is employed. In contrast to other *Clostridium* species, however, in the case of Phe and Tyr there is an atypical equal preference for codons ending in U and C, while in the wobble position for those codons specifying Asn, Gln, and His the use of C or G is clearly preferred over U or A. These latter anomalies in codon usage, compared to other clostridial genes, may be a consequence of the G + C content of the gene (36.8%), which is relatively higher than that normally associated with the genes of other saccharolytic clostridia. A similar preference in codons is also exhibited by an unknown gene, ORF A (Figure 2) which immediately precedes the β-methylaspartase gene on the *C. tetanomorphum* chromosome.

At the 3′-end of the gene the translational stop codon is closely followed by a sequence exhibiting substantial dyad symmetry, a feature characteristic of ρ independent transcriptional termination signals. The stem loop structure of a mRNA molecule transcribed from this region would have a ΔG of −17.6 kcal mol^{-1} (Tinoco et al., 1973). A second region of dyad symmetry occurs some 101 nucleotides 3′ to the gene's stop codon. A transcript produced from this region could form a stem loop structure with a predicted ΔG of −12.6 kcal mol^{-1}. The high levels of recombinant enzyme detected in cells carrying plasmid pSG3 clearly demonstrate that the sequenced *Hin*dIII fragment contains nucleotide sequences which mediate efficient transcription of the β-methylaspartase gene in *E. coli*. Examination of the 126-bp region of intervening DNA, between the stop codon of the unknown ORF A and the start codon of the β-methylaspartase gene, demonstrates the presence of a number of sequence motifs corresponding to typical *E. coli* promoter elements. Most notable is a sequence at position 701 which perfectly conforms to the consensus *E. coli* −10 promoter element, TATAAT. However, even if this sequence does play a role in transcription of the gene in *E. coli*, it is not necessarily used in *C. tetanomorphum*. Definitive assignment of transcriptional elements will require the isolation of mRNA from the natural host and its use in RNA transcript mapping.

Size and Subunit Structure. Translation of the determined nucleotide sequence shows that methylaspartate ammonia-lyase is composed of 413 amino acid residues. The predicted amino acid composition (Table IV) exhibits a high degree of agreement with that determined experimentally on protein hydrolysates by Williams and Libano (1966) and by Hsiang and Bright (1967). The predicted molecular weight of the

Table III: Variation of Kinetic Parameters for (2S,3S)-3-Methylaspartic Acid with K^+ Concentration[a] for Natural (C) and Recombinant (R) Enzyme

		(2S,3S)-3-methylaspartic acid		(2S,3S)-[3-^{2}H]-3-methylaspartic acid			
[KCl]/(mM)	enzyme	K_M (mM)	V_{max}[b] (10^{-6} mol dm^{-3} min^{-1})	K_M (mM)	V_{max}[b] (10^{-6} mol dm^{-3} min^{-1})	^DV	$^D(V/K)$
0.3	C	2.80 ± 0.80	309	2.63 ± 0.50	274	1.13	1.06
0.3	R	2.54 ± 0.52	231	2.38 ± 0.41	195	1.19	1.11
1.6	C	2.37 ± 0.20	654	2.35 ± 0.25	385	1.70	1.68
1.6	R	2.17 ± 0.15	462	2.14 ± 0.20	283	1.63	1.70
50.0	C	0.67 ± 0.07	2089	0.67 ± 0.07	2089	1.00[c]	1.00[c]
50.0	R	0.61 ± 0.09	1597	0.61 ± 0.09	1597	1.00[c]	1.00[c]

[a] Incubations contained 0.5 M Tris (pH 9.0), 0.02 M $MgCl_2$, substrate, and KCl in a total volume of 3 mL. Reactions were run at 30 ● 0.1 °C. [b] Corrected for one unit of natural enzyme assayed at pH 9.0. This batch of recombinant enzyme showed 75% of the specific activity of the natural enzyme and V_{max} values are corrected accordingly. Errors on V_{max} values are <8%. [c] Both isotopomers lie on the same plot.

protein (M_r 45 539) is consistent with that estimated by SDS–PAGE (M_r 49 000) for the enzyme purified from *C. tetanomorphum* (Figure 3). The established molecular weight of the protein, $M_r \sim 100\,000$, (Hsiang & Bright, 1967) indicates that the active enzyme is a simple homodimer rather than a tetramer as was originally thought (Wu & Williams, 1968).

The recombinant enzyme purified from pSG4 was isolated in 22% yield using the same protocols as those used for the purification of the enzyme from *C. tetanomorphum* (Table II). At least 5–8% of all of the soluble protein expressed in pSG4 is active methylaspartase.

The homogeneous recombinant enzyme was indistinguishable from methylaspartase isolated from *C. tetanomorphum* as judged by SDS–PAGE and migrated as a single band of 49 000 amu (Figure 3). The elution times for the two enzymes from the TSK-5PW anion-exchange FPLC column used during the purification were also identical.

Kinetic Properties. The recombinant protein showed very similar kinetic properties to the protein isolated from *C. tetanomorphum* (Table III). The values of K_m for each protein with (2S,3S)-3-methylaspartic acid as the substrate were identical although the recombinant protein showed a slightly lower specific activity.

The primary deuterium isotope effects for V and V/K for the two enzymes were also the same. The effects of the concentration of the monovalent cationic cofactor, K^+, on the kinetic constants and on the magnitude of the deuterium isotope effects were identical for both enzymes. The K^+ concentration dependence of these parameters is a very sensitive test for the binding and the debinding orders of the substrate, products, and metal ions (Botting & Gani, 1992).

Interestingly, the recombinant protein did catalyze the slow conversion of mesaconic acid to (2S,3R)-3-methylaspartic acid in the presence of ammonia, as well as the rapid conversion of mesaconic acid to the natural substrate (2S,3S)-3-methylaspartic acid (Figure 4). The formation of the (2S,3R)-epimer, a formal syn-addition process is catalyzed by the enzyme purified from *C. tetanomorphum*. The finding that the recombinant protein displays an identical activity rules out the possibility that the operation of a contaminating protein in *C. tetanomorphum* was responsible for the formation (or deamination) of the minor (2S,3R)-epimer in the work originally reported by Barker and co-workers (1959).

Location of Active Site Peptide. Examination of the primary structure of methylaspartase (Figure 2) indicates there are seven cysteine residues (Table IV). This number is consistent with the 14 cysteine residues per mole of enzyme that could be titrated by photooxidation (Williams & Libano, 1966). However, Wu and Williams also showed that eight uniformly labeled peptides were formed upon the treatment of methylaspartase with [^{14}C]-*N*-ethylmaleimide followed by tryptic digestion and assumed that eight cysteine residues in the protein had been labeled (Wu & Williams, 1968). This result led these researchers to believe that their earlier estimate of the number of Cys residues in the molecule had been too low. Bright also provided evidence for the presence of 16 Cys residues per mole of enzyme on the basis of *p*-chloromercuribenzoate titrations (Hsiang & Bright, 1967). Further work in Williams' laboratory allowed an active site peptide to be isolated (Wu & Williams, 1968). When the enzyme was treated with unlabeled *N*-ethylmaleimide in the presence of substrate, dialysis of the protein solution gave an active enzyme. Treatment of the dialyzed enzyme with [^{14}C]-*N*-ethylmaleimide caused inactivation, and treatment of this labeled protein with trypsin gave a single active site labeled peptide.

This peptide corresponded to one of the eight uniformly labeled peptides obtained from the earlier experiment and was assumed to contain cysteine. The amino acid composition of the active site peptide was determined by total acid catalyzed hydrolysis, and the peptide was estimated to be a 39–42-mer (Wu & Williams, 1968). Note that the structure of the modified cysteine hydrolysis product, *S*-succinocysteine, was not proved in these experiments.

When we compared the possible tryptic peptide sequences and amino acid compositions for 35–45-mers for each of the seven cysteine residues in methylaspartase (Figure 2) with the reported amino acid composition of the active site *N*-ethylmaleimide-labeled peptide, there was no correlation whatsoever. This analysis indicates that the active site peptide does not contain a cysteine residue and that the other seven uniformly labeled peptides detected in the Wu and Williams experiment were (probably) cysteine-containing peptides.

When all of the possible tryptic peptide sequences and amino acid compositions for 35–45-mers were compared with the reported amino acid composition of the active site *N*-methylmaleimide-labeled peptide, relaxing the requirement for the presence of cysteine, there was one very good correlation. This possible peptide, a 37-mer, which spans Lys-147 to Lys-183 (Figure 2), contains a unique serine residue (Table IV). Note that this peptide would arise from cleavage at Arg-146 and Lys-183. There is another site for trypsin cleavage at Lys-144, and cleavage here would give a 39-mer containing an extra Thr and Arg residue (see Figure 2 and Table IV).

The correlation of the deduced 37-mer peptide composition with the amino acid composition of the tryptic peptide was perfect for Arg, Asp, and Asn; Glu and Gln; His; Ile; Lys; Met; Phe; Pro; Ser; and Thr and good for Ala. Indeed, glycine was the only amino acid which correlated very poorly. On the basis of this analysis, it seems almost certain that the so-called active site cysteine-containing peptide isolated by Wu and Williams actually contained serine or a posttranslationally modified serine residue.

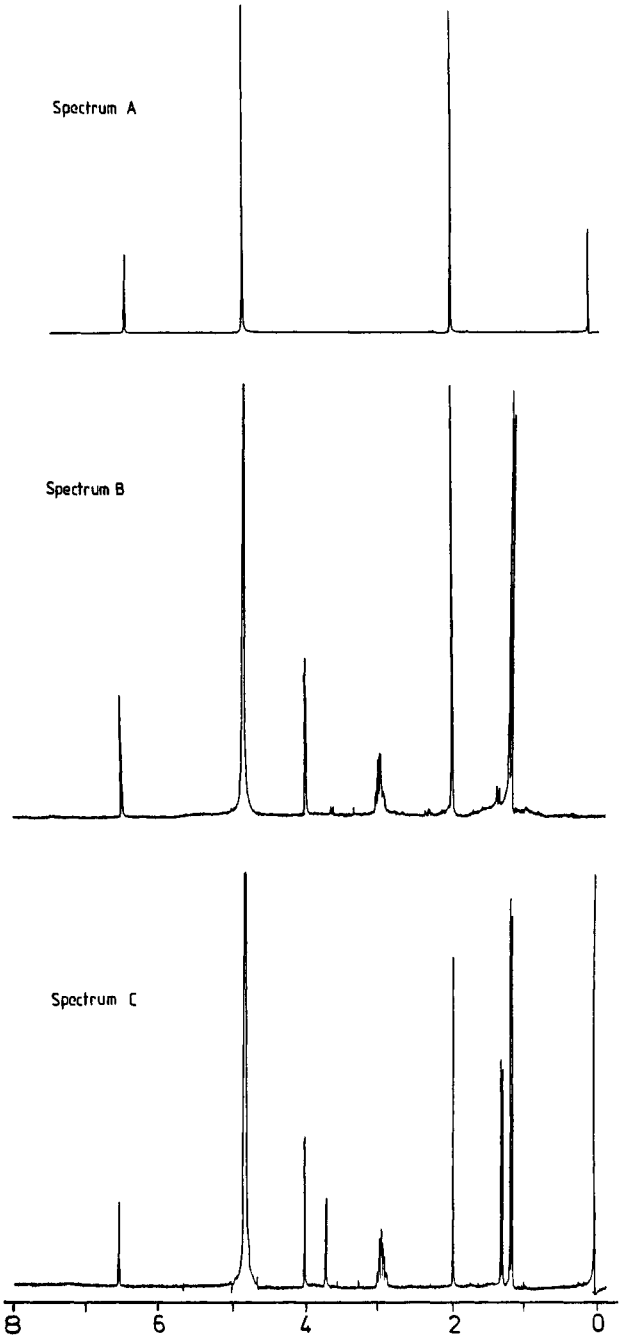


FIGURE 4: 270 MHz ^{1}H-NMR spectra showing the recombinant methylaspartase-catalyzed formation of (2*S*,3*S*)-3-methylaspartic acid [the L-*threo*-isomer] and (2*S*,3*R*)-3-methylaspartic acid [the L-*erythro*-isomer] from mesaconic acid and ammonia, see text for details. Spectrum A was recorded before the addition of the enzyme, spectrum B after 8 h, and spectrum C after 172 h. The methyl signals for mesaconate, (2*S*,3*S*)-3-methylaspartate, and (2*S*,3*R*)-3-methylaspartate are well separated and occur at 2.0, 1.15, and 1.30 ppm, respectively, using the HO^{2}H signal at 4.6 ppm as an internal reference. Other signals are assigned as follows: ^{1}H 6.60 (=CH– of mesaconate); 4.02 (C^α-H of (2*S*,3*S*)-3-methylaspartate); 3.78 (C^α-H of (2*S*,3*R*)-3-methylaspartate); 2.92 (C^β-H of both 3-methylaspartates).

To understand why the product of the serine codon should react with *N*-ethylmaleimide (Wu & Williams, 1968) is not easy if serine is the final product since the β-hydroxyl group of serine does not usually react with Michael acceptors to give conjugate addition products. Also, the finding by Bright that treatment of the enzyme with eight molar equivalents of *p*-chloromercuribenzoate, but not with less, caused peptide chain cleavage is curious (Hsiang & Bright, 1967, 1969).

Table IV: Comparison of Determined and Deduced Amino Acid Composition of β-Methylaspartase

	β-methylaspartase			NEM peptide	
amino acid	deduced	determined[a]		deduced	determined[b]
alanine	41	43	43	4	5
arginine	23	23	29	2	2
asparagine	18			3	
aspartic acid	33			5	
(Asp + Asn)	51	54	54	8	8
cysteine	7	8	8	0	0
glutamine	12			1	
glutamic acid	29			3	
(Glu + Gln)	41	45	44	4	4
glycine	38	42	40	2	8
histidine	7	8	9	0	0
isoleucine	29	30	29	2	2
leucine	27	30	29	0	1
lysine	28	33	36	2	2
methionine	17	16	18	1	1
phenylalanine	14	16	14	1	1
proline	13	12	13	2	2
serine	6	9	7	1	1
threonine	22	22	22	1	1
tryptophan	2	2		0	
tyrosine	12			2	1
valine	35	37	36	5	3

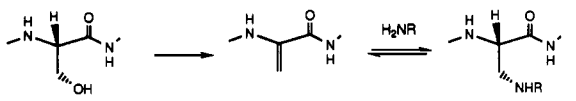
[a] Determinations taken from Williams and Libano (1966) and Hsiang and Bright (1967), respectively. [b] Determinations taken from Wu and Williams (1968), see text for details.

Recent studies in our laboratory have shown that phenylhydrazines are potent irreversible inactivators of methylaspartase, although negatively charged nucleophiles such are borohydride and cyanide are not so effective. The substrate completely protects against inactivation by phenylhydrazines (M. Ahktar and D. Gani, unpublished results), and the findings suggest that the enzyme may contain an electrophilic residue, such as a dehydroalanine residue. Indeed, a dehydroalanine residue has been implicated in each of the deamination reactions catalyzed by phenylalanine (Havir & Hanson, 1975; Hermes et al., 1985) and histidine (Givot et al., 1969) ammonia-lyase. Conversely, the aspartate ammonia-lyase reaction does not appear to involve the formation of covalent intermediates, and in fact, hydrazine behaves as a substrate (Hanson & Havir, 1972).

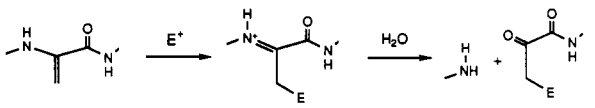
For methylaspartase, further substantial support for the operation of a dehydroalanine residue, which can act as covalent binding site for ammonia, is provided by the results of our previous kinetic studies (Botting et al., 1988b, 1989; Botting & Gani, 1992). For example, although the ^{15}N-isotope effect for V/K for the deamination of (2*S*,3*S*)-3-methylaspartic acid at pH 6.5 increased from 1.0255 to 1.0417 when deuterium was introduced at C-3 of the substrate (Botting et al., 1989) [a result which indicates that a concerted deamination mechanism operates (Hermes et al., 1982)], ammonia was known to increase the rate of C-3 substrate hydrogen exchange with the solvent, without itself becoming incorporated into the substrate pool (Bright, 1964; Botting & Gani, 1992); a result most easily accommodated by a stepwise carbanionic mechanism. These results and many others, including the finding that product inhibition by ammonia is nonlinear and uncompetitive, led us to propose that there was an extra binding site for ammonia, in addition to the product binding site, and that under certain conditions the vacation of this extra binding site was kinetically significant (Botting & Gani, 1992).

If Ser-173 is indeed converted to a dehydroalanine residue, the posttranslational modification must be catalyzed by the protein. This must be true (unless the host possesses the

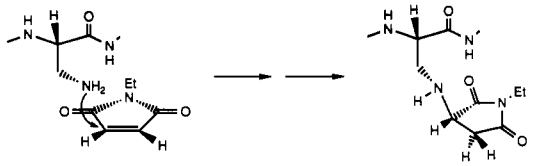
Scheme II



Scheme III



Scheme IV



necessary processing activities) because only one complete ORF is present in the cloned clostridial fragment.

A dehydroalanine residue would be expected to react with hydrazines, to give inactive enzyme, in the same manner as substrate and ammonia, via β-addition of the nitrogen nucleophile to the α,β-unsaturated amide (Scheme II).

The dehydroalanine residue is also an acylated enamine and, as such, would be nucleophilic at the β-carbon atom and prone to reactions with electrophiles. To this end, the reaction of the dehydroalanine residue with mercury salts would be expected to result in amide bond cleavage as indicated in Scheme III. Alternatively, the addition of the eighth equivalent of *p*-chloromercuribenzoate, which causes peptide bond cleavage (Hsiang & Bright, 1969), might activate a methionine residue for lactonization-peptide chain cleavage in much the same way as cyanogen bromide.

The product of ammonia addition to the putative dehydroalanine residue, a 2,3-diaminopropionic acid residue, whether formed directly or through its elimination from an intermediate substrate complex, would be expected to add to *N*-ethylmaleimide via its β-amino group. The reaction is almost identical to the microscopic reverse of the catalytic elimination reaction in which the maleimide replaces a fumarate (Scheme IV).

Experiments designed to gain further information on the existence and origin of the putative dehydroalanine residue in methylaspartase are underway.

Comparison with Other Proteins. Previous studies (Woods et al., 1986) have shown that a number of bacterial enzymes, which carry out a functionally similar reaction to that catalyzed by methylaspartate ammonia-lyase (i.e., the elimination of water or ammonia from a substituted succinic acid to give fumaric acid), exhibit substantial amino acid homology. Thus, *E. coli* fumarase (EC 4.2.1.2) and aspartase (EC 4.3.1.1) share 37.5% identical residues and exhibit 62.8% and 37.8% identity, respectively, with fumarase from *Bacillus subtilis* (Woods et al., 1986). Comparative alignment of the deduced primary amino acid sequence of methylaspartase with the three enzymes did not, however, reveal any significant conservation of amino acids between sequences. Indeed, a search of current protein data libraries showed that β-methylaspartase displayed no convincing homology to any characterized protein.

The identity of the putative polypeptide encoded by ORF A is presently unknown, and its primary structure exhibits no homology to any characterized protein. The possibility exists that it could code for a second enzyme involved in the mesaconate pathway of glutamic acid metabolism. Evidence has been obtained to indicate that the gene(s) encoding mesaconase reside immediately 3′ to the β-methylaspartase gene (S. Goda and N. P. Minton, unpublished results). Thus ORF A may encode glutamate mutase. This possibility is being investigated.

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