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Mechanism of 3-Methylaspartase Probed Using Deuterium and Solvent Isotope Effects and Active-site Directed Reagents: Identification of An Essential Cysteine Residue†

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Abstract—The mechanism of the L-*threo*-3-methylaspartate ammonia-lyase (EC 4.3.1.2) reaction has been probed using deuterium and solvent isotope effects with three different substrates, (2*S*,3*S*)-3-methylaspartic acid, (2*S*)-aspartic acid and (2*S*,3*R*)-3-methylaspartic acid. Each substrate appears to form a covalent adduct with the enzyme through the amination of a dehydroalanine (DehydAla-173) residue. The true substrates are *N*-protonated and at low pH, the alkylammonium groups are deprotonated internally in a closed solvent-excluded pocket after K^+ ion, an essential cofactor, has become bound to the enzyme. At high pH, the amino groups of the substrates are able to react with the dehydroalanine residue prior to K^+ ion binding. This property of the system gives rise to complex kinetics at pH 9.0 or greater and causes the formation of dead-end complexes which lack Mg^{2+} ion, another essential cofactor. The enzyme–substrate adduct is subsequently deaminated in two elimination processes. Hydrazines act as alternative substrates in the reverse reaction direction in the presence of fumaric acid derivatives, but cause irreversible inhibition in their absence. Borohydride and cyanide are not inhibitors. *N*-Ethylmaleimide also irreversibly inactivates the enzyme and labels residue Cys-361. The inactivation process is enhanced in the presence of cofactor Mg^{2+} ions and Cys-361 appears to serve as a base for the removal of the C-3 proton from the natural substrate, (2*S*,3*S*)-3-methylaspartic acid. The dehydroalanine residue appears to be protected in the resting form of the enzyme by generation of an internal thioether cross-link. The binding of the substrate and K^+ ion appear to cause a conformational change which requires hydroxide ion. This is linked to reversal of the thioether protection step and generation of the base for substrate deprotonation at C-3. The deamination reaction displays high reverse reaction commitments and independent evidence from primary deuterium isotope effect data indicates that a thiolate acts as the base for deprotonation at C-3.

Introduction

Methylaspartase (3-methylaspartate ammonia-lyase, EC 4.3.1.2) catalyses the reversible α,β-elimination of ammonia from L-*threo*-(2*S*,3*S*)-3-methylaspartic acid (**1**) in an *anti*-fashion to give mesaconic acid (Scheme 1).[1] The enzyme lies on the main catabolic pathway for glutamate in *Clostridium tetanomorphum*[2] and a number of other species.[3,4] The clostridial enzyme is a 91,000 Da homodimer of 413-residue subunits and requires monovalent and divalent cation cofactors for activity.[5] Potassium and magnesium ions serve as the best metal ion cofactors. The enzyme has been shown to deaminate the L-*erythro*-(2*S*,3*R*)-diastereomer of methylaspartic acid[1,6] as well as (2*S*)-aspartic acid and a number of 3-alkyl homologues.[7] Moreover, the enzyme was shown to be able to catalyse the exchange of the C-3 hydrogen atom of the physiological substrate with hydrogen derived from the solvent, at rates greater than for the overall deamination reaction, under some conditions.[8] No primary deuterium isotope effect was detected for the deamination reaction over the pH range from 5.5 to 10.5 and, on the basis of these observations, a mechanism involving the intermediacy of a carbanion was proposed,[8,9] (see Hansen and Havir for a review of the early work[10]). Since Bright's work, the methylaspartase system has been regarded as the archetypal example of an enzyme which operates via a carbanion elimination

Key words: Enzymes and enzymic reactions; enzyme inhibitors; kinetics; isotope effects.

Abbreviations: Bis Tris propane, 1,3-bis [tris(hydroxymethyl)-methylamino]propane; HPLC, high performance liquid chromatography; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; UV, ultra-violet; TLC, thin layer chromatography; Tris, (Tris[hydroxymethyl]aminomethane); GC–MS, gas chromatography–mass spectrometry; SIR-GC–MS, single ion recording-gas chromatography–mass spectrometry.

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†Dedicated to the memory of Sir Derek H. R. Barton and his intellectual impact in the chemical sciences.

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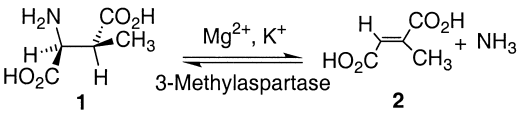


Scheme 1.

mechanism,[11] and indeed, more recent studies of the related systems, L-aspartase,[12] phenylalanine ammonia-lyase[13] and argininosuccinate lyase[14] 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[15–17] and, furthermore, prompted an examination of the primary deuterium isotope effects for the deamination of these substrate analogues.[18] In our hands, (2*S*,3*S*)-3-methylaspartic acid, the natural substrate, showed a significant isotope effect of ~1.7 on V and on V/K over a wide pH range, in contrast to the reported findings.[8] Further work established that K^+ ion binds to the enzyme–substrate–Mg^{2+} complex last in an ordered binding sequence and debinds before the products or Mg^{2+} ion are released, indicating that both ^{D}V and $^{D}(V/K)$ might be suppressed at high K^+ concentration.[19] It was also noted that double reciprocal plots of initial rate versus K^+ ion concentration were not linear but concave down. The repetition of Bright's experiments at high K^+ concentration confirmed that no C-3 primary deuterium isotope effect was apparent and provided an explanation for the dichotomy.[19] In view of these findings, other evidence in support of the carbanion mechanism was challenged.

The mechanism of the C-3 hydrogen exchange reaction was examined by measuring the primary deuterium isotope effect for tritium wash-in into the substrate from the solvent.[19] The reaction displayed a significant pH-independent isotope effect on V_{max} of 1.5–1.6 which mirrored the isotope effects determined for the deamination reaction in the same experiments. However, the ratios of the rates for the exchange versus deamination reaction (v_{ex}/v_{deam}) varied widely (> fivefold) over the pH-range of the study, but were not affected by the presence of deuterium. These results are not in accord with a simple C-3 carbanion hydrogen exchange mechanism. The mechanism for the *syn*-elimination of ammonia from the L-*erythro*-(2*S*,3*R*)-diastereomer of methylaspartic acid[6,20] was probed using C-3 deuteriated and unlabelled substrate by measuring the rate of mesaconic acid formation and C-3 hydrogen exchange and epimerisation by NMR spectroscopy. The results indicated that epimerisation and hydrogen exchange could not occur before the L-*erythro*-substrate had first undergone elimination to give mesaconic acid. Again these findings do not support the operation of a carbanion mechanism, but do not exclude the possibility that the proton removed from the C-3 position is shielded from the solvent until the products are formed. Preliminary $^{15}(V/K)$ data at low pH also indicated that the reaction did not follow a simple carbanion mechanism[21] (see Discussion and following article).

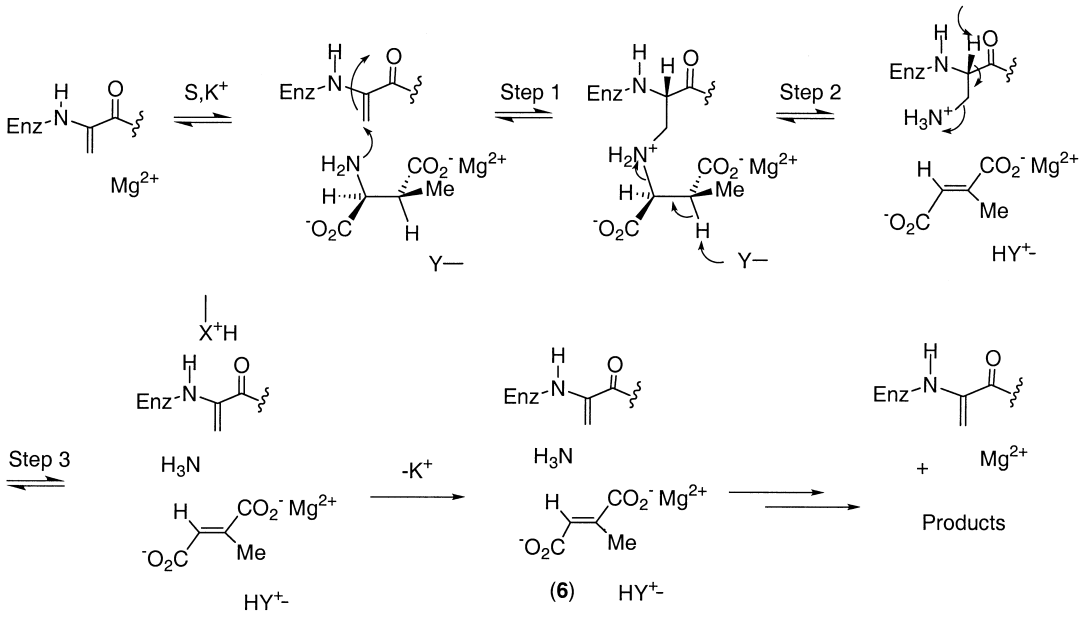
More recently evidence was provided to suggest that methylaspartase might contain an electrophilic dehydroalanine prosthetic group derived from Ser residue 173.[5] In accord with this suggestion, some other ammonia-lyase enzymes[22,23] have since been shown to utilise a modified Ser residue. In methylaspartase the Ser-173 residue occurs in the sequence Ser-Gly-Asp which also occurs in histidine and phenylalanine ammonia-lyase.[24,25] For methylaspartase it has been proposed that the substrate could attack the dehydroalanine moiety in a Michael fashion (Scheme 2) to give a covalent enzyme–substrate complex which is subsequently deaminated twice via two α,β-elimination reactions.[5] Model studies with dehydroalanine peptides have shown that nitrogen nucleophiles can attack the dehydroalanine residue at C-3, to give a Michael adduct, or at C-2, which involves trapping an imine (or iminium) intermediate.[26] The regioselectivity is dependent upon the electron withdrawing or releasing properties of the groups attached to both the amino and the carboxyl group of the dehydroalanine residue. Since enzymes have the potential to delicately 'tune' the electronic properties of each of the carboxamide moieties in the *N*-acyldehydroalanyl peptide at their active site, there are two chemically feasible pathways to covalent intermediates for the ammonia-lyase reactions (Scheme 3). Regardless of the exact regiochemistry, any such covalent adduct formation between the substrate and the enzyme would involve extra ^{15}N-sensitive transition states which would complicate the interpretation of $^{15}(V/K)$ isotope effects[21] (see also following article[27]).

Here, and in the following article, we develop a unified model for the mechanism of methylaspartase and describe the results of solvent deuterium isotope effect and ^{15}N-fractionation experiments designed to detect chemical steps that might be involved in forming a covalent enzyme–substrate complex. It is shown that the methylaspartase reaction is extremely complicated because the enzyme releases its products very late and that large reverse reaction commitments dominate the kinetics of the system. It is nevertheless demonstrated that a covalent intermediate complex exists and that the chemical mechanism for the deamination of the physiological substrate, (2*S*,3*S*)-3-methylaspartic acid, is concerted. It is shown that the thiolate anion of a Cys residue serves as the base which removes the C-3 hydrogen. We also present the results of chemical modification studies designed to elucidate details of the active-site structure, identify the Cys residue which serves as a base and discover whether the dehydroalanine moiety is protected or unprotected from exogenous nucleophiles in the resting state of the enzyme.

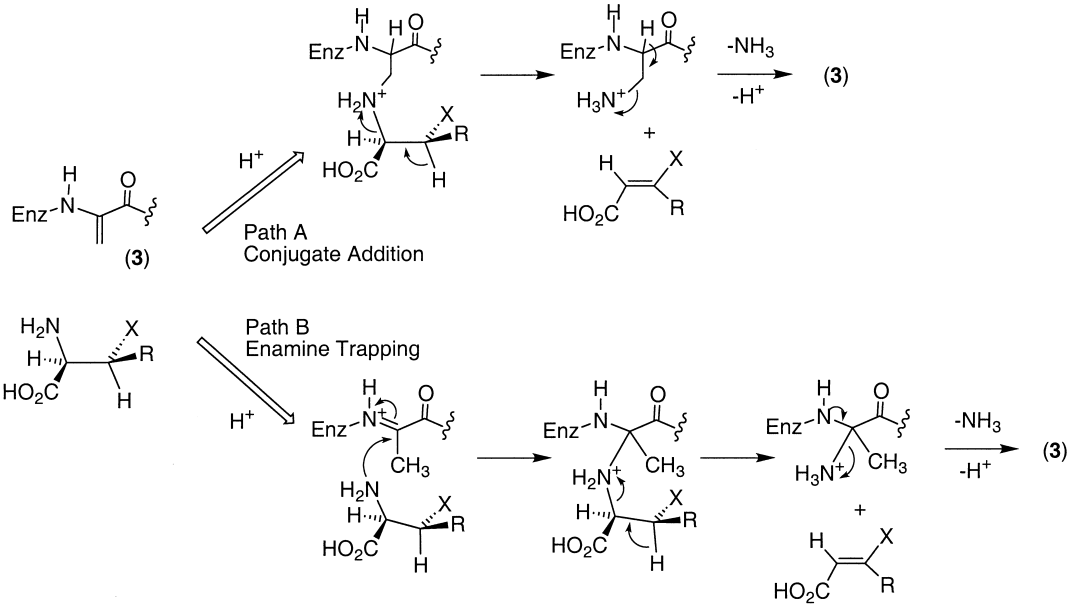
Results

Deuterium and solvent isotope effects for deamination

(2*S*,3*S*)-3-Methylaspartic acid. The kinetic parameters K_m (K_{Measp}), V_{max} and V/K for unlabelled and 3-deuteriated



Scheme 2.



Scheme 3.

(2*S*,3*S*)-3-methylaspartic acid for the deamination reaction performed in water and deuterium oxide at pL 6.5, 7.6, 9.0 and 9.4 are shown in Table 1. The derived isotope effects are also given for each study pL value. The parameter x represents V or V/K and leading superscripts refer to the varied label; D represents substrate C-3 deuteriation and S represents deuteriated solvent. Trailing subscripts refer to the presence of other non-varied labelled sites. The primary deuterium isotope effects for V and V/K, ^{D}V and ^{D}(V/K), respectively, are not pH sensitive and are similar in value at ~1.7 (Table 1). This indicates that C-3 hydrogen abstraction is kinetically important across the pL range of the study. The solvent deuterium isotope effects ^{S}V and ^{S}(V/K), respectively, are not similar in value to each other and increase in magnitude with increasing pL. The maximum value of ^{S}(V/K) is 3.5 (Fig. 1) and this occurs at pL 9.0 which corresponds to the optimum pH for V/K (Fig. 2). The maximum value of ^{S}V is 3.4 and this occurs at pL 9.4 which corresponds to the optimum pH for V (Figs 1 and 2).

Table 1. Primary deuterium and solvent isotope effects for the deamination of (2*S*,3*S*)-3-methylaspartic acid

	pL 6.5			pL 7.6			pL 9.0			pL 9.4			pL 10.0		
Solvent, substrate	V[a]	K[a]	V/K	V	K	V/K	V	K	V/K	V	K	V/K	V	K	V/K
1. H_2O, H-MeAsp	7.51	2.3	3.27	70.1	2.4	29.2	654	2.4	277	850	6.5	130	850	9.0	94.4
2. H_2O, D-MeAsp	4.42	2.3	1.92	41.1	2.4	17.1	385	2.4	164	434	6.5	68	491	9.0	54.6
3. D_2O, H-MeAsp	4.95	1.5	3.34	56.2	1.9	29.6	210	2.6	80.8	247	2.6	96	nd		
4. D_2O, D-MeAsp	nd			nd			194	2.2	88.2	191	2.3	83	nd		
Isotope effects	V	V/K		V	V/K		V	V/K		V	V/K		V	V/K	
Entry 1/2 $^{D}(x)$	1.70	1.70		1.71	1.71		1.70	1.69		1.95	1.95		1.73	1.73	
Entry 3/4 $^{D}(x)_S$							1.08	0.91		1.29	1.15				
Entry 1/3 $^{S}(x)$	1.52	0.98		1.25	0.99		3.11	3.46		3.44	1.35				
Entry 2/4 $^{S}(x)_D$							1.95	1.85		2.27	0.82				
Entry 1/4 $^{SD}(x)$							3.38	3.10		4.45	1.56				

[a]Errors in V and K are ±10% or less.

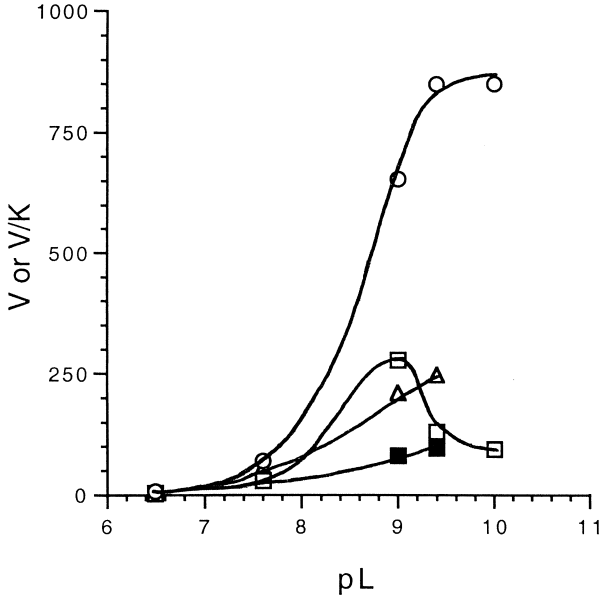


Figure 1. Dependence of the kinetic parameters V and V/K on pL for the deamination of (2*S*,3*S*)-3-methylaspartic acid in water and in deuterium oxide. V-H_2O (○), (V/K)-H_2O (□), V-D_2O (△), (V/K)-D_2O (■). Plots are drawn free-hand.

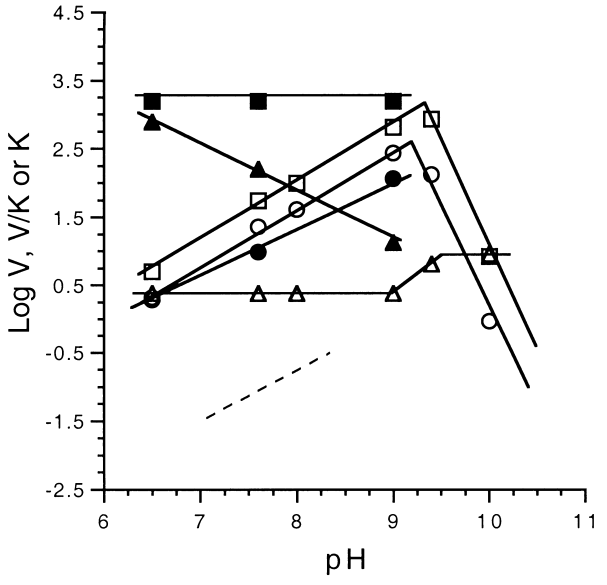


Figure 2. Non-filled dependence of the kinetic parameters for (2*S*,3*S*)-3-methylasparic acid and potassium ion on pH as a logarithm plot. V (□), V/K (○), K (△), V_K (■), $(V/K)_K$ (●), K_K (▲). Unit slope (- - - -) is also shown.

At pL 6.5 and 7.6, ^{S}V and $^{S}(V/K)$ are small, 1.5 or less and 1.0, respectively, indicating that the solvent deuterium transfer step(s) that are rate limiting at pL 9.0–9.4 are apparently not so important compared to C-3–H bond cleavage at low pL.‡

At pL 9.0 and 9.4, the combined primary deuterium and solvent isotope effects upon V and V/K, ^{SD}V and $^{SD}(V/K)$ respectively, are very similar to the values for ^{S}V and $^{S}(V/K)$. Examined in a different way this analysis indicates that the primary deuterium isotope effects in deuteriated solvent, $^{D}V_S$ and $^{D}(V/K)_S$, respectively, are suppressed compared to those obtained in water. Indeed, $^{D}V_S$ and $^{D}(V/K)_S$ are very close to unity at pL 9.0 and 9.4. The solvent isotope effects for the deuteriated substrate $^{S}V_D$ and $^{S}(V/K)_D$ are also suppressed compared to those for the unlabelled material, ^{S}V and $^{S}(V/K)$, again indicating that the primary deuterium and solvent isotope effects operate on different rate limiting steps in the deamination process.

‡Note that the pL independent solvent isotope effects are 2.65 for ^{S}V and 2.90 for $^{S}(V/K)$ and that the pL profile in deuteriated solvent is shifted to higher pL by ca. 0.4 units.

(2*S*)-Aspartic acid

The kinetic parameters K_m (K_{Asp}), V_{max} and V/K for unlabelled and 3-deuteriated (2*S*,3*R*)-[3-$^{2}H_1$]-aspartic acid for the deamination reaction performed in water and deuterium oxide at pL 9.0 and 9.4 are shown in Table 2. The derived isotope effects are also given and these are large compared to the effects observed for the natural substrate. The pH dependencies of the kinetic parameters for (2*S*)-aspartic acid over the range from 7.6 to 10.0 are shown in Figure 3.

Table 2. Primary deuterium and solvent isotope effects for the deamination of (2*S*)-aspartic acid

	pL 9.0			pL 9.4		
Solvent, substrate	V[a]	K[a]	V/K	V	K	V/K
1. H_2O, H-Asp	4.80	10.5	0.46	5.07	15.6	0.33
2. H_2O, D-Asp	4.80	10.5	0.46	4.73	13.0	0.36
3. D_2O, H-Asp	2.81	37.9	0.074	2.40	23.0	0.104
4. D_2O, D-Asp	1.41	32.9	0.043	1.18	27.8	0.043
Isotope effects	V		V/K	V		V/K
Entry 1/2 $^{D}(x)$	1.0		1.0	1.07		0.92
Entry 3/4 $^{D}(x)_S$	2.0		1.7	2.03		2.4
Entry 1/3 $^{S}(x)$	1.71		6.18	2.11		3.17
Entry 2/4 $^{S}(x)_D$	3.4		10.6	4.3		7.7
Entry 1/4 $^{SD}(x)$	3.4		10.6	4.0		8.4

[a]Errors in V and K are ±14% or less.

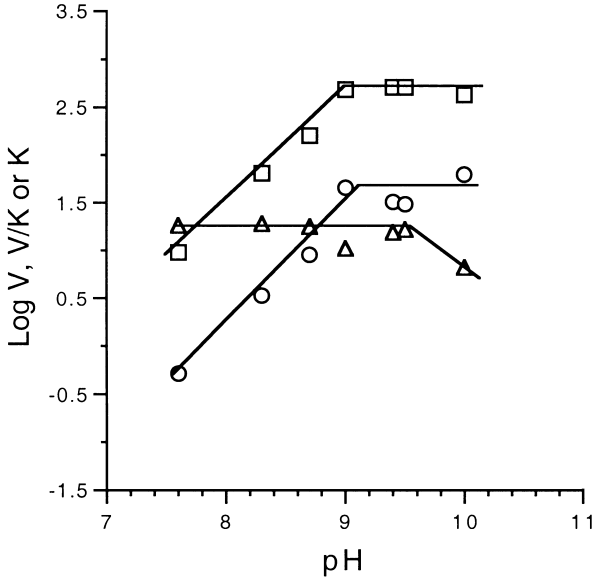


Figure 3. Non-filled dependence of the kinetic parameters for (2*S*)-aspartic acid on pH as a logarithm plot. V (□), V/K (○), K (Δ).

C-3 Hydrogen exchange

Experiments were conducted to simultaneously, in the same experiment, measure the rate of deamination and C-3 solvent tritium exchange into (2*S*)-aspartic acid and (2*S*,3*R*)-[3-$^2H_1$]-aspartic acid at pH 9.0. The rates of deamination and exchange were similar for each substrate and the ratio v_{Ex}/v_{Deam} was 16.5 for the unlabelled material and 15.1 for (2*S*,3*R*)-[3-$^2H_1$]-aspartic acid. The findings indicate that the exchange reaction is much faster than for the natural substrate (see discussion)[19] and that, for aspartic acid, neither the deamination or the exchange reaction display a primary deuterium isotope effect.

(2*S*,3*R*)-3-Methylaspartic acid

The kinetic parameters and the primary deuterium isotope effects at pH 9.0 at 1 mM and at 50 mM K^+ ion are given in Table 3. At 1 mM K^+ ion $^{D}V = 7.15$ and $^{D}(V/K) = 3.39$; and, at 50 mM K^+ ion $^{D}V = 6.79$ and $^{D}(V/K) = 4.10$ indicating that there are very large primary deuterium isotope effects for the *syn*-elimination.[6]

Solvent isotope effects for amination

The kinetic parameters K_m (K_{Mesa}), V_{max} and V/K for the amination of mesaconic acid performed in water and deuterium oxide at pL 6.5, 9.0 and 9.4 are shown in Table 4. The derived isotope effects are also given for each study pL value. Since these reflect both the solvent isotope effects and the C-3 deuterium isotope effect for forming the deuteriated natural substrate, these are labelled $^{SD}V_{Rev}$ and $^{SD}(V/K)_{Rev}$ and can be compared with ^{SD}V and $^{SD}(V/K)$ in Table 1. As for the corresponding effects in the deamination reaction direction, at high pL both $^{SD}V_{Rev}$ and $^{SD}(V/K)_{Rev}$ are significant. The value of $^{SD}V_{Rev}$ is 6.7 and maximal at pL 9.4 and the value of $^{SD}(V/K)_{Rev}$ is 1.9 and maximal at pL 9.0. At lower pL the magnitude of both isotope effects diminishes to give values similar to those corresponding to the primary deuterium isotope effects for the forward reaction, ^{D}V and $^{D}(V/K)$ in Table 1.

The kinetic parameters K_m (K_{Fum}), V_{max} and V/K for the amination of fumaric acid performed in water and deuterium oxide at pL 9.0 and 9.4 are shown in Table 5.

Covalent modification of methylaspartase

Inhibition by hydrazines. The relative rates of the inactivation of methylaspartase at pH 8.0 in Tris buffer by various hydrazines are shown in Figure 4 under the specified conditions. Phenylhydrazine, methylhydrazine and 4-nitrophenylhydrazine are the most effective irreversible inhibitors. Complete protection from inactivation is afforded by 2 mM (2*S*,3*S*)-3-methylaspartic acid. Sodium borohydride, sodium cyanide, phenylphosphine and nitromethane are ineffective as irreversible inhibitors. The presence of magnesium ions (0.4 mM) increased the rate of inactivation by 4-nitro- and 2,4-dinitro-phenylhydrazine, but had little effect on the rate of inactivation by phenylhydrazine. Potassium ions (250 mM) and ammonium ions (10 and 20 mM) had no effect on the rate of the inhibition of the enzyme by phenylhydrazine at pH 9.0 in 50 mM Tris buffer (data not shown), but the rate of inactivation was faster than at pH 8.0. At pH 9.0 the inactivation reaction showed saturation kinetics although the inactivation itself was biphasic (Fig. 5). The value of K_S calculated from the initial faster phase was approximately 2.13 mM.

The pH-dependence of the rate for the initial phase of the inactivation showed increases in log rate versus pH of greater than unit slope (Fig. 6) indicating that more than one species must be deprotonated for inactivation to occur.

Inhibition by *N*-ethylmaleimide. Treatment of the enzyme with NEM irreversibly inactivated the enzyme and gave simple first-order kinetics (Fig. 7). The rate of reaction showed saturation kinetics at pH 9.0. K_S for

Table 3. Primary deuterium effects for the deamination of (2*S*,3*R*)-3-methylaspartic acid at pH 9.0

	pH 9.0, 1.0 mM K^+			pH 9.0, 50.0 mM K^+		
Solvent, substrate	V	K	V/K	V	K	V/K
1. H_2O, H-MeAsp	17.2 ± 0.3	40 ± 0.8	0.43	41.1 ± 4.0	5.2 ± 1.1	7.90
2. H_2O, D-MeAsp	2.4 ± 0.78	18.8 ± 8.4	0.13	6.1 ± 0.23	3.14 ± 0.29	1.93
Isotope effects	V	V/K		V	V/K	
Entry 1/2 $^{D}(x)$	7.15 ± 2.74	3.39 ± 1.6		6.79 ± 0.92	4.10 ± 1.3	

Table 4. Solvent isotope effects for the amination of mesaconic acid in the presence of 0.4 M ammonium chloride

	pL 6.5			pL 9.0			pL 9.4		
Solvent	V[a]	K[a]	V/K	V	K	V/K	V	K	V/K
1. H_2O	8.18	1.18	6.93	894	1.24	721	492	1.87	263
2. D_2O	4.25	0.78	5.48	326	1.10	297	73.7	0.36	205
Isotope effect	V	V/K		V	V/K		V	V/K	
Entry 1/2 $^{SD}(x)_{Rev}$	1.93	1.27		2.12	1.88		6.70	1.30	

[a]Errors in V and K are ± 10% or less.

Table 5. Solvent isotope effects for the amination of fumaric acid in the presence of 0.4 M ammonium chloride

	pL 9.0			pL 9.4		
Solvent	V[a]	K[a]	V/K	V	K	V/K
1. H_2O	1702	23.2	73.4	1565	2.96	524
2. D_2O	205	5.95	34.5	479	2.78	172
Isotope effect	V	V/K		V	V/K	
Entry 1/2 $^{SD}(x)_{Rev}$	8.32	2.13		3.27	3.04	

[a]Errors in V and K are ± 10% or less.

NEM was determined to be 34.5 ± 5.4 mM at pH 7.0 in the presence of 5 mM sodium phosphate where k_{Inact} was 5.0 ± 0.6 min^{-1}. Phosphate served as a linear competitive inhibitor for the inactivation reaction and displayed a K_I value of 1.5 mM in the presence of 0.5 mM NEM at pH 7.0. Acetate had no protective effect. Mg^{2+} ions served as an activator for the irreversible inhibition of the enzyme and at saturating concentrations increased the rate by ~10-fold. The activation by Mg^{2+} ions showed simple saturation kinetics (after correction for the non-catalysed inactivation by NEM), and K_{Mg} was 117 ± 22 μM. Mg^{2+} ions enhanced the affinity of the enzyme for NEM by 10-fold and at saturating concentrations of Mg^{2+} ions K_S for NEM was decreased to 2.76 ± 0.37 mM and k_{Inact} was 3.31 ± 0.68 min^{-1}. Thus, the increase in activity for NEM as an inhibitor in the presence of Mg^{2+} ions results largely from its increased affinity for the enzyme and not from an increase in the rate of alkylation.

(2*S*,3*S*)-3-Methylaspartic acid and mesaconic acid protected the enzyme against inactivation by NEM. At pH 7.0 the values of K_I for the inhibition of the inactivation reaction by the two substrates were 0.6 ± 0.1 mM and 0.5 ± 0.1 mM, respectively. The K_I value of mesaconic acid was not altered by the presence of Mg^{2+} ions.

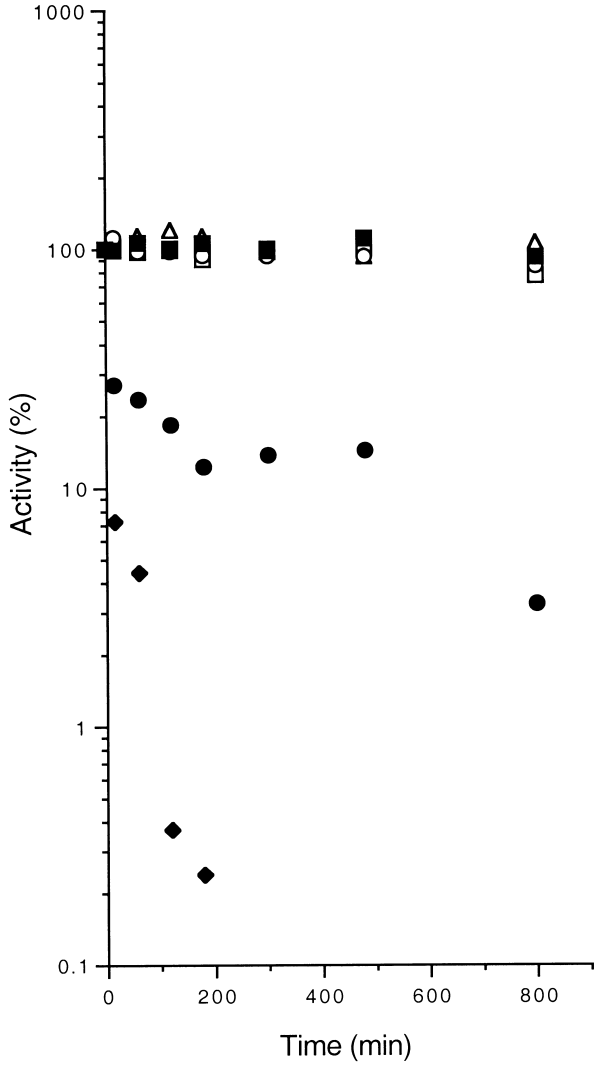


Figure 4. Time course for the inhibition of methylaspartase by phenylhydrazine (◆), 4-nitrophenylhydrazine (●), 2,4-dinitrophenylhydrazine (○), 3,6-dioxaoctane dioic acid dihydrazide (Δ), acetylhydrazine (□) and a control with no inhibitor (■).

Potassium ions protected the NEM alkylation reaction and K_S for K^+ at pH 9.0 was 4.89 ± 1.26 mM. Ammonium ion was less effective at protecting the enzyme and K_S was 10.17 ± 3.68 mM.

The pH-dependence of the inactivation rate is shown in Figure 8. The enzyme itself tritrates at pH 9.3 to a form that reacts rapidly with NEM, since NEM cannot deprotonate under the conditions of the experiment.

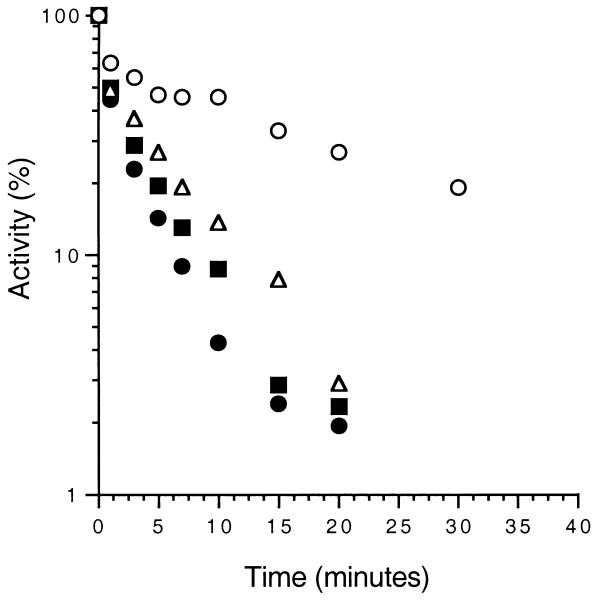


Figure 5. Time course for the inhibition of methylaspartase by phenylhydrazine: 2.5 mM (○), 10 mM (△), 15 mM (■) and 20 mM (●).

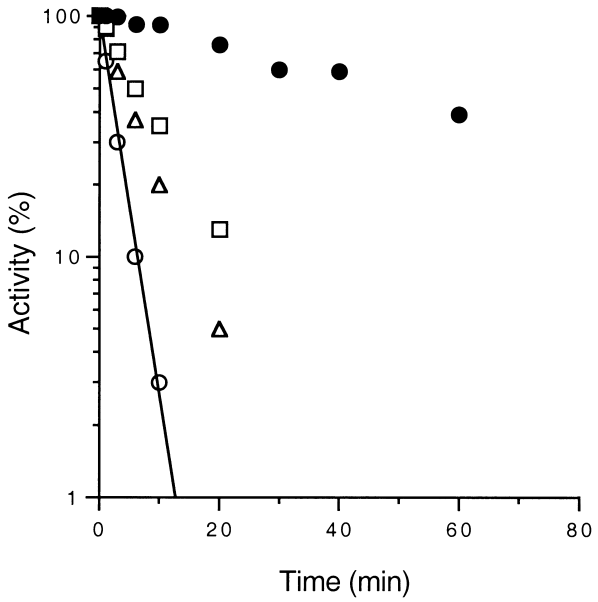


Figure 7. Time course for the inhibition of methylaspartase by *N*-ethylmaleimide: 0.05 mM (●), 0.25 mM (□), 0.5 mM (△), 1.0 mM (○).

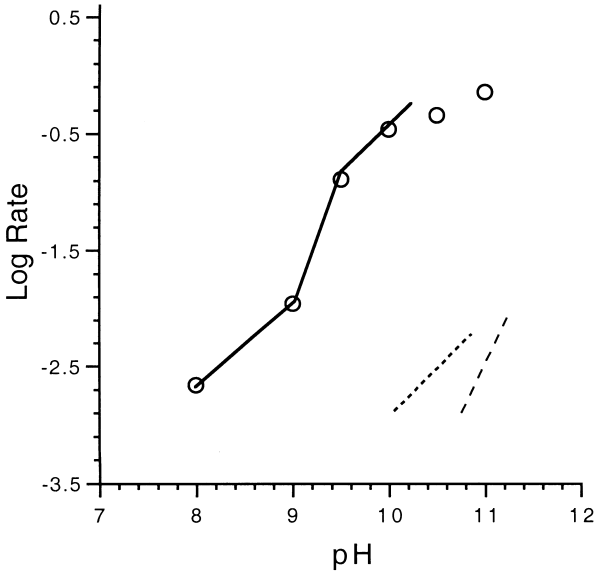


Figure 6. Dependence of the inhibition of methylaspartase by phenylhydrazine (○) on pH as a logarithm plot and unit slope (----).

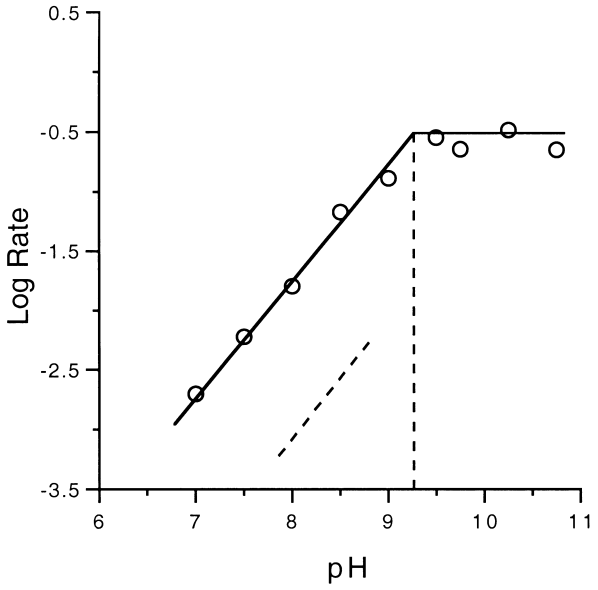


Figure 8. Dependence of the inhibition of methylaspartase by *N*-ethylmaleimide (○) on pH as a logarithm plot and unit slope (----).

Proteolysis with trypsin

The sites of modification by both *N*-ethylmaleimide and 2,4-dinitrophenylhydrazine were investigated by a series of peptide mapping experiments. For *N*-ethylmaleimide the enzyme was protected with substrate and was then treated with unlabelled inhibitor under the specified conditions. Following repeated dialysis steps, to remove substrate and excess unreacted *N*-ethylmaleimide, the protein (which had retained >95% of the original activity) was incubated with *N*-[2-^{3}H]-ethylmaleimide. All activity was lost after 60 min. The inactive protein was then subjected to repeated dialysis and treated with the endoproteinase enzyme trypsin (which is known to cleave the peptide chain at the C-terminus of lysine and arginine residues) at pH 7.8 for 4 h at room temperature (see Experimental for full details). The resulting peptide mixture was subsequently lyophilised to afford a white powder in preparation for analysis by HPLC. In the case of 2,4-dinitrophenylhydrazine modification, the native enzyme was incubated with the inhibitor for 45 h, after which time all of the activity had been lost. The inactive protein was subjected to repeated dialysis and was then treated with trypsin using the procedure outlined above.

HPLC Analysis of tryptic peptides

Unmodified methylaspartase. In order to compare directly the HPLC elution profiles of modified methylaspartase with the native recombinant protein (see Experimental), unmodified enzyme was digested with trypsin and the peptide mixture was analysed by reversed-phase HPLC using a 10 R2 POROS column (Fig. 9). A number of peptides were isolated and were subjected to N-terminal amino acid analysis by Edman degradation. The sequences for all of the isolated peptides were matched with the deduced amino acid sequence[5] and will be reported on in full elsewhere. The retention time of peptides corresponding to the phenylhydrazine and NEM modified peptide sequences described below were noted.

Methylaspartase labelled with 2,4-dinitrophenylhydrazine. The peptide mixture resulting from tryptic digestion of 2,4-dinitrophenylhydrazine modified protein was analysed by reversed phase HPLC, using a 10 R2 POROS column (100×4 mM). The eluent was monitored directly by UV spectroscopy at 220 and 360 nm. The elution profile (Fig. 10) showed the presence of ca. 25 resolved peaks possessing a UV absorbance at 220 nm and one major peak possessing an absorbance at 360 nm with a retention volume of 12.4 column volumes. The material giving rise to the peak was collected and was further purified by a repeat HPLC step. In this case the peak absorbing at 360 nm coincided exactly with a peak absorbing at 220 nm of retention volume 5.8 column volumes. A final HPLC purification step using a microbore system (100×1 mM column) resulted in an elution profile comprising two peaks absorbing at 360 nm. Both were isolated and the first five residues of the N-terminal sequence were determined. The peak which eluted late in the gradient did not exhibit any amino acid sequence, and was evidently not a peptide. The single peak with a lower retention time was subjected to 18 cycles of N-terminal analysis by Edman degradation. Three peptide sequences were recorded consistent with the bis-disulfide tripeptide composed of SAEVTTNIGMACGAR, ANGMGAYCGGTCNETNR, and GVDAELVADEWCNTVEDVK. Presumably the ANG peptide formed one disulfide bridge with each of its two Cys residues to the single Cys residue in each of the other peptides. Note no such oxidised peptides were isolated in digests of enzyme that had not been treated with hydrazines.

Methylaspartase labelled with *N*-[2-^{3}H]-ethylmaleimide. The HPLC trace for the peptide mixture resulting from tryptic digestion of *N*-ethylmaleimide modified protein (Fig. 11) showed the presence of ca. 50 UV-absorbing peaks, consistent with the 52 anticipated peptides.[5] In addition, one major peak displaying radioactivity was observed with a retention volume of 7.3 ± 0.3 column volumes. Fractions associated with this radioactivity were pooled, concentrated under reduced pressure and then subjected to a second HPLC purification step (Fig. 11, inset). In this case just four UV-absorbing peaks and a single peak possessing radioactivity were observed. The radioactive peak coincided with a UV-absorbing peak possessing a retention volume 7.3 ± 0.3 column volumes. The material giving rise to the peak was collected and was subjected to Edman N-terminal amino

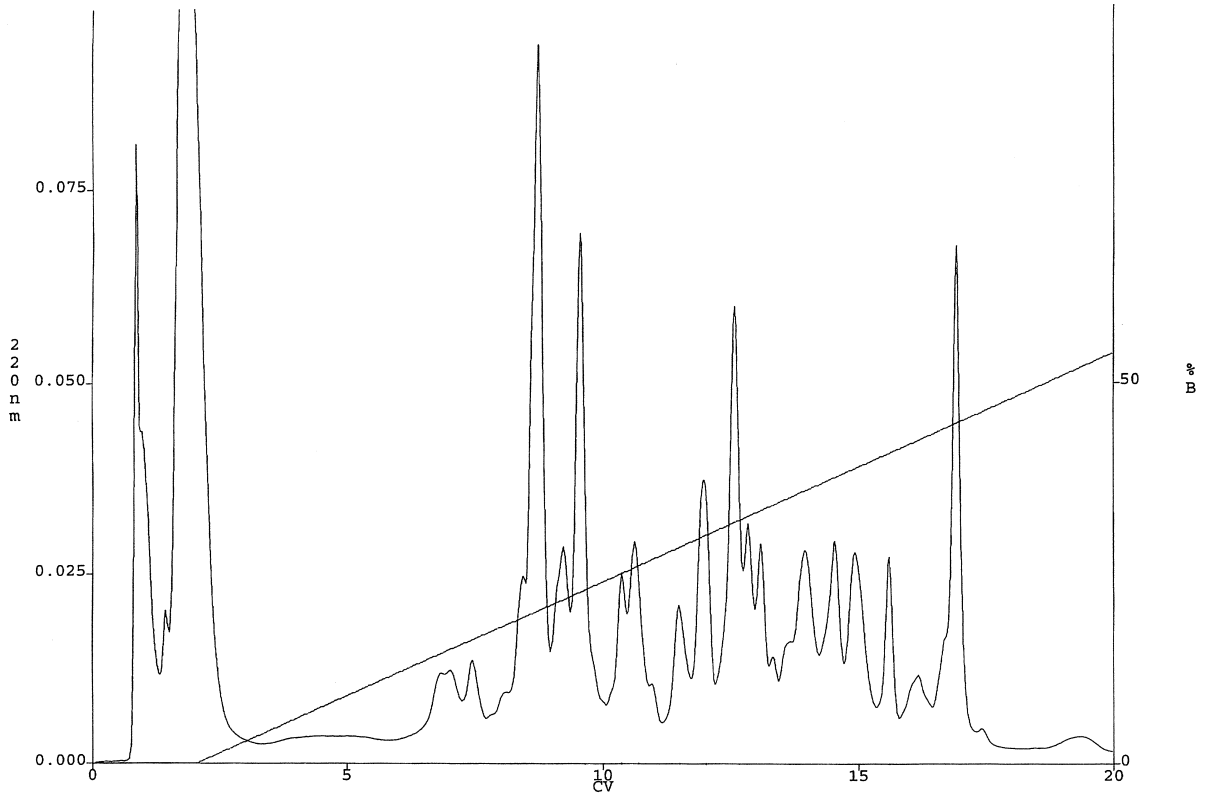


Figure 9. HPLC chromatograph (10 R2 POROS column) for the tryptic digest of native methylaspartase.

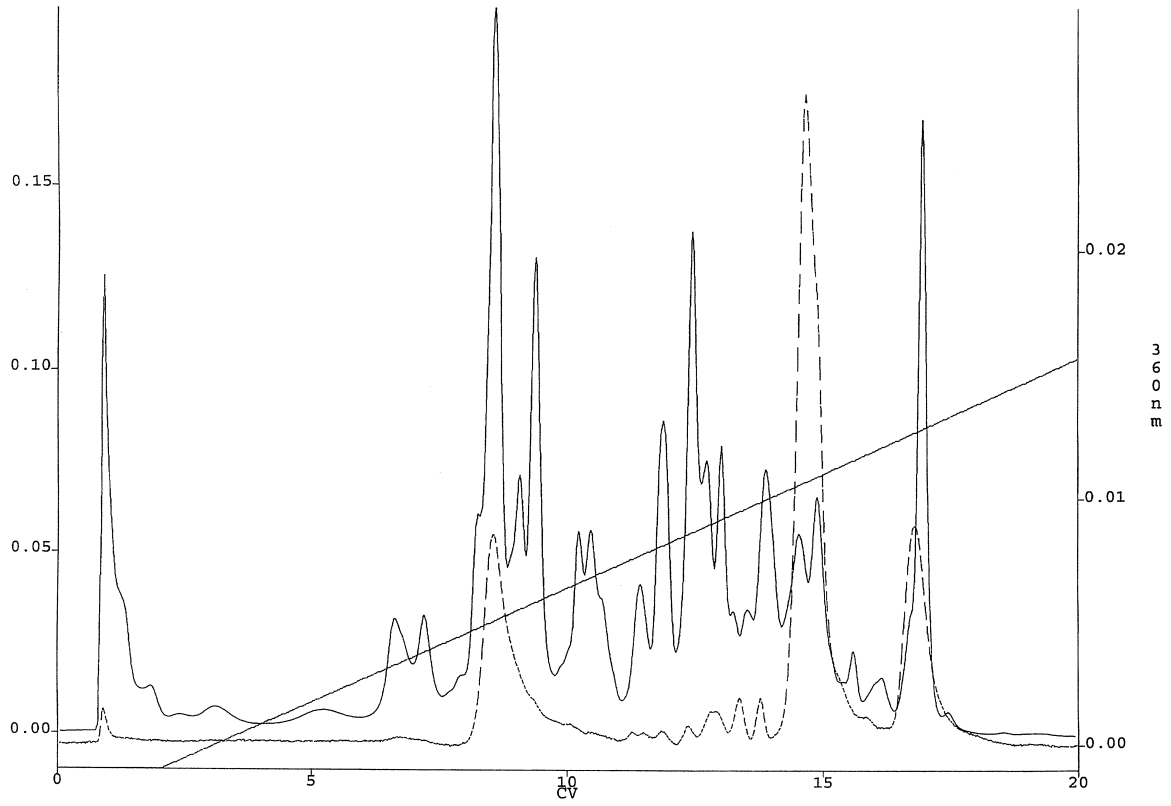


Figure 10. HPLC chromatograph (10 R2 POROS column) for the tryptic digest of 2,4-dinitrophenylhydrazine inhibited methylaspartase. Detection at 220 nM (——) and 360 nM (- - - -).

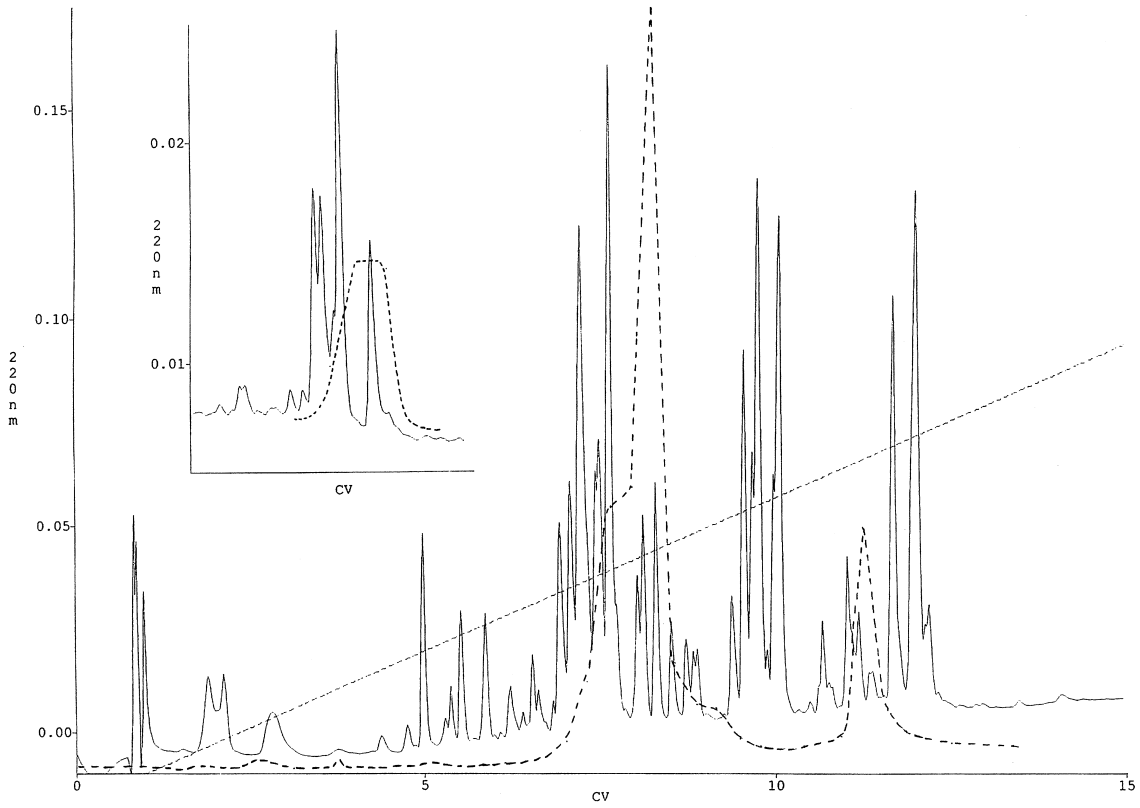


Figure 11. HPLC chromatograph (C^{18} column) for the tryptic digest of *N*-ethylmaleimide inhibited methylaspartase. Detection at 220 nm (——), ^{3}H content of fraction (- - - -). Inset chromatograph shows the further HPLC purification of the major peak in radioactivity.

acid sequencing. Aliquots of each sequencing cycle were retained in order to determine the ^{3}H-content of individual residues. A single peptide sequence ANGMG AYXGGTZNETNR was observed. Cycle 8 (X) showed no HPLC elution profile, consistent with a Cys residue and cycle 12 (*Z*) gave a radioactive eluent and showed two bands in the HPLC trace of the Edman derivative running between Pro and Val, indicative of a modified amino acid. Note that the published sequence of the enzyme contains Cys residues at positions 8 and 12 in the only peptide commencing with ANG.[5]

Discussion

Before commencing to attempt to interpret the isotope effect data it is necessary to construct a model for the catalytic process. Early investigations of the mechanism of the L-*threo*-methylaspartase reaction by Bright and co-workers indicated that the enzyme operated via a carbanionic elimination mechanism[8,9,28–31] (see Introduction). However, work in our own laboratory revealed that there is a significant primary deuterium isotope effect for the deamination reaction with (2*S*,3*S*)-3-methylaspartic acid as the substrate,[18] contrary to the earlier reports. It was also demonstrated that at the high concentrations of K^+ ion used in Bright's original experiments the observed isotope effects were suppressed by increasing the forward and reverse reaction commitments.[19] This work also showed that the exchange of the C-3 hydrogen of the substrate with solvent derived hydrogen did not occur at the level of the putative carbanion but after a slow step or steps involving both C-3 hydrogen and C-2 nitrogen bond cleavage.

Product debinding order

Using unlabelled substrate and the labelled products, [^{15}N]-ammonia and [methyl-$^{2}H_3$]-mesaconic acid, it was shown that the products were released from the enzyme in an apparently random fashion and that neither of the species could be trapped on the enzyme and converted back, through reverse steps, to labelled substrates.[19] The use of methylamine as an inhibitor also suggested that product release occurred in a random fashion. However, ammonia itself (in the presence of high concentrations of potassium ion) acted as a noncompetitive product inhibitor at high concentration, indicating that it is released before mesaconic acid, and as a nonlinear uncompetitive inhibitor at lower concentration (Fig. 12), indicating that more than one molecule can bind to the same enzyme species. If ammonia or ammonium ion can bind to an intermediate enzyme complex in a site other than the product or activator site, as appears to be the case (see Fig. 12 and the discussion below), then exogenous labelled ammonium ion would not, necessarily, be able to trap mesaconic acid on the enzyme. Since the effects of ammonium ion are further complicated by the fact that it can serve as a cationic activator (as a surrogate for potassium ion), the debinding order is difficult to refine further. Nevertheless, it seems certain that either product release is random, or, that ammonium ion is released first. This situation is very different to that which has been ascribed for phenylalanine ammonia-lyase where the product, cinnamate, can dissociate from the enzyme before ammonia is released from the covalent amino–enzyme complex.[13] In the analysis below we shall assume that mesaconic acid cannot escape before ammonium ion. In the light of the suggestion that the enzyme operates via the intermediacy of a dehydroalanine residue[5] (see below), several potential sites for the addition of a second ammonia molecule to an enzyme–intermediate complex become apparent. For example, ammonia might react directly with the enzyme to give a covalent complex which is still able to bind to the substrate. Some evidence to support this idea is offered by the finding that the transition state inhibitor, (1*S*,2*S*)-1-methylcyclopropane-1,2-dicarboxylic acid (**4**) was a good inhibitor ($K_i = 20\,\mu M$) for the enzyme, but was even more potent in the presence of ammonium ion.[32] Thus, there is clearly enough room for extra heavy atoms (Scheme 4). Alternatively, ammonium ion may be able to trap a covalent enzyme–substrate complex that does not contain a bound Mg^{2+} ion. Such complexes would cause dead-end inhibition and the importance of the covalent interactions are discussed below.

Active site dehydroalanine moiety

At the time of the original work on the metal ion and product binding and debinding orders,[19] we had not completed a primary structural analysis of the enzyme. When the deduced amino acid sequence of methylaspartase became available,[5] it was possible to compare the sequence with the known amino acid composition of isolated active site tryptic peptides that had been labelled with [^{14}C]-*N*-ethylmaleimide.[33] From this comparison it appeared that a serine residue (or its post-translationally modified product) rather than a cystein residue, as had been originally proposed, had reacted with the electrophilic species. In Wu and Williams' original experiments, the enzyme was treated with unlabelled *N*-ethylmaleimide in the presence of substrate and dialysis of the protein solution gave an active chemically modified enzyme.[33] When this modified enzyme was treated with [^{14}C]-*N*-ethylmaleimide the resulting protein was completely inactive. Treatment of this labelled protein with trypsin gave a single active site labelled peptide (a ca. 40-mer) which corresponded to one of the eight uniformly labelled peptides that had been obtained earlier by the same workers by treating the enzyme in the absence of substrate with [^{14}C]-*N*-ethylmaleimide.[33] Note that there are only seven Cys residues in native methylaspartase,[5] and presumably each of these had been covalently modified, to give the *S*-succinimide derivatives in the active modified enzyme referred to above. We suggested that the eighth peptide obtained was a 37-mer generated by tryptic cleavage at Arg-146 and Lys-183 (see Table 6), because it was the only single peptide sequence that displayed a close match to the published amino acid composition. However, we will show below that the Wu and Williams' peptide[33] may have possessed a more complex structure.

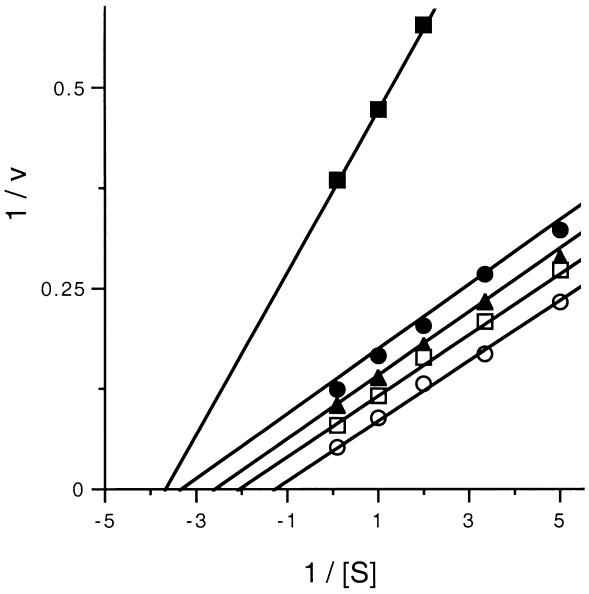
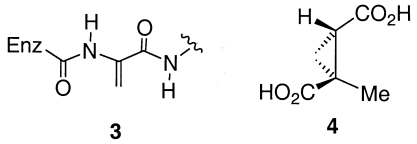
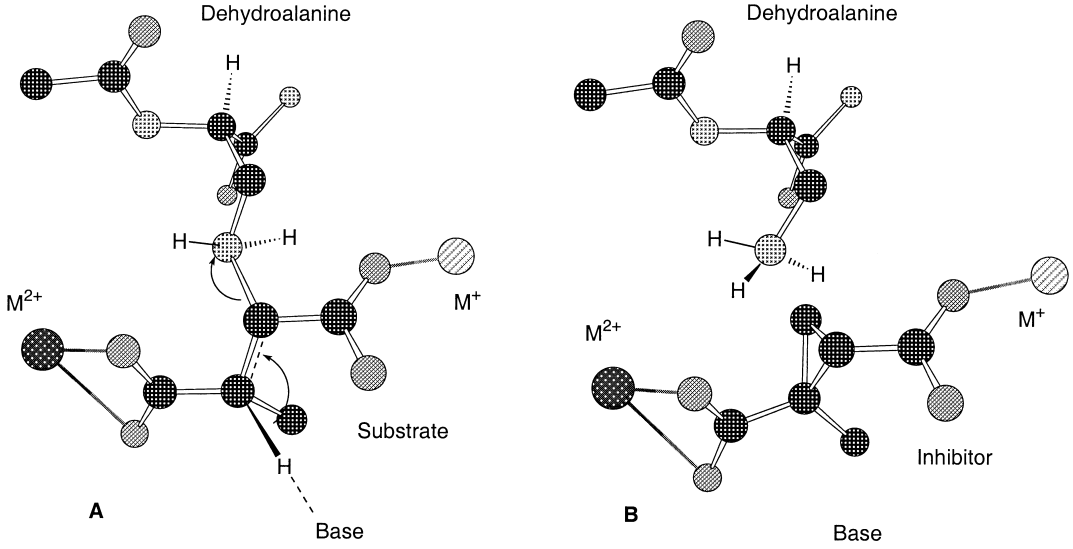


Figure 12. Ammonia inhibition of the deamination reaction at 50 mM potassium ion concentration. Incubations contained; 0.5 M Tris (pH 9.0), 20 mM $MgCl_2$, (2*S*,3*S*)-3-methylaspartic acid and ammonium chloride at (a) zero; (b) 10 mM; (c) 50 mM, and (d) 400 mM concentrations. Reactions carried out at 30.0 ± 0.1 °C and are corrected for one unit of enzyme.





Scheme 4. (A) Expected transition state for C–N bond cleavage in the substrate. (B) Possible interaction of (1*S*,2*S*)-1-methyl-1,2-cyclopropanedioic acid (**4**) with the enzyme.

As was suggested previously[5] to understand why the product of the serine codon should react with *N*-ethylmaleimide[33] is not easy if serine is the final product given that the β-hydroxyl group of serine does not usually react with Michael acceptors to give conjugate addition products. To accommodate the observation it was proposed that a dehydroalanine residue could be formed and then undergo reaction with the amino group of the substrate to give a covalent enzyme–substrate adduct.[5] The elimination of the 3-amino group of the 2,3-diaminopropanoic acid moiety from the substrate would then give mesaconic acid and the amino-

Table 6. Amino acid composition for peptides derived from 3-methylaspartase. (A) Determined by acid hydrolysis from the purified peptide isolated from *N*-[2-^{14}C]-ethylmaleimide inhibited enzyme by Wu and Williams.[33] (B) Deduced from the inferred amino acid sequence of the enzyme for the Val-148-Lys-183 peptide.[5] (C) Determined by N-terminal amino acid sequencing of the peptide isolated from *N*-[2-^{3}H]-ethylmaleimide-inhibited enzyme in this study. (D) Amino acid content of the putative bis-oligo-peptide thioether formed by cross-linking Ala-350-Arg-366 (column B) and Asp-156-Lys-177 (the smallest tryptic peptide containing Ser-173) via Ser-173 and Cys-357. The numbers in brackets refer to the expected outcome of an amino acid analysis of the bis-oligo-peptide, see column A

Amino acid	A	B	C	D
A	5	4	2	5
R	2	2	1	2
N + D	8	8	3	8
C	1	0	2	2 (1)
Q + E	4	4	1	3
G	8	2	4	6
H	0	0	0	0
I	2	2	0	1
L	1	0	0	0
K	2	2	0	0 (2)
M	1	1	1	1
F	1	1	0	1
P	2	2	0	2
S	0	1	0	1 (0)
T	1	1	2	2
W	0	0	0	0
Y	1	2	1	2
V	3	5	0	2

enzyme. While we have shown that in the native enzyme the amino-enzyme cannot be trapped,[19] the same does not necessarily apply to the active unlabelled NEM-modified enzyme which was treated with labelled *N*-ethylmaleimide by Wu and Williams.[33] In an alternative mechanism, it is not unreasonable to believe that the dehydroalanine moiety could act as an enimine and, therefore, as a nucleophile at C-3 and react with NEM to form a C−C bond to label the protein. Indeed, since many Cys residues did react with unlabelled NEM, it is reasonable to expect that the ability of the modified enzyme to deaminate itself would be impaired compared to the native enzyme. Thus, the amino-enzyme form might be able to exist long enough to react with electrophiles such as NEM. In accord with these ideas, chemical model studies have shown that the addition of amines including the dimethyl ester of (2*S*,3*S*)-3-methyl-aspartic acid to various *N*-acyl dehydroalanine esters and amides is quite rapid at 30 °C and occurs at C-3 or C-2 depending on the electronic properties of the protecting groups.[26] It is also established that N^{2}-protected 2,3-diaminopropanoic acid amides react with *N*-methylmaleimide to give the conjugate addition products.[34] Thus, there is now ample evidence to support the proposals that dehydroalanine residues in proteins could serve as electrophilic amine group acceptor moieties as has been proposed for ammonia-lyase enzymes for many years[22,23] (see below).

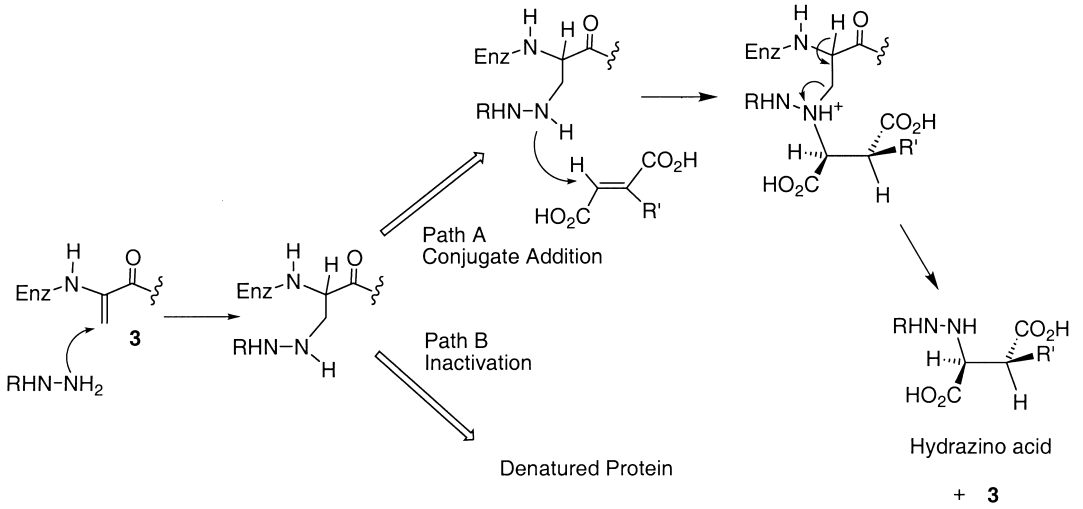
Support for the existence of a dehydroalanine residue at the active site of methylaspartase is also provided by the finding that aryl and alkyl hydrazines are potent irreversible inhibitors of the enzyme (Fig. 5 and Results). However, hydrazine[35,36] and methyl hydrazine serve as extremely slow co-substrates for the formation of hydrazino acids from substituted fumaric acids. Thus, the enzyme can be rescued from irreversible inactivation by fumaric acids (Scheme 5).

Dehydroalanine residues have also been implicated in the deamination reactions catalysed by phenylalanine[13,22] and histidine[23,37] ammonia-lyase. Remarkably the precursor serine residue for both enzymes occurs as part of the triad Ser-Gly-Asp[24,25] as it does in methylaspartase.[5] Conversely, the aspartate ammonia-lyase reaction does not appear to involve the formation of covalent intermediates.[10,12]

Although there is a growing body of evidence and now also good chemical support for the role of dehydroalanine residues in reacting with the amino groups of substrates in the ammonia-lyase systems, direct proof has yet to be provided for any system. Indeed, recent work from Retey's laboratory on phenylalanine ammonia-lyase questions whether the amino group of the substrate (via a conjugate addition), or, the aromatic ring (via Friedel–Crafts alkylation) reacts with the dehydroalanine residue.[38] These workers, in fact, come out in favour of the Friedel–Crafts reaction. This new proposal in which C−C bond formation and then C−C bond cleavage occurs either side of a proton abstraction step, and before C−N bond cleavage, does not fit well with deuterium and nitrogen isotope effects observed by Cleland and co-workers.[13] If the same side chain reaction of the substrate with the dehydroalanine residue did occur for methylaspartase, the β-carboxylate group would react to give a β-carboxylic ester. While such an adduct might be expected to possess more acidic C-3 hydrogen atoms, the idea does not fit in with the results of several other experiments including the ability of the enzyme to form α-hydrazino acids rather than β-acyl hydrazides from fumaric acids and hydrazine.[36] Thus, for the purpose of interpreting the data described in this paper we shall first consider a mechanism in which a dehydroalanine residue[38] exists at the active site and serves to covalently anchor the amino group of the substrate and the product, through its β-C-3 rather than it's α-C-2 carbon atom[26] (Scheme 2).

Metal ion cofactor binding/debinding orders

The sequence of binding for the two metal ion cofactors Mg^{2+} and K^{+} has been investigated in some detail but again, at the time of the work the possible involvement of a dehydroalanine residue was not appreciated.[19] The analysis of the results indicated that at all pH values at which experiments were performed, pH 6.5 through to pH 9.0, the substrate and Mg^{2+} ion added to the enzyme in a random fashion before K^{+} ion. However several features of the analysis were not easy to accommodate including the fact that the patterns for double reciprocal plots for substrate and Mg^{2+} ion binding changed from an intersecting one below pH 9.0 to a parallel one at pH 9.0 (Fig. 13). Thus, at pH 9.0 Mg^{2+} acted as an uncompetitive activator and the values of V/K at different Mg^{2+} concentrations were the same



Scheme 5.

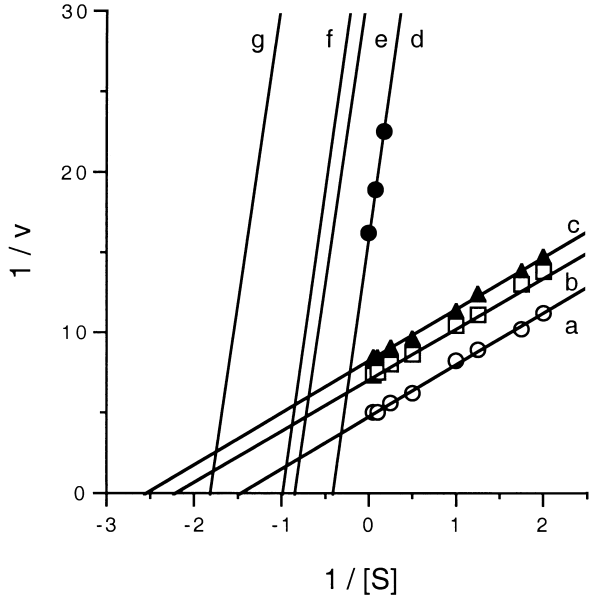
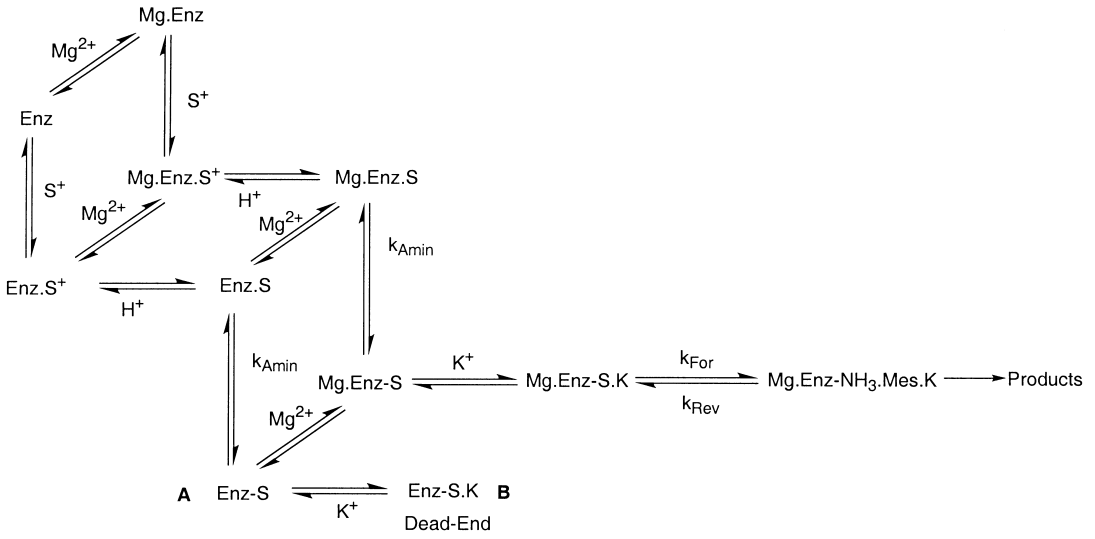


Figure 13. Effect of Mg^{2+} concentrations on deamination at pH 9.0. Incubations contained; 0.5 M Tris–HCl (pH 9.0), $MgCl_2$, KCl and 3-methyl-aspartic acid, in a total volume of 3 mL. Reactions carried out at 30.0 ± 0.1 °C and are corrected for one unit of enzyme. Some data points are off scale. (a) 20 mM $MgCl_2$, 50 mM KCl; (b) 1.0 mM $MgCl_2$, 50 mM KCl; (c) 0.4 mM $MgCl_2$, 50 mM KCl; (d) 20 mM $MgCl_2$, 1.6 mM KCl; (e) 1.0 mM $MgCl_2$, 1.6 mM KCl; (f) 0.4 mM $MgCl_2$, 1.6 mM KCl; (g) 0.2 mM $MgCl_2$, 1.6 mM KCl.

and as a consequence the apparent K_M values for the substrate decreased with decreasing Mg^{2+} concentration. That is, substrate was increasingly tightly bound as the Mg^{2+} ion concentration was decreased. This is certainly consistent with the formation of a covalent substrate–enzyme complex. Furthermore, the primary deuterium isotope effects upon V and V/K were suppressed at low Mg^{2+} concentrations, but not at high concentrations. Given that the substrate could form a dead-end complex if it reacts with the dehydroalanine before Mg^{2+} binds to the enzyme as depicted in Scheme 6, it is possible to account for both of these previously unexplained observations. The change in the initial velocity double reciprocal plot patterns with pH would then arise because the reaction of the substrate with the dehydroalanine residue would be more rapid at high pH where the amino group of the substrate would be unprotonated. Where the substrate was covalently attached to the enzyme to give adduct **A** and then trapped on the enzyme by K^+ ion, to give dead-end complex **B**, there would be no escape apart from through reverse steps since Mg^{2+} cannot bind after K^+ ion.[19] It would seem possible that Mg^{2+} ion could bind to the covalent aminated adduct (**A** in Scheme 6) but if it could not, **A** would also be a dead-end complex. Thus, the steady-state concentration of the dead-end complex(es) at any given concentration of Mg^{2+} would be high at high pH and/or high K^+ ion concentration. If it is assumed that the rate of breakdown of the covalent substrate–enzyme complex is slower in the absence of



Scheme 6.

Mg^{2+} ions then high concentrations of Mg^{2+} ion would be required to suppress the formation of the dead-end complex and rescue the substrate from a pathway possessing a high forward reaction commitment. This analysis indicates that for the formation of active complexes, Mg^{2+} must be bound to the enzyme before the formation of the covalent substrate–enzyme complex and remain bound. Otherwise, at low Mg^{2+} concentration, the substrate would be sticky and, in its covalent adduct form, unable to freely dissociate from the enzyme. This is consistent with the suppressed values of $^{D}(V/K)$ for the substrate at low Mg^{2+} and the lower apparent values of K_m for the substrate.[19]

Another consideration that must be accommodated within the mechanism involving the intermediacy of a dehydroalanine residue to complete the construction of a kinetic model concerns the timing of potassium ion binding. Whilst it is clear that K^+ only binds after the substrate across the pH range 6.5–9.0,[19] whether it binds before the addition of the amino group of the substrate to the dehydroalanine residue or after the formation of the covalent complex, could not be determined from the results of previous work. In Scheme 6, for the sake of simplicity, it is assumed that K^+ binds after the formation of the covalent Mg.E–S complex. Nevertheless, it was previously established that the concentration of K^+ ion had a profound effect on the apparent values of ^{D}V and $^{D}(V/K)$ and suppressed the isotope effects at high concentration, as is expected for a species which binds after the labelled one (see ref 40 for a useful review, and references cited therein). However, very low concentrations of K^+ ion also suppressed ^{D}V and $^{D}(V/K)$. Furthermore, the activator ion did not show simple saturation kinetics at pH 9.0 and two apparent dissociation constants were detected. From the results presented here (Table 7, Fig. 1 and Results) and in the following article, it is clear that K^+ does not suppress the value of the solvent isotope effect or the value of $^{15}(V/K)$ at pH 9.0. These findings are more readily consistent with the addition of K^+ ion to the enzyme after the formation of the covalent enzyme–substrate complex. Under different conditions, however, some other results indicate that the addition of K^+ ion to the enzyme occurs before the formation of the covalent enzyme–substrate complex (see below).

Some clues to the puzzle come from the knowledge that K^+ ion does show simple saturation kinetics well below pH 9.0. Using the high K^+ ion asymptote of double reciprocal plots of initial rate versus activator concentration at pH 9.0 gives values of V and V/K which lie on a zero and unit slope plot, respectively, for graphs of these kinetic parameters versus pH (Fig. 2). The parameter V with respect to K^+ is pH independent meaning that the slow rate of reaction observed at low pH can be completely overcome by increasing the activator concentration. This fact alone indicates that the potassium ion-bound form of the enzyme is closed and not accessible to solvent derived protons during the course of the reaction since the pH of the solution is irrelevant. The substrate amino group must be protonated at low pH and, since the K_m value of the substrate is pH independent above pH 6.5 and below pH 9.0 but increases above pH 9.0 (see Fig. 2), it is evident that the protonated form of the substrate is the true substrate. Thus, there must also be a pathway in which the amino group of the substrate attacks the dehydroalanine residue after K^+ ion has bound (Scheme 7). In this scheme the low pH site for K^+ would be site **A** and instead of removing a proton from the substrate, a proton must be removed from the enzyme to provide a base for the deprotonation of the alkylammonium group after the enzyme has closed.

It is also evident from the curved double reciprocal plots of initial rate versus activator concentration determined at pH 9.0[19] that at low substrate concentration, the apparent binding constant for K^+ is high (ca. 20 mM). This value of the apparent binding constant for K^+ is in accord with values extrapolated for K_K determined at low pH where the substrate must be protonated. Conversely, the apparent value at high substrate concentration

is low (ca. 0.2 mM). The result suggests that at high pH, when the substrate can deprotonate, the nucleophilic amino group can directly attack the dehydroalanine residue and, in so doing, creates a new binding site for K^+ (site **B** in Scheme 7), with a very high affinity. Hence, at pH 9.0 or above, the dependence of the rate of reaction on K^+ would be dependent upon the substrate concentration and its reaction with the dehydroalanine residue and, double reciprocal plots of rate versus K^+ activator ion concentration would be non-linear, as was observed.[19]

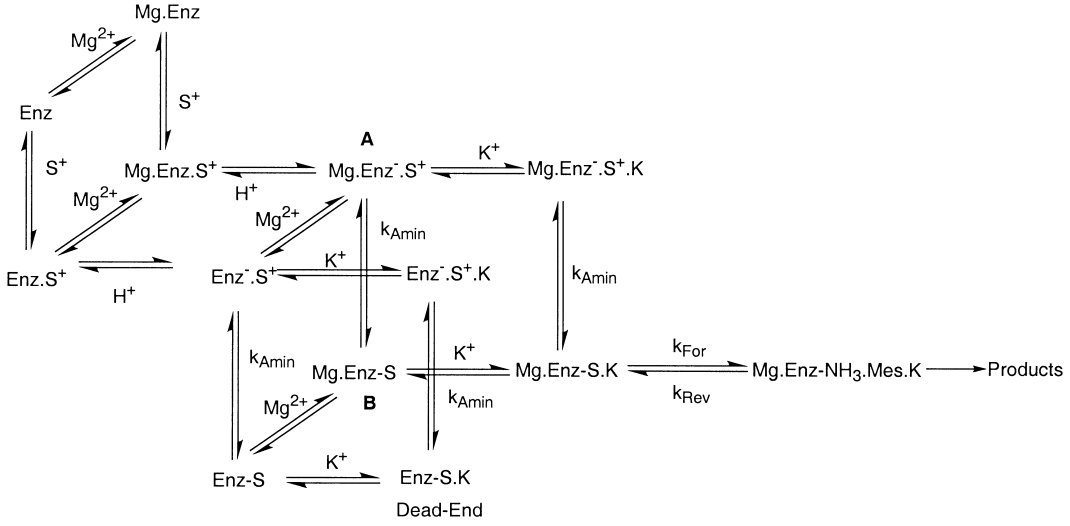
The titrations in the deuterium solvent isotope effects [and ^{15}(V/K) effects, which are discussed in the following article] at or just above pL 9.0 (Fig. 1) are also consistent with the exposure of the first part of the reaction sequence to the solvent. The uncatalysed (or at least less efficiently catalysed) addition of the amino group to the dehydroalanine would be expected to show a substantial isotope effect for the protonation of the conjugate base. Moreover, the attack of a deprotonated amino group would be expected to show a substantially reduced ^{15}(V/K) effect by analogy with the phenylalanine ammonia-lyase system.[13] Both of these properties are observed at high pH (see Results and the following article). Note that the external reaction of the amino group of the substrate with the open form of the enzyme in Scheme 7 is entirely analogous to the formation of the dead-end complexes lacking a bound Mg^{2+} ion referred to above and shown in Scheme 6. Applying the same reasoning to the reverse reaction with the knowledge that mesaconate, ammonium ion and Mg^{2+} bind in a random fashion[19] and that the solvent isotope effects are largest at high pH (Fig. 1) gives Scheme 8.

Assumptions implicit in kinetic Scheme 8

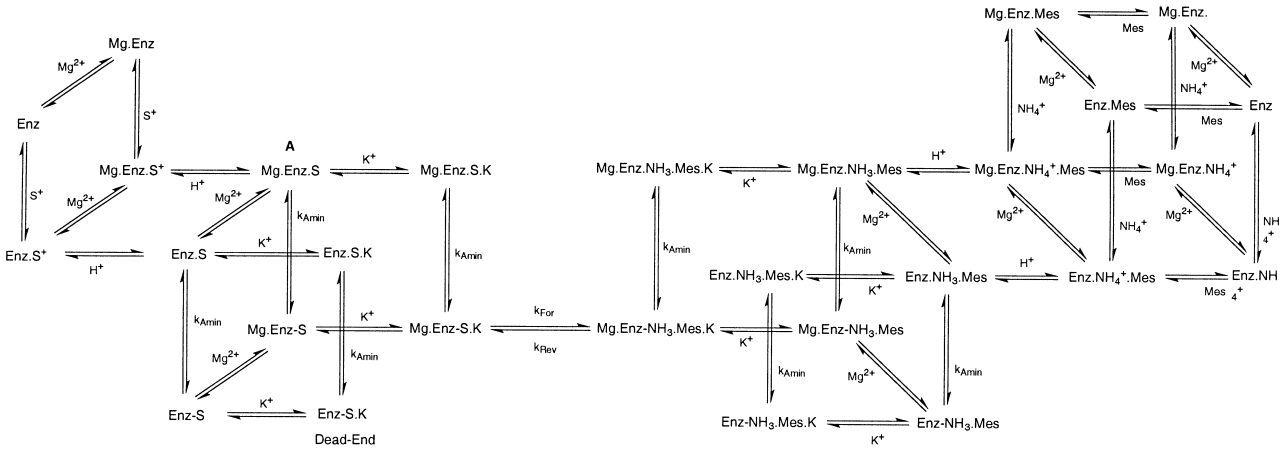
Scheme 8 embodies the culminated results of all previous work and analyses. At high Mg^{2+} concentration, low K^+ concentration and at pH 9.0 or above, the substrate binds after the Mg^{2+} ion and then is deprotonated to give the species which attacks the dehydroalanine residue. At low Mg^{2+} concentration some dead-end complex is formed. At high K^+ concentration and at pH 9.0 or below, the substrate binds after the Mg^{2+} ion and is then trapped on the enzyme by K^+ and deprotonated internally, without the influence of solvent pH, to give the species which attacks the dehydroalanine residue.

The ability of the enzyme to perform its function in a pH-independent manner at saturating K^+ ion concentration over the range pH 6.5 to 9.0 raises some interesting questions concerning the protonation states of the various acid–base groups on the enzyme in the closed form. Clearly, a deprotonated form of the enzyme and the presence of substrate are required to create a binding site for K^+. The increasing affinity of the site **A** in Scheme 7 with increasing pH with unit slope in the logarithmic plot (Fig. 2), indicates that a single acid possessing a pK_a value of greater than 9.0 is required in its unprotonated form for K^+ binding. Above pH 9.3 the plot of log V versus pH plummets with a negative slope of 3.0 and the reaction appears to be limited by other steps involving external proton transfers in both the forward and reverse reaction. The solvent isotope effects in both directions are large for V, 4.5 and 6.7, respectively, and small for V/K, 1.6 and 1.3, respectively (see Tables 1 and 4, and Fig. 1). The kinetic parameters for the slow substrate, (2*S*)-aspartic acid, show identical properties with respect to pH below pH 9.0 and V and V/K increase with unit slope and then level out to zero gradient revealing that an acid possessing a pK_a value of 9.1 must be deprotonated for activity (Fig. 3). The difference in behaviour of the slow substrate above pH 9.4 compared to the natural substrate (Fig. 2) suggests that the slow substrate can access an acid at high pH (see Discussion).

Aspartic acid shows the same type of non-linear double reciprocal plots for initial rate versus K^+ concentration, but the curvature is more pronounced (data not shown) and the differences in the K^+ binding affinities of the two sites (**A** and **B** in Scheme 7) are larger. This may



Scheme 7.



Scheme 8.

reflect that fact that the substrate is not such a good fit, so that complex **A** is destabilised but, at the same time, is less sterically hindered and less protonated at low pH and more able to attack the dehydroalanine in the open form of the enzyme than the natural substrate. The rate of the deamination of aspartic acid is not limited above pH 9.0 in the same way as the natural substrate and very large solvent isotope effects of 10.8 and 6.4 are expressed on V/K at pH 9.0 and 9.4, respectively (see Table 2). The reverse reaction, the amination of fumaric acid, is faster than the corresponding amination of mesaconic acid and interestingly, as for mesaconic acid, the solvent isotope effect is largest for V at pH 9.0 (ca. 8.3) and equally expressed on V and V/K at pH 9.4 (ca. 3.3).

Active site acid–base groups

At least three acid–base groups are required for the deamination of (2*S*,3*S*)-3-methylaspartic acid, two to deprotonate the amino group of the substrate and aminated adducts of the enzyme, B-1 and B-2, and one to remove the C-3 proton from the substrate, B-3. The latter base, B-3, should not be able to access the amino group of the substrate directly because the C-3 hydrogen and amino group must be disposed in an *anti*-conformation upon opposite faces of the double bond in the nascent product, mesaconic acid. However, a polyprotic base, possibly B-1, should be able to access the conjugate base B-3, if the acid form is a monoprotic thiol in order to account for the exchange reaction (see below). It should be noted that the requirements for the *syn*-deamination of (2*S*,3*R*)-3-methylaspartic acid are different and are considered below.

Two of the acid–base groups, B-3 and either of B-1 or B-2, would need to be unprotonated in the closed substrate bound form (Scheme 9). Interestingly, only V/K for K^+ shows a pH-dependent increase when all species are saturating. The unit slope in the log plot of V/K versus pH indicates that a single acid must deprotonate in order to bind K^+ and that its pK_a is ca. 9.3 (see discussion above). This analysis indicates that one of the acids must be unprotonated at or below pH 6.5, the low pH limit of the study. If the acid–base groups cannot exchange protons with each other on opposite faces of the product, mesaconic acid, in the closed form of the enzyme, the base B-3 must be unprotonated. Furthermore, the generation of a tight-binding site for K^+ appears to be associated with the generation of the other base, B-1 or B-2.

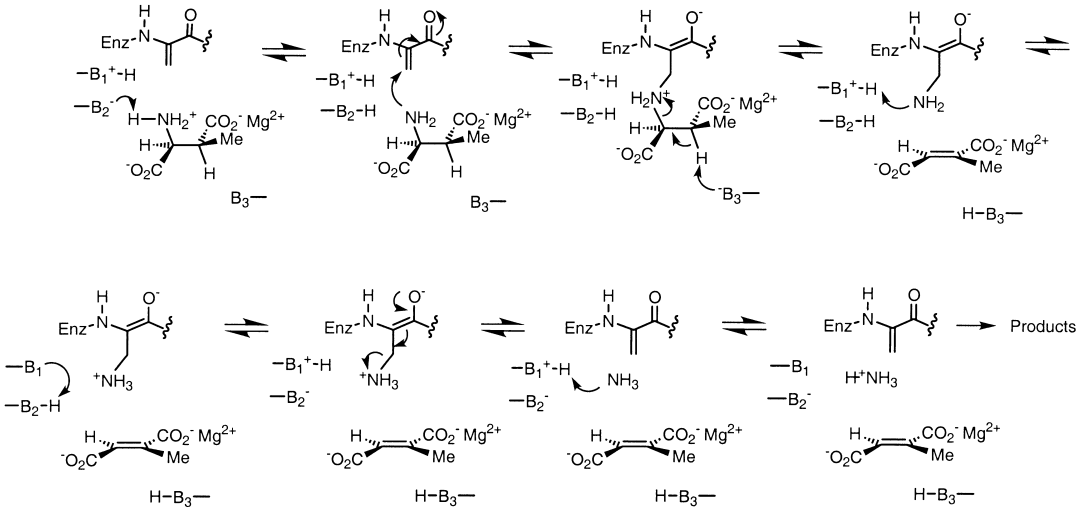
In order to gain further information on the properties of the active site, a number of experiments were performed using the irreversible inhibitors phenylhydrazine and *N*-ethylmaleimide (see Experimental and Results). A plot of the pH dependence of the log of the rate inhibition by NEM gave a line with unit slope and a pK_a value of 9.3 (Fig. 8). Since NEM does not ionise in the pH range of the study, the result indicates that an acid with a pK_a of 9.3 must be deprotonated to react directly with NEM or that such an ionisation must occur in order to unmask a nucleophile that is sterically hindered in the protonated form of the enzyme. The value of the pK_a correlates well with the pK_a value of 9.3 derived from the binding of K^+ ion (see above). Intriguingly, phenylhydrazine displayed a similar pH-dependence for inhibition except between pH 9.0 and 9.5 the plot displayed a slope of 2.0 indicating that two acid groups need to be deprotonated for the reaction (Fig. 6). This might be expected for a reaction that occurs in the open form of the enzyme and which requires a base catalysed conformational change and a nucleophilic attack on a dehydroalanine. However, the reaction of hydrazines with the dehydroalanine residue is reversible initially[35,36] and the enzyme can be rescued with fumaric acids (Scheme 5). Thus, the inactivation kinetics we observe may represent slow steps involved in the slower conversion of the β-*N*-aminoalanine residue into a permanently inactivated system.

The properties of NEM are more simple to analyse. The substrate protection afforded by methylaspartic acid and mesaconic acid and the derived K_S values for the substrates are in complete accord with the independent findings of Bright who used alternative thiol attacking reagents, including mercury benzoates, to inactivate the enzyme.[8]

The discovery that Mg^{2+} ions activate the inhibition process (see Results) is of particular interest because NEM could bind very close to the position of the base responsible for the removal of the proton from the carbon acid at C-3 of the substrate. It is believed that the role of Mg^{2+} ion is to activate the C-3 hydrogen to proton abstraction by the formation of a magnesium 4-carboxylate interaction (Fig. 14). Indeed, it is possible that the protection from inactivation afforded by phosphate dianion is attributable to the ability of the dianion to occupy the site for the 4-carboxylate group of the substrate. Note, acetate was totally ineffective in affording protection at up to 50 mM. Hence, it was of interest to determine the nature of the group that was modified by NEM and its position within the sequence of the enzyme.

Wu and Williams claimed that NEM modification of the enzyme, followed by tryptic digest gave a large peptide containing two lysine residues.[33] Previously we suggested that an amino-enzyme form of the protein derived from a dehydroalanine residue would match the amino acid composition of the Wu and Williams' peptide[5] (Table 6). However, we were completely unable to detect a trypsin resistant Lys-Xaa bond in our own proteolysis studies. Indeed, the short 17 amino acid sequence (ANGMGAYCGGTCNETNR) obtained for the peptide labelled with tritiated NEM possessed two Cys residues (Cys-357 and Cys-361) and no Lys residues. One of the Cys residues, Cys-361, was modified and radiolabelled, and the other was unalkylated (see Results).

From the observed protection against alkylation by NEM offered by substrates and phosphate dianion, together with the activation of inhibition caused by Mg^{2+} ions, it is reasonable to propose that the NEM



Scheme 9.

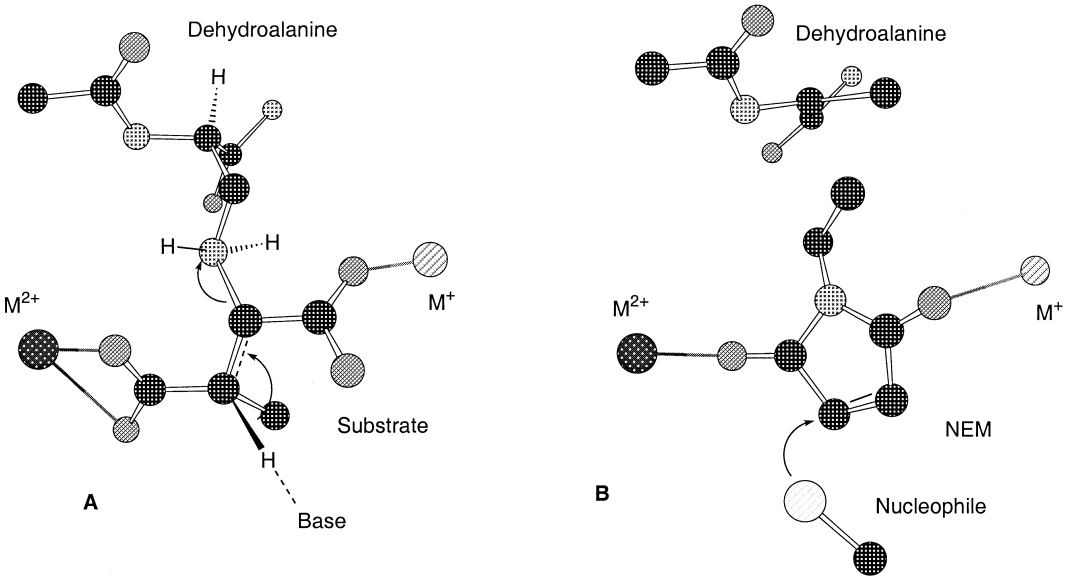


Figure 14. Showing A) the expected arrangement of the base (B-3) for deprotonation of C-3 in the sbstrate, and; B) the reaction of enzyme–Mg^{2+}-bound NEM with the base in the inactivation reaction.

molecule binds to the enzyme–Mg^{2+} complex and alkylates one of the catalytically essential acid–base groups (Fig. 14). Given that Mg^{2+} ions increase the binding affinity of NEM >10-fold but do not enhance the maximum rate of the inactivation, k_{Inact}, by much, it would seem that the conjugate addition of the thiolate group of Cys-361 is not catalysed through the Mg^{2+} ion serving as a Lewis acid. Thus, it is likely that the thiolate group attacks at a position analogous to the C-3 site of the substrate which, in turn suggests that it is the C-3 base (B-3 as defined in Scheme 9) that is modified (Fig. 14).

In order to rationalise our own sequencing results with the amino acid analyses performed on tryptic peptides by Wu and Williams (Table 6), it is necessary to suggest that the unlabelled Cys residue was protected throughout the reaction with NEM, since it remained unmodified. One attractive manner would be by forming an internal conjugate addition adduct with the dehydroalanine residue; a thioether bridge linking Cys-357 and dehydroAla-173. If in our experiments the re-elimination of thiolate occurred to give a tryptic digest of the open form of the enzyme, whilst in Wu and Williams' experiment[33] a tryptic digest of the closed form of the enzyme was obtained, there would be no dichotomy. One sensible explanation for the difference could be that in our proteolytic studies, recombinant enzyme containing a polyHis tag was used instead of wild-type enzyme.[41] It is possible that the proteins behave differently during proteolysis and that the His tag was capable of catalysing the sulphydryl elimination. Interestingly, the theoretical conjugate is the correct size and possesses the correct amino acid residues if it is accepted that the hydrolysis product of the Cys-dehydroAla thioether might have appeared to Wu and Williams to look like two Lys residues (see Table 6).

A further line of evidence that links the dehydroAla residue with the NEM labelled peptide comes from studies of the inhibition of the enzyme with nitrophenylhydrazine. Here it was noted that the tryptic digest of a modified peptide possessing a 360 nm absorbance was a bis-disulfide adduct of the ANG peptide containing the Cys residues 357 and 361. It appeared that these Cys residues were linked to the Cys-containing peptide SAEVTTNIGMACGAR, spanning residues 367–382, and the peptide GVDAELVADEWCNTVEDVK, which spans residues 299–317. Indeed, all of the Cys containing peptides sequenced as disulfide adducts with the ANG peptide indicating that the hydrazine was oxidising one of the thiol groups in the ANG peptide and that other Cys containing peptides were then displacing and exchanging with the oxidised species. One possible reaction pathway is depicted in Scheme 10. It is important to note that neither ourselves or previous workers have identified disulfide bridges in the native form of the enzyme.[8,33]

If the dehydroalanine residue is protected by Cys-357 then many features of the system become easier to understand.[§] For example, the failure of sodium borohydride and cyanide to react with the dehydroalanine residue; the fact that substrates in the presence of K^+ ion and inhibitors require the same base-catalysed step to enter the active site, and the fact that the two Cys residues could be appropriately placed to serve as bases for the deprotonation of the alkylammonium group (B-1 or B-2, see above) and C-3 of the substrate (B-3), respectively, within the closed enzyme form.[‖]

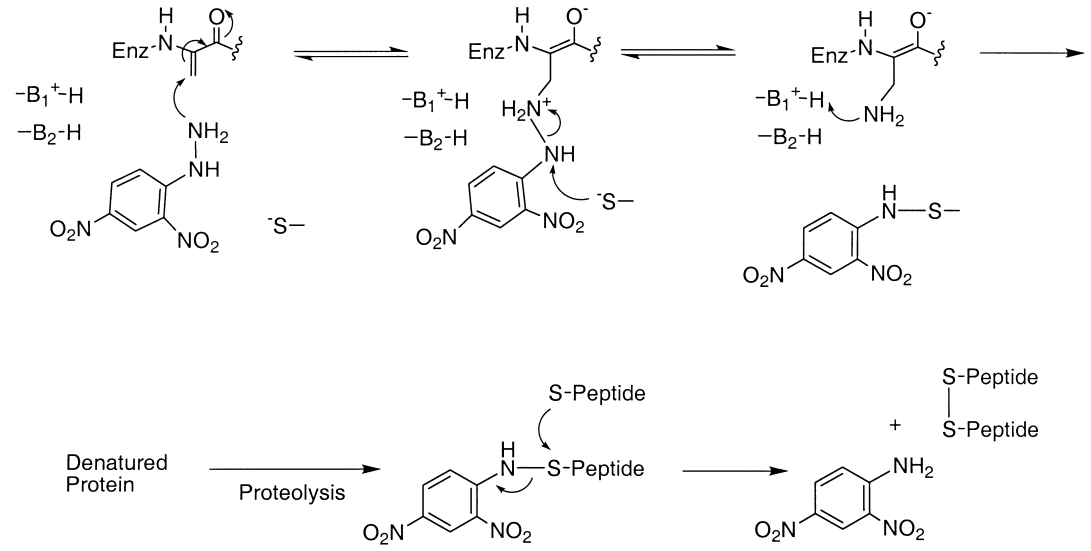
Primary deuterium isotope effects

It would appear from the analysis above that below pH 9.0, the enzyme catalyses all of its chemistry internally within a closed structure after K^+ ion has bound. The fact that ^{D}V and $^{D}(V/K)$ are pH independent across the entire pH range at 1.70 and the fact that the enzyme displays solvent hydrogen exchange, measured as tritium incorporation, at rates equal to or faster than the rate of deamination, and that at different pH these exchange reactions show a primary deuterium isotope effects of ~1.7, indicates that the C-3 hydrogen is removed at equilibrium. The ratio of the exchange reaction to deamination (ν_{Ex}/ν_{Deam}) is pH sensitive[8,19] and varies from much less than 1.0 above pH 9.0 and below pH 6.0, to about 5.0 at pH 7.6. The rate of deamination increases by 85-fold from pH 6.5 to 9.0. However, both reactions show similar primary deuterium isotope effects for V_{max} of ~1.7 across the pH range 6.5 to 9.0 and the ratio ν_{Ex}/ν_{Deam} for the deuteriated substrate is identical to that for the unlabelled material at any given pH.[19] Although the exchange reaction complicates the analysis of some of the experiments described here, the pH-independent magnitude of the isotope effects for exchange of ~1.6 and the similar pH-independent magnitude of the isotope effects for deamination of ~1.7 for ^{D}V and $^{D}(V/K)$ (which are identical within experimental error), together with the pH-dependent ratio ν_{Ex}/ν_{Deam} require rather well defined conditions.

If the exchange reaction requires an external acid–base group, the exchange must occur after K^+ has been released from the enzyme, so that the active site can open to exchange hydrogen with the solvent. The rebinding of K^+ to the product complex would then allow the reverse reaction to occur to give C-3 hydrogen exchanged substrate. There is much additional evidence to indicate that the enzyme cannot exchange hydrogen before the deamination reaction is complete including the finding that the slow substrate (2*S*,3*R*)-3-methylaspartic acid does not epimerise but rather deaminates to give mesaconic acid and then re-aminates to give (2*S*,3*S*)-3-methylaspartic acid[20] (see below). Since tritium incorporation into the substrate pool could display a very large isotope effect, the exchange rates may be gross underestimates for the reverse reaction commit-

[§]Methylaspartase from *Citrobacter amalonaticus* strain YG-1002 has recently been cloned and the gene sequenced (Kato, Y.; Asano, Y. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 468). Comparison of the inferred amino acid sequence with that from *Clostridium tetanomorphum*, highlights that Cys-357 is not conserved and is glutamine in *C. amalonaticus*. This suggests that Cys-361 or possibly an alternative Cys residue is involved in the formation of the thioether cross-link.

[‖]Note that we are not advocating that the pK_a value of Cys 361 is ca. 9.3, as it is believed that a conformational change associated with K^+ binding titrates at pH 9.3 (see above).



Scheme 10.

ments to C-3 deprotonation. Nevertheless, the reverse commitments are large and ^{D}V and $^{D}(V/K)$ could only be equal in magnitude and vary synchronously with changing cofactor and activator concentrations if there were no commitments or very large and dominant reverse commitments and a deuterium isotope equilibrium constant ($^{D}K_{eq}$) for C-3 deprotonation that is not equal to 1.0 (eqs (1) and (2)).

$$^{D}(V/K) = 1.7 = \frac{^{D}k + C_f + {}^{D}K_{eq}(C_r)}{1 + C_f + C_r} \quad (1)$$

$$^{D}V = 1.7 = \frac{^{D}k + C_{Vf} + {}^{D}K_{eq}(C_r)}{1 + C_{Vf} + C_r} \quad (2)$$

C_f is the forward reaction commitment for the deuterium sensitive step, C_r is the reverse reaction commitment and C_{Vf} is the forward reaction commitment ratio used for V_{max} isotope effects as defined by Cleland.[40]

Only one type of base, a thiolate, which possesses a fractionation factor of 0.4 to 0.65,[42–44] could give rise to such a large equilibrium isotope effect.[45] The fractionation for the removal of the C-3 proton from methylaspartic acid is expected to be ca. 1.15[46] and therefore, the equilibrium isotope effect $^{D}K_{eq}$ would be $\phi_{C\text{-}3}$ (the fractionation factor estimated for the C-3 proton of the substrate) divided by ϕ_{BH} (the fractionation factor for the C-3 base); that is ca. 1.15/(0.5 to 0.65) = 1.77 to 2.30.

In order to estimate the size of the commitments in eqs (1) and (2) an expression was derived to account for the exchange reaction (eq (3)) where C_f' and C_r' are equal to C_f and C_r in eq (1) if exchange occurs immediately after the deprotonation at C-3 of the substrate.

$$\nu_{Ex}/\nu_{Deam} = C_r'/C_f' \quad (3)$$

Now the commitments describe the partitioning of the deprotonated enzyme-bound substrate in the forward direction (to give at least one unbound product) and in the reverse direction to give C-3 hydrogen exchanged substrate. Assuming for now that exchange occurs immediately after the removal of the C-3 proton of the substrate (i.e. at the earliest time that it could happen in any mechanism), the values of C_f' and C_r' would be the same as those for C_f and C_r in eqs (1) and (3). At pH 9.0, ν_{Ex}/ν_{Deam} (measured as tritium wash-in from tritiated water[19]) = 1.0 such that $C_r' = C_f'$. Substituting for C_f into eq (1) and assuming that $^{D}k = 7.0$ gives the expression $C_r = 5.3/(2.4 - {}^{D}K_{eq})$. Thus, reaction commitments can be estimated for assumed values of $^{D}K_{eq}$. At pH 7.6, $\nu_{Ex}/\nu_{Deam} = 5.0$ such that $C_r' = 5C_f'$. Substituting for C_f into eq (1) now gives the expression $C_r = 5.3/(1.84 - {}^{D}K_{eq})$. Both expressions give a common value of 7.57 for C_f' at pH 9.0 and 7.6 when $^{D}K_{eq} = 1.7$ and the values of C_r' are then 7.57 and 37.9 at pH 9.0 and 7.6, respectively. Recall that at pH 6.5 $\nu_{Ex}/\nu_{Deam} = 1.0$, also, and that ν_{Ex}/ν_{Deam} falls-off below pH 6.5 and above pH 9.0.[8,19] This analysis clearly indicates that the reverse commitments are large across a wide pH-range and makes assumptions that would probably underestimate the size of C_r for the deuterium sensitive step in eq (1). This is because an allowance for the tritium isotope effect was omitted, and because C-3 exchange with the solvent should occur after the release of K^+ ion from the product complex (i.e. later in the mechanism than we have assumed). Hence, C_r is so large that the primary deuterium kinetic isotope effect data can only be consistent with the operation of a thiolate base for the removal of the C-3 proton, base B-3. This finding accords with the notion that Cys-361 is B-3.

Timing of C-3 solvent hydrogen exchange

The products ammonia and mesaconic acid appear to debind in a random order, or ammonium ion debinds first (see above), after the deamination of the intermediate 2,3-diaminopropanoic acid residue, since an amino-enzyme intermediate cannot be trapped with labelled mesaconic acid.[19] K^+ ion must debind and/or

there must be a conformational change before there is enough room for mesaconic acid to escape from the enzyme. Note that this aspect of the mechanism differs from that for phenylalanine ammonia-lyase system where the amino-enzyme form can exist after the other product, cinnamic acid, has escaped.[13]

Since for the methylaspartase system K^+ ion should be released before solvent can enter the active site and exchange protons with acid–base groups, the cause of the exchange reaction should derive from the competition between product release in the forward direction and K^+ rebinding (after proton exchange between B-3 and the solvent) in the reverse direction. The ratio of the exchange reaction to deamination (ν_{Ex}/ν_{Deam}) is pH sensitive but both reactions show similar primary deuterium isotope effects for V_{max} of ~1.7 across the pH range from 6.5 to 9.0.[19] In the presence of 150 mM K^+ ion the rate of deuterium wash-out from (2*S*,3*S*)-3-methylaspartic acid into the substrate pool shows a bell-shaped dependence on pH.[8] The wash-out rate increases with the deprotonation of an enzyme-bound acid group possessing a molecular pK_a value of ca. 6.5 and decreases with the deprotonation of a species possessing a pK_a value of ca. 9.2. Since the base B-3 (Cys-361) must be protonated after the removal of the C-3 proton in the product complex, it would appear that the lower pK_a value corresponds to the deprotonation of Cys-361 in the product complex. Note that earlier it was suggested that the base responsible for the deprotonation of the C-3 position of the substrate should possess a pK_a value of 6.5 or below because, there is only one ionisation required for deamination reaction in the range 6.5–9.4 (Fig. 2) and this corresponded to K^+ ion binding.

The high pK_a of ca. 9.2 associated with a decrease in the deuterium wash-out rate may represent the ionisation of the group which protonates the B-3 thiolate. Several candidate acids exist including the protonated form of either of B-1 or B-2, or the protonated product ammonium ion. It is interesting to note that the deprotonation of the B-3 thiolate and the deprotonation of either B-1 or B-2 must occur before a new substrate molecule binds in the active site because, in the product complex, two of these bases would be in the wrong ionisation state.

Effect of ammonium ion

In the discussion concerning product release (above), it was noted that the involvement of a dehydroalanine residue provides, potentially, many sites to which a second ammonium ion could bind. All experiments performed to date are consistent with the random binding of ammonium ion and mesaconate in the reverse reaction[19] where two molecules of ammonium ion are required in the absence of K^+. One serves as the activator and double reciprocal plots of initial rate versus the square of ammonium ion concentration are linear.[19] Increasing potassium ion concentration does not increase the exchange rate for C-3 deuterium wash-out even though K^+ ion binding in the reverse reaction direction must compete with product release in the forward deamination reaction direction (see above). Presumably this is because the increase in K^+ ion concentration must also increase the forward reaction commitments (and retards the release of methylaspartate) which cancels the effect where the binding affinities of the enzyme–product and enzyme–substrate complexes for K^+ ion are similar. However, ammonium ion displays a pronounced effect in increasing the C-3 deuterium wash-out rate at low and at high K^+ ion concentrations and also inhibits the deamination reaction. At high K^+ concentration, the increase in wash-out rate is accompanied by non-linear uncompetitive inhibition at low concentrations of ammonium ion and by mixed uncompetitive–noncompetitive inhibition at high concentrations of ammonium ion (Fig. 12). If ^{15}N-ammonium ion is used, no ^{15}N-label is incorporated into the substrate pool (through reverse amination) and it is evident that the ammonium ion is not binding simply as a product.[19] Most consistent with these results is the notion that the activator site for K^+ ion has a high affinity for ammonium ion in the product complex and simply traps the products and re-commits them to partition in the reverse reaction direction. Since K^+ ion must have been released to open up the active site to solvent, the proton derived from C-3 of the substrate would be able to exchange. This site for ammonium ion is, of course, the same site that ammonium ion occupies as an activator in the reverse (amination) reaction where there is a squared dependence on the ammonium ion concentration.[19]

The interpretation above indicates that the reverse commitments estimated for the purpose of evaluating $^{D}K_{eq}$ are probably very much larger than the values deduced from the C-3 exchange reaction. However, this does not spoil the analysis. The values, as before, correspond to the reaction commitments for the hydrogen exchange step but, because the exchange takes place later in the mechanism, C_r for the exchange is now equal to the rate constant for the first product to debind divided by the second order rate constant for K^+ ion binding to the product complex. If ammonium ion binds faster than K^+, C_r and the observed ratio ν_{Ex}/ν_{Deam} will increase. C_f for the exchange is the product of the net rate constants leading to the release of K^+ ion in the forward direction.

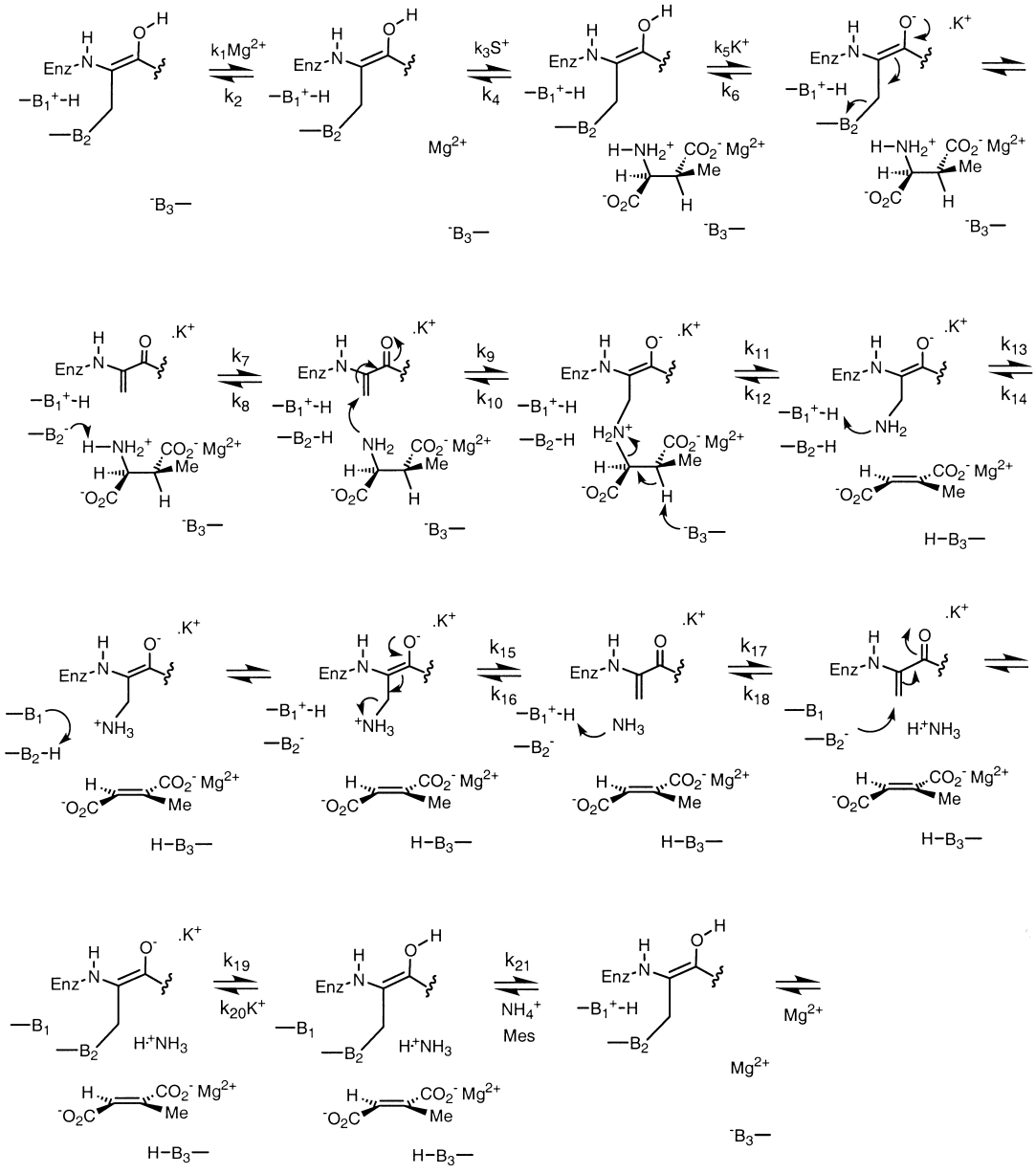
Conformational changes associated with K^+ ion binding

The requirements for K^+ binding are a substrate-bound enzyme complex, whether or not Mg^{2+} ions are bound, and hydroxide ions. At neutral pH the dissociation constant for K^+ is large and decreases with increasing pH but, the value of V is pH independent at saturating K^+ concentrations. Thus, at low pH, higher concentrations of K^+ are required to compensate for the low concentration of hydroxide ions. In our steady-state kinetic experiments we have used 0.1–1.0 nM enzyme solution so that even at pH 6.5 there is a several 100-fold excess of hydroxide ions. The binding of K^+ ion brings about a conformational change which appears to release a base, B-1 or B-2, and close up the active site so that it is no longer accessible to

solvent-derived protons. Several other monovalent cations will serve as surrogates for potassium ion, including rubidium, lithium, ammonium and sodium, in order of decreasing effectiveness.[1] Thus, the enzyme is not specific for K^+ and the role of the activator ions seems to be to stabilise a closed conformation. The true substrates are *N*-protonated aspartates and the first step, following the closure of the K^+-bound complex, is to deprotonate the alkylammonium group with B-1 or B-2 (Scheme 9).

As has been alluded to above, B-1 and B-2 should be in opposite protonation states. One base is required to deprotonate the alkylammonium group of the substrate to generate the nucleophile to attack the dehydroalanine residue. Thus, the dehydroalanine residue must be formed and ready to react with the N-nucleophile if it is pre-protected as a thioether (with Cys-357, as suggested above) or by some other means. The pK_a value of the base in the open form of the enzyme must be less than 6.5 or higher than 9.3 since there are no titrations in that range other than for the binding of K^+ ion. Another attractive possibility is that the binding of K^+ ion stabilises the unprotected form of the dehydroalanine residue such that the titration of the binding affinity of K^+ causes the elimination of, for example, the thiolate moiety of Cys-357 to generate a base (B-2) in the closed form of the enzyme which removes the proton from the alkylammonium group of the substrate (Scheme 11). The nucleophilic attack by the amino group would then give a covalent substrate–enzyme complex possessing a dialkylammonium moiety. Both B-1 and B-2 would now exist in their protonated forms.

The elimination of the C-3 proton and ammonium group from the substrate (step 11) could now proceed



Scheme 11.

aided by the base B-3 (Cys-361) to give an unprotonated 2,3-diaminopropionic acid moiety in place of dehydroAla-173, enzyme-bound mesaconate and three protonated bases. The acidic form of B-2 would then protonate the 3-amino group of residue 173 either directly, or indirectly, via B-1. The protonated residue would undergo elimination (step 15) to regenerate the dehydroalanine residue and form enzyme-bound ammonia. Still in the closed form of the enzyme, the protonated base B-1 would protonate the ammonia molecule to give enzyme bound ammonium ion, such that both B-1 and B-2 are now free bases. B-2 (Cys-357) would then attack the dehydroalanine residue to give a thioether and allow K^+ ion to debind. Following the release of the metal ion, water could enter the active site and exchange hydrogen with the bases B-2 (unprotonated) and B-3 (protonated), each of which would need to swap ionisation states in order to rebind and process a new substrate molecule. The reverse reaction would lead to hydrogen exchange, as has been discussed above, and forward steps would allow the products, mesaconate and ammonium ion, to escape. The entire proposed mechanism is shown in Scheme 11.

Methylaspartase can also process the *erythro*-substrate, (2*S*,3*R*)-3-methylaspartic acid via a *syn*-elimination process, at low rates. The reaction displays very large primary deuterium isotope effects upon V of ca. 7.0 and upon V/K of ca. 4.0 at 1 mM and 50 mM K^+ ion at pH 9.0. The reaction shows a similar dependence on K^+ ions as for the natural substrate and (2*S*)-aspartate, but does not show an exchange reaction for the C-3 proton with solvent hydrogen.[6,20] Note that the natural substrate shows a ν_{Ex}/ν_{Deam} ratio of 1.0 at pH 9.0 and recall from Results that (2*S*)-aspartic acid displays a ν_{Ex}/ν_{Deam} ratio of ~16 at pH 9.0.

At first, the lack of exchange for the *erythro*-substrate might seem to be trivial since the substrate shows a near intrinsic primary deuterium isotope effect upon V, and therefore, the reverse commitments must be very low, indeed, less than 1.0. However, the product of the *syn*-elimination of the *erythro*-substrate is also mesaconic acid and if the protonation state of the product complex was the same as for the deamination of the natural substrate, the reverse reaction would occur very rapidly to give the *threo*-isomer possessing a C-3 proton derived from the solvent. Such a reaction does not occur and the first product to form is mesaconic acid.[6,20] This is a highly informative result as it indicates that the enzyme is tightly closed during the reaction.

The results also indicate that there is a base suitably disposed to remove the C-3 hydrogen from the *erythro*-substrate which cannot interact with the C-3 base (B-3) for the *threo*-substrate when the substrate and K^+ ion are bound. If it could, a C-3 epimerisation reaction would not be observed.

¶Note that in a *syn*-elimination process, the unprotected amino group of the amino-enzyme residues, on the same face of the product as the C-3 hydrogen, could also serve as a base in a carbonium ion substrate elimination mechanism (see *Bioorg. Med. Chem.*, **1999**, *7*, 977, this issue).

It is interesting to note that at pH 9.0 at equilibrium, the enzymic amination of mesaconate by ammonia in the presence of Mg^{2+} ions gives an almost 1:1 (actually 1.05:1) mixture of the *threo:erythro* methylaspartic acid diastereomers. Thus, their free energies are very similar. The relative values of V/K for the two substrates are 277 and 0.43, for the *threo*- and *erythro*-diastereomers, respectively, and their ratio is 644. This would represent the differences in the correctly protonated states of the enzyme for the reaction with the two substrates if their k_{cat} values were equal, but V_{Thr} is 38 times larger than V_{Ery}. Hence $38/644 = 5.9\%$ of the enzyme at pH 9.0 exists in the correctly protonated form for the deamination of the *erythro*-substrate and incorrectly protonated for the deamination of the *threo*-substrate. For the *syn*-addition/elimination to work B-3 must be retained in its unprotonated form and both B-1 and B-2 must be unprotonated after K^+ ion binds and closes the active site in the deamination reaction direction. Either B-1 or B-2 (or, indeed, the amino group of the amino-enzyme)¶ should serve as the base for the removal of the C-3 proton in the elimination reaction (Scheme 12) and the pK_a value of the conjugate acid must be higher than that of 6.5 for B-3. The analysis above indicates that the pK_a value should be 1.14 pK units higher than the pH of the experiment (i.e. ca. 10.1). Interestingly, at pH 9.76 Barker found that the ratio of the values of V/K for the two substrates were 106 and the ratio for the values of V were also 106.[1] This would indicate that 100% of the enzyme was in the correct form for processing the *erythro*-isomer. However, we now know that the activity of the *threo*-isomer falls-off dramatically at pH 9.4 (Fig. 2), and therefore, that the values of V and V/K for the *threo*-substrate would not correspond to those for the correctly protonated form of the enzyme. Thus, from Figure 2, corrections applied to Barker's data give a reduction for V/K of 3.3-fold. That is 33% of the enzyme would be in the correct form for processing the *erythro*-isomer and the pK_a value of the acid would be 0.48 pK units higher than the pH of the experiment (i.e. 10.24), which is close to the value of 10.1 estimated above. For the *threo*-substrate, the fall-off in activity with increasing pH is almost certainly assisted by the deprotonation of the conjugate acid which must be able to protonate the ammonia molecule after its elimination from the 2,3-diaminopropionic acid residue, thereby preventing it from re-aminating the nascent dehydroalanine residue. We ascribe this pK_a of ca. 10.1 to the conjugate acid of B-1 and tentatively suggest that the functional group could be the ε-ammonium group of a Lys residue in close proximity to the B-2 base, which we believe might be a Cys-357 thiolate. Interestingly we have isolated an active-site tryptic peptide (AIK) and an active-site chymotryptic peptide (DDQRAIKKGAGHDGF) containing the same Lys residue upon treatment with [^{14}C]-acetic anhydride. The side-chain modifications are completely protected by substrate. Further work will be required to determine the pK_a value for its modification. The only other modified peptide isolated from the active site upon treatment with [^{14}C]-acetic anhydride was the dipeptide LR. If potassium ion is not associated with the 1-carboxy group of the substrate, an Arg residue would be a

strong contender for its binding site. Full details of these protein modification experiments will be reported elsewhere.

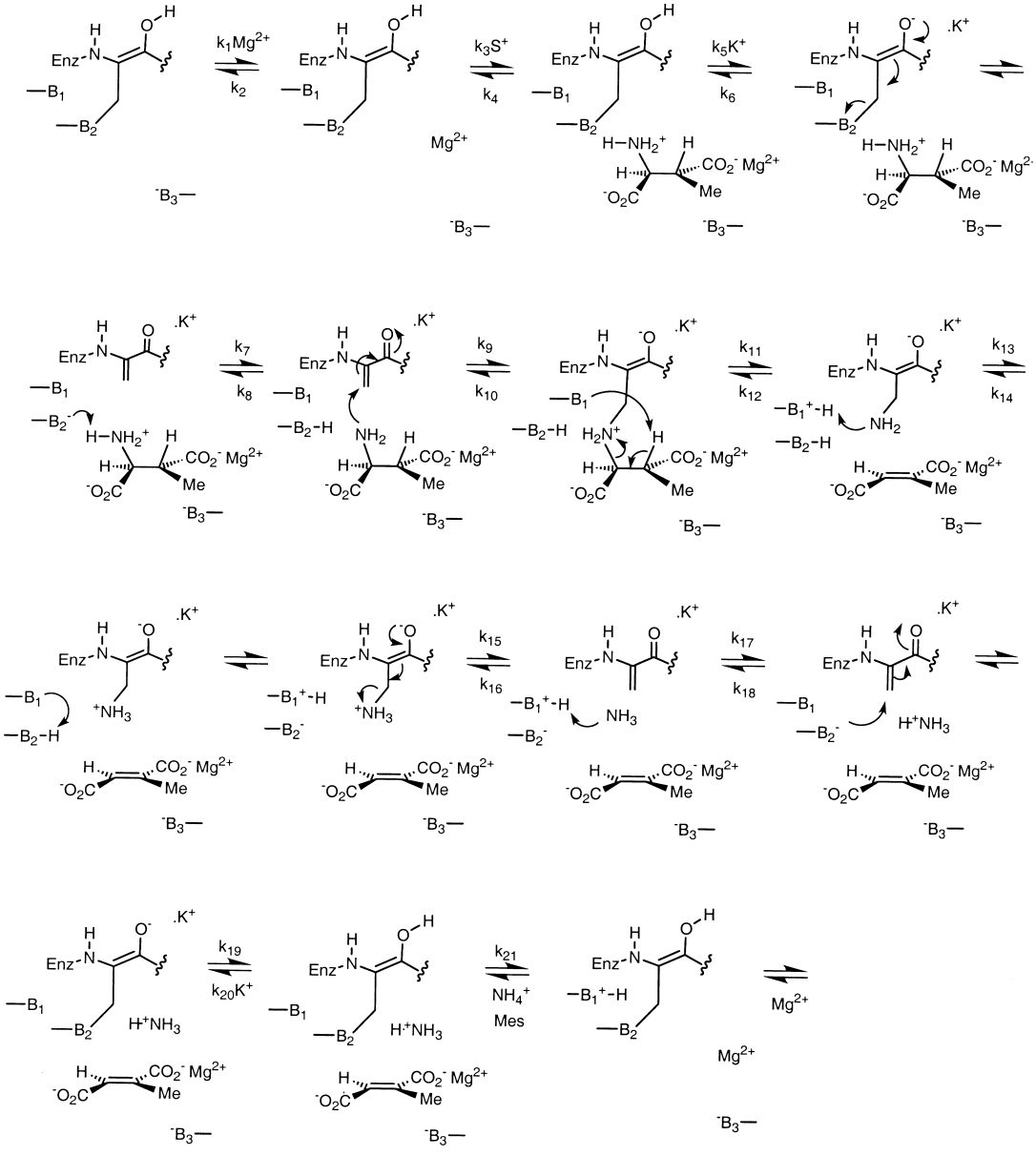
The explanation for the role of the acid–base group B-1 in the mechanism for both the *threo*- and *erythro*-methylaspartic acid substrates also explains why the V and V/K profiles for aspartic acid do not plummet above pH 9.4 (compare Figs 2 and 3). It is suggested that when B-1 is not protonated, the base can remove the 3-*pro*-S hydrogen from aspartic acid to give a *syn*-elimination similar to the *erythro*-diastereomer of methylaspartic acid (Scheme 12).

The results described here and the proposed mechanistic schemes are used to help interpret ^{15}N-isotope effects measured for (2*S*,3*S*)- and (2*S*,3*R*)-3-methylaspartic acid and (2*S*)-aspartic acid in the following article.

Experimental

Materials

Tris(hydroxymethyl)aminomethane (Tris), magnesium chloride hexahydrate and deuterium oxide (99.8 atom%) were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium chloride and ammonium chloride were obtained from British Drug Houses (Poole, Dorset, U.K.). (2*S*,3*R*)-[3-^{2}H$_1$]-Aspartic acid, (2*S*,3*S*)-3-methylaspartic acid and (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid were prepared as previously reported.[16,48] (2*S*,3*R*)-3-Methylaspartic acid and (2*S*,3*R*)-[3-^{2}H]-3-methylaspartic acid were prepared as described by Archer et al.[20] 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. Substrates, buffers, ammonium chloride and hydrated salts for use in rate determinations performed in deuterium oxide were separately pre-dissolved in deuterium



Scheme 12.

oxide and were lyophilised prior to use. The pD of all deuteriated buffer solutions was adjusted using either deuterium chloride solution or sodium deuteroxide.

Enzyme

The enzyme used in all kinetic experiments was purified from *Clostridium tetanomorphum* strain H1 (ATCC 15920), obtained from the American Typed Culture Collection, grown according to the method of Barker et al. using a modification of literature procedures[1,49] as previously described.[5,19] The specific activity of the enzyme used in these studies was at least 40–200 units (mg of protein)$^{-1}$. Irreversible inhibition studies using NEM and hydrazines and peptide mapping experiments were carried out using a polyhistidine-tagged recombinant enzyme expressed in *Escherichia coli* strain BL21 (DE3).[41] This protein differed to the wild type enzyme in possessing additional residues (MGHHHHHHHHHHSSGHIEGRHMLEDP) upstream and additional residues (YGSGC) downstream of the native sequence.[5] The recombinant enzyme was prepared in 2-L batches and purified as described below.

A 2-L culture of *E. coli* BL21 (DE3) was grown at 37 °C in Luria broth, to an OD_{600} of 0.6 prior to induction with 1 mM IPTG and then grown for a further 5 h at 37 °C. The cells were harvested by centrifugation for 20 min at 9000×*g* and stored at −20 °C. Frozen cell pellet was resuspended in 50 mM sodium phosphate buffer at pH 8.0 containing 50 mM imidazole, 100 mM NaCl, 1 mM PMSF and 1 mM 2-mercaptoethanol at a concentration of 1 g of cell paste/mL of buffer. Cell lysis was carried out by sonication (140 W, 20 kHz) in 10×30 s bursts at 4 °C and the cell debris removed by centrifugation for 30 min at 20 000×*g*. Cell free extract was applied to a Ni-chelate column (10×1.6 cm), pre-equilibrated with the purification buffer and washed with 2 column volumes of the purification buffer. A 0–1 M imidazole concentration gradient was then applied (10 column volumes) and collected as 5 mL fractions. 3-Methylaspartase activity (11 000 units, 126 u/mg) which was eluted in fractions 10–15 (300 mM imidazole concentration) was pooled and concentrated by ultra-filtration (Amicon ultra-filtration apparatus) under a 45 psi pressure of nitrogen. This was then dialysed overnight at 4 °C against 1 L of the purification buffer, retaining 50% activity. The dialyased pool was then applied to an anion exchange HPLC column (Bio-Cad HQ Poros 20 media, 10×3 mM) in aliquots of 1 mL and washed with 3 column volumes of the purification buffer. A 50–1000 mM NaCl concentration gradient was then applied (20 column volumes) where upon 3-methylaspartase activity was found in those fractions eluted with 200 mM NaCl concentration (4125 units, 257 u/mg). Analysis by silver stain 12% SDS-polyacrylamide gel electrophoresis showed the presence of a single protein band at 46 kDa. This protein showed very similar (but not identical) kinetic properties to the wild-type enzyme.[5,17,19]

Enzyme assay

Methylaspartase activity was assayed according to the method of Barker[1] where 1 unit of enzyme catalyses the formation of 1 μmol of mesaconic acid min^{-1} at pH 9.0 at 30 °C[17,19] as determined by the increase in OD_{240} under the assay conditions.

Determination of kinetic parameters

Deamination of (2*S*,3*S*)-3-methylaspartic acid and (2*S*)-aspartic acid. Experiments to determine V_{max} and K_m for the substrates, where pH and K^+ concentration were varied, were performed in the presence of 20 mM $MgCl_2$ as described previously.[19] Some of these determinations were repeated with (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid and (2*S*,3*R*)-[3-^{2}H]-aspartic acid. Experiments were also performed with each substrate using deuterium oxide as the solvent at pD values which corresponded to the pH values for those experiments conducted in water. pD values were determined using a standard pH electrode according to the equation pD−0.4 = pH.[44] The final deuterium content of all incubation solutions was estimated to be greater than 95 atom% and for all rate determinations the addition of the enzyme (typically 10–25 μL, in water, to a cuvette containing 2.8 mL of the buffered substrate solution) did not significantly reduce this figure. Experiments performed at pH 6.5 contained 0.1 M PIPES buffer, those performed at pH 7.6 contained 0.1 M Bis Tris propane, those performed at pH 9.0 and 9.4 contained 0.5 M Tris buffer and those performed at pH 9.5 and 10.0 contained 0.1 M CAPS buffer.

Amination of mesaconic and fumaric acid. Experiments to determine V_{max} and K_m for mesaconic acid were conducted at saturating concentrations of ammonium ion as described previously[17,18] at pH 6.5, 9.0 and 9.4. Experiments were also performed using deuterium oxide as the solvent at corresponding pD values. Deuteriated solutions of specific pD were prepared as described above. Similar experiments were performed using fumaric acid at pL 9.0 and 9.4.

Initial rate assays were conducted as described previously[17] and each rate measurement was carried out in triplicate. All incubations were performed at 30 ± 0.1 °C and reactions were followed directly spectrophotometrically at 240 nm, on a Pye-Unicam SP8-500 or Shimadzu 2101 instrument, under the stated conditions. Reactions were linear over the time course measured (up to 5% of total conversion). The initial rate data obtained was analysed by non-linear regression analyses using the computer program Enzfitter, by Leatherbarrow.[50] Values for V and K were obtained and primary deuterium isotope effects and solvent deuterium isotope effects for V and V/K were deduced.

C-3 Hydrogen exchange

Experiments were conducted to simultaneously measure the rate of deamination and C-3 solvent tritium exchange into (2*S*)-aspartic acid using protocols previously described for (2*S*,3*S*)-methylaspartic acid.[19] Incubations contained 0.1 M substrate, 0.5 M Tris (pH 9.0), 20 mM $MgCl_2$, 1.0 mM KCl and tritium oxide 1×10^9 dpm containing 5 units of enzyme in a total volume of 5 mL.

Reactions were started by the addition of enzyme, the extent of deamination was measured spectrophotometrically at 240 nm and the extent of exchange was measured by the periodic removal of aliquots of the incubation solution, acid denaturation, and lyophilisation to constant radioactivity, as described previously. The experiment was repeated using (2*S*,3*R*)-[3-^{2}H]-aspartic acid as the substrate.

Covalent modification of methylaspartase

Inhibition by hydrazines. Inhibition of 3-methylaspartase by hydrazines was performed by treating buffered enzyme solution (0.5 units in 50 mM Tris–HCl, pH 9.0) with various hydrazines and hydroxylamines including phenyl-, 4-nitrophenyl-, 2,4-dinitrophenyl-, methyl- and acetylhydrazine and unsubstituted hydrazine, over a concentration range of 0.01 to 1 mM. The solutions were incubated at 30 °C and aliquots (5 μL) were removed at regular time intervals and assayed for enzyme activity. The rate of inhibition followed biphasic first order kinetics and the initial faster rates were estimated by plotting the logarithm of the activity (relative to the activity at zero time) for early times in the inactivation process (up to 50% inactivation) against time and calculating the gradient of the best fit line. At pH 9.0 the inhibition reaction showed concentration dependent saturation kinetics and value of K_S for phenylhydrazine was calculated from the initial faster phase. The inhibition rate data was analysed using non-linear regression analysis.[50] The pH-dependence of the rate for the initial phase of the inactivation was determined using phenylhydrazine in 50 mM Tris buffer (pH 8.0–9.5) and 50 mM CAPS buffer at higher pH.

The effect on the rate of inactivation at pH 9.0 in 50 mM Tris buffer of potassium ions (250 mM) and ammonium ions (10, 20 and 40 mM) was also tested.

Inhibition by *N*-ethylmaleimide. Inhibition of 3-methylaspartase by *N*-ethylmaleimide was performed by treating buffered enzyme solution (0.5 units in 50 mM Tris–HCl, pH 9.0) with various amounts of *N*-ethylmaleimide to give final concentrations of 20 μM–0.5 mM. The solutions were incubated at 30 °C and aliquots (5 μL) were removed at regular time intervals and assayed for enzyme activity. The rate of inhibition followed first order kinetics and the rates were determined by plotting the logarithm of the activity (relative to the activity at zero time) against time and calculating the gradient of the best fit line.

The value of K_S for *N*-ethylmaleimide was determined in potassium phosphate buffer (pH 7.0, 5 mM) using the above methods over the concentration range 0–10 mM with and without 0.01 mM $MgCl_2$. The inhibition rate data was analysed using non-linear regression analysis.[49] The pH dependence of the inhibition reaction was studied using the above method in Tris–HCl buffer (50 mM) in 0.5 pH intervals over the range 7.0–10.0. An *N*-ethylmaleimide concentration of 0.5 mM was used for the experiments performed at pH 7.0 and 8.5 and a concentration of 0.02 mM was used at pH 8.5 and 10.0. CAPS buffer (50 mM) was used in experiments conducted at pH 9.75, 10.25 and 10.75 with a concentration of 0.01 mM *N*-ethlymaleimide.

The effect on the rate of inhibition of L-*threo*-(2*S*,3*S*)-3-methylaspartic acid and mesaconic acid was investigated using the methods described above in potassium phosphate buffer (pH 7.0, 5 mM) in the presence and absence of 0.01 mM $MgCl_2$. The concentration of both L-*threo*-(2*S*,3*S*)-3-methylaspartic acid and mesaconic acid was varied over the range 0–5 mM with 0.5 mM *N*-ethylmaleimide concentration. The intial rate of inhibition data was analysed by plotting reciprocal rate versus the concentration of either L-*threo*-(2*S*,3*S*)-3-methylaspartic acid or mesaconic acid.

Selective active site labelling

With 2,4-dinitrophenylhydrazine. To 2 mL of potassium phosphate buffer (50 mM, pH 9.0) containing 2 mg of 3-methylaspartase (500 units) was added 0.2 mL of a 50 mM solution of 2,4-dinitrophenylhydrazine in acetone (5 mM final concentration) and the solution was maintained at 30 °C. Aliquots (5 μL) were removed at regular time intervals and the enzyme activity determined using the standard assay, see above. After 45 h almost all of the original activity had been inactivated. The small amount of precipitate that had formed was removed by centrifugation for 5 min at 13 000×*g* and the supernatent solution was dialysed at 4 °C against two 1 L changes of water. The inactivated enzyme was subjected to digestion with trypsin as described below.

With *N*-[2-^{3}H]-ethylmaleimide. To 1 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 50 mg of 3-methylaspartase (12 500 units) was added 1 mL of a 1 M L-*threo*-(2*S*,3*S*)-3-methylaspartic acid solution (pH 7.8) to protect the active site. The solution was stored at 30 °C for 30 min prior to the addition of 0.1 mL of a 0.2 M *N*-ethylmaleimide solution (14 mM final concentration). After a further 30 min at 30 °C, 2-mercaptoethanol was added to a final concentration of 20 mM in order to remove any excess, unreacted *N*-ethylmaleimide. The solution was subsequently dialysed at 4 °C against 250 mL of 50 mM potassium phosphate buffer at pH 7.8 containing 5 mM L-*threo*-(2*S*,3*S*)-3-methylaspartic acid and then 2×500 mL of 50 mM, pH 7.8 potassium phosphate buffer and, finally, 500 mL of 10 mM potassium phosphate buffer at pH 7.0 containing 1 mM $MgCl_2$. To the product of dialysis, which had retained 95% of the original activity, was added 0.1 mL of a 40 mM solution of *N*-[2-^{3}H]-ethylmaleimide (14 mCi/mmol specific activity). A 10 μL aliquot was removed at regular time intervals and the enzyme activity was determined. After 60 min almost all activity had been lost. Excess unreacted inhibitor was removed by the addition of 2-mercaptoethanol to a final concentration of 1.25 mM and the solution was dialysed at 4 °C against two 500 mL changes 50 mM potassium phosphate buffer at pH 7.0 and four 1 L changes of water.

Proteolytic digestion of methylaspartase

An aqueous solution of methylaspartase or inactivated methylaspartase was adjusted to pH 7.8, denatured in a water bath at 100 °C for 15 min and then allowed to cool to room temperature. An aliquot of a 4 mg mL^{-1} solution of trypsin in 1 mM $CaCl_2$ solution was added, in an amount equal to 2.5% by weight of 3-methylaspartase. The pH of the solution was maintained at pH 7.8 by the addition of small aliquots of a 0.1 M NaOH solution. After 2 h at room temperature a second equivalent amount of trypsin was added and the solution incubated at room temperature for a further 2 h. The digestion reaction was then quenched by the addition of glacial acetic acid to pH 2–3 and any precipitated protein was removed by centrifugation at 13 000×g for 5 min. The supernatant solution was lyophilised and the white amorphous tryptic digest was stored at −20 °C.

HPLC Analysis of tryptic peptides

Methylaspartase labelled with 2,4-dinitrophenylhydrazine. The product of tryptic digestion was dissolved in 0.1 mL of water filtered through a Millipore filter and then analysed by reversed-phase HPLC using a Perseptive Bio-Cad HPLC system fitted with a 10 R2 POROS column (4×100 mM). The column was eluted with a gradient of 100% H_2O:TFA (99.9:0.1) to a 60:40 mixture of MeCN:TFA (99.95:0.05) and H_2O:TFA (99.9:0.1) over 20 column volumes. The eluent was monitored directly by UV spectroscopy at 220 and 360 nm collecting 0.75 mL fractions. Fraction 21 coincided with the major peak in absorbance at 360 nm (retention volume of 12.4 ± 0.3 column volumes) and was further purified by the same method (elution gradient from a 75:25 mixture of H_2O:TFA (99.9:0.1) and MeCN:TFA (99.95:0.05) to a 60:40 mixture of MeCN:TFA (99.95:0.05) and H_2O:TFA (99.9:0.1) over 20 column volumes). Fraction 36, which coincided with the major peaks in absorbance at both 220 and 360 nm, was concentrated under vacuum and further purified by a final microbore HPLC step (1 mM column). Two peaks in absorbance at 360 nm were observed and collected. These were submitted for N-terminal amino acid sequencing using a Procise Edman sequencer.

Methylaspartase labelled with *N*-[2-^{3}H]-ethylmaleimide. The product of tryptic digestion was taken up in 0.1 mL of water and analysed by reversed-phase HPLC using a Perseptive Bio-Cad HPLC system fitted with a C^{18} pep-map column (4×250 mM). The column was eluted with a gradient of 100% H_2O:TFA (99.9:0.1) to a 60:40 mixture of MeCN:TFA (99.95:0.05) and H_2O:TFA (99.9:0.1) over 15 column volumes. The eluent was monitored directly by UV spectroscopy at 220 nm and 0.75 mL fractions were collected, from which 50 μL aliquots were removed in order to determine the ^{3}H-incorporation by scintillation counting. The major peak in radioactivity was observed in fraction 28 (retention volume of 7.3 ± 0.3 column volumes) and was further purified by the same method (elution gradient from a 75:25 mixture of H_2O:TFA (99.9:0.1) and MeCN:TFA (99.95:0.05) to a 60:40 mixture of MeCN:TFA (99.95:0.05) and H_2O:TFA (99.9:0.1) over 20 column volumes). A single peak in radioactivity was located in fractions 25 and 26 (retention volume of 7.3 ± 0.6 column volumes) which coincided with one of 4 major peaks in absorbance at 220 nm. The 2 fractions were pooled and subjected to N-terminal amino acid sequencing. An aliquot of each cycle from the sequencer (about 100 μL) was retained and ^{3}H-incorporation determined by scintillation counting.

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