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The 3-Methylaspartase Reaction Probed using ^{2}H- and ^{15}N-Isotope Effects for Three Substrates: A Flip from a Concerted to a Carbocationic Amino-Enzyme Elimination Mechanism upon Changing the C-3 Stereochemistry in the Substrate from *R* to *S*

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

Abstract—The mechanisms of the elimination of ammonia from (2*S*,3*S*)-3-methylaspartic acid, (2*S*)-aspartic acid and (2*S*,3*R*)-3-methylaspartic acid, catalysed by the enzyme L-*threo*-3-methylaspartase ammonia-lyase (EC 4.3.1.2) have been probed using ^{15}N-isotope effects. The ^{15}N-isotope effects for V/K for both (2*S*,3*S*)-3-methylaspartic acid and aspartic acid are 1.0246 ± 0.0013 and 1.0390 ± 0.0031, respectively. The natural substrate, (2*S*,3*S*)-3-methylaspartic acid, is eliminated in a concerted fashion such that the C^{β}–H and C^{α}–N bonds are cleaved in the same transition state. (2*S*)-Aspartic acid appears to follow the same mechanistic pathway, but deprotonation of the conjugate acid of the base for C-3 is kinetically important and influences the extent of ^{15}N-fractionation. (2*S*,3*R*)-3-Methylaspartic acid is deaminated via a stepwise carbocationic mechanism. Here we elaborate on the proposed model for the mechanism of methylaspartase and propose that a change in stereochemistry of the substrate induces a change in the mechanism of ammonia elimination.

Introduction

The enzyme 3-methylaspartase (3-methylaspartate ammonia-lyase, EC 4.3.1.2) catalyses the reversible elimination of ammonia from a number of acidic amino acids,[1] including the natural substrate L-*threo*-(2*S*,3*S*)-3-methylaspartic acid, its diasteroisomer L-*erythro*-(2*S*,3*R*)-3-methylaspartic acid and (2*S*)-aspartic acid (Scheme 1).[2,3] In common with phenylalanine and histidine ammonia-lyase (other members of the true ammonia-lyase family of enzymes), the mechanism of methylaspartase is thought to proceed via a covalent enzyme–substrate complex involving an electrophilic dehydroalanine prosthetic group.[4,5] For methylaspartase it has been proposed that the dehydroalanine residue results from a post-translational dehydration of Ser-173.[6] In the article immediately preceding this paper we reported on the results of deuterium and solvent isotope effect experiments along with studies of the irreversible inhibitors *N*-ethylmaleimide and a range of hydrazine analogues, designed to probe the mechanism and active site structure of the enzyme.[7]

It was shown that at pH 9.0, or lower, the true-substrates were protonated on nitrogen and that, following the binding of the cofactor K^{+} ion, the alkylammonium groups were deprotonated in a solvent-excluded pocket by an active site base (B-2), Scheme 2. Subsequently, it was proposed that the addition of the alkylamino group to the β-(C-3)-methylene moiety of a dehydroalanine resulted in the formation of a covalent enzyme–substrate adduct. With the natural substrate, it was further suggested that the antiperiplanar elimination of ammonia occurred through the removal of the C-3 proton by the base B-3, to give enzyme-bound mesaconic acid and a 2,3-diaminopropionic form of the enzyme. It was also proposed that protonation of the 3-amino group of the 2,3-diaminopropionic acid residue (by the conjugate

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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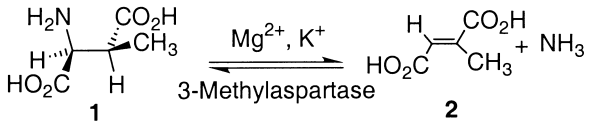
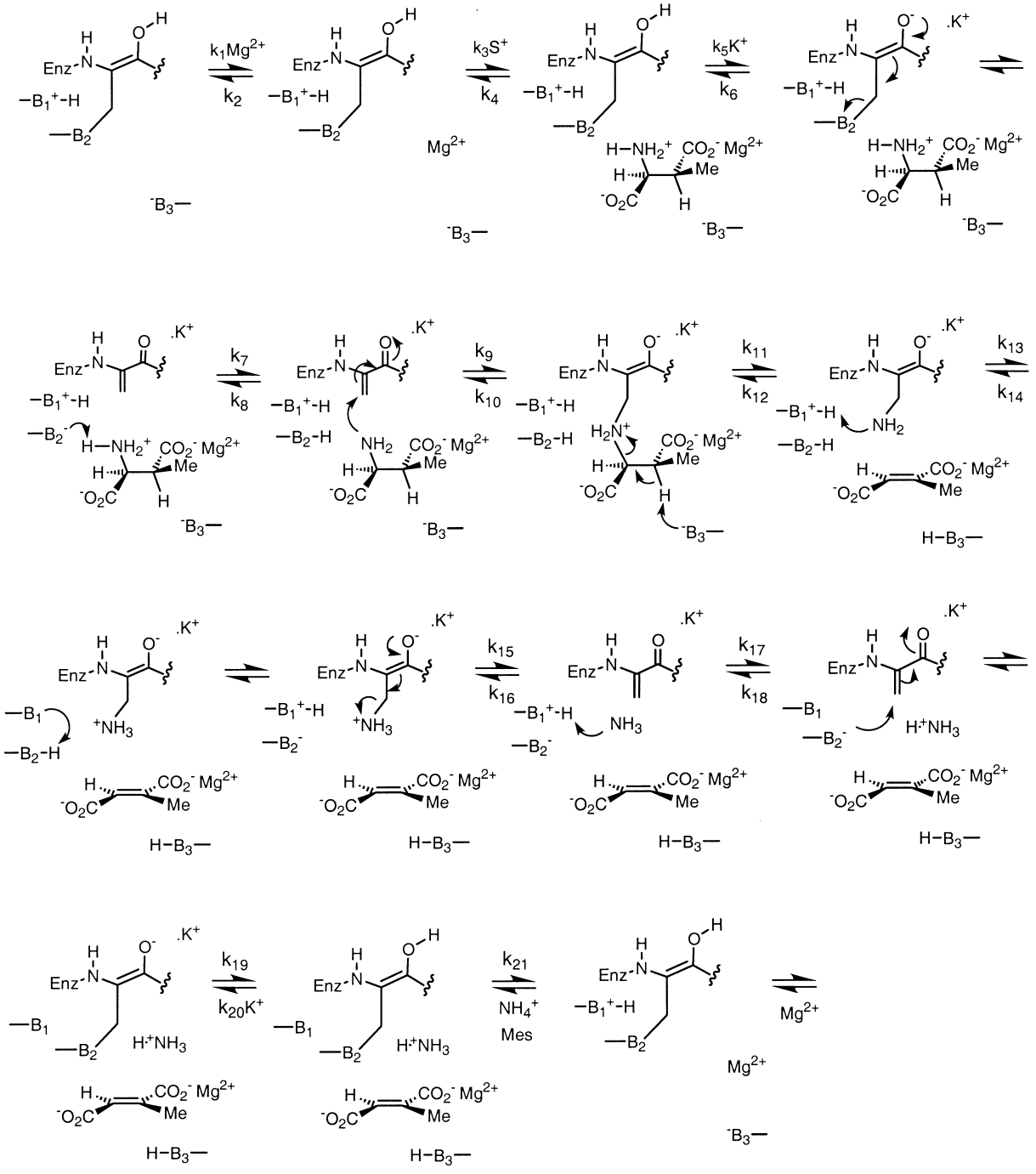


Scheme 1.

acid of B-1) facilitated the elimination of ammonia, allowing the re-formation of the dehydroalanine prosthetic group and the formation of a bound molecule of ammonia. Following the protonation of ammonia, it appeared that a potassium ion was released from the active site to allow the non-ordered release of an ammonium ion and mesaconic acid.

The group-specific chemical-modifying agent *N*-ethylmaleimide (NEM) was found to be an irreversible inhibitor ($K_S = 34.5\,mM$, $k_{inact} = 5\,min^{-1}$ in sodium phosphate buffer at pH 9.0) in accord with the finding of Wu and Williams.[8] Incubation of the enzyme with substrate and the inhibitor, followed by dialysis, gave an active modified protein which was subsequently inactivated with labelled NEM (*N*-[2-^{3}H]-ethylmaleimide). A radiolabelled peptide was isolated (Ala-350–Arg-366) and the site of modification was determined to



Scheme 2.

be Cys-361.[7] In addition a second cysteine (Cys-357), present in the only modified peptide, was shown to be protected throughout the NEM inactivation reaction. The rate of inactivation by NEM was enhanced in the presence of the cofactor, Mg^{2+}, ions and protection from inactivation was effected by either the substrate or phosphate dianion. Further considerations led to the proposal that Cys-361 might serve as the base (B-3) for removal of the C-3 proton from the natural substrate. A detailed analysis of primary deuterium isotope effects[7,9,10] indicated that the removal of the C-3 proton occurred at equilibrium and required a base possessing a low fractionation factor of ~0.5–0.6.[10–12] It was noted that the result was consistent with a thiolate serving as the base responsible for removal of the C-3 proton from the natural substrate, further supporting the proposed role of Cys-357.[7]

Hydrazines, in the presence of fumaric acid derivatives, act as alternative substrates in the reverse reaction direction, however in their absence they lead to irreversible inactivation.[6,14,15] HPLC analysis of a tryptic digest for nitrophenyl hydrazine inhibited enzyme, showed the presence of a single band in UV absorbance at 360 nm. Further analysis of the HPLC by Edman degradation showed the presence of three peptide sequences, consistent with a bis-disulphide linked tripeptide comprising Gly-299–Lys-366 (GVDAELVADEWCNTVEDVK); Ala-350–Arg-366 (ANGMGAYCGGTCNETNR) and Ser-367–Arg-381 (SAEVTTNIGMACGAR). In the absence of hydrazines no disulphides were formed.[7,8] In order to explain the protection of Cys-357 from NEM inhibition and the presence of disulphides with hydrazines, it was suggested that the enzyme, in the absence of substrate, exists in a closed protected form. Under such circumstances Cys-357 could, reversibly, form an internal thioether cross-link with the dehydroalanine prosthetic group, thereby, protecting it from modification by NEM. The first step in the reaction scheme would then require cleavage of the thioether cross-link, presumably accompanied by a conformational change and substrate binding. This would give a free dehydroalanine Michael acceptor and an active site thiolate anion which, it was proposed, might be able to serve as the base (B-2), responsible for removal of a proton from the (now bound) *N*-protonated substrate. To account for the formation of disulphides it was suggested that hydrazines were capable of oxidising one of the Cys residues in Ala-350–Arg-366 to give an R-N-S-R′ species, in which the N-atom could be displaced by *S*-nucleophiles from other Cys residues, to result in the formation of the observed bis-disulphide tripeptide.[7]

The natural (2*S*,3*S*)-enantiomer of the substrate is known to undergo exchange at C-3 with the bulk solvent at a rate greater than that for deamination under certain conditions,[10] however the disatereoisomer, L-*erythro*-(2*S*,3*R*)-3-methylaspartic acid, does not display C-3 hydrogen exchange.[2,16] These findings prompted an investigation into the kinetic significance of C^{α}–N bond cleavage for both diastereomers. Here we describe the results of ^{15}N-fractionation studies designed to probe C^{α}–N bond cleavage in three different substrates and to detect $^{15}N/^{14}N$ isotope fractionation-sensitive chemical steps that might be involved in formation of the covalent enzyme–substrate complexes.

Results

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

The fraction of the reaction and the $^{15}N/^{14}N$-atom ratios in the substrate and in the product used for the calculation of $^{15}(V/K)$ and $^{15}(V/K)_D$ for (2*S*,3*S*)-3-methylaspartic acid in, one experiment at pH 9.0, in water, are given in Table 1. The deduced ^{15}N-isotope effects are given in Table 2, where each entry represents the average of at least three experiments/isotope ratio determinations, see Experimental Section. The nitrogen isotope effects are all for V/K where the trailing subscript refers to the isotope of hydrogen at C-3 in the substrate (H or D) and in the solvent, where S refers to the use of deuterium oxide. At pL 9.0 $^{15}(V/K)$ for the unlabelled substrate, $^{15}(V/K)_H$, is large at 1.0246 ± 0.0013 and the value of $^{15}(V/K)_D$ for the C-3 deuteriated substrate is almost identical. In deuterium oxide $^{15}(V/K)_{HS}$ is 1.0197 ± 0.0014 and is suppressed compared to $^{15}(V/K)_H$. However, $^{15}(V/K)_{DS}$ for the deuteriated substrate is larger than $^{15}(V/K)_{HS}$ indicating that the presence of deuterium at C-3 increases the importance of a nitrogen sensitive step in the presence of deuterium oxide. At pL 9.4 all of the $^{15}(V/K)$ values are suppressed relative to the sizes at pL 9.0, see Figure 1, but other kinetic differences are also apparent. In water at pL 9.4 the small value of $^{15}(V/K)_H$ increases upon the introduction of deuterium at C-3. In deuterium oxide $^{15}(V/K)_{HS}$ is larger than $^{15}(V/K)_H$ indicating possibly that the motion of a solvent derived hydrogen atom is associated with a transition state involving bond cleavage/formation with the heavy atom, see discussion below. There is no large effect on the nitrogen fractionation upon introducing deuterium at C-3 at pL 9.4 and the value of $^{15}(V/K)_{DS}$ is the same as that for $^{15}(V/K)_{HS}$ within the error of the experiments.

Table 1. Full data for $^{15}(V/K)$ isotope effect determinations for the deamination of (2*S*,3*S*)-3-methylaspartic acid and (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid in water at pH 9.0[a]

Substrate	Concentration (mM)	f[b]/%	R	$^{15}(V/K)$ Isotope effect
H-Me Asp	0.17	22.6	0.3641	1.02430
($R_0 = 0.37180$)	0.20	18.0	0.3633	1.02607
	0.17	24.0	0.3644	1.02355
				Average = 1.0246 ± 0.0013
D-MeAsp	0.17	17.8	0.3611	1.02530
($R_0 = 0.36937$)	0.20	23.4	0.3619	1.02365
	0.17	16.9	0.3619	1.02330
	0.17	23.0	0.3617	1.02423
				Average = 1.0241 ± 0.0009

[a]Incubations (10 mL) carried out at 30 ± 0.1 °C in 0.5 M Tris (pH 9.0) with 0.02 M $MgCl_2$, 0.001 M KCl and substrate.
[b]Measured as increase in optical density at 240 nm.

Table 2. Nitrogen isotope effects for the deamination of (2*S*,3*S*)-3-methylaspartic acid

Solvent	Substrate	pL	[K^+]	Isotope effect
1. H_2O	H-MeAsp	9.0	1.0	$^{15}(V/K)_H = 1.0246 \pm 0.0013$
2. H_2O	D-MeAsp	9.0	1.0	$^{15}(V/K)_D = 1.0241 \pm 0.0009$
3. D_2O	H-MeAsp	9.0	1.0	$^{15}(V/K)_S = 1.0197 \pm 0.0014$
4. D_2O	D-MeAsp	9.0	1.0	$^{15}(V/K)_{SD} = 1.0274 \pm 0.0006$
5. H_2O	H-MeAsp	9.0	50.0	$^{15}(V/K)_H = 1.0222 \pm 0.0022$
6. H_2O	D-MeAsp	9.0	50.0	$^{15}(V/K)_D = 1.0150 \pm 0.0013$
7. H_2O	H-MeAsp	9.4	1.0	$^{15}(V/K)_H = 1.0032 \pm 0.0001$
8. H_2O	D-MeAsp	9.4	1.0	$^{15}(V/K)_D = 1.0051 \pm 0.0010$
9. D_2O	H-MeAsp	9.4	1.0	$^{15}(V/K)_S = 1.0105 \pm 0.0049$
10. D_2O	D-MeAsp	9.4	1.0	$^{15}(V/K)_{SD} = 1.0078 \pm 0.0027$
11. H_2O	H-MeAsp	6.5	1.0	$^{15}(V/K)_H = 1.0255 \pm 0.0014$
12. H_2O	D-MeAsp	6.5	1.0	$^{15}(V/K)_D = 1.0417 \pm 0.0010$

At pL 9.0 and in the presence of a high concentration of potassium ion (50 mM), the value of $^{15}(V/K)$ of 1.0222 is similar to that at 1 mM K^+ ion but is depressed when deuterium is present at C-3, to a value of 1.0150. This result suggests that the deuterium atom can also exert an effect on a step other than one which causes a fractionation of the heavy atom, see discussion below.

At pL 6.5 where solvents isotope effects are now known to be small,[7] $^{15}(V/K)_H$ is 1.0255 ± 0.0011 in water. The C-3 deuteriated substrate gives a value for $^{15}(V/K)_D$ of 1.0417 ± 0.0010 clearly indicating that the nitrogen fractionation under these conditions occurs in a deuterium sensitive step.

(2*S*)-Aspartic acid

The deduced ^{15}N-isotope effects for (2*S*)-aspartic acid are given in Table 3. At pL 9.0 in water $^{15}(V/K)_H$ for the unlabelled substrate is large at 1.0390 ± 0.0031 and the value of $^{15}(V/K)_D$ for the C-3 deuteriated substrate is

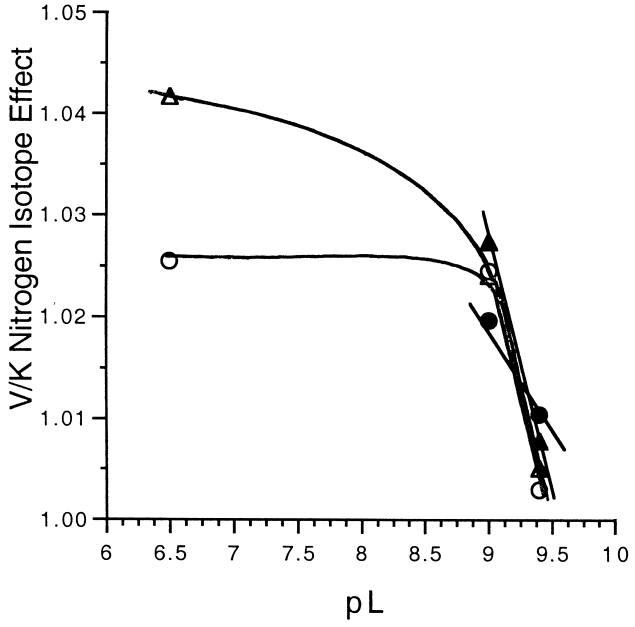


Figure 1. pL Dependence of ^{15}N-isotope effects on pL for the deamination of (2*S*,3*S*)-3-methylaspartic acid. $^{15}(V/K)_H$ (○) and $^{15}(V/K)_D$ (△) in water and $^{15}(V/K)_S$ (●) and $^{15}(V/K)_{SD}$ (▲) in deuterium oxide.

Table 3. Nitrogen isotope effects for the deamination of aspartic acid

Solvent	Substrate	pL	Isotope effect
1. H_2O	H-Asp	9.0	$^{15}(V/K)_H = 1.0390 \pm 0.0031$
2. H_2O	D-Asp	9.0	$^{15}(V/K)_D = 1.0240 \pm 0.0023$
3. D_2O	H-Asp	9.0	$^{15}(V/K)_S = 1.0168 \pm 0.0057$
4. D_2O	D-Asp	9.0	$^{15}(V/K)_{SD} = 1.0255 \pm 0.0035$
5. H_2O	H-Asp	9.4	$^{15}(V/K)_H = 1.0213 \pm 0.0044$
6. H_2O	D-Asp	9.4	$^{15}(V/K)_D = 1.0210 \pm 0.0018$

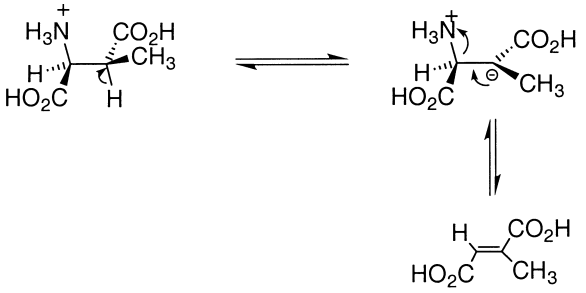
smaller at 1.024 ± 0.0023. This indicates that a deuterium sensitive step does not occur on the same step (or steps) which cause the significant heavy atom fractionation. Note that the substrate does not display primary deuterium isotope effects upon V or V/K under these conditions. In deuterium oxide $^{15}(V/K)_{HS}$ is 1.0197 ± 0.0014 and is suppressed compared to $^{15}(V/K)_H$ (1.0213 ± 0.0044). However, $^{15}(V/K)_{DS}$ is larger than $^{15}(V/K)_{HS}$ indicating that in deuterium oxide the fractionation of the heavy atom is greater in the presence of deuterium at C-3 of the substrate. At pL 9.4 in water $^{15}(V/K)_H$ is 1.0213 ± 0.044 and is reduced compared to its value at pL 9.0, as is the case for the natural substrate. $^{15}(V/K)_D$ (1.021 ± 0.0023) possesses a similar value and no change in the fractionation of the heavy atom is caused by the introduction of deuterium at C-3.

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

The deduced ^{15}N-isotope effects for (2*S*,3*R*)-3-methylaspartic acid at pL 9.0 in water are $^{15}(V/K) = 1.0050 \pm 0.0023$ for the unlabelled substrate and $^{15}(V/K)_D = 1.0058 \pm 0.0028$ for the C-3 deuteriated substrate which are the same within experimental error. The slightly larger value of $^{15}(V/K)_D$ might be interpreted to indicate that the deuterium sensitive step occurs on the same step that causes heavy atom fractionation and is rate limiting, because the substrate displays very large primary deuterium isotope effects upon V or V/K under these conditions. However the absolute values of $^{15}(V/K)$ are too low to be consistent with a concerted mechanism, see discussion below.

Discussion

Aspartase and phenylalanine ammonia-lyase have been shown to operate via a stepwise carboanionic mechanism in which the removal of the 3-pro-S hydrogen occurs before C–N bond cleavage,[17] Scheme 3. Previously we showed that methylaspartase displayed a primary deuterium isotope effect for removal of the C-3 proton from both (2*S*,3*S*)-3-methylaspartic acid and (2*S*,3*S*)-3-ethylaspartic acid but not for (2*S*)-aspartic acid.[9,18] We further showed that for (2*S*,3*S*)-3-methylaspartic acid the fractionation of $^{15}N/^{14}N$ increased very significantly upon the introduction of deuterium at C-3 at low pH and interpereted this result in favour of a concerted mechanism.[19] At the time we were unaware that the enzyme might operate via a covalent amino-enzyme intermediate but were aware that a simple elimination mechanism could not account for all of the observed kinetic properties of the system. Unaccounted



Scheme 3.

for, or, poorly accounted for features included the observation of solvent hydrogen exchange into the C-3 position of the substrate which varied with pH differently to the rate of deamination; the pH independence of the primary isotope effects for exchange and deamination; and the parallel line patterns obtained for double reciprocal plots of initial rate versus substrate concentration at different Mg^{2+} ion concentrations.[10] Moreover, only at low pH did the introduction of deuterium at C-3 of the substrate lead to a significant increase in the extent of $^{15}N/^{14}N$ fractionation.[19] The further finding that hydrazines could irreversibly inactivate the enzyme prompted the consideration of more complex mechanisms involving a dehydroalanine residue and the formation of covalent amino-enzyme intermediates.[6,14,15,20] In the previous article it was shown that a substituted enzyme mechanism is able to accommodate many of the properties of the system that did not fit in with simple elimination mechanisms, see Scheme 2. However, it was also evident that the added complexity of the substituted enzyme mechanism which also incorporates steps for the deprotonation of the electrophilic dehydroalanine residue and reformation of an internal thioether linkage (Scheme 2), precluded a simple interpretation as to upon which steps both primary deuterium and solvent isotope effects are expressed. Here we use nitrogen isotope effects to probe the processing mechanisms in an attempt to construct a unified scheme for the reaction sequence and gain an understanding of the 3-D environment of the active site.

^{15}N-Isotope effects

(2*S*,3*S*)-3-Methylaspartic acid. The ^{15}N-isotope effects for V/K observed for (2*S*,3*S*)-3-methylaspartic acid are given in Table 2. Interpretation of the data is extremely difficult because V/K isotopes provide information on the reaction mechanism from the time the labelled substrate binds up until the time that the first irreversible step occurs, usually release of the first product. For methylaspartase we have shown that the products are released late in the reaction sequence and, apparently, at the same time.[10] The enzyme operates by a complex substituted amino-enzyme mechanism where no less than six of the steps, leading to the release of the products, involve making or breaking bonds with the N-atom of the amino group derived from the substrate.[7] Moreover, it is evident from the discussion in the preceding article that the reaction displays significant solvent hydrogen exchange activities with the C-3 proton of the substrate and appears to catalyse all of its chemistry below pH 9.0 in a closed, solvent inaccessible form. It is, therefore, apparent that, under such conditions, the dissociation of the products from solvent hydrogen-exchanged central complexes is slower than partitioning through reverse steps, resulting in the release of solvent-exchanged substrate.[7] Hence, with the natural substrate, the reaction will show very large reverse commitments to the step for the removal of the C-3 proton and/or the C^{α}–N bond cleavage step in the substrate, regardless of whether the actual chemical mechanism is concerted or stepwise.

The interpretation of the ^{15}N-isotope effect data is made even more difficult by the fact that intrinsic ^{15}N-kinetic isotope effects ($k_{(14N)}/k_{(15N)} = {}^{15}k$), are of the same magnitude as those for the ^{15}N-equilibrium isotope effects ($^{15}k_{For}/^{15}k_{Rev} = {}^{15}K_{eq}$), in contrast to those for deuterium which show much larger kinetic isotope, rather than equilibrium isotope, effects. However, by using ^{15}N-isotope effect data in conjunction with pH variation, alternative substrates and the use of deuterium isotope effects (double isotope fractionation) it is possible to gain further information on the system without necessarily quantifying every single rate constant in the rate equation. Double isotope fractionation experiments have proved to be extremely powerful in unravelling the complexities of multi-step reactions[17,21–23] although interpretations are usually simpler than for the methylaspartase system because fewer steps report on the heavy isotope fractionation.

The ^{15}(V/K) isotope effect for the simplified concerted mechanism (Scheme 2), developed in the previous article, is given by eq (1).

$$\begin{aligned}{}^{15}(V/K) = \{ & {}^{15}k_{11}\,{}^{15}K_7\,{}^{15}K_9 + [(k_{11}/k_{10})({}^{15}k_9 + (k_9/k_8) \\ & ({}^{15}k_7 + (k_7/k_6)(1 + k_5K^+/k_4)))] \\ & + [{}^{15}K_7\,{}^{15}K_9\,{}^{15}K_{11}(k_{12}/k_{13}) \\ & ({}^{15}k_{13} + {}^{15}K_{13}(k_{14}/k_{15})({}^{15}k_{15} + {}^{15}K_{15}(k_{16}/k_{17}) \\ & ({}^{15}k_{17} + {}^{15}K_{17}(k_{18}/k_{19}) \\ & (1 + k_{20}K^+/k_{21}))))]\}/(1 + C_f + C_r)\end{aligned} \quad (1)$$

where $C_f = (k_{11}/k_{10})(1 + (k_9/k_8)(1 + (k_7/k_6)(1 + k_5K^+/k_4)))$, and where $C_r = (k_{12}/k_{13})(1 + (k_{14}/k_{15})(1 + (k_{16}/k_{17})(1 + (k_{18}/k_{19})(1 + k_{20}K + /k_{21}))))$.

For $^{15}(V/K)_D$, k_{11} in the numerator and denominator of eq (1) should be divided by the intrinsic deuterium isotope effect for C-3 deprotonation, $^{D}k_{11}$. In addition, k_{12} in the numerator and denominator of eq (1) should be divided by the intrinsic deuterium isotope effect for C-3 deprotonation, and multiplied by the deuterium equilibrium isotope effect, $^{D}K_{eq}$.[21–23]

The equation assumes that the enzyme amination/deamination steps are not associated with coupled protonation/deprotonation and also that the hydrogen that is removed by base B-3 (to give the conjugate acid as was discussed in the preceding article) does not display an

isotope effect for deprotonation. The latter issue and the influence of possible conformational changes are considered below.

The term $^{15}k_{11}{}^{15}K_7{}^{15}K_9$ should be normal and approximately equal to $^{15}k_{11}$. The value of $^{15}k_{11}$ must be normal and therefore larger than $^{15}K_{11}$, which is the equilibrium isotope effect for the deamination of the substrate‡ and should possess a value of 1.033 as deduced by Hermes et al.,[17] see Figure 2. $^{15}K_7$ is the equilibrium isotope effect for deprotonation of the amino group of the substrate and, by analogy to the phenylalanine ammonia-lyase system, should possess a value of 1.0167.[17] $^{15}K_9$ is the equilibrium isotope effect for amination of the dehydroalanine residue and should be inverse with a value of 0.979.[17] Thus, $^{15}K_7{}^{15}K_9$ should be very close to unity and equal to 0.995. The rates of hydrogen exchange, which are discussed in the preceding paper, indicate that the reverse commitment is much larger than C_f, therefore, we will assume that at low K^+ ion concentration, the influence of C_f is small and insignificant. The terms in C_r, however, are both complex and significant. The product $^{15}K_7{}^{15}K_9{}^{15}K_{11}$ multiplies all terms in C_r in the numerator of eq (1) and should possess a value of 1.027.

The ratio k_{12}/k_{13} may be less than 1.0 because step 13 is an amino-enzyme protonation step. The intrinsic isotope effect $^{15}k_{13}$ should be just normal (~1.005), see discussion below, but the equilibrium isotope effect $^{15}K_{13}$ would be inverse (0.984).[17] Thus, if the deprotonation of the product, represented by k_{14}, was much larger than the rate constant k_{15} for the elimination of ammonia (to regenerate the dehydroalanine moiety), the value of $^{15}(V/K)$ would be suppressed. The intrinsic isotope effect $^{15}k_{15}$ would be large and normal and greater than the equilibrium isotope effect ($^{15}K_{15}$) for the step (1.033). However, step 16 Scheme 2 (re-amination of the enzyme), would not be expected to be faster than the protonation of ammonia, step 17, so that the contribution of $^{15}K_{15}$ could be quite small. Finally, the value of $^{15}k_{17}$ for the protonation of ammonia would be ~1.000 while the value of $^{15}K_{17}$ is known to be strongly inverse at ~0.980,[17] see Fig 2. If either step 18, the deprotonation of the ammonium ion is faster than the release of K^+ ion (step 19), which is surely the case, or the rebinding of K^+ ion ($k_{20}K^+$) is faster than the release of the first product (k_{21}), (which it must be at high K^+ concentration) the influence of the inverse equilibrium isotope effect, $^{15}K_{17}$, would be large. Thus, one might expect to observe small normal values, or even inverse values, for $^{15}(V/K)$ if both $^{15}K_{13}$ and $^{15}K_{17}$ dominate in the term for C_r in the numerator of eq (1).

‡The equilibrium isotope effect $^{15}K_{eq}$ for the elimination of ammonia from a protonated monoalkylammonium ion to give an alkene and neutral ammonia is 1.033.[17] This value contains two components, one for dealkylation of the N-atom to give ammonium ion $^{15}K_{Dealk} = 1.0138$ (determined for the Asp⇌Fumarate + NH_4^+ equilibrium[17]) and one for the deprotonation of ammonium ion $^{15}K_{Deprot} = 1.019$.[17] Hermes et al. found that $^{15}K_{eq}$ for the deprotonation of the N-atom from phenylalanine was 1.0167[17] which indicates that the dealkylation of a neutral monoalkylamine should possess an equilibrium isotope effect of 1.0161, see Figure 2.

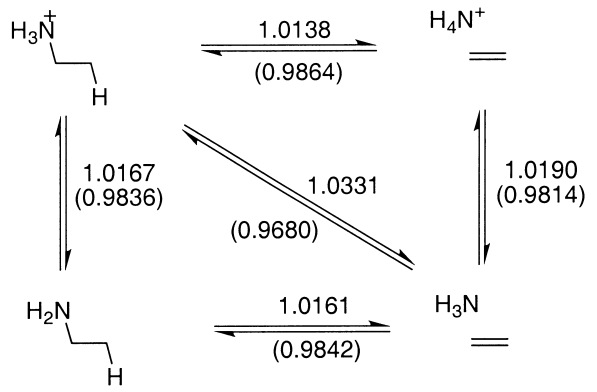


Figure 2. Summary of ^{15}N equilibrium isotope effects[17] used to estimate the importance of chemical steps in the methylaspartase system. Values for reverse steps are given in parenthesis.

Four "probes" can be used to interrogate the importance of terms in C_r. First, the effect of introducing a deuterium atom at C-3 can be examined, as alluded to above. Second, deuteriated solvent can be used. Third, the concentration of the monovalent metal ion activator can be varied; and fourth, alternative substrates can be employed. In addition, the pL of the solutions can be varied and the probes can be used in combination, where appropriate.

The observed ^{15}N-isotope effects for (2*S*,3*S*)-3-methylaspartic acid, Table 2, show that at pL 9.0 $^{15}(V/K)$ for the unlabelled substrate ($^{15}(V/K)_H$) is large at 1.0246 ± 0.0013. The value of $^{15}(V/K)_D$ is identical, within experimental error, which might be expected to indicate that the reaction mechanism is not step-wise carbanionic or carbocationic. If this were not the case, the presence of deuterium would be expected to increase the reaction commitments to all of the ^{15}N-sensitive steps and reduce the observed value of $^{15}(V/K)$. For a concerted reaction where the transition state for C–H bond cleavage is the same as for C–N bond cleavage, the value of $^{15}(V/K)_D$ should increase, unless the step is already cleanly rate determining.[21] This is clearly not the case for the *threo*-substrate where ^DV and $^D(V/K)$ are 1.7 and are dominated by large reverse reaction commitments to the deuterium sensitive step which should be identical to those defined for C_r in eq (1). Thus, there appear to be two effects due to the presence of deuterium which balance under the reaction conditions. One which enhances ^{15}N-fractionation and a second effect which suppresses ^{15}N-fractionation and which must occur on a non-^{15}N-sensitive step, see below.

In deuterium oxide at pL 9.0, $^{15}(V/K)_S$ is 1.0197 ± 0.0014 and is slightly suppressed in value compared to $^{15}(V/K)_H$. For a concerted reaction, the result is consistent with the observed reduction of the size of the C-3 primary isotope effects in deuterium oxide [$^DV_S \sim {}^D(V/K)_S = 1.0$] compared to that in water [$^DV = {}^D(V/K) = 1.7$] as was shown in the preceding article. Together the results indicate that the solvent deuterium isotope effects are expressed on steps other than those

associated with nitrogen fractionation. Thus, the amination of the enzyme by the substrate and the deamination of the amino enzyme appear to be step-wise processes. If they were concerted, the value of $^{15}(V/K)_S$ should increase because the deuterium solvent isotope effects at pL 9.0 are significant at 3.11 for ^{D}V and 3.46 for $^{D}(V/K)$.[7] $^{15}(V/K)_{SD}$ for the deuteriated substrate is larger than $^{15}(V/K)_{SH}$ indicating that the presence of deuterium at C-3 increases the importance of a nitrogen sensitive step (step 11) in the presence of deuterium oxide. Thus, the influence of the second effect which suppresses the size of the increase in ^{15}N-fractionation in the presence of a C-3 deuterium atom is decreased in deuteriated solvent.

At pL 9.4 all of the $^{15}(V/K)$ values are suppressed relative to the sizes at pL 9.0. This observation is in accord with the removal of the term $^{15}K_7$ for the deprotonation of the substrate in eq (1) as, in this case, the unprotonated amino group of the substrate adds directly to the dehydroalanine residue. Since C_f is small compared to C_r, the reduction in the size of $^{15}(V/K)$ at pL 9.4 should be substantial, as is observed (see Fig. 1), since the term is a component of C_r. It is interesting to note that very similar effects in the reduction of the values of $^{15}(V/K)$ with increasing pH, were observed for the phenylalanine ammonia-lyase system, although the sizes of the ^{15}N-isotope effects were somewhat smaller.[17]

For methylaspartase, other kinetic differences are also apparent upon increasing the reaction pL from 9.0 to 9.4. In water the small value of $^{15}(V/K)_H$ increases upon the introduction of deuterium at C-3, which is as expected for a concerted reaction. This result is also consistent with results discussed in the preceding article which show that although the solvent isotope effect for V/K was suppressed (relative to that for V) at pL 9.4, the magnitude of the primary deuterium isotope effect $^{D}(V/K)$ was not.[7] As noted above in the Results section, the effect upon the nitrogen fractionation of introducing deuterium at C-3 is small and the values of $^{15}(V/K)_{SD}$ and $^{15}(V/K)_{SH}$ are the same within the error of the experiments.

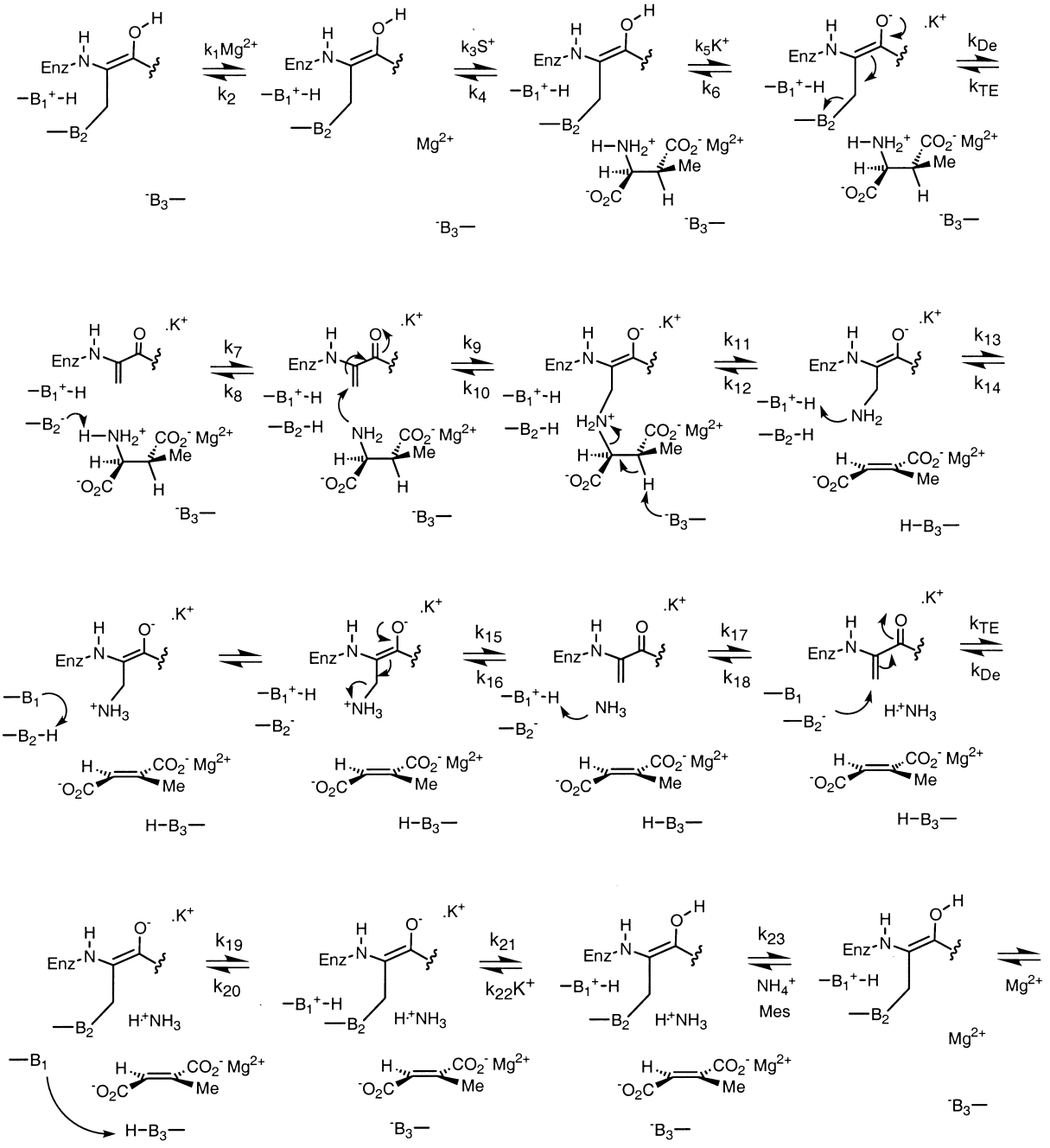
At pL 6.5 where the activity of the enzyme is low and both V and V/K are small at 1 mM K^+ ion,[7,10] $^{15}(V/K)_H$ is 1.0255 ± 0.0011 in water. The C-3 deuteriated substrate gives a value for $^{15}(V/K)_D$ of 1.0417 ± 0.0010 clearly indicating that the nitrogen fractionation under these conditions occurs in a deuterium sensitive step. Indeed, collectively all of the $^{15}(V/K)$ isotope effects are consistent with the concerted elimination of the amino-enzyme from the covalent enzyme–substrate complex since the introduction of deuterium at C-3 maintains or increases the extent of ^{15}N-fractionation. However, it is not clear why the values of $^{15}(V/K)_D$ do not always increase above those for $^{15}(V/K)_H$, since the concerted step is not cleanly limiting. As noted above, a second effect which reduces ^{15}N-fractionation seems to express itself upon the introduction of deuterium at C-3 under certain conditions (when V and V/K are large) and evidently, the kinetic model for the mechanism needs further refinement. Some clues come from the experiments conducted at high K^+ ion concentration, discussed below and from the results obtained in deuterium oxide at pL 9.0 noted above.

At pL 9.0 and in the presence of 50 mM K^+ ion, where it is known that the primary deuterium isotope effects upon V_{max} and V/K are 1.0 and are completely suppressed,[10] the value of $^{15}(V/K)_H$ is 1.0222 ± 0.0022 which is close to the value of 1.0246 obtained at 1 mM K^+. Clearly, K^+ ion must suppress the deuterium isotope effects by increasing C_f (as was originally proposed),[10] but this result highlights the fact that the terms for C_r are not the same for the calculation of primary deuterium isotope effects as for those required for the calculation of primary ^{15}N-isotope effects. Thus, K^+ ion binds to the product complex (in the reverse reaction direction) before the labelled product (^{15}N-ammonium ion) has been released from the enzyme such that C_r for the calculation of $^{15}(V/K)$ isotope effects is increased by increasing concentrations of K^+ ion. However, when K^+ ion is released in the forward reaction direction, the deuterium label derived from C-3 of the substrate can equilibrate with the solvent (this gives rise to the exchange reaction) and the label is irreversibly lost. Therefore, increasing K^+ concentrations cannot increase C_r in the equations for ^{D}V and $^{D}(V/K)$, eqs (2) and (3) below, and the K^+ dependent term in C_r (but not in C_f) for $^{D}(V/K)$ needs to be removed.[7]

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

$$^{D}V = 1.7 = \frac{^{D}k + C_{Vf} + {}^{D}k_{eq}(C_r)}{1 + C_{Vf} + C_r} \quad (3)$$

Most intriguingly at 50 mM K^+ the nitrogen isotope effect is depressed from 1.0222 to 1.0150 by the presence of deuterium at C-3. This result indicates that the deuterium atom is exerting an effect on a step which is not causing the fractionation of the heavy atom and this must be associated with the transfer of deuterium from the conjugate acid of B-3 to another site. This appears to provide an explanation as to why the full effect of increasing the ^{15}N-fractionation for the concerted step is suppressed upon the introduction of deuterium at C-3 of the substrate (i.e., why for the deuterated substrate, $^{15}(V/K)$ did not increase as much as was expected at pL 9.0). This chemistry coincides with the requirement to return the enzyme to the correct protonated form to process a new substrate molecule where B-3 is a base and B-1 is an acid, Scheme 2. The process could occur immediately after the protonation of the ammonia, (which is produced upon the deamination of the 2,3-diaminopropionic acid residue), see Scheme 4 below. The consequent reduction in the rate of deuteron transfer from the conjugate acid of B-3 would then bring the step for ammonia protonation into equilibrium. The $^{15}K_{17}$ isotope effect is inverse (0.980) and its increased importance would decrease the $^{15}(V/K)$ isotope effect, eq (4). Note that this reaction, the transfer of deuterium from the acid B-3 to B-1 could be essentially irreversible if the conjugate acid of B-1 is a monodeuteriated



Scheme 4.

ε-ammonium Lys side-chain, as was suggested in the preceding article. This is because the two hydrogens could compete with back-transfer of deuterium to the thiolate group and because the value of $^{D}K_{eq}$ for the transfer of deuterium from an alkyl ammonium group to a thiolate is ~2.4.[24,25] Thus, the effect of the deuterium transfer from B-3 could be, solely, to slow the formation of a complex that could release K^+ ion. It seems likely that any conformational changes that should occur to allow the conjugate acid of B-3 to contact the base B-1 must occur before the hydrogen transfer, simply because B-1 and B-3 must reside on opposite faces of the bound mesaconic acid (at least up until the time that the diaminopropionic acid residue eliminates ammonia). This fits in well with the notion, proposed in the preceding article, that the dehydroalanine moiety is protected through an internal thioether cross-link, since, following the protonation of the nascent ammonia by the conjugate acid of B-1, the nucleophilic form of B-2 could attack the dehydroalanine residue to give a thioether and cause the conformational change required to facilitate a B-3 to B-1 proton transfer.

Scheme 4 shows the fully refined mechanism with all proton transfers and conformational change steps. The scheme gives rise to eq (4), which is modelled on all of the experimental observations that we know of to date for the natural substrate, (2*S*,3*S*)-3-methylaspartic acid,

and differs from eq (1) in possessing terms for the internal proton transfer steps and for conformational changes.

$$
\begin{aligned}
{}^{15}(V/K) = \{ & {}^{15}k_{11}\,{}^{15}K_7\,{}^{15}K_9 + [(k_{11}/k_{10})({}^{15}k_9 + (k_9/k_8) \\
& ({}^{15}k_7 + (k_7/k_{TE})(1 + (k_{De}/k_6) \\
& (1 + k_5K^+/k_4))))] + [{}^{15}K_7\,{}^{15}K_9\,{}^{15}K_{11}(k_{12}/k_{13}) \\
& ({}^{15}k_{13} + {}^{15}K_{13}(k_{14}/k_{15})({}^{15}k_{15} + {}^{15}K_{15}(k_{16}/k_{TE}) \\
& ({}^{15}k_{17} + {}^{15}K_{17}(k_{De}/k_{17})(1 + (k_{18}/k_{19}) \\
& (1 + (k_{20} + /k_{21}) \\
& (1 + (k_{22}K^+/k_{23})))))))]\}/(1 + C_f + C_r)
\end{aligned}
\tag{4}
$$

where $C_f = (k_{11}/k_{10})(1 + (k_9/k_8)(1 + (k_7/k_{TE})(1 + (k_{De}/k_6)(1 + k_5K^+/k_4))))$, and; where $C_r = (k_{12}/k_{13})(1 + (k_{14}/k_{15})(1 + (k_{16}/k_{TE})(1 + (k_{De}/k_{17})(1 + (k_{18}/k_{19})(1 + (k^+_{20}/k_{21})\ (1 + (k_{22}K^+/k_{23}))))))))$.

In eq (4) conformational changes are represented by k_{TE} and k_{De} for the formation of the thioether (TE) and dehydroalanine (De) forms of residue 173, respectively. As for eq (1) (for $^{15}(V/K)_D$), k_{11} in the numerator and denominator of eq (4) should be divided by the intrinsic deuterium isotope effect for C-3 deprotonation ($^Dk_{11}$), and k_{12} in the numerator and denominator of eq (1) should be divided by the intrinsic deuterium isotope effect for C-3 deprotonation ($^Dk_{11}$) and multiplied by the deuterium equilibrium isotope effect ($^DK_{eq}$). Additionally, the rate constant k_{19} for the deprotonation of the conjugate acid of B-3 should be multiplied by the intrinsic isotope effect ($^Dk_{19}$) in C_r. If the proton transfer gives a monodeuteriated polyprotic alkylammonium group, such that it is essentially irreversible, the equation requires no further corrections and the reverse commitment will be increased by $^Dk_{19}$, which could be as large as 3.0.

In conclusion, the natural substrate forms a covalent enzyme–substrate complex which undergoes elimination via a concerted mechanism. The concerted step is flanked by other slow steps and the reaction shows a primary deuterium equilibrium isotope effect consistent with the operation of a thiolate base at C-3 of the substrate. The unequal magnitude of the forward and reverse reaction commitments vary over the pL range 6.5 to 9.0 and the large reverse commitment is not solely due to product release. The enzyme releases its products in competition with the rebinding of the activator, K^+, and thus displays C-3 hydrogen exchange with the solvent.

(2*S*)-Aspartic acid. The ^{15}N-isotope effects for V/K observed for (2*S*)-aspartic acid are given in Table 3. At pL 9.0 $^{15}(V/K)_H$ is large at 1.0390 ± 0.0031. Aspartic acid does not display primary deuterium isotope effects in water at pH 9.0[7] but the value of $^{15}(V/K)_D$ is 1.0240 ± 0.0023, significantly lower than in the absence of deuterium at C-3. It appears that the elimination of ammonia from the substrate is step-wise. However, first it is necessary to consider whether the transfer of deuterium from the conjugate acid of B-3 might be causing the depressed valued of $^{15}(V/K)$. Aspartic acid shows a very high C-3 exchange rate of 16.0 at pH 9.0 (measured as tritium wash-in).[7] In deuterium oxide the values of ^DV_S and $^D(V/K)_S$ are significant at 2.0 and 1.7, respectively. It would appear that exchange of deuterium from the acid B-3 to B-1 is responsible for the isotope effects in deuterium oxide and that in water, the transfer of deuterium is masked by the equilibration with other protons on the polyprotic base B-1. The effect of suppressing the ^{15}N-fractionation should be reduced in deuterium oxide where the polyprotic base would be deuteriated and would transfer back in the reverse step the same isotope of hydrogen that it received. For example, if protium was transferred from a thiol to a dideuteriated alkylamino group, protium should be transferred back, (due to the low fractionation factor for thiols) and if deuterium was transferred to the same dideuteriated alkylamino group, deuterium must be transferred back. In deuterium oxide $^{15}(V/K)_{SD}$ was 1.0255 and, therefore larger than $^{15}(V/K)_{SH}$ (1.0168) which is, indeed, consistent with the result obtained for the natural substrate and indicative of a concerted mechanism. At pL 9.4, the presence of deuterium at C-3 did not alter the value of $^{15}(V/K)$. Nevertheless, the value for the non-deuteriated substrate was lower than at pL 9.0, in accordance with the expected deprotonation of the ammonium group of the substrate and the removal of the term for the equilibrium isotope effect ($^{15}K_7$), from eq (4). Further analysis is not possible for the (2*S*)-aspartic acid system as the isotope effects and exchange reactions were not investigated at lower pH. Nevertheless, it is clear that the release of products from the enzyme–product complex is very slow compared to the rebinding of K^+ and progression through all of the reverse steps to substrate release, because the exchange reaction is so facile. In broad terms the reaction seems to follow the same pathway as for the natural substrate, Scheme 4, except at high pL when some *syn*-elimination may occur as is observed with the *erythro*-substrate, (2*S*,3*R*)-3-methylaspartic acid.

(2*S*,3*R*)-3-Methylaspartic acid. The *erythro*-substrate is processed very slowly via a *syn*-elimination mechanism and displays massive primary deuterium isotope effects of ~7.0 and 4.0 for V and V/K, respectively, at low and high K^+ concentration.[2,7,16] Thus, there is a small forward reaction commitment but, the elimination step is cleanly rate limiting and V reflects the full value of the intrinsic deuterium isotope effect. In the previous article it was suggested that the substrate reacted with an anionic form of the enzyme where both B-3 and B-1 are in their basic form. It was of interest then, to determine the ^{15}N-isotope effects for the substrate to see if the *syn*-elimination process was also concerted. The reaction displayed a $^{15}(V/K)$ isotope effect of 1.0050 ± 0.0023 for the unlabelled substrate whilst the C-3 deuteriated substrate gave a slightly larger value for $^{15}(V/K)_D$ of 1.0058 ± 0.0028. These values originally appeared to be very low because the reaction does not display a C-3 hydrogen exchange reaction and because only C_f is significant in the calculation of the reaction commitments

for the primary deuterium isotope effects, eqs (2) and (3).

Scheme 4 and eq (4) are inappropriate for the *syn*-elimination reaction because B-3 is not used as a base. As there is no substrate C-3 hydrogen exchange activity and C_r in eqs (2) and (3) and C_{Vf} in eq (3) must be near zero at 1 mM K^+, the value of C_f can be calculated quite accurately. Scheme 2, however, appears to be suitable to model a concerted elimination. Thus, substituting the values of ^Dk derived from ^DV (7.15)[2,10] into eq (2) gives $C_f = 1.6$ at 1 mM K^+ ion concentration. The value for the forward commitment should be the same as for the ^{15}N-isotope effect, eq (1), and since there are no reverse commitments, eq (1) simplifies to give eq (5).

$$^{15}(V/K) = \{^{15}k_{11}\,{}^{15}K_7\,{}^{15}K_9 + [(k_{11}/k_{10})(^{15}k_9 + (k_9/k_8)(^{15}k_7 + (k_7/k_6)(1 + k_5K^+/k_4)))]\}/(1 + C_f) \quad (5)$$

where $C_f = (k_{11}/k_{10})(1 + (k_9/k_8)(1 + (k_7/k_6)(1 + k_5K^+/k_4))) = 1.6$.

The value of 0.995 for $^{15}K_7{}^{15}K_9$ has been considered above ($^{15}K_7 = 1.0167$; $^{15}K_9 = 0.979$) and is totally appropriate for the *erythro*-substrate, if it is protonated. Since $^{15}(V/K)$ is small at 1.005, it is evident that the substrate is not protonated and reacts directly in its unprotonated form with the dehydroalanine residue. Thus, the term $^{15}K_7$ vanishes and by the analysis described in the preceding article[7] K^+ ion seems to bind after the substrate has attacked the dehydroalanine residue. Moreover, because the effect of increasing K^+ ion concentration does not significantly increase the reaction commitments for the *erythro*-substrate, eq (5) simplifies further to give eq (6).

$$^{15}(V/K) = \{^{15}k_{11}\,{}^{15}K_9 + [(k_{11}/k_{10})(^{15}k_9)]/(1 + C_f) \quad (6)$$

The kinetic isotope effect for adding the amino group of the substrate to the dehydroalanine residue ($^{15}k_9$), should be 1.010, by analogy to the phenylalanine ammonia-lyase system.[17] Therefore, $^{15}(V/K) = \{^{15}k_{11}(0.979) + 1.616\}/(2.6) = 1.005$, and $^{15}k_{11}$ would be 0.992 ! Since the kinetic isotope effect for the elimination process must be greater or equal to the equilibrium isotope effect of 1.033 determined previously,[7] a concerted deamination mechanism does not fit with the experimental data. Moreover, a step-wise carbanionic mechanism does not fit with the data as it would show a large decrease in the value of $^{15}(V/K)$ for the substrate possessing deuterium at C-3, due to the large observed primary deuterium isotope effect of 3.4 expressed upon V/K.

In order to test the fit for a carbonium ion mechanism, Scheme 5 was devised (starting from the mechanism for concerted *syn*-elimination described in the preceding article). Here it is assumed that the substrate binds in its protonated form, as for the *threo*-isomer. After the addition of K^+ ion and deprotonation, formation of a covalent enzyme–substrate complex occurs. The N-atom would be protonated and the C-2–N bond would break to give a carbonium ion which then loses a proton. Since there are no reverse commitments to the deuterium sensitive step, no further reaction steps need to be considered. Where the release of the protonated substrate from the Michaelis complex is fast compared to reaction steps (which it is for this very slow substrate, as has been reasoned in the preceding paper[7]), the expressions for a carbonium ion elimination mechanism simplify. We will assume that flux through k_{11} and the subsequent steps through to products is much greater than k_{10} because there is no exchange reaction and no C_r term for ^DV or $^D(V/K)$. Moreover, because the effect of increasing K^+ ion concentration does not significantly increase the reaction commitments as was noted above, the equations simplify further to give eqs (7)–(9).

$$^D(V/K) = [^{15}k_9 + (k_9/k_8)(1 + (k_7/k_6))]/[1 + (k_9/k_8)(1 + (k_7/k_6))] \quad (7)$$

where C_f for step 9 $= (k_9/k_8)(1 + (k_7/k_6))$ or $(1 + (k_7/k_6))/(k_8/k_9) = 1.6$

$$^{15}(V/K) = [^{15}K_5\,{}^{15}k_7 + (k_7/k_6)^{15}k_5 + {}^{15}K_5\,{}^{15}K_7(k_8/k_9)]/[1 + (k_7/k_6) + (k_8/k_9)] \quad (8)$$

$$^{15}(V/K)_D = [^{15}K_5\,{}^{15}k_7 + (k_7/k_6)^{15}k_5 + {}^{15}K_5\,{}^{15}K_7(k_8/k_9)\,{}^Dk_9]/[1 + (k_7/k_6) + (k_8/k_9)^Dk_9] \quad (9)$$

The ^{15}N-kinetic isotope effect ($^{15}k_7$) for breaking the C–N bond to give the carbonium ion and amino-enzyme should be almost the same as that for breaking the C–N bond between the substrate and the enzyme ($^{15}k_6$) to give the dehydroalanine residue and the unprotonated substrate. Thus, $^{15}K_7 = {}^{15}k_7/{}^{15}k_8 = {}^{15}k_6/{}^{15}k_8$; such that the term $^{15}K_5{}^{15}K_7$ in eqs (8) and (9) is equal to $^{15}k_5/{}^{15}k_8$ and the term $^{15}K_5{}^{15}k_7$ is equal to $^{15}k_5$. Dividing all terms by k_8/k_9 and substituting by C_f gives eqs (10) and (11).

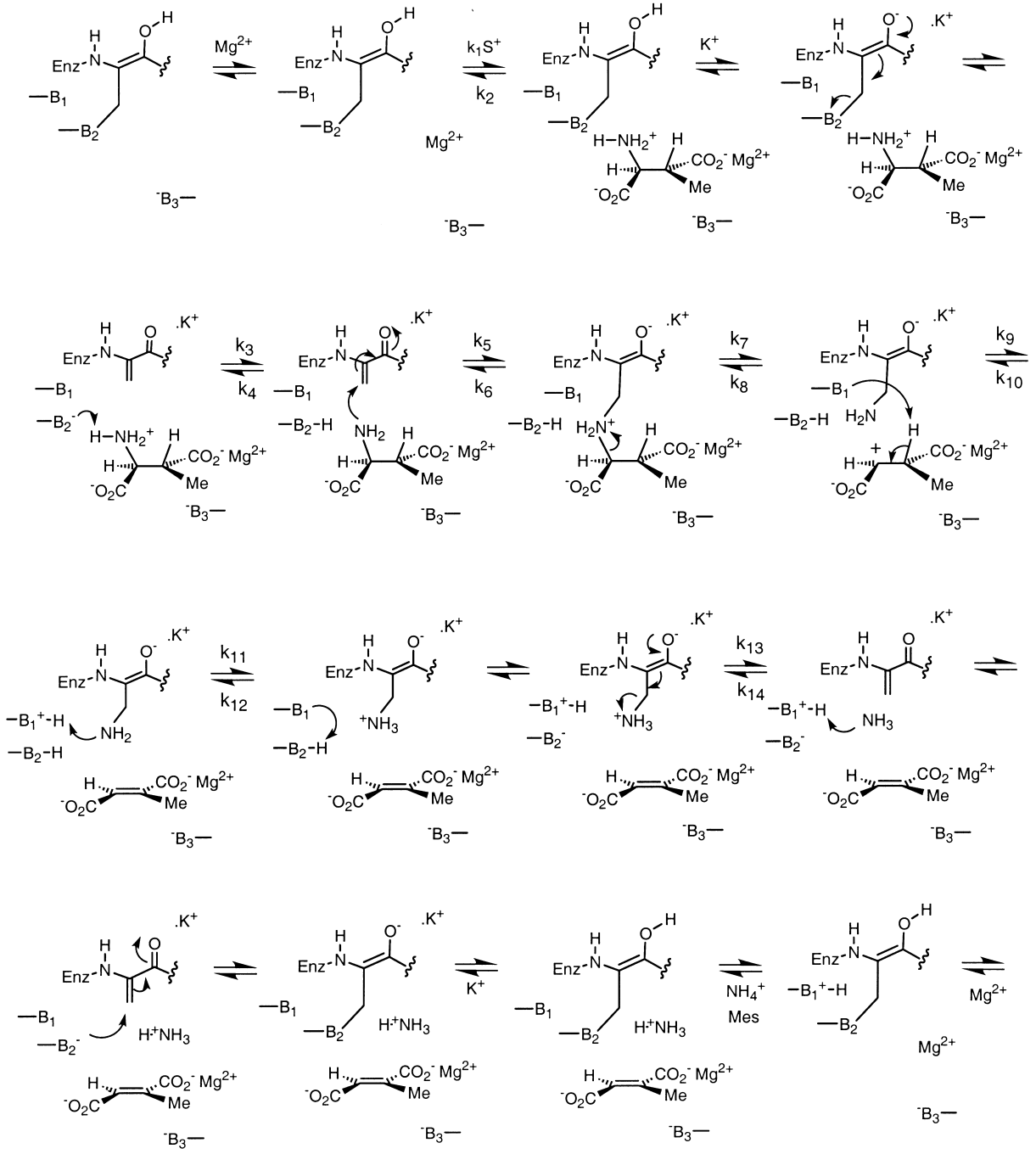
$$^{15}(V/K) = {}^{15}k_5[C_f + (1/{}^{15}k_8)]/[C_f + 1] \quad (10)$$

$$^{15}(V/K)_D = {}^{15}k_5[C_f + ({}^Dk_9/{}^{15}k_8)]/[C_f + {}^Dk_9] \quad (11)$$

The term $^{15}k_5$ is a factor in both equations and represents the primary ^{15}N-isotope effect for the amination of the dehydroalanine residue by the substrate to give a secondary (dialkyl) ammonium group. The equilibrium isotope effect $^{15}k_5$ should be strongly inverse (see Fig. 2) and Hermes et al. have calculated that $^{15}k_5$ possesses a value of ~1.010 in the phenylalanine ammonia-lyase system.[17] Note that $^{15}k_8$ also represents the alkylation of a primary amine (the amino enzyme) by the product to give the same dialkylammonium group such that its

value should be slightly smaller than $^{15}k_5$ because the amino group in the amino enzyme is bonded 'less stiffly' to only one C-atom bearing one C-atom and two H-atoms, whereas, in the substrate it is bonded to one C-atom bearing two C-atoms and one H-atom. Using the experimentally determined values of $^{15}(V/K)$ and the deduced values of $^{D}k_9$ and C_f (7.15 and 1.6, respectively) allows the size of $^{15}k_8$ to be calculated for assumed values of $^{15}k_5$, using eqs (10) and (11). However, since the values of $^{15}(V/K) = 1.0050 \pm 0.0023$ and $^{15}(V/K)_D = 1.0058 \pm 0.0028$ are essentially identical, the value of $^{15}k_8$ can be solved without assuming a value for $^{15}k_5$ by setting $^{15}(V/K) = {}^{15}(V/K)_D$ for eqs (10) and (11). This gives a value of 1.000 for $^{15}k_8$, here substitution back into the individual eqs (10) and (11) gives values for $^{15}k_5$ of 1.0055 ± 0.0026. Importantly $^{15}k_8$ is slightly smaller than $^{15}k_5$ (see discussion above) and both values are well within the expected range.[17] The analysis indicates that a covalent enzyme–substrate intermediate is formed with the L-*erythro*-substrate and that the deamination reaction occurs via a carbonium ion mechanism. A full summary of the mechanism is given in Scheme 5.

The enzyme apparently, has "pulling power" and is able to strain the *N*-protonated covalent enzyme–substrate complex to the point that the C–N bond breaks to give a



Scheme 5.

carbonium ion. Presumably the reaction requires the high pK_a base (B-1 Scheme 5) to be unprotonated in order to receive the C-3 proton in the *syn*-elimination process. The conjugate acid (B-2) needs to be protonated later in order to protonate the diaminopropionic acid residue, prior to the elimination of ammonia. However, if the base (B-1) is the ε-amino group of a Lys residue, it may also be important for the side-chain to be unprotonated and of neutral charge to prevent the formation of adverse interactions in the transition state leading to the formation of the carbonium ion. Alternatively, the neutral diaminopropionic acid residue, in the amino enzyme intermediate, may serve as the base. Note that although the *erythro*-substrate is processed very slowly, the k_{cat} value is quite respectable at 4.6 s^{-1}.

The ability of the enzyme to process the *erythro*-substrate via a step-wise carbonium ion mechanism is totally consistent with the concerted pathway displayed by the *threo*-substrate. Simply, a small increase in the rate of proton removal from the *erythro*-substrate where C–N bond is not rate limiting and the difference in k_9 and k_8 is less than twofold would merge the two transitions states into one single barrier and concerted C–H and C–N bond motion.

Experiments designed to resolve the role and determine the location of the acid/base groups in methylaspartase are presently in progress. It will be interesting to compare these roles and sites with the location of acid/base groups in the aspartase system which catalyses very similar chemistry via an apparently non-covalent mechanism.[22,26,27] Currently the methylaspartase system is being crystalised in both our own laboratory (with the recombinant *E. coli* version of the *Clostridium tetanomorphum* protein) and the laboratory of Asano with enzyme isolated from the two facultative anaerobes *Citrobacter* sp. and *Morganella morganii*.[28]

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, UK). (2*S*,3*R*)-[3-$^2H_1$]-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.[21,29] (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.[16] 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 oxide and were lyophilised prior to use. The pH 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] as previously described.[6,10] The specific activity of the enzyme used in these studies was at least 40–200 units (mg of protein)$^{-1}$.

Enzyme assay

Enzyme was assayed according to the method of Barker where 1 unit of enzyme catalyses the formation of 1 μmol of mesaconic acid min^{-1} at pH 9.0 at 30 °C as determined by the increase in OD_{240} under the assay conditions.[1,18]

$^{15}N/^{14}N$-Fractionation experiments

The ^{15}(V/K) isotope effects were determined by isotope ratio mass spectrometric analysis and comparison of the $^{15}N/^{14}N$ ratio in dinitrogen derived from ammonia obtained from the starting substrate and dinitrogen derived from the ammonia collected after the partial conversion of the substrate (10–20% of the reaction) to products using natural abundance ^{15}N-substrates. The ^{15}(V/K) isotope effect was then calculated using eq (12), where R_0 and R are the measured $^{15}N/^{14}N$ isotope ratios in substrate and product after the fraction of reaction, f, respectively.

$$^{15}(V/K) = \log(1 - f)/\log[1 - (f.R/R_0)] \qquad (12)$$

The $^{15}N/^{14}N$ ratios R_0 and R were measured using a VG SIRA 10 dual-inlet isotope ratio mass spectrometer.

Determination of R_0 and R

R_0 was determined by digesting a sample of the substrate using the Kjeldahl procedure[31] to yield ammonia which was oxidised to dinitrogen and subjected to mass ratio analysis. To a sample of the substrate (2*S*,3*S*)-3-methylaspartic acid (50 mg, 0.34 mmol) was added potassium sulphate (0.3 g, 1.72 mmol), concentrated sulphuric acid (1.2 ml, 21.6 mmol), a solution of mercury(II) sulphate (0.3 ml) [prepared by dissolving red mercury(II) oxide (2.5 g, 11.54 mmol) in concentrated sulphuric acid (3 ml) and diluting to 25 ml with distilled water] and anti-bumping granules. The solution was boiled for 2 h, after which time dissolution was complete. The solution was cooled to room temperature and distilled water (5 ml) was added. After re-cooling, zinc dust (0.12 g, 1.84 mmol) was added and the mixture was allowed to stand for 30 min. The remaining zinc dust was removed by filtration and the solution was concentrated to a volume of ca. 1 ml. The residual solution was basified with concentrated aqueous potassium hydroxide solution to liberate the ammonia and this was distilled into dilute sulphuric acid at room temperature by passing air through the solution. The sample of

ammonia in sulphuric acid was then oxidised to dinitrogen using excess sodium hypobromite solution. The evolved dinitrogen was introduced directly into one port of the dual-inlet isotope ratio mass spectrometer and the $^{15}N/^{14}N$ ratio (R_0) was determined against a reference standard of dinitrogen connected to the other port. Three complete duplicate determinations were carried out for the sample. The entire procedure was repeated for the substrate (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid.

R was determined using ammonia produced from a partial deamination reaction performed under the specific conditions described below. Incubations (total volume 10 ml) were performed at 30 ± 0.1 °C, in buffered solution with 20 mM $MgCl_2$, and KCl as specified below. The substrate concentration was in the range 0.15 to 0.2 M and the reaction was initiated by addition of enzyme solution (50 µl, ca. 6 units). The extent of the deamination reaction (f) was measured spectrophotometrically by withdrawing small aliquots of the solution at various time intervals and determining the concentration of mesaconic acid from the UV absorbance at 240 nm.[9,18] After 10–20% of the methylaspartic acid had been deaminated, the reaction was quenched by the addition of concentrated sulphuric acid (1.0 ml) and the product ammonia was distilled into dilute sulfuric acid in a stream of air, see above. The dinitrogen obtained upon hypobromite oxidation was analysed as described above to give the $^{15}N/^{14}N$ ratio in the product (R). Three duplicate incubations were carried out for each deamination experiment.

Unlabelled substrate in water

Initial experiments were performed, at least four times, under the standard assay conditions (1 mM KCl, 20 mM $MgCl_2$, 500 mM Tris, pH 9.0) using unlabelled substrate. The entire experiment and analysis was repeated for enzymic deamination reactions performed at pH 6.5 (1 mM KCl, 20 mM $MgCl_2$, 100 mM PIPES, pH 6.5) and at 9.4 (1 mM KCl, 20 mM $MgCl_2$, 500 mM Tris, pH 9.4) and at pH 9.0 (50 mM KCl, 20 mM $MgCl_2$, 500 mM Tris, pH 9.0).

C-3 Deuteriated substrate in water

Similar experiments to those described above were performed at pH 6.5, 9.0 and 9.4 in the presence of 1 mM KCl and 20 mM $MgCl_2$ using C-3 deuteriated substrate to determine the $^{15}(V/K)_D$ isotope effects.

$^{15}N/^{14}N$-Fractionation in deuteriated solvent

Similar experiments to those described above were performed at pD 6.5, 9.0 and 9.4 to determine the $^{15}(V/K)$ isotope effects for the unlabelled and C-3 deuteriated substrate in deuterium oxide. Buffer solutions were prepared and their pD values were adjusted as described above. Values for pD were determined using a standard pH electrode according to the equation pD–0.4 = pH.[13]

(2*S*)-Aspartic acid

Similar experiments to those described above were performed using (2*S*)-aspartic acid and (2*S*,3*R*)-[3-$^2H_1$]-aspartic acid at pL 9.0 and 9.4 in water and in deuterium oxide in the presence of 1 mM KCl and 20 mM $MgCl_2$.

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

Similar experiments to those described above were performed using (2*S*,3*R*)-3-methylaspartic acid and (2*S*,3*R*)-[3-^{2}H]-3-methylaspartic acid at pH 9.0 in water in the presence of 1 mM KCl and 20 mM $MgCl_2$.

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