Mechanism of the Enzymic Elimination of Ammonia from 3-Substituted Aspartic Acids by 3-Methylaspartase

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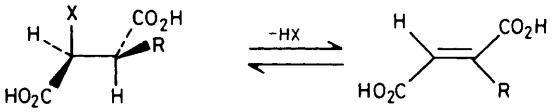
Kinetic experiments with 3-methylaspartase, using aspartic, 3-methylaspartic, and 3-ethylaspartic acid and the appropriate C-3 deuteriated isotopomers as substrates, reveal that C(3)–H bond cleavage is partially rate-limiting for 3-methylaspartic acid, much less rate-limiting for 3-ethylaspartic acid, and not rate-limiting at all for aspartic acid.

Study of the mechanism of the reactions catalysed by the ammonia-lyases [*e.g.* aspartase (Scheme 1; X = NH_2, R = H), methylaspartase (Scheme 1; X = NH_2, R = Me) and phenylalanine ammonia-lyase] and the dehydrases [*e.g.* fumarase (Scheme 1; X = OH, R = H)] have attracted much interest in recent years;[1—4] however, they are still poorly understood. Methylaspartase appears to act *via* a carbanion mechanism ($E1_{cb}$), as C-3 hydrogen exchange occurs more rapidly than C–N bond cleavage for the physiological substrate (2*S*,3*S*)-3-methylaspartic acid.[5,6] Also, no primary isotope effect has been detected for the elimination of ammonia from the C-3 deuteriated substrate.[6] Carbocation mechanisms have been suggested for both aspartase[7] and fumarase,[8] largely because the enzyme-catalysed reactions show no primary isotope effect with C-3 deuteriated substrates and do not catalyse the exchange of C-3 hydrogen with the

Table 1. Kinetic parameters.

Substrate	K_M/mM	$10^7 V_{max}$/dm^{-3} s^{-1} [a]	V/K
(2*S*)-Aspartic acid	10.50 ± 0.82	0.80	0.076
(2*S*,3*R*)-[3-$^2H_1$]-Aspartic acid	10.50 ± 0.82	0.80	0.076
(2*S*,3*S*)-3-Methylaspartic acid	2.37 ± 0.2	109.0	46.0
(2*S*,3*S*)-[3-^{2}H]-3-Methylaspartic acid	2.35 ± 0.25	64.2	27.3
(2*S*,3*S*)-3-Ethylaspartic acid	17.08 ± 1.4	48.7	2.85
(2*S*,3*S*)-[3-^{2}H]-3-Ethylaspartic acid	17.66 ± 1.6	41.8	2.37

[a] Corrected for 16.7 nKat (1 unit) enzyme assayed at pH 9 (*cf.* ref 1); error ± 10% for all $V_{max.}$ values.



Scheme 1

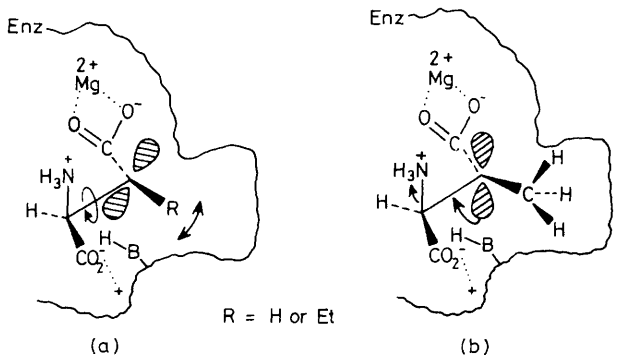


Figure 1

solvent more rapidly than the overall reaction. Recent evidence points to a carbanion mechanisms for both aspartase and fumarase.[3] The enzymes show a remarkable degree of protein amino acid homology.[4]

During our recent studies of the amination of substituted fumaric acid (**1**; R = H, Me, Cl, or Br) using 3-methylaspartase (EC 4.3.1.2) to catalyse the retro-physiological reaction, it was noted that the reaction rates ($V_{max.}$) for all substrates were similar.[9] These findings were of particular interest because the published rate for the deamination of (2*S*)-aspartic acid (**2**; R = H) is about 100 times less than that of the physiological substrate, (2*S*,3*S*)-3-methylaspartic acid (**2**; R = Me).[10] Indeed, in our hands $V_{max.}$ for (2*S*)-aspartic acid was 137 times less than for the homologue.[11]

In order to determine the the mechanistic basis for the large differences in deamination reaction rates we set out to synthesize three pairs of substrates, each pair consisting of the C-3 deuteriated substrate and its non-deuteriated analogue. It was expected that comparison of the $V_{max.}$ values for the substrates would provide a reliable guide to the contribution to the overall rate of individual rate constants for the chemical steps only, since, for the best (fastest-reacting) substrate for the deamination reaction, a chemical step, C–N bond cleavage, rather than debinding of either mesaconic acid or ammonia, was known to be rate-limiting.[6]

(2*S*,3*R*)-[3-$^2H_1$]Aspartic acid was prepared through enzymic amination of fumaric acid in deuterium oxide using 3-methylaspartase, in 65% yield (*cf.* ref. 12); $[\alpha]_D^{20}$ + 23.9° (*c* 0.6, 6 M-HCl) [lit.,[13] for non-deuteriated material +24.6° (in 6 M-HCl)]. (2*S*,3*S*)-3-Methylaspartic acid was obtained in a similar manner using mesaconic acid in protium oxide, in 61% yield; $[\alpha]_D^{20}$ + 13.4° (*c* 0.6, 6 M-HCl), −10.3° (*c* 0.6, H_2O) [lit.,[14] + 13.3° (*c* 3.0, 5 M-HCl), −10° (*c* 0.42, H_2O)]. (2*S*,3*S*)-[3-^{2}H]-3-Methylaspartic acid was prepared as for the unlabelled material, by conducting the incubation in deuterium oxide, in 60% yield; δ_H (360 MHz; ^2H_2O; pH 1) 4.90 (1H, s, 2-H) and 1.78 (3H, s, CH_3), $[\alpha]_D^{20}$ + 12.0° (*c* 0.6, 6 M-HCl).

In order to prepare the 3-ethyl homologues, ethylfumaric acid was first prepared through treatment of ethyl 2-ethylacetoacetate (obtained through ethylation of acetoacetic ester) with bromine/sodium hydroxide, to effect a Favorskii-type rearrangement.[15] After acidic work-up the product was obtained in 65% overall yield, m.p. 194—195 °C (lit.,[15] 193—195 °C). (2*S*,3*S*)-3-Ethylaspartic acid† was prepared through enzymic amination of ethylfumaric acid in 60% yield; m.p. 245—246 °C, δ_H (360 MHz; ^2H_2O; pH 1) 4.89 (1H, d, *J* 4.2 Hz, 2-H), 3.50 (1H, m, 3-H), 2.2 (2H, m, CH_2Me), and 1.48 (3H, t, *J* 7.4 Hz, CH_2CH_3), $[\alpha]_D^{20}$ + 15.0° (*c* 0.6, 6 M-HCl}. (2*S*,3*S*)-[3-^{2}H]-3-Ethylaspartic acid was prepared in 58% yield by conducting the incubation with ethylfumaric acid in deuterium oxide; δ_H (360 MHz; ^2H_2O; pH 1), 4.87 (1H, s, 2-H), 2.23 (2H, brq, *J* 7.4 Hz, CH_2Me), and 1.45 (3H, t, *J* 7.4 Hz, CH_2CH_3), $[\alpha]_D^{20}$ + 14.5° (*c* 0.6, 6 M-HCl). All analytical and spectroscopic data for the synthetic compounds confirmed their structures and purity. All deuteriated compounds contained >95 atom % heavy isotope.

Each of the synthetic substrates and commercial (2*S*)-aspartic acid was incubated with 3-methylaspartase at a variety of concentrations; the kinetic parameters (K_M and $V_{max.}$) obtained are shown in Table 1. From these values it was evident that C(3)–H bond cleavage is not rate-limiting for the deamination of (2*S*)-aspartic acid and is only marginally limiting for (2*S*,3*S*)-3-ethylaspartic acid. However, the physiological substrate (2*S*,3*S*)-3-methylaspartic acid showed an isotope effect of 1.7 on $V_{max.}$ and V/K for C–H bond cleavage; thus for this substrate, contrary to previous reports (see before), C–H bond cleavage is *partially* rate-limiting.

† This is the expected stereoisomer, by analogy with the enzymic amination of four other fumaric acids.[16,17]

Bright and his co-workers have reported that there is no isotope effect for the deamination of (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid.[6] However, their substrates contained *ca.* 14% unlabelled compound and thus it is possible that under these circumstances $V_{max.}$ was identical with that of the undeuteriated material within experimental error.

Since it has been established that C–N cleavage is rate-limiting[6] for 3-methylaspartic acid deamination, it is possible to rationalize both the slow rates of deamination of (2*S*)-aspartic acid and (2*S*,3*S*)-3-ethylaspartic acid and also the lack of any observable isotope effects for these substrates. Presumably for the two slowly reacting substrates removal of the C-3 hydrogen generates a carbanion in which the torsion angle HC(2)C(3)NH_2 is not optimal for the elimination of ammonia; hence no primary isotope effect is expected. This situation probably arises as a result of weak [3-H of the (2*S*)-aspartic acid carbanion] or strained [3-Et of the (2*S*,3*S*)-3-ethylaspartic acid] interaction with the hydrophobic methyl-binding pocket of the enzyme [Figure 1(a)]. This analysis suggests that in the physiological reaction catalysed by 3-methylaspartase, hydrophobic binding of the methyl group of the substrate ensures that the carbanion is restrained in the optimum conformation for minimization of the activation energy for C–N bond cleavage [Figure 1(b)]. This reaction, therefore, would be expected to show the most *E*2 character, and since C-3 hydrogen exchange with the solvent takes place at only about one-third of the rate of the overall elimination reaction at pH 9,[6] a small primary isotope effect would also be expected.

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References

1 (a) K. R. Hanson and E. A. Havir, 'The Enzymic Elimination of Ammonia,' in 'The Enzymes,' ed. P. Boyer, 3rd edn., Academic Press, New York, 1972, vol. 7, ch. 3, p. 75; (b) R. L. Hill and J. W. Teipel, 'Fumarase and Crotonase,' in 'The Enzymes' (see ref. 1a), 1971, vol. 5, ch. 19, p. 539.
2 M. A. Marletta, Y. Cheung, and C. Walsh, *Biochemistry*, 1982, **21**, 2637.
3 (a) D. J. T. Porter and H. J. Bright, *J. Biol. Chem.*, 1980, **255**, 4772; (b) I. I. Nuiry, J. D. Hermes, P. M. Weiss, C. Chen, and P. F. Cook, *Biochemistry*, 1984, **23**, 5168.
4 S. A. Woods, J. S. Miles, R. E. Roberts, and J. R. Guest, *Biochem. J.*, 1986, **237**, 547.
5 H. J. Bright, L. L. Ingraham, and R. E. Lundin, *Biochim. Biophys. Acta*, 1964, **81**, 576.
6 H. J. Bright, *J. Biol. Chem.*, 1964, **239**, 2307.
7 T. B. Dougherty, V. R. Williams, and E. S. Younathan, *Fed. Proc.*, 1971, **30**, 1239; J. P. Klinman, *Adv. Enzymol.*, 1977, **44**, 456.
8 J. N. Hansen, E. C. Dinovo, and P. D. Boyer, *J. Biol. Chem.*, 1969, **244**, 6270.
9 M. Akhtar, M. A. Cohen, and D. Gani, *J. Chem. Soc., Chem. Commun.*, 1986, 1290.
10 H. A. Barker, R. D. Smyth, R. M. Wilson, and H. Weissbach, *J. Biol. Chem.*, 1959, **234**, 320; M. F. Winkler and V. R. Williams, *Biochim. Biophys. Acta*, 1967, **140**, 284.
11 N. P. Botting, M. A. Cohen, M. Akhtar and D. Gani, *Biochemistry*, submitted for publication.
12 S. J. Field and D. W. Young, *J. Chem. Soc., Perkin Trans. 1*, 1983, 2387.
13 'Handbook of Chemistry and Physics,' ed. R. C. Weast, 55th edn., Chemical Rubber Publishing Co., Cleveland, 1974, p. 124.
14 H. A. Barker, R. D. Smyth, E. J. Wawszkiewicz, M. N. Lee, and R. M. Wilson, *Arch. Biochem. Biophys.*, 1958, **78**, 468.
15 P. Walden, *Ber*, 1891, **24**, 2038.
16 H. J. Bright, R. E. Lundin, and L. L. Ingraham, *Biochemistry*, 1964, **3**, 1224.
17 M. Akhtar, M. A. Cohen, and D. Gani, *Tetrahedron Lett.*, 1987, **28**, 2413.