Kinetics and Mechanism of *syn*-Elimination of Ammonia from (2*S*,3*R*)-3-Methylaspartic Acid by Methylaspartase

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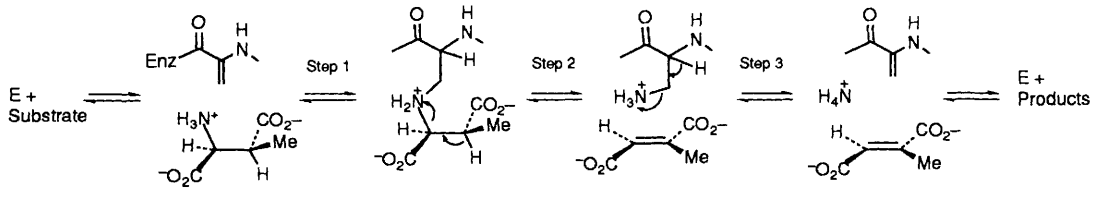
Methylaspartase catalyses the slow *syn*-elimination of ammonia from the (2*S*,3*R*)-[L-*erythro*]-diastereoisomer of the natural substrate (2*S*,3*S*)-3-methylaspartic acid, to give mesaconic acid; the reaction does not involve C-3 epimerisation followed by normal *anti*-elimination, ruling-out the possibility of a carbanion intermediate, but, displays large primary deuterium isotope effects consistent with concerted C–H and C–N bond cleavage.

Methylaspartase catalyses the second step in the catabolism of (2*S*)-glutamic acid in *Clostridia* and several other bacteria, the *anti*-elimination of ammonia from (2*S*,3*S*)-3-methylaspartic acid **1** to give mesaconic acid **2**. Early reports by Barker claimed that methylaspartase could also catalyse the *syn*-deamination of the L-*erythro*-isomer, (2*S*,3*R*)-3-methylaspartic acid **3**, at *ca.* 1% of the rate for the natural substrate, to give mesaconic acid.[1] Based on this and other observations, including the finding that C-3 solvent hydrogen exchange into (2*S*,3*S*)-3-methylaspartic acid **1** occurred at a rate faster than the natural deamination reaction, Bright suggested that each of the deamination reactions might proceed *via* a C-3 carbanion intermediate.[2] However, it was later shown that the natural substrate **1** displayed significant primary deuterium isotope effects for both the deamination[3] and the C-3 hydrogen-exchange reaction.[4] The existence of a carbanion was finally discounted when subsequent work, using double $^{15}N/^{14}N$–$^{2}H/^{1}H$-isotope fractionation measurements, showed that the elimination reaction was concerted[5] and was followed by a slow step (step 3), which could account for the apparent rapidity of C-3 hydrogen exchange, Scheme 1.[4,6]

In the absence of a carbanion intermediate derived from the L-*threo*-substrate **1**, it was difficult to understand why methylaspartase should process the L-*erythro*-diastereoisomer. (2*S*,3*R*)-3-Methylaspartic acid, however, had been identified as an intermediate in the biosynthesis of (2*S*)-glutamate in

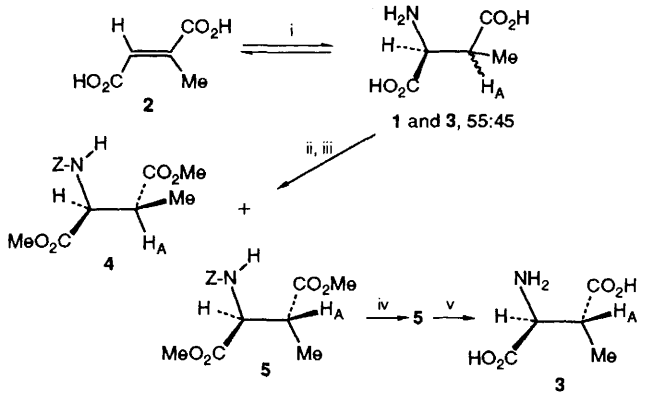


Scheme 1

Acetobacter suboxydans implying the existence of a distinct L-*erythro*-specific methylaspartase activity.[7] As contamination with an L-*erythro*-specific enzyme might account for Barker's original observations[1] we set out to identify such an enzyme and investigate the mechanism of the reaction.

L-*threo*-3-Methylaspartase was isolated and purified to homogeneity (as judged by SDS-PAGE) from *Clostridium tetanomorphum* H1[8] using modifications of Barker's original procedures.[1] Incubation of the enzyme with diammonium mesaconate at pH 9.0 in the presence of K^+ and Mg^{2+} ions gave initially L-*threo*-3-methylaspartic acid, and, after extended incubation times, L-*erythro*-3-methylaspartic acid, as judged by ^1H NMR spectroscopic examination of the incubation solution. Thus, methylaspartase did possess an L-*erythro*-3-methylaspartase or a C-3 L-*erythro*-*threo*-3-methylaspartic acid epimerase activity.[8] To exclude the possibility of contamination with other active proteins, the L-*threo*-3-methylaspartase gene was cloned and over-expressed in *Escherichia coli* and the experiment described above was repeated using the pure recombinant enzyme.[6] The results were identical indicating that L-*erythro*-3-methylaspartase (or epimerase) activity was an inherent property of L-*threo*-3-methylaspartase.

In order to distinguish between epimerase and L-*erythro*-3-methylaspartase activities, access to stereochemically pure (2*S*,3*R*)-3-methylaspartic acid and (2*S*,3*R*)[3-^{2}H]-3-methylaspartic acid was required. Accordingly, the C-6 alkylation of commercially available (3*R*)-2,5-dimethoxy-3-isopropyl-3,6-dihydropyrazine with methyl (2*R*)-2-bromopropanoic acid [derived from (2*R*)-alanine in two steps] was assessed. The reaction was successful and the subsequent deprotections occurred smoothly[9] but the chemical yields and the chiral integrity at C-3 were too low to be of utility in the planned investigations.† Accordingly, the unlabelled and deuteriated



Scheme 2 *Reagents and conditions*: i, NH_4Cl (0.2 mol dm^{-3}), $MgCl_2$ (20 mmol dm^{-3}), KCl (10 mmol dm^{-3}) in H_2O, pH 9.0 or D_2O, pD 8.6, methylaspartase (500 units for 5 g of mesaconic acid), 30 °C, several days required to reach equilibrium, 86%; ii, *N*-(benzyloxycarbonyloxy)-succinimide, K_2CO_3 (aq), 99%; iii, CH_2N_2, Et_2O, 98%; iv, column chromatography on flash silica, eluting with Et_2O–light petroleum (1 : 1), *N*-Z-(2*S*,3*R*)-3-methyl aspartate dimethyl ester is obtained in quantitative recovery, after recycling, and elutes first; v, AcOH–HCl (1 : 1), reflux, 55%, Z = benzyloxycarbonyl

L-*erythro*-3-methylaspartic acids were obtained by resolving the (2*S*,3*RS*)-*N*-benzyloxycarbonyl-3-methylaspartic acid dimethyl esters (**4** and **5** H_A = H or ^{2}H) derived from enzymically prepared (2*S*,3*RS*)-3-methylaspartic acid as shown in Scheme 2.

The (2*S*,3*R*)-3-methylaspartic acids (**3**, H_A = H) and (**3**, H_A = ^{2}H) showed the expected spectral and analytical data and contained less than 2% of the (2*S*,3*S*)-diastereoisomer.‡

To distinguish between epimerase and L-*erythro*-3-methylaspartase activities, (2*S*,3*R*)-3-methylaspartic acid (**3**, H_A = H) was incubated with the enzyme in deuterium oxide in the presence of K^+ and Mg^{2+} ions and the reaction was monitored by 200 MHz ^1H NMR spectroscopy. The deamination reaction proceeded smoothly to give mesaconic acid and then, much later, (2*S*,3*S*)[3-^{2}H]-3-methylaspartic acid. No solvent hydrogen incorporation into C-3 of the substrate (2*S*,3*R*)-3-methylaspartic acid was detected over the course of the reaction (24 h). The estimated limit of detection by 200 MHz ^1H NMR spectroscopy is ~2% of deuterium incorporation. Moreover, it was evident that the L-*threo*-isomer was not formed *via* direct epimerisation at C-3 since its formation depended on the amination of mesaconic acid generated during the initial phase of the reaction. Hence, methylaspartase is able to catalyse, directly, the *syn*-elimination of ammonia from the L-*erythro*-substrate.

Examination of the kinetic properties of the L-*erythro*-substrate **3** in the presence of 1 mmol dm^{-3} K^+ and 20 mmol dm^{-3} Mg^{2+} at pH 9.0 revealed that V_{max} was 17.2 × 10^{-6} mol dm^{-3} min^{-1} (k_{cat} 12.9 s^{-1}), 38 times slower, and K_m was 40 mmol dm^{-3}, 17 times larger, than the corresponding parameters for the L-*threo*-diastereoisomer.† As with the L-*threo*-substrate, increasing the K^+ concentration from 1 to 50 mmol dm^{-3} increased the apparent value of V_{max} and also decreased the apparent value of K_m. For the L-*erythro*-substrate, these factors were 2.4 and 7.6, respectively.

Our earlier work on the mechanism of elimination of ammonia from the L-*threo*-substrate had indicated that the reaction involved three chemical steps: (*i*) fast reversible conversion of the Michaelis complex to a covalent substrate–enzyme complex; (*ii*) partially rate-limiting, concerted C^β–H and C^α–N bond cleavage; and (*iii*) partially rate-limiting regeneration of the electrophilic prosthetic group, the putative dehydroalanine residue, Scheme 1. The kinetics for the L-*erythro*-substrate were expected to differ only in the rates for the first two steps since step 3, regeneration of the electrophilic prosthetic group, should be identical.[4]

In order to determine which step(s) was slowed by the difference in stereochemistry at C-3, the kinetic parameters were reexamined using the deuteriated substrate (**3**, H_A = ^{2}H) and the isotope effects on V_{max} and V/K were calculated. At 1 mmol dm^{-3} K^+, ^DV [or V_H/V_D] was 7.15 ± 2.74 clearly indicating that C–H bond cleavage is the rate-determining step in the reaction. The value of $^D(V/K)$ [or $(V_H/V_D)/(K_H/K_D)$] was somewhat smaller, 3.39 ± 1.6, indicating that a reverse step(s) preceding the isotopically sensitive transition state in the reaction coordinate is slow and exerts a forward reaction commitment. As substrate debinding is fast for the L-*threo*-isomer[4] and is likely to be fast for the L-*erythro*-isomer, the most probable cause for the depressed value of $^D(V/K)$ is the

† This rather cumbersome route provided (2*S*,3*R*)- and (2*S*,3*S*)-3-methylaspartic acid in a ratio of 95 : 5. This material was used in preliminary experiments, which indicated that it was too impure for kinetic studies. The ratio of the specificity constants (k_{cat}/K_m) for (2*S*,3*R*)- and (2*S*,3*S*)-3-methylaspartic acid at pH 9.0 is 1 : 650 at 1 mmol dm^{-3} K^+ and at 1 : 400 at 50 mmol dm^{-3} K^+ (see text), which explains why a very high diastereoisomeric excess was required. Small levels of contamination with the (2*S*,3*S*)-isomer (≤2%) can be tolerated as the (2*S*,3*S*)-isomer is consumed rapidly, in a burst, before the steady-state rate is established. This behaviour is a consequence of the fact that the substrate binds to the enzyme in rapid equilibrium[4] and is processed much more quickly than the unnatural isomer.

‡ All compounds and intermediates gave the expected spectral and analytical data. For (2*S*,3*R*)-3-methylaspartic acid; m.p. 257–259 °C (decomp.); $[\alpha]_D^{25}$ +36.3° (*c* 1.0, 5 mol dm^{-3} HCl), lit[10a] +38.7° (*c* 1.83, 5 mol dm^{-3} HCl), lit[10b] +32.9° (*c* 0.8, 5 mol dm^{-3} HCl); δ_H (200 MHz; ^2H_2O) 1.20 (3H, d, *J* 7.5 Hz, 3-CH_3), 2.76 (1H, m, *J* 7.5 and 5.0 Hz, 3-H) and 3.50 (1H, d, *J* 5.0 Hz, 2-H). For (2*S*,3*R*)[3-^{2}H]-3-methylaspartic acid; m.p. 258–259 °C (decomp.); $[\alpha]_D^{25}$ +30.5° (*c* 1.0, 5 mol dm^{-3} HCl); δ_H (200 MHz; ^2H_2O) 1.20 (3H, s, 3-CH_3), and 3.50 (1H, s, 2-H).[9] The (2*S*,3*S*)-diastereoisomer can be easily distinguished from the (2*S*,3*R*)-diastereoisomer in ^1H NMR spectra by examining the chemical shifts for the signals due to the methyl groups which occur at δ 1.05 and 1.20, respectively.

slow break-down of the covalent L-*erythro*-substrate–enzyme complex to give the Michaelis complex, the reverse of step 1 (compare with Scheme 1).

At 50 mmol dm^{-3} K^+, ^DV was 6.79 ± 0.92 and $^D(V/K)$ was 4.10 ± 1.30, although the absolute apparent values of V_{max} and K_m were increased and decreased, respectively. These findings (faster rates, lower K_m values, similar magnitudes of isotope effects to those obtained at 1 mmol dm^{-3} K^+) are in accord with the notion that the reverse of step 1, rather than substrate debinding, accounts for the depressed value of $^D(V/K)$ compared with ^DV. Note that at 50 mmol dm^{-3} K^+, the isotope effects for the *threo*-isomer were completely suppressed.[4]

Collectively the results presented here show that methylaspartase possesses an intrinsic ability to slowly deaminate the L-*erythro*-diastereoisomer of its natural substrate; that this ability is not associated with an epimerase activity, and that a C-3 carbanion is not an intermediate. The reaction shows a very large primary deuterium isotope effect for V_{max} and a smaller, but significant effect on V/K under conditions where the rate and the apparent binding constants vary widely. Thus, C–H bond cleavage is cleanly rate-limiting and is preceded by a step that is slow in the reverse direction, which is not substrate debinding. Given that the facile formation of a C^{α}-carbonium ion carboxylic acid intermediate seems unlikely, the results suggest that the *syn*-elimination reaction is concerted.

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