^{15}N-Isotope and Double Isotope Fractionation Studies of the Mechanism of 3-Methylaspartase: Concerted Elimination of Ammonia from (2*S*,3*S*)-3-Methylaspartic Acid

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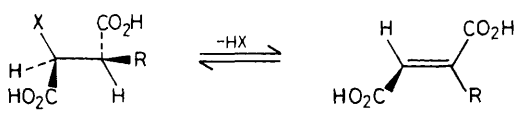
The mechanism of the L-*threo*-3-methylaspartate ammonia-lyase reaction has been investigated using double isotope (^{2}H/^{1}H, ^{15}N/^{14}N) fractionation techniques; the physiological substrate is deaminated *via* a concerted mechanism in which C$^\beta$–H and C$^\alpha$–N bond cleavage occur in the same transition state.

The mechanism for the enzymic elimination of ammonia or water from substituted succinic acids (Scheme 1) has been an area of much interest in recent years.[1] Investigation of a number of systems including aspartase[2] (E.C. 4.3.1.1), 3-methylaspartase[3,4] (E.C. 4.3.1.2.), argininosuccinate lyase[5] (E.C. 4.3.2.1.), and fumarase[6] (E.C. 4.2.1.2.) have led to the conclusion that the reactions occur *via* carbanion or even carbonium ion mechanisms.

Until recently it was believed that none of these enzymic reactions showed a significant primary deuterium isotope effect. Work in our own laboratory led us to question the results obtained for 3-methylaspartic acid with the enzyme 3-methylaspartase[3] and to discover that the reaction showed a primary isotope effect of ~1.7 for V_{max} and V/K in incubations conducted at a range of pH values.[7] Although it had been assumed that C–N bond cleavage is at least partially rate-limiting in the 3-methylaspartase catalysed reaction, the

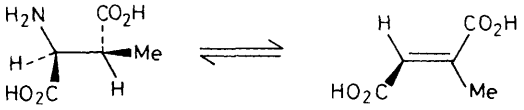


R = H, X = NH_2: aspartase
R = Me, X = NH_2: methylaspartase
R = H, X = arginino: argininosuccinate lyase
R = H, X = OH: fumarase

Scheme 1

^{15}N-isotope effect had not been reported. Here we report on the use of the primary deuterium isotope effect in double isotope fractionation experiments which measure the ^{15}N-isotope effect for C–N bond cleavage in the absence and presence of deuterium at C-3.

3-Methylaspartase from *Clostridium tetanomorphum* requires both monovalent and divalent cations for full activity; K^+ and Mg^{2+} are the best. In the absence of monovalent cations, the reaction is extremely slow, but ammonium ion is able to fulfil the role of K^+. Since ammonium ion is able to serve as a monovalent cationic activator and is also a deamination product, the enzyme behaves autocatalytically in incubations conducted at low K^+ concentration, especially near the pH optimum for the enzyme, pH 9.0. Indeed, at pH 9.0, increasing monovalent cation concentrations decrease the apparent dissociation constant K_m for the substrate in addition to increasing V_{max}, while at pH 6.5 only V_{max}, which is already relatively low, compared to pH 9.0, increases.[4] While low K^+ concentrations ensure that the chemical steps are slow compared to the substrate and product on/off rates, a desirable situation for measuring isotope effects, autocatalysis complicates the analysis and interpretation of the kinetic isotope data. This is because it is usual to determine deuterium isotope effects under non-competitive conditions where the values of V and V/K are derived from *initial* reaction rate data with the fully protiated and deuteriated substrates separately, while conversely, for heavy atoms, the fractionation of heavy isotopes (*e.g.* ^{13}C/^{12}C or ^{15}N/^{14}N) in 'natural abundance' substrate is measured competitively as an isotope ratio before



Scheme 2

and after 10—20% of reaction has occurred.[8a] Clearly, if ammonium ion activates the reaction as it proceeds and causes autocatalysis, then the commitments to the rate-limiting chemical step(s) must increase as these steps become faster, leading to increasingly suppressed isotope effects. (Note: the forward commitment is the partition ratio through the step of interest, compared to substrate dissociation, through reverse steps, and is related to the first-order rate constants for chemical and binding steps as defined by Cleland[9]). In essence then, it is expected that the observed deuterium and nitrogen isotope effects will reflect different commitments; the deuterium isotope effects representing the commitments at the start of the reaction and the suppressed nitrogen isotope effects representing an average of the commitments over the course of the reaction. Fortunately, these complications do not preclude a qualitative interpretation of such effects since it is evident that under autocatalytic conditions (low K^+, and especially near the pH optimum) the true nitrogen isotope effects will be underestimated.

The ^{15}N-isotope effect for (2*S*,3*S*)-3-methylaspartic acid was determined by measurement of the $^{15}N/^{14}N$ ratio in the initial substrate and in the ammonia produced after ~17% of the enzyme catalysed deamination was complete at both pH 6.5 and 9.0. From the change in the ratio the $^{15}(V/K)$ isotope effect was determined using equation (1), where R_0 and R are the measured isotope ratios in the substrate and in the ammonia produced after the fraction of the reaction (f) respectively. The extent of the reaction in each incubation was measured spectrophotometrically and the reactions were quenched by the addition of acid. The ammonia produced by enzymic deamination was distilled into dilute sulphuric acid and then oxidized to dinitrogen using hypobromite. The $^{15}N/^{14}N$ ratio (R) was determined using a VG SIRA 10 dual-inlet isotope ratio mass spectrometer. R_0 was determined by digesting a sample of the substrate using the Kjeldahl procedure.[10] The ammonia produced was analysed as dinitrogen as described above. The enzyme incubations and the analyses were repeated 3 times and the average values for the isotope effects were found to be 1.0246 ± 0.0013 at pH 9.0 and 1.0255 ± 0.0011 at pH 6.5.

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

These observed values of ~2.5% are quite large,[8] especially if they underestimate the true $^{15}(V/K)$ values, *vide supra*, and reflect a significant fraction of the intrinsic ^{15}N-isotope effect of ~5%, the deduced lower limit from equilibrium isotope studies.[11] Thus, for (2*S*,3*S*)-3-methylaspartic acid both C^{β}–H and C^{α}–N bond cleavage show significant primary isotope effects and are, therefore, both kinetically important.

In order to distinguish between the two plausible elimination mechanisms that would be expected to show significant V/K isotope effects for both chemical steps,[12] balanced stepwise carbanion and concerted mechanisms, the ^{15}N-isotope effects for the reaction were re-assessed using (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid. For a reaction following a balanced stepwise mechanism, the isotope effect for the second step, C–N bond cleavage, should be sensitive to the presence of a heavy isotope in the bond cleaved in the first step. This would lead to a decrease in the observed $^{15}(V/K)$ isotope effect. A concerted mechanism, however, predicts that the observed $^{15}(V/K)$ isotope effect will increase towards the intrinsic value in the presence of deuterium as the kinetic importance of the single transition state is increased, unless it is already maximal, in which case the value will remain unchanged, Figure 1.

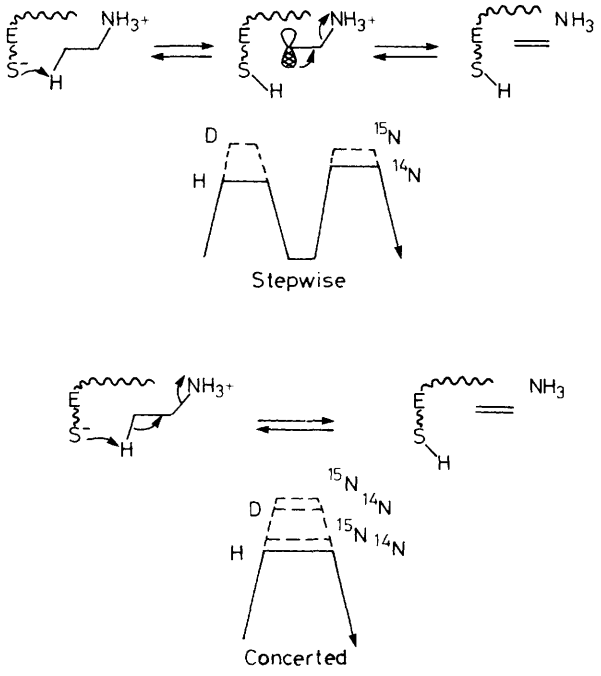


Figure 1. Free energy profiles for the possible reaction mechanisms for 3-methylaspartase. (A) Balanced step-wise; (B) concerted. Free energy differences are shown in the transition state only for purposes of illustration.

The incubations using the deuteriated isotopomer were conducted 4 times and the results were analysed as outlined above. The average observed value for $^{15}(V/K)$ at pH 9.0 was found to be 1.0241 ± 0.0009. Within experimental error the result is identical to that obtained with the undeuteriated substrate, and together with the known identity of ^{D}V and $^{D}(V/K)$ of 1.7 at pH 9.0, *vide supra*, indicates that the *actual* value of $^{15}(V/K)$ is large and already maximal and that the observed value is a considerable underestimate owing to autocatalytic suppression, as proposed above.

At pH 6.5 the value of $^{15}(V/K)$ was 1.0417 ± 0.0010 revealing a substantial increase in nitrogen fractionation compared to that observed in the absence of deuterium. The increased kinetic importance of C–N bond cleavage clearly demonstrates that both parts of the elimination process occur on a single transition state. The results, therefore, indicate that methylaspartase operates *via* a concerted mechanism and, furthermore, that the chemistry of the reaction is not affected by gross changes in pH. This is particularly interesting since a concerted reaction pathway with its single transition state for both bond-breaking processes is a unique route from substrate through to product and might be expected to be rather sensitive to changes in reaction conditions which slow down or speed up either of the two individual components.

To our knowledge this is the first example of a concerted enzymic elimination reaction, and indeed, methylaspartase has been hitherto regarded as the archetypal example of an enzyme which operates *via* a carbanion mechanism.

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