Substrate Specificity of the 3-Methylaspartate Ammonia-Lyase Reaction: Observation of Differential Relative Reaction Rates for Substrate–Product Pairs†

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Received October 1, 1987; Revised Manuscript Received December 7, 1987

ABSTRACT: A range of substituted fumaric and aspartic acid substrates for the enzyme 3-methylaspartate ammonia-lyase (EC 4.3.1.2) have been synthesized and used to study the kinetics of the catalyzed reaction in both the forward (deamination) and reverse (conjugative amination) reaction directions. The rates of amination for all of the α,β-unsaturated substrates studied (bearing substituents the size of an ethyl group or smaller) were similar under [s] $\gg K_M$ conditions although K_M values for the substrates varied by a factor of 25. The rates of deamination for the corresponding 3-substituted amino acid substrates varied widely with structure under [s] $\gg K_M$ conditions, and thus for substrate–product pairs the ratio for V(forward)/V(reverse) also varied. These differential reaction rates indicate that there is a step in the deamination direction that is especially sensitive to the size of the 3-substituent of the substrate and that a relatively large group (methyl to ethyl in size) is required for binding in order to reduce the activation energy for this step. Given that it is proposed that the enzyme operates via an $E1_{cb}$-type mechanism where C–N bond cleavage is rate limiting, it is likely that binding of the C-3 substituent of aspartic acid substrates affects the alignment of the nascent carbanion with the C–N bond for elimination.

3-Methylaspartate ammonia-lyase catalyzes the reversible α,β-elimination of ammonia from L-*threo*-3-methylaspartic acid (**1**) produced from L-glutamic acid in *Clostridium tetanomorphum* (Barker et al., 1959) (Scheme I).

The enzyme also catalyzes the deamination of L-*erythro*-3-methylaspartic acid and L-aspartic acid (Barker et al., 1959) and also some other 3-alkylaspartic acids (Winkler & Williams, 1967). Since it was known that the enzyme could be used to catalyze the amination of mesaconic acid (**2**, X = CH_3) to give (2*S*,3*S*)-3-methylaspartic acid (the L-*threo*-diastereomer), in good yield (Barker et al., 1958), we undertook to assess the utility of the enzyme in the enantiospecific synthesis of 3-halogeno- and 3-alkyl-L-aspartic acids (Akhtar et al., 1986, 1987a). The amination reactions occurred as predicted to give products possessing the expected absolute configuration (Akhtar et al., 1987a,b) (Scheme II).

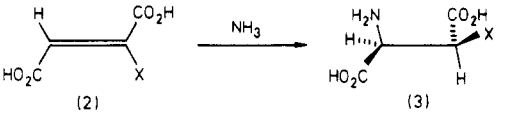
During these synthetic studies (Akhtar et al., 1986) it was noted that the amination rates were similar for 3-substituted fumaric acids (**2**, X = H, CH_3, Cl and Br) although it was known that the enzymic deamination of (2*S*)-aspartic acid (**3**, X = H) was about 100 times slower than the deamination of the physiological substrate (2*S*,3*S*)-3-methylaspartic acid (**3**, X = CH_3) under similar conditions (Barker et al., 1959; Winkler & Williams, 1967). This observation prompted us to investigate, in detail, the rates of reaction for a range of substrate/products in both the forward (deamination) and reverse (amination) reaction directions. Here we present the results of these studies and provide a mechanistic rationale for the observed differential relative rates of deamination/amination for substrate/product pairs.

Materials and Methods

Chemicals and Reagents. Chemicals, buffers, and reagents were of analytical grade or were otherwise recrystallized or redistilled to high purity. Organic solvents were distilled and water for all aqueous solutions was distilled from glass once or twice as appropriate.

Scheme I: 3-Methylaspartate Ammonia-Lyase Reaction

Scheme II: Amination Reaction Catalyzed by 3-Methylaspartate Ammonia-Lyase



Substrates. Fumaric acid and acetylenedicarboxylic acid were obtained from British Drug Houses (Poole, Dorset, U.K.); mesaconic acid was obtained from Sigma Chemical Co. (St. Louis, MO). Fluoro-, chloro-, bromo-, iodo-, ethyl-, isopropyl-, *n*-propyl-, and *n*-butylfumaric acid were prepared as described earlier (Akhtar et al., 1987a). (2*S*)-Aspartic acid was obtained from British Drug Houses (Poole, Dorset, U.K.) and (2*R*,3*S*)-3-chloroaspartic acid, (2*S*,3*S*)-3-methylaspartic acid, and (2*S*,3*S*)-3-ethylaspartic acid were prepared through enzymic amination of the appropriate fumaric acids (Akhtar et al., 1987a).

Enzyme. 3-Methylaspartate ammonia-lyase 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. (1959) using a modification of literature procedures (Barker et al., 1959; Hsiang & Bright, 1969). The specific activity of the enzyme used in these studies was 25–40 units (mg of protein)$^{-1}$.

Enzyme Assay. Enzyme was assayed according to the literature procedure. One unit catalyzes the formation of 1 μmol of mesaconic acid min^{-1} at pH 9.7 at 25 °C as determined by the increase in OD_{240} under the assay conditions (Barker et al., 1959).

† This work was supported by Science and Engineering Research Council grants to D.G.; M.A. and M.A.C. are grateful for studentships. Contribution from the Institute of Biomolecular Sciences, University of Southampton.
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0006-2960/88/0427-2953$01.50/0

Table I: Extinction Coefficients for Substituted Fumaric Acids at 240 nm[a]

substrate	ϵ (mol^{-1} dm^3 cm^{-1}) 240 nm	270 nm
fumaric acid	2530	555.0
mesaconic acid	3850	482.5
ethylfumaric acid	3323	431.0
chlorofumaric acid	4434	487.0
bromofumaric acid	4216	521.0
n-propylfumaric acid	3920	
isopropylfumaric acid	3753	

[a] Extinction coefficients measured in 0.5 M Tris at pH 9 and at 30 °C.

Table II: Kinetic Parameters for the Amination of Substituted Fumaric Acids[a]

substrate	K_M (mM)	V_{max} ($\times 10^{-6}$ mol dm^{-3} min^{-1})[b]
fumaric acid	23 ± 2.2	1702
mesaconic acid	1.24 ± 0.085	894
ethylfumaric acid	1.05 ± 0.2	583
chlorofumaric acid	3.52 ± 0.71	382
bromofumaric acid	2.64 ± 0.53	425
n-propylfumaric acid	2.1 ± 1.3	4.2
isopropylfumaric acid	5.5 ± 3.0	5.3
n-butylfumaric acid		<0.05
iodofumaric acid		<0.05

[a] Incubation mixtures contained 0.5 M Tris (pH 9), 0.02 M $MgCl_2$, 0.4 M NH_4Cl, and substrate, in a total volume of 3 mL. Reaction was initiated by addition of enzyme solution (20 μL), which was preassayed. Reactions were carried out at 30 ± 0.1 °C. Loss of starting material was observed spectrophotometrically at 240 nm. [b] Corrected for 1 unit of enzyme assayed at pH 9. Error is ±10% for all V_{max} values.

Determination of Kinetic Parameters. All rate measurements were conducted at pH 9 at 30 °C. The extinction coefficients used for each substituted fumaric acid are given in Table I. These were obtained by preparing buffered solutions of each of the compounds at pH 9 from analytically pure commercially obtained or synthetic samples as appropriate. Two extinction coefficients were used in order to allow the direct spectrophotometric rate determinations for the wide range of concentrations of the fumaric substrates required for studying the reaction in the amination direction.

Amination Direction. Incubations contained 500 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 9.0, 20 mM $MgCl_2$, 400 mM NH_4Cl, and substrate, 10–14 different concentrations ranging from 0.5 to 20 mM. Reactions were initiated by the addition of enzyme and were followed directly spectrophotometrically on a Pye-Unicam SP8 500 instrument at 240 or 270 nm in 10- or 1-mm quartz cuvettes as appropriate to the concentration of the substrate. Each rate determination was measured in triplicate. The reactions were linear over the time course measured (up to ca. 5% of total conversion). Kinetic data were analyzed with Hanes or Eadie–Hofstee plots. Regression analyses gave the best straight lines.

Deamination Direction. Incubations contained 500 mM Tris-HCl, pH 9.0, 20 mM $MgCl_2$, 1 mM KCl, and substrate, 10–14 different concentrations ranging from 0.5 to 20 mM. Reactions were initiated by the addition of enzyme and were followed at 240 nm in 10-mm quartz cuvettes. Each determination was measured in triplicate. Kinetic data were analyzed as described, vide supra.

Results

The kinetic parameters for the 3-methylaspartase-catalyzed amination of substituted fumaric acids are given in Table II and are shown in Lineweaver–Burk form in Figure 1. The values of V_{max} for each substrate containing a substituent the size of an ethyl group or smaller are similar although the K_M values vary by a factor of 25. Thus, the rate of conjugate addition of ammonia to fumaric acid substrates is not sensitive to electronic or steric changes at C-3 of the substrate.

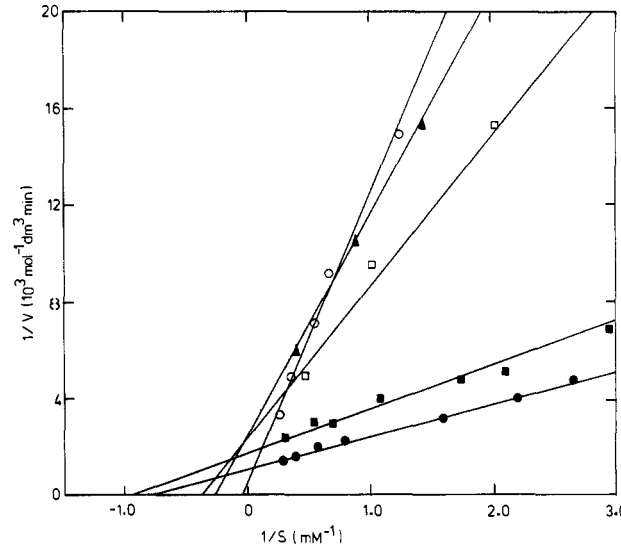


FIGURE 1: Lineweaver–Burk plots for the amination of substituted fumaric acids. For reaction conditions see Table II: (●) mesaconic acid; (■) ethylfumaric acid; (□) bromofumaric acid; (▲) chlorofumaric acid; (○) fumaric acid. Note: several points are off-scale. The propylfumaric acids react too slowly to be shown on the same diagram.

Table III: Kinetic Parameters for the Deamination of 3-Substituted Aspartic Acids[a]

substrate	K_M (mM)	V_{max} ($\times 10^{-6}$ mol dm^{-3} min^{-1})[b]
3-methylaspartic acid	2.37 ± 0.2	654.0
aspartic acid	10.5 ± 0.82	4.8
3-ethylaspartic acid	17.08 ± 1.4	292.0
3-chloroaspartic acid	>50	

[a] Incubation mixtures contained 0.5 M Tris (pH 9), 0.02 M $MgCl_2$, 0.001 M KCl, and substrate in a total volume of 3 mL. Reaction was initiated by addition of enzyme solution (20 μL). Reactions were carried out at 30 ± 0.1 °C. Formation of product was observed spectrophotometrically at 240 nm. [b] Corrected for 1 unit of enzyme assayed at pH 9. Error is ±10% for all V_{max} values.

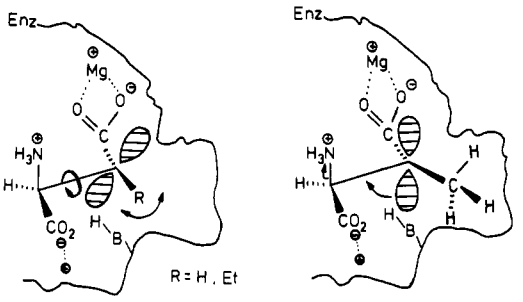
The kinetic parameters for the deamination of substituted aspartic acids are given in Table III. The values of V_{max} vary widely whereas the values of K_M are similar. The ratios of the rates in each direction under V_{max} conditions show that the two extreme results are for the substrate/product pairs mesaconic/methylaspartic acid, $V_{max}(for)/V_{max}(rev) = 0.73$, and fumaric/aspartic acid, $V_{max}(for)/V_{max}(rev) = 0.0028$, with ethylfumaric acid/ethylaspartic acid at an intermediate value of 0.50.

Discussion

It has been proposed (Bright, 1964) that the rate of the overall reaction with (2*S*,3*S*)-methylaspartic acid as substrate is governed by the rate of C–N bond cleavage. No primary isotope was observed in these studies using 86% C-3-deuteriated substrate (Bright, 1964) while rapid wash-out of label through C-3 hydrogen exchange occurred without apparent C–N bond cleavage [for a review, see Hanson and Havir (1972)].

Both of these findings support the proposed $E1_{cb}$-type mechanism providing that C-3 hydrogen exchange occurs at the carbanion intermediate. Our recent results (Akhtar et al.,

Chart I: Proposed Effect of the Size of the 3-Substituent of the Substrate upon Orbital Alignment before C–N Bond Cleavage



1987; Botting et al., 1988) do not support all of Bright's conclusions, but in essence, the enzyme does appear to operate via a carbanion mechanism with some substrates, and thus our present results can be rationalized in terms of Bright's model of the mechanism. Our results indicate that relative to methylaspartic acid there is a slow step in the deamination reaction direction for aspartic acid. Since the observed rate is 137 times slower than for the physiological substrate and since a chemical step is thought to be rate limiting for this fast substrate, it is unlikely that a step other than a chemical step is rate limiting for the deamination of aspartic acid. Also, it should be noted that in the reverse reaction direction fumaric acid has a larger value for V_{max} than the methyl homologue. Two processes are involved in an $E1_{cb}$-type elimination: removal of a proton from C-3 and then C–N bond cleavage, resulting in the expulsion of ammonia. In principle, either or both of these two processes would be expected to be sensitive to changes in the protein-substrate binding interaction adjacent to C-3. For example, a conformation change could result in a lower rate of proton abstraction from the C-3 position if the change decreased the acidity of the proton (through poor *aci*-carboxylate stabilization) or made the proton less accessible to the enzyme-bound base. Alternatively, or additionally, the C–N bond cleavage step could become more rate limiting through nonoptimal alignment of the intermediate carbanion with the leaving N atom (Chart I).

Given the C–N bond cleavage is believed to be rate limiting for 3-methylaspartate ammonia-lyase (Bright 1964) and is known to be rate limiting for the related enzymic reactions catalyzed by aspartase (Porter & Bright, 1980; Nuiry et al., 1984) and argininosuccinate lyase (Kim & Raushel, 1986a,b), we expect that in the present study poor orbital alignment of the carbanion accounts for the low rate of deamination for (2*S*)-aspartic acid. Support for this view is now available (Botting et al., 1987, 1988).

ACKNOWLEDGMENTS

We thank Prof. C. Greenwood and A. Thompson, University of East Anglia, for growing the *Clostridium tetanomorphum*.

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