Primary Deuterium Isotope Effects for the 3-Methylaspartase-Catalyzed Deamination of (2*S*)-Aspartic Acid, (2*S*,3*S*)-3-Methylaspartic Acid, and (2*S*,3*S*)-3-Ethylaspartic Acid†

Nigel P. Botting, Mark A. Cohen, Mahmoud Akhtar, and David Gani*,‡
Department of Chemistry, The University, Southampton SO9 5NH, U.K.
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ABSTRACT: 3-Methylaspartate ammonia-lyase catalyzes the deamination of (2*S*)-aspartic acid 137 times more slowly than the deamination of (2*S*,3*S*)-3-methylaspartic acid but catalyzes the amination of fumaric acid 1.8 times faster than the amination of mesaconic acid [Botting, N. P., Akhtar, M., Cohen, M. A., & Gani, D. (1988) *Biochemistry* (preceding paper in this issue)]. In order to understand the mechanistic basis for these observations, the deamination reaction was examined kinetically with (2*S*)-aspartic acid, (2*S*,3*S*)-3-methylaspartic acid, (2*S*,3*S*)-3-ethylaspartic acid, and the corresponding C-3-deuteriated isotopomers. Comparison of the double-reciprocal plots of the initial reaction velocities for each of the three pairs of substrates revealed that the magnitude of the primary isotope effect on both V_{max} and V/K varied with the substituent at C-3 of the substrate. 3-Methylaspartic acid showed the largest isotope effect (1.7 on V_{max} and V/K), 3-ethylaspartic acid showed a smaller isotope effect (1.2 on V_{max} and V/K), and aspartic acid showed no primary isotope effect at all. These results, which are inconsistent with earlier reports that there is no primary isotope effect for 3-methylaspartic acid [Bright, H. J. (1964) *J. Biol. Chem. 239*, 2307], suggest that for both 3-methylaspartic acid and 3-ethylaspartic acid elimination occurs via a predominantly concerted mechanism whereas for aspartic acid an $E1_{cb}$ mechanism prevails. It is expected that the change in mechanism arises as a result of the loss of the hydrophobic interaction between the C-3-alkyl substituent of the substrate and the complementary active site pocket of the enzyme, which leads to poor orbital alignment of the nascent negative charge with the C–N bond in the carbanion derived from aspartic acid. Substrate C-3 hydrogen exchange experiments suggest that the intermediate carbanion/conjugate acid (of the enzyme-bound base) is not accessible to solvent during catalysis.

Study of the mechanism of the reactions catalyzed by the ammonia-lyases [e.g., aspartase (Scheme I, X = NH_2, R = H) (Porter et al., 1980; Nuiry et al., 1984) and methylaspartase (Scheme I, X = NH_2, R = CH_3) (Hanson & Havir, 1972; Bright, 1964)] and the dehydrases [e.g., fumarase (Scheme I, X = OH, R = H) (Hill & Teipel, 1971; Marletta et al., 1982; Jones et al., 1980)] has attracted much interest in recent years, yet they are still poorly understood. It was proposed that methylaspartase operates via a carbanion mechanism ($E1_{cb}$) because C-3 hydrogen exchange of the substrate with the solvent occurs more rapidly than C–N bond cleavage and because no primary isotope effect was observed for the deamination of the C-3-deuteriated substrate. Carbonium ion mechanisms were once proposed for the aspartase- (Doughertey et al., 1971; Klinman, 1977) and fumarase- (Hansen et al., 1969) catalyzed reactions largely because no primary deuterium isotope effects or rapid solvent hydrogen exchanges were observed. Recent studies support a carbanion mechanism for both enzymes (Porter & Bright, 1980; Nuiry et al., 1984). Interestingly, these enzymes catalyze reactions of similar stereospecificity and also show a remarkable degree of protein sequence homology (Woods et al., 1986).

During our recent studies of the amination of substituted fumaric acids, using 3-methylaspartase to catalyze the retrophysiological reaction, it was noted that the reaction rates (V_{max}) for each of the substrates (3, X = H, CH_3, Et, Cl, Br) were similar (Botting et al., 1988). These findings were of particular interest because the published rate for deamination of (2*S*)-aspartic acid (Barker et al., 1959; Winkler et al., 1967) was about 100 times slower than that of the physiological substrate. Indeed, in our hands V_{max} for (2*S*)-aspartic acid was 137 times slower than that for the methyl homologue (Botting et al., 1988). In order to account for these observations, we suggest that with (2*S*)-aspartic acid as substrate the lack of a C-3 substituent–active site lipophilic interaction could distort the E·S complex so that the β-proton acidity was increased (due to poor *aci*-carboxylate stabilization) and/or that nonoptimal alignment of the nascent carbanion with the C–N bond reduced the rate of deamination.

Scheme I: Ammonia-Lyase/Dehydrase Reaction (See Text for Details)

Since the enzyme was reported to operate via an $E1_{cb}$ mechanism with rate-limiting C–N bond cleavage for (2*S*,3*S*)-3-methylaspartic acid (Bright, 1964), the fastest substrate, we set out to determine the mechanism for the elimination of ammonia from both (2*S*)-aspartic acid and (2*S*,3*S*)-3-ethylaspartic acid. Here we present the results of studies using C-3-deuteriated substrates which reveal not only that the mechanism of the deamination changes as the size of the C-3 substituent is varied but also that the physiological substrate shows a primary deuterium isotope effect (Botting et al., 1987), contrary to previous reports (Bright, 1964). The results are discussed in terms of the mechanism of the elim-

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ination process, and evidence is provided to suggest that enzyme-bound carbanions cannot exchange solvent hydrogen without C–N bond cleavage, contrary to previous reports.

Materials and Methods

Materials. Tris(hydroxymethyl)aminomethane (Tris), magnesium chloride hexahydrate, and (2*S*)-aspartic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium chloride was obtained from British Drug Houses (Poole, Dorset, U.K.). (2*S*,3*S*)-3-Methylaspartic acid and (2*S*,3*S*)-3-ethylaspartic acid were prepared as previously described (Botting et al., 1987; Akhtar et al., 1987). (2*S*,3*R*)-[3-^{2}H]Aspartic acid, (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid, and (2*S*,3*S*)-[3-^{2}H]-3-ethylaspartic acid were prepared through enzymic amination of the corresponding fumaric acids in deuterium oxide as previously reported (Botting et al., 1987; Akhtar et al., 1987). Each of the deuteriated substrates contained >95 atom % deuterium at the appropriate position.

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) with a modification of literature procedures (Barker et al., 1959; Hsiang & Bright, 1969) as described in the preceding article (Botting et al. 1988). 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 method of Barker where 1 unit of enzyme 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).

Determination of Kinetic Parameters. Three sets of incubations (conducted at pH 9.0, 8.0, and 7.6) contained 500 mM Tris-HCl, 20 mM $MgCl_2$, 1 mM KCl, and substrate, 10–20 different concentrations ranging from 0.5 to 20 mM. Reactions were initiated by the addition of enzyme and were followed directly spectrophotometrically at 240 nm (Pye-Unicam SP8-500) in 10-mm quartz cuvettes (Botting et al., 1988). The reactions were linear over the time course measured, up to ca. 5% of the overall reaction. Each rate determination was measured in triplicate. Kinetic data were analyzed with Eadie–Hofstee plots. Regression analyses gave the best straight lines. The entire analysis for each substrate was conducted 2 or 3 times.

^{1}\{H\}^{13}C NMR Spectroscopic β-Hydrogen Exchange Assay. (A) Exchange in Protium Oxide. Incubations contained 100 mM Tris-HCl, pH 9.0, 20 mM $MgCl_2$, 4.5 mM NaCl, and C-3-deuteriated substrate (200 mg), (2*S*)-aspartic acid or (2*S*,3*S*)-3-methylaspartic acid, in H_2O–^2H_2O–MeOH (88:10:2, 2.2 mL) in 10-mm tubes; ^2H_2O was present to "lock" the spectrometer (Bruker AM 360), and MeOH served as a ^{13}C NMR chemical shift reference. The broad-band proton decoupler power was set to 11 H. Reactions were initiated by the addition of enzyme (16 units), and the reactions were followed over a period of several hours.

(B) Exchange in Deuterium Oxide. Incubations were identical with those described above except that the substrates used were nonlabeled, and the solvent was ^2H_2O–MeOH (98:2).

The peak height and multiplicity of signals corresponding to starting material and product were used to *approximate* the extent of the reactions. Poor signal to noise ratios and added complications due to changing nuclear Overhauser enhancement prevented any kinetic analysis other than a *qualitative assessment* of the relative reaction rates for β-hydrogen exchange.

Table I: Kinetic Parameters for the Deamination of 3-Substituted Aspartic Acids and Their Corresponding C-3-Deuteriated Isotopomers[a]

substrate	K_M (mM)	V_{max} ($\times 10^{-6}$ mol dm^{-3} min^{-1})[b]	V/K
(2*S*)-aspartic acid	10.50 ± 0.82	4.8	0.46
(2*S*,3*R*)-[3-^{2}H]aspartic acid	10.50 ± 0.82	4.8	0.46
(2*S*,3*S*)-3-methylaspartic acid	2.37 ± 0.2	654.0	276.0
(2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid	2.35 ± 0.25	385.2	163.9
(2*S*,3*S*)-3-ethylaspartic acid	17.08 ± 1.4	292.0	17.1
(2*S*,3*S*)-[3-^{2}H]-3-ethylaspartic acid	17.66 ± 1.6	250.6	14.7

[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), which was preassayed. Reactions were carried out at 30 ± 0.1 °C. Formation of product was observed spectrophotometrically. [b] Error ± 10% for all V_{max} values.

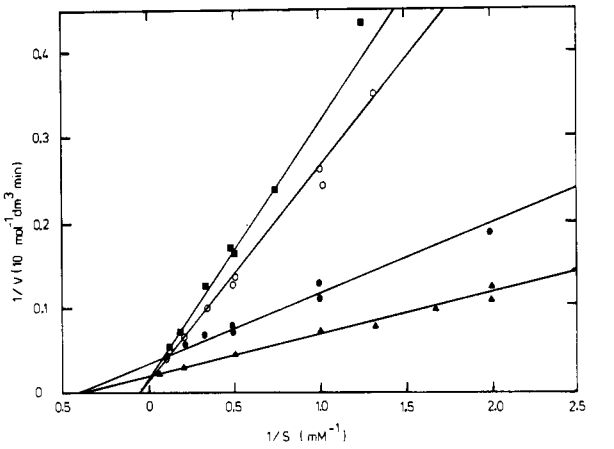


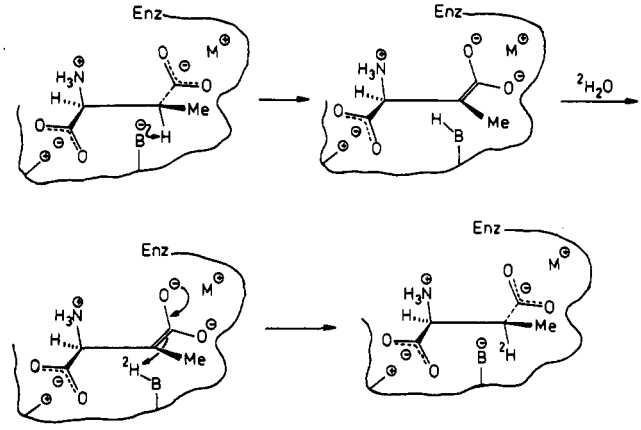
FIGURE 1: Lineweaver–Burk plot for the deamination of 3-methylaspartic acids and 3-ethylaspartic acids. For reaction conditions, see Table I: (1) (2*S*,3*S*)-3-methylaspartic acid (■); (2) (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid (○); (3) (2*S*,3*S*)-3-ethylaspartic acid (●); (4) (2*S*,3*S*)-[3-^{2}H]-3-ethylaspartic acid (▲). (Values for 3-methylaspartic acids are multiplied by 3 to fit on the same scale.)

Results

The kinetic parameters as determined from initial rate values for the deamination of (2*S*)- and (2*S*,3*R*)-[3-^{2}H]aspartic acid are given in Table I. At pH 9.0 no primary isotope effect was observed on V_{max} or V/K. (2*S*,3*S*)-3-Methylaspartic acid and (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid were deaminated at different rates (Figure 1) although the value of K_M was the same for both substrates. The primary isotope effect upon V_{max} and V/K is 1.7 ± 0.3. (2*S*,3*S*)-3-Ethylaspartic acid and (2*S*,3*S*)-[3-^{2}H]-3-ethylaspartic acid showed similar K_M values but were also deaminated at different rates (Figure 1). The primary isotope effect upon V_{max} and V/K is 1.16 ± 0.2. The results of experiments conducted at pH 8 and pH 7.6 were similar.

The relative rates for β-hydrogen exchange for (2*S*)-aspartic acid and (2*S*,3*S*)-3-methylaspartic acid were difficult to access from the ^{13}C NMR spectroscopic experiments. The incubations conducted in 88:10:2 H_2O–^2H_2O–MeOH with deuteriated substrates were most informative. From these experiments it was possible to deduce that the rate of hydrogen exchange with solvent hydrogen (measured as deuterium wash-out) was much slower for aspartic acid than for 3-methylaspartic acid. While deuterium wash-out from the methylaspartic acid after 1 h had occurred to the extent of at least 50% of total methylaspartic acid (the remainder was

Scheme II: Mechanism Proposed by Bright To Account for the β-Hydrogen Exchange Reaction



mesaconic acid), only 5% of wash-out had occurred for deuteriated aspartic acid.

Discussion

The mechanism of the 3-methylaspartate ammonia-lyase reaction has been investigated by Bright (1964). Using samples of (2*S*,3*S*)-[3-^{2}H]-3-methylaspartic acid and unlabeled material, Bright showed that the rate of the deamination reaction was not sensitive to substitution by deuterium at C-3 at pH 5.5, 7.5, and 9.5. It should be noted that in these experiments the deuteriated substrate contained a considerable amount of protium at C-3, and thus a small isotope effect may have been difficult to distinguish from the limits of experimental error. Bright also found that the enzyme catalyzed the exchange of the C-3 hydrogen atom of the substrate with solvent hydrogen at significant rates and that the ratio of the rate of exchange versus the rate of deamination varied with pH.

Using the results of these experiments, Bright proposed that the enzyme operated by a carbanion mechanism, whereby an enzyme-bound base, probably a cystein thiolate group, removes the C-3 hydrogen of the substrate to give a stabilized carbanion, Scheme II. It was proposed that the active site thiol of the enzyme/carbanion intermediate could exchange hydrogen with the solvent to give, via reverse steps, labeled 3-methylaspartic acid or could alternatively eliminate ammonia via C–N bond cleavage, the rate-limiting process for the deamination reaction, to give mesaconic acid.

Further support for the carbanion mechanism came from studies of the exchange reaction (Bright, 1964). Bright found that in the presence of increasing concentrations of ammonia the ratio of exchange over deamination increased. The possibility that dissociation of an enzyme–mesaconate complex might be determining V_{max} for the overall reaction was, however, ruled out by the finding that in the presence of [^{15}N]-ammonia no ^{15}N label was detected in the solvent hydrogen exchanged product. In the presence of the other deamination product, mesaconic acid, no significant effect was noted upon the rate of either exchange or deamination, a result which rules out the possibility of rate-determining enzyme–ammonia dissociation. Thus hydrogen exchange could apparently occur without C–N bond cleavage. Bright concluded that the enzyme–substrate carbanion intermediate underwent hydrogen exchange before the elimination of ammonia and that after rate-determining C–N bond cleavage ammonia and mesaconic acid were released from the active site rapidly.

Our present results indicate that this analysis cannot be correct. We have found that 3-methylaspartic acid shows the same primary deuterium isotope effect of 1.7 ± 0.3 at three different pHs. While a pure carbanion mechanism should not show any isotope effect, the similar values at three different pHs where the ratios of the exchange versus deamination reaction are known to vary, vide supra, rules out the possibility that any enzyme-bound intermediate prior to the deaminated complex can exchange with solvent hydrogen.

The modification to Bright's proposed mechanism raises an interesting question regarding the nonincorporation of [^{15}N]ammonia into hydrogen-exchanged 3-methylaspartic acid. One possibility is that ammonia released from the immediate vicinity of the active site does not dissociate to equilibriate with the endogenous material but rather occupies another binding site. Thus the product of the enhanced exchange reaction in the presence of [^{15}N]ammonia would contain solvent-derived hydrogen and substrate-derived ^{14}N, that is, the original N atom. As the overall reaction in Bright's original experiments contained a large excess of substrate, the form of the enzyme capable of catalyzing the amination exchange reaction would always contain [^{14}N]ammonia bound at the unique site. Experiments designed to determine the exact nature of the interaction of ammonia with the enzyme are under way in our laboratory.

The values of the primary deuterium isotope effect for the deamination of (2*S*)-aspartic acid and (2*S*,3*S*)-3-ethylaspartic acid are smaller, 1.0 and 1.15, respectively. These results together with the decrease in deamination rates are consistent with a shift in mechanism toward an $E1_{cb}$ mode due to decreased orbital alignment of the C–N bond and the nascent carbanion, thus making C–N bond cleavage relatively more rate limiting.

The relative rates of C-3 hydrogen exchange for 3-methylaspartic acid and aspartic acid are also consistent with the inability of the enzyme-bound carbanion to exchange directly. As aspartic acid is deaminated 137 times more slowly than 3-methylaspartic acid and because the former substrate shows no isotope effect and thus probably resides predominantly as the carbanion in the steady state, a direct carbanion hydrogen exchange mechanism (prior to C–N bond cleavage) would predict a massive exchange rate. Note V_{max} for fumarate is 1.8 times the V_{max} for mesaconate (Botting et al., 1988) so that all of the reverse steps are fast and K_M for aspartate is only 4 times higher than for the physiological substrate. Our ^{13}C NMR spectroscopic data sets the rates of exchange for aspartic acid approximately 10 times lower than that for the physiological substrate. In accord with these results, recent work with the related enzymes L-aspartase (Nuiry et al., 1984) and argininosuccinate-lyase (Kim & Raushel, 1986) has revealed that direct solvent hydrogen exchange with the corresponding enzyme-bound carbanions does not occur.

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Synthesis of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate (AppppA) from Adenosine 5′-Phosphosulfate and Adenosine 5′-Triphosphate Catalyzed by Yeast AppppA Phosphorylase†

Andrzej Guranowski,*,‡ Gerald Just,§ Eggehard Holler,§ and Hieronim Jakubowski*,‡,∥

Katedra Biochemii, Akademia Rolnicza, ul, Wolynska 35, 60-637 Poznan, Poland, Institut für Biophysik und physikalische Biochemie, Universität Regensburg, 8400-Regensburg, FRG, and Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey—New Jersey Medical School, Newark, New Jersey 07103

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ABSTRACT: A novel way of enzymatic synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA), which does not involve aminoacyl-tRNA synthetases, has been discovered. Yeast AppppA α,β-phosphorylase catalyzes irreversible conversion of adenosine 5′-phosphosulfate (APS) and ATP into AppppA according to the equation APS + ATP → AppppA + sulfate. In this reaction, the enzyme exhibits a broad pH optimum (between 6 and 8) and requires Mn^{2+}, Mg^{2+}, or Ca^{2+} ions for activity, with Mn^{2+} being twice as effective as Mg^{2+} or Ca^{2+} at optimal concentration (0.5 mM). The K_m values computed for APS and ATP are 80 μM and 700 μM, respectively. The rate constant for the AppppA synthesis is 3 s^{-1} (pH 8.0, 30 °C, 0.5 mM $MgCl_2$). Some ATP analogues like ppppA, GTP, adenosine 5′-(α,β-methylenetriphosphate), and adenosine 5′-(β,γ-methylenetriphosphate), but not dATP, UTP, or CTP, are also substrates for AppppA phosphorylase and accept adenylate from APS with the formation of AppppppA, AppppG, Appp(CH_2)pA, and App(CH_2)ppA, respectively. Functional versatility of yeast AppppA phosphorylase may provide a link between metabolism of AppppA on one hand and metabolism of APS and phosphate on the other and raises the possibility of participation of AppppA in regulation of metabolism of APS and/or inorganic phosphate in yeast.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA)[1] α,β-phosphorylase discovered in the extracts of yeast *Saccharomyces cerevisiae* (Guranowski & Blanquet, 1985) catalyzes the reaction

$$\text{AppppA} + \text{P*}_i \rightleftarrows \text{pppA} + \text{p*pA} \qquad \text{(I)}$$

The equilibrium of the reaction strongly favors phosphorolysis of AppppA, and by use of sensitive detectors combined with a HPLC system, only traces of enzymatic formation of AppppA could be detected as a result of the reverse reaction (Guranowski & Blanquet, 1985). The equilibrium constant $K = [\text{AppppA}][\text{P}_i]/[\text{ATP}][\text{ADP}]$ is very sensitive to pH and increases 100-fold from $K = 0.0003$ at pH 8.0 to $K = 0.028$ at pH 5.5 (Brevet et al., 1987). The enzyme supports also an exchange between the β-phosphate of nucleoside 5′-diphosphates and inorganic phosphate (Guranowski & Blanquet, 1986a):

$$\text{NDP} + \text{P*}_i \rightleftarrows \text{NDP*} + \text{P}_i \qquad \text{(II)}$$

Among many ADP analogues tested in the exchange reaction,

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‡ Akademia Rolnicza.
§ Universität Regensburg.
∥ University of Medicine and Dentistry of New Jersey—New Jersey Medical School.

[1] Abbreviations: AppppA, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; HPLC, high-performance liquid chromatography; P_i, inorganic phosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; NDP, unspecified nucleoside 5′-diphosphate; APS, adenosine 5′-phosphosulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; TLC, thin-layer chromatography.

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