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Mechanistic Characterization of *Escherichia coli* L-Aspartate Oxidase from Kinetic Isotope Effects

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Abstract

L-Aspartate oxidase, encoded by the *nadB* gene, is the first enzyme in the *de novo* synthesis of NAD⁺ in bacteria. This FAD-dependent enzyme catalyzes the oxidation of L-aspartate to generate iminoaspartate and reduced flavin. Distinct from most amino acid oxidases, it can use either molecular oxygen or fumarate to reoxidize the reduced enzyme. Sequence alignments and the three-dimensional crystal structure have revealed that the overall fold and catalytic residues of NadB closely resemble those of the succinate dehydrogenase/fumarate reductase family rather than those of the prototypical D-amino acid oxidases. This suggests that the enzyme can catalyze amino acid oxidation via typical amino acid oxidase chemistry, involving the removal of protons from the α -amino group and the transfer of the hydride from C2, or potentially deprotonation at C3 followed by transfer of the hydride from C2, similar to chemistry occurring during succinate oxidation. We have investigated this potential mechanistic ambiguity using a combination of primary, β -secondary, solvent, and multiple deuterium kinetic isotope effects in steady state experiments. Our results indicate that the chemistry is similar to that of typical amino acid oxidases in which the transfer of the hydride from C2 of L-aspartate to FAD is rate-limiting and occurs in a concerted manner with respect to deprotonation of the α -amine. Together with previous kinetic and structural data, we propose that NadB has structurally evolved from succinate dehydrogenase/fumarate reductase-type enzymes to gain the new functionality of oxidizing amino acids while retaining the ability to reduce fumarate.

Graphical abstract

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Supporting Information

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Notes

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NAD⁺ and NADP⁺ are essential coenzymes in biological oxidation and reduction reactions and in maintaining redox balance in all prokaryotic and eukaryotic organisms. The first two steps of *de novo* NAD⁺ biosynthesis are unique to bacteria as eukaryotes synthesize quinolinate from tryptophan through the kynurenine pathway.¹ Aspartate oxidase (NadB) converts L-aspartate to iminoaspartate, which is then condensed with dihydroxyacetone phosphate by quinolinate synthase (NadA) to generate the pyridine dicarboxylate, quinolinate, in a highly unusual reaction (Scheme 1). Quinolinate is subsequently converted to NAD⁺ and NADP⁺ through a universal pathway common to all organisms. Most bacteria also encode the enzymes of the Preiss–Handler salvage pathway, allowing them to grow on nicotinamide by first hydrolyzing nicotinamide to nicotinic acid and then catalyzing the formation of nicotinic acid mononucleotide using PRPP.

L-Aspartate oxidase is a flavoenzyme containing one molecule of noncovalently bound flavin adenine dinucleotide (FAD). Its three-dimensional structure, and active site architecture, is most similar to that of members of the succinate dehydrogenase/fumarate reductase family.^{2–4} Its kinetic mechanism has been reported to be a Ping-Pong bi-bi mechanism using fumarate as the electron acceptor, because the iminoaspartate product must dissociate before fumarate can bind. However, the initial velocity pattern with oxygen as the oxidant revealed a small effect on V/K_{asp} , suggesting a sequential mechanism in which oxygen can bind before the release of iminoaspartate and reoxidize the reduced flavin.⁵ In the reductive half-reaction, L-aspartate binds to the FAD-bound NadB to form a Michaelis complex, followed by the transfer of a hydride ion from aspartate to FAD, resulting in the formation and release of iminoaspartate and the reduced flavin (FADH₂). The initial step of L-aspartate oxidation could be either deprotonation of the α -amino group or the removal of a proton from either C2 or C3 to generate the corresponding carbanions (Scheme 2). These intermediates could then collapse with the transfer of a hydride ion to FAD from C2, N1, or C2. In the oxidative half-reaction, FADH₂ is reoxidized to FAD using molecular oxygen to generate hydrogen peroxide. Alternatively, NadB is capable of regenerating FAD using fumarate as the electron acceptor to form succinate.

The ability to use either molecular oxygen or fumarate as the oxidant is physiologically relevant in that NadB can function in both aerobic and anaerobic environments.^{4,5} It has been shown that the mammalian aspartate oxidase is specific for D-aspartate and cannot oxidize L-aspartate,⁶ making the bacterial NadB an attractive drug target against pathogenic bacteria such as *Mycobacterium tuberculosis* and *Haemophilus influenzae*.⁷

The crystal structure of *Escherichia coli* NadB determined by Mattevi et al. revealed a folding topology similar to that of the succinate dehydrogenase and fumarate reductase (SDH/FRD) family of oxidoreductases.³ In addition, many conserved residues are clustered within the FAD-binding and aspartate-binding sites. A significant difference in the active site

architectures (Figure 1) is the presence of Glu121 in aspartate oxidase that is an apolar residue in other SDH/FRD family members and is positioned to interact with the α -amino group of aspartate.³ In a mechanism analogous to succinate oxidation, deprotonation of aspartate by NadB at C3 would allow for the transfer of the hydride from C2, generating the enamine tautomer of the iminoaspartate product (Scheme 2C).⁸ This mechanism differs substantially from those of other flavin-dependent amino acid oxidases in which deprotonation instead occurs at the α -amino group of aspartate, and the transfer of the hydride from C2 directly generates the imino form of the product (Scheme 2A).^{9,10}

In the absence of quinolinate synthase, iminoaspartate is then hydrolyzed to generate oxaloacetate and ammonia.¹¹ Despite mechanistic conclusions drawn from mutagenesis studies, crystal structures, and docking studies, no kinetic evidence validating the chemical mechanism of the reductive half-reaction and its resemblance to that of SDH/FRD or D-amino acid oxidase-type (DAAO-type) enzymes has been published.

In this study, we report a mechanistic analysis of NadB using steady state primary solvent and multiple kinetic isotope effects, as well as primary kinetic isotope effect experiments of the aspartate oxidation half-reaction. We show that NadB oxidation of aspartate in D₂O in the presence of malate dehydrogenase yields unlabeled malate. These results indicate that the reductive half-reaction proceeds by a mechanism most similar to DAAO-catalyzed amino acid oxidation involving a concerted amine deprotonation–hydride transfer mechanism.

MATERIALS AND METHODS

Materials

All chemicals were of analytical or reagent grade and were used without further purification; 99.9% deuterated water was from Cambridge Isotope Laboratories. Native porcine glutamate oxaloacetate transaminase (aspartate transaminase) was from Cell Sciences. L-Aspartic acid sodium salt monohydrate, perdeuterated aspartic acid, and porcine heart malate dehydrogenase were from Sigma-Aldrich. All restriction enzymes, calf intestinal phosphatase, and T4 DNA ligase were from New England Biolabs.

Production and Purification of NadB

The *nadB* gene was amplified from *E. coli* K12 genomic DNA and cloned into pET28a(+) using *Nde*I and *Hind*III restriction sites. This plasmid was subsequently transformed into *E. coli* BL21(DE3) (EMD Bioscience catalog no. 69387-3) and cultured in Luria-Bertani (LB) medium containing kanamycin (30 μ g/mL) at 37 °C until the OD₆₀₀ reached 0.6. Expression of the *nadB* gene was induced for 20 h at 16 °C by addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside. Cells were harvested by centrifugation and resuspended in 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 10 mM imidazole and then lysed by sonication. The supernatant containing soluble protein was purified using a Ni²⁺-NTA agarose column. We further purified the protein by loading it onto a Superdex-200 column (GE Life Sciences) and separating the monomeric NadB from contaminant protein using a running buffer consisting of 50 mM HEPES (pH 7.5) and 100 mM NaCl. The protein was

concentrated to approximately 20 mg/mL as determined by the Bradford method. The final NadB stock was supplemented with a 2-fold excess of FAD and stored in 40% (v/v) glycerol at -20°C until use.

Measurement of Enzymatic Activity

NadB activity assays were performed at 25°C in air-saturated solutions ($\sim 250\ \mu\text{M}$) under initial rate conditions in a coupled assay following the conversion of NADH to NAD^{+} ($\epsilon = 6220\ \text{M}^{-1}\ \text{cm}^{-1}$) by malate dehydrogenase at 340 nm. The reaction mixture contained 50 mM HEPES/NaCl (pH 7.5), $100\ \mu\text{M}$ NADH, 8 units of malate dehydrogenase, $2\ \mu\text{M}$ NadB, and variable amounts of L-Asp in a final volume of 1 mL. Initial kinetic parameters were obtained by fitting to the Michaelis–Menten equation (eq 1)

$$v = (VS)/(K_m + S) \quad (1)$$

where v is the initial velocity, V is the maximal velocity, and K_m is the Michaelis constant for substrate, S . Because oxygen was not saturating, all reported kinetic parameters are apparent ones under the experimental conditions.

Dependence of pH on Rate

The pH dependence of the kinetic parameters was determined by measuring initial rates at varying concentrations of L-aspartate. The experiments were conducted at 25°C in 50 mM buffer at the indicated pH values: MES (pH 6.8), HEPES (pH 6.8–7.8), TAPS (pH 7.8–8.7), and CHES (pH 8.7–10). The resulting k_{cat} data were fit to eq 2 to obtain the $\text{p}K_b$, the negative log of the base dissociation constant, where c is the pH-independent plateau value. The k_{cat}/K_m data also were fit to eq 2, which describes the pH dependence where deprotonation of a single group decreases k_{cat}/K_m at high pH values.

$$\log k_{\text{cat}} \text{ or } \log k_{\text{cat}}/K_m = \log [c/(1 + 10^{\text{pH} - \text{p}K_a})] \quad (2)$$

[2- ^2H]Aspartic Acid

[2- ^2H]Aspartic acid was prepared enzymatically from aspartate and D_2O in the presence of aspartate transaminase at 25°C , as previously described.¹² L-Aspartate (5 g) was incubated in a 50 mL solution of D_2O containing 100 mM phosphate buffer (pD 7.5) and 0.1 mM α -ketoglutarate. The reaction was initiated by the addition of 1000 units of aspartate aminotransferase containing $50\ \mu\text{M}$ added pyridoxal 5-phosphate. The exchange of the C2 proton of aspartate was monitored by proton nuclear magnetic resonance (NMR) spectroscopy at 300 MHz. The reaction was quenched after 24 h when the solution was heated at 80°C for 10 min. [2- ^2H]Aspartic acid was purified using an anion exchange resin (Dowex AG 1-X8, mesh 100–200) with a linear gradient from 0 to 6 N formic acid. Aspartate-containing fractions were identified by spotting on a thin layer chromatography plate and staining with ninhydrin. For isotope effect studies, the [2- ^2H]aspartate stock solution was adjusted to pH 7.5 by NaOH titration.

L-Aspartic Acid Quantification

Prior to the performance of kinetic isotope effect studies, concentrations of protiated and deuterated L-aspartate were carefully measured. The concentrations of L-aspartate stock solutions were measured using a burnoff assay containing 50 mM HEPES/NaCl (pH 7.5), 200 μ M α -ketoglutarate, 100 units of aspartate transaminase, 1 mM NAD⁺, 0.5 unit of glutamate dehydrogenase, 1 mM MTT, 2 units of diaphorase, and 50 μ M L-aspartate. The aspartate concentration was calculated by fitting the net absorbance change from the reduction of MTT to formazan at 565 nm on a standard curve.

Steady State Kinetic Isotope Effects

Isotope effect studies were performed at 25 °C in 50 mM HEPES/NaCl (pH 7.5) with varying concentrations of L-Asp. The results were globally fit to eq 3

$$\nu = \frac{VS}{K_m \{1 + F_i(E_k) + S[1 + F_i(E_v)]\}} \quad (3)$$

where ν is the initial velocity, V is the maximal velocity, K_m is the Michaelis constant for substrate S , E_k is the isotope effect on V/K (minus 1), E_v is the isotope effect on V (minus 1), and F_i is the fractional percentage of isotopic labeling.

Solvent Kinetic Isotope Effects

Solvent kinetic isotope effects were measured by varying concentrations of aspartate and fit to eq 3, where $F_i = 0$ for H₂O and $F_i = 0.95$ for 95% D₂O. A viscosity control of 9% (w/w) glycerol¹³ did not show any effect on either V or V/K (data not shown). Proton inventories were determined under saturating concentrations of aspartate at 10% volume increments of D₂O from 0 to 90%.

Primary Kinetic Isotope Effects

Primary KIEs were measured by varying concentrations of aspartate and fit to eq 3, where $F_i = 0$ for L-aspartate and $F_i = 0.99$ for 99% [2-²H]-L-aspartate.

Multiple Kinetic Isotope Effects

Multiple KIEs were measured using two methods by (a) varying concentrations of L-aspartate or [2-²H]-L-aspartate in 95% D₂O and fit to eq 3, where $F_i = 0$ for L-aspartate and $F_i = 0.99$ for 99% [2-²H]-L-aspartate, and (b) using [2-²H]-L-aspartate in H₂O or 95% D₂O and fit to eq 3, where $F_i = 0$ for H₂O and $F_i = 0.95$ for 95% D₂O.

Deuterium Oxide Incorporation Experiment

L-Aspartate (40 mg) was incubated in a 3 mL D₂O solution containing 100 mM phosphate buffer (pD 7.5), purified NadB, 160 units of malate dehydrogenase, and 80 mM NADH. The formation of malate was monitored by proton NMR spectroscopy. The reaction was quenched after 24 h when the solution was heated at 80 °C for 10 min. Malate was purified using an anion exchange resin (Dowex AG 1-X8, mesh 100–200) with a linear gradient from

0 to 6 N formic acid. Malate-containing fractions were identified and analyzed by ^1H NMR spectroscopy (Figure S1). Deuterium incorporation of the malate product was quantitated by ESI mass spectrometry (Figure S2).

Pre-Steady State Primary Kinetic Isotope Effects

Pre-steady state experiments were conducted on an SX-20 (Applied Photophysics) stopped-flow, rapid-mixing spectrophotometer at 25 °C in 50 mM HEPES/NaCl (pH 7.5). The two drive syringes were filled with deoxygenated buffer, and 10 shots were performed to deoxygenate the mixing chamber. Deoxygenated NadB was prepared by dialyzing in HEPES buffer with N_2 purging via a fritted sparger (Tech Air, Purity Plus, 99.9999% N_2) for 3 h to remove glycerol and excess unbound FAD. The enzyme concentration was measured by the Bradford method before dilution to a final concentration of 15 μM with deoxygenated HEPES buffer. The enzyme was removed from the dialysis bag, while still being purged, via one of the drive syringes that was directly attached to the spectrophotometer. Aspartate stock solutions (10–80 mM) were deoxygenated by continuous Ar purging for 10 min and then another 5 min purge immediately before being loaded onto the spectrophotometer. The individual substrate solutions were taken up directly in the drive syringe that was then immediately attached to the spectrophotometer. Primary isotope effects of the reductive half-reaction were measured under anaerobic conditions. FAD reduction was monitored at 452 nm using either L-Asp or $[2\text{-}^2\text{H}]\text{-L-Asp}$. At each substrate concentration, two shots were performed to cleanse the mixing chamber followed by four shots, which were averaged to generate the final traces at each substrate concentration (Figure S3). Stopped-flow absorbance traces were fit to eq 4

$$A(t) = (A_o - A_i)e^{-k_{\text{obs}}t} + A_i \quad (4)$$

where A_o is the initial absorbance, A_i is the absorbance at infinite time t , and k_{obs} is the observed rate constant. Together with steady state values for $^{\text{D}}V/K$ and $^{\text{D}}V$, the intrinsic kinetic isotope effects determined by pre-steady state studies were fit to eq 5¹⁴

$$^{\text{D}}(V/K)_{\text{Asp}} = \frac{{}^{\text{D}}k_2 + C_f + C_r}{1 + C_f + C_r} {}^{\text{D}}K_{\text{eq}} {}^{\text{D}}V_{\text{Asp}} = \frac{{}^{\text{D}}k_2 + C_{V_f} + C_r}{1 + C_{V_f} + C_r} {}^{\text{D}}K_{\text{eq}} \quad (5)$$

where C_f and C_{V_f} are the forward commitments on V/K and V respectively, C_r is the reverse commitment, ${}^{\text{D}}K_{\text{eq}}$ is the deuterium isotope effect on the equilibrium constant, ${}^{\text{D}}k_2$ (or ${}^{\text{D}}k_{\text{red}}$) is the intrinsic kinetic isotope effect on E-FAD reduction, and rate constants k refer to those in Scheme 3. When C_r is zero or negligible, eq 5 may be simplified to eq 6. C_f and C_{V_f} are defined by eq 7.

$$^{\text{D}}(V/K)_{\text{Asp}} = \frac{{}^{\text{D}}k_2 + C_f}{1 + C_f} {}^{\text{D}}V_{\text{Asp}} = \frac{{}^{\text{D}}k_2 + C_{V_f}}{1 + C_{V_f}} \quad (6)$$

$$C_f = \frac{k_{\text{chem}}}{k_{\text{off}}} C_f = \frac{k_2}{k_{-1}} C_{v_f} = \frac{k_2}{k_5} \quad (7)$$

Data Fitting

All data fitting was performed with GraphPad Prism version 6.0e. In all graphs, the points are the means of experimental triplicate measurements and the error bars are the standard deviation of the triplicate measurements. The solid lines are the result of fitting to the denoted equation.

RESULTS AND DISCUSSION

The chemical mechanism of L-aspartate oxidase has been debated because of its unique characteristics that make it distinct from other flavin-dependent oxidases. This distinction is based mainly on sequence similarity, the structural resemblance of NadB to succinate dehydrogenase/fumarate reductases, and the ability of NadB to utilize both oxygen and fumarate as electron acceptors in the oxidative half-reaction. Typical amino acid oxidases utilize molecular oxygen as the sole oxidant in the conversion of FADH₂ to FAD.

The chemical mechanism of amine oxidation by flavoenzymes, such as D-amino acid oxidase (DAAO), has been a topic of debate for many years.⁹ It was initially proposed that the reductive half-reaction involves abstraction of the α -proton from the amino acid to generate a carbanion intermediate that could collapse with the transfer of the hydride from N1 to FAD (Scheme 2B).^{15–17} This proposal was supported by studies of β -chloroalanine and DAAO using substrate kinetic isotope effects.^{18,19} The elimination of HCl to generate pyruvate and the oxidation of β -chloroalanine to generate chloropyruvate were consistent with the cleavage of the C2–H bond to generate a carbanion that could collapse with chloride elimination (generating the enamine and then imine) or the transfer of the hydride from N1 to generate the imine of chloropyruvate.²⁰ Hersh and Jorns argued that the mechanism of DAAO was instead consistent with a mechanism for the direct transfer of the hydride from C2 (Scheme 2A). They showed that replacement of FAD with 5-deaza-FAD results in the transfer of the amino acid α -proton to C5 of 5-deaza-FAD.¹⁰ Crystal structures of pig kidney²¹ and yeast²² DAAO support this, and the C2 hydride transfer mechanism has now been accepted as the mechanism of catalysis by DAAO.²³

The mechanistic analysis of L-amino acid oxidases (LAAOs) is similarly unclear and supported by contradictory mechanistic data. Structurally, LAAOs belong to the monoamine oxidase (MAO) flavoenzyme family as opposed to the DAAO family. Mutagenesis studies of MAO-A and MAO-B enzymes highlight the role of two tyrosine residues forming an “aromatic cage”, which has been used to support a carbanion mechanism of amine oxidation.²⁴ However, it has also been demonstrated that some LAAOs do not contain aromatic cages, such as the LAAO isolated from snake venom. The crystal structure of snake venom LAAO supports initial unprotonated amine deprotonation by a histidine residue followed by direct transfer of the hydride from C2 to N5 of the flavin ring.²⁵ In parallel with their earlier DAAO studies, Walsh and co-workers have shown that L-amino acid oxidase

also produces pyruvate and L-chloropyruvate from L-chloroalanine, suggesting a carbanion mechanism of action similar to that of DAOs. More recent structural studies of bacterial LAO from *Rhodococcus opacus* provide additional support for a concerted deprotonation-hydride transfer mechanism.²⁶

E. coli L-aspartate oxidase provides further ambiguity because its structure is similar to that of members of the succinate dehydrogenase/fumarate reductase structural family, exhibiting 30% primary sequence homology. SDH/FRD are membrane-localized flavoproteins of the respiratory chain that also have the ability utilize fumarate as the electron acceptor.¹ From mutagenesis studies of NadB, it appears that substrate recognition of the α -amino group is due to a hydrogen bonding interaction with Glu121.^{3,8} In addition, it has been proposed that Arg290 may function as a general acid/base,^{27,28} and the NadB crystal structure also suggests the importance of a second arginine group (Arg386) for binding or catalysis.⁸ In the oxidative half-reaction, the reduced flavin can react with oxygen, presumably via a 4a-peroxy intermediate. However, under anaerobic conditions, fumarate reduction must be accomplished by reverse transfer of the hydride from FADH₂ to generate a succinate carbanion that is protonated. Either Arg290 or Glu121, in the case of NadB, could be the general acid for succinate carbanion protonation. The reverse of this reaction, equivalent to succinate oxidation, suggests a third mechanistic possibility for the C2 carbanion and N1 deprotonation-C2 hydride transfer mechanisms, a mechanism invoking C3 deprotonation and C2 hydride transfer (Scheme 2C).

pH Dependence of NadB

The pH dependence of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ was determined to identify pK values of ionizable groups involved in catalysis and binding and to determine an appropriate pH value for performing kinetic isotope effects, especially solvent effects that are sensitive to pH. As shown in Figure 2, the two pH dependencies are similar, the points are the experimental data, and the smooth curves are the fits of the data to eq 2. At pH >8, both kinetic parameters decreased because of the deprotonation of a group participating in catalysis and/or binding, most likely catalysis. Experimental traces fit to eq 2 determined the pK value of the ionizable group to be 8.2 ± 0.1 for k_{cat} and 8.4 ± 0.1 for $k_{\text{cat}}/K_{\text{m}}$. These results suggest the presence of a general acid required for catalysis, and on the basis of structural studies and mutagenesis studies demonstrating their essential roles in catalysis and/or binding, this group is likely to be Arg290, which has been shown to be appropriately positioned for fumarate reduction^{2,27} and may serve a similar role in enzyme reduction.

Determination of pK values for either kinetic parameter at lower pH values was limited by the coupling assay used, as malate dehydrogenase precipitation below pH 6.4 was observed as has been reported.²⁹ We would expect to observe the signature of a general base, most likely based on the structure, Glu121, required for substrate recognition and potentially L-aspartate amine deprotonation.⁸ We would also expect to observe an active site base, presumably Arg290 if deprotonation of aspartate occurs at C3 prior to hydride transfer.³ A previous pH profile determined by Nasu et al.³⁰ using radiolabeled aspartate to measure enzyme activity indeed revealed a bell-shaped profile representing the requirement for both an active site acid and base. In agreement with the previously determined pH profile, we

observe an optimal rate of activity of pH 7.5, the pH used in all the kinetic isotope effect studies described below.

Solvent Kinetic Isotope Effects

Solvent kinetic isotope effects are useful in analyzing the potential rate-limiting nature of proton transfer reactions during enzymatic catalysis. The following solvent kinetic isotope effects for L-aspartate oxidation at pH 7.5 were determined: $^{D_2O}V_{Asp} = 1.8 \pm 0.2$, and $^{D_2O}(V/K_{Asp}) = 2.1 \pm 0.3$ (Figure 3A). The large effects on both V and V/K indicate that proton transfer reactions during catalysis are slow and partially rate-limiting. The proton inventory is linear (Figure 3B), indicating that a single solvent-derived proton is transferred in the oxidation of aspartate as has been previously reported with DAAO.³¹

In a Ping-Pong bi-bi kinetic mechanism, the expression for V/K_{Asp} includes rate constants from the binding of aspartate, k_1 , to the first irreversible step, in this case the dissociation of iminoaspartate, k_3 . The solvent kinetic isotope on V/K_{Asp} therefore reports only on the proton transfer steps occurring during aspartate oxidation (Scheme 3). The observed value of 2.1 suggests that general base-catalyzed substrate deprotonation is partially rate-limiting in the reaction. This appears to rule out a mechanism involving C3 deprotonation and the transfer of the hydride to FAD, because these hydrogen atoms are not solvent-exchangeable.

The expression for V contains rate constants for all steps after the formation of the E-Asp Michaelis complex through the final step of the reaction, release of hydrogen peroxide, k_6 . Therefore, solvent kinetic isotope effects can and will include contributions from proton transfer reactions occurring during aspartate oxidation via N1 deprotonation and molecular oxygen reduction. Assigning the step that is responsible for the solvent kinetic isotope effect on V is therefore more difficult and will also depend on the relative rates of the two half-reactions.

Deuterium Oxide Incorporation

A specific prediction of a succinate dehydrogenase-like mechanism for aspartate oxidase is that after C3 deprotonation and the transfer of the hydride from C2, the resulting intermediate enamine will tautomerize to generate the required imine product (Scheme 2C). If the aspartate oxidase reaction is performed in D_2O , tautomerization will result in the incorporation of deuterium at C3 of iminoaspartate and oxaloacetate, generated from the hydrolysis of the imine. In either of the C2-N1 mechanisms, no such incorporation is anticipated. To “trap” the oxaloacetate product, which itself relatively rapidly exchanges deuterium into the C3 position via enolization, we performed the reaction in D_2O in the presence of a high concentration of malate dehydrogenase and NADH to rapidly reduce the oxaloacetate product. The produced L-malate was analyzed for deuterium content by 1H NMR and ESI-MS. 1H NMR revealed that the integrated intensity of the C3:C2 protons was 2:1 as expected for all protium-containing malate (Figure S1). Mass spectrometry revealed a mass of 133.1 (Figure S2), also confirming the lack of deuterium incorporation. Together with the C3 kinetic isotope effects, these results allow us to consider as untenable a succinate dehydrogenase-like mechanism for aspartate oxidase and define the C3 effects as “secondary” kinetic isotope effects. This small, but normal, C3 secondary kinetic isotope

effect is expected for the mechanisms shown in panels A and B of Scheme 2, where rehybridization of C2 from sp^3 to sp^2 is occurring. Finally, deprotonation of C3 to generate a carbanion would potentially allow for the elimination of ammonia to generate fumarate, a reaction catalyzed by aspartase.

C2 Kinetic Isotope Effects

Kinetic isotope effects were measured by comparing the rates of oxidation of aspartate and $[2-^2H]$ aspartate. The measured values for the C2 kinetic isotope effects were as follows: $^D V = 2.1 \pm 0.2$, and $C^2D(V/K_{Asp}) = 2.6 \pm 0.3$ (Figure 4A). These large effects on V and V/K are consistent with the large primary isotope effects observed with both d-amino acid oxidases^{20,31} and L-amino acid oxidases.³² However, the magnitude of these effects is not sufficient to distinguish among the three possible mechanisms because in each the C2–H bond is broken (Scheme 2).³³

Multiple Kinetic Isotope Effects

Having removed one potential mechanism from further consideration, we are left to try to distinguish between the N1 deprotonation–C2 hydride transfer and C2 carbanion–N1 hydride transfer mechanisms and therefore wished to obtain information about the stepwise or concerted nature of the proton and hydride transfer mechanisms. Measurement of multiple isotope effects provides a method of determining whether the two effects arise from the same step if both effects are significant. The kinetic isotope effect will increase or remain the same if the reaction is concerted but decrease if it is stepwise.³⁴ This occurs because in a stepwise mechanism, there are two transition states (for deprotonation and hydride transfer) that are independently affected (increased transition state energy) by solvent isotopic composition and C2 deuteration, respectively. In this case, where both solvent and C2 kinetic isotope effects are substantial (> 2), if the solvent-sensitive reaction occurs before hydride transfer, it will make the hydride transfer reaction relatively less rate-limiting and decrease the C2 kinetic isotope effect in D_2O . In contrast, in a concerted mechanism with a single transition state, the presence of D_2O raises the energy barrier for both hydrogen- and deuterium-containing species equally; however, the energy barrier difference for C–H and C–D cleavage is unaffected, and thus, the kinetic isotope effect (a manifestation of the zero-point energy difference) for C2 deuteration is identical to that in water. Similar multiple-kinetic isotope effect approaches have been applied to the flavoenzymes choline oxidase³⁵ and D-amino acid oxidase.³⁶

Multiple kinetic isotope effects were measured by varying L-aspartate and $[2-^2H]$ -L-aspartate concentrations in D_2O , yielding the following values: $^D V = 1.6 \pm 0.2$, and $^D(V/K_{Asp}) = 2.3 \pm 0.5$ (Figure 4B,C). Compared to the C2 kinetic isotope effects observed in H_2O [$^D V = 2.1 \pm 0.2$, and $^D(V/K_{Asp}) = 2.6 \pm 0.3$], the $^D V/K_{Asp}$ values in H_2O and D_2O are statistically equivalent, while the $^D V$ is somewhat smaller, perhaps reflecting additional rate limitation in D_2O in the second half-reaction, not described in $^D V/K_{Asp}$. These values are consistent with a concerted hydride transfer mechanism in which the C2 (primary) and solvent isotope effect report on the same chemical step. A second, multiple solvent kinetic isotope effect was measured using a second method by varying the $[2-^2H]$ aspartate concentration in either H_2O or 95% D_2O . These studies yielded the following values:

$^{D_2O}V = 1.8 \pm 0.2$, and $^{D_2O}(V/K_{[2-^2H]Asp}) = 2.4 \pm 0.3$. Compared to the solvent kinetic isotope effects measured with aspartate [$^{D_2O}V_{Asp} = 1.8 \pm 0.2$, and $^{D_2O}(V/K_{Asp}) = 2.1 \pm 0.3$], a similar conclusion can be reached in support of the concerted deprotonation–hydride transfer mechanism.

Pre-Steady State Kinetic Isotope Effects

The magnitude of steady state kinetic isotope effects is influenced by the kinetic complexity of the reaction. This may include the kinetic mechanism, the order of substrate binding, the substrate that is isotopically labeled, and most importantly the partitioning of the labeled substrate in the Michaelis complex. These partitioning ratios represent the propensity of the labeled substrate to undergo chemistry versus dissociating from the Michaelis complex. The Ping-Pong bi-bi mechanism is one of the simplest in that while two chemical reactions are involved, each substrate reacts consecutively with the alternating enzyme forms (in the case of NadB, E–FAD and E–FADH₂). We performed an anaerobic stopped-flow analysis of the half-reaction involving oxidation of aspartate or [2-²H]-L-aspartate by measuring the rate of reduction of enzyme-bound FAD at 452 nm. As shown in Figure 4D, the first-order rate constants of FAD reduction were dependent on substrate concentration and isotope. The isotope effect for enzyme reduction ($^Dk_{red}$) was determined to be 2.7 ± 0.3 (Figure 4D). This intrinsic isotope effect is similar to the value previously reported for a related flavoenzyme oxidase, tryptophan 2-monooxygenase ($^Dk_{red} = 2.4$).³⁷ The reductive isotope effects determined previously for DAOs²⁰ are dependent on the amino acid substrate ($^Dk_{red} = 1.2$ for alanine, $^Dk_{red} = 3.4$ for glycine, and $^Dk_{red} = 4.8$ for serine) but bracket the value reported here for NadB.

The magnitude of the pre-steady state primary isotope effect for NadB is similar to the value of $^D(V/K_{Asp})$ that we observed in steady state studies, suggesting that aspartate exhibits a negligible “commitment to catalysis”. The commitment to catalysis is defined for aspartate in a Ping-Pong bi-bi mechanism as k_2/k_{-1} (eqs 6 and 7), suggesting that aspartate, once bound to the enzyme, preferentially dissociates rather than undergoing oxidation. The determination of $^Dk_{red}$ additionally allows us to calculate a ratio for the reductive and oxidative half-reactions. Using a value for ^{c_2D}V of 2.1 and a value for $^Dk_{red}$ of 2.7, we can calculate the V commitment factor, defined as k_2/k_5 (k_{red}/k_{ox}), as 0.54 under our experimental conditions (air-saturated buffer). The reoxidation of FADH₂ by molecular oxygen is thus twice as rapid as FAD reduction by aspartate under these roughly physiological conditions.

CONCLUSION

Our interest in the bacterial *nadB*-encoded aspartate oxidase was piqued by the determination of the three-dimensional structure of the *E. coli* enzyme, revealing structural similarity to the SDH/FRD family of flavoenzymes, rather than “typical” flavoprotein amino acid oxidases.² The accepted chemical mechanism for succinate dehydrogenation by SDH involves proton abstraction at a position equivalent to C3 of aspartate followed by the transfer of the hydride from C2, based on the nanomolar inhibition by nitropropionate,³⁸ a chemistry theoretically possible for L-aspartate oxidase. The ability of the enzyme to use

alternate electron acceptors, molecular oxygen and fumarate, suggested a mechanistic bifunctionality not commonly observed in flavoenzymes. The results reported here (solvent and C3 kinetic isotope effects and deuterium wash-in) clearly argue persuasively against a carbanionic intermediate, succinate dehydrogenase-like mechanism for aspartate oxidation and for a concerted mechanism for deprotonation and hydride transfer. However, we do not have data that can unambiguously distinguish between a C2 carbanion–N1 hydride transfer sequence and a N1 deprotonation–C2 hydride transfer sequence. The unique presence of Glu121 in L-aspartate oxidase in a position to bind to the protonated α -amino group of L-aspartate and perform base-catalyzed deprotonation suggests that this may be the initiating step in catalysis, followed by the transfer of the hydride from C2 of L-aspartate to N5 of FAD. Our data do not distinguish between deprotonation of the protonated (as shown in Scheme 2A) and unprotonated α -amino group of L-aspartate. On the basis of our multiple kinetic isotope effect data, these two steps are concerted.

L-Aspartate oxidase likely coevolved with the next enzyme in the *de novo* NAD biosynthetic pathway, the *nadA*-encoded quinolinate synthase, which is an oxygen-sensitive, [4Fe-4S] cluster-containing enzyme. The enzyme is most structurally similar to the respiratory SDH/FRD enzymes and has retained most of the catalytic machinery to perform fumarate reduction, especially Arg290, which functions as a general acid in the fumarate reduction half-reaction after the transfer of the hydride from N5 of FADH₂ to C2 of fumarate. NAD⁺ and NADP⁺, and their two-electron-reduced counterparts, NADH and NADPH, respectively, serve as substrates for dozens of essential redox reactions in both anabolic and catabolic transformations. Their reduced forms are uniquely oxygen-stable and thus serve as stores for reducing equivalents under both anaerobic and, as oxygen concentrations increased in the primitive atmosphere, aerobic conditions. L-Aspartate oxidase thus may have mechanistically evolved from SDH/FRD-like enzymes by incorporating Glu121 into the active site to perform amino acid oxidase chemistry, while also retaining the primordial ability to use fumarate as an oxidant under the anaerobic conditions under which quinolinate synthase is active.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ATP	adenosine triphosphate
CHES	<i>N</i> -cyclohexyl-2-amino-ethanesulfonic acid

DAAO	D-amino acid oxidase
DHAP	dihydroxyacetone phosphate
FAD	flavin adenine dinucleotide
FRD	fumarate reductase
HEPES	<i>N</i> -(2-hydroxyethyl)-piperazine- <i>N'</i> -2-ethanesulfonic acid
ImAsp	iminoaspartate
KIE	kinetic isotope effect
LA AO	L-amino acid oxidase
MAO	monoamine oxidase
MDH	malate dehydrogenase
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAAD	nicotinic acid dinucleotide
NAD⁺	nicotinamide adenine dinucleotide
NAMN	nicotinic acid mononucleotide
SDH	succinate dehydrogenase
TAPS	<i>N</i> -[tris-(hydroxymethyl)methyl]-3-aminopropanesulfonic acid

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Figure 1.
Active site of *E. coli* L-aspartate oxidase (Protein Data Bank entry 1KNP⁸) in complex with succinate.

Figure 2. pH dependence of NadB on $\log k_{cat}$ ($\blacklozenge$) and $\log k_{cat}/K_m$ ($\blacklozenge$). Fitting $\log k_{cat}$ to eq 2 yielded a pK_b of 8.2 ± 0.1 and $\log k_{cat}/K_m$ to eq 2 a pK_b of 8.4 ± 0.1 .

Figure 3.

Solvent kinetic isotope effects and proton inventory. (A) Solvent KIE with a varying (0.25–5 mM) L-aspartate concentration in (●) H₂O or (○) 95% D₂O provided the following values:

$D_2O V = 1.8 \pm 0.2$, and $^{D_2O} (V/K) = 2.1 \pm 0.3$. (B) Proton inventory in which k_{cat} was measured at a saturating L-aspartate concentration at 10% increments of D₂O and fit to a straight line.

Figure 4.

Kinetic isotope effects. (A) Primary kinetic isotope effect at a varying (0.25–5 mM) (●) L-aspartate or (○) $[2\text{-}^2\text{H}]\text{-L-aspartate}$ concentration yielded the following values: $^D V = 2.1 \pm 0.2$, and $^D(V/K) = 2.6 \pm 0.3$. (B) Multiple kinetic isotope effects at a varying (0.25–5 mM) (●) L-aspartate or (○) $[2\text{-}^2\text{H}]\text{-L-aspartate}$ concentration in 95% D_2O yielded the following values: $^D V = 1.6 \pm 0.2$, and $^D(V/K) = 2.3 \pm 0.5$. (C) Multiple kinetic isotope effects at a varying (0.25–5 mM) $[2\text{-}^2\text{H}]\text{-L-aspartate}$ concentration in (●) H_2O or (○) 95% D_2O yielded the following values: $^D V = 1.8 \pm 0.2$, and $^D(V/K) = 2.4 \pm 0.3$. (D) Pre-steady state primary kinetic isotope effect at a varying (10–80 mM) (●) L-aspartate or (○) $[2\text{-}^2\text{H}]\text{-L-aspartate}$ concentration with 15 mM NadB under anaerobic conditions yielded the following value: $^D k_{\text{red}} = 2.7 \pm 0.3$.

Scheme 1.
Bacterial Biosynthesis of NAD⁺ and NADP⁺

Scheme 2.

Proposed Chemical Mechanisms for NadB Catalysis: (A) N1 Deprotonation–Hydride Transfer Mechanism, (B) C2 Deprotonation–Hydride Transfer Mechanism, and (C) C3 Deprotonation–Hydride Transfer Mechanism

Scheme 3.
Catalytic Mechanism of NadB