Nitrogen Isotope Effects As Probes of the Mechanism of D-Amino Acid Oxidase

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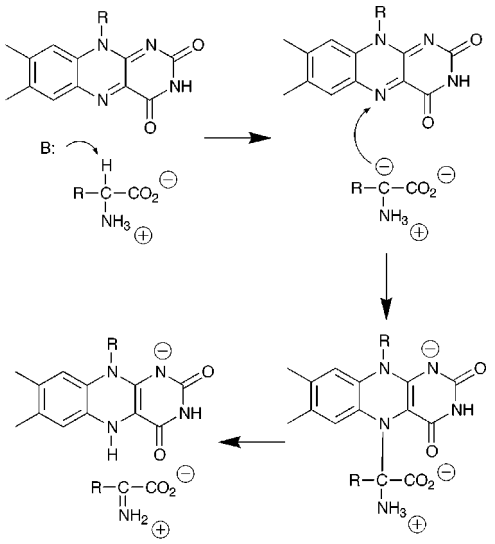
Flavoprotein oxidases catalyze the removal of a hydride equivalent from their substrates, transferring the electrons to the flavin cofactor and ultimately to molecular oxygen. The mechanisms of flavoprotein oxidases which oxidize α-hydroxy and α-amino acids have been extensively studied due to their ubiquity in metabolism and the high pK_a values of the protons which must be removed.[1] D-Amino acid oxidase has long served as the paradigm for these enzymes.[2] A seminal contribution to understanding the mechanism of this enzyme was the characterization of its ability to catalyze the elimination of HCl from *β*-chlorinated amino acids to form the respective keto acids.[3] This reaction implied the catalytic intermediacy of a carbanion formed by removal of the α-hydrogen as a proton (Scheme 1). Subsequent studies of α-hydroxy acid oxidizing flavoenzymes have been consistent with a similar mechanism for those enzymes.[4] In the case of the latter group of enzymes, extensive structural analyses have provided further support for a carbanion intermediate.[5]

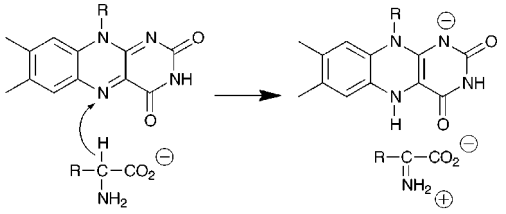
An alternative mechanism for carbon−hydrogen bond cleavage by D-amino acid oxidase involves direct hydride transfer from the substrate to the flavin (Scheme 2); this would be similar to the reactions of the pyridine nucleotide dependent alanine and glutamate dehydrogenase.[6] Indeed, the recently described three-dimensional structure of pig kidney D-amino acid oxidase is consistent with a hydride transfer mechanism. In contrast to the structures of the α-hydroxy acid oxidizing enzymes glycolate oxidase and flavocytochrome b_2,[5] the active site of D-amino acid oxidase appears to lack a residue capable of acting as the base which would abstract the α-proton to form the proposed carbanion.[7] Thus, mechanistic conclusions drawn from the structure and those drawn from solution studies are contradictory.

A critical difference between the mechanisms of Schemes 1 and 2 is that carbon−hydrogen bond cleavage and formation of the imine double bond are stepwise in the former and concerted in the latter. We have utilized secondary nitrogen kinetic isotope effects to distinguish the relative timing of these two events as a probe of the structure of the transition state for carbon−hydrogen bond cleavage by D-amino acid oxidase.[8] The isotope effects were determined with D-serine as substrate because the intrinsic isotope effects for the carbon−hydrogen bond cleavage step are fully expressed in the *V*/*K* value for this amino acid.[9] Thus, *V*/*K* isotope effects with this substrate report directly on the transition state for carbon−hydrogen bond cleavage. In the case of carbanion formation, there should be no ^{15}N isotope effect on the V/K_{ser} value, since there is no change in the bond order to nitrogen,[10] while hydride transfer should result in a significant inverse $^{15}V/K_{ser}$ effect. The results are summarized in Table 1.[11]

Scheme 1



Scheme 2



The $^{15}V/K_{ser}$ value is significantly different from unity under all the conditions examined. The measured effect clearly increases in D_2O and decreases at high pH. The primary deuterium isotope effect with D-serine is independent of pH or solvent,[8] so that the changes in the observed ^{15}N effects are not due to changes in transition state structure or values of commitments. A reasonable explanation for the changes in the measured ^{15}N effect is that the amino group of the amino acid substrate must be uncharged for catalysis. The $^{15}K_{eq}$ value for deprotonation of the amino group is 1.016 in H_2O, increasing to 1.022 in D_2O.[12] The increase in the observed effect in D_2O can be ascribed to the larger

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(8) D-Amino acid oxidase was purified from hog kidney as described by Fitzpatrick, P. F.; Massey, V. M. *J. Biol. Chem.* **1982**, *257*, 1166−1171.

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(10) A reviewer has suggested that there may be negative hyperconjugation between the nitrogen lone pair and the α-hydrogen on the basis of effects of deuterium substitution on amine pK_a values (Northcott, D.; Robertson, R. E. *J. Phys. Chem.* **1969**, *73*, 1559−1603; Norrman, K.; McMahon, T. B. *Int. J. Mass Spectrom.* **1999**, 381−402). If so this could result in a slightly normal ^{15}N effect for carbanion formation.

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Table 1. ^{15}N Isotope Effects for D-Amino Acid Oxidase

pL	solvent	measured $^{15}V/K_{ser}$	corrected $^{15}V/K_{ser}$[a]
7.5	H_2O	1.0128 ± 0.0003	0.9969 ± 0.0003
7.5	D_2O	1.0175 ± 0.0015	0.9957 ± 0.0015
7.9	D_2O	1.0171 ± 0.0004	0.9955 ± 0.0004
10.1	H_2O	0.9991 ± 0.0001	0.9971 ± 0.0001
		average	0.9963 ± 0.0016

[a] The measured isotope effects were corrected for the contribution of the ^{15}N equilibrium isotope effect on deprotonation of the substrate amino group by dividing the observed effect by $1 + (^{15}K_{eq} - 1)f_{NH3}$; here, f_{NH3} is the fraction of the amino acid in the zwitterionic form at a given pH. Values of 1.0163 and 1.0214 were used for $^{15}K_{eq}$ in H_2O and D_2O, respectively, and values of 9.21 and 9.79 were used for the pK_a of the D-serine amino group in H_2O and D_2O, respectively.

equilibrium isotope effect for deprotonation in that solvent, and the decrease in the effect at high pH to an increase in the amount of correctly protonated substrate with increasing pH. For each set of conditions, the contribution made by the equilibrium isotope effect to the observed isotope effect is given by $1 + (^{15}K_{eq} - 1)f_{NH3,}$ Here, f_{NH3} is the molar fraction of the substrate in which the amino group is ionized for a specific pH and solvent, and $^{15}K_{eq}$ is the equilibrium isotope effect for deprotonation of the amino group in H_2O or D_2O. The results of dividing the observed isotope effects by this correction factor are shown in the last column of Table 1. Critically, the corrected effects are independent of the pH and the solvent, supporting the assignment of the zwitterionic form of the substrate as the active species, and have an average value of 0.996. The expected value for formation of an imino acid from an unprotonated amino acid is 0.997,[13] while that for formation of the carbanion is expected to be 1.000 or a value slightly above unity. Thus, the ^{15}N effects with D-serine as substrate are most consistent with direct transfer of the α-hydrogen as a hydride without the intermediacy of a carbanion, as shown in Scheme 2.[14]

The pH dependence of the V/K value with D-serine as substrate has been determined previously.[8] The pH curve is bell-shaped, showing the effects of groups with pK_a values of 8.5 and 9.3. These two values are too close together to unambiguously ascertain whether the pK_a value of 9.3 corresponds to a group which must be protonated and the pK_a value of 8.5 to a group which must be unprotonated or if the reverse is true. The pK_a value of 9.3 matches the pK_a value for the amino group of the substrate D-serine. The earlier analysis assumed that this moiety must be protonated, in line with the expectations of a carbanion mechanism, and assigned the lower pK_a value to an amino acid residue on the enzyme which must be unprotonated, as would be required of an active site base.[9] However, since the ^{15}N isotope effects establish instead that the amino acid substrate must be unprotonated for catalysis, it is the pK_a value of 8.5 which must be for an enzyme residue which must be protonated for catalysis. A likely candidate for this residue is Tyr228, which forms a hydrogen bond to the carboxylate of the substrate.[7]

The catalysis of HCl elimination by D-amino acid oxidase and previously measured isotope effects have been interpreted as favoring a carbanion mechanism. HCl elimination can be reconciled with a hydride transfer mechanism if the elimination reaction occurs by nucleophilic displacement of the chloride by a hydride from the reduced flavin. This model is consistent with the observation that deuterium or tritium removed from C2 of 3-Cl-alanine or 3-Cl-aminobutyrate is found in place of the chloride in the keto acid produced by HCl elimination.[3] It has previously be shown that there is no solvent isotope effect on the V/K_{ser} value;[9,15] this is fully consistent with a hydride transfer mechanism in which the substrate amino group must uncharged for productive binding. Finally, the relatively small secondary tritium isotope effect seen with glycine can be attributed to either an internal commitment or an early transition state with this substrate; both would be consistent with the much smaller primary deuterium effect with this substrate compared to that with D-serine or D-alanine.[9]

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(11) To determine isotope effects, enzyme (0.2−25 μM), 100 mM D-serine, 10 μM FAD, 0.06 mg/mL catalase, in 200 mL either H_2O or D_2O was stirred in a round-bottom flask surrounded by aluminum foil at 23 °C. The buffer was 50 mM sodium phosphate at pH 7.5−7.9 and 50 mM sodium bicarbonate at pH 10.1. Before initiating the reaction with enzyme, 50 mL was removed for work up. The reaction was allowed to proceed to approximately 50% completion, determined as described in ref 8, at which time it was quenched by adding concentrated HCl until a pH of 1 was reached. The denatured protein was removed by centrifugation. Fifty-milliter aliquots of the supernatant were loaded separately onto a Dowex-50W column, and the column was washed with 500 mL of distilled water. The amino acid was eluted with a 0 to 0.3 M NH_4OH linear gradient. The ninhydrin-positive fractions and the two fractions before and after the ninhydrin-positive fractions were pooled. The solvent was removed by rotary evaporation. The amino acid substrate was redissolved in distilled water. The solvent was removed by rotary evaporation. This was repeated three times. After the third wash, the solvent was removed under vacuum. The residue was then lyophilized. The lyophilized amino acid samples were placed in quartz tubes (24 cm × 9 mm o.d., 7 mm i.d.) with CuO (10 g), Cu (0.2 g), and silver (0.2 g). After cooling, the tubes were evacuated, sealed with a torch, and heated to 850 °C for 2 h. The tubes were cracked on high-vacuum lines. The nitrogen gas obtained from combustion of the amino acids was distilled through dry ice-2-propanol and liquid nitrogen traps and collected on molecular sieves chilled with liquid nitrogen. The purified nitrogen gas was liberated from the sieves by heating to 200 °C and analyzed by isotope ratio mass spectrometry. The isotope effects were calculated using eq 1

$$\text{isotope effect} = \log(1 - f)/\log((1 - f)(R_s/R_0)) \quad (1)$$

where R_0 is the abundance of ^{15}N in unreacted starting material, R_s is the amount in the remaining substrate, and f is the fraction of the reaction.

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(13) Determined from comparison of the fractionation factors for an unprotonated NH_2 group (1.0163) and for a protonated imine (1.0192) Rishavy, M. A.; Cleland, W. W. *Can J. Chem.* **1999**, *77*, 967−977.

(14) An alternative possibility is a mechanism in which an electron is transferred first, followed by a hydrogen atom. The radical intermediate can be detected only if the intermediate is populated sufficiently to be identified. Otherwise, a rapid, but reversible, electron transfer to give an intermediate in very low population which then loses a hydrogen atom in the rate-limiting step cannot be distinguished from hydride transfer. In an effort to test this mechanism, the reductive half reaction of D-amino acid oxidase was carried out with allylglycine, which should give a stabilized radical. No semiquinone could be detected in this experiment (Giovanni, G.; Fitzpatrick, P. F., unpublished observations).

(15) There is a report of a solvent isotope effect on the V/K value for the enzyme from *Trigonopsis variabilis* with phenylglycine as substrate (Pollegioni, L.; Blodig, W.; Ghisla, S. *J. Biol. Chem.* **1997**, *272*, 4924−4934). However, this was determined at a pH below the pH optimum, and no correction was made for the effect of D_2O on the pH dependence.