pH and Secondary Kinetic Isotope Effects on the Reaction of D-Amino Acid Oxidase with Nitroalkane Anions: Evidence for Direct Attack on the Flavin by Carbanions

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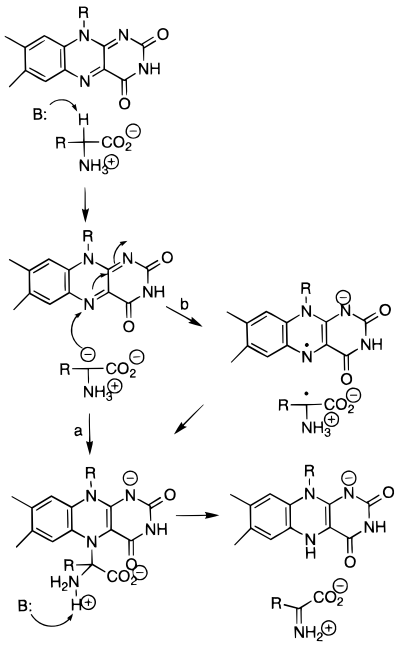
Received August 9, 1996

D-Amino acid oxidase catalyzes the oxidation of D-amino acids to their respective imino acids by transfer of a hydride equivalent to the tightly bound FAD (reductive half-reaction) and subsequent transfer of electrons to molecular oxygen to form hydrogen peroxide (oxidative half-reaction). The chemical mechanism of the reductive half-reaction has been proposed to involve the initial formation of a carbanion by abstraction of the α-proton from the amino acid (Scheme 1).[1] The carbanion would then attack the N(5) of FAD to form a covalent adduct, which subsequently decays to form the reduced flavin and imino acid.[1,2] While formation of the carbanion is supported by several studies,[2−4] the mechanism of the subsequent electron transfer to the enzyme−flavin has been much less amenable to experimental examination.

The reaction of nitroalkane anions with D-amino acid oxidase has been considered a model for the proposed carbanion mechanism with D-amino acids.[1,5,6] Several authors have pointed out that an alternative mechanism to direct nucleophilic attack of the carbanion on the flavin is one involving a radical intermediate, as shown in path b of Scheme 1.[1c,7,8] Indeed, Novak and Bruice concluded from model studies that flavins oxidize carbanions via such a radical mechanism.[8b,c] The reaction of glucose oxidase with nitroalkane anions has been shown to involve a radical intermediate;[9] however, the reaction of this enzyme with its normal substrates is not likely to proceed via a carbanion.[10] While it has not been possible to analyze directly the reaction of an amino acid carbanion with the flavin in D-amino acid oxidase because carbon−hydrogen bond cleavage is much more rapid than the subsequent steps,[4] the reaction with nitroalkanes begins with the preformed carbanion. We have measured secondary kinetic isotope effects for the reaction of nitroalkane anions with D-amino acid oxidase in order to distinguish between direct nucleophilic attack and a radical mechanism.

Scheme 1



The V/K_a values for the anions of nitroethane and 1-nitropropane as substrates for D-amino acid oxidase were determined over the pH range 6−11.[11,12] The effects of pH on the V/K_a values are shown in Figure 1. For nitroethane anion, the data indicate that a group with an apparent pK_a value of 6.8 ± 0.1 must be unprotonated and a group with an apparent pK_a value of 9.9 ± 0.1 must be protonated for activity. For 1-nitropropane anion, a group with an apparent pK_a value of 6.9 ± 0.1 must be unprotonated and a group with an apparent pK_a value of 9.6 ± 0.1 must be protonated for activity. The pK_a values obtained with these two nitroalkane anions are not significantly different.

Secondary deuterium kinetic isotope effects were then determined with nitroethane anion as a substrate for D-amino acid oxidase.[13] At pH 8.5, the pH optimum, the $^{\alpha D}V/K_{nea}$ value was 0.86 ± 0.05. At pH 6 and 11, the $^{\alpha D}V/K_{nea}$ values were 0.82 ± 0.09 and 0.85 ± 0.12, respectively. These values are essentially identical, giving an average secondary isotope effect of 0.84 ± 0.02. This result is indicative of significant sp^2 to

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(11) D-Amino acid oxidase was purified from hog kidney as described by Fitzpatrick and Massey (*J. Biol. Chem.* **1982**, *257*, 1166−1171). The anions of nitroethane (pK_a = 8.6) and 1-nitropropane (pK_a = 9.0) were prepared just before use by equilibration of the corresponding nitroalkane (0.1 M) with an equivalent amount of potassium hydroxide. The reaction was followed by monitoring the change in pH. The final pH of the reaction (usually between 10 and 10.5) was reached in approximately 2 h. To measure initial rates for kinetic studies, a coupled enzymatic assay using aldehyde dehydrogenase and NAD was used to convert the product aldehyde formed upon nitroalkane oxidation to the corresponding acid. The increase of absorbance at 340 nm due to NAD reduction was monitored at 25 °C. Concentrations of aldehyde dehydrogenase (0.03−1.1 U/mL) and NAD (1 mM) were chosen such that the coupling reaction was not rate-limiting and the traces showed no lags. All assays contained 10 mM FAD, 5 mM dithiothreitol, and 1 mM D-amino acid oxidase. The buffer was 0.1 M *N*-(2-acetamido)-2-aminoethanesulfonic acid, 0.052 M Tris, and 0.052 M ethanolamine. The pH was adjusted with NaOH or acetic acid as necessary. Assays were initiated by the addition of 2−20 μL of nitroalkane anion from a stock solution. The concentrations of anion substrates were determined from their absorbances at 228 nm in base.

(12) Initial rate data were fit to $v = VA/(K + A)$ and pH−rate data to $\log (V/K) = \log [C/(1 + K_2/H + H/K_1)]$ using the programs of Cleland in *Methods Enzymol.* **1979**, *63*, 103−138 and the KinetAsyst software (IntelliKinetics, State College, PA).

(13) A modification of the procedure of Bell, R. P.; Goodall, D. M. *Proc. R. Soc. London, Ser. A* **1966**, *294*, 273−297 was used to synthesize [1,1-$^2H_2$]nitroethane. The ^1H NMR of the product showed >96% α-deuteration. The anion was formed by incubating [1,1-$^2H_2$]nitroethane in D_2O and KOD. The $^{\alpha D}V/K_{nea}$ values were calculated by direct comparison of the V/K_{nea} values obtained with deuterated and nondeuterated substrates.

S0002-7863(96)02783-7 CCC: $14.00

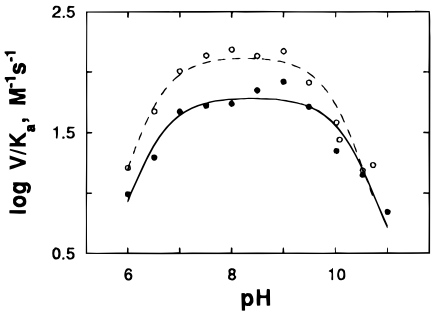

Figure 1. pH dependence of the *V*/*K* values for nitroalkane anions as substrates for D-amino acid oxidase at 25 °C: (●) nitroethane anion; (○) 1-nitropropane anion. The lines are fits of the data to log (*V*/*K*) = log [*C*/(1 + K_2/*H* + *H*/K_1)].

sp^3 rehybridization in the transition state for the reaction of nitroethane anion with D-amino acid oxidase.[14] The structure of a nitroalkane anion in solution is known (**I**); the α-carbon is

I

sp^2-hybridized.[15] Consistent with such a structure, the deprotonation of nitroethane to form the carbanion shows a secondary deuterium kinetic isotope effect of 1.15,[15] very close to the inverse of the value reported here. In contrast, formation of a radical from the carbanion would be expected to generate a small and normal secondary kinetic isotope effect.[14,16] Thus, path a, but not path b, of Scheme 1 is fully consistent with the inverse isotope reported here, and the oxidation of nitroalkane anions by D-amino acid oxidase proceeds by a direct attack of the carbanion on the N(5) position of the flavin.[17] This strongly suggests that the reaction of the amino acid carbanion with the flavin similarly involves a direct nucleophilic attack rather than two single electron transfers.

The pH dependence of the reaction of D-amino acid oxidase with nitroalkanes can be compared with results obtained with inhibitors and amino acid substrates. The $^{\alpha D}V/K$ values for nitroethane anion are pH independent. This indicates that there is no external commitment with this substrate; that is, the isotope-sensitive chemical step is much slower than binding steps. Therefore, the pK_a values of 6.8−6.9 and 9.6−9.9 are likely to be the intrinsic values.[18] There are two previously determined pK_a values which could correspond to the lower value determined here. An ionizable group with a pK_a value of 6.3−6.6 must be unprotonated for binding of the competitive inhibitor benzoate.[19] The value of 6.8−6.9 determined here is sufficiently close to the previously measured value to suggest that they are due to the same residue. An alternative possibility is the ionization observed when glycine is a substrate.[20] With this substrate, CH bond cleavage is reversible, so that the pH dependence of subsequent steps in catalysis is seen in pH profiles. Since the reaction with nitroalkane anions only involves steps subsequent to CH bond cleavage, it is reasonable that the rate is affected by the same ionization. Denu and Fitzpatrick were unable to assign a specific pK_a value to this ionization because of the kinetic complexity of the reaction with glycine;[20] a value of 6.9 would be consistent with the earlier results. The second pK_a value of 9.6−9.9 reported here agrees well with the value of 9.6 determined for the 3-imino position of the enzyme-bound flavin in D-amino acid oxidase.[19a] It is chemically reasonable that reaction of a nitroalkane anion with the FAD would require that the flavin be in the neutral rather than the ionic form, so this ionization can be assigned to the flavin.

Cleavage of the CH bond of an amino acid substrate by D-amino acid oxidase requires that a group with a pK_a value of 8.8 be unprotonated and a group with a pK_a value of 10.7 be protonated.[20] There is no evidence in Figure 1 for a group with a pK_a value of 8.8. This is the expected result if this pK_a value is due to the amino acid residue which acts as a base to remove the α-proton of the amino acid substrate, as previously suggested.[20] The involvement of a group with a pK_a value of 10.7 would not be detectable from the data shown in Figure 1 because of the decrease in activity above pH 10. Thus, the pH dependency of the reaction with nitroalkanes is consistent with the behavior seen with other substrates and inhibitors.

Acknowledgment. This research was supported in part by Grant MCB-9506060 from the National Science Foundation.

JA962783N

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(17) A radical mechanism would be consistent with these data if transfer of the first electron were rapid and fully reversible. The rate-limiting step would then be collapse of a radical pair. The observed isotope effect would be the product of the equilibrium isotope effect for the first electron transfer, which would effectively be 1, and the inverse effect due to collapse of the radicals. However, such a mechanism would be unlikely given the requirement for a stable radical pair involving a nitroalkane radical and a flavin semiquinone.

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