Use of 5-DeazaFAD to Study Hydrogen Transfer in the D-Amino Acid Oxidase Reaction*

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The apoprotein of hog kidney d-amino acid oxidase was reconstituted with 5-deazaflavin adenine dinucleotide (5-deazaFAD) to yield a protein which contains 1.5 mol of 5-deazaFAD/mol of enzyme. The deazaFAD-containing enzyme forms complexes with benzoate, Z-aminobenzoate, and 4-aminobenzoate which are both qualitatively and quantitatively similar to those observed with native enzyme. The complex with 2-aminobenzoate exhibits a new long wavelength absorption band characteristic of a flavin charge-transfer complex.

The reconstituted enzyme exhibits no activity when assayed by D-alanine oxidation. However, the bound chromophore can be reduced by alanine, phenylalanine, proline, methionine, and valine, but not by glutamate or aspartate, indicating the deazaFAD enzyme retains the substrate specificity of the native enzyme. Reduction of the enzyme by d-alanine exhibits a 1.6-fold deuterium isotope effect. Reoxidation of the reduced enzyme occurred in the presence of pyruvate plus ammonia, but not with pyruvate alone or ammonia alone. D-Phenylpyruvate and a-ketobutyrate, but not a-ketoglutarate could replace pyruvate.

Reduced enzyme isolated following reaction with [α-3H]alanine was found to contain 0.5 mol of tritium/mol of deazaFADH₂. After denaturation of the tritium-labeled enzyme, the radioactivity was identified as deazaFADH₂. Reaction of the reduced tritium-labeled enzyme with pyruvate plus ammonia prior to denaturation yields [α-3H]alanine and unlabeled deazaFAD. These results suggest that reduction and reoxidation of enzyme-bound deazaFAD involves the stereo-specific transfer of α-hydrogen from substrate to deazaFAD.

The similarity in chemical properties of deazaflavins as compared to normal flavins (1-3), has provided the basis for using these analogues to study both nonenzymatic (3-5) and flavoenzyme reactions (6-8). Jorns and Hersh (2, 6) showed that 5-deazaFMN functions co-enzymatically in the N-methylglutamate synthetase reaction, while Fisher and Walsh (7) used 5-deazariboflavin as a substrate in the Beneckea harveyi oxidoreductase reaction. Most recently, Averill et al. (8) have demonstrated a half-reaction with deazaFMN-lactate oxidase, while Jorns and Hersh obtained similar results with glycolate oxidase. In all of these studies it has been shown that hydrogen transfer from substrate to deazaflavin occurs during the course of the reaction.

During the past few years a considerable amount of evidence has been presented which suggests that proton abstraction from the substrate is one of the initial steps in flavoprotein oxidase reactions (9-16). Since it has been demonstrated previously that deazaflavins can be used to detect hydrogen transfer from substrate to flavin (2, 6-8) it seemed of considerable interest to determine whether or not the α-hydrogen is transferred to flavin in the d-amino acid oxidase reaction. We have prepared, therefore, deazaFAD-containing d-amino acid oxidase and have studied the fate of the α-hydrogen of alanine during reduction. The results of these studies are the subject of this paper.

MATERIALS AND METHODS

D-Amino acid oxidase from pig kidney was prepared in crystalline form according to the procedure of Brumby and Massey (17). Benzolate-free enzyme was obtained by adding d-alanine to the enzyme-benzoate complex and passing the reaction mixture through a Sephadex G-25 column (16). Apo-d-amino acid oxidase was prepared by dialysis of the benzoate-free enzyme against potassium bromide (18). The concentration of apoenzyme was determined by the Lowry method (19). The absorbance ratio A₄₅₀/A₂₆₀ of the holoenzyme-benzoate complex was 10 ± 0.2, while the specific activity at 25°C in 0.1 M sodium pyrophosphate buffer, pH 8.3, 40 mM DL-alanine, and 4 μM FAD was 4.5 to 5 μmol of alanine utilized/min/mg.

Preparation of FAD Pyrophosphorylase—FAD pyrophosphorylase (EC 2.7.7.2) was purified from rat liver. We used as the starting material the supernatant obtained from rat liver microsomal preparations (20) which were stored frozen until use.
The supernatant solutions were thawed and pooled to give a starting volume of between 400 to 1000 ml. Solid ammonium sulfate was added to 40% saturation (231.1 g/liter) and the solution was then stirred for 30 min. The precipitate was collected by centrifugation at 10,000 x g for 30 min. The precipitate was collected by centrifugation at 10,000 x g for 30 min, redissolved in a minimum volume of 20 mM potassium phosphate buffer, pH 7.4, containing 5 mM 2-mercaptoethanol. After dialysis against this buffer, the enzyme was applied to a DEAE-cellulose column (3.6 x 40 cm) previously equilibrated with the same buffer. The nonadsorbed protein was washed through the column, then a linear gradient consisting of 1 liter of starting buffer in the mixing chamber and 1 liter of starting buffer containing 0.5 M potassium chloride in the reservoir was applied to the column. The fractions containing activity were pooled and concentrated by precipitation with solid ammonium sulfate (526.6 g/liter). The precipitate was collected by centrifugation at 10,000 x g for 1 liter of starting buffer containing 0.5 M potassium chloride in the reservoir was applied to the column. The fractions containing activity were pooled and concentrated by precipitation with solid ammonium sulfate (526.6 g/liter). The precipitate was collected by centrifugation at 10,000 x g for 30 min and redissolved in a minimal volume of 20 mM potassium phosphate buffer, pH 7.4, containing 5 mM 2-mercaptoethanol.

The redissolved protein was applied to a Bio-Gel P200 column (2.5 x 90 cm) previously equilibrated with this buffer and the enzyme eluted with this same buffer. The active fractions were pooled, concentrated by lyophilization, and then chromatographed on a Bio-Gel P-10 column (1.5 x 115 cm) using water as the solvent. The enzyme was dialyzed against either frozen until further use, or stored at 4°C for no longer than 2 weeks.

Deazariboflavin and deazaFMN were synthesized as previously described (2). DeazaFAD was prepared enzymatically from deazaFMN utilizing the partially purified FAD pyrophosphorylase. A reaction mixture containing 4 μmol of deazaFMN, 2.0 mmol of ATP, neutralized to pH 7.5, 9.1 mmol of magnesium sulfate, 100 μmol of FAD pyrophosphorylase, and 2.0 mmol of Tris-hydrochloride buffer, pH 7.5, in a final volume of 40 ml was incubated at 37°C for 6 hours in the dark.

The reaction mixture was then passed through an Amicon ultrafiltration apparatus using a UM-10 membrane. The effluent was chromatographed on a DEAE-cellulose column equilibrated with 10 mM potassium phosphate buffer, pH 6.5. Two deazariboflavin peaks were eluted with this buffer. Paper chromatography, with 1-butanol/acetic acid/water (4/1/5 v/v) along with water as the solvent, was used to identify the first peak as deazariboflavin, and the second peak as a mixture of deazaFMN and deazaFAD. The impure deazaFAD-containing fractions were pooled, lyophilized, and then chromatographed on a Bio-Gel P-10 column (1.5 x 115 cm) using water as the solvent. The enzyme was dialyzed against either frozen until further use, or stored at 4°C for no longer than 2 weeks.

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Another property characteristic of native D-amino acid oxidase is the quenching of FAD fluorescence upon binding of the coenzyme to apoenzyme (18). Upon adding an excess of apo-D-amino oxidase to deazaFAD, no decrease in fluorescence was observed but rather a small increase (10 to 20%) in fluorescence occurred. In order to determine whether the fluorescence of the reconstituted enzyme preparation was in fact due to enzyme-bound deazaFAD, the fluorescence emission spectrum of deazaFAD enzyme was compared to that obtained with free deazaFAD released from the enzyme by boiling. As shown in Fig. 3, bound deazaFAD exhibits slightly more fluorescence than the free flavin with the emission maximum of the bound material shifted about 10 nm hypochromically as compared to the free flavin. Further evidence that enzyme-bound deazaFAD is fluorescent was obtained by showing that the fluorescence intensity decreases proportionally to the time-dependent decrease in absorption at 400 nm upon reduction of the bound chromophore by D-alanine, Fig. 3.

Catalytic Properties of DeazaFAD D-Amino Acid Oxidase—Although deazaFAD-containing D-amino acid oxidase does not catalyze the oxygen-dependent oxidation of D-alanine to pyruvate, the bound chromophore can be reduced by a number of amino acids. Table I lists the pseudo-first order rate constants for reduction of bound deazaFAD by a number of DL-amino acids. With the exception of proline, glutamate, and aspartate, reduction proceeded to over 85% completion. The lack of reactivity of glutamate and aspartate is consistent with the known specificity of native D-amino acid oxidase (29). In the case of proline, reduction only proceeded to 33% completion at 20 mM DL-proline, and to 50% completion at 120 mM DL-proline. The incomplete reduction by proline is not fully understood at the present time, but possibly relates to reversibility of the reaction (30) by Δ-pyline-2-carboxylate. Over 95% inhibition of the rate of reduction by 20 mM D-alanine was observed when either benzoate or 2-amino-benzoate was added at a concentration of 0.1 mM.

The spectral changes occurring when deazaFAD D-amino acid oxidase is reduced by DL-alanine can be described as a decrease in absorbance at 400 nm, an increase in absorbance at 432 nm, and a decrease in the fluorescence intensity of the enzyme-bound chromophore.

We observed FAD fluorescence to be quenched 80% upon binding to apo-D-amino acid oxidase as compared to approximately 70% quenching noted in Ref. 18.

![Fig. 2. Titration of deazaFAD D-amino acid oxidase with benzoate. The increase in absorbance at 432 nm was monitored upon the addition of sodium benzoate to 0.04 mM deazaFAD D-amino acid oxidase (with respect to the concentration of enzyme-bound deazaFAD) in 0.1 M sodium pyrophosphate buffer, pH 8.5.](image)

![Fig. 3. Fluorescent properties of deazaFAD D-amino acid oxidase. Curve 1, fluorescence emission spectrum of deazaFAD D-amino acid oxidase, 5 x 10^{-7} M with respect to deazaFAD in 0.1 M sodium pyrophosphate buffer, pH 8.4; Curve 2, fluorescence emission spectrum after 30 min of incubation with 10 mM D-alanine. At this time 50% of the deazaFAD was reduced as determined spectrophotometrically on a withdrawn aliquot (400 nm); Curve 3, same after 80 min of incubation with 10 mM D-alanine. At this time 85% of the deazaFAD was reduced as judged spectrophotometrically on a withdrawn aliquot (400 nm); Curve 4, fluorescence emission spectrum of 5 x 10^{-7} M deazaFAD, obtained by boiling deazaFAD D-amino acid oxidase for 1 min in a sealed tube, and then separating the denatured protein by centrifugation. Essentially the same curve was obtained if 5 x 10^{-7} M deazaFAD was used prepared from a stock deazaFAD preparation. The excitation wavelength was 400 nm.](image)

<table>
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<th>Substrate</th>
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<tr>
<td>DL-Alanine</td>
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<tr>
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</tr>
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<tr>
<td>DL-α-Deutero alanine</td>
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</tr>
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*Estimated from initial rates. Only 33% of the bound flavin was reduced.

* No reduction was observed after 30 min reaction time.

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**Table I**

Reduction of deazaFAD D-amino acid oxidase by various substrates

Reaction mixtures contained 100 mM sodium pyrophosphate buffer, pH 8.5, 0.1 mM dithioerythreitol, 0.06 mM deazaFAD enzyme, and substrates as indicated in a final volume of 1.0 ml. Reduction was monitored by the decrease in absorbance at 400 nm at 20°C. Rate constants were determined from pseudo-first order rate plots. At a concentration of 20 mM we observed the following relative rates of oxygen uptake using our preparation of native D-amino acid oxidase: DL-alanine, 1.0; DL-methionine, 1.2; DL-phenylalanine, 1.6; DL-valine, 0.6; DL-proline, 2.3. No reaction was observed with DL-glutamate.
323 nm, and an isosbestic point at 333 nm (Fig. 4). The product of the reaction, has a spectrum characteristic of 1,5-dihydrodeazaflavin (2). In order to determine whether or not cleavage of the α-hydrogen bond is rate-determining, the rate of reduction of deazaFAD d-amino acid oxidase by DL-alanine was compared to the rate of reduction by DL-α-deuterolalanine. As can be seen in Table I, the rate of the reaction with the deuterated substrate is considerably slower. At saturating alanine and α-deuterolalanine a deuterium isotope effect of 1.6 is observed. This value is similar to the deuterium isotope effect observed with serine and threonine as substrates for the native enzyme (9, 10), and suggests that cleavage of the α-C—H bond is also partially rate-determining in the reaction of the deazaFAD enzyme.

Radhakrishnan and Meister (30) have shown that under anaerobic conditions d-amino acid oxidase catalyzes a transhydrogenation reaction. As seen in Fig. 5, the addition of pyruvate plus ammonia, but not pyruvate alone or ammonia alone, causes the reoxidation of the reduced deazaflavin. Reoxidation was also observed with ammonia plus β-phenylpyruvate or α-ketobutyrate but not with ammonia plus α-ketoglutarate. The pseudo-first order rate constant for reoxidation of isolated reduced deazaFAD d-amino acid oxidase by 50 mM ammonia plus 50 mM pyruvate is 0.5 min⁻¹ compared to a rate of 0.039 min⁻¹ for reduction by d-alanine. This observation is similar to that noted for the relative rates of reduction and reoxidation of deazaFMN N-methylglutamate synthetase (2).

The ability of the enzyme to undergo cyclic reduction-reoxidation is shown in Fig. 6. In this experiment the enzyme was reduced by d-alanine, re-oxidized by pyruvate plus ammonia, and then further reduced by increasing the concentration of alanine. These results suggest that like the native enzyme (30) deazaFAD d-amino acid oxidase catalyzes hydrogen transfer according to Equations 1 and 2.

\[
\begin{align*}
E\text{-deazaFAD} + R &\rightarrow E\text{-deazaFADH} + R-C-COO^- \\
 &\rightleftharpoons E\text{-deazaFADH} + R-C-COO^- + NH_3 \\
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\end{align*}
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From the data obtained in the experiment shown in Fig. 6, and the equilibrium constant for imine formation from pyruvate and ammonia (31) an equilibrium constant

\[K_eq = \frac{[E\text{-deazaFADH}][Pyruvate] + NH_3}{[E\text{-deazaFAD}][alanine]}\]

den of 1 × 10⁻⁴ can be calculated with alanine and pyruvate-imine as substrates.

In order to determine whether reduction of the enzyme by alanine results in the transfer of the α-hydrogen to flavin, deazaFAD d-amino acid oxidase was reduced with DL-[α-³H]alanine, and then separated from unreacted alanine by chromatography on a Sephadex G-25 column. As shown in Fig. 7A, tritium was found to co-chromatograph with the enzyme. From the data obtained in the experiment shown in Fig. 6, and the equilibrium constant for imine formation from pyruvate and ammonia (31) an equilibrium constant

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FIG. 6. Reduction-reoxidation cycles with deazaFAD D-amino acid oxidase. Curve 1, D-amino acid oxidase (6.25 x 10^{-6} M with respect to deazaFAD) in 0.1 M sodium pyrophosphate buffer, pH 8.5; Curve 2, 90 min after addition of 15 mM D-alanine; Curve 3, 45 min after addition of 5 mM pyruvate and 5 mM ammonia; Curve 4, 60 min after increasing the D-alanine concentration to 35 mM.

FIG. 7. Incorporation of tritium from DL-a-[3H]alanine into deazaFAD D-amino acid oxidase. A, a 1-ml reaction mixture containing 40 nmol of deazaFAD D-amino acid oxidase in 0.1 M sodium pyrophosphate buffer, pH 8.5, was reacted for 6 min at 20°C with 25 mM DL-a-[3H]alanine, specific activity 1.1 x 10^6 cpm/μmol. A total of 34.7 nmol of reduced deazaFAD were produced during this time. The reaction mixture was then chromatographed on a Sephadex G-25 column (1.2 x 50 cm) previously equilibrated with 1 mM sodium pyrophosphate buffer, pH 8.5. Fractions of 1.3 ml were collected and assayed for protein by absorbance at 280 nm (●) and tritium content (O). The total radioactivity in the protein-containing fractions corresponds to 17.2 nmol of tritium incorporated. B, deazaFAD D-amino acid oxidase was treated identically as described above except that 25 mM DL-[U-14C]alanine, specific activity 6.6 x 10^5 cpm/μmol was substituted for tritiated alanine. In this experiment 35.8 nmol of reduced deazaFAD were formed. Following chromatography on Sephadex G-25 as described above, no significant amount of radioactivity (O) was observed in the protein (●)-containing fractions.

DISCUSSION

Although deazaFAD-containing D-amino acid oxidase does not catalyze the oxygen-dependent conversion of amino acids to imino acids, this form of the enzyme is active in catalyzing the same type of transhydrogenation reaction observed anaerobically with the native enzyme. This finding is not surprising in view of the fact that the oxygen reactivity of reduced deazaflavin is lowered by more than a factor of 10^4 over that of reduced FMN (3). On the other hand, Brüstlein and Bruice have shown that the rate of reduction of 3,10-dimethyl-5-deaza-isoalloxazine by NADH or reduced N-methylnicotinamide is 3.5 to 7% the rate of the reaction with 3,10-dimethylisoalloxazine (4). Thus, the extreme stability of reduced deazaflavins toward reaction with oxygen can account for these results.

The observation that deazaFAD fluorescence is not quenched upon binding to D-amino acid oxidase suggests that the environment or interactions of the deazaflavin analogue on the protein may differ somewhat from that in the native enzyme. On the other hand, the ability of benzoate and other carboxylic acids to perturb the visible spectrum of the native product was identified exclusively as tritiated alanine (Fig. 8). Since there are two potential hydrogens in reduced deazaFAD, either of which could be removed during reoxidation, the observed transfer of all of the tritium to alanine indicates stereospecificity at position 5 of deazaFAD.
The enzyme has been interpreted to suggest the presence of a positively charged amino acid residue near the flavin which can either undergo hydrogen-bonding with the flavin or function in binding anions (28). Clearly, these same or similar interactions also exist for deazaFAD bound to D-amino acid oxidase. The long wavelength absorption seen in the presence of 2-aminobenzoate indicates the ability of deazaflavins to form charge-transfer complexes similar to those observed with flavin. 

In the case of native D-amino acid oxidase the E-FADH₂·AA complex reacts with oxygen during turnover, although this intermediate will decay to yield E-FADH₃·IA under anaerobic conditions (29). The E-FADH₃·IA complex, as indicated by its spectral properties, exists as a charge-transfer complex between reduced flavin and imino acid (32). The inability to detect such an intermediate in the reaction of deazaFAD enzyme cannot be due to the inability of deazaflavins to form charge-transfer complexes since a charge-transfer complex between oxidized deazaFAD enzyme and 2-aminobenzoate has been observed, but rather appears to be most probably explained by a low steady state level of this intermediate. This hypothesis seems tenable since the rate of reoxidation of reduced deazaFAD D-amino acid oxidase by pyruvate-imine is considerably faster than the rate of its formation with D-alanine and the overall equilibrium constant of the reaction lies in the direction of oxidized deazaFAD. Therefore, it seems quite reasonable to assume the pathway leading to reduced deazaFAD is the same as that leading to reduced FAD.

It is clear from these studies that transfer of the α-hydrogen from substrate to flavin is an integral part of the reaction sequence in going from oxidized deazaFAD to reduced deazaFAD. Evidence presented previously (9-18) strongly suggests that one of the initial steps in the D-amino acid oxidase reaction is the abstraction of the substrate α-hydrogen as a proton. Although the substrate α-hydrogen is ultimately transferred to position 5 in reduced flavin, it is very unlikely that the oxidized flavin itself could serve as a general base to abstract a proton since the pK for protonation of oxidized flavin is on the order of zero (33). It thus seems probable that if proton abstraction is an initial step in the D-amino acid oxidase reaction this hydrogen transfer is mediated by a basic residue on the protein.

Subsequent formation of reduced flavin must involve transfer of this hydrogen to flavin and transfer of 2 electrons from substrate to flavin. The latter could occur via formation of a covalent substrate carbanion-flavin complex (16, 34) or could be mediated through a noncovalent carbanion-flavin complex (55).

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REFERENCES
5. Shinkai, S., and Bruice, T. C. (1973) J. Am. Chem. Soc. 95, 7526-7528
34. Walsh, C. T., Schonbrunn, A., Lockridge, O., Massey, V., and Abeles, R. H. (1972) 247, 6004-6006
35. Bruce, T. C., Main, I., Smith, S., and Bruce, P. Y. (1971) J. Am. Chem. Soc. 93, 7827-7828
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