Proton Release during the Reductive Half-reaction of D-Amino Acid Oxidase*

Paul F. Fitzpatrick† and Vincent Massey

From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

(Received for publication, February 8, 1982)

Changes in the net protonation of d-amino acid oxidase during binding of competitive inhibitors and during reduction by amino acids have been monitored using phenol red as a pH indicator. At pH 8.0, no uptake or release of protons from solution occurs upon binding the inhibitors benzoate, anthranilate, picolinate, or l-leucine. The \( K_d \) values for both picolinate and anthranilate were determined from pH 5.4 to 8.0. The results are consistent with a single group on the enzyme having a \( pK \) of 6.3 which must be unprotonated for tight binding, as is the case with benzoate binding (Quay, S., and Massey, V. (1977) Biochemistry 16, 3348–3354) and with tight binding of the inhibitor form with an unprotonated amino group.

Upon reduction of the enzyme by amino acid substrates, two protons are released to solution. The first is released concomitantly with reduction to the reduced enzyme-imino acid charge transfer complex. The second is released only upon dissociation of the charge transfer complex to free reduced enzyme and imino acid. The first proton is assigned as arising from the amino acid amino group and the second from the amino acid \( \alpha \)-hydrogen. These results are consistent with the flavin in reduced d-amino acid oxidase being anionic.

The mechanism of reduction of the flavin in flavoprotein oxidases has been extensively studied. Both steady state and rapid reaction methods have been used, employing a variety of modified amino acid substrates. The general conclusion from all of the studies is that reduction proceeds through a carbanion transient formed by abstraction of the amino acid \( \alpha \)-proton followed by reduction of the flavin (Porter et al., 1973; Ghisla and Massey, 1980).

Experiments in which the \( \alpha \)-hydrogen has been replaced by deuterium supply strong evidence for abstraction of the \( \alpha \)-hydrogen during reduction. There is a kinetic isotope effect of two to five on \( k_b \), the rate of reduction at infinite substrate concentration, when \( \alpha \)-deuterated substrates are used (Yagi et al., 1970; Yagi et al., 1973; Walsh et al., 1973; Porter et al., 1977). The most complete study of kinetic isotope effects in the reductive half-reaction with d-amino acid oxidase was done by Porter et al. (1977). With \( \alpha \)-phenylalanine and d-alanine, they found identical deuterium isotope effects on both \( k_b \) and \( k_a \), the rate of the reverse reaction, from which they concluded that the \( \alpha \)-proton was retained in the charge transfer complex and not released until the imino acid dissociated.

Additional evidence for a carbanion mechanism comes from work with \( \beta \)-halogenated substrates. When either \( \beta \)-Cl-[\( \alpha \]-H] aminobutyrate or \( \beta \)-Cl-[\( \alpha \]-H]alanine was used as substrate, the products contained label at the \( \beta \)-carbon (Walsh et al., 1973; Cheung and Walsh, 1976). When d-amino acid oxidase was incubated with [\( \alpha \]-H]proline, pyruvate, and ammonia anaerobically in an attempt to produce labeled d-alanine, the label was lost to water and the alanine which was produced was unlabeled, consistent with loss of the \( \alpha \)-proton to solvent from the free reduced enzyme (Walsh et al., 1971).

Both the kinetic isotope data and the results with \( \beta \)-Cl-substrates suggest that the \( \alpha \)-proton is shielded on the enzyme after formation of the reduced enzyme-imino acid charge transfer complex and cannot exchange with solvent until after product release. The loci of proton release steps during the reductive half-reaction were, therefore, determined directly using phenol red as an indicator. Changes in enzyme protonation during binding events were also monitored using several inhibitors.

**EXPERIMENTAL PROCEDURES**

FAD and d-alanine were purchased from Sigma Chemical Co. d-proline was from Calbiochem. Sodium benzoate was from Mallinckrodt. Anthranilic acid was from Matheson, Coleman, and Bell and was recrystallized from water before use. Rapid reaction measurements were made in a temperature-controlled, anaerobic stopped flow spectrophotometer interfaced with a Nova (Data General) minicomputer system as described by Beaty and Bellou (1981). Visible spectra were recorded with temperature-controlled spectrophotometers. Measurements of pH were performed with a Radiometer model 25 pH meter using a Radiometer electrode model GK 2321C. Anaerobiosis was obtained by repeated evacuation and flushing with purified nitrogen from which residual oxygen had been removed by treatment with Fieser's solution (Fieser, 1924) or by passage over a Riddox column. Anaerobic titrations were made in an apparatus similar to that described by Foust et al. (1969).

\( \beta \)-Amino acid oxidase was purified by a combination of previous methods (Brumby and Massey, 1968; Curti et al., 1973; Jenkins et al., 1979) as previously described (Fitzpatrick and Massey, 1982). Benzoate was removed by the method of Brumby and Massey (1968).

Phenol red was used to measure proton release during reduction of d-amino acid oxidase by substrate in the stopped flow spectrophotometer. Each sample of substrate in 25 \( \mu \)M phenol red, 0.1 M NaCl, and 25 \( \mu \)M sodium pyrophosphate was purged of oxygen and carbon dioxide by extensive equilibration with purified nitrogen until the spectrum of the phenol red was stable. The sample was then titrated to pH 8.0 in an anaerobic titrator by adding measured aliquots of 10 \( \mu \)L NaOH. After each addition, the spectrum of the mixture was taken and compared with a standard curve to determine the pH.

From a series of such additions, a standard curve could be constructed which allowed determination of the stoichiometry of proton release or uptake from the absorbance changes seen in the stopped flow spectrophotometer. This procedure was repeated for each sample of substrate or ligand used. The d-amino acid oxidase to be used in these experiments was first dialyzed overnight against two changes of 0.1 M NaCl, 25 \( \mu \)M sodium pyrophosphate, pH 8, using 100 to 200 volumes change. The enzyme was then diluted with 0.1 M NaCl, 25 \( \mu \)M sodium pyrophosphate, pH 8, and the spectrum taken to determine the concentration. Phenol red was next added from a 1 \( \mu \)M stock to give
25 μM phenol red. The enzyme solution was purged of oxygen and carbon dioxide by extensive equilibration with purified nitrogen and the pH adjusted to 8.0 by the anerobic addition of 10 mM NaOH.

RESULTS

Lack of Binding of Phenol Red by D-Amino Acid Oxidase—The possibility that D-amino acid oxidase binds phenol red was tested in several ways. Aliquots of phenol red were added to a sample of enzyme (27 μM) in a cuvette at 19 °C, pH 8.5, and the spectrum was taken using a reference cuvette containing the same concentration of phenol red. At concentrations of phenol red up to 25 μM there was no change in the absorbance spectrum of D-amino acid oxidase. The pK of phenol red at 19 °C was measured as 7.78 by spectrophotometric titration. When the titration was done in the presence of 11 μM enzyme, the pK was 7.83. Finally, steady state kinetics using D-alanine as substrate at concentrations of 0.67 mM to 10 mM (the \( K_a \) for D-alanine in air-saturated buffer is 2 mM) were unaffected by the addition of 100 μM phenol red.

Proton Uptake during Binding—Benzoate was used as a substrate analogue to monitor proton uptake or release during binding events. Since neither free D-amino acid oxidase nor benzoate-bound enzyme had any absorbance beyond 530 nm, the absorbance peak of phenol red at 558 nm could be used to follow proton uptake. Spectrophotometric titrations showed that phenol red was isosbestic at 366 nm with changes in pH, so the time course of benzoate binding was followed at this wavelength. The results at each wavelength when D-amino acid oxidase was mixed with benzoate (50 μM after mixing) in the stopped flow spectrophotometer are shown in Fig. 1. When benzoate binding was followed at 366 nm, there was a rapid, monophasic decrease in absorbance (Fig. 1A), with a rate constant of 10.1 s\(^{-1}\). When phenol red was monitored at 558 nm, there was a rapid increase in absorbance (Fig. 1B), with a rate constant of 10.6 s\(^{-1}\). At all of the concentrations tested, both benzoate binding and changes in phenol red absorbance occurred at the same rate, as shown in Fig. 2. The second order rate constant obtained from Fig. 2 (3 × 10\(^{5}\) M\(^{-1}\)s\(^{-1}\)) is identical with that previously determined for benzoate binding at pH 8.0 in 20 mM sodium pyrophosphate (Quay and Massey, 1977), confirming that neither the buffer system used nor the presence of phenol red had any effect on the benzoate-binding properties of the enzyme. The increase in absorbance at 558 nm corresponded to a net uptake of 0.065 ± 0.016 proton by D-amino acid oxidase during benzoate binding at pH 8.0. This uptake is consistent with the known pK values of groups required for benzoate binding (Quay and Massey, 1977) as considered in more detail under "Discussion."

Similar experiments were done with anthranilate, picolinate, and L-leucine in order to examine the effect of a ligand amino group on proton uptake or release. Anthranilate and picolinate have amino group pK values of 4.95 and 5.4 and are, therefore, predominantly uncharged at pH 8, while L-leucine has a pK of 9.7 and is predominantly the zwitterion (Dawson et al., 1969). The results were similar to those with benzoate, with both binding and proton uptake occurring as rapid, monophasic reactions. In all cases, less than 0.1 proton/enzyme-bound flavin was taken up from solution upon binding.

To further examine what effect the protonation state of the amino group of ligands has on binding by D-amino acid oxidase, the effect of pH on the \( K_a \) values for anthranilate and picolinate was measured from pH 5.4 to 9.8. The results with both ligands were similar to those with benzoate (Quay and Massey, 1977) with a single group on the enzyme with a pK of 6.3 being unprotonated for binding. There was no evidence for preferential binding of ligands with protonated amino groups. The loose binding of L-leucine (\( K_a = 1.75 \text{ mM at pH 8} \)) and

**FIG. 1**. Absorbance changes upon mixing D-amino acid oxidase (15.5 μM after mixing) with benzoate (50 μM after mixing) in the presence of 25 μM phenol red at 19 °C in CO₂-free 0.1 M NaCl, 25 μM pyrophosphate, pH 8.0. A, time course of changes in the flavoprotein spectrum, monitored at 366 nm. B, time course of changes in pH, monitored at 558 nm using phenol red as a pH indicator.

**FIG. 2** (left). Rates of (A) benzoate binding to D-amino acid oxidase, followed at 366 nm, and of (C) proton uptake from solution, followed at 558 nm using phenol red as a pH indicator, as a function of benzoate concentration at 19 °C, pH 8.0. **FIG. 3** (center). Absorbance changes upon anaerobically mixing D-amino acid oxidase (16 μM after mixing) with D-alanine (0.28 mM after mixing) in the presence of 25 μM phenol red in CO₂-free 0.1 M NaCl, 25 μM pyrophosphate, pH 8.0, at 19 °C. A, reduction of the flavoprotein to form the reduced enzyme-imino acid charge transfer complex, followed at 640 nm. B, change in pH of the solution, followed at 502 nm using phenol red as a pH indicator. Both traces have been corrected for the slow decay of the charge transfer complex.

**FIG. 4** (right). Double reciprocal plot of (C) rate of reduction of D-amino acid oxidase, followed at 640 nm, and of (C) rate of proton release, followed at 502 nm using phenol red as a pH indicator, as a function of the concentration of D-alanine. Conditions as in Fig. 3. It should be noted that the true intercept in such plots is ±0.001 s (Massey and Gibson, 1984; Porter et al., 1977).
the presence of two groups on the enzyme with pK values between 9 and 10 made it impossible to do meaningful experiments with L-leucine.

Proton Release during Reduction with D-Alanine—Changes in protonation of D-amino acid oxidase upon reduction by D-amino acid substrate were examined with D-alanine. D-Alanine was chosen because the formation of the reduced enzyme-imino acid charge transfer complex is much more rapid than its breakdown and because D-alanine has been well characterized as a substrate for D-amino acid oxidase. Changes in the absorbance of the enzyme were followed at 640 nm, where only the charge transfer complex absorbs (Massey and Gibson, 1964). Changes in the absorbance of phenol red were followed at 502 nm, where oxidized enzyme and the charge transfer complex are isosbestic.

Upon mixing D-alanine with D-amino acid oxidase anaerobically in the presence of phenol red, there was a rapid increase in long wavelength absorbance, corresponding to the formation of the charge transfer complex (Fig. 3A). There was also a corresponding decrease in absorbance at 502 nm (Fig. 3B) due to release of protons into solution. Proton release and reduction occurred at identical rates at all substrate concentrations tested. This is shown in Fig. 4 as a double reciprocal plot. The stoichiometry of the proton release was 0.8 ± 0.05 proton/enzyme-bound flavin.

Proton Release during Reduction with D-Proline—To determine whether there were any changes in the net protonation of the enzyme upon dissociation of the charge transfer complex to free reduced enzyme and imino acid, it was necessary to use an amino acid other than D-alanine. This was because the imino acid product formed with D-alanine hydrolyzes to free ammonium ion and pyruvate after release from the enzyme, with a rate comparable to the rate of release. With D-proline, the imino acid, Δ⁻-pyrroline-2-carboxylic acid, is stable after release into solution so it was used instead.

The enzyme was again monitored at 640 nm, where the charge transfer complex has considerable absorbance, and at 502 nm (Fig. 5A), where only the charge transfer complex absorbs (Massey and Gibson, 1964). Reduction of D-amino acid oxidase in the stopped flow apparatus by D-proline showed that reduction of the charge transfer complex was isosbestic at 502 nm (Fig. 5A). The changes at 502 nm corresponded to a release into solution of 1.2 ± 0.1 protons/enzyme-bound flavin during reduction of D-amino acid oxidase by D-proline. The stoichiometry was independent of the concentration of D-proline used. The rapid increase at 640 nm was followed by a slow decrease due to the breakdown of the charge transfer complex to free reduced enzyme and imino acid (Fig. 5A). The absorbance of phenol red at 385 nm increased at the same rate (Fig. 5B). This increase at 385 nm corresponded to a release into solution of 1.06 ± 0.03 protons/enzyme-bound flavin during the dissociation of the charge transfer complex.

DISCUSSION

Changes in protonation upon binding of inhibitors to D-amino acid oxidase were used as simple models for binding of ligands to the enzyme. Quay and Massey (1977) showed that groups on the enzyme with pK values of 6.3, 9.2, and 9.6 are involved in benzoate binding. For tight binding of benzoate, the pK 6.3 group must be unprotonated and both the pK 9.2 and the pK 9.6 groups must be protonated. At pH 8.0, the pK 6.3 group would be 1.9% protonated, while the pK 9.2 and 9.6 groups would be 5.9% and 2.4% protonated, respectively. This predicts net uptake of 0.058 ± 0.024 = 0.019 = 0.064 protons from solution upon benzoate binding. Binding of benzoate resulted in the uptake of 0.065 proton, in excellent agreement with prediction.

Similar experiments with anthranilate, picolinate, and L-leucine were done to investigate the effect of an amino group on these protonation changes. Of interest was whether D-amino acid oxidase binds the charged or neutral form of the amino group. If the predominant form of picoinate or anthranilate in the enzyme-anthranilate complex had a charged amino group, binding of anthranilate would have resulted in the uptake of one proton from solution since the amino group of each ligand is predominantly uncharged at pH 8.0. Instead, only a very slight uptake of protons was seen. That D-amino acid oxidase is indeed binding the uncharged form of the amino group strongly was confirmed by the effect of pH on the Kd values of these two inhibitors. If the charged form of the amino group were bound preferentially, the Kd would decrease with decreasing pH from pH 9 to pH 7.5. Instead,
the pH dependency is identical with that seen with benzoate. Finally, as expected (Massey and Ghisla, 1974), model studies show that the anthranilate amino group in anthranilate-flavin charge transfer complexes is uncharged (Shieh et al., 1981).

While the experiments with anthranilate and picolinate show that it is not necessary for the amino group to be protonated in order to have tight binding to the enzyme, they do not preclude the binding of compounds with a charged amino group. Indeed, the lack of any proton release on binding of L-leucine, which at pH 8 is predominantly in the zwitterionic form, indicates that the protonation state of signal amino groups must be relatively unimportant for binding. In a paper on L-amino acid oxidase, Porter and Bright (1980) report that the amino group must be protonated for binding of D-amino acids to D-amino acid oxidase during catalysis. In the present work, a single proton was released to solution on reduction of D-amino acid oxidase by D-alanine to form the charge transfer complex between reduced enzyme and imino acid. In agreement with these results; however, they interpreted this as evidence for binding to the enzyme of the form of the amino acid with an uncharged amino group prior to reduction. The results of a study of the effect of pH on the reduction of L-amino acid oxidase by phenylalanine are consistent with a charged substrate amino group being required for reduction; substrate binding results in acidification of the enzyme base and increased basicity of the substrate amino group (Porter and Bright, 1980). Extrapolation of these results to D-amino acid oxidase and the results of the present study suggest that the proton released upon reduction with D-alanine is the amino group proton. The presence of a positive charge on the amino group would be expected to make formation of an α-carbanion much more favorable, so that it is quite reasonable that the protonation state of the amino group is unimportant for binding but crucial for catalysis. This is summarized in Scheme 1.

The experiments with D-proline were done to determine the protonation state of the charge transfer complex and to determine when the α-hydrogen is lost to solvent. The results show that a single proton is lost during reduction to form the charge transfer complex, as was the case with D-alanine, and a second is lost when the imino acid dissociates. The reasoning above with D-alanine suggests that this second proton is the α-proton. Furthermore, the results of Walsh and co-workers with β-C1-α-amino acids showed that the α-proton can be transferred to the β-position of product so that it must exchange slowly or not at all with solvent (Walsh et al., 1973; Cheung and Walsh, 1976), and the work of Porter et al. (1977) shows that the α-hydrogen is retained in the charge transfer complex with D-alanine long enough to result in a deuteron isotope effect on k vowel, the rate of the reversal of reduction. This is also consistent with the second proton originally being the α-hydrogen.

It is difficult to determine from the spectra of reduced flavins whether the dihydroflavin is in the anionic or neutral form. Indeed, the spectrum of the reduced flavin is often more indicative of the degree of bend in the reduced flavin (Tauscher et al., 1973) than the protonation state (Mayhew et al., 1969; Massey and Hemmerich, 1980). As a result, some confusion has arisen as to whether the reduced flavin in D-amino acid oxidase is neutral or anionic because the spectrum has some similarities to both the neutral and anion forms of dihydrotetraflavin free in solution (Ghisla et al., 1974). The results with D-proline are consistent with the flavin in reduced D-amino acid oxidase being anionic, since there would only be a single proton released during the entire reaction with D-proline if the reduced flavin were neutral.

Finally, the question of the location of the α-proton in the charge transfer complex must be addressed. The likely possibilities are on the flavin itself or on the enzyme base which originally abstracted the proton. The former possibility would be consistent with the results with 5-deazaflavin and D-amino acid oxidase, in which reduction results in transfer of the α-proton of substrate to the flavin (Hersh and Jorns, 1975). These results are complicated by the fact that 5-deazaflavin may be a better analogue for pyridine nucleotides than for flavins (Hemmerich et al., 1977), so the mechanism of reduction may be different with the altered flavin. There is undoubtedly at least one proton on the flavin in the charge transfer complex, but it may have come from either the α-proton or the N-proton of the imino acid. These possibilities are summarized in Scheme 2.

Although it has not been possible to determine the exact location in the charge transfer complex of the proton abstracted from the amino acid, it has proved possible to determine at which points in reduction protons are released to solvent. It has been shown that two protons are released during reduction. The first is released concomitantly with the formation of the reduced enzyme-imino acid charge transfer complex and probably arises from the substrate amino group. This requirement is consistent with the proposed carbanion transient intermediate. The second proton is released to solvent only on dissociation of the imino acid product from the charge transfer complex.

REFERENCES


Proton Release from D-Amino Acid Oxidase

Hersh, L. B., and Jorns, M. S. (1975) J. Biol. Chem. 250, 8728-8734
Quay, S., and Massey, V. (1977) Biochemistry 16, 3348-3354