Oxidation-Reduction Potentials of D-Amino Acid Oxidase

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SUMMARY

This paper reports a study of the oxidation-reduction equilibrium of D-amino acid oxidase, a flavoprotein containing FAD.

The oxidation-reduction potential at 50% oxidation ($E_1$) is $-0.004$ volt at pH 7.0 and 20°, and therefore about 180 mv higher than that of the free coenzyme (FAD). This difference in oxidation-reduction potential may be described in terms of relative affinity of the apoenzyme for the reduced and oxidized forms of the coenzyme. On this basis the affinity constant for the binding of reduced FAD to the apoenzyme is about 105 higher than that of oxidized FAD.

The curve relating $E_1$ to pH is in the alkaline range consistent with a slope of about $-0.058$ volt per pH unit which corresponds to the difference of 1 proton between the oxidized and reduced forms of the enzyme. The apparent $pK$ of the oxidation-linke group, which belongs to the oxidized form, is $\sim 7.1$.

The shape of the oxidation-reduction equilibrium curve of D-amino acid oxidase is pH dependent, the value of $n$ increasing from about 1 at pH 8.6 to about 3, or more, at pH 6.6. Under these conditions, therefore, one must consider the existence of functional homotropic interactions between at least 2 FAD molecules. The pH dependence of the cooperative oxidation-reduction equilibrium is discussed in the framework of the theory of linked functions.

Structural and functional properties of the FAD-dependent D-amino acid oxidase (D-amino acid:O2 oxidoreductase (deaminating), EC 1.4.3.3), an enzyme which catalyzes the oxidation of D-amino acids, have been investigated over the past years in several laboratories (1–6). The interest has been largely polarized on the reaction mechanism, and several important contributions involving kinetic methods as applied to the catalytic function have shed light on the properties of the enzyme (7). However no report has appeared so far on the oxidation-reduction properties of D-amino acid oxidase, in spite of the potentialities of this approach to yield pertinent information on the system.

This paper reports the results of a study on the oxidation-reduction equilibrium of D-amino acid oxidase as measured by a direct potentiometric method. On one hand, the experiments allow the determination of the oxidation-reduction potential of the enzyme, which is obviously relevant to its biological function; on the other hand, they yield new and pertinent information to define the role of the protein moiety in controlling the oxidation-reduction properties of the coenzyme. In this perspective the work reported here has to be considered preliminary, since only a limited number among all possible variables has been explored.

EXPERIMENTAL

D-Amino acid oxidase was purified in the presence of benzoate from pig kidney, freed from benzoate according to Brumby and Massey (8), and stored at $-20°$. Several batches of the enzyme were used during the course of the work; their FAD content varied from 1.9 to $2.1 \times 10^{-5}$ moles per mg of protein, except in one case in which a preparation containing $1.2 \times 10^{-5}$ moles of FAD per mg of protein was employed. The apoprotein, obtained according to Massey and Curti (9), was used immediately after the preparation.

The FAD content of the enzyme was calculated with a millimolar extinction coefficient of 11.3 at 455 nm. Protein concentration was determined either by a modification of the biuret method (10) or by spectroscopy with the following extinction values: $E_{274} = 23.0$ for the holoenzyme and $E_{278} = 15.4$ for the apoenzyme. D-Alanine was obtained from Merck and FAD from Serva. All other chemicals used were of analytical grade.

Spectra were obtained with a Cary 14 recording spectrophotometer.

Catalytic activity was measured under standard conditions (8) either with an oxygen electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio) or spectrophotometrically, with D-phenylglycine as substrate according to Fonda and Anderson (11). Oxidation-reduction equilibria were determined according to the standard procedure already described (12). The electrode potentials were read against a saturated calomel half-cell ($E_b$)
0.248 volt at 20°), and referred to the normal hydrogen electrode.
All measurements were performed in an atmosphere of
purified argon at constant temperature (20 ± 0.2°).

The equilibrium curves were determined by two different
procedures: (a) The enzyme in the oxidized form was progressively
reduced with either benzyl viologen or anthraquinone-β-sulfate,
previously reduced by hydrogen and palladium. (b) The
enzyme reduced with anthraquinone-β-sulfate was subse-
quently oxidized stepwise with degassed potassium ferricyanide.

In order to facilitate attainment of equilibrium at the platinum
electrode, methylene blue was used as mediator (14); its molar
concentration varied, in the different experiments, between 3 and
5% of that of the protein (expressed in terms of FAD content).

RESULTS

Treatment of Experimental Data—The potentiometric data
have been treated with the electrode equation which, at constant
pH, is given by

\[ E_h = E_1 + \frac{RT}{nF} \ln \left( \frac{\text{Ox}}{\text{Red}} \right) \]  

(1)

It is well established that the value of \( n \), which formally repre-
sents the number of electrons exchanged in the oxidation reduc-
tion process (i.e. the stoichiometry of the reaction), is not neces-
sarily an integral value. Thus for biological systems \( n \) acquires
the value of a parameter which reflects both the heterogeneity
and the functional interaction between the (electron)-binding
sites (15-17). Solving Equation 1 for \( n \), we have

\[ n = \frac{RT}{F} \frac{d \ln \left( \frac{\text{Ox}}{\text{Red}} \right)}{dE_h} \]  

(2)

A plot of \( \ln \left( \frac{\text{Ox}}{\text{Red}} \right) \) against the oxidation-reduction poten-
tial at 20° should yield a straight line the slope of which,
multiplied by 0.058 volt, gives the apparent value of \( n \). This
value is an empirical parameter which can be related to the extent
of the homotropic interactions between the electron binding sites
in the system (16).

General Behavior and Reversibility of System—The oxidation-
reduction equilibrium of \( \alpha \)-amino acid oxidase was investigated
at a protein concentration from 0.7 to 1.0 mg per ml. Under
these conditions stable potentials were obtained during the oxid-
ation-reduction titration only in the presence of an electromotively
active mediator (methylene blue).

In a series of experiments the enzyme in the oxidized state was
reduced by stepwise addition either of reduced anthraquinone-
β-sulfate or of the semiquinone of benzyl viologen. Neither
one of the two reagents fulfills the requirements for an ideal re-
ductant, the first because of its oxidation-reduction potential, and
the second because of the uncertainties in achieving the same
level of reduction in different experiments. In spite of this the
results of the reductive titrations with the two reagents were gen-
erally reproducible. Alternatively the enzyme was reduced by
adding an excess of anthraquinone-β-sulfate and thereafter
oxidized by progressive additions of potassium ferricyanide.
This procedure generally yielded very satisfactory results and
the amount of oxidant used corresponded, within experimental
to the expected stoichiometry.

Fig. 1 reports the oxidation-reduction equilibrium curves of
\( \alpha \)-amino acid oxidase at pH 6.5 to 8.7 in 0.1 m sodium pyrophos-
phosphate buffer at 20°. The results obtained by the reductive
titrations (with benzyl viologen) and the oxidative titration (with
ferricyanide) are compared in the figure, and appear to be in ex-
cellent agreement. This finding, together with the stability of
the measured potential at any stage along the titration curve
shows that the system is in a condition of true thermodynamic
equilibrium.

It is worthwhile to recall that, according to expectations, the

![Fig. 1 (left). Oxidation-reduction equilibrium curve of \( \alpha \)-amino acid oxidase at pH 6.5 in 0.1 m sodium pyrophosphate buffer and 20°. ○ —○, oxidative titration with potassium ferricyanide; ∙, reductive titration with benzyl viologen; — — — —, theoretical curve for \( n = 1 \). The three experiments were made to coincide at 50% oxidation to compensate for the slight difference in pH. Ordinate, fractional oxidation. Abscissa, potential referred to the hydrogen electrode, in volts.](http://www.jbc.org/)

![Fig. 2 (center). Relation of the midpoint potential (\( E_1 \)) to pH for \( \alpha \)-amino acid oxidase at 20°. ○, results in sodium phosphate buffer 0.1 m; ∙, results in sodium pyrophosphate buffer 0.1 m. The lower continuous line shows the results for FAD and FMN at 30°, from Lowe and Clark (18); the broken line, those for the old yellow enzyme, also at 30°, from Vestling (19). ∆, an experiment performed on FAD at 20° and pH 7.0 in 0.1 m sodium phosphate buffer.](http://www.jbc.org/)

![Fig. 3 (right). Oxidation-reduction equilibrium curve of \( \alpha \)-amino acid oxidase at pH 8.0 in 0.1 m sodium phosphate buffer (○, points from two experiments) and pH 8.6 in 0.1 m sodium pyrophosphate buffer (●, points from three experiments). Temperature 30°. Ordinate, fractional oxidation. Abscissa, potential referred to the hydrogen electrode, in volts.](http://www.jbc.org/)
color of the enzyme solution changes progressively during the titration from a bright yellow (typical of the oxidized form) to a very pale yellow (in the reduced form).

Further checks were performed to test for possible irreversible processes caused by the action of the reagents used in the potentiometric experiments. The specific activity of the material recovered after the oxidation-reduction experiments was found to be unchanged, thereby excluding irreversible damages of the enzyme. Additional experiments performed reducing d-amino acid oxidase with dithionite, both in the presence and absence of sodium phosphate, showed no significant decrease in specific activity and no change in the absorption spectrum.

**Effect of pH on Oxidation-Reduction Equilibrium**—The potentiometric data obtained at different pH values from 6.25 to 8.72 are summarized in Table I in terms of the two parameters $E_i$ and $n$. The results show that both the midpoint potential and the shape of the oxidation-reduction equilibrium curve of d-amino acid oxidase are pH dependent.

The dependence on pH of the oxidation-reduction potential of d-amino acid oxidase at 50% oxidation ($E_i$) is shown in Fig. 2, where similar results on FAD or FMN (18) and on the old yellow enzyme (19) are also reported. In the alkaline pH region the results on d-amino acid oxidase are consistent with a slope of about $-58$ mvolts per pH unit. At lower pH values the potential becomes essentially independent of pH, and levels off at a value of about zero volt.

Fig. 3 reports the oxidation-reduction equilibrium curves of d-amino acid oxidase at two pH values, namely 8.6 and 6.6. At pH 8.6 the titration corresponds closely to a 1-electron process over the whole range; as the pH is lowered there is an obvious increase in the slope of the equilibrium curve, the value of $n$ at pH 6.6 being higher than 3. The increase in the value of $n$ with decrease in pH appears to be monotonic (see Table I).

Control experiments were performed to exclude the possibility that irreversible changes in the properties of the enzyme were caused by the pH of the solvent. In the region of interest (namely between pH 6 and 10) the activity of the enzyme was essentially independent of the time of exposure to any particular condition.

**Interaction of FAD with Apoenzyme**—A direct measure of the shift in the oxidation-reduction potential of FAD upon binding to the protein was obtained in a differential experiment. The titration of FAD performed under our experimental conditions is in good agreement with previous results (18) if the difference in temperature is taken into account (see Fig. 2). The potential of a solution of FAD, brought to 50% reduction by addition of sodium dithionite, shifted from $-0.20$ volt towards positive values upon addition of apoenzyme. In the presence of roughly stoichiometric amounts of apoenzyme and FAD, the potential of the solution was about $-0.08$ volt (at pH 7.1) and therefore still significantly lower than the potential of the native enzyme under the same conditions ($E_i = -0.01$ volt) (see Table I). However, in view of the difficulty of the experiment and of the instability of the apoenzyme, which may not have been enough to bind all the FAD, the change in potential realized in the differential experiment, which corresponds to two-thirds of the expected change, can be considered to be satisfactory.

**DISCUSSION**

Analysis of the oxidation-reduction properties of d-amino acid oxidase greatly benefits from the possibility of comparing the oxidation-reduction behavior of the enzyme with that of the free coenzyme, FAD. In so far as the absolute value of the oxidation-reduction potential is concerned, the comparison is shown in Fig. 2 for the pH range of interest. The change in the oxidation-reduction potential of FAD occurring upon binding to the macromolecule can be envisaged, on the basis of simple thermodynamic considerations, as a difference in the free energy of interaction of the apoenzyme with the two forms of the coenzyme (13). The positive shift in $E_i$ observed upon interaction of FAD with d-amino acid oxidase implies that the equilibrium constant for the formation of the apoenzyme-reduced FAD complex is larger than that for the formation of apoenzyme-oxidized FAD complex. A shift similar in direction, but smaller in magnitude, has been reported upon binding of FMN to the old yellow enzyme (19). The change in $E_i$ ($\Delta E_i$) between free and bound FAD is related to the equilibrium (association) constants for the interaction of the apoprotein with the oxidized ($K_o$) and reduced ($K_r$) coenzyme by the following:

$$\Delta E_i = \frac{RT}{nF} \ln \frac{K_r}{K_o}$$

The applicability of this simple equation may be doubtful in

**Table I**

| Preparation | pH | Buffer | Reagents | $E_i$ (volt) | $n$ |
|-------------|----|--------|----------|-------------|----|
| 4           | 6.25 | Sodium phosphate 0.1 M | Ferricyanide | $-0.007$ | 2-3 |
| 5           | 6.51 | Sodium phosphate 0.1 M | Ferricyanide | $-0.017$ | 2-3 |
| 4           | 6.67 | Sodium phosphate 0.1 M | Ferricyanide | $-0.004$ | 3.5 |
| 4           | 6.65 | Sodium phosphate 0.1 M | Ferricyanide | $-0.004$ | 3.5 |
| 3           | 7.09 | Sodium phosphate 0.1 M | Benzyl viologen | $+0.004$ | 2.8 |
| 3           | 7.13 | Sodium phosphate 0.1 M | Benzyl viologen | $-0.006$ | 1.8 |
| 3           | 7.14 | Sodium phosphate 0.1 M | Ferricyanide | $-0.006$ | 1.8 |
| 5           | 7.65 | Sodium phosphate 0.1 M | Ferricyanide | $-0.03$ | 1-2 |
| 5           | 7.94 | Sodium pyrophosphate 0.1 M | Ferricyanide | $-0.055$ | 1 |
| 2           | 8.5  | Sodium pyrophosphate 0.1 M | Ferricyanide | $-0.081$ | 1 |
| 1           | 8.6  | Sodium pyrophosphate 0.1 M | Ferricyanide | $-0.085$ | 1 |
| 3           | 8.79 | Sodium pyrophosphate 0.1 M | Benzyl viologen | $+0.077$ | 1 |

* Different numbers refer to different preparations of the enzyme all of which contained from 1.9 to $2.1 \times 10^{-5}$ moles of FAD per mg of protein.

* The values of $n$ are those reckoned near 50% oxidation. Whenever the equilibrium curves were heterogeneous, higher and lower estimates of $n$ are reported.
the present case since, at some pH values, the shape of the equilibrium curve of free and bound FAD is different; thus at pH 8.5, \( n \approx 1.5 \) for the free coenzyme and \( n \approx 1 \) for \( n \)-amino acid oxidase. However, if we consider the situation at pH 7, where \( n = 2 \) in both cases, the positive shift in potential (\( \Delta E^\circ = 0.180 \) volt) yields a value of \( \log (K_R/K_0) = 4 \). Since \( K_R \) has been estimated to be \( -5 \times 10^4 \) m\(^{-1} \) (1), \( K_R \) should be \( 5 \times 10^2 \) m\(^{-1} \), although this estimate should be taken with great caution. In the case of the old yellow enzyme, the affinity of the apoenzyme for reduced FMN is considerably larger than that for oxidized form, although the difference between the two equilibrium constants is not as large as that obtained for \( n \)-amino acid oxidase (\( K_R = 10^8 \) m\(^{-1} \) and \( K_R = 2 \times 10^5 \) m\(^{-1} \)) (19). The large change in oxidation-reduction potential of FAD once bound to a specific protein moiety has an obvious significance in connection with the biological properties of the enzyme.

The decrease in the mid-point potential at pH \( >7 \) (\( \Delta F_1/ \Delta pH = 55 \) to 60 mvols per pH unit) is consistent, within the errors of the measurements, with that expected for 1 oxidation-reduction-linked proton (i.e. \(-58 \) mvols per pH unit at \( 20^\circ \)). The shape of the \( E_1 \)-pH curve indicates the presence of one oxidation-linked group which is operative in the oxidized form of the molecule, with an apparent pK of \( \sim 7.1 \).

The most intriguing result is the dependence on pH of the shape of the oxidation-reduction equilibrium curve of \( n \)-amino acid oxidase. The value of \( n \) near the midpoint of the titration, which is essentially 1 at pH \( \sim 8.6 \), becomes 3 or higher at pH \( \sim 6.6 \). This is brought out in a clearer fashion by Fig. 4, where oxidation-reduction equilibrium curves are plotted in terms of Equation 2 to emphasize the dependence on pH of the steepness of the equilibrium curve. In this respect as well, the behavior of the enzyme is at variance with that of free FAD, for which a value of \( n \) of about 1.8 is reported at different pH values (18).

This again points to the essential role of the protein in controlling the oxidation-reduction properties of the coenzyme.

A general interpretation of the behavior of \( n \)-amino acid oxidase can be envisaged in the framework of the theory of linked functions (16). In the case of \( n \)-amino acid oxidase the presence of a prosthetic group which contains two oxidation-reduction sites per molecule suggests that a distinction between heterotropic interactions within 1 FAD molecule and between FAD molecules should eventually be made. In free FAD (or FMN), the high value of \( n \) (\( n \approx 2 \)) implies the presence of very strong interactions between the two electron-exchanging sites in a coenzyme molecule which means that the fraction of FAD (or FMN) present as the semiquinone is very small (18). This situation is probably the one which is operative in the case of the oxidase-reduction equilibrium of the old yellow enzyme, for which the shape of the titration curve resembles closely that of free FMN (\( n = 2 \) and pH independent) (19). In the case of \( n \)-amino acid oxidase at low pH, the value of \( n > 2 \) implies that, in addition to the strong stabilization within 1 FAD molecule, there must be additional site-site interactions between at least 2 FAD molecules. It is worthwhile to recall that, as found in the course of this work, the value of \( n \) for the oxidation-reduction equilibrium curve of \( n \)-amino acid oxidase is significantly dependent upon the ratio of FAD to protein, being considerably smaller if the amount of FAD per protein unit is reduced to about half. A measure of the minimum value of the free energy of interaction between the sites can be made from the data in Fig. 4, along the lines of the treatment developed by Wyman for hemoglobin (16). In the case of the oxidation-reduction equilibrium of \( n \)-amino acid oxidase at pH 6.6 an approximate value of \( \Delta F_1 \approx 1.6 \) kcal per site can be estimated. This value of course is inclusive of the contributions arising from all possible molecular processes which are linked to the oxidation reduction reaction, such as the oxidation-linked association-dissociation phenomena.

The drop in \( n \) with increase in pH is not unequivocally interpretable at this stage. It may be caused either by a change with pH of the total free energy of interaction or by a pH-dependent functional heterogeneity between the various oxidation-reduction sites. In any case the low value of \( n \) observed at alkaline pH implies stabilization of forms of the enzyme in states of partial oxidation. The pH dependence of the shape of the equilibrium curve in turn suggests that the (heterotropic) interactions between the oxidation-reduction sites and ionizable groups are different for the various oxidation-reduction intermediates. Thus, the stabilization of the intermediates might be visualized as a direct consequence of different pK changes associated with addition of electrons to the various sites. The situation is sus-
ceptible of quantitative treatment in the framework of linkage theory; however, such a treatment appears premature at this stage in view of the uncertainties about the number of interacting FAD molecules and about the distribution of the semiquinone intermediates.

The oxidation-reduction behavior of d-amino acid oxidase is, in its very general features, strikingly similar to that of hemoglobin. In the latter case as well the dependence on pH of the oxidation-reduction behavior has been rationalized on the basis of two opposing effects, i.e. homotropic interaction between the sites and functional heterogeneity which increases with pH, reflecting the different pH dependence of the oxidation-reduction reaction as between α and β chains (20). In the case of d-amino acid oxidase the occurrence of cooperative phenomena in the oxidation of 2 FAD molecules and the controlling effects of pH may be of significance for the catalytic function of the enzyme and may lead to a more efficient way of utilizing the oxidation-reduction equivalents of O2.

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