Interpretation of the pH Dependence of Flavin Reduction in the L-Amino Acid Oxidase Reaction*

David J. T. Porter and Harold J. Bright

From the Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The pH dependence of flavin reduction by phenylalanine in the L-amino acid oxidase reaction at 25°C requires the following scheme, where $E_0$ and $E_r$ are, respectively, fully oxidized and reduced enzyme and the superscripts denote relative net charge.

$$(E_0 + S)^* \xrightleftharpoons[k_{-1}]{k_1} (E_r + S)^* \xrightarrow[k_{-2}]{k_2} E_r \cdots \mathbf{P}$$

The pH profiles of the apparent biomolecular rate constants for the reaction of free $E_0$ with [2-1H]- and [2-1H]phenylalanine (evaluated as $(k_h)_{1D}$ and $(k_h)^{1S}$, respectively) from rapid kinetic measurements and as the equivalent steady state coefficients $(\phi_i^{-1})_{1D}$ and $(\phi_i^{-1})^{1S}$ show that $k_i = k_i^{-1}$, $k_2 > k_1 < k_2^*$ and that the ionization controlled by $K_i$ (i.e., $k_i^{-1}/k_i$) is at virtual equilibrium. The expression for $(k_h)_{1D}$ is, therefore,

$$(k_h)_{1D} = (\phi_i^{-1})_{1D} = k_i K_i = k_i K_i/k_i^{-1} + [H^+]$$

The apparent ionization constant $(K_{as_{app}} = k_i K_i/k_i^{-1})$ is a kinetic, rather than thermodynamic, quantity and $(k_h)_{1D}$ reduces to $k_i = 10^5 \text{ m}^{-1} \text{s}^{-1}$ when pH $\approx pK_{as_{app}}$. Furthermore, the deuterium kinetic isotope effect originates in $k_2$ ($k_2/k_2^* = 5$, $k_2 > 10^5 \text{ s}^{-1}$). Consequently, there is a 5-fold kinetic isotope effect on the apparent ionization constant $(pK_{as_{app}} = 7.9, pK_{as_{app}} = 8.6)$ and $(k_i)_{1D}/(k_i)_{1S}$ varies from a maximum value of 5 at low pH to values approaching unity at high pH. Thus, the rate-limiting step in flavin reduction, as measured by $(k_h)_{1D}$, changes at $k_2$ from low pH values (where the substrate is not sticky) to $k_1[S]$ at high pH values (where the substrate is sticky). Similar conclusions apply in the event that $(E_0 + S)^*$ cannot be formed from $(E_0 + S)^*$.

Possible assignments of the ionizations corresponding to $K_i$ and $K^*$ are discussed.

The simple flavoprotein oxidases, such as glucose oxidase and the amino acid oxidases, catalyze reactions having the stoichiometry of Equation 1, where $X = O$ or NH:

$$H \rightarrow C \xrightarrow{XH + O_2} \xrightarrow{C = X + H_2O_2} \xrightarrow{(S)} \xrightarrow{(P_1)}$$

The product $P_1$ (a lactone or imine) undergoes solvolysis after it is released from the enzyme (1). The reaction pathway for this entire class of enzymes (1), without specification of ionization states, conforms to the general scheme of Equation 2 where $E_0$ represents fully oxidized enzyme (E-FAD) and $E_r$ represents fully reduced enzyme (E-FADH$_2$ = E-FADH + H$^+$).

$$(E_0 + S)^* \xrightarrow{k_1[S]} E_r \xrightarrow{k_1} E_r \cdots \mathbf{P}$$

Unlike most schemes of this complexity, Equation 2 can be deduced by transient kinetic techniques in a step by step procedure, making use of the unique and often intense absorbance of the intermediates and of dead-end binary complexes. Consequently, both the number and sequence of elementary steps, as well as the value of their associated rate constants, are known with great precision and none of the ad hoc assumptions characteristic of steady state kinetic analyses is required. We have summarized the required kinetic protocols that have been developed in several laboratories over the last 15 years or so (1).

Investigative effort is now focused on the chemical mechanisms by which the 2 electrons from S enter the flavin nucleus via $k_2$ and leave it to reduce $O_2$ via $k_4$. The rate-determining step in flavin reduction by physiological substrates, with one exception (2), is the cleavage of the substrate C-H bond in the $E_r \cdots S$ complex. This was first demonstrated by stopped flow spectrophotometry in the glucose oxidase reaction through the occurrence of a large (10- to 15-fold) deuterium kinetic isotope effect on $k_2$ when 3-[1-1H]glucose was compared to D-[1-1H]glucose (3) and was subsequently shown also to be the case in the L-amino acid (4) and D-amino acid oxidase (2) reactions. Evidence that C-H cleavage might represent proton abstraction and the formation of a transient enzyme-bound substrate carbanion was first obtained from studies of the nonoxidative elimination of HX from 3-halogenated substrates (5, 6), although transient kinetic studies of these reactions have shown that they are far more complicated and, hence, more difficult to interpret, than their steady state behavior would suggest (7). Direct evidence for a carbanion intermediate was obtained for the oxidation of nitroethane by D-amino acid oxidase (8). A complete chemical...
mechanism, involving rate-determining formation of a covalent N-5 flavin-carbanion adduct following by three rapid rearrangements of this adduct, was established for this reaction. Evidence for a transient N-5 adduct has been obtained more recently for the lactate oxidase reaction (9).

In view of the proton abstraction hypothesis, it is important to seek evidence for the required enzyme base in flavinprotein oxidase reactions. We have initiated such studies in the case of n-amino acid oxidase and have identified tyrosine as a catalytic residue in that enzyme through a combination of active site-directed chlorination experiments and transient kinetic analysis (10, 11).

The kinetic mechanism of the L-amino acid oxidase reaction has been shown by Curti and Massey, using transient kinetic techniques, to be very similar to that of D-amino acid oxidase (12). Page and Van Etten (13-15) used substrate and solvent deuterium isotope effects to probe the pH dependence of the reduction of L-amino acid oxidase and concluded that a histidyl residue in its conjugate base state was kinetically significant in flavin reduction. The purpose of the present study is to re-examine the pH dependence of flavin reduction in the L-amino acid oxidase reaction. We show, inter alia, that the pK assigned by Page and Van Etten (13-15) to a residue in the free enzyme is a kinetic, rather than thermodynamic, quantity, and that the true pK of the group responsible for this ionization (which may be the 2-ammonium group of the free substrate rather than a residue in the free enzyme) is greater than 8.6.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Enzyme—L-amino acid oxidase (EC 1.4.3.2, L-amino acid-O2 oxidoreductase, deaminating** from the lyophilized venom of *Crotalus adamanteus* (Ross Allen Reptile Institute, Florida) was purified by a three-step procedure which utilized the first step of previous protocols (16, 17) but involved the substitution of Sephadex G-100 gel filtration for @&@ (PO4)4 fractionation (16) because we were unable to reproduce the latter step. The details of our protocol can be found elsewhere (18). The purified enzyme, obtained in 78% overall yield and stored at 5°C and pH 7.0 in a light-proof tube as a solution containing 0.2 M KCl, 0.02 M imidazole-HCl, and a drop of toluene, had specific activity (assuming coo = 1.14 x 10^9 M cm^-1 as reported in Ref. 19) and A260/A490 values which were identical to those described previously (17). Three protein bands, each staining for enzyme activity and corresponding to isoenzymes A, B, and C (17), were obtained in disc electrophoresis using 7% polyacrylamide gel.

**nit-[^2H]Phenylalanine—This was prepared by allowing pyridoxal phosphate to catalyze the exchange of the 2-hydrogen of phenylalanine with medium[^2H]O. DL-PheHalanine (6.6 g), pyridoxal 5'-phosphate (1.0 g), and KOH (4.0 g) were added to 100 ml of[^2H]O to give a pH meter reading of 13.0. After heating at 60°C for 48 h, the solution was cooled to 0°C and the pH was adjusted to 5.0 with 10.0 N HCl. Ethanol was added to the extent of 50% by volume, and after 20 min at 0°C the solution was filtered. The precipitate was washed with methanol and then suspended in a minimal volume of[^2H]O at 80°C. This was cooled to 0°C and filtered. The precipitate was dissolved in a minimal volume of[^2H]O at 25°C, after which an equal volume of ethanol was added. After storage at ~10°C overnight, the white needle-shaped crystals were collected and washed with cold ethanol. This product had a UV spectrum identical to that of nit-[2-[^2H]Phenylalanine and both it and the precursor DL-[2-[^2H]Phenylalanine gave the calculated phenylpyruvate (elab = 1.4 x 10^9 M cm^-1) stoichiometry in spectrophotometric measurements of the l-amino acid oxidase reaction with excess O2. No 2'H resonance could be detected by NMR, indicating that at least 95% exchange with[^2H]O had occurred at the 2-carbon. Yields varied between 30 and 40%.

**Other Reagents**—The commercial origins of other major reagents were as follows: [H]O (99.8 mole %). Bio-Rad; phenylpyruvate and L-phenylalanine, Calbiochem; dl-phenylalanine, Schwarz/Mann; imidazole, Baker and Eastman; anthranilic acid, Eastman; benzoic acid, Fisher; Tris and ammonium sulfate (enzyme grade), Schwarz/Mann; pyridoxal 5'-phosphate and phenazine methosulfate, Sigma; p-nitroblue tetrazolium (Na+ salt). Nutritional Biochemicals; glucose oxidase and catalase, Schwarz/Mann; glucose, Franzthiel. All other chemicals were of reagent grade.

Both samples of phenylalanine were chromatographically homogeneous, as was the phenylpyruvate. The imidazole was recrystallized from benzene in order to remove a yellow contaminant.

**Methods**

Initial steady state velocity measurements were obtained with the Clark O2 electrode and also through spectrophotometric detection of ketophenylpyruvate at 235 nm (Amax = 1.12 mM cm^-1 with respect to phenylalanine). The product (P1) actually released by the enzyme is the 2-imino acid, which converts nonenzymically to ketophenylpyruvate by a way of a 2-carbinolamine (1, 20). Measurements were done at a given substrate concentration and were routinely analyzed (21) by using kE at high substrate concentration and then utilizing the expression

\[
k_{	ext{Eo}} = \frac{k_{	ext{Eo}}}{k_{	ext{Eo}} - k_{	ext{Eo}}} \left( \frac{k_{	ext{Eo}}}{k_{	ext{Eo}}} \right)^{-1}
\]

(where kEo is the time at which the concentration of E- . P is maximal) to determine kEo, where kEo is given by Equation 5. The validity of this method was confirmed by computer fitting of data sets using the statistical method of Deming (22) and a program written by Dr. Richard Viale (Department of Pathology, University of Pennsylvania). A Gibson-Durrum apparatus was used for all stopped flow measurements.

All kinetic measurements were carried out at 25°C in solutions containing 0.2 M KCl. The steady state turnover experiments utilized 0.02 M buffer (acetate below pH 6.0, imidazole from pH 6.0 to 7.5, Tris from 7.5 to 9.0, and ethanolamine above pH 9.0) and enzyme concentrations varying between 0.5 µM at low pH to 0.05 µM at high pH. This combination of enzyme concentrations and low buffer concentrations prevented the accumulation of the 2-imino acid, as well as the tautomerization of this species and of ketophenylpyruvate, so that the initial rate of absorbance increase at 235 nm (due to ketophenylpyruvate) accurately reported the initial steady state flux of the reaction (18). Conditions for the anaerobic stopped flow experiments were identical, except that buffer and enzyme concentrations were 0.2 M and about 10^-3 N, respectively. Methods for achieving desired O2 concentrations, including anaerobiosis, have been described previously (8).

**RESULTS**

We shall first examine the stopped flow transient kinetics of the anaerobic half-reaction (Eo + S → E+ + Pi). Then we shall show how these are related to the results of steady state kinetic measurements of the enzyme in turnover. Finally, we shall briefly describe the behavior of competitive inhibitors. The interpretation of the pH dependence of flavin reduction will be presented under "Discussion."

**Stopped Flow Spectrophotometric Studies of the Anaerobic Half-reaction with Phenylalanine**—When substrate is mixed anaerobically with Eo, the scheme under consideration is the following segment of Equation 2:

\[
S \rightarrow \text{Eo} \rightarrow \text{E} \rightarrow \text{E- . P} \rightarrow \text{E+ + Pi}
\]

The transient kinetics of such a scheme can, in principle, be
monitored through any of the enzyme species. In practice, the time course of states of the enzyme absorbs at this wavelength (see Fig. 1). The analysis of such traces has been described in the stopped flow technique. Furthermore, the rise of 550 nm absorbance is sufficiently well separated from its decay to be a function of substrate concentration, whereas the decay is not, showing that the rise is associated with the first two steps of Equation 3, while the decay corresponds to the third step. The transient accumulation of E...P, obtained from the first phase of the anaerobic half-reaction (see Fig. 1) corresponding to the formation of E...P from Eo + S (Equations 3 and 5). Concentrations after mixing were 7 μM enzyme, 0.2 M KC1, and 0.1 M imidazole-HC1. Values of (k1)H and (k2)H were obtained as the reciprocals of the slopes of the lines corresponding to pH and [2-1H]phenylalanine, respectively. Inset shows resolving of ordinate intercept (corresponding to (k1)H as discussed in text) with 1-[2-1H]phenylalanine under other identical conditions.

Fig. 1. Transient kinetics of the anaerobic formation and decay of E...P (see Equation 3) monitored at 550 nm in the stopped flow spectrophotometer after mixing Eo with S at pH 6.0 and 25°C. Concentrations after mixing were 25 μM enzyme, 25 mM l-phenylalanine, 0.2 M KC1, and 0.1 M potassium citrate. The major purpose of this figure is to illustrate the uniphasic decay of E...P corresponding to k3 of Equation 3 and to demonstrate the stability of the reaction trace following the decay of E...P. For the purpose of data acquisition in the first phase and computation of (k1)H (see "Methods" and "Results") considerably shorter time scales were used. The inset shows the electronic spectra of Eo, E...P, and E. at pH 6.2 (---, 0.1 M imidazole-HC1) and pH 8.1 (——, 0.1 M Tris-HC1) in 0.2 M KC1, 25°C. The spectra of E...P were obtained from anaerobic stopped flow half-reaction data, such as those shown in the main figure, and were corrected to correspond to complete observation of E...P. Corrected spectra of E...P obtained from stopped flow measurements (1.0 s after mixing) of the enzyme in turnover were identical to those shown in the inset.

The pH Dependence of (k1)H

The pH dependence of the apparent bimolecular rate constant (k1)H that determined from the anaerobic stopped flow experiments, is shown in Fig. 3 for both [2-1H]- and [2-2H]phenylalanine.

The pH dependence of (k1)H could only be determined at low pH values. The data of the inset of Fig. 2 show why this is so. Thus, (k1)H increases from a value of 25 s⁻¹ at pH 5.0 to

\[
\tau_2 = \frac{1}{k_{\text{obs}}} = \frac{k[S] + k_{-1} + k_2 + k_{-2}}{k[S](k_2 + k_{-2}) + k_{-1}k_2}
\]  

(4)

Equation 4 predicts that unless k1 or k₂, or both, are effectively 0, the plot of τ₂ versus [S]⁻¹ will be nonlinear, having 1/(k₂ + k₃) as ordinate intercept and asymptotically approaching (k₁ + k₂ + k₃)/k₂ at low values of [S]. The experimental data, as shown in Fig. 2 for pH 6.2, clearly show no curvature at this or any other pH value. The question of whether k₁ or k₂ is effectively 0 can be decided by considering the effect of substitution of ¹H and ²H at the 2-carbon of the substrate. As shown in Fig. 2, for experiments at pH 6.2, deuterium substitution causes a large slope effect. This will only occur (Equation 4) if the kinetic isotope effect is on k₂ and if k₃, but not k₁, is effectively 0. Thus, the data of Figs. 1 and 2 (and of all other such experiments across the entire pH range investigated) conform to Equation 5:

\[
\tau_2 = \frac{1}{k_{\text{obs}}} = \frac{k[S] + k_{-1} + k_2 + k_{-2}}{k[S](k_2 + k_{-2}) + k_{-1}k_2}
\]  

(5)

The pH dependence of (k1)H, (k2)H*, (k3)H, and (k4)H* at 25°C. The values of (k1)H and (k2)H* were obtained with pH-2-[2-1H]- and pH-2-[2-2H]phenylalanine, respectively. The slopes of reciprocal plots of aerobic turnover data (see Fig. 4) of aerobic stopped flow half reaction data. The values of (k1)H and (k3)H* were obtained with pH-2-[2-1H]- and pH-2-[2-2H]phenylalanine, respectively, from the slopes of reciprocal plots of aerobic turnover data (see Fig. 4). Solutions were buffered as described under "Methods" and contained 0.2 M KC1 in all cases.
a value of about 375 s\(^{-1}\) at pH 6.2 in the case of L-[\(^{2-}\)H]phenylalanine. This corresponds almost exactly to a 10-fold increase for each pH unit so that at pH 7, for example, \(k_2\) is expected to be about 3750 s\(^{-1}\) and, hence, unresolvable by the stopped flow technique. In accord with this expectation, the value of the ordinate intercept in plots such as Fig. 2 was indistinguishable from 0 at pH 7 and above. This experimental difficulty is even greater in the case of DL-phenylalanine (in which form the [\(^2-\)H]phenylalanine is prepared) because of the limited solubility of the racemate. Consequently, no reliable estimation of \((k_5)_{\text{H}}^{*}\) for [\(^2-\)H]phenylalanine could be obtained directly from the stopped flow data. However, from the arguments used to derive Equation 5 from Equation 4, there can be no doubt that the deuterium kinetic isotope effect, with a value of about 5-fold, originates in \(k_2\). Moreover, as we shall show, this value for the kinetic isotope effect on \(k_2\) can be deduced in several ways.

The slow relaxation associated with the decay of the 550 nm absorbance in Fig. 1, and corresponding to the conversion of \(E_{\text{R}}\cdot P\) to \(E_{\text{R}} + P\), is simply \(\tau_{\text{R}}^{-1} = k_6\) because the data can be plotted as a single, substrate-independent, first order process at all pH values. The imino acid (\(P\)), therefore, never accumulates to a steady state level which is comparable to \(k_3/k_5\), in these anaerobic half-reaction experiments and \(k_2[\text{P}]\) may be neglected. However, \(k_2[\text{P}]\) cannot be neglected in stopped flow turnover experiments using similar enzyme concentrations (24). The value of \((k_3)_{\text{H}}^{*}\) rises from 1 s\(^{-1}\) at pH 5.5 to a plateau value of 10 s\(^{-1}\) between pH 7 and 9 (with an inflexion point at pH 6.5) and then increases linearly above pH 9 to a value of 30 s\(^{-1}\) at pH 10.5.

**O\(_2\)-monitored Steady State Turnover**—The initial steady state rate equation corresponding to the scheme of Equation 2 is given by Equation 6:

\[
E_T \text{ (sec)} = \frac{1}{v} = \frac{k_{-1} + k_1}{k_3} + \frac{k_2 [O_2]}{k_3} + \frac{k_4 [O_2]}{k_3} + \frac{k_6}{k_3} + \frac{k_6}{k_3}
\]

(6)

Since initial velocities are used, \(k_4[\text{P}]\) and \(k_6[\text{P}]\) are neglected. Furthermore, \(k_2 > k_{-2}\) as has been shown already. When the reaction flux occurs predominantly through the lower loop of Equation 2 (\(k_6\) is large) \(k_3/k_5\) can be neglected.

When reaction flux occurs largely through the upper loop of Equation 2 (\(k_5\) is large) \(k_4\) can be neglected. This situation is illustrated in Fig. 2, that substrate inhibition during aerobic turnover does not vitiate the analysis given here. A second feature of the turnover data, having also to do with the occurrence of a branch point in the pathway (Equation 2), is the fact that plots of \(E_{\text{R}}/v\) with \(O_2\) as variable substrate are bimodal such that only at very high \([O_2]\) (achieved in a pressure cell) can slopes and intercepts be interpreted in terms of \(k_2, k_4\) and \(k_5\) of Equation 7 (upper loop of Equation 2). Correspondingly, \(k_2, k_4\) and \(k_5\) of Equation 8 (lower loop of Equation 2) are approximated only at low \([O_2]\), while at intermediate \([O_2]\) weighted averages of \(k_2, k_4\) and \(k_5\) are obtained. But here should the detailed theoretical analysis be too detailed for the text.

**The pH Dependence of L-Amino Acid Oxidase**

A plot of \((\phi_2')_{\text{H}}/\phi_2'\) versus pH shown in Fig. 5. Two additional features of the steady state turnover data (Fig. 4) deserve mention. First, the inhibition observed at high substrate concentrations in Fig. 4 is a well known phenomenon, the origin of which is now understood (18, 19, 25) to be due to the steady state accumulation of \(E_{\text{R}}\cdot \cdot \cdot \cdot \cdot S\) which reacts much more slowly with \(O_2\) than does \(E_{\text{R}}\cdot \cdot \cdot P\) or \(E_{\text{R}}\) in Equation 2. The most important reason, among several, for the onset of inhibition at high rather than low pH values (compare the data sets of Fig. 4) is the pH dependence of \(k_2\), described in the previous section. In any case, it can be formally shown, as well as appreciated from the correspondence of \(k_2\) and \(\phi_2'\) in Fig. 3, that substrate inhibition during aerobic turnover does not vitiate the analysis given here. A second feature of the turnover data, having also to do with the occurrence of a branch point in the pathway (Equation 2), is the fact that plots of \(E_{\text{R}}/v\) with \(O_2\) as variable substrate are bimodal such that only at very high \([O_2]\) (achieved in a pressure cell) can slopes and intercepts be interpreted in terms of \(k_2\) and \(\phi_2'\) of Equation 7 (lower loop of Equation 2). Correspondingly, \(k_2\) and \(\phi_2'\) of Equation 8 (upper loop of Equation 2) are approximated only at low \([O_2]\), while at intermediate \([O_2]\) weighted averages of \(k_2\) and \(\phi_2'\) are obtained. But here should the detailed theoretical analysis be too detailed for the text.
The pH Dependence of l-Amino Acid Oxidase

The steady state velocity data obtained on the O2 electrode with five concentrations of DL-phenylalanine and inhibitor. Simple competitive patterns were obtained in all cases. Reaction conditions were 0.2 M KCl, 0.02 M buffer (see "Methods") and 0.24 mM O2 at 25°C.

Table 1

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<th>φ-DH</th>
<th>φ-O2</th>
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<th>φ-CH2CO2</th>
<th>φ-CH2CO2(1)</th>
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<td>5.0</td>
<td>200</td>
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</table>

K values (mM) for substrate-competitive inhibitors

The K values were computed from double reciprocal plots of initial steady state velocity data obtained on the O2 electrode with five concentrations of O2-phenylalanine and inhibitor. Simple competitive patterns were obtained in all cases. Reaction conditions were 0.2 M KCl, 0.02 M buffer (see "Methods") and 0.24 mM O2 at 25°C.

Again, as is clear from Equations 7 and 8, kinetic analysis of the sequence E0 → E1 → P, which is common to the two loops, is uncomplicated by the existence of a branch point.

The pH Dependence of Substrate-competitive Inhibition—

The K values of six compounds structurally related to phenylalanine were measured in the conventional fashion with phenylalanine as variable substrate in O2-monitored turnover experiments. These values are given in Table 1.

DISCUSSION

We shall show that, taken together, our kinetic data conform to the following model (Equation 9), where superscripts denote relative net charge and the dashed arrows represent equilibrium only for the ionization controlled by k1.

The data from the anaerobic stopped flow experiments (Equation 5 and Fig. 3) conform to Equation 10:

\[
\frac{1}{(k_{\text{obs}})_n} = \frac{1}{(k_0)_n} + \frac{1}{(k_0)_n[S]}
\]

The pH dependence of the bimolecular term, assuming virtual equilibrium only for the ionization controlled by K_a and setting k_1/k_h = K_1, is given by Equation 11:

\[
(k_{\text{obs}})_n = \frac{(k_0 + k_1)(k_0 + k_1')K_a + (k_1 + k_1')K_1}{k_1'}\]

Equation 11 does not generate the curves of Fig. 3 unless k_1 = k_1' and k_1'/k_1 > k_1' (26). The latter condition is tantamount to setting (E_0' - S)' and (E_0' - S)'1 at virtual equilibrium via K_a. Thus, using these conditions and setting k_1'/k_1' = K_1', we obtain Equation 12:

\[
(k_{\text{obs}})_n = \frac{k_0K_a}{K_1'} \]
The pH Dependence of L-Amino Acid Oxidase

Fig. 6. Graphical construction showing that pkaw and pk*a correspond to the pH values at which (kS)H and (kS)H*, respectively, equal k'. See text for arguments leading to this construction.

The good agreement, therefore, between the values of pkaw and pk*a determined from Fig. 6 and those obtained from Fig. 3 demonstrates the validity of the arguments leading to Equation 13. Fig. 6 suggests that pk* is no less than 9.0 and that k2 is at least 2.5 × 105 s⁻¹. The fact that pkaw ≥ 6.6 follows from the value of pk*a in Fig. 3 and also from the observation that (kS)H* = 0.63 (kS)H at the highest pH used in Fig. 5.

Using the values of pkaw app and k2/k1 determined independently from Fig. 6 and Fig. 2, respectively, and expressing Equation 13 as Equation 18, the pH dependence of (kS)H/(kS)H* can be predicted as is shown by the curve in Fig. 5.

\[
\frac{(kS)H}{(kS)H*} = \frac{k2 k2'/h + k[H^*]/k1' + k[H]/k1'}{k2 k2'/h + k[H^*]/k1' + k[H]/k1'} \tag{18}
\]

The pH at the midpoint of the curve corresponds to pkaw app, while a plot of the inverse of Equation 18 suggests (kS)H/(kS)H* can be predicted as shown by the curve in Fig. 5.

It should be noted that it is the existence of (Eo + S)* and (Eo - S)*, together with its rapid protonic equilibrium with (Eo + S)H and (Eo - S)H, which are the crucial features of the scheme of Equation 9 in explaining the kinetic isotope effect data. Thus, at high pH values the substrate is sticky because the fraction of the Michaelis complex which is unprotonated is near unity and flux forward, k2(Eo + S)*, will greatly exceed the substrate dissociation rate, k2(Eo - S)*, because k2 > k1. At low [S], under which condition (kS)H and (kS)H* are measured, k2 > k[S] + k[S] becomes the rate-determining step in flavin reduction, and no kinetic isotope effect on (kS)H is observed. However, at low pH values the substrate is no longer sticky because free Eo and free S can rapidly equilibrate with (Eo + S)* through (Eo - S)* via K1 and K*. Flux forward, k2 (Eo - S)*, is now small because the fraction of unprotonated Michaelis complex is small, whereas the dissociation rate, k2(Eo - S)*, is large. Since the system is at virtual equilibrium, the kinetic isotope effect is fully expressed in (kS)H = k2K*/K[H*]. Other variants of the scheme of Equation 9 which predict loss of activity at low pH values (such as those in which (Eo - S)* either cannot be formed from (Eo + S)* and (Eo - S)* or is formed only as a dead-end complex which is unable to interconvert with (Eo - S)*) do not permit the stickiness of the substrate to vary with pH and are, therefore, ruled out.

The requirement that k2' > k2 is for the derivation of Equations 13 and 14 is of some concern in view of the fact that k2 = 10^2 s⁻¹. In the absence of buffer catalysis, the conversion of (Eo - S)* to (Eo - S)* will proceed by parallel pathways involving OH⁻ and H2O as proton acceptors, with the water pathway predominant at low pH values. The symmetry observed when k2' > k2 is that the experimental curve falls more than 0.3 log unit below the point of intersection of the asymptotes in Fig. 3 (26). However, the breakdown is limited to this region and is not particularly severe even when k2' = 9 k2. The localization of this problem to pH values in the region of pkaw app is due to the fact that the system can easily equilibrate at lower pH values via K1 and K* (because (kS)H is small), while at higher pH values little reaction flux occurs through (Eo - S)*. Using an approximate expression (27) to compute the bimolecular rate constant for proton transfer between a base and a conjugate acid (k = 10^10/(1 + 10^−pK)), and assuming that pKaw < 14, we compute a first order rate constant of 10^6 s⁻¹ at pH 8 for deprotonation of (Eo - S)* by OH⁻ (and a much smaller rate constant for H2O). This might be as large as 5 × 10^5 s⁻¹ if the conjugate acid species in (Eo - S)* is cationic. In view of these numbers, and because of the relative insensitivity of the data to violation of the condition k2' > k2, we conclude that the analysis is physically valid. Furthermore, buffer catalysis could raise the effective value of k2'.

An interesting variant of the scheme of Equation 9 is that in which (Eo + S)* and (Eo - S)* interconvert slowly or, if K1 = ∞, not at all. The (kS)H profile for the case is approximately bell-shaped with ionization constants K1 app = k2(k2' + k2')/(k2' + k2) and K* (assuming rapid equilibration only for the ionization controlled by K*). To be compatible with the experimental results, K1 app and K* must be assigned to the acidic and basic limbs, respectively, of the (kS)H profile. The low pH asymptote is log k2K*/K[H*] while the high pH asymptote is log k2(k2' + k2')/k2'K* of the ionization controlled by K*. In the plateau region, (kS)H will approach (depending on the values of pkaw app and pk*a) k2K*/K'H* or k2'K*/K'H*. Furthermore, (kS)H will exhibit the required pH dependence with apparent ionization constant (k2 + k2')/k2'. Thus, in the acidic and plateau regions of the (kS)H profile, the scheme responds to 2-deuteration of the substrate in precisely the same way as does the full scheme of Equation 9 since the kinetic isotope effect originating in k2 is fully expressed in (kS)H when [H*] > K2* app and in pkaw app itself, while in the plateau region the kinetic isotope effect on (kS)H will tend to unity. Unfortunately, we were unable to extend the kinetic measurements to pH values greater than 9.4 because of the instability of Eo. Consequently, we cannot be certain if, and with what pH dependence, falls at pH values greater than 9.4. This mechanistic variant of Equation 9, therefore, cannot be ruled out. Furthermore, as noted, the experimentally observed ionization constants in the (kS)H and (kS)H* profiles (Fig. 3) will be kinetic, rather than thermodynamic, quantities.

Although a great deal more kinetic and chemical effort is required before definitive pK assignments can be made for this enzyme, the following represent two of the more obvious possibilities.

1. K1 and K* represent the ionizations of the 2-ammonium group in free and enzyme-bound phenylalanine, respectively. The value of pkaw would be 9.2 (28), giving k2/k1 = 30 and k2*/k1 = 6. Since pkaw ≥ 9 for the full scheme of Equation 9, any significant perturbation of the ionizations of the 2-ammonium group in the (Eo - S)* complex would result in an increase in the basicity of this group (in which case k2' > k2).
2. $K_a$ represents the ionization of the 2-ammonium group of the free substrate ($pK_a = 9.2$) as in Case 1, while $K_s$ represents the ionization of an enzymic general base in the Michaelis complex. This assignment requires compensatory perturbations so that substrate binding acidifies the enzyme base and increases the basicity of the substrate 2-ammonium group. This sequence of ionizations would also fit the variant of the scheme of Equation 9 in which $(Eo + S)$ is not formed from $(Eo + S)^{2-}$. The enzyme, according to this view, would be capable of binding only the form of the substrate in which the 2-amino group is protonated. We have found this to be the case in the d-amino acid oxidase reaction.

The fact that the $K_i$ values for a variety of competitive inhibitors (Table I) are pH-independent in the range tested is consistent with the conclusion that $pK_a$ is a kinetic, rather than thermodynamic, constant, and that both $pK_s$ and $pK_i$ are probably greater than 9.0. However, it should be noted that there must be a perturbation of an enzyme ionization by a competitive inhibitor in order that it be detected by this technique.

Page and Van Etten, using [2-1H]- and [2-1H]leucine, were the first to observe a pH-dependent kinetic isotope effect in the L-amino acid oxidase reaction (13-15). They did not carry out systematic transient and steady state kinetic analyses but relied for the most part on measurements of $k_{obs}$ (a bimolecular parameter intended to be equivalent to the coefficient $1/2$ and obtained from pseudo-first order plots of $O_2$ consumption at low leucine concentrations) and of the $K_s$ for leucine (obtained at a single O2 concentration). Since these parameters are likely to be complicated and variably weighted functions of several steady state coefficients (as well as $O_2$ in the case of the $K_s$ values), it is difficult to compare our results in any detail. Experimentally, the deuterium kinetic isotope effect on $k_{obs}$ was shown to be pH-dependent in much the same way as we have shown here for (1/2) (and $k_{ij}$) (Fig. 5) and it is, therefore, likely that leucine, like phenylalanine, is a sticky substrate at high pH values and nonsticky at low pH values. However, the mechanism suggested by Page and Van Etten (13-15) is inconsistent with their (and our) findings because it predicts that $k_{obs}$ would be pH-independent and would not show a pH-dependent kinetic isotope effect. Moreover, their suggestion of histidyl as an active site general base did not take into account the fact that the ionization seen in the $k_{obs}$ profile is almost certainly a kineic, rather than thermodynamic, quantity. We should also note that the biphasic decay of $E_2$ in the anaerobic stopped flow experiments observed by Page and Van Etten (13-15) and attributed by them to slow deprotonation of the Michaelis complex was not observed in our experiments with phenylalanine (see Fig. 1) or in the studies of Curti and Massey (19). It should also be noted that the latter authors obtained intersecting plots of their steady state data, whereas we obtained parallel line patterns (Fig. 4). We have shown (29) in the case of d-amino acid oxidase that, for reactions where flux occurs preponderantly through the lower loop of the scheme of Equation 2 (which, formally, are sequential rather than ping-pong mechanisms) the degree of convergence of steady state data is controlled by the relative magnitude of $k_2$ in the scheme of Equation 2. The experiments of Curti and Massey (19) were carried out at 1°C, while ours were conducted at 25°C. It is, therefore, possible that the reversibility of the reductive half-reaction, through a relative increase in $k_2$, is greater at the lower temperature.

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