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

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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.

$$
(E_0 + S)^* \xrightarrow{k_1} (E_r \cdot S)^* \xrightarrow{k_2} E_r \cdot P
$$

The pH profiles of the apparent biomolecular rate constants for the reaction of free $E_0$ with [2-$^3$H]- and [2-$^3$H]phenylalanine (evaluated as $(k_h)_n$ and $(k_h)_n^*$, respectively, from rapid kinetic measurements and as the equivalent steady state coefficients $(\phi_1^{-1})_h$ and $(\phi_1^{-1})_h^*$) show that $k_1 = k_1^*$, $k_2 > k_2^*$ and that the ionization controlled by $K'_{a} = (k_1/k_1^*)$ is at virtual equilibrium. The expression for $(k_h)_n$ is, therefore,

$$(k_h)_n = (\phi_1^{-1})_h = \frac{k_2K_a/K_1}{k_2K_a/K_1 + [H^+]}
$$

The apparent ionization constant $(K_{a,app} = k_2K_a/k_1)$ is a kinetic, rather than thermodynamic, quantity and $(k_h)_n$ reduces to $k_1 = 10^5$ m$^{-1}$ s$^{-1}$ when pH $\gg pK_{a,app}$. Furthermore, the deuterium kinetic isotope effect originates in $k_2$ ($k_2/k_2^* = 5$, $k_2 > 10^5$ s$^{-1}$). Consequently, there is a 5-fold isotope effect on the apparent ionization constant $(pK_{a,app} = 7.9$, $pK_{a,app}^* = 8.6$) and $(k_h)_n/(k_h)_n^*$ 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 $(k_h)_n$, changes from $k_2$ at 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 $(E_0 + S)^n$. Possible assignments of the ionizations corresponding to $K_a$ and $K_a'$ 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:

$$
H-C-XH + O_2 \rightarrow C = X + H_2O_2
$$

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 ($E-FADH_2$) and $E_r$ represents fully reduced enzyme ($E-FADH_2 = E-FADH + H^+$).

$$
E_0 \xrightarrow{k_3} E_{-}[P_1] \xrightarrow{k_4} E_{-} \cdot P
$$

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 focussed 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 $k_1$ and $k_4$. 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 $E_{-} \cdot S$ 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-$^2$H]glucose was compared to 3-[1-$^3$H]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 followed 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:On oxi-
ducatease, deaminating) from the lyophilized venom of Crotaulus adamantans (Ross Allen Reptile Institute, Florida) was purified by a three-step procedure which utilized the first step of previous pro-
tocols (16, 17) but involved the substitution of Sephadex G-100 gel filtration for Sephadex G-100 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 KC1, 0.02 M imidazole-HCI, and a drop of toluene, had specific activity (assuming free substrate rather than a residue in the free enzyme) is 1.0 mmol of substrate oxidized per minute of enzyme at a given substrate concentration and were routinely analyzed (21) by measuring k, directly at high substrate concentration and then utilizing the expression

\[
\text{Eo} = \text{Eo} - \text{P} + \text{E} + \text{P}_i
\]

at a given substrate concentration and were routinely analyzed (21) by measuring k, directly at high substrate concentration and then utilizing the expression

\[
k_{\text{cat}} = \frac{k_{\text{cat}}}{k_{\text{cat}} - k_i} \ln \frac{[\text{Eo}]}{[\text{Eo}] + [\text{P}]] (\text{where } \text{Eo} \text{ is the time at which the concentration of } \text{E} \cdot \text{P} \text{ is maximal) to determine } k_{\text{cat}}, \text{where } \text{Eo} \text{ 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 Pennsyl-
vania). A Gibson-Durrum apparatus was used for all stopped flow measurements.

All kinetic measurements were carried out at 25°C in solutions containing 0.3 M KC1. 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 and 10.0 nM. Methods for achieving desired pH concentrations, including anaerobiosis, have been described previ-
ously (8).

RESULTS

We shall first examine the stopped flow transient kinetics of the anaerobic half-reaction (Eo + S → E + P). 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:

\[
Eo + S \underset{k_{-2}}{\overset{k_{-1}}{\longrightarrow}} E + P \quad \text{(3)}
\]

The transient kinetics of such a scheme can, in principle, be
monitored through any of the four enzyme species. In practice with L-amino acid oxidase, however, it is easiest to follow the time course of the enzyme absorbs at this wavelength (see Fig. 1). The analysis of such traces has been described in the main figure, and were corrected to correspond to complete turnover were identical to those shown in the inset.

Equation 3 predicts three relaxation times, whereas only two are observed in Fig. 1. This result is expected if \( \tau_1 \) will not be resolved by the stopped flow technique. Furthermore, the rise of 550 nm absorbance is sufficiently well separated from its decay to be considered to be kinetically uncoupled from the latter. The fact that the rise time is a function of substrate concentration, whereas the decay is not, shows 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 \( E_{\cdot \cdot \cdot \cdot} \) is described by the following expression (Equation 4):

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

Equation 4 predicts that unless \( k_{-1} \) or \( k_{-2} \), or both, are effectively 0, the plot of \( \tau_2 \) versus \([S]\)^{-1} will be nonlinear, having 1/\((k_2 + k_{-2})\) as ordinate intercept and asymptotically approaching \((k_{-1} + k_2 + k_{-2})/k_{-2}\) 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 \( k_{-1} \) or \( k_{-2} \) is effectively 0 can be decided by considering the effect of substitution of \(^2\)H and \(^3\)H 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 \( k_2 \) and if \( k_{-2} \), but not \( k_{-1} \), 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:

\[
\tau_3 = \frac{1}{k_{obs}} = \frac{k[S] + k_{-1} + k_2}{k_2 + \frac{1}{k[S]}}
\]
a value of about 375 s⁻¹ at pH 6.2 in the case of L-[2-³H]-
phenylalanine as variable substrate

![Figure 4](http://www.jbc.org/)

**Figure 4.** Reciprocal plots of initial velocities of aerobic turnover monitored by the O₂ electrode at pH 8.1 (●) and pH 6.15 (○) using 0.02 M Tris-HCl and 0.02 M imidazole-HCl, respectively, at 25°C. Other concentrations were 0.05 mM (pH 8.1) and 0.3 mM (pH 6.15) enzyme and 0.2 M KCl.

![Figure 5](http://www.jbc.org/)

**Figure 5.** The pH dependence of the deuterium kinetic isotope effect on (φH⁻¹)H and (kH). The data were taken from Fig. 3 while the curve was generated from Equation 18 as explained in the text.
The pH Dependence of l-Amino Acid Oxidase

Table 1

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<th>φ-SPH</th>
<th>φ-PO4</th>
<th>φ-PH2</th>
<th>φ-CH2CO2H</th>
<th>φ-CH2CO2E</th>
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</table>

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 a complete inventory of the ionizable residues directly involved in flavin reduction:

\[
\begin{align*}
(E_0 + S)^0 & \xrightleftharpoons{K_a} (E_0 + S)^+ \xrightarrow{k_1} (E_0 + S)^{+}\text{P} \\
K_a [H'] & \xrightarrow{k_1} k'_{1}^{*} [H'] \\
(E_0 + S)^{+} & \xrightarrow{k_1^{*}} (E_0 + S)^{+}
\end{align*}
\]

(9)

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

\[
\frac{1}{(k_{obs})_H} = \frac{1}{(k_0)_H} + \frac{1}{(k_1)_H[S]}
\]

(10)

The pH dependence of the bimolecular term, assuming virtual equilibrium only for the ionization controlled by K_a and setting k_{-1}/k_1 = K_1 is given by Equation 11:

\[
(k_1)_H = \frac{(k_{-1} + k_1)(k_{-1} + k_1 + k_1^{*})K_a}{k_1^{*}K_a} [H'] + \frac{(k_{-1} + k_1 + k_1^{*})K_a}{k_1^{*}K_a} [H']
\]

(11)

Equation 11 does not generate the curves of Fig. 3 unless k_1 = k_1^{*} and k_1^{*} > k_1. (26) The latter condition is tantamount to setting (E_0 + S)^0 and (E_0 + S)^+ at virtual equilibrium via K_a. Thus, using these conditions and setting k_{-1}/k_1 = K_1, we obtain Equation 12:

\[
(k_1)_H = \frac{k_1K_a}{K_1} [H'] + \frac{k_1^{*}K_a}{K_1} [H']
\]

(12)

However, the amplitudes of the (k_1)_H and (k_1^{*})_H profiles are experimentally identical, or nearly so, because their ratio approaches unity at high pH values (see Fig. 5). Since the kinetic isotope effect originating in k_1, this will occur only if k_1 > k_1^{*} > k_2. The desired relationship is, therefore, Equation 13, having asymptotes given by Equations 14 and 15:

\[
(k_1)_H = \frac{k_2K_a}{K_1} \left(1 - \frac{[H']}{K_a} \right) \frac{k_1K_a}{K_1} \left(1 + \frac{[H']}{K_a} \right)
\]

(13)

\[
\lim_{[H'] \to 0} \log (k_1)_H = \log k_2 - \log k_1
\]

(14)

\[
\lim_{[H'] \to 0} \log (k_1)_H = \log k_2 - \log k_1
\]

(15)

Equations 13 to 15 explain three major findings. First, when (k_1)_H and (k_1^{*})_H become pH-independent at high pH values (Fig. 3), the rate of flavin reduction at low [S] is limited by k_2[S], the rate of combination of S with E_0. Since k_2 is not pH-dependent, the amplitudes of the k_1[H'] and k_1^{*}[H'] profiles are kinetic, rather than thermodynamic, quantities corresponding to pK_a. The latter condition is tantamount to the two loops, which agree well with the data of Fig. 2. The behavior of (k_1)_H and (k_1^{*})_H, therefore, reflects a change in rate-limiting step within k_2 and k_2 at high pH values to k_2[S] at high pH values.

The pH dependence of (k_2)_H can now be expressed as Equation 16:

\[
(k_2)_H = \frac{k_2K_a}{K_0 + [H']}
\]

(16)

We were able to resolve (k_2)_H only at pH 6.2 and below (see inset of Fig. 2) because, as shall show, k_2 probably exceeds 10^6 s^{-1}. Consequently, (k_1)_H varied experimentally as k_2[K_a]/[H'] and k_2 and K_0 could not be evaluated independently. However, lower limits can be placed on these constants.

A useful and independent method for visualizing the change in rate-limiting step within (k_1)_H is given in Fig. 6, for which we require values of k_{-1}/k_1 = K_1 (= k_1^{*}), (k_1)_H and (k_1^{*})_H. First, the observed rate of dissociation of the Michaelis complex in Equation 9 is given by Equation 17:

\[
(k_{obs})_H = k_1^{*} \left(\frac{K_0 + [H']}{{K_a + [H']}}\right)
\]

(17)

This shows that, when K_a << [H'] >> K_0, the Michaelis complex dissociates predominantly through k_1^{*} [E_0 + S]^+ [H']. It can be readily shown that the abscissa intercept in the reciprocal plot of the anaerobic half-reaction data when [H'] >> K_0, such as is shown in the inset of Fig. 2, corresponds to 1/K_1. Since K_1 = 0.1 M (from experiments at both pH 5.0 and 6.2) and k_1^{*} = 10^6 M^{-1} s^{-1} from arguments given previously, we compute k_{-1}/k_1 > 10^5 s^{-1}. Second, values of (k_1)_H corresponding to k_2K_a/[H'] are also available for pH 5.0 and 6.2 and we can set (k_1)_H/[(k_1)_H] = 5 from the data of Fig. 2. In the plot of Fig. 6 the values of pK_a = 7.7 and pK_a = 8.4 are determined as the pH values at which (k_1)_H and (k_1^{*})_H, respectively, intercept k_1^{*} (i.e. k_1^{*} = k_2K_a/[H'] and k_1^{*} = k_2K_a/[H']), and from Equation 13, pK_a = 5K_0/k_1^{*} and pK_a = 5K_0/k_1^{*}. The construction of Fig. 6 is largely independent of the arguments used to generate Equation 13.
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Fig. 6. Graphical construction showing that $pK_{s\text{app}}$ and $pK^*_s\text{app}$ correspond to the pH values at which $(k_3)_{\text{H}}$ and $(k_3)_{\text{H}}^*$, respectively, equal $k'-1$. See text for arguments leading to this construction.

The good agreement, therefore, between the values of $pK_{s\text{app}}$ and $pK^*_s\text{app}$ determined from Fig. 6 and those obtained from Fig. 3 demonstrates the validity of the arguments leading to Equation 13. Fig. 6 suggests that $pK^*_s$ is no less than 9.0 and that $k_2$ is at least $2.5 \times 10^5$ s$^{-1}$. The fact that $pK_{s\text{app}} \approx 9.6$ follows from the value of $pK^*_s\text{app}$ in Fig. 3 and also from the observation that $(k_3)_{\text{H}}^* = 0.63 (k_3)_{\text{H}}$ at the highest pH used in Fig. 5.

Using the values of $pK_{s\text{app}}$ and $k_2/k_3^*$ determined independently from Fig. 6 and Fig. 2, respectively, and expressing Equation 13 as Equation 18, the pH dependence of $(k_3)_{\text{H}}/(k_3)_{\text{H}}^*$ can be predicted as is shown by the curve in Fig. 5.

$$\frac{(k_3)_{\text{H}}}{(k_3)_{\text{H}}^*} = \frac{(k_3)_{\text{H}}/(k_1 + k_3^* \text{H}^+)}{(k_3^* \text{H}^+)/(k_3^* \text{H}^+ + k_3 \text{H}^+ \text{H}^+)/k_3^*} = \frac{(k_3\text{K}_a/k_1 + k_3^* \text{H}^+)/k_3^*}{(k_3\text{K}_a/k_1 + k_3 \text{H}^+ \text{H}^+)/k_3^*}$$

(18)

The pH at the midpoint of the curve corresponds to $pK_{s\text{app}}$ while a plot of the inverse of Equation 18 (not shown) will yield $pK^*_s\text{app}$ as the midpoint pH.

It should be noted that it is the existence of $(E_o - S)^+$, together with its rapid protonic equilibration with $(E_o - S)^-$, 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, $k_1(E_o - S)^+$, will greatly exceed the substrate dissociation rate, $k_1(E_o - S)^-$, because $k_2 > k_1$. At low [S], under which condition $(k_3)_{\text{H}}$ and $(k_3)_{\text{H}}^*$ are measured, $k_2 > k_1[S]$. $k_1[S]$ becomes the rate-determining step in flavin reduction, and no kinetic isotope effect on $(k_3)_{\text{H}}$ is observed. However, at low pH values the substrate is no longer sticky because free $E_o$ and free S can readily equilibrate with $(E_o - S)^+$ through $(E_o - S)^-^+$ via $K_1$ and $K_2$. Flux forward, $k_2 (E_o - S)^-$, is now small because the fraction of unprotonated Michaelis complex is small, whereas the dissociation rate, $k_1^*(E_o - S)^-$, is large. Since the system is at virtual equilibrium, the kinetic isotope effect is fully expressed in $(k_3)_{\text{H}} = k_3^* \text{H}^+/ K_1^*[\text{H}^+].$ Other variants of the scheme of Equation 9 which predict loss of activity at low pH values (such as those in which $(E_o - S)^-^+$ either cannot be formed from $(E_o + S)^-^+$ and $(E_o - S)^-$ or is formed only as a dead-end complex which is unable to interconvert with $(E_o - S)^-$) do not permit the stickiness of the substrate to vary with pH and are, therefore, ruled out.

The requirement that $k_2 > k_1$ for the derivation of Equations 13 and 14 is of some concern in view of the fact that $k_1 = 10^4$ s$^{-1}$. In the absence of buffer catalysis, the conversion of $(E_o - S)^+$ to $(E_o - S)^-$ will proceed by parallel pathways involving $OH^-$ and $H_2O$ as proton acceptors, with the water pathway predominant at low pH values. The symptoms observed when $k_1 > k_2$ 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 $k_1 > 3 k_2$. The localization of this problem to pH values in the region of $pK_{s\text{app}}$ is due to the fact that the system can easily equilibrate at lower pH values via $K_1$ and $K_2$ (because $(k_2)_{\text{H}}$ is small), while at higher pH values little reaction flux occurs through $(E_o - S)^-^+$. Using an approximate expression (27) to compute the bimolecular rate constant for proton transfer between a base and a conjugate acid ($k = 10^{14}/(1 + 10^{10-pH})$ m$^{-1}$ s$^{-1}$), and assuming that $pK_{s\text{app}} < 4$, we compute a first order rate constant of $10^8$ s$^{-1}$ at pH 8 for deprotonation of $(E_o - S)^-$ by $OH^-$ (and a much smaller rate constant for $H_2O$). This might be as large as $5 \times 10^2$ s$^{-1}$ if the conjugate acid species in $(E_o - S)^-$ is cationic. In view of these numbers, and because of the relative insensitivity of the data to violation of the condition $k_2 > k_1$, we conclude that the analysis is physically valid. Furthermore, buffer catalysis could raise the effective value of $k_1$.

An interesting variant of the scheme of Equation 9 is that in which $(E_o + S)^0$ and $(E_o - S)^0$ interconvert slowly or, if $K_1 = \infty$, not at all. $(k_3)_{\text{H}}$ profile for the case is approximately bell-shaped with ionization constants $K_{s\text{app}} = k_2(k_2^* + k_2) \text{H}^+/(k_2^* + k_2)K_1$ and $K_2$ (assuming rapid equilibration only for the ionization controlled by $K_1$). To be compatible with the experimental results, $K_{s\text{app}}$ and $K_2$ must be assigned to the acidic and basic limbs, respectively, of the $(k_3)_{\text{H}}$ profile. The low pH asymptote is log $k/k_2\text{d} + K_1^*[\text{H}^+]$ while the high pH asymptote is log $k_2(k_2^* + k_2) \text{H}^+ K_2/(k_2^* + k_2)K_1$. In the plateau region, $(k_3)_{\text{H}}$ will approach (depending on the values of $pK_{s\text{app}}$ and $pK_2$) $k_2^* \text{H}^+/(k_2^* + k_2)$. Furthermore, $(k_3)_{\text{H}}$ will exhibit the required pH dependence with apparent ionization constant $(k_2 + k_2')/k_2'$. This, in the acidic and plateau regions of the $(k_3)_{\text{H}}$ profile, this scheme responds to 2-deuteriation of the substrate in precisely the same way as does the full scheme of Equation 9 since the kinetic isotope effect originating in $k_3$ is fully expressed in $(k_3)_{\text{H}}$ when $[\text{H}^+] > K_{s\text{app}}$ and in $pK_{s\text{app}}$, itself, while in the plateau region the kinetic isotope effect on $(k_3)_{\text{H}}$ 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 $E_o$. 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 $(k_3)_{\text{H}}$ and $(k_3)_{\text{H}}^*$ 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. $K_1$, and $K_2$ represent the ionizations of the 2-ammonium group in free and enzyme-bound phenylalanine, respectively. The value of $pK_2$ would be $9.2$ (28), giving $k_2/k_3 = 30$ and $k_2/k_3 = 6$. Since $pK_{s\text{app}} \geq 9$ for the full scheme of Equation 9, any significant perturbation of the ionizations of the 2-ammonium group in the $(E_o - S)^-^+$ complex would result in an increase in the basicity of this group (in which case $k_1 > k_2$).
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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 $(E_0 + S)$ is not formed from $(E_0 + S)_0$. 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.\footnote{D. Purdy, N. G. Rudie, D. J. T. Porter, and H. J. Bright, manuscript in preparation.}

The fact that the $K_i$ values for a variety of competitive inhibitors (Table 1) are pH-independent in the range tested is consistent with the conclusion that $pK_{a, app}$ is a kinetic, rather than thermodynamic, constant, and that both $pK_a$ and $pK'_a$ 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-'H]- and [2-'H]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 $\phi_1$ and obtained from pseudo-first order plots of $O_2$ consumption at low leucine concentrations) and of the $K_a$ for leucine (obtained at a single O$_2$ 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 $K_a$ values), it is difficult to compare our results in any detail. Experimentally, the deuteron kinetic isotope effect on $k_{obs}$ was shown to be pH-dependent in much the same way as we have shown here for $(\phi_1^{-1})_2$ and $(k_{II})_2$ (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 kineric, rather than thermodynamic, quantity. We should also note that the biphase decay of $E_0 \cdots P$ 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 predomi-

REFERENCES


Interpretation of the pH dependence of flavin reduction in the L-amino acid oxidase reaction.
D J Porter and H J Bright