Mechanistic Features of the \(\alpha\)-Amino Acid Oxidase Reaction Studied by Double Stopped Flow Spectrophotometry*

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The catalytic cycle of the amino acid oxidase reactions is initiated by reduction of the FAD moiety by the amino acid substrate (the reductive half-reaction) and completed by oxidation of the reduced FAD by \(O_2\) (the oxidative half-reaction). In the oxidative half-reaction, the \(\alpha\)-amino acid (\(S\)) is converted to the \(2\)-amino acid (\(P\)) which is released and hydrolyzed nonenzymatically to the \(2\)-keto acid and ammonia (1).

\[
E_o \xrightleftharpoons[k_{-1}]{k_1} [S] \xrightarrow{k_2} E_r \cdot \cdot \cdot P \xrightarrow{k_3} E_r + P,
\]

where \(E_o\), \(E_r \cdot \cdot \cdot P\), and \(E_r\) are oxidized enzyme, the transient species absorbing at 550 nm and free reduced enzyme, respectively. The oxidative half-reaction can be initiated either at \(E_r \cdot \cdot \cdot P\) or \(E_r\), (Equation 2) depending on the value of the \(k_1[O_2]/k_3\).

![Mechanistic Features Diagram](http://www.jbc.org/)

With most substrates, and under most experimental conditions, the partition of \(E_r \cdot \cdot \cdot P\) into \(E_r\) and \(E_r \cdot \cdot \cdot P\) strongly favors the latter and the lower loop of Equation 2 is therefore the major pathway for oxidation of the enzyme (2-4).

We address ourselves in this paper to the following issues in the \(\alpha\)-amino acid oxidase reaction. (a) What is the rate of conversion of \(E_r \cdot \cdot \cdot P\) to \(E_o\) through Steps 2 and 1 of Equation 1 (i.e. the reverse of the reductive half-reaction)? This issue is related to the question of why the sequential pathway represented by the lower loop of Equation 2 yields parallel line patterns in double stopped flow plots of turnover data from substrates (like \(\alpha\)-alanine, for example) which are oxidized almost exclusively by the lower loop. (b) Is the 2-hydrogen of the amino acid substrate conserved in the species \(E_r \cdot \cdot \cdot P\) or \(E_r\)? (c) Is negative charge developed at the 2-
carbon by physiological substrates in the transition state of Step 2 of Equation 1? This would be expected, by extrapolation, from results of studies with nitroalkanes and 3-chloro-2-amino acids which have been interpreted to mean that the 2-hydrogen is removed as a proton (9-11).

In answering these questions, we have made extensive use of the double stopped flow technique. The rationale here is to rapidly generate the transient \( E_2 \cdots P \) species in the first mix and then react this in the second mix with a reagent which reacts irreversibly and very rapidly with another enzyme species (e.g., \( E_2 \cdots P \) connected to \( E_2 \cdots P \) then becomes a starting reagent for direct kinetic studies of either the reverse of the reductive half-reaction (\( E_2 \cdots P \rightarrow E_0 + S \)) or the oxidative half-reaction (\( E_2 \cdots P + O_2 \rightarrow E_0 + P_2 + H_2O_2 \)). Neither of these pathways is accessible to single stopped flow measurements.

**MATERIALS AND METHODS**

\( \alpha \)-Amino acid oxidase was purified as described previously (12) with the modification suggested by Curti et al. (13). The ratio \( A_\text{het} / A_\text{het} \) for the benzoate-holoenzyme complex was 9.8, whereas that for the holoenzyme prepared as described previously (9) was 10.0. Reagent grade amino acids were used without purification and were supplied as follows: \( \alpha \)-alanine and \( \alpha \)-phenylalanine, Mann; p-NO\(_2\), p-NHS, p-OH, p-CH\(_2\)SH, and DL-phenylalanine, Sigma; p-Br-phenylalanine, Calbiochem. All other reagents and amino acids were of the highest quality commercially available. DL-[2-\( ^2 \)H]Alanine and DL-[2-\( ^3 \)H]phenylalanine were prepared as described previously (14). Substitution of the 2-hydrogen by deuterium was estimated by NMR to be greater than 90% as judged by the disappearance of the 2-proton resonance and of the splitting of the 2-protons.

All reactions were performed in 0.1 M sodium pyrophosphate at pH 7.0 or 8.3 and 25° ± 0.1°. Anaerobic conditions were maintained as previously described (9). Rapid kinetics were measured on the Gilson-Durrum stopped-flow spectrophotometer. Double stopped flow measurements were made on the Durrum Multi-Mixing system in the interrupted flow mode as described in the Multi-Mixing manual (15). Typically, the volume of the delay line was 0.15 ml, giving a delay time (in addition to that preset on the dial) of 28 ms with a flow rate of 10.5 ml/s. The volume of the first mix was 0.42 ml, and the volume discharged in the second flow was 0.21 ml. The age time was calibrated using a first order reaction. The observed age time was obtained from the amount of the reaction missed before observation began. It was found that the calculated delay time (dial setting plus the time due to flow) agreed with the observed delay time for times greater than 0.1 s. For shorter delay settings, the experimentally observed delay times were used. When changes in absorbance were less than 0.1 (2 cm)^-1, the percentage of T mode was employed. For larger changes in absorbance, the Durrum log amplifier was employed. All concentrations are reported for the final reaction mixture. First order constants were obtained from semilog plots of the data.

**RESULTS**

**Steady State Turnover Kinetics with \( \alpha \)-Alanine**

The kinetics of the \( \alpha \)-amino acid oxidase reaction are enzyme concentration-dependent (16, 17). Thus, in order to correlate rate constants obtained from stopped flow measurements of enzyme transients with steady state coefficients, we measured the latter in stopped flow turnover experiments at the same concentrations of enzyme used for the transient kinetic measurements. The steady state coefficients were obtained by monitoring oxidized enzyme species (\( E_0 \), \( E_2 \cdots S \), and \( E_2 \cdots P \)) at 372 nm. The rationale of this method has been fully described (18). The results, as shown in Fig. 1, conform to Equation 3.

\[
\frac{[E_i]}{v} \sim \frac{\phi_0}{[S]} + \frac{\phi_1}{[O_2]} \tag{3}
\]

We shall subsequently prove, to a very good approximation, that in terms of the scheme of Equation 2, \( \phi_0^{-1} = k_0, \phi_1^{-1} = k_1 \) and \( \phi_2^{-1} = k_2 \) (see Table I).

**Transient Kinetics of Reductive Half-reaction with \( \alpha \)-Alanine**

When \( E_2 \cdot P \) is mixed with \( \alpha \)-alanine anaerobically in single mix stopped flow experiments, the appearance of \( E_2 \cdots P \) occurs as a single exponential increase of absorbance at 550 nm. This is followed in a second phase by the slower decay of \( E_2 \cdots P \) to \( E_0 \) plus \( P_2 \) via \( k_3 \). Fig. 2 shows that the double reciprocal plot of \( k_{obs} \) for the first phase versus alanine concentration is linear. We have shown that \( k_{obs} \) for these experiments, when interpreted in terms of Equation 1, is given by Equation 4 (18).

\[
\text{Fig. 1. Steady state turnover of \( \alpha \)-alanine monitored by the stopped flow technique at pH 8.3 and 25° with 36 \mu M \( \alpha \)-amino acid oxidase. The inset is a replot of the ordinate intercepts with alanine as variable substrate. The values of } \phi_0, \phi_1, \text{ and } \phi_2 \text{ (see Equation 3) obtained from these data are given in Table I. In this and all following figure legends, the buffer used in all solutions is 0.1 M sodium pyrophosphate.}
\]

\[
\text{Fig. 2. Double reciprocal plot of reductive half-reaction data for } \alpha \text{-alanine (first phase only, see text) at pH 8.3 and 25° according to Equation 4. Substrate was mixed anaerobically with enzyme in the stopped flow spectrophotometer and the exponential increase of absorbance at 520 nm, corresponding to } E_2 \cdots P \text{ formation, was monitored. After mixing, the enzyme concentration was 10 \mu M. The dashed lines are computed with the assumption that } b d \text{ (} = k_{obs} \text{) from Equation 3 is } 0.5 \text{ s}^{-1} \text{ or } 0.1 \text{ s}^{-1}. \text{ The inset shows, on an expanded scale, the deuterium kinetic isotope effect on } k_1 \text{ obtained when } \alpha \text{-[2-}^{2} \text{H]alanine is compared with } \alpha \text{-[2-}^{3} \text{H]alanine.}
\]
Mechanism of D-Amino Acid Oxidase

\[
k_{\text{obs}} = \frac{k[S]k_1 + k_2 + k_3 + k_4}{k[S] + k_1 + k_2 + k_3 + k_4}
\]

(4)

Defining \(a = k_1(k_2 + k_3), b = k_4, c = k_1,\) and \(d = k_2 + k_3 + k_4,\) Equation 4 becomes, after rearrangement

\[
\frac{1}{k_{\text{obs}} - b/d} = \frac{cd}{ad - bc} + \frac{d^2}{(ad - bc)[S]}
\]

(5)

Since no curvature is observed (Fig. 2), \(b/d\) must be much less than any observed value of \(k_{\text{obs}}.\) The curves expected when \(b/d\) is 0.5 s\(^{-1}\) and 0.1 s\(^{-1}\) are included in Fig. 2. Thus, \(b/d\) (which, as will be fully discussed, represents the apparent first order rate constant, \(k_{\text{rev}},\) for reversal of the reductive half-reaction \(E_r \to E\)) must be less than 0.1 s\(^{-1}\). This condition will be confirmed by direct double stopped flow measurements, which show that \(k_{\text{obs}} = 0.021\) s\(^{-1}\). Furthermore, the transient spectrum of the enzyme at saturating \(S\) has no oxidized character, suggesting that \(k_5 \gg k_2.\) This conclusion is directly verified by the fact that \(\Delta E_{\text{obs}}\) (see inset of Fig. 2) is numerically identical to the value of \(k_2\) obtained from double stopped flow experiments (see later). This can only occur if \(k_5 \gg k_2.\) When \(D\)-alanine is substituted with \(^3\)H at the 2-carbon, the double reciprocal plot is linear and parallel to that observed with normal substrate (see inset of Fig. 2). This means that \(d^2/(ad - bc)\) from Equation 5 must be independent of the deuterium substitution and hence independent of \(k_2.\) The condition for this is \(k_2 + k_3 \gg k_4.\) Taken together, these results show that \(k_{12} \ll k_{23}\) and that the anaerobic half-reaction with \(D\)-alanine behaves to a good approximation as

\[
E_o + S \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2 \xrightarrow{k_3} E_3 \xrightarrow{k_4} P
\]

(6)

with \(k_1 = \phi_{\text{H}}\) (see Table 1). The values of \(k_2\) are given by the ordinate intercepts of Fig. 2 and are also included in Table 1. It should be noted that Strickland et al. (19) have also analyzed the patterns obtained from transient kinetic experiments such as those illustrated in Figs. 2 and 9. The value of \(k_4,\) obtained from the decay of \(E_3 \to P\) and measured at 550 nm in the same experiments, is independent of substrate concentration and 2-deuterium substitution (Table 1).

Test of Double Stopped Flow Technique: Direct Measurements of \(k_2\) and \(k_6\)

The kinetics of the lower loop of Equation 2 are not amenable to direct measurement by single stopped flow spectrophotometry because both \(E_r \to P\) and \(E_o \to P\), are transient intermediates and therefore cannot be used as initiating reagents. The assignment and evaluation of \(k_1\) and \(k_2,\) consequently, has been a matter of deductive elimination (2, 3). Thus, in the case of \(D\)-alanine, neither \(k_1\) (see inset 2) nor \(k_2,\) (0.017 s\(^{-1}\)) can account for \(\phi_{\text{D}}\) (10 s\(^{-1}\)), and it must be assumed that \(E_2 \to P\) reacts directly with \(O_2\) (\(k_5 = \phi_{\text{D}}\)) and that the release of \(P\) from \(E_3 \to P,\) \((k_4 = \phi_{\text{D}})\) represents the limiting first order process in turnover. Stopped flow monitored studies of oxidized enzyme species \((E_o, E_o \to S,\) and \(E_o \to P,\) see Fig. 1) during turnovers are entirely consistent with these assumptions and provide firm values for \(k_5\) and \(k_2\) provided that the assumed mechanism is correct. The double stopped flow experiments about to be described provide direct evidence for the lower loop of Equation 2 in the case of \(D\)-alanine. Furthermore, the fact that the directly measured values of \(k_5\) and \(k_2\) are in excellent agreement with \(\phi_{\text{D}}\) and \(\phi_{\text{D}}\), respectively, validates the double stopped flow technique for the purpose of this paper.

The rationale for the double stopped flow experiment is to first mix \(E_o\) with \(S\) anaerobically. At times \(<k_{\text{obs}}\) this converts virtually all of \(E_o\) to \(E_2 \to P\) because \(k_5 \gg k_2.\) \(E_2 \to P\) is then mixed, at a predetermined time, with \(O_2.\) However, since \(E_o \to P\) \((T) < [O_2]\) turnover will ensue unless \(E_o\) is trapped rapidly and irreversibly. We have used benzoate and anthranilate as ligands (I) in the second mix (together with \(O_2\)) to trap \(E_o\) because both of these are substrate-competitive inhibitors, suggesting that they combine with \(E_o\) and no other enzyme intermediates (20). Furthermore, the spectral characteristics, kinetics, and equilibria of the binding of benzoate and anthranilate to \(E_o\) have been thoroughly studied (21, 22) so that conditions can readily be chosen which ensure that the rate of trapping of \(E_o\) as \(E_o \to I\) is much faster than any other enzymic process of interest and which allow either \(E_o \to I,\) \(E_o \to P\) plus \(E_o \to I,\) or \(E_2 \to P\) to be monitored independently. The reactions of \(E_o \to P\) occurring after the second mix are the following (Equation 7). We have omitted \(k_3\) because the level of \(P_1\) is always insignificant compared to the value of \(k_3/k_2.\)

\[
\begin{align*}
E_o + I & \quad \xrightarrow{k_3} E_3 + E_1 + P_1 \\
E_o + S & \quad \xrightarrow{k_1} E_1 + P_1 \\
E_o & \quad \xrightarrow{k_4} E_3 + P_1
\end{align*}
\]

(7)

When \(k_3\) is to be evaluated, benzoate (\(K_d = 3 \mu M\)) is used as the trapping agent in the second mix because \(k_5 \gg k_2.\) \(E_2 \to P\) can then be uniquely monitored at 550 nm. Alternatively, the rate of formation of oxidized enzyme species \((E_o \to I,\) plus \(E_o \to I,\) can be monitored at 450 nm. In either case, the observed exponential absorbance change after the second mix is related to the three pathways for degradation of \(E_o \to I\) as follows (Eq. 8).

\[
k_{\text{obs}} = \frac{k_2 + k_3 + k_4}{k_2 + k_3 + k_4}
\]

(8)

Fig. 3 shows typical traces from such experiments monitored at 550 nm and the inset gives \(k_{\text{obs}}\) as a function of \(O_2\) according to Equation 8. The slope of this plot gives \(k_4,\) which is in

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**Table 1**

**Steady state coefficients and specific rate constants for D-Alanine and D-amino acid oxidase at pH 9.3 and 25°**

For further information, see Equations 3 and 12 (steady state coefficients) and the scheme of Equation 2 (specific rate constants).

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Value</th>
<th>Rate constant</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\phi_{\text{H}}) ((=k_1))</td>
<td>10 s(^{-1})</td>
<td>(k_3 = 9.8 \times 10^7 \text{M}^{-1} \text{s}^{-1})</td>
<td>(k_4 = 1.0 \times 10^9 \text{M}^{-1} \text{s}^{-1})</td>
</tr>
<tr>
<td>(\phi_{\text{D}}) ((=k_2))</td>
<td>0.68 \times 10^4 \text{M}^{-1} \text{s}^{-1}</td>
<td>(k_1(12-\text{H}) = 1.7 \times 10^7 \text{M}^{-1} \text{s}^{-1})</td>
<td>(k_1(13-\text{H}) = 4000 \text{M}^{-1} \text{s}^{-1})</td>
</tr>
<tr>
<td>(\phi_{\text{D}}) ((=k_4))</td>
<td>1.4 \times 10^5 \text{M}^{-1} \text{s}^{-1}</td>
<td>(k_1(12-\text{H}) = 1000 \text{M}^{-1} \text{s}^{-1})</td>
<td>(k_1(13-\text{H}) = 1000 \text{M}^{-1} \text{s}^{-1})</td>
</tr>
<tr>
<td>(k_1) ((\text{O} = \text{H}))</td>
<td>(1.7 \times 10^7 \text{M}^{-1} \text{s}^{-1})</td>
<td>(k_1 = 0.017 \text{s}^{-1})</td>
<td>(k_1 = 0.71 \text{s}^{-1})</td>
</tr>
<tr>
<td>(k_1) ((\text{O} = \text{H}))</td>
<td>(4.7 \times 10^5 \text{M}^{-1} \text{s}^{-1})</td>
<td>(k_1 = 0.017 \text{s}^{-1})</td>
<td>(k_1 = 0.71 \text{s}^{-1})</td>
</tr>
</tbody>
</table>
The rate-determining dissociation of the $E \cdot \cdot P$ after a 0.2-s delay, this solution was mixed with an aerobic solution of 100 mM benzoate. The absorbance traces have been corrected for complete observation of the reaction. Concentrations after the second mix were 10 mM enzyme, 10 mM dioxygen, and 50 mM benzoate. The concentrations of $O_2$ after the second mix are given by the experimental traces and as the $abcissa$ values of the inset, which is a plot of the data according to Equation 8.

The design of the double stopped flow experiments to measure the kinetics of reversal of the reductive half-reaction involved anaerobic mixing of $E_o$ with $S$ to generate $E_o \cdot \cdot P$ followed by anaerobic mixing of the latter with benzoate. The scheme applicable here is that of Equation 9.

$$E_o \cdot \cdot P + B_2 \rightarrow E_o \cdot \cdot P + B_2$$

The disappearance of $E_o \cdot \cdot P$ after the benzoate quench occurs as a single exponential process and can be monitored at 550 nm according to Equation 10.

$$k_{obs} = \frac{k_{fast} + k_{slow}}{k_{fast} + k_{slow} + k_{benzoate}} = k_{rev} + k_3$$

$p$-Alanine—Fig. 5 shows the effect of including benzoate in the second mix. The value of $k_{obs}$ (0.038 s$^{-1}$, lower trace) in the presence of benzoate corresponds to $k_{rev} + k_3$. When benzoate is omitted (upper trace), $E_o \cdot \cdot P$ decays via $k_3$ only (0.017 s$^{-1}$). Neither $k_{rev}$ nor $k_3$ is changed when $p$-[2$^3$H]alanine is substituted for $p$-[2$^3$H]alanine. These results are not affected by 5-fold increases in the concentrations of benzoate or benzoate. Nor was $k_{obs}$ affected by the time of addition of benzoate after formation of $E_o \cdot \cdot P$. The increased rate of disappearance of $E_o \cdot \cdot P$ caused by benzoate cannot be attributed to trace $O_2$ contamination for the following reasons. Since the enzyme concentration was $2 \times 10^{-5}$ M and $k_3$ is $1.7 \times 10^6$ M$^{-1}$ s$^{-1}$, contamination by $O_2$ to the extent of $10^{-6}$ M would cause $E_o \cdot \cdot P$ to disappear in a biphasic manner. The rate constant for the fast phase would be 3 s$^{-1}$ and the slow phase would correspond to $k_3$ (0.017 s$^{-1}$). Thus, the fact that the traces were uniphasic rules out $O_2$ contamination and establishes that the enhancement of the rate of $E_o \cdot \cdot P$ breakdown by benzoate is due to reversal of the reductive half-reaction via $k_{rev}$. 

**Mechanism of $\alpha$-Amino Acid Oxidase**

**Fig. 3. Evaluation of $k_r$ (see Equation 7) by the double stopped flow technique at pH 8.3 and 25°C using benzoate (I) as quenching agent in the second mix. The experimental traces represent the decay of $E_o \cdot \cdot \cdot P$ at 550 nm as it returns to $E_o$ (which is trapped as $E_o \cdot \cdot \cdot i$) via the three pathways shown in Equation 7. In the first mix, 40 mM $p$-alanine was added anaerobically to 75 mM enzyme to form $E_o \cdot \cdot \cdot P$. After a 0.2-s delay, this solution was mixed with an aerobic solution of 100 mM benzoate. The absorbance traces have been corrected for complete observation of the reaction. Concentrations after the second mix were 10 mM enzyme, 10 mM dioxygen, and 50 mM benzoate. The concentrations of $O_2$ after the second mix are given by the experimental traces and as the $abcissa$ values of the inset, which is a plot of the data according to Equation 8.**

**Fig. 4. Evaluation of $k_r$ (see Equation 7) by the double stopped flow technique at pH 8.3 and 25°C using anthranilate (I) as quenching agent in the second mix. The experimental trace represents the accumulation of $E_o \cdot \cdot \cdot i$ at 550 nm. $E_o \cdot \cdot \cdot P$ was generated in the first mix as described in Fig. 3. After a 0.2-s delay, this solution was mixed with 1.2 mM $O_2$ and 0.1 mM anthranilate. Concentrations after the second mix were 22 mM enzyme, 0.6 mM $O_2$, 5 mM $p$-alanine, and 50 mM anthranilate.**
Other Amino Acids—The values of $k_{nv}$ (see Equation 10) and $k_s$ were determined for nine amino acid substrates in all, as shown in Table II. In each case, we used the double stopped flow technique exactly as has been described for $a$-alanine. The value of $k_{nv}$ for phenylalanine is relatively large and a complete study was therefore made of this substrate (see next section).

Since $k_{nv}$ for phenylalanine is large, it would be predicted that the anaerobic addition of alanine (which forms $E_s \cdot P$ essentially irreversibly) to the phenylalanine-derived $E_s \cdot P$ in a double stopped flow experiment would trap $E_n$ as $E_s \cdot P$ and give an $E_s \cdot P$ decay rate ($k_s$) characteristic of alanine rather than phenylalanine. This prediction was confirmed, since the first order decay of $E_s \cdot P$ at 550 nm in such an experiment was 0.017 s⁻¹ rather than 3.2 s⁻¹ (see Table II).

Kinetics of Reversal of Reductive Half-reaction with Ring-substituted Phenylalanines

Typical 550 nm absorbance traces are shown in Fig. 6 for the double stopped flow experiments with phenylalanine. When benzoate is included in the second mix (lowest trace), a single exponential process is observed and Equations 9 and 10 apply. When benzoate is omitted from the second mix (top trace), only $k_s$ is observed (see Equation 9). Table III summarizes the control experiments necessary to show that $k_{nv}$ is not significantly affected by relatively large variations in the concentrations of benzoate and substrate or in the time of the benzoate quench. It should also be noted (Table III) that the presence of L-phenylalanine does not affect $k_{nv}$. The value of $k_{nv}$ was unchanged when anthranilate, rather than benzoate, was used as the quenching agent (reaction monitored at 450 nm) and was also independent of wavelength.

Fig. 7 is a linear free energy plot of $k_{nv}$ versus $\alpha^+$ (23) for a series of ring-substituted phenylalanines. The $p$ value is 1.14 ($r = 0.913$). The respective phenylalanines were used as their $d$-racemates. Since the $d$ isomer of unsubstituted phenylalanine (see Table III) did not affect $k_{nv}$, it is reasonable to assume that the $d$ isomer of each of the racemic ring-substituted phenylalanines was similarly inert.

Evidence for Conservation of Substrate 2-Hydrogen in $E_s \cdot P$
The rationale for these experiments was to choose $d$-deuterated substrates which demonstrate a kinetic isotope effect on $k_s$ and then search, using the double stopped flow technique, for a corresponding kinetic isotope effect on $k_{nv}$ as $E_s \cdot P$ is pulled back to $E_n \cdot I$.

In the case of $d-[2-2H]lalanine, no deuterium kinetic isotope effect was detected in either $k_s$ or $k_{nv}$ (see Fig. 5), although $k_2$ (Fig. 2 and Table I) is so affected. On the other hand, $d-[2-2H]phenylalanine gives a large kinetic isotope effect on $k_{nv}$ (see Fig. 6) and $k_s$ (see Fig. 9) but no effect on $k_s$ (see Fig. 6). These results, which are summarized in Table IV and more fully discussed later, clearly show that $k_{nv} = k_s$ in the case of phenylalanine and that the 2-hydrogen of the amino acid substrate is conserved in the species $E_s \cdot P$ on the time scale of these experiments.

Kinetic Consequences of Facile Reversal of Reductive Half-reaction with Phenylalanine

The large value of $k_{nv}$ obtained with phenylalanine (Table III) is predicted to cause two interesting effects on the kinetic behavior of this substrate. First, $k_{nv}$ may be competitive with $k_s[O_2^+]$, in which case, catalytic flux through the lower loop of Equation 2 will give rise to intersecting double reciprocal turnover plots, rather than the parallel line patterns commonly found with this and other flavoprotein oxidases. See
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TABLE III
Control experiments for determination of \( k_{\text{obs}} \) for D-phenylalanine at pH 8.3 and 25°C using benzoate as a trap for \( E_2 \)

| II. Effect of D-phenylalanine concentration (age 0.08 s, 25 mM benzoate) |
|-----------------|-----------------|
| D-Phenylalanine | \( k_{\text{obs}} \) s\(^{-1}\) |
| 0               | 3.1             |
| 2.5             | 15.0            |
| 25              | 15.7            |

| III. Effect of age time (12.5 mM D-phenylalanine, 25 mM benzoate) |
|-----------------|-----------------|
| Age time        | \( k_{\text{obs}} \) s\(^{-1}\) |
| 0.03            | 15.6            |
| 0.08            | 15.7            |
| 0.23            | 16.0            |
| 0.53            | 14.4            |

| IV. Comparison of rate of formation of \( E_2 \cdot \text{P} \) and disappearance of \( E_2 \cdot \text{P} \) upon benzoate addition to \( E_2 \cdot \text{P} \) (25 mM benzoate, 12.5 mM D-phenylalanine, age 0.08 s) |
|-----------------|-----------------|
| Wavelength      | \( k_{\text{obs}} \) s\(^{-1}\) |
| 550             | 15.6            |
| 450             | 15.6            |

| V. Effect of \( \omega \)-phenylalanine on \( k_{\text{obs}} \) (25 mM benzoate, 12.5 mM D-phenylalanine, age 0.08 s) |
|-----------------|-----------------|
| \( 12.5 \text{mM \( \omega \)-phenylalanine} \) | 15.0 |
| \( 25 \text{mM \( \omega \)-phenylalanine} \) | 15.7 |

| VI. Comparison of two \( E_2 \) traps (12.5 mM \( \omega \)-phenylalanine, age 0.08 s) |
|-----------------|-----------------|
| Trap            | \( k_{\text{obs}} \) s\(^{-1}\) | Observation wavelength |
| 25 mM Benzoate  | 15.7            | 550          |
| 50 mM Anthranilate | 18.4           | 450          |

Fig. 7. The dependence of \( k_{\text{obs}} \) on electron withdrawing or donating substituents in the phenyl ring of phenylalanine presented as a linear free energy plot using \( \sigma^t \). The values of \( k_{\text{obs}} \) were measured for the substituted phenylalaines at pH 8.3 and 25°C as described in Fig. 5. The slope of the line is 1.14 with an \( r \) value of 0.913.

Fig. 8. Steady state turnover of D-phenylalanine monitored by the \( \text{O}_2 \) electrode at pH 7.0 and 25°C with 0.05 \( \mu \text{M} \) enzyme and 10 \( \mu \text{M} \) FAD.

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TABLE IV
Summary of values for \( k_{\text{obs}} \) and other kinetic constants obtained with D-alanine and D-phenylalanine at 25°C

<table>
<thead>
<tr>
<th>Method and substrate</th>
<th>Rate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate quench*</td>
<td></td>
</tr>
<tr>
<td>pH 8.3</td>
<td></td>
</tr>
<tr>
<td>D-[2-\text{H}]alanine</td>
<td>0.020</td>
</tr>
<tr>
<td>D-[2-\text{H}]benzaline</td>
<td>0.020</td>
</tr>
<tr>
<td>D-[2-\text{H}]phenylalanine</td>
<td>11.8</td>
</tr>
<tr>
<td>D-[2-\text{H}]phenylalanine</td>
<td>3.6</td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
</tr>
<tr>
<td>D-[2-\text{H}]phenylalanine</td>
<td>20.0</td>
</tr>
<tr>
<td>D-[2-\text{H}]phenylalanine</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Forward Reaction*

| pH 8.3              |               |
| D-[2-\text{H}]phenylalanine | 84            |
| D-[2-\text{H}]phenylalanine | 1.9           |
| D-[2-\text{H}]phenylalanine | 15.5          |
| D-[2-\text{H}]phenylalanine | 3.0           |

| pH 7.0              |               |
| D-[2-\text{H}]phenylalanine | 14.0          |
| D-[2-\text{H}]phenylalanine | 2.8           |

---

* Double stopped flow experiments such as are shown in Figs. 5 and 6.

| Single stopped flow experiments such as are shown in Figs. 2, 9, and 10.

| Corrected for competitive inhibition by the \( \text{L-[2-\text{H}]phenylalanine} \) present in racemic \( \text{[2-\text{H}]phenylalanine} \).
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comparable to measured values of \(k_{\text{max}}\). In fact, Equation 4 predicts that the limiting value of \(k_{\text{obs}}\) as \(S \to 0\) will be \(k_{\text{max}}\) (which, as we show under "Discussion," is equal to \(k_{-2}\) in the case of phenylalanine). Fig. 9 shows that this is indeed the case with both D-[2-\(^{2}H\)]- and D-[2-\(^{2}H\)]-phenylalanine. These studies were conducted at pH 7.0 because the value of \(k_{1}\) is smaller at pH 7.0 than at pH 8.3 and \(k_{\text{max}}\) is more easily evaluated. The limiting values of \(k_{\text{obs}}\) (= \(k_{\text{max}}\), see Equation 4) at small substrate concentrations is estimated to be 28 s\(^{-1}\) and 7.5 s\(^{-1}\) for [2-\(^{2}H\)] and [2-\(^{2}H\)]-phenylalanine, respectively. The inset of Fig. 9 shows that Equation 5 (the linear form of Equation 4) fits both sets of data well. Thus, single stopped flow measurements of the reductive half-reaction provide an independent check of the evaluation of \(k_{\text{max}}\) by the double stopped flow quenching technique used heretofore. Table IV summarizes the values of \(k_{2}\) and \(k_{\text{max}}\) determined by the single and double stopped flow techniques at pH 8.3 and pH 7.0. The absolute values of \(k_{\text{max}}\) determined by the two techniques agree reasonably well.

A further interesting consequence of the reversibility of the conversion of \(E_{1} \cdots \cdot S\) to \(E_{1} \cdots \cdot P\) in the case of phenylalanine is that the observed rate of conversion of \(E_{1} \cdots \cdot P\) to \(E_{1} + P\), in a single stopped flow experiment is highly substrate concentration-dependent. This is not so in the case of alanine, where \(E_{1} \cdots \cdot S\) is converted irreversibly to \(E_{1} \cdots \cdot P\) and \(k_{1}\) may be directly measured in a single experiment provided that the substrate concentration is high enough to clearly separate the exponential rise and decay of 550 nm absorbance. Values of \(k_{1,\text{app}}\) are plotted reciprocally versus phenylalanine in Fig. 10. The abscissa and ordinate intercepts are given by \(-(1 + K_{3})/K_{1}\) and \((1 + K_{3})/K_{3}k_{1}\), respectively, where \(K_{1} = k_{-1}/k_{1}\) and \(K_{2} = k_{2}/k_{-2}\). Since \(K_{1}\) can be evaluated independently from \(k_{2}\) and \(k_{-2}\), \(K_{1}\) can be calculated. These values are summarized in Table IV.

**DISCUSSION**

The experiments described here, to our knowledge, represent the first application of double stopped flow spectrophotometry to flavoenzyme reactions. The interaction of aequorin with Ca\(^{2+}\) was studied in this fashion (24). The technique is very useful for the generation and kinetic study of otherwise inaccessible transient intermediates in a partial reaction (e.g., the use of benzoate or anthranilate in studies of the reductive half-reaction as described herein) or in a complete reaction when the catalytic cycle can be quenched nondestructively during or after one turnover by inclusion in the second mix of an inhibitor (benzoate or anthranilate in our case) which reacts specifically, rapidly, and essentially irreversibly with an enzyme species (\(E_{0}\) in our case) derived from the transient intermediate which was generated in the first mix. Several new features of the D-amino acid oxidase reaction have been revealed by these studies.

The "ping-pong" turnover patterns of flavoprotein oxidase reactions might be (and often have been) interpreted to mean that the enzyme oscillates between \(E_{1}\) and \(E_{1}\), during catalysis, with the required irreversible step between the binding of \(S\) and \(O_{2}\) being provided by the release of \(P\), from \(E_{1}\). That this is not so in the case of D-amino acid oxidase was evident from the earliest transient kinetic studies which established that the rate of formation of \(E_{1}\) from \(E_{1} \cdots \cdot P\) is much smaller than the maximum turnover number for many substrates (2, 3). Despite the fact that several groups have provided convincing evidence that the lower loop of Equation 2 is the major pathway for catalysis, neither the irreversibility of the sequence \(E_{1} \to E_{1} \cdots \cdot P\) (which is required in order to explain the parallel line double reciprocal plots of steady state turnover data) nor the reaction of \(E_{1} \cdots \cdot P\) with \(O_{2}\) have been studied directly before now.

The ordered sequential mechanism of the lower loop of Equation 2 gives the following steady state rate law (Equation 11).

\[
\frac{v}{c} = \frac{k_{2} + k_{1} + k_{2} + k_{-2}}{k_{2}k_{3} + k_{-1} + k_{2} + k_{-2}}
\]

The presence of the fourth term requires that the families of lines intersect to the left of the ordinate of Fig. 1. However, the intersection will be undetectable, and the lines will appear to be parallel, if the fourth term is negligible compared to the other three terms over the ranges of \(S\) and \(O_{2}\) employed. The coefficient \(\phi_{4}\) of the fourth term is equal to \(k_{\text{max}}k_{2}/k_{-2}\), which from the data of Table I for D-alanine has a value of 1.38 x
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10⁻¹¹ M² s⁻¹. If we take midrange values of 5 x 10⁻⁸ M and 10⁻⁴ M for S and O₂, respectively, from Fig. 1, the value of the fourth term of Equation 10 becomes 2.46 x 10⁻⁵ s. This is 5000 times smaller than ϕ₄ and the points of intersection with S and O₂ as variable substrates in Fig. 1 are predicted to be at −5 x 10⁵ M⁻¹ and −10⁻⁸ M⁻¹, respectively. Clearly, such intersection points cannot be detected in experiments such as those of Fig. 1 and the ordered sequential mechanism of the lower loop of Equation 2 will appear to give ping-pong steady state patterns.

Conditions necessary to cause this behavior were discussed previously (25). The stopped flow experiments show that the remaining steady state coefficients are, practically speaking, considerably simpler than those given in Equation 11. Thus, k₂ is much larger than k₁, k₃, or k₄ with the result that the D-alanine reaction behaves to a very good approximation as the following (Equation 12). It should be noted that we have experimentally shown that the locus of H₂O₂ release is at the k₃ step, as shown in Equation 12 (18).

\[
\begin{align*}
E₀ \rightarrow k₄[S] E₀ \rightarrow k₃[S] E₃ \rightarrow k₂[P, H₂O₂] k₄[O₂] (F₄ = 1/k₄ + 1/k₃ = 1/k₄[O₂]) (12)
\end{align*}
\]

Other, less direct, experimental estimates of the rate at which E₃·P converts to E₄ anaerobically in the case of alanine have given conflicting results. Yagi et al. (26, 27), from experiments in which benzoate was added to E₄, ammonia, and pyruvate and from measurements of the exchange of solvent deuteron at the 2-carbon of D-alanine concluded that the rate of formation of E₄ from E₃·P must be very slow. Similarly, Massey et al. (28) showed that the anaerobic addition of D-methionine to E₃·P made from D-alanine did not affect the rate of conversion of E₃·P to E₄. Although those experiments were not designed to evaluate k₄, they in fact demonstrate that the anaerobic rate of conversion of alanine-derived E₃·P to E₄ must be slow. Walsh et al. (10) showed that, after a 10-min anaerobic incubation of enzyme with amino acid and ammonium sulfamate in H₂O, no solvent hydrogen had been incorporated into the 2 position of alanine, proline, or 2-chloroalanine. They regarded the irreversibility of formation of E₄·P from E₃·P as an unlikely explanation and argued instead that E₃·P either converts much more rapidly to E₄·P than to E₃·S (which is not the case with alanine, as shown in Table I) or does not exchange its substrate-derived proton with solvent (which is the case, at least on the time scale of milliseconds, as shown by the present studies with alanine and phenylalanine). It was deduced from other experiments that E₄, in contrast to E₃·P, must exchange its substrate-derived proton with solvent (10) and one would have expected E₄ to have been present under the anaerobic conditions employed by Walsh et al. (10). We should note that the distribution and kinetics of interconversion of S and P₁ and of enzyme species (E₀, E₀·S, E₃·P, E₄, E₄·S, E₄·NH₃, E₄·NH₃ etc.) in such experiments would be very complicated, and it is possible, for example, that E₄ is substantially trapped as E₄·S. Thus, while our findings are in qualitative agreement with one of the explanations given by Walsh et al. (10) for the lack of solvent exchange (namely, proton conservation at the level of E₃·P), it is quite possible that, in a quantitative sense, irreversibility of E₃·P formation from E₃·S, coupled perhaps with trapping of E₄, as E₄·S, is the major reason for their results.

On the other hand, Koster and Veeger (29) concluded from converging double reciprocal patterns of steady state data obtained from D-alanine turnover in the presence of benzoate, that k₄ should be 50 s⁻¹. This is clearly at variance with our direct measurements of k₄, and with the less direct estimations just described. The reason for the discrepancy could perhaps be due to the fact that Koster and Veeger (29) used lower enzyme concentrations (1 μM) while higher concentrations (10 μM) were used in our experiments. E₄ is known to undergo dimerization (30, 31) and certain kinetic properties of the monomeric and dimeric species differ (16, 17). It is possible that k₄ is greatly diminished in the case of the dimer. From our value of k₄, it is unlikely that converging double reciprocal patterns could be obtained in the absence of benzoate under our conditions.

When k₄ is relatively large, as in the case with phenylalalnine, the ϕ₂₁ coefficient of Equation 11 (i.e. the fourth term) becomes experimentally significant and the ordered sequential mechanism of the lower loop of Equation 2 is clearly reflected in the convergence of the steady state patterns (Fig. 8). It may well be that the reduction in k₄ (compared to alanine) is the single factor leading to the enhancement of k₄, and thereby to the converging steady state patterns and to the curvature in the plot of the reductive half-reaction data (Fig. 9). In any case, it is clear that the presence or absence of these phenomena is fully explained by the magnitude of k₄ as determined from the double stopped flow experiments.

Our evidence for conservation of the substrate 2-hydrogen in the intermediate E₃·P consists of the fact that both k₃ and k₄ in the case of phenylalanine show deuteron kinetic isotope effects. In order for the kinetic isotope effect to be carried over to k₄, the substrate 2-deuterium must be removed and conserved between E₃ + S and E₃·P and the rate-determining step in the reversal of the reductive half-reaction must be k₂ (i.e. k₄v = k₄ in the case of phenylalanine). The latter will occur if k₂ ≫ k₃ ≫ k₄. There cannot be a primary kinetic isotope effect on k₄ because, in the forward direction of the reductive half-reaction, the 2-C–H bond is consistently cleaved in the k₃ step for substrates tested here. The reason why k₄v shows no deuteron kinetic isotope effect in the case of alanine is that k₂ ≪ k₃ ≫ k₄, as we have already shown. Consequently, k₄v = k₃/k₄ = k₃/k₄ and the isotope effects on k₃ and k₄ exactly cancel. It should be noted that, at pH 7.0, the kinetic isotope effects (−4-fold) on k₃ and k₄ for phenylalanine are indeed almost identical. If the 2-deuterium of alanine had not been conserved in E₃·P, owing to rapid exchange with solvent, an inverse isotope effect on k₄v would have been observed. It seems likely, therefore, that after cleavage of the 2-C–H substrate bond in the k₃ step and transfer of the hydrogen to the enzyme, conservation of this hydrogen at a nonexchangeable site in E₃·P is a general characteristic of the physiological substrates. Many lines of evidence (summarized in Ref. 18) support the hypothesis that the 2-hydrogen is removed as a proton. Conservation of carbon-bound substrate hydrogen after transfer to the enzyme as a proton is a common feature of many enzymes during catalysis (32–34). This feature has also been demonstrated, by methods substantially different from those described here, for flavoprotein reactions (10, 11). In the case of p-amino acid oxidase, the substrate hydrogen is not conserved in the sequence E₄ → E₅ (10). Our data, therefore, suggest that the enzyme-bound hydrogen is lost to the solvent in the step E₃·P → E₃·P + E₄ (10). Significant conservation of the substrate 2-hydrogen has been shown to occur during 2,5 elimination of HCl from 3-chloro amino acids catalyzed by α amino acid oxidase (10, 11, 35). In this case the...
hydrogen is transferred to the 3-carbon of the product. Although it is not clear whether flavin reduction must precede the elimination of Cl- in the reactions of these unphysiological substrates, the rate at which the proton originating at the substrate 2-carbon is transferred to the 3-carbon within the enzyme–enamine complex was found to be 91 s⁻¹ in the case of 3-chloro-o-
aproline (36). The half-time for this process is a good deal shorter than the half-time of the substrate-derived proton in E,···P demonstrated here for o-phthalaldehyde (which must be greater than 0.69/k₂ = 0.06 s at pH 8.3, see Table IV). Therefore, it would appear that the proton conservation seen with 3-chloro-2-amino acids is not a special attribute of these unphysiological substrates. Rather, the substrate-derived proton is to be regarded as a reagent which is substantially trapped during the lifetime of the E,···P complex and which is presumably located at the center of greatest basicity within this complex (either a general base such as tyrosyl, which has been implicated in active site chlorination studies (37), or, more likely, the N-5 position of reduced flavin after transfer from the general base).

One practical consequence of proton conservation is that tests for solvent hydrogen exchange at the 2-carbon of the substrate during aerobic turnover will give negative results even if E,···P were to contain a 2-carbanion component which is in rapid equilibrium with E, + S (e.g., phenylalanine).

The question of exactly where in E,···P the substrate-derived 2-hydrogen is located cannot be decided by these studies. It is clear, however, that this proton resides at some position on the apoprotein or on the flavin nucleus. If the oxidation-reduction state of the flavin in E,···P is considered to be fully reduced, then the N-5 position is an obvious candidate. However, in contrast to demonstrations of direct hydrogen transfer to the C-4 position of dihydronicotinamides (38), the rate of solvent exchange at the N-5 position of reduced flavin in E, during catalysis and subsequent workup would vitiate experimental evidence for such direct transfer to the flavin nucleus. For this reason, Brustlein and Bruice (39) used 5-deazaalloxazine as a model flavin for the demonstration of direct hydrogen transfer to the C-5 position during nonenzymic oxidation of NADH. Subsequently, several flavoenzymes, after resolution and reconstitution with the appropriate deazaflavin co-factor, were shown to catalyze direct transfer of substrate hydrogen to the C-5 position of the deazaflavin (5–8).

Although the rates of reduction of such deazaflavoproteins are extremely sluggish and the reduced deazaflavoprotein oxidases, by about the same factor, are poorly oxidized by O₂, such studies suggest that the substrate 3-hydrogen, after abstraction as a proton by an amino acid side chain acting as a general base, is transferred intact to the N-5 position of the native flavin coenzyme during or after reduction of the latter. If covalent adducts derived from the substrate 2-carbanion and the N-5 position of the flavin are obligatory intermediates in the oxidation of physiological substrates, as they have been proved to be in the case of nitroalkanes (9), the formation and subsequent rearrangement of such adducts are extremely rapid compared to proton abstraction from the 2-carbon (k₂) and to the lifetime of the E,···P complex and its conserved hydrogen.

In summary, we cannot decide from these studies whether the conserved substrate proton in E,···P is located on the general base or at the N-5 position of reduced flavin, although the deazaflavin studies suggest the latter to be a good possibility. It should be noted that the intrinsic rate of exchange with solvent (unassisted by buffer species) at pH 8.3 of such a weak conjugate acid as the 5-NH of FADH₂ is at least 10⁴-fold smaller than the rate (k₂) with which the substrate-derived proton in E,···P is trapped by E,···S.¹ Thus, no special mechanism which involves solvent exclusion from the active site need be invoked to explain proton retention in E,···P. It is interesting that the values of k₂, at pH 8.3 and 7.0 are 11.8 and 15.5 s⁻¹, respectively, in the case of phenylalanine. This shows that an ionizable group with a pKₐ value above 8.0 and which is able to rapidly equilibrate its ionization state with solvent, is not involved in k₂. The report by Yagi et al. (40) that the rate of oxidation of E,···P by O₂ (via k₂) is unchanged when the substrate is 2-deuterated suggests that the substrate 2-hydrogen conserved in E,···P is not involved in the rate-determining step for oxidation of E,···P by O₂.

Taken together, or separately, the values of k₂ and kₜ for the amino acid substrates listed in Table II show no consistent pattern of behavior. Thus, a linear free energy plot of kₑ, using Taft's σ⁺ values (41), gives considerable scatter. One reason for this must be the fact that the rate-determining steps comprising kₑ, differ from substrate to substrate (e.g., k₂ for phenylalanine and kₜ for alanine). These data are therefore not suitable for discussions of structure-function relationships.

In the case of the ring-substituted phenylalanines, where differences in hydrophobic, steric, and hydrogen-bonding interactions with the active site would be expected to be attenuated, there is a reasonably linear free energy relationship between kₑ and the electron-withdrawing power of the R group as measured by σ⁺. The parameter σ⁺ was chosen because the reaction center is separated by a methylene from the phenyl ring (23). The fit of the data in Fig. 7 would probably improve if hydrophobic (42) and charge transfer (43) interactions were also taken into account. Nevertheless, the magnitude of kₑ increases fairly consistently with increasing electron-withdrawing power of the substituent with a ρ value, after correction for the methylene intervening between the reaction center and the phenyl ring (41), of +3.2. It has been demonstrated for many reactions that a positive ρ value is consistent with the development of negative charge in the transition state (44). The transition state referred to here clearly involves migration and (in the rutagol) formation of the covalent bond between the 2-carbon and the 2-hydrogen because of the kinetic isotope effects on k₂ and kₜ obtained with 2-H-substituted phenylalanine. However, the difficulty with the interpretation of substituent effects on terms of ρ is that one is attempting to deduce the character of the transition state from measurements of the free energy difference between ground and transition states. It need not generally be true that the ground state free energy is unaffected in the series. On the other hand, if the Hammett plot is better satisfied by σ⁻, it has been suggested that the substituent effect is entirely on the ground state (45). The latter argument has been followed in the interpretation of substituent effects on the reduction of benzaldehydes by yeast alcohol dehydrogenase (46). In our case, the superior correlation of log kₑ with σ⁻ suggests that a transition state effect is being measured. If this is so, then the value of ρ = 3.2 is consistent with the development of partial negative charge on the substrate 2-hydrogen.

¹ The pKₐ value for the N-5 position of reduced flavin (\(\text{NH} = \text{N}⁺ + \text{H}⁺\)) almost certainly exceeds 20 (T. C. Bruice, personal communication). Thus, according to the Eigen principles (Eigen, M. 1964 Angew. Chem. Int. Ed. Engl. 3, 1) the maximum rate for abstraction of the N-5 proton by OH⁻, at pH 8, is about 10⁻¹⁰ s⁻¹.
carbon in the transition state for bond breaking/making to hydrogen. This suggests that the substrate 2-carbon develops carbanion character during the conversion of $E_0 \rightarrow S$ to $E$, $\cdots$ - P. This is fully consistent with the evidence obtained from studies with substrate analogues and derivatives in this and other flavoprotein oxidase reactions (18). Other positive $p$ values have been obtained for the D-amino acid oxidase reaction (47, 48). However, these studies did not focus on an isolated rate constant and cannot be interpreted unequivocally.

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D J Porter, J G Voet and H J Bright


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