Studies on the Mechanism of Action of L-Amino Acid Oxidase*

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The L-amino acid oxidase of Crotalus adamanteus venom, which has recently been crystallized and shown to have a molecular weight of approximately 130,000, contains 2 moles of flavin adenine dinucleotide per mole of enzyme (1). The enzyme catalyzes the oxidation of a number of L-amino acids according to the following equation:

\[
\text{R} + \text{NH}_2 + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{COOH} + \text{COOH} + \text{H}_2\text{O} + \text{NH}_3 + \text{H}_2\text{O}_2
\]

This reaction takes place in at least two steps, reduction of the enzyme by amino acid, and reoxidation of the enzyme by molecular oxygen. It has been shown in this laboratory (2) that the first of these steps is reversible. The available data indicate that the first oxidation product of the amino acid is an imino acid, which is hydrolyzed to the corresponding keto acid and ammonia. Reaction 1 may therefore be considered to be the sum of the following reactions (E is enzyme):

\[
\text{R} \rightarrow \text{C} = \text{NH} + E - \text{FAD} \rightarrow E - \text{FADH}_2 + \text{COOH}
\]

\[
\text{R} \rightarrow \text{C} = \text{NH} + \text{H}_2\text{O} \rightarrow \text{C} = \text{O} + \text{NH}_3 + \text{H}_2\text{O}_2
\]

We postulated previously (4) that Reaction 2 takes place in two steps, the first of which leads to the formation of a half-reduced enzyme capable of being further reduced by substrate or of being reoxidized by molecular oxygen more rapidly than the completely reduced enzyme. Such a mechanism would explain the marked substrate inhibition exhibited by this enzyme. Furthermore, inasmuch as the enzyme has two flavins, the complete reduction of the enzyme would involve the dehydro-
calculated on the basis of a molecular weight of 130,000 and a specific activity of 7,600 for the pure enzyme. On this basis, 1.77 x 10^7 units are equivalent to 1 mole of enzyme.

In the experiments in which the gas phase was oxygen, the flasks were flushed while shaking with pure oxygen saturated with water at 38° for 15 to 20 minutes. When a gas phase of 5% oxygen was used, each flask was flushed with 2 liters of the gas mixture over a 4-minute period, while shaking at 38°. Readings were started 5 minutes after mixing the components and continued for 15 or 20 minutes, during which time the rate was linear within experimental error. The values obtained may therefore be considered to be “initial velocities.” In the plotting of the data, the amino acid concentration present in the middle of the period of measurement was used.

RESULTS

Figs. 1, 2, and 3 describe the effect of varying the concentration of L-leucine, L-methionine, and L-valine, respectively, on the rate of oxidation of these amino acids in air and in oxygen. It may be seen that L-leucine and L-methionine inhibited the reaction markedly at high concentrations. The velocity exhibited a sharp maximum at a substrate concentration of about 0.008 M. In contrast, L-valine produced little or no inhibition at any of the concentrations tested, either in oxygen or in air. In order to see whether L-valine would produce inhibition at lower oxygen tensions, the effect of varying the concentration of this amino acid was tested under a gas phase of 5% oxygen. Under these conditions, as shown in Fig. 4, L-valine began to inhibit the reaction at concentrations comparable to those at which L-leucine and L-methionine inhibited in air or oxygen.

Inasmuch as the conditions of the velocity measurements were steady state, rather than equilibrium conditions, it may not be assumed that the oxygen concentration in the solution was in equilibrium with the gas phase. Under a given set of conditions (rate of shaking, size and shape of the vessel, amount of liquid, etc.), the rate of oxygen uptake, R, is a function of its steady state concentration in the liquid phase, (O_2)_liquid, and of the partial pressure of oxygen in the gas phase, (O_2)_gas, as expressed by the relation, R = K_1 (O_2)_gas - K_2 (O_2)_liquid (K_1 and K_2 are proportionality constants) (11). When R = 0, (O_2)_liquid = K_1/K_2 (O_2)_gas. The ratio, K_1/K_2, therefore, represents the solubility of oxygen, which is 23.6 µl ml^{-1} atmosphere^{-1} at 38° (12). The maximal rate of uptake would be obtained when (O_2)_liquid = 0; thus, R_max = K_1 (O_2)_gas. To determine K_1, the rate of oxygen uptake was measured in the presence of very high enzyme concentrations, as shown in Fig. 5. From these results it was concluded that R_max is close to 18 µl min^{-1} when the gas phase is air, i.e. when (O_2)_gas = 0.196 atmospheres (taking the vapor pressure of water into account). Therefore, K_1 = 18/0.196 = 92 µl min^{-1} atmosphere^{-1}, and K_2 = 92/23.6 = 3.90 ml min^{-1}. The concentration of oxygen in solution may thus be obtained for any experiment from the relation, R = 92 (O_2)_gas - 3.90 (O_2)_liquid. It may be seen that the curve given in Fig. 5 is linear, within experimental error, when the rate of oxygen uptake was below 6 µl min^{-1}. Deviation from linearity at higher rates of oxygen uptake is due to decreased concentration of oxygen in solution. Since, in the experiments in which the effect of amino acid concentration was tested, the amount of enzyme was adjusted to yield rates varying from 2 to 6 µl min^{-1} (corresponding to oxygen concentrations of 4.1 to 3.1 µl ml^{-1} when the gas phase was air, and 21.6 to 20.6 µl ml^{-1} for oxygen), the oxygen concentration was taken as constant in these experiments, and the average values, 3.6 µl ml^{-1} (1.60 x 10^{-4} M) for air and 21.1 µl ml^{-1} (9.4 x 10^{-4} M) for oxygen were used in the calculations given below. In the experiments carried out under 5% O_2 the concentration of oxygen in solution was extremely low and may not be taken as being equal in all vessels. However, the inhibition observed was evidently not due to this variation since the oxygen concentration was highest in the vessels in which the rate of uptake was lowest.

It is of interest that if the oxygen concentration is raised by a factor of 5.9, the rate of oxidation of L-leucine and L-methionine is only increased 2.5 to 3.5-fold. With L-valine, at concentrations lower than 0.02 M, substitution of oxygen for air had no effect, whereas such substitution increased the reaction rate at higher concentrations of valine.

DISCUSSION

In their original studies on L-amino acid oxidase, Zeller and Maritz (5) observed a characteristic substrate inhibition. Zeller suggested (7) that this inhibition was due to the combination of two amino acid molecules with the enzyme to yield an...
FIG. 2. Effect of substrate concentration on the oxidation of L-methionine. Ordinate, turnover number \( \times 10^{-3} \); abscissa, L-methionine concentration. \( \circlearrowleft \circlearrowleft \), gas phase, air; \( \square \square \), gas phase, oxygen. Other conditions as in Fig. 1. Curves are calculated (see the text).

FIG. 3. Effect of substrate concentration on the oxidation of L-valine. Ordinate, turnover number \( \times 10^{-3} \); abscissa, L-valine concentration. \( \circlearrowleft \circlearrowleft \), gas phase, air; \( \square \square \), gas phase, oxygen. Other conditions as in Fig. 1.
inactive complex. It may be shown with the classical steady-state treatment of enzymatic mechanisms (13), that a mechanism involving such an inactive complex, expressed symbolically as follows,⁵,⁶

$$E + AA \xrightarrow{k_1} EAA \xrightarrow{k_2} EH_2 + IA$$ (5)

$$EAA + AA \xrightarrow{k_3} E(AA)_2$$ (6)

$$EH_2 + O_2 \xrightarrow{k_3} EHHO_2 \xrightarrow{k_3} E + H_2O_2$$ (7)

would yield the following kinetic equation:

$$\frac{(E_i)}{v} = \frac{a}{(AA)} + b + \frac{c}{(O_2)} + \frac{d(IA)}{v}$$ (8)

in which

$$a = \frac{k_1}{k_2}, \quad b = \frac{k_3}{k_2}, \quad c = \frac{k_3}{k_3}, \quad d = \frac{k_3}{k_3}$$

It may be seen from this equation that when $\frac{(E_i)}{v}$ is plotted against $(AA)$ at constant $(O_2)$ the curve obtained must always be concave upwards (i.e. the slope must always increase as the amino acid concentration increases) and it must approach an asymptote at high amino acid concentration, the slope of which $(d)$ is independent of the oxygen concentration. Plots of $\frac{(E_i)}{v}$ against $(AA)$ for the oxidation of L-leucine and L-methionine in an atmosphere of air and pure oxygen are shown, respectively, in Figs. 6 and 7. It is clear that the conditions required by

⁵ The abbreviations used are: $E$, enzyme; AA, amino acid; IA, imino acid; KA, keto acid; $(E_i)$, total concentration (moles per liter) of enzyme; $v$, initial velocity (moles per liter per minute).

⁶ The derivation of the equations given below is described in detail elsewhere (14).
decrease observed on addition of excess dithionite to either the enzyme (83%), or to free FAD (91% at 400 μM) (15). If the reaction takes place by the mechanism represented by Equations 13 to 16, it would be sufficient to postulate that the dihydroenzyme is reoxidized more rapidly than the completely reduced enzyme (i.e. that $k_3 > k_4$) to account for the inhibition. With amino acids that react very slowly with the enzyme (e.g. L-valine, L-alanine) it may not be possible to obtain concentrations high enough to cause inhibition, unless the oxygen concentration is reduced to a very low level. This hypothesis is consistent with the studies on L-valine (Figs. 3 and 4); thus, inhibition occurred when the oxygen concentration in the gas phase was reduced to 5%.

In order to see whether the mechanism described by Equations 13 to 16 is consistent with the results obtained with L-leucine and L-methionine, a kinetic equation was derived (Equation 17), and the theoretical curves calculated from this equation were compared with the experimental data.

$$\frac{(E_t)}{v} = \frac{a}{(AA)} + \frac{b}{(O_2)} + \frac{c}{(AA) + d(O_2)}$$  \hspace{1cm} (17)

in which $a = \frac{1}{k_1}$, $b = \frac{1}{k_4}$, $c = \frac{1}{k_2} \left(1 - \frac{k_2}{k_1} - \frac{k_3}{k_4}\right)$, and $d = \frac{k_3}{k_2}$. If $k_3 > k_4$, $c$ must be negative, whereas $a$, $b$, and $d$ are positive. A curve calculated from Equation 17 for the oxidation of L-leucine in air is shown in Fig. 1 (lower curve) and Fig. 6 (upper curve). The oxygen concentration was taken as $1.60 \times 10^{-4}$ M (see above), and the values of the constants were taken as: $a = 8.80 \times 10^{-4}$ min M; $b = 14.69$ min M; $c = 0.678$ min M; $d = 351$. These values were selected to obtain the best fit of the experimental data. A curve for L-methionine oxidation in air, calculated with the following values of the constants, $a = 9.73 \times 10^{-4}$ min M; $b = 9.99$ min M; $c = -0.471$ min M; $d = 394$, and the same oxygen concentration is plotted in Fig. 2 (lower curve) and Fig. 7 (upper curve). It may be seen that both curves fit the experimental values rather closely.
According to Equation 17, the velocity of the reaction at very high amino acid concentrations \((\text{AA} \rightarrow \infty)\) should be proportional to the concentration of oxygen. However, this is not the case. When pure oxygen was substituted for air in the gas phase, leading to about a 8-fold increase in the oxygen concentration in the solution, the velocity of the reaction at high concentrations of L-leucine and L-methionine increased only approximately 3-fold. This suggests that oxygen forms a complex with the reduced enzyme before the hydrogen transfer takes place to yield hydrogen peroxide. Kinetic data which are consistent with the formation of such a reduced enzyme-oxygen complex before reoxidation of the flavin have also been obtained by Gutfreund and Sturtevant (16) for xanthine oxidase. Even the reduced forms of free FAD and flavin mononucleotide appear to form such complexes with oxygen (17). Since enzyme-substrate complexes are undoubtedly formed with the amino acids as well, the above mechanism must be modified to include these intermediates. Thus,

\[
E + \text{AA} \xrightarrow{k_1} \text{EAA} \xrightarrow{k_2} \text{EH}_2 + \text{IA}
\]  

\[
\text{EH}_2 + \text{AA} \xrightarrow{k_4} \text{EHH}_2 \xrightarrow{k_5} \text{EH}_4 + \text{IA}
\]

\[
\text{EH}_4 + \text{O}_2 \xrightarrow{k_6} \text{EHH}_4 \xrightarrow{k_7} \text{EHH}_2 + \text{H}_2\text{O}_2
\]

The equation derived from this mechanism may be written in the form:

\[
\frac{(E)}{v} = \frac{a}{(\text{AA})} + b_1 + \frac{b_2}{(\text{O}_2)} + c_1 + c_2 (\text{O}_2)
\]

where:

\[
a = \frac{k_2 + k_3}{k_1 k_3}, \quad b_1 = \frac{1}{k_6 + \frac{k_1}{k_2}}, \quad b_2 = \frac{k_{11} + k_{12}}{k_{10} k_{11}}
\]

\[
c_1 = \frac{k_4 k_5}{k_4 k_5} - \frac{k_4 + k_5}{k_4 k_5} - \frac{k_4 k_9}{k_4 k_9 + k_12(k_9 + k_4)}
\]

\[
c_2 = \frac{k_7 k_9(k_4 + k_6)}{k_4 k_9(k_4 + k_3)}
\]

\[
d = \frac{k_5 k_9(k_4 + k_6)}{k_4 k_9(k_4 + k_3)}
\]

This equation contains six constants the values of which must be known in order to calculate the velocity of the reaction at any amino acid and oxygen concentration. Their values are a function of the affinities of the enzyme for the amino acid and for oxygen, and the rates of the several hydrogen transfers. The constants may be determined by comparing curves calculated from the equation with experimental curves. For any constant value of \((\text{O}_2)\), Equation 22 is equivalent to Equation 17. The constants found to give the best fit of the experimental data obtained for the oxidation of L-leucine and L-methionine are given in Table I. The curves calculated from Equation 22, with the constants given in Table I, are plotted in Figs. 1, 2, 6,
and 7 (upper and lower curves). It may be noted that the calculated curves for air in these figures are identical with those obtained from Equation 17.

It may be concluded from these results that the data are consistent with a mechanism involving the formation of a half-reduced enzyme which is reoxidized by oxygen more rapidly than the fully reduced enzyme. Although the kinetic data provide no information on the exact nature of this intermediate, other studies on the mechanism of the amino acid oxidase reaction suggest that it may be a free radical form of the enzyme. Thus, Beinert (18) observed the transient appearance of a broad absorption band with a maximum near 550 nm during oxidation of L-leucine by L-amino acid oxidase in the presence of air, and concluded that it was due to a free radical intermediate. Free radicals have also been detected directly by electron spin resonance during the reaction catalyzed by D-amino acid oxidase and other flavoproteins (19). In view of the known reactivity of oxygen towards free radicals, such an intermediate may be expected to be reoxidized more rapidly than the fully reduced enzyme.

The following mechanism, based on the above considerations, is tentatively suggested. The amino acid forms a complex with the enzyme and donates two hydrogen atoms, one to each FAD, yielding a dihydroydenzyme, and an imino acid which is hydrolyzed to ammonia and the corresponding keto acid (Fig. 8, steps 1, 2, and 3). This half-reduced enzyme is rapidly reoxidized by oxygen (steps 4 and 5). In the absence of oxygen, or in the presence of high concentrations of amino acid, a second amino acid molecule reacts with the half-reduced enzyme to yield a fully reduced enzyme (steps 6, 7, and 8), which reacts slowly with oxygen (steps 9 and 10). It is also possible that oxygen may react with the fully reduced enzyme by removing two hydrogen atoms from the same FAD (step 11) to yield an enzyme containing one reduced and one oxidized FAD. This possibility is considered in view of the results reported earlier (10) that demonstrated the formation of an intermediate on reoxidation of the fully reduced enzyme by small amounts of oxygen. If the amino acid always donates one hydrogen atom to each FAD, the intermediate formed in step 11 would not be expected to be reduced by amino acid, because one FAD is already fully reduced. The intermediate reported previously (10) has the properties to be expected of such a form of the enzyme; thus, it is not reduced by amino acids, but it may be reduced by dithionite. Furthermore, it reacts with oxygen to yield the fully oxidized enzyme. Although one might expect the spectrum of such a form of the enzyme to be the average of that of the fully oxidized and fully reduced enzyme, provided the two prosthetic groups do not interact, it seems probable that if they were in close proximity, the spectrum would be considerably affected.

The mechanism from which Equation 22 has been derived includes only one pathway for the reoxidation of the fully reduced enzyme (Fig. 8, steps 9 and 10). Inasmuch as the fully reduced enzyme could be reoxidized, alternatively, by another pathway (Fig. 8, steps 11, 12, and 13), or perhaps by both pathways, it may be asked whether these possibilities may be distinguished kinetically. Kinetic equations have been derived for these two additional possible mechanisms, and it has been found that each has the form of Equation 22. These three possibilities are therefore not distinguishable by a steady state kinetic analysis.

Fig. 8. Tentative scheme for the mechanism of L-amino acid oxidase.

It has been shown above that the mechanism suggested in Fig. 8 is consistent with the available kinetic and spectrophotometric data concerning L-amino acid oxidase. However, it is evident that no mechanism can be unambiguously proven by kinetic data alone. The final proof of such a mechanism must depend on more direct identification or isolation of the intermediates involved, and a detailed knowledge of the structure of the enzyme.

**SUMMARY**

These studies were undertaken in an effort to elucidate the striking substrate inhibition of the L-amino acid oxidase reaction observed with certain amino acids and the previously reported evidence for an intermediate formed in the reoxidation of the reduced flavoprotein. A kinetic study of the reaction was carried out with L-leucine, L-methionine, and L-valine in the presence of various concentrations of oxygen. In air or in oxygen, L-leucine and L-methionine, in concentrations above 0.01 M, inhibited the reaction markedly, whereas little or no inhibition was observed with L-valine. With 5% oxygen, L-valine also produced inhibition above 0.01 M. The kinetic data are consistent with a mechanism involving a two-step reduction of the enzyme by the amino acid. We propose that the enzyme, which possesses two prosthetic groups, reacts with a molecule of amino acid to yield a half-reduced enzyme. This may react with oxygen or reduced further by another molecule of amino acid. The inhibition is accounted for if the half-reduced intermediate reacts with oxygen more rapidly than the fully reduced enzyme. This transient intermediate may be similar to the free radical suggested by other investigators as an intermediate in this and other flavoprotein-catalyzed reactions. The intermediate we reported previously, which exhibits a characteristic spectrum, is formed on reoxidation of the fully reduced enzyme, and is different from that formed on reduction of the enzyme by the amino acid. It may be a form of the enzyme containing one reduced and one oxidized flavin. Such an interpretation appears to be consistent with all of the available data.
REFERENCES

14. Wellner, D., Doctoral dissertation, Department of Biochemistry, Tufts University School of Medicine, 1960.
CORRECTIONS

In the paper by Bruce N. Ames, Robert G. Martin, and Barbara J. Garry on page 2025, Vol. 236, No. 7, July 1961, the third paragraph under “Discussions” should read “The inhibition of the phosphorylase by low concentrations of histidine etc.”

In the paper by Daniel Wellner and Alton Meister on page 2359, Vol. 236, No. 8, August 1961, in some copies of the issue the first letter of the second and third lines of the legend to Fig. 2 are missing. These should read “$L$-methionine” and “calculated.”

In the Preliminary Communication by Eraldo Antonini, Jeffries Wyman, Romano Zito, Alessandro Rossi-Fanelli, and Antonio Caputo on page PC69, Vol. 236, No. 9, September 1961, lines 1, 11, and 12, “hydroxyl-terminal” should read “carboxyl-terminal.” On page PC62 in the legend to Fig. 1, the last line should read “8.2 to 11.” On page PC63, the paragraph on “Observations on Myoglobin” should read “When horse myoglobin etc.” Also on page PC63, reference 8 should read “FOLK, J. E., PIEZ, K. A., CARROLL, W. R., AND GLADNER, J. A., J. Biol. Chem., 235, 2972 (1960).”
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