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L-AMINO-ACID OXIDASE

I. EFFECT OF pH

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SUMMARY

The effect of pH on K_m and upon the rate constants associated with the reduction of oxidized enzyme by substrate and with the oxidation of reduced enzyme has been examined for the case of purified L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) of Eastern Diamondback Rattlesnake *(Crotalus Adamanteus)*, using L-leucine as a substrate at 25°, 30° and 35°.

The effect of pH on K_m yields two pK's: one in the range 5.7–5.9 corresponding to the enzyme–substrate (ES) intermediate and one in the range 8.2–8.4 corresponding to the free enzyme. The variation of the two sets of pK's with temperature both yield an apparent enthalpy of ionization of 7.5 kcal/mole. These data lead to the conclusion that histidyl is most likely involved in the catalysis at the active center.

The variation of the reduction rate constant k_{obs} with pH parallels the variation of K_m with pH, indicating that the variation of k_{obs} with pH depends on the variation in the availability of ES intermediate with pH.

The oxidation rate constant, k_{ox}, does not vary appreciably with pH, suggesting that the oxidation process proceeds *via* a direct combination of oxygen and reduced form or forms of the enzyme without the mediation of catalytic groups which may be titrated in the range of pH studied.

INTRODUCTION

The effect of pH on the kinetic parameters of an enzyme–substrate system is an important area of study, since it may enable one to gain some insight into the nature of the protein groups participating in the catalytic interactions at the enzyme active center.

L-Amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) is an important flavin enzyme suitable for the investigation of the effect of pH on appropriate kinetic parameters and is readily available in purified form.

The reaction whose pH dependence is being considered is shown in Eqn. 1, where $[E]_{ox}$ and $[E]_{red}$ are oxidized and reduced forms of the flavoprotein enzyme, respectively.

$$[E]_{ox} + \text{L-leucine} \rightleftarrows \text{intermediate state(s)} \rightleftarrows [E]_{red} + \text{product} \quad (1)$$

Because the enzyme exhibits substrate inhibition, it is necessary to perform such studies of pH dependence at substrate concentrations where inhibition is not encountered[1]. Eqn. 1 represents the sequence in the action mechanism which is rate limiting under the conditions of low substrate concentration where substrate inhibition does not occur. The steps in the reaction are written as equilibria since the overall reaction has been shown to be reversible[2]. The classical *ES* complex is termed an intermediate state in order not to imply any specific number of kinetically distinct bound forms of enzyme and substrate.

MATERIALS AND METHODS

L-Amino-acid oxidase was obtained in purified form from Worthington Biochemical Corp. Purified L-leucine was obtained from Schwartz Bioresearch Corp. The buffers used were 0.05 M Tris–maleate for pH 5.3–8.8 and 0.05 M glycine–NaOH for pH 9.0–9.6. All solutions were 0.2 M in KCl. The pH of all buffers was determined as a function of temperature using a Sargent Model DR pH meter.

The concentration of active enzyme, $[E]_0$, was initially determined using a Cary 14 spectrophotometer by measuring the absorbance difference at 450 nm between the completely oxidized and completely reduced forms of the enzyme arising from the addition of an amount of 0.1 M leucine in great excess of the stoichiometric amount under anaerobic conditions. An approximate (maximal) molar absorbance coefficient for the absorbance difference between oxidized and reduced forms of the enzyme was taken to be $2.2 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 450 nm, assuming two FAD groups per active enzyme molecule[3]. The total active enzyme concentration determined in this manner was related to the slope of the straight line obtained at each temperature studied when $\log [O_2]_t$ was plotted *vs.* t for the reaction of enzyme and 0.100 M leucine in Tris–maleate buffer (pH 7.4) initially saturated with oxygen. Under these conditions the overall kinetics are very nearly first-order in oxygen and constitute a convenient way to determine the active enzyme concentration before each experiment with reference to that of the spectrophotometrically standardized enzyme. This procedure was necessary because the concentration of active enzyme decreased slowly from day to day during storage at 4°.

The rate of oxygen uptake was measured using a biological oxygen monitor (Yellow Springs Instrument Co.). The total volume of reaction mixture was 3 ml. Leucine solutions in buffer were saturated with oxygen at 25°, 30° or 35°. At pH values less than 6.5, the reaction was sufficiently slow that it was necessary to correct the primary data for oxygen leakage from the cell assembly of the oxygen monitor and for oxygen consumption by the polargraphic oxygen sensor.

The enzyme preparations used did not contain any detectable amount of catalase. Experiments were performed at several pH values using $8.0 \cdot 10^{-4}$ M leucine dissolved in buffer solutions initially saturated with oxygen at 25°, 30° and 35°. At all three temperatures, the initial concentration of oxygen was greater than the initial substrate concentration. It was found that, within experimental error, a stoichiometric amount of oxygen was consumed during the course of the reaction. If catalase were present in significant amount, then the amount of oxygen consumed would have been less than $8.0 \cdot 10^{-4}$ M.

All velocity measurements were made at an oxygen percent saturation corresponding to $8.6 \cdot 10^{-4}$ M at each temperature. K_m values were determined from Lineweaver–Burk plots. The substrate concentrations were chosen to give the best intervals of $1/[S]$ in the range where substrate inhibition did not occur. This range varied from 0.1 M or less (pH 5.3) to $1.5 \cdot 10^{-3}$ M or less (pH 9.6).

The apparent enthalpy of ionization corresponding to each set of pK values was calculated from a simple Van 't Hoff plot of log K *vs.* $1/T$ by multiplying the slope by the factor $-2.3\,R$.

The second-order forward rate constant, k_{obs}, associated with the reduction of oxidized enzyme by leucine was determined from experiments in which the initial substrate concentration was nearly equal to the initial oxygen concentration. Assuming the active enzyme concentration, $[E]_0$, is approximately equal to the steady-state concentration of the oxidized form of the enzyme, a plot of ln $[S]_t$ *vs.* t gives a straight line with slope $-k_{obs}\,[E]_0$, where $[S]_t = [S]_{t=0} - ([O_2]_{t=0} - [O_2]_t)$.

The second-order forward rate constant, k_{ox}, associated with the overall reaction at substrate concentrations of approx. 0.05 M or greater where the reoxidation of the reduced form or forms of the enzyme determines the overall reaction rate, was determined at 38° using 0.1 M leucine in buffers initially saturated with oxygen. Recent work by MASSEY AND CURTI[4] suggests that substrate inhibition in this system can be explained by invoking an oxidation step of a reduced enzyme–amino acid complex which is slower than the reoxidation of other reduced enzyme species. If such is the case, then k_{ox} would correspond to the specific rate of the reoxidation of this reduced enzyme–amino acid species. At pH values greater than 6.5, a plot of ln $[O_2]_t$ *vs.* t was initially linear. At pH values less than 6.5, the first-order plots exhibited a downward curvature, and the tangent to the curve at time zero was taken as an estimate of the slope for the purpose of comparison with values at pH values greater than 6.5. The slope of the first-order plot at each pH was taken to be equal to $-k_{ox}\,[E]_0$.

The rate constants, k_{obs} and k_{ox}, are second-order rate constants in that they correspond to the reaction of enzyme *plus* substrate, either leucine or oxygen, respectively. Since the total enzyme concentration is a constant during the course of a given experiment, the overall kinetics appear to be pseudo-first-order in substrate.

RESULTS

Effect of pH on K_m

Figs. 1, 2 and 3 show the effect of pH on pK_m for leucine at 25°, 30° and 35°, respectively. Each solid curve was calculated using Eqn. 2 (ref. 5) and pK values which gave the best agreement with the experimental observations.

$$pK_m = \text{constant} + \log\left[1 + \frac{H^+}{K_{ES}}\right] - \log\left[1 + \frac{H^+}{K_E}\right] \qquad (2)$$

Table I tabulates these pK values. Useful data beyond pH 9.6 were not obtainable under these experimental conditions, since the enzyme rapidly loses activity at higher pH. At pH values lower than 5.3 the reaction rate becomes too slow to be measured accurately with the apparatus used.

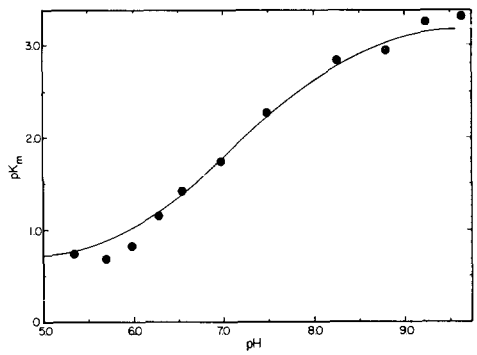

Fig. 1. pH dependence of $-\log K_m$ or pK_m of the L-amino-acid oxidase catalyzed reaction of L-leucine at 25°. Each point was obtained from a Lineweaver–Burk plot.

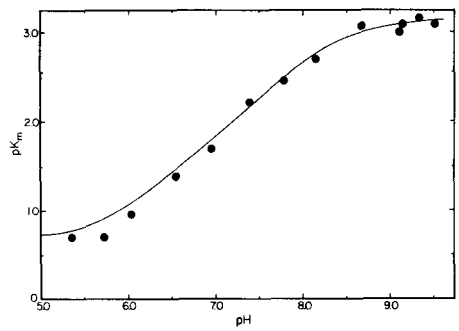

Fig. 2. pH dependence of pK_m of the L-amino-acid oxidase catalyzed reaction of L-leucine at 30°. Each point was obtained from a Lineweaver–Burk plot.

Effect of pH on k_{obs}

The effect of pH on the forward rate constant of the reaction studied is shown in Fig. 4 for the three temperatures used. Within the precision of k_{obs}, the curves are parallel and have the same general features of the pK_m *vs.* pH curves.

Effect of pH on k_{ox}

Fig. 5 shows the variation of k_{ox} with pH. Within the pH range studied there seems to be little change in the second-order rate constant associated with the oxidation of reduced enzyme.

TABLE I

pK VALUES DETERMINED KINETICALLY WITH PURIFIED L-AMINO-ACID OXIDASE AND L-LEUCINE AT 25°, 30° AND 35°

The given pK values are considered accurate to ± 0.10 pK unit. Values beyond this latitude did not permit a satisfactory theoretical representation of the experimental data. Moreover, the pK_m *vs.* pH curves indicate a consistent displacement of 0.1 pK unit for each 5° temperature interval, thus giving added weight to the accuracy of the calculated enthalpy of ionization.

	pK values at		
	25°	*30°*	*35°*
ES intermediate	5.90	5.80	5.70
Free enzyme	8.40	8.30	8.20

DISCUSSION

A theoretical treatment of the effect of pH on enzyme–substrate affinity has been discussed in detail by DIXON[6] and by DIXON AND WEBB[7]. According to theory, the observed pH effect on K_m and the maximal velocity V can be interpreted in terms of the pK values of groups situated in the free enzyme, in the free substrate and in the enzyme–substrate intermediate state *ES*. The theory of DIXON states that a plot of

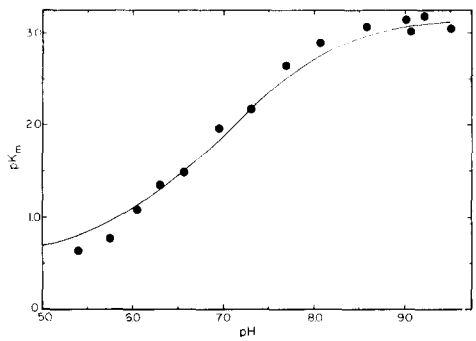

Fig. 3. pH dependence of pK_m of the L-amino-acid oxidase catalyzed reaction of L-leucine at 35°. Each point was obtained from a Lineweaver–Burk plot.

pK_m *vs.* pH will be composed of straight line segments having integral slopes connected by short curved segments which indicate the pK of an ionizing group in one of the components of the components of the system. An upward bend in the curve is produced by each pK of a group situated in the *ES* intermediate. Each pK of a group in the free enzyme or free substrate produces a downward bend.

The graphs of pK_m *vs.* pH exhibit changes generally as predicted by theory; the calculated curves (solid lines in Figs. 1–3) describe the behavior of the experimental data quite well. The upward bend in acid pH for each of the curves corresponds to the pK of a group situated in the *ES* intermediate. The experimental curve appears to break more sharply at acidic pH values than is predicted theoretically for a simple

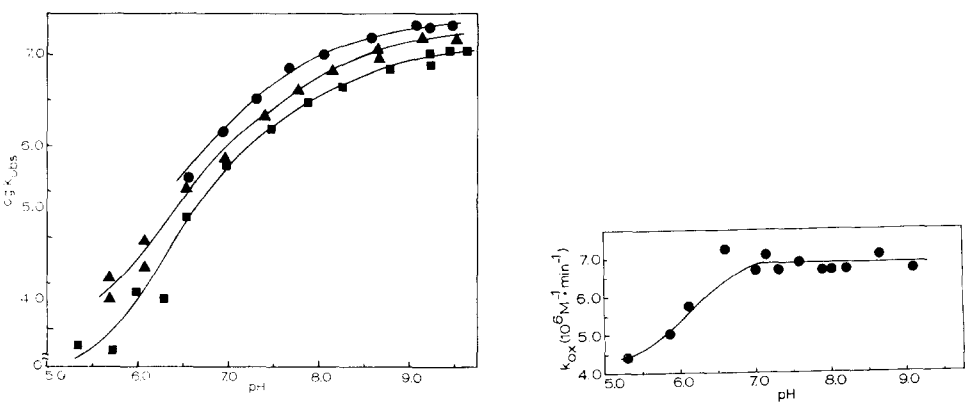

Fig. 4. pH dependence of log k_{obs}, the second-order forward rate constant associated with the reduction of oxidized L-amino-acid oxidase by L-leucine at 25° (■—■), 30° (▲—▲) and 35° (●—●). Each point at 25° was determined from the slope of a plot of ln $[S]_t$ *vs.* t determined from oxygen uptake data using $1.2 \cdot 10^{-3}$ M leucine in buffers initially saturated with oxygen at 25° ($[O_2]_0 = 1.2 \cdot 10^{-3}$ M). Data at 30° and 35° were determined in a similar manner (see MATERIALS AND METHODS) using $1.0 \cdot 10^{-3}$ M leucine in buffers initially saturated with oxygen at the temperature studied.

Fig. 5. pH dependence of k_{ox}, the pseudo-second-order rate constant associated with the oxidation of reduced L-amino-acid oxidase at 38°. Each point was obtained from the slope of a plot of ln $[O_2]_t$ *vs.* t as determined from oxygen uptake data using 0.10 M L-leucine in buffers initially saturated with oxygen at 38°.

ionization of a single group. This might be due to the influence of other charged groups near the group responsible for the measured pK.

The variation of these pK values with temperature yield an enthalpy of ionization of 7.5 kcal/mole for the group situated in the ES intermediate. The unit slope of the rising straight segments of the curves indicates a $+1$ change of charge when the ES intermediate dissociates into free enzyme and free substrate. This process would correspond to: $(ES)^n \rightleftarrows E^{n+1} + S$. Since leucine would be in the zwitterionic form throughout the pH range under consideration and since the free enzyme appears to have a pK at 8.2–8.4, the example above is a reasonable representation of the actual process of dissociation.

The downward bend in basic pH corresponds to the pK of a group in the free enzyme. These pK values yield an enthalpy of ionization of 7.4 kcal/mole. It is unlikely that these pK values are associated with an ionizable group of free leucine since they are more than one pK unit too low for the pK of the amino acid ammonium group (which is 9.6). Furthermore, the enthalpy of ionization of such an ammonium group is on the order of 11 kcal/mole, which is significantly greater than that which is observed in the present case[8]. Curves of the nature of Figs. 1–3 generally correspond to an ionizing group not directly involved in the bonding between enzyme and substrate in the ES intermediate, whose pK value in the free state is perturbed to a different pK value in the different environment of the ES intermediate. In the present case, the ionizing group in the ES intermediate has a pK of 5.7–5.9. As the intermediate dissociates to give free enzyme and free substrate in the pH range 6–8, the pK_m *vs.* pH curves indicate that a group of the free enzyme gains a positive charge. The group of the free enzyme corresponding to the pK values of 8.2–8.4 is most likely this group. After being freed from the influence of substrate, the pK values of 8.2–8.4 would render this group capable of gaining a positive charge in the pH range of 6–8. Therefore, the two sets of pK values observed in the present case most likely correspond to the same group in different environments.

Although the observed ΔpK of 2.5 units between pK_{ES} and pK_E is large, it is not unreasonable to assign the same group in different charge environments as being responsible for both. Since the enzyme exhibits reversible inactivation in the absence of mono-negative anions, it was necessary to perform all experiments in the presence of 0.2 M Cl^-. It is conceivable that chloride binding at the active site could account for the rather high pK assigned to histidyl in the free enzyme active center, the effect of the negatively charged species being to increase the pK of the imidazole group of histidyl. The proximity of the dipolar amino acid zwitterion to the imidazole side chain of a histidyl residue in the ES intermediate could have a profound effect on the pK of the imidazole group in comparison to the same group in the free enzyme. It is well known that the adjacency of a positively charged body to an ionizable group can lower its pK_a drastically. For example, the pK_a of free imidazole has been determined[9] to be 6.95. Substitution of an 2-ethylammonium side chain in the 4(5) position results in a depression[10] of the pK_a to 5.78*.

The pK values of 5.7–5.9 for a group in the ES intermediate and of 8.2–8.4 for a group in the free enzyme, together with the fact that both groups exhibit apparent enthalpies of ionization of 7.5 kcal/mole, all lead to the conclusion that histidyl is a

* A differentiation between electrostatic and inductive effects is not possible with these data but it is worth noting that the pK_a of 4(5)-methylimidazole is perturbed upwards[9] to 7.52.

group which is probably involved with the catalytic interactions at the active center. From a consideration of the reversible inactivation process exhibited by L-amino-acid oxidase, KEARNEY AND SINGER[11] have postulated earlier that histidyl might be present at the active center. The present study serves to strengthen this hypothesis.

DE KOK AND VEEGER[12] recently found a group with an apparent pK of 7.8 in the free form of L-amino-acid oxidase which participates in the binding of the inhibitor *o*-aminobenzoate. They suggested the group might be a histidyl group. In view of the close similarity between the pK value which they observed and those reported for the free enzyme in the present work, it is probable that the same group, possibly a histidyl, is involved in the binding of the inhibitor and of the substrate.

The variation of log k_{obs} with pH, as shown in Fig. 4, indicates that the specific rate of the overall reaction parallels the availability of the intermediate form of enzyme and bound substrate as a function of pH. This suggests that effects on binding rather than base catalysis may explain the overall pH dependence of the forward reaction (Eqn. 1), but this point deserves further investigation.

The variation of k_{ox} with pH, as shown in Fig. 5, shows that the oxidation process in the action mechanism seems to be insensitive to changes in pH. The slight downward trend in the curve at low pH is probably a manifestation of the fact that the absolute rate of the reduction step becomes less than that of the oxidation process due to the great decrease in k_{obs} at the low end of the pH range.

The application of the theory of DIXON appears to successfully explain the pH behavior of the L-amino-acid oxidase–L-leucine kinetic system. In view of the variable pH dependence of the D-amino-acid oxidase system with different substrates[13], it should be noted that there is a possibility that the present results may not necessarily represent the pH behavior of L-amino-acid oxidase with amino acids other than leucine. In particular, one might not expect the value of pK_{ES} to be the same with substrates other than leucine. However, the probable involvement of a histidyl residue in the binding of at least one substrate leads to additional interesting experiments on solvent isotope effects, on histidine photooxidation experiments and with possible specific irreversible inhibitors. Further studies are in progress on this system to investigate the nature of the elementary processes involved in the reduction of oxidized enzyme by substrate.

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