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L-AMINO ACID OXIDASE

II. DEUTERIUM ISOTOPE EFFECTS AND THE ACTION MECHANISM FOR THE REDUCTION OF L-AMINO ACID OXIDASE BY L-LEUCINE

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SUMMARY

A study was made of the effect of p^2H and p^2H upon the rate of reduction of L-amino acid oxidase (L-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.2) by L-leucine and by DL-[α-^{2}H]leucine. The reduction process was studied with the system in turnover using a Clark oxygen electrode and also with the anaerobic combination of enzyme and substrate using stopped-flow techniques. A striking pH-dependent deuterium kinetic isotope effect is observed which is consistent with a change in rate determining step as the pH is changed over the range 6 to 9. A deuterium solvent isotope effect is also observed and is shown to be the result both of an upward shift of 0.6 unit in the pK_a of an active site group and of additional effects on the specific rates of the component steps of the reduction process. The experimental results are consistent with the interpretation that a proton transfer step is mediated by a basic group on the enzyme during the reduction reaction. The basic group is tentatively identified as a histidyl residue. The reduction process is seen to involve the very rapid formation of a transient, spectrophotometrically distinct intermediate followed by the rapid decay of this intermediate into the fully reduced enzyme, consistent with earlier reports. In addition to the rapid decay, a much slower decay is noted which may be due to the dissociation of an imidazolium ion in the hindered environment of the active site to form the catalytically active charge form of the enzyme–substrate complex, or to the presence of isoenzymes possessing different catalytic specificities. An action mechanism is presented which is consistent with the experimental data for the reduction reaction.

INTRODUCTION

Recently we reported some of the results of our studies of the pH dependence

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of the L-amino acid oxidase-catalyzed reaction of L-leucine[1]. These studies led to the conclusion that the basic form of an ionizable group at the active center of the enzyme participates in the interaction between oxidized enzyme and leucine. The pK and enthalpy of ionization of this group were consistent with the identification of this group as the imidazole side-chain of a histidyl residue. We have also communicated some preliminary results of our studies of the striking pH- and p^{2}H-dependent deuterium isotope effects in this system[2]. PORTER AND BRIGHT[3] have described some preliminary results of their study of the hydrogen transfer steps in the reduction of oxidized L-amino acid oxidase by L-phenylalanine. They found that this system can be described (at a given pH) by the sequence shown in Scheme 1. In Scheme 1, E_{ox}

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} ES_{540} \overset{k_3}{\rightarrow} E_{red}$$

Scheme 1. Sequence for the reduction of L-amino acid oxidase by phenylalanine according to PORTER AND BRIGHT[3].

and E_{red} are the oxidized and fully reduced forms of the enzyme, respectively and ES_{540} is a transient, spectrophotometrically distinct intermediate[4,5]. Using DL-[α-^{2}H]-phenylalanine as a substrate they found that the α-hydrogen transfer from substrate to enzyme occurs during the formation of ES_{540}. The purpose of the present work is to examine the action mechanism of the reduction reaction in detail by examining the effects of pH, of p^{2}H, and of deuterium substitution in the substrate upon the kinetics of the reduction process in order to determine if possible the catalytic interactions occurring during the hydrogen transfer step and other steps in this process.

MATERIALS AND METHODS

L-Amino acid oxidase used in this study was obtained in part from Worthington Biochemical Corp. in purified form, and in part was purified by the method of WELLNER AND MEISTER[6] from *Crotalus adamanteus* venom obtained from Sigma Chemical Corp. (Type I). Identical results were obtained with both enzyme preparations. The determination of the concentration of active enzyme was carried out as described earlier[1]. Purified L-leucine was obtained from Schwartz Bioresearch Corp. DL-[α-^{2}H]-leucine was synthesized by hydrolysis of ethyl α-carbethoxy-α-cyano-γ-methylpentanoate[7] in a 38% ^{2}HCl solution (99% deuterium content; Stohler Isotope Chemicals). Analysis of the recrystallized product by NMR showed the presence of 8.5% DL-α-[^{1}H]leucine. All buffers used were 0.2 M in KCl; 0.05 M Tris–maleate was used in the pH (p^{2}H) range 5.5–8.8; 0.05 M glycine–NaOH was used in the pH (p^{2}H) range 9.0–9.5. The relationship[8] $p^2H = pH_{meter} + 0.40$ was used in the preparation of buffers in ^2H_2O. Deuterium oxide of 99.5% isotopic purity was obtained from Columbia Chemical Co. and was glass-distilled before use.

Turnover experiments were performed by measuring the rate of oxygen uptake of enzyme and substrate solutions in buffers initially saturated with oxygen before the addition of enzyme using a Yellow Springs Instrument Co. Biological Oxygen Monitor. All rate determinations were made at an oxygen percent saturation corresponding to $8.6 \cdot 10^{-4}$ M. K_m values were determined from Lineweaver–Burk plots. Under conditions such as those employed in this study the K_m for L-leucine is known to be independent of oxygen concentration[5]. The method of determining k_{obs}, the

overall specific rate of the reduction sequence has been given elsewhere[1]. Both K_m and k_{obs} values were determined from experiments in which the substrate concentrations were less than those at which substrate inhibition is encountered at each pH value.

Stopped flow studies were performed at 15° using a Durrum–Gibson stopped-flow spectrophotometer. In agreement with the earlier work of BEINERT[4], MASSEY AND CURTI[5] have shown that when oxidized enzyme and substrate combine anaerobically a transient intermediate is formed during the course of the reduction process which, unlike the fully oxidized or fully reduced forms of the enzyme, absorbs light at 540 nm; this intermediate corresponds to ES_{540} in Scheme 1. In the present study, the solutions of substrates in buffers were deoxygenated by repeated degassing and equilibration with high purity nitrogen after which traces of oxygen were consumed by making each solution $1 \cdot 10^{-8}$ M in glucose oxidase (EC 1.1.3.4; Mann Biochemical Corp.) and $1 \cdot 10^{-3}$ M in β-D-glucose. Enzyme solutions in 0.2 M KCl could not be degassed satisfactorily due to frothing and were, therefore, deoxygenated by making each solution $1 \cdot 10^{-8}$ M in glucose oxidase and $1 \cdot 10^{-3}$ M in β-D-glucose[3]. Both enzyme and substrate solutions so treated were placed in syringes, the ends of which were sealed by miniature valves (Hamilton Co.), and allowed to remain at 4° for 12 h under nitrogen. The contents of the syringes were further protected from oxygen diffusion by nitrogen-filled plastic bags secured so that only the valve-end of the syringe protruded. The concentration of active enzyme used in the stopped-flow experiments ranged from $8.5 \cdot 10^{-6}$ to $1.13 \cdot 10^{-5}$ M. The pH (p^2H) of the solutions were determined after mixing using a Sargent model DR pH meter.

RESULTS

The results of the turnover experiments will be given first.

Effect of p^2H upon K_m

Fig. 1 shows the effect of p^2H and pH upon K_m using non-deuterated leucine. The dashed curve gives the corresponding curve in H_2O as reported previously. It is clear that the pK values of the ionizable group at the active center are shifted upward in ^2H_2O. Table I gives these pK values in H_2O and ^2H_2O. The experimental points for ^2H_2O were fitted with a curve of the same shape as for the data in H_2O.

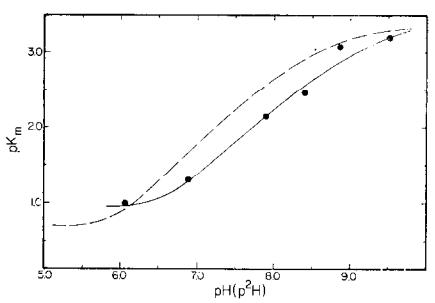


Fig. 1. pH dependence of pK_m for the L-amino acid oxidase catalyzed reaction of L-[α-^{1}H]leucine at 25° in H_2O (— — —) and in ^2H_2O (●—●).

TABLE I

pK VALUES OF A CATALYTICALLY IMPORTANT IONIZABLE GROUP OF L-AMINO ACID OXIDASE IN H_2O AND IN ^2H_2O AT 25°

Solvent	pK_{ES}	pK_E
H_2O	5.9	8.4
^2H_2O	6.5	9.0

Fig. 2 shows the effect of pH and p^{2}H on K_m using DL-[α-^{2}H]leucine giving also the same curve for non-deuterated leucine in H_2O for reference. The effect of pH (p^{2}H) upon K_m when measured using deuterated substrate may be seen to parallel that

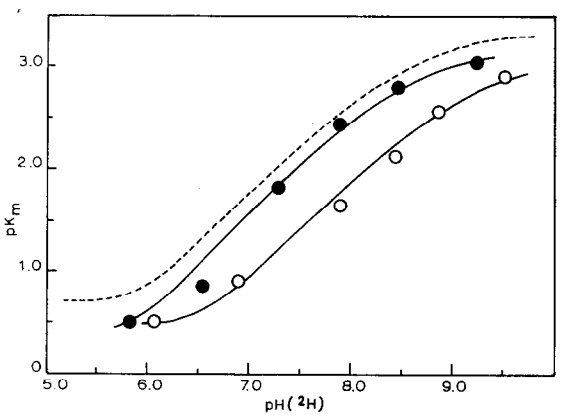


Fig. 2. pH dependence of pK_m for the L-amino acid oxidase catalyzed reaction of DL-[α-^{2}H]leucine at 25° in H_2O (●—●) and in ^2H_2O (○—○). The dashed curve corresponds to the same data for L-[α-^{1}H]leucine in H_2O at 25°.

obtained with non-deuterated substrate except that the Michaelis constant K_m for deuterated substrate is lower at any given pH value; this may reflect either a true binding effect (if $K_m = K_s$) or the complex kinetic nature of K_m.

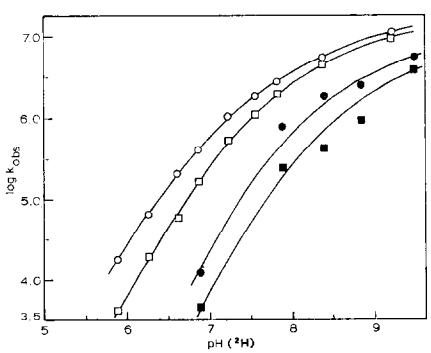


Fig. 3. pH dependence of log k_{obs} for the L-amino acid oxidase catalyzed oxidation of L-[α-^{1}H]leucine in H_2O (○—○) and in ^2H_2O (●—●) at 25°, and of the oxidation of DL-[α-^{2}H]leucine in H_2O (□—□) and ^2H_2O (■—■) at 25°. Points are determined as described in the text.

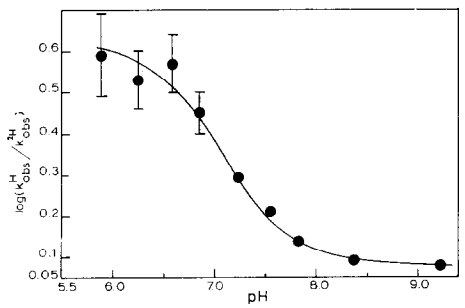


Fig. 4. The pH dependence of the deuterium kinetic isotope effect on k_{obs} for the L-amino acid oxidase catalyzed oxidation of L-[α-^{1}H]leucine and DL-[α-^{2}H]leucine in H_2O at 25°. The larger relative errors associated with the data at low pH values are due to the low overall reaction rate at these pH values, particularly when deuterated substrate was used.

The pH- and p^{2}H-dependence of k_{obs}

Fig. 3 shows the pH (p^{2}H) dependence of k_{obs} for both deuterated and non-deuterated substrate at 25°. The interesting feature of this graph is that both the deuterium kinetic isotope effect and the deuterium solvent isotope effect are strongly pH- and p^{2}H-dependent. Fig. 4 shows the pH dependence of the deuterium kinetic isotope effect as expressed by the ratio: $k^{H}_{obs}/k^{^2H}_{obs}$.

Stopped-flow experiments

Fig. 5 shows the absorbance change at 540 nm when enzyme and substrate combine anaerobically under a number of different experimental conditions. These curves are characterized by a very rapid initial formation of an intermediate and the subsequent rapid decay of this intermediate into the fully reduced form of the enzyme.

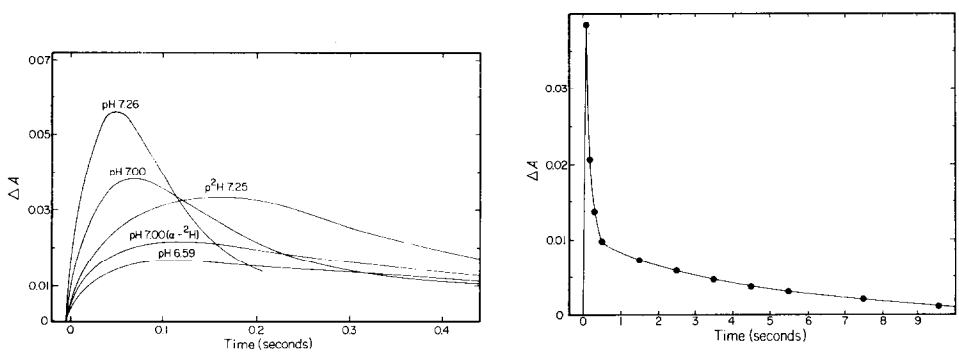


Fig. 5. The change in absorbance at 540 nm *vs.* time for L-amino acid oxidase and L-leucine or DL-[α-^{2}H]leucine interacting anaerobically in a Durrum-Gibson stopped-flow spectrophotometer at 15°. The pH (or p^{2}H) of the reaction mixture is labelled over the corresponding curve. The substrate used was 0.01 M L-leucine for all curves except the curve labelled "(α-^{2}H)", where the concentration of substrate was 0.01 M in the L-isomer. Since several of these curves were obtained using different active enzyme concentrations, the absorbance values used to plot the curves were normalized to a common active enzyme concentration.

Fig. 6. The change of absorbance at 540 nm graphed as a function of time for the anaerobic interaction of L-amino acid oxidase and L-leucine at 15° and pH 7.00. This curve is analogous to the corresponding curve in Fig. 5 except that the time scale is increased to illustrate the slow first order decay of the transient intermediate.

Fig. 6 shows data similar to Fig. 5 except that the time scale is chosen to show much slower rate processes. Here it is quite apparent that a slow decay process is occurring in addition to the rapid decay. All of the data corresponding to the formation of the first 30% of the transient intermediate was analyzed using a Hewlett-Packard 2115A digital computer by an iterative method since the infinity absorbance value for this process had to be estimated. This iterative method varied the differences between an estimated A_∞ value and the experimental A_t values until the best linear first-order plot of $(A_\infty - A_t)$ *vs.* t was obtained as determined by a simple linear least-squares method. The initial estimate of A_∞ was obtained from a consideration of the relative amount of the active charge form of *ES* as calculated from an approximate pK_{ES} value at 15° of 6.1 in H_2O and 6.7 in ^2H_2O (estimated from previous data[1] and as determined in the present work), and of a molar absorptivity for the 540 nm transient intermediate of $8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ as estimated from the data of MASSEY AND CURTI[5]. A first-order plot of the data corresponding to the decay of the intermediate exhibited two linear segments, corresponding to a rapid decay and to a slow decay, at all pH and p^{2}H values noted in Table II except pH 7.87. A first-order plot of the slow decay

TABLE II

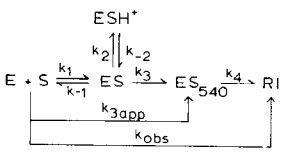
THE VALUES OF THE RATE CONSTANTS OF THE KINETICALLY ACCESSIBLE STEPS OF SCHEME 2

The method of evaluating the rate constants from the primary data has been described in the text (see RESULTS). The quantity k_{3app} is the apparent specific rate of formation of ES_{540} (see Scheme 2) and approaches k_3 in the limit of infinite substrate concentration. All of the rate constants given here correspond to a substrate concentration of 0.01 M in the L-form of leucine.

Substrate	*pH (p^{2}H)*	*k_{-2app} (sec^{-1})*	*k_{3app} (sec^{-1})*	*k_4 (sec^{-1})*
L-Leucine	pH 6.59	0.36	3.4	2.6
	7.00	0.28	16.6	8.6
	7.26	0.18	29.0	16.0
	7.87	—	82.0	23.0
DL-[α-^{2}H]Leucine	pH 7.00	0.25	5.7	5.3
	7.87	—	39.0	16.0
L-Leucine	p^{2}H 7.02	0.22	2.7	2.8
	7.25	0.20	7.9	6.1

data yielded the first-order rate constant for this process directly. The slow decay first-order plot was extrapolated to time zero and the slow decay absorbance contribution was subtracted from the overall absorbance values at each time point in the time range of the rapid ES_{540} decay. The data which resulted gave good first-order plots, thus allowing the calculation of the rate constants corresponding to the rapid decay of ES_{540} into the fully reduced enzyme.

Scheme 2 shows an action mechanism consistent with the data presented here



Scheme 2. Sequence for the reduction of L-amino acid oxidase by L-leucine.

for the reduction of L-amino acid oxidase by L-leucine. In Scheme 2, E is the oxidized form of the enzyme, RI is the complex between fully reduced enzyme and imino acid, ES is the initial enzyme-substrate complex, and ES_{540} is the spectrophotometrically distinct transient intermediate. The fully reduced form of the enzyme is given as a reduced enzyme–imino acid complex in accord with the work of MASSEY AND CURTI[5]. This mechanism is consistent with the data obtained for the pH range of 6.5 to 7.9, where the system was studied in detail. The equilibrium described by k_1/k_{-1} was reached at too rapid a rate to be measured experimentally. The apparent slow decay of ES_{540} may be interpreted as arising from the dissociation of an inactive charge form (ESH^+) to give the active charge form (ES) which can then proceed to form the fully reduced enzyme (see DISCUSSION). The acid–base equilibrium described by k_2/k_{-2} is considered to correspond to a pK value of 6.1 at 15° (pK_{ES} of ref. 1). The apparent first-order rate constant associated with this slow ES_{540} decay process is given in Table II as k_{-2app}, where an approximate definition is:

$$1/k_{-2app} = (1/k_{-2})\ (1 + k_2/k_3)$$

The exact definition of k_{-2app} is given in APPENDIX.

The experimental quantity corresponding to the specific rate of formation of ES_{540} is denoted as k_{3app}, the value of which depends on substrate concentration, and approaches the value of k_3 in the limit of infinite substrate concentration. Solving the steady state equations for k_{obs} and for k_{3app} (see APPENDIX) one obtains the following relationships:

$$1/k_{3app} = 1/k_3 + (k_{-1} + k_3)/(k_1k_3\,[S])$$
$$k_{obs} = k_1k_3/(k_{-1} + k_3)$$

Table II gives the values of the rate constants for the kinetically accessible steps of the action mechanism shown in Scheme 2.

DISCUSSION

As shown in Figs. 1 and 2, the pK_{ES} value of an ionizable group at the enzyme active center is shifted upward by 0.6 pK units in ^2H_2O, irrespective of whether deuterated or non-deuterated substrate is used. The observed pK shifts in ^2H_2O are consistent with the suggestion[1] that a histidyl residue is important in the catalytic interaction between enzyme and substrate, because it is known that the pK_a of imidazole changes from 7.09 to 7.65 on going from H_2O to ^2H_2O[9]. One important consequence of this at any value of p^{2}H is a 4-fold reduction in the equilibrium concentration of the active charge form[1] of ES, leading to as much as a 4-fold reduction in the overall rate of the reduction process. This explains, in part, the behavior of k_{obs} as a function of p^{2}H as shown in Fig. 3. This observation is similar to that made for the case of a shift in the pK_a of an essential sulfhydryl group of glyceraldehyde 3-phosphate dehydrogenase[10]. The presence of an important histidine residue at the active site of L-amino acid oxidase has also been suggested by KEARNEY AND SINGER[11] on the basis of reversible inactivation studies, and by DEKOK AND VEEGER[12] on the basis of inhibitor binding experiments.

Additional kinetic factors must be invoked to fully explain the solvent isotope effect on k_{obs} because the overall solvent isotope effect on k_{obs} in the region of pH 7.0

is a 20- to 30-fold reduction in rate as compared to k_{obs} at pH 7.0. The fact that the ^2H_2O effect on k_{obs} using deuterated substrate parallels the ^2H_2O effect using non-deuterated substrate rules out the possibility that the observed solvent isotope effect on k_{obs} arises from a prior equilibrium between ^2H_2O and an exchangeable *alpha* substrate hydrogen. The stopped-flow experiments show that the additional kinetic contributions to the overall solvent isotope effect reside both in k_{3app} and k_4. The behavior of k_{3app} and k_4 for the system in ^2H_2O suggests the possibility that the ionization state of other groups, for example the flavin moiety, might have an influence on the kinetics of reduction in the pH (p^2H) range studied. While the isoalloxazine pK_1 and pK_2 values of oxidized flavins are approx. 0 and 10, and thus of negligible influence here, the fully reduced flavin has an ionizable group with a pK value of approx. 6.5 corresponding to the deprotonation of N_1 of the isoalloxazine to form an anionic species[13] (The pK_a for ionization of the reduced flavin is expected to be highly dependent upon the nature and dielectric constant of the surrounding medium and would probably be perturbed upwards compared to the value of the pK_a in water). It is, therefore conceivable that the state of ionization of the ES_{540} form of the flavin prosthetic group could influence the reduction process, although there is no direct evidence for this.

The quantity k_{-2} can reasonably be identified with the specific rate constant for the dissociation reaction of the ES = ESH^+ equilibrium as shown in Scheme 2. The rate constant for dissociation of imidazolium ion (in H_2O at 25°) to free imidazole plus hydronium ion has been measured[14] to be $3 \cdot 10^3$ sec^{-1} which is within three orders of magnitude of the observed values of k_{-2app} (Table II). This process could correspond to the dissociation of an imidazolium ion which is located in a sterically hindered environment (caused in part by the close proximity of bound substrate molecules). It should be noted (see APPENDIX) that k_{-2app} predominantly depends not only on the dissociation rate of the enzyme-bound imidazole but also on the values of k_2 and k_3, if this explanation is correct. Thus, it is possible that the true value of k_{-2} is much greater than k_{-2app}.

The observed slow decay of ES_{540} might be thought to be an artifact caused by small amounts of oxygen in the system, because this would allow a small amount of the total enzyme present to be reoxidized and then to undergo reduction again. However, it does not appear that the slow decay can be explained in such a manner, as the following discussion would indicate. If k_{-2app} represents the apparent rate of reoxidation of reduced enzyme at very low oxygen concentrations then k_{-2} should be pH independent because the reoxidation process has been shown[1] to be largely independent of pH in the pH range studied here. Furthermore, this process does not represent a small fraction of the system undergoing turnover with a specific rate of k_{obs} since comparison of k_{obs} with k_{-2app} indicates that k_{obs} is about three orders of magnitude less than k_{-2app}. Moreover, the relative amount of material undergoing this slow apparent decay increases with decreasing pH in a manner that correlated well with an estimated pK for $ESH^+ = ES$ of 6.1. The apparent slow decay disappears at pH 7.87 as would be predicted, since there would be a very small amount of ESH^+ in equilibrium with ES at this pH. Therefore, it appears that the slow decay of ES_{540} is not an artifact caused by small amounts of oxygen in the system.

One other explanation which may be suggested for the apparent slow decay process is that it is due to the presence in the enzyme preparations of two (or more)

components having different catalytic activities. At the present time there is no conclusive evidence on this point. WELLNER[15] has previously described the separation of the enzyme into three electrophoretically distinct components, but he reported that they have "similar" enzymatic activities. More recently HAYES AND WELLNER[16] have described the further separation of snake venom L-amino acid oxidase into a large number of catalytically active bands (at least 18) using gel electrophoresis and isoelectric focusing. Some of these fractions appear to have different amino acid compositions, but unfortunately no data are reported for their comparative catalytic activities with various amino acid substrates. We suggest this could be a fruitful line of investigation. Such an explanation would be consistent with the fact that there does not appear to be an experimentally significant deuterium solvent isotope effect upon k_{-2app}.

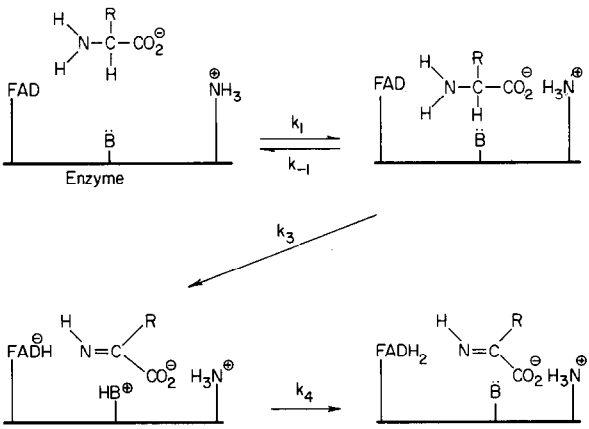
The variation in the kinetic isotope effect on k_{obs} as a function of pH is shown in Fig. 4. A limiting value for $k^{H}_{obs}/k^{^2H}_{obs} = 4.0$ is reached at pH values less than 6.5 while at pH values greater than 8.5 the ratio approaches unity. This marked pH dependence emphasizes the importance of testing for the existence of such isotope effects under a variety of experimental conditions. TRIGGLE AND MORAN[17] observed an isotope effect of 1.4 for the L-amino acid oxidase catalyzed reactions of DL-[α-^{2}H]-tyrosine and DL-[α-^{2}H]phenylalanine at pH 7.2 and suggested that this small isotope effect was related to "simple considerations of the mass difference between H and ^{2}H ($k_H/k^2_H = \sqrt{2} = 1.42$)". They concluded that the α–C to α–^{2}H (or H) bond was stretched to only a small extent in the transition state. More recently PORTER AND BRIGHT[3] by means of stopped-flow spectrophotometry have observed an isotope effect of 2.3 associated with the formation of ES_{540} during the L-amino acid oxidase catalyzed reaction of deuterated phenylalanine at pH 7.2. The magnitudes of the individual isotope effects observed by these groups of workers are in agreement with our study of the pH dependence of the isotope effect as shown in Fig. 4.

The variation of the isotope effect with pH clearly establishes that there is a change in the rate determining step of the overall turnover sequence. At pH 7, the kinetic isotope effect on k_{3app} is equal to that on k_{obs}, while at pH 7.87, the kinetic isotope effect on k_{obs} is less than that on k_{3app}. The pH dependence of the deuterium isotope effect on k_{obs} and, in more detail, on k_{3app}, is consistent with a proton transfer from some group of leucine to an acceptor group on the enzyme which could possibly be the base form of the imidazole group present on a histidyl residue. This suggestion is supported by the fact that as the equilibrium concentration of the conjugate acid increases with decreasing pH, the values of k_{3app} and k_{obs} decrease correspondingly, since the protonated form of this base would no longer catalyze proton removal from a substrate group. At basic pH values some step other than the transfer becomes rate limiting, and the isotope effect vanishes.

The overall sequence of action of L-amino acid oxidase as outlined by MASSEY AND CURTI[5] involves, by analogy to that for D-amino acid oxidase[18] a rate-limiting dissociation of an oxidized enzyme–imino acid (EI) complex after reoxidation of the reduced enzyme-imino acid species of Scheme 2. Since the turnover experiments, which yield the k_{obs} values, are conducted on the system essentially in the steady state, the rate of dissociation of the EI complex would equal the rate of reduction of oxidized enzyme by substrate. In the stopped-flow experiments, the system of course is not in the steady state and the overall forward rate of the reduction process measur-

ed in such experiments need not correspond to the turnover rate. At pH 7, the data suggests that the reduction sequence is rate determining, and at pH 7.9 some other step, possibly the dissociation of *EI*, is rate determining.

It is tempting to consider possible detailed mechanisms for the reduction of L-amino acid oxidase. One mechanism which is consistent with the evidence currently available is shown in Scheme 3. In Scheme 3 a general base catalyst (such as the



Scheme 3. Proposed mechanism for the reduction of L-amino acid oxidase by substrate.

histidyl residue previously implicated) acts to catalyze removal of a proton from the α-carbon atom of the substrate. Concerted with this proton abstraction is a hydride transfer from the substrate amino group to the flavin prosthetic group. This concerted proton abstraction and hydride transfer is characterized by the rate constant k_3. The initial proton abstraction could be considered to be the rate-limiting elementary process in the k_3 step in keeping with the observed primary deuterium kinetic isotope effect on this step. Some measure of the possibility of formation of such a transitory carbanionic species may be gained from a consideration of the work of BUCKINGHAM and coworkers[19]. They found proton exchange and mutarotation of amino acids chelated to Co (II) to proceed *via* an intermediate carbanion arising from the ionization of the α-hydrogen of the amino acid. This ionization is aided by electron withdrawal from the amino group and the carboxylate group by the complexed Co (II), which reduces the electron density on the α-carbon atom and thus increases the acidity of the α-hydrogen. A carbanion intermediate such as that proposed by BUCKINGHAM *et al.*[19] is, however, formally analogous to that proposed in Scheme 3. Although L-amino acid oxidase is apparently not a metalloenzyme, it is easy to envisage an effectively similar interaction occuring in the interaction between leucine and the oxidized enzyme. An ionic interaction between the carboxylate group of the substrate and a cationic site on the enzyme (an interaction which would be greatly facilitated in a region of low dielectric constant) would have the apparent effect of reducing the electron density on the carboxylate group to a level more resembling that of an ester. The presence of a metal ion-chelated amino group on the substrate would also have the effect of increasing the acidity of the α-hydrogen. Concerted with

a general base-catalyzed removal of the α-hydrogen, a carbanion-like transition state can reasonably be pictured as transferring a hydride ion to the flavin, thus accomplishing the two-electron transfer directly. It is probable that steric factors in the active site facilitate the formation of the coplanar arrangement of atoms found in the intermediate imine and, most importantly, in the transition state leading to imine formation. Such a coplanar arrangement should facilitate hydride transfer. The final product of the k_3 step in Scheme 3 can possibly be envisaged as a tautomer of a fully reduced flavin anion. The fully reduced neutral form of the flavin could then be formed by protonation of this anionic species.

In the detailed reduction sequence, a primary deuterium kinetic isotope effect is evidenced in the formation reaction of ES_{540}, which is in agreement with the work of PORTER AND BRIGHT[3]. In addition to the kinetic isotope effect on k_{3app}, there is a kinetic isotope effect k^{H}_{4}/k^{2H}_{4} of 1.6 at pH 7.0 and of 1.4 at pH 7.9 in the decay of ES_{540} to fully reduced form of the enzyme. This small but reproducible effect suggests that the active site proton acceptor (histidyl) in Scheme 3 may be mediating a proton transfer between the substrate alpha position and some other active site group, possibly an acceptor site on the isoalloxazine part of the flavin moiety, or that the dissociation of the protonated base is important in the forward reaction. The step corresponding to k_4 need not imply a direct proton transfer from the protonated base to the flavin moiety, only that some type of catalytically important dissociation does occur in keeping with the observed deuterium kinetic isotope effect on k_4.

The significant deuterium solvent isotope effect associated with both k_3 and k_4 is consistent with the involvement of proton transfer processes. This observation is similar to that recently reported by COLMAN AND CHU[20] for the case of the enzyme isocitrate dehydrogenase, a nicotinamide adenine dinucleotide-requiring enzyme. These workers found that the rate of the isocitrate dehydrogenase reaction proceeded five times more rapidly in H_2O than in ^2H_2O, and ascribed this solvent isotope effect to rate limiting proton transfer steps in the dehydrogenation reaction.

The reaction shown in Scheme 3 represents one mechanism which is consistent with the available evidence. This evidence included the kinetic studies by BEINERT[1], MASSEY AND CURTI[5], PORTER AND BRIGHT[3] as well as the present work[2]. The suggested mechanism is consistent with the requirement for a critical histidyl residue in L-amino acid oxidase [11,12,1] and is in agreement with our data on the pH-dependent deuterium primary and deuterium solvent kinetic isotope effects as well as with other experiments currently in progress in our laboratory. There may, however, be other ionic mechanisms which would be consistent with the available data, including four-center transfer reactions or reaction processes in which electron transfer proceeds *via* an intramolecular reaction of a covalent flavin–amino acid adduct similar to that proposed for D-amino acid oxidase by COFFEY *et al.*[21]. We look forward with great interest to experiments which will further support or which may disprove any of these specific possibilities.

APPENDIX

DERIVATION OF EXPRESSIONS FOR k_{3app}, k_{obs} AND k_{-2app} IN TERMS OF THE RATE CONSTANTS IN SCHEME 2

(1) Derivation of k_{3app} describing the rate of formation of ES_{540}

Given the transformation

$$E_{ox} \xrightarrow{k_{3app}} ES_{540}$$

and since $[E_{ox}] = [E_{total}] \times [ES_{540}]$
then

$$\frac{d[ES_{540}]}{dt} = k_{3app}([E_{total}] - [ES_{540}]) \tag{1}$$

From Scheme 2,

$$\frac{d[ES_{540}]}{dt} = k_3[ES] \tag{2}$$

and

$$[E_{ox}] = [E_{total}] - [ES] - [ESH^+] - [ES_{540}] \tag{3}$$

If $[ES]$ and $[ESH^+]$ are assumed to be in the steady-state then:

$$0 \simeq \frac{d[ES]}{dt} = k_1[E_{ox}][S] + k_{-2}[ESH^+] - (k_{-1} + k_2 + k_3)[ES] \tag{4}$$

$$0 \simeq \frac{d[ESH^+]}{dt} = k_2[ES] - k_{-2}[ESH^+]$$

or

$$k_2[ES] = k_{-2}[ESH^+] \tag{5}$$

From Eqns. 4 and 5

$$k_1[E_{ox}][S] = (k_{-1} + k_3)[ES] \tag{6}$$

From Eqn. 3, Eqn. 6 becomes:

$$k_1[S]([E_{total}] - [ES_{540}]) = \left(k_1[S] + \frac{k_1k_2[S]}{k_{-2}} + k_{-1} + k_3\right)[ES]$$

Solving for $[ES]$ and substituting into Eqn. 3 yields:

$$\frac{d[ES_{540}]}{dt} = \frac{k_1k_3[S]([E_{total}] - [ES_{540}])}{k_1[S] + \frac{k_1k_2}{k_{-2}}[S] + (k_{-1} + k_3)} \tag{7}$$

Comparison of Eqns. 1 and 7 yields:

$$k_{3app} = \frac{k_1k_3[S]}{k_1[S] + \frac{k_1k_2[S]}{k_{-2}} + (k_{-1} + k_3)}$$

or

$$\frac{1}{k_{3app}} = \frac{1}{k_3} + \frac{k_2}{k_{-2}k_3} + \frac{k_{-1} + k_3}{k_1k_3[S]} \tag{8}$$

The term $k_2/(k_{-2}k_3)$ in Eqn. 8 arises from the $ES \rightleftharpoons ESH^+$ equilibrium. At pH's greater than 7 this term will drop out since $[ESH^+]$ becomes very small compared with the other terms in the conservation equation (3); the result is the expression for $1/k_{3app}$ given in the text.

(II) Derivation of an expression for k_{obs}

For the transformation

$$E_{ox} + S \xrightarrow{k_{obs}} RI$$

we may write the expression

$$-\frac{d[S]}{dt} = k_{obs}[S]\,[E_{ox}] \tag{9}$$

for the reaction under conditions (such as at pH values less than 7–8) where the reduction reaction is slow relative to the reoxidation process. Under these conditions the enzyme is predominantly in the oxidized form[1]. From Scheme 2, assuming ES, ESH^+ and ES_{540} are in the steady-state:

$$-\frac{d[S]}{dt} = k_4[ES_{540}] \tag{10}$$

$$0 \simeq \frac{d[ES_{540}]}{dt} = k_3[ES] - k_4[ES_{540}] \tag{11}$$

$$0 \simeq \frac{d[ESH^+]}{dt} = k_2[ES] - k_{-2}[ESH^+] \tag{12}$$

$$0 \simeq \frac{d[ES]}{dt} = k_1[E_{ox}]\,[S] + k_{-2}[ESH^+] - (k_{-1} + k_2 + k_3)\,[ES] \tag{13}$$

On substitution of Eqn. 12 into Eqn. 13 we obtain:

$$\frac{k_1[E_{ox}]\,[S]}{k_{-1} + k_3} = [ES] \tag{14}$$

Then from Eqns. 10 and 11 together with the expression (14) for $[ES]$, we obtain

$$-\frac{d[S]}{dt} = \frac{k_1k_3[E_{ox}]\,[S]}{(k_{-1} + k_3)} \tag{15}$$

Thus, comparison of Eqns. 9 and 15 leads to an expression for k_{obs} in terms of the reactions of Scheme 2:

$$k_{obs} = \frac{k_1k_3}{k_{-1} + k_3}$$

(III) Derivation of expressions for k_{-2app}

Consider the transformation:

$$ESH^+ \xrightarrow{k_{-2app}} RI$$

$$\frac{d[RI]}{dt} = k_{-2app}[ESH^+] = k_{-2app}([E_{total}] - [RI]) \quad (16)$$

If ES, E and ES_{540} are assumed to be in the steady-state, then from Scheme 2:

$$o \simeq \frac{d[E]}{dt} = k_{-1}[ES] - k_1[E][S] \quad (17)$$

$$o \simeq \frac{d[ES]}{dt} = k_1[E][S] + k_{-2}[ESH^+] - (k_{-1} + k_2 + k_3)[ES] \quad (18)$$

$$o \simeq \frac{d[ES_{540}]}{dt} = k_3[ES] - k_4[ES_{540}] \quad (19)$$

$$[ESH^+] = [E_{total}] - [E] - [ES] - [ES_{540}] - [RI] \quad (20)$$

$$\frac{d[RI]}{dt} = k_4[ES_{540}] = k_3[ES] \quad (21)$$

From Eqn. 17,

$$[E] = \frac{k_{-1}[ES]}{k_1[S]} \quad (22)$$

From Eqn. 19,

$$[ES_{540}] = \frac{k_3[ES]}{k_4} \quad (23)$$

From Eqn. 18:

$$[ES](k_{-1} + k_2 + k_3) = k_1[E][S] + k_{-2}[ESH^+]$$

Substituting Eqn. 20:

$$[ES](k_{-1} + k_2 + k_3) = k_1[E][S] + k_{-2}([E_{total}] - [E] - [ES] - [ES_{540}] - [RI])$$

Substituting Eqn. 23:

$$[ES](k_{-1} + k_2 + k_3) = k_1[E][S] - k_{-2}[E] - \frac{k_{-2}k_3[ES]}{k_4} - k_{-2}[ES] + k_{-2}([E_{total}] - [RI])$$

$$[ES]\left(k_{-1} + k_2 + k_3 + \frac{k_{-2}k_3}{k_4} + k_{-2}\right) = k_1[E][S] - k_{-2}[E] + k_{-2}([E_{total}] - [RI])$$

Substituting Eqn. 22:

$$[ES]\left(k_{-1} + k_2 + k_3 + \frac{k_{-2}k_3}{k_4} + k_{-2}\right) = k_{-1}[ES] - \frac{k_{-2}k_{-1}[ES]}{k_1[S]} + k_{-2}([E_{total}] - [RI])$$

$$[ES]\left(k_2 + k_3 + \frac{k_{-2}k_3}{k_4} + k_{-2} + \frac{k_{-2}k_{-1}}{k_1[S]}\right) = k_{-2}([E_{\text{total}}] - [RI])$$

$$[ES] = \frac{k_{-2}([E_{\text{total}}] - [RI])}{k_2 + k_3 + \frac{k_{-2}k_3}{k_4} + k_{-2} + \frac{k_{-2}k_{-1}}{k_1[S]}} \tag{24}$$

Substituting Eqn. 24 into Eqn. 21 and comparing with Eqn. 16 gives:

$$k_{-2\text{app}} = \frac{k_{-2}k_3}{\left(k_2 + k_3 + k_{-2} + \frac{k_3k_{-2}}{k_4} + \frac{k_{-1}k_{-2}}{k_1[S]}\right)}$$

and

$$\frac{1}{k_{-2\text{app}}} = \frac{1}{k_{-2}}\left(1 + \frac{k_2}{k_3} + \frac{k_{-2}}{k_3} + \frac{k_4}{k_{-2}} + \frac{k_1[S]k_{-2}}{k_{-1}k_3}\right) \tag{25}$$

Eqn. 25 is the exact expression for $1/k_{-2\text{app}}$. The actual physical situation permits some simplifications to be made. Since most of the enzyme is reduced *via* the rapid decay of ES_{540} except at pH values below about 6.6, then all of the terms in Eqn. 20 will be very small during the time of the slow decay with the exceptions of ESH^+ and RI. Thus, Eqn. 5 becomes

$$[ESH^+] \simeq [E_{\text{total}}] - [RI] \tag{26}$$

If Eqn. 26 is utilized as the conservation equation then the above derivation leads to:

$$\frac{d[RI]}{dt} = \frac{k_{-2}k_3}{k_2 + k_3}([E_{\text{total}}] - [RI])$$

and the corresponding expression for $k_{-2\text{app}}$ becomes:

$$\frac{1}{k_{-2\text{app}}} = \frac{1}{k_{-2}}\left(1 + \frac{k_2}{k_3}\right) \tag{27}$$

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