PRELIMINARY NOTES

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pH-dependent deuterium isotope effects on L-amino-acid oxidase

As part of a detailed study of the elementary steps involved in the reduction of the oxidized form of L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) by L-leucine, we have conducted an investigation of the ^2H solvent and kinetic isotope effects on the turnover kinetics as a function of pH and ^2H. We find a striking pH dependence of both the ^2H solvent isotope effect (as measured in 95% ^2H_2O solution) and the ^2H kinetic isotope effect (as measured with 92.5% isotopically pure DL-[α-^2H]leucine). These pH- and ^2H-dependent isotope effects have important mechanistic implications.

L-Amino-acid oxidase was obtained in purified form from Worthington Biochemical Corp. The molar concentration of active enzyme was determined as described previously[1] on the basis of 2 moles of FAD per mole of enzyme. L-Leucine was obtained from Schwartz Bioresearch Corp. and DL-[α-^2H]leucine was prepared by hydrolysis of ethyl α-carbethoxy-α-cyano-γ-methyl pentanoate[2] in a 38% ^2HCl solution (99% deuterium content; Stohler Isotope Chemicals). Analysis of the resulting DL-[α-^2H]-leucine by NMR showed the presence of 8.5% DL-[α-^1H]leucine. All buffers used were 0.2 M in KCl; 0.05 M Tris–maleate was used in the pH (p^2H) range 5.5–8.8 while 0.05 M glycine–NaOH was used for the pH (p^2H) range 9.0–9.5. The relationship $p^2H = pH_{meter} + 0.40$ was used for the preparation of buffers in ^2H_2O (ref. 3). ^2H_2O of 99.9% isotopic purity was obtained from Columbia Chemical Co. and was glass distilled before use. The rate of O_2 uptake was measured at 25° using a Yellow Springs Biological Oxygen Monitor and buffered solutions of substrate which were initially saturated with O_2 before addition of enzyme. All rate determinations were made at a percent O_2 saturation corresponding to $8.6 \cdot 10^{-4}$ M. K_m values were determined from Lineweaver–Burk plots. The rate constant k_{obs} corresponds to the overall specific rate of the reduction sequence shown in Eqn. 1.

$$E_{ox} + S \rightleftarrows ES_{540} \rightleftarrows E_{red} + \text{products} \qquad (1)$$

Here E_{ox} and E_{red} are the oxidized and reduced form of the enzyme, respectively. The species ES_{540} is the intermediate state shown previously by MASSEY AND CURTI[4] to be formed upon combination of enzyme and substrate. The method of evaluating k_{obs} from the primary kinetic data has been described in detail elsewhere[1].

TABLE I

pK VALUES OF A CATALYTICALLY IMPORTANT IONIZABLE GROUP AT THE ACTIVE CENTER OF L-AMINO-ACID OXIDASE[1] 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

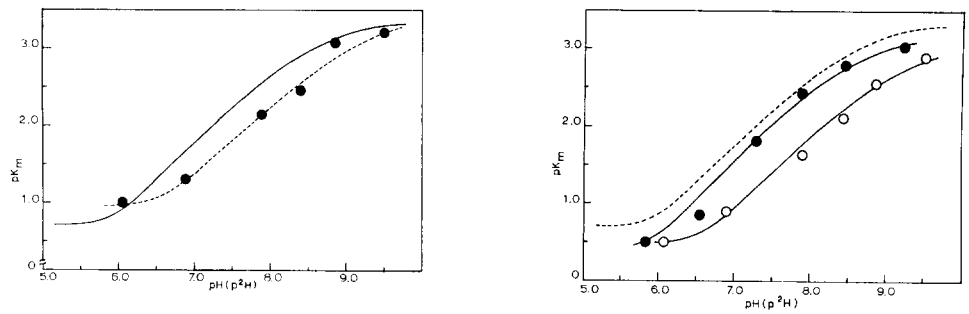

Fig. 1. pH dependence of pK_m for the L-amino-acid oxidase catalyzed reaction of L-[α-^{1}H]leucine at 25° in H_2O (———) and in ^2H_2O (●— — —●). Each point was obtained from a Lineweaver–Burk plot.

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°. Each point was obtained from a Lineweaver–Burk plot.

Some results of the present investigation are illustrated in Figs. 1–4. Fig. 1 shows a graph of pK_m *vs.* pH for L-leucine in H_2O and in ^2H_2O. Fig. 2 shows a similar graph for the reaction of DL-[α-^{2}H]leucine in H_2O and ^2H_2O. Important features of Figs. 1 and 2 are that the pK_E and pK_{ES} values of a catalytically important ionizable group at the active site are shifted upward by 0.6 unit in ^2H_2O. These pK's are summarized in Table I. The perturbation in pK caused by ^2H_2O results in a 4-fold decrease in the concentration of the active charge form of the enzyme at any given p^{2}H value as compared to the concentration of that enzyme species at corresponding pH values. Previous work from this laboratory led to the suggestion that a histidyl residue is important in the catalytic interaction between the enzyme and L-leucine[1]. The observed pK shifts in ^2H_2O are consistent with this conclusion because it is known that the pK of imidazole changes from 7.09 to 7.65 on going from H_2O to ^2H_2O [5]. Thus, part of the ^{2}H solvent isotope effect apparent in the graph (Fig. 3) of log k_{obs} *vs.*

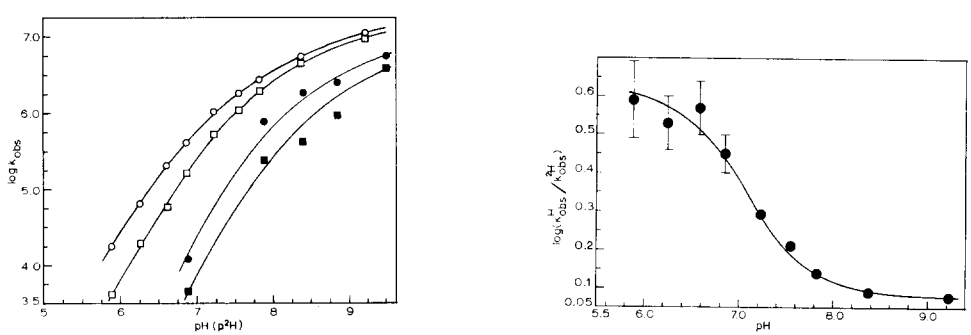

Fig. 3. pH dependence of log k_{obs} for the L-amino-acid oxidase catalyzed oxidation of L-[α-^{1}H]-leucine in H_2O (○—○) and in ^2H_2O (●—●) at 25°, and of the oxidation of DL-[α-^{2}H]-leucine in H_2O (□—□) and in ^2H_2O (■—■) at 25°. Points are determined as described in the text.

Fig. 4. The pH dependence of the ^{2}H 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 errors associated with the data at low pH values are based on the uncertainties arising from the extreme slowness of the overall reaction rate at low pH, particularly when ^{2}H-labeled substrate was used.

pH(p^{2}H) for the four substrate–solvent systems studied may be ascribed to a shift in the pK of a catalytically important ionizable group, probably histidyl.

The pH dependence of the ^{2}H kinetic isotope effect is shown in Fig. 4. A limiting value of $k^{H}_{obs}/k^{^2H}_{obs} = 4.0$ 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[6] 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. These authors suggested that this small isotope effect was related to "simple considerations of the mass difference between H and ^{2}H ($k_H/k_{2H} = \sqrt{2} = 1.42$)" and concluded that the α-C–α-^{2}H (or H) bond was stretched to only a small extent in the transition state. More recently PORTER AND BRIGHT[7] 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 magnitude of the isotope effect observed by these workers is in agreement with our study of the pH dependence of the isotope effect as shown in Fig. 4.

The variation of $k^{H}_{obs}/k^{^2H}_{obs}$ with pH taken together with the pH dependence of K_m and k_{obs} strongly suggests that a basic group (such as an imidazole) at the active site of the enzyme is mediating a proton transfer step in which the α-H (or α-^{2}H) is transferred to some basic acceptor. This is consistent with the observation that a rate-limiting hydrogen transfer step (as measured by the kinetic isotope effect) is observed only at low pH where the concentration of the free base form of such a catalytic group would become small and apparently rate-limiting. Thus, the pH dependence of the kinetic isotope effect is ascribed to a change in the rate-limiting step of the reaction sequence.

Further work is in progress which is designed to identify the steps responsible for the observed kinetic isotope effects and to explain if possible the solvent isotope effect observed at pH 7.

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