Location of Hydrogen Transfer Steps in the Mechanism of Reduction of L-Amino Acid Oxidase*

David J.T. Porter** and Harold J. Bright***

Department of Biochemistry, School of Medicine
University of Pennsylvania, Philadelphia, Pennsylvania, 19104

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We show by means of a specific kinetic isotope effect that the bond between hydrogen and the 2-carbon of the substrate is broken in forming the 540 mμ complex of L-amino acid oxidase. In addition, this process is considerably slowed in ^2H_2O. The conversion of the 540 mμ complex to the fully reduced enzyme appears not to involve the rate-limiting transfer of a species of hydrogen of any kind.

The anaerobic reduction of L-amino acid oxidase by a variety of substrates involves the transitory formation of an intermediate which can be monitored spectrophotometrically by rapid reaction techniques at 540 mμ (1,2,3). In the case of L-valine, a limiting rate of formation of E_{540} could be measured by stopped-flow spectrophotometry (3) and it was therefore concluded that the mechanism of reduction of the enzyme is the following

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_{ox}\text{-}S \xrightarrow{k_2} E_{540} \xrightarrow{k_3} E_{red} \qquad (1)$$

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Abbreviations used are: S-^{1}H, 1-^{1}H-L-phenylalanine; S-^{2}H, 1-^{2}H-L-phenylalanine.

In other studies, deuterium kinetic isotope effects on the turn over of 2-^{2}H-phenylglycine and 2-^{2}H-tyrosine were noted (4). Since the formation and/or disappearance of E_{540} may well involve the rate-limiting transfer of a species of hydrogen from substrate to enzyme, we have compared the kinetics of reduction of the enzyme using 2-^{1}H- and 2-^{2}H-DL-phenylalanine. In addition, the effect of deuterium substitution in the solvent was studied.

L-amino acid oxidase from Crotalus adamanteus was prepared by a method similar to that of Wellner and Meister (5). The enzyme was stored at 5° in 0.2 M Tris-HCl, pH 7.2, containing 0.1 M KCl, with no loss of activity over a period of a month. The 2-^{2}H-DL-phenylalanine was prepared by a method (6) involving the use of catalytic amounts of pyridoxal phosphate in $^{2}H_2O$. The purified product had an ultraviolet spectrum identical to that of 2-^{1}H-DL-phenylalanine and both the 2-^{2}H- and 2-^{1}H-DL-phenylalanine preparations used gave the calculated phenylpyruvate ($\epsilon_{235} = 1.14 \times 10^{3}$ $M^{-1}cm^{-1}$) stoichiometry in spectrophotometric measurements of the L-amino acid oxidase reaction with excess O_2. Comparison of turnover rates of 2-^{1}H-DL-phenylalanine and 2-^{1}H-L-phenylalanine showed that the D-isomer has no apparent effects on the kinetics of the reaction.

The kinetics of formation and disappearance of the 540 mμ complex was measured with the Gibson-Durrum stopped-flow spectrophotometer thermostatted at 8°. Solutions of the enzyme and substrates, containing 0.2 M Tris-HCl, pH 7.2, and 0.1 M KCl, were made anaerobic in tonometers by the addition of 10^{-8} M glucose oxidase and 5×10^{-3} M D-glucose. Attempts to deaerate by repeated evacuation and flushing with N_2 caused excessive precipitation of the enzyme.

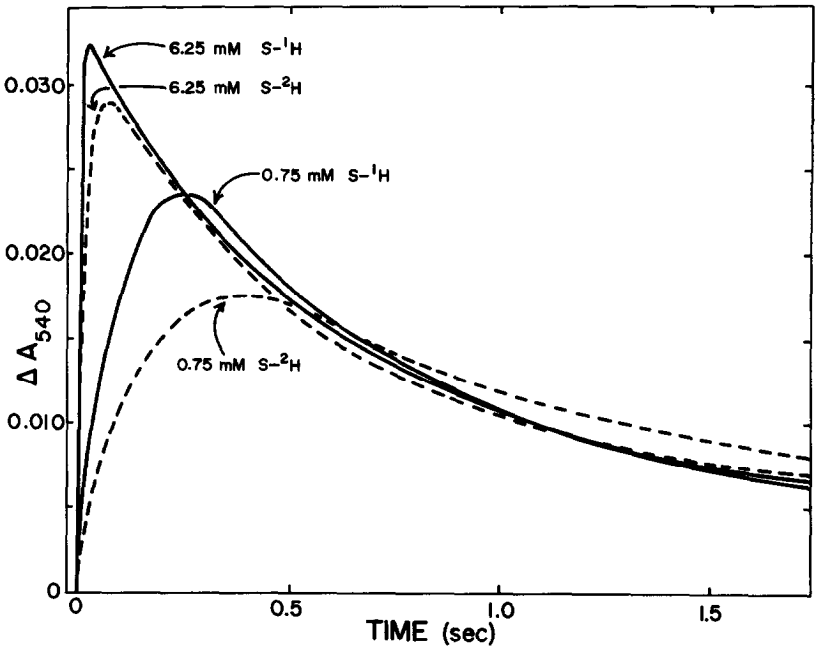

Figure 1. Kinetics of the formation and decay of E_{540} measured by stopped-flow spectrophotometry at 8° and pH 7.2, showing the kinetic isotope effect with 2-^{2}H-DL-phenylalanine. The concentration of L-amino acid oxidase after mixing, in terms of FAD (3), was 7.5×10^{-6} M, while the concentrations of L-phenylalanine after mixing were as indicated by the traces. Other conditions are given in the text.

A typical set of experiments is given in figure 1. These traces resemble those reported previously (2,3). It is qualitatively evident that deuterium substitution in the substrate decreases the rate of appearance of E_{540}, but has no significant effect on its rate of disappearance. By curve fitting it was found that the reaction could be described in all cases by

$$E \xrightarrow{k_{obs}} E_{540} \xrightarrow{k_3} E_{red} \qquad (2)$$

as was reported by Massey and Curti (3). The two rate constants are plotted in figure 2 in double reciprocal form as a

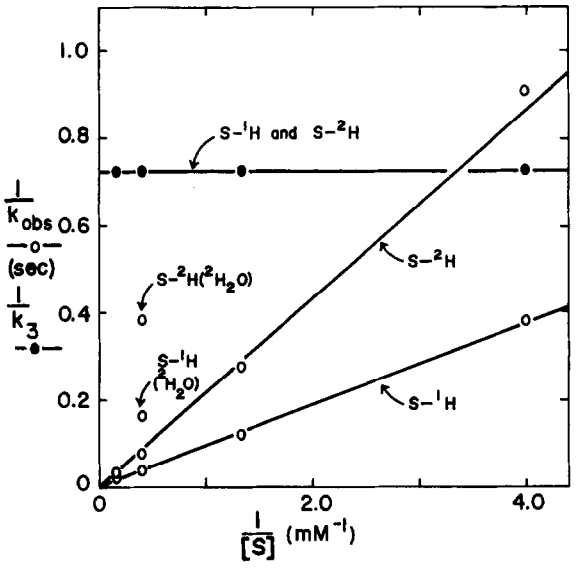

Figure 2. Double reciprocal plot of k_{obs} and k_3, from experiments such as those in figure 1, versus the concentration of L-phenylalanine (see equation 2). The reciprocal of the slope of the line in the case of k_{obs} (see equation 3) is 10.6×10^3 $M^{-1}sec^{-1}$ for 2-^{1}H-phenylalanine and 4.6×10^3 $M^{-1}sec^{-1}$ for 2-^{2}H-phenylalanine, both calculated on the basis of the L-isomer. The value of k_3 is 1.38 sec^{-1} for both 2-^{1}H- and 2-^{2}H-phenylalanine. The values of k_{obs} in ^2H_2O for 2-^{1}H-phenylalanine and 2-^{2}H-phenylalanine (both 2.5 mM) are decreased by four- and five-fold, respectively.

function of substrate concentration.[1]

The steady state derivation of k_{obs} for the formation of E_{540} in equation 1 is the following

$$\frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{k_{-1}+k_2}{k_1 k_2 [S]} \qquad (3)$$

Since the ordinate intercepts of $\frac{1}{k_{obs}}$ for both S-^{1}H and S-^{2}H are indistinguishable from zero (figure 2), $k_2 \geq 200$ sec^{-1} for both substrates as was concluded previously in the case of S-^{1}H at 1.5° (3). We consider it unlikely that the isotope

[1] We have noted, in experiments to be published, that the double reciprocal initial velocity patterns of S-^{1}H and S-^{2}H at 25° show an effect which is almost identical to that seen for k_{obs} in figure 2.

effect on k_{obs} originates in k_1, because this would imply that the breaking of the bond between hydrogen and the 2-carbon of the substrate occurs concomitantly with the formation of E_{ox}-S. For this reason we favor the interpretation that the isotope effect is on k_2, in analogy (at least kinetically) with the glucose oxidase reaction (7).

Similar experiments were performed in ^2H_2O, using a suitable pH meter correction (8). The enzyme was incubated in a ^2H_2O solution for two days. As shown in figure 2, there is a marked solvent deuterium effect[2] on k_{obs}, while there is no effect on k_3. Furthermore the substrate and solvent kinetic isotope effects on k_{obs} are approximately additive. Whether the solvent isotope effect originates in k_1 or k_2, or both, is not clear.

Although the substrate kinetic isotope effect of 2.3 fold on k_{obs} indicates that the 1-hydrogen of the substrate is removed by the enzyme in the formation of E_{540}, the question as to whether the species removed is a proton, hydrogen atom or hydride ion requires further experimentation. The interpretation of the solvent kinetic isotope effect on k_{obs} is more ambiguous, since it may arise in principle from the modification of the acidity of acid-base residues concerned in catalysis, from the slowing of proton transfer in a general acid-base catalysed process or from a general influence on protein reactivity.

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[2] D.S. Page and R.L. VanEtten (private communication, 1968) have observed a solvent deuterium isotope effect of similar magnitude in turnover studies with L-leucine.

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