KINETIC ISOTOPE EFFECT ON THE REACTION OF D-AMINO-ACID OXIDASE

Kunio YAGI, Morimitsu NISHIKIMI, Nobuko OHISHI and Akiko TAKAI
Institute of Biochemistry, Faculty of Medicine,
University of Nagoya, Nagoya, Japan

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1. Introduction

Upon anaerobic mixing of D-alanine and D-amino-acid oxidase (EC 1.4.3.3), by rapid reaction technique, transitory appearance of long-wavelength absorbing intermediate was observed [1, 2], which was found to be a single species and was identified spectrophotometrically with the purple complex observed in an equilibrium state [2]. The absorbance at 550 nm increases rapidly and subsequently decreases slowly, indicating the rapid formation of the purple intermediate and its slow conversion to the fully reduced enzyme. The reaction sequence in anaerobic reduction of the enzyme with the substrate is expressed as follows [1, 2]:

$$E_{ox} + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox}.S \xrightarrow{k_{+2}} \text{purple intermediate}$$

$$\xrightarrow{k_{+3}} E_{red}.P.$$

To obtain kinetic and chemical information regarding the reaction, it is profitable to examine the occurrence and the magnitude of kinetic isotope effects by using the isotopically substituted substrates. Since NMR study revealed that D-H exchange of the substrate by this enzyme in the equilibrium state occurred at the α-carbon but not at the β-carbon [3], the present study was done with α-deuterated DL-alanine and DL-leucine, to investigate the details of the reduction of the enzyme.

* Since L-leucine inhibits the enzymic reaction in competition with D-leucine (K_i = 1.44 mM) in the concentrations investigated, exact estimation of k_{+2} was not possible.

2. Materials and methods

D-Amino-acid oxidase holoenzyme was prepared according to Yagi et al. [4]. α-^{2}H-DL-Alanine and α-^{2}H-DL-leucine were prepared by the reaction of the respective racemic amino acid with salicylaldehyde and $CuSO_4$ in D_2O [5]. Their purity was checked by paper chromatography, NMR and IR; it was found that the isotopic substitution of α-hydrogen was almost complete.

The transmittance change at 550 nm caused by anaerobic mixing of the enzyme and the substrate was measured using a Yanaco SPS-1 stopped-flow spectrophrotometer at 20°C as described previously [2]. Solutions of the enzyme and of the substrate, containing M/60 pyrophosphate buffer, pH 8.3, were made anaerobic by bubbling with argon gas which had been washed with alkaline pyrogallol solution.

3. Results and discussion

The formation of the purple intermediate and its conversion to the fully reduced enzyme are easily distinguishable in the reaction trace at 550 nm due to the large difference between the rates of formation and decay. The formation of the purple intermediate was a first-order reaction in accord with the report of Massay and Gibson [1]. The pseudo first-order rate constant (k_{obs}) of formation of the purple intermediate was plotted versus the concentration of substrate as shown in figs. 1 and 2 for the reactions with alanine and leucine*, respectively. With leucine, the rate of formation of the purple intermediate

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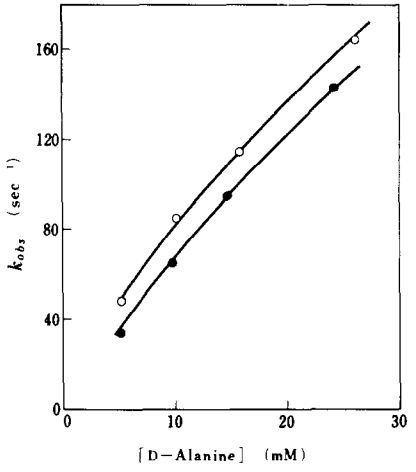

Fig. 1. Kinetic isotope effect on the formation of the purple intermediate of D-amino-acid oxidase with D-alanine. The anaerobic solutions of the enzyme and of DL-alanine containing M/60 pyrophosphate buffer, pH 8.3, were mixed and the transmittance change at 550 nm was followed using a Yanaco SPS-1 stopped-flow spectrophotometer with an observation chamber of 2.0 mm light path, at a controlled temperature of 20°C. The concentration of the enzyme after mixing was 5.0×10^{-5} M with respect to FAD. The pseudo first-order rate constant (k_{obs}) for the reaction was plotted versus the concentration of D-alanine; α-^{1}H-DL-alanine (——○——), α-^{2}H-DL-alanine (——●——).

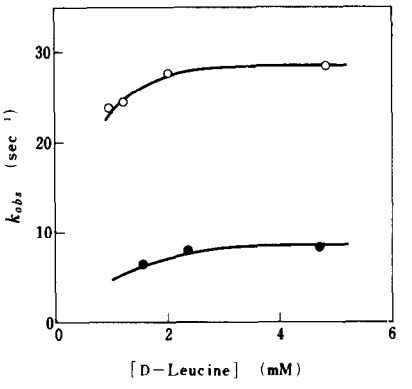

Fig. 2. Kinetic isotope effect on the formation of the purple intermediate of D-amino-acid oxidase with D-leucine. Using α-^{1}H-DL-leucine (——○——) and α-^{2}H-DL-leucine (——●——), the formation of the purple intermediate was examined in conditions similar to those specified in fig. 1 except that the length of light path was 1.0 cm and that the final concentration of the enzyme was 1.4×10^{-5} M with respect to FAD.

levelled off in the range of the substrate concentration investigated, so that the rate is considered to be controlled by the process, $E_{ox}.S \rightarrow$ purple intermediate. In this case, the substitution of the α-hydrogen of leucine by deuterium made the rate of formation of the purple intermediate fall to approx. 1/3. Therefore, the decrease in the rate due to isotopic substitution should be due to the decrease in the value of k_{+2}. With alanine, however, the substitution of the α-hydrogen of alanine by deuterium caused only a slight decrease in the observed rate of formation of the purple intermediate. Possible interpretations available at present for the different behavior or isotopic substitution in leucine and in alanine are (1) the different kinetic isotope effect on k_{+2} in the two cases, and (2) the difference in the rate-controlling step in the two cases, viz., $E_{ox}.S \rightarrow$ purple intermediate with leucine and $E_{ox} + S \rightarrow E_{ox}.S$ with alanine.

The conversion of the purple intermediate to the fully reduced enzyme followed first-order kinetics, and was almost independent of the substrate concentration in agreement with the report of Massey and Gibson [1]. First-order rate constants for the reactions with alanine and leucine were not affected significantly by the substitution of the α-hydrogen by deuterium, indicating that the conversion of the purple intermediate to the fully reduced enzyme does not involve the rate-limiting transfer of a species of hydrogen of any kind. Therefore, the possibility that the proton abstraction from the α-carbon may be involved in the conversion of the purple intermediate to the fully reduced enzyme [6] could be ruled out. From these results, it is concluded that the removal of hydrogen from the substrate occurs in the process, $E_{ox}.S \rightarrow$ purple intermediate.

Although the present results do not determine whether the species removed is a proton, hydrogen atom or hydride ion, it should be considered that in the purple intermediate the substrate moiety interacts with the flavin moiety after the breaking of the bond between hydrogen and the α-carbon of the substrate. Recently, Porter and Bright [7] have reported a similar isotope effect with L-amino-acid oxidase, however, they could not obtain any direct evidence of a kinetic isotope effect on the step of $E_{ox}.S \rightarrow$ purple intermediate.

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