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## Nuclear magnetic resonance study on D-amino acid oxidase reaction

In our recent work[1] on the mechanism of action of D-amino acid oxidase (D-amino acid:$O_2$ oxidoreductase (deaminating), EC 1.4.3.3), rate-determining removal of the $\alpha$-hydrogen of the substrate was observed during the formation of the purple complex, the rapidly appearing intermediate. For obtaining further information on the fate of the substrate, the nuclear magnetic resonance (NMR) measurement was attempted with special attention paid to the behavior of the protons at the $\beta$-carbon of the substrate.

The holoenzyme of D-amino acid oxidase was prepared according to the method of YAGI *et al.*[2]. $^2H_2O$ (99.76%) was obtained from E. Merck AG, Darmstadt. DL-[$\alpha$-$^2$H]Alanine was prepared by heating DL-alanine with $^2H_2O$, salicylaldehyde and $CuSO_4$ in a sealed tube at 120–125° for 72 h[3]. The NMR spectrum of this preparation in $^2H_2O$ showed a singlet signal centered at $\delta$ 1.48, indicating that the isotopic substitution of the $\alpha$-hydrogen was almost complete. The solution of the purple complex was prepared[4] by mixing anaerobically 0.05 M D-alanine (or 0.1 M DL-[$\alpha$-$^2$H]alanine), 0.1 M lithium pyruvate and 0.05 M $(NH_4)_2SO_4$ with D-amino acid oxidase (50 $\mu$M with respect to FAD) in 0.017 M pyrophosphate buffer prepared with $^2H_2O$ (or with $H_2O$) of p$^2$H 8.3 (or pH 8.3). Incubation was carried out at 0–2° in the dark. The aerobic reaction of this enzyme with D-alanine in $^2H_2O$ (or with DL-[$\alpha$-$^2$H]alanine in both $^2H_2O$ and $H_2O$) was performed in 0.017 M pyrophosphate buffer of p$^2$H 8.3 (or pH 8.3), at 20° while bubbling oxygen. The isotopic composition of the substrate and the product in the solution of the purple complex or of the aerobic reaction product was determined by NMR spectroscopy using a Varian A-60 spectrometer at 25°. Chemical shift values were estimated relative to sodium 2,2′-dimethyl 2-silapentane 5-sulfonate.

Signals from the protons at the $\alpha$-carbon and $\beta$-carbon of D-alanine and from the protons at the $\beta$-carbon of pyruvate in the solution of the purple complex prepared in $^2H_2O$ appeared at $\delta$ 3.8 (qualtet), $\delta$ 1.48 (doublet) and $\delta$ 2.38 (singlet), respectively, as shown in Fig. 1, Curve I. The exchange of a hydrogen atom for a deuterium atom at the $\alpha$-carbon of alanine would be expected to make the qualtet disappear and to convert the doublet arising from the $\beta$-carbon protons to a singlet due to the absence of significant coupling of the $\beta$-carbon protons with the $\alpha$-carbon deuterium. This was experimentally observed when the NMR spectrum of the solution of the purple complex was repeatedly scanned. The signal from the proton at the $\alpha$-carbon diminished and the doublet signal from the $\beta$-methyl group converted to the singlet with time of incubation, as shown in Fig. 1. However, the shape of the signal associated with the methyl group of pyruvate remained unchanged.

Conversely, in the measurement made with the solution of the purple complex prepared with DL-[$\alpha$-$^2$H]alanine in $H_2O$, the doublet signal associated with the methyl group of alanine appeared with time of incubation, indicating that the deuterium atom at the $\alpha$-carbon is replaced by a hydrogen atom (see Fig. 2). The signal associated with the methyl group of pyruvate was not changed in this case either. In the absence of the enzyme, the hydrogen–deuterium exchange at the $\alpha$-carbon of the substrate observed in the above experiments did not occur. These results indicate that, in an anaerobic equilibrium state, the hydrogen–deuterium exchange at the $\alpha$-carbon of

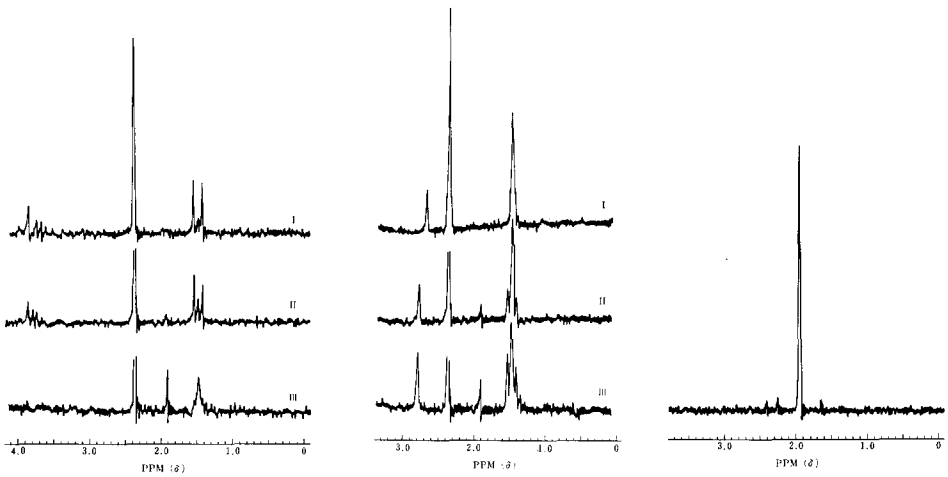


Fig. 1. The NMR spectra of [$\alpha$-$^1$H]alanine and pyruvate in the solution of the purple complex of D-amino acid oxidase prepared in $^2H_2O$. Curve I, before mixing of the enzyme; Curve II, 48 h after mixing of the enzyme; Curve III, 165 h after mixing of the enzyme. The solution contained 0.05 M D-[$\alpha$-$^1$H]-alanine, 0.1 M lithium pyruvate, 0.05 M $(NH_4)_2SO_4$ and D-amino acid oxidase (50 $\mu$M with respect to FAD), in 0.017 M pyrophosphate buffer of p$^2$H 8.3. Incubation was carried out at 0–2°. After a long time of incubation, a minor peak appeared at $\delta$1.95 which is ascribed to acetate produced from pyruvate.

Fig. 2. The NMR spectra of [$\alpha$-$^2$H]alanine and pyruvate in the solution of the purple complex of D-amino acid oxidase prepared in $H_2O$. The details of reaction mixture and conditions were similar to those specified in Fig. 1 except that D-[$\alpha$-$^1$H]alanine was replaced by 0.1 M DL-[$\alpha$-$^2$H]-alanine. The qualtet from the proton at the $\alpha$-carbon could not be examined because in the measurements in $H_2O$, the enormous peaks from the solvent, $H_2O$, obscured the spectrum in the region around $\delta$5 and produced massive spinning side bands near $\delta$3.

Fig. 3. The NMR spectrum of the product of the aerobic reaction of [$\alpha$-$^1$H]alanine with D-amino acid oxidase in $^2H_2O$. Reaction mixture contained 0.1 M D-[$\alpha$-$^1$H]alanine and D-amino acid oxidase (2 $\mu$M with respect to FAD) in 0.017 M pyrophosphate buffer of p$^2$H 8.3 and was incubated with bubbling of oxygen gas for 30 min at 20°.

alanine occurred slowly. Considering that a methyl group of $CH_2{}^2H$ type will show a triplet with the same amplitude, the present results with the methyl group of pyruvate indicate that a hydrogen–deuterium exchange did not occur at the $\beta$-carbon of the substrate in an anaerobic equilibrium state.

To investigate a hydrogen–deuterium exchange at the $\beta$-carbon of the substrate under aerobic conditions, an enzymic reaction was performed in $^2H_2O$ in the presence of catalase. At the end of the reaction, the peaks from the protons at the $\alpha$- and $\beta$- carbon of alanine disappeared and a singlet appeared at $\delta$ 2.38*. This peak corresponds to the methyl group of pyruvate. In the absence of catalase, acetic acid was found to be formed from alanine as verified by the appearance of a singlet at $\delta$ 1.95, associated with the methyl group of the acid (see Fig. 3). Considering the nature of the signal of $CH_2{}^2H$ type, it is concluded that the incorporation of a deuterium atom from the solvent to the $\beta$-methyl group does not occur during the oxidation of D-alanine with D-amino acid oxidase.

* Besides this, another singlet signal was found near $\delta$2.25 which is under investigation.

In another experiment, when [$\alpha$-$^2$H]alanine was oxidized to pyruvate or to acetate both in $H_2O$ and in $^2H_2O$, the signals of the methyl groups of these products were also singlet. This indicates that the intramolecular transfer of a hydrogen atom (from the $\alpha$- to $\beta$-carbon in this case) is not involved in the enzymic oxidation of the amino acid, in contrast to the other reactions which are catalyzed by $B_{12}$ enzymes[5,6].

In the case of L-amino acid oxidase, it has already been shown that a hydrogen–deuterium exchange does not occur at the $\beta$-carbon of the substrate[7]. Since it has recently been shown that L-amino acid oxidase has a similar reaction mechanism to D-amino acid oxidase[8], it may be expected analogically that the intramolecular transfer of a hydrogen atom does not occur in the L-amino acid oxidase reaction either.

From these results, it is concluded that the participation of the group at the $\beta$-position of the substrate is not involved in the D-amino acid oxidase reaction and that the occurrence of an $\alpha,\beta$-unsaturated intermediate is ruled out.

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## The ternary complexes of fatty acid amides with horse liver alcohol dehydrogenase and NAD$^+$

Stable ternary complexes of fatty acid amides, NADH and horse liver alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) were first reported by WINER AND THEORELL[1]. At that time, no evidence was obtained in their equilibrium fluorescence titrations for the existence of the corresponding ternary complexes composed of alcohol dehydrogenase, NAD$^+$ and amides. The failure to demonstrate alcohol dehydrogenase–NAD$^+$–amide complexes suggested an absolute specificity

Abbreviations: PHMB, *p*-hydroxymercuribenzoate; $K_{EO,I}$, the dissociation constant of ternary complex into alcohol dehydrogenase–NAD$^+$ and free amide; $K_{ER,I}$, the dissociation constant of ternary complex into alcohol dehydrogenase–NADH and free amide.