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RELEASE OF α-IMINO ACID AS PRIMARY PRODUCT IN D-AMINO-ACID OXIDASE REACTION

KUNIO YAGI, MORIMITSU NISHIKIMI, NOBUKO OHISHI AND AKIKO TAKAI
Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya (Japan)

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SUMMARY

Due to the formation of oxidation products from noncyclic amino acids, such as D-alanine and D-leucine, on catalysis with D-amino-acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) in slightly alkaline solution, the pH of the solution decreased and subsequently increased towards the initial level. In the case of cyclic amino acids such as D-proline, on the other hand, only a decrease in pH was observed.

These results can be interpreted to mean that an imino acid, of which the imine group is unprotonated, is released as the primary oxidation product from the enzyme before its hydrolysis to keto acid and NH_4^+.

INTRODUCTION

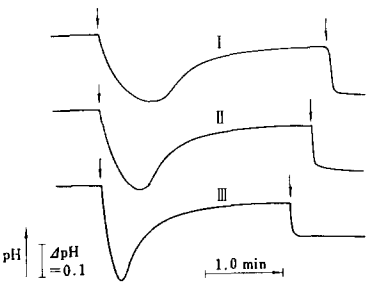
It has been widely accepted that the initial oxidation product of amino acids in flavin enzyme catalysis is an unstable α-imino acid which is hydrolyzed to the corresponding keto acid nonenzymically. This hypothesis resulted from reasonable chemical considerations[1,2]. In support of this view, the occurrence of an α,β-unsaturated intermediate (enamine) has been denied in both L- and D-amino-acid oxidase reactions[3,4]. Although direct demonstration of the formation of imino acid in the enzymic oxidation of amino acids is difficult due to its instability, PITT[5] has presented evidence for its occurrence in the L-amino-acid oxidase (EC 1.4.3.2) reaction by detecting spectrophotometrically an enamine produced from the imino acid on reaction with tautomerase. In our present study on the D-amino-acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) reaction, liberation of a proton was observed, which implied that the product released from the enzyme is an imino acid. Details of the results are described herein. Our conclusion is not consistent with the hypothesis of HELLERMAN AND COFFEY[6], however, who considered that the product is released from the enzyme as a keto acid through the intervention of the ε-amino group of the lysyl residue of the enzyme.

EXPERIMENTAL

The holoenzyme of D-amino-acid oxidase, which was free from catalase, was prepared according to the method of YAGI *et al.*[7]. Catalase was purchased from Sigma Chemical Co. Ltd. D-Alanine was purchased from Nakarai Chemicals Ltd., D-leucine and D-phenylalanine from Tanabe Amino Acid Research Foundation, and DL-proline, DL-pipecolic acid and DL-arginine $HCl \cdot H_2O$ from Tokyo Kasei Kogyo Co. Ltd.

In most of the experiments, the reaction was initiated by addition of a minor volume (10–100 μl) of D-amino-acid oxidase solution (in 0.017 M pyrophosphate buffer, pH 8.1) to 3.0 ml of the solution, containing $1.0 \cdot 10^{-2}$ M D-amino acid, $1.0 \cdot 10^{-5}$ M FAD and 0.1 M KCl, which was adjusted to pH 8.1 by the addition of a dilute aqueous solution of KOH. In order to mix the reactants the solution was agitated for a short period, by means of a small magnetic stirrer. After the addition of the enzyme solution, the concentration of pyrophosphate was less than $5.6 \cdot 10^{-4}$ M. In the case of lower substrate concentration ($1.0 \cdot 10^{-3}$ M D-amino acid), the concentration of pyrophosphate was increased to $1.7 \cdot 10^{-3}$ M in order to maintain a slight buffer action. The change in pH caused by the reaction was recorded by means of a Hitachi–Horiba F-5 pH meter connected to a pen-recorder at 22°. The concentration of D-amino-acid oxidase was expressed with respect to enzyme-bound FAD.

In order to examine the interrelationship between pH change and oxygen consumption, simultaneous recordings were performed using the pH meter and an oxygen electrode (Beckman oxygen sensor) whilst constant stirring of the reaction mixture with the magnetic stirrer was maintained. For the calibration of the amount of proton liberated, 10 μl of 0.0188 M HCl were added to the reaction mixture after the reaction was completed.



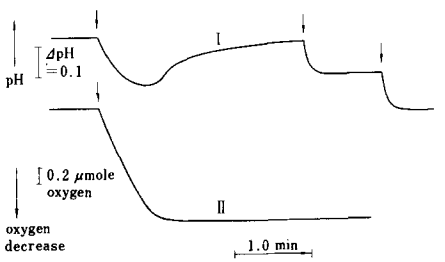


Fig. 1. Transitory pH change during the reaction of D-amino-acid oxidase with D-leucine. The reaction mixture, which contained $1.0 \cdot 10^{-2}$ M D-leucine, $1.0 \cdot 10^{-5}$ M FAD and 0.1 M KCl in a total volume of 3.0 ml, was adjusted to pH 8.1 by the addition of a dilute solution of KOH. The reaction was started by the addition of the enzyme at 22°. The pH change was recorded with a Hitachi–Horiba F-5 pH meter connected to a pen-recorder. Final concentrations of the enzyme are $4.34 \cdot 10^{-6}$ M for Curve I, $7.18 \cdot 10^{-6}$ M for Curve II and $14.1 \cdot 10^{-6}$ M for Curve III. Left-hand arrows show the addition of the enzyme and right-hand arrows that of 0.188 μmole of HCl.

Fig. 2. Simultaneous recording of pH change and oxygen consumption during the reaction of D-amino-acid oxidase with D-leucine. The reaction mixture, which contained $1.0 \cdot 10^{-3}$ M D-leucine, $1.0 \cdot 10^{-6}$ M FAD and $1.7 \cdot 10^{-3}$ M sodium pyrophosphate in a total volume of 5.0 ml, was adjusted to pH 8.3. The reaction was started by the addition of the enzyme ($8.65 \cdot 10^{-6}$ M in final concentration) at 22°. The change of pH and oxygen consumption were recorded simultaneously with a pH meter and an oxygen electrode connected to a recorder. Curve I: change of pH, Curve II: oxygen consumption. Left-hand arrows show the addition of the enzyme and right-hand arrows that of each 0.188 μmole of HCl.

RESULTS

Upon addition of the enzyme ($4.34 \cdot 10^{-6}$ M in final concentration) to the aqueous solution of D-leucine, the pH of the solution decreased and subsequently increased towards the initial level, as shown by Curve I in Fig. 1. This behavior indicates a transitory liberation of proton followed by its neutralization. Upon increasing the amount of the enzyme, the curve became steep (see Curves II and III in Fig. 1), indicating rapid liberation of the proton. Although the amount of proton liberated at the time of maximum pH depression increased slightly, it was present to a greater extent than was the added enzyme, *viz.* the molar ratios of the proton liberated to the enzyme were 20, 13 and 11 for Curves I, II and III in Fig. 1, respectively. On further addition of enzyme to the reaction mixture after the recovery of the pH value, no significant change in pH was observed. At this time, the reaction mixture was practically anaerobic (see Fig. 2). When unbuffered solution saturated with oxygen was added, the transitory liberation of proton again occurred. These results indicate that the proton liberation during the reaction is closely related to the formation of the product. Extending this consideration, the amount of proton liberated at the point of maximum pH depression should not exceed that of the oxygen present. This was the case for these experimental data.

This theory was further confirmed by recording the pH change and oxygen consumption simultaneously. As shown in Fig. 2, the time-course of the pH decrease corresponds to that of the oxygen consumption. When the initial concentration of oxygen was elevated by bubbling oxygen gas through the solution, proton liberation was prolonged, as revealed by comparing Curve II in Fig. 1 with Curve I in Fig. 3. A similar effect was observed when the reaction mixture contained catalase which decomposed H_2O_2 to regenerate oxygen in the solution (see Fig. 3).

This transitory liberation of proton and its neutralization were also observed when D-alanine was used as substrate, as shown in Fig. 4. However, the shape of the curve was different from that obtained in the case of D-leucine. This may be ascribed

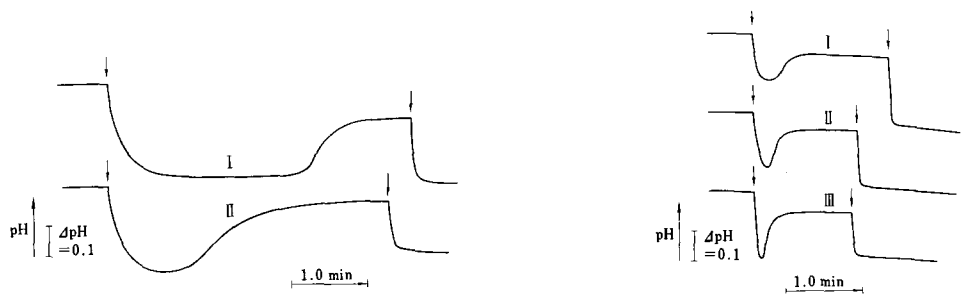


Fig. 3. The effect of oxygen on the transitory pH change during the reaction of D-amino-acid oxidase with D-leucine. The reaction mixture was the same as that specified in Fig. 1, Curve II, except that it was previously aerated with oxygen gas for 10 min (Curve I) or was added together with 7000 units of catalase (Curve II). Left-hand arrows show the addition of the enzyme and right-hand arrows that of 0.188 μmole of HCl.

Fig. 4. Transitory pH change during the reaction of D-amino-acid oxidase with D-alanine. The reaction mixture was the same as that specified in Fig. 1, except that $1.0 \cdot 10^{-2}$ M D-alanine was used as substrate in place of D-leucine. Final concentrations of the enzyme are $4.34 \cdot 10^{-6}$ M for Curve I, $7.18 \cdot 10^{-6}$ M for Curve II and $14.1 \cdot 10^{-6}$ M for Curve III. Left-hand arrows show the addition of the enzyme and right-hand arrows that of 0.188 μmole of HCl.

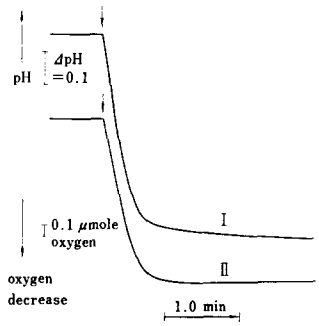


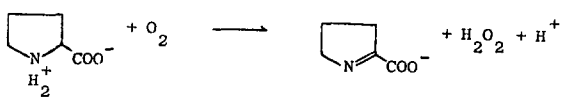
Fig. 5. Simultaneous recording of pH change and oxygen consumption during the reaction of D-amino-acid oxidase with D-proline. The reaction mixture and reaction procedure were the same as those specified in Fig. 2, except that $2 \cdot 10^{-3}$ M DL-proline was used in place of D-leucine. The final concentration of the enzyme was $2.63 \cdot 10^{-5}$ M. Curve I, change of pH; Curve II, oxygen consumption. Arrows show the addition of the enzyme. The calibration of the amount of proton liberated was performed by the addition of 0.188 μmole of HCl to another reaction mixture of which the pH level reached the initial pH level of the main experiment after the initial release of proton. Similar calibration procedures were performed in order to cover the range of pH decrease of the main experiment.

to the different rate of formation of the product. These phenomena also occurred in the reaction of other noncyclic amino acids, such as D-phenylalanine and D-arginine.

In contrast to the above type of proton liberation, however, only the initial release of proton was observed in the case of D-proline, as shown in Fig. 5. Also, in this case, the amount of proton liberated was far in excess of that of the enzyme added and the time-course of the proton liberation corresponded directly to that of the oxygen consumption. As distinct from the case of the oxidation of noncyclic amino acids, an almost stoichiometric relation was found to exist between the proton liberated and the oxygen consumed in this case. This type of release was also observed in the reaction with D-pipecolate.

DISCUSSION

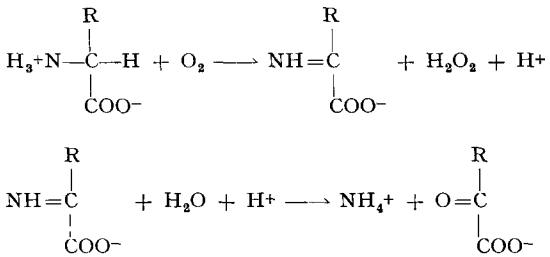
The observed fact that only the initial release of proton occurred in the case of D-proline can provide key information regarding the origin of the pH change. In the oxidation of D-proline, the substrate is a zwitter ion and the product, Δ^1-pyrroline 2-carboxylate, is practically unprotonated on its imine nitrogen atom (pK approx. 6.0) (ref. 8) in the pH range over which the reaction was performed; the overall reaction is written as follows:



Therefore, the amount of proton to be liberated should be practically equimolar to that of the oxygen present initially. In fact, this was found to be the case for the present results on the oxidation of D-proline.

On the other hand, in the case of noncyclic amino acids, the primary product of the oxidation is considered to be an α-imino acid from the fact that H–D exchange

did not occur at the β-position[4]. Assuming that the imine group is unprotonated, the reaction process could be written as:



According to this reaction process, the transitory decrease in pH is ascribed to the formation of the unprotonated imino acid which is hydrolysed to the corresponding keto acid and NH_4^+.

HELLERMAN AND COFFEY[6] proposed a hypothesis which suggests the release of the oxidation product from the enzyme as a keto acid through the intervention of the ε-amino group of the lysyl residue, based upon the isolation of a reduced ^{14}C-labeled substrate–enzyme intermediate after treatment with sodium borohydride. However, the release of proton demonstrated in the present study rules out the possibility of release of the oxidation product as a keto acid. Further, MASSEY *et al.*[9] showed that the sodium borohydride treatment did not result in any loss of catalytic activity. Taking these results into account, the participation of the lysyl ε-amino group is not essential for the catalytic oxidation of substrate by this enzyme.

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