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Spectroscopic Demonstration of an Initial Stage of the Complex of D-Amino Acid Oxidase and its Substrate D-α-Aminobutyric Acid

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SUMMARY

D-Amino acid oxidase [D-amino acid : O_2 oxidoreductase (deaminating), EC 1.4.3.3] was anaerobically mixed with its substrate D-α-aminobutyric acid at -10°C and pa$_H^*$ 7 which were apart from their maxima for the enzymatic reaction. By an ordinary self-recording spectrophotometer, the absorption spectrum of an initial stage of the complex could be observed. The spectrum was in principle similar to that of the complex of this enzyme with benzoate, the enzyme-substrate complex model. The spectroscopic observation revealed that this species is in an equilibrium with the purple intermediate, a strong charge transfer complex between the enzyme and its substrate neutral D-amino acid.

INTRODUCTION

An intermediate of the reaction between D-amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3] and its substrate, D-alanine, had been isolated by crystallization (1). This purple colored crystal is an equimolar complex of the enzyme and the substrate, and involves a strong charge transfer interaction between them (2). Accordingly, we must suspect the occurrence of an enzyme-substrate complex prior to the electron transfer in an anaerobic reaction shown in the following formula :

$$E_{ox} + S \xrightarrow{k_1} E_{ox}\cdot S \xrightarrow{k_2} E'\cdot S' \xrightarrow{k_3} E_{red}\cdot P \xrightarrow{k_4} E_{red} + P$$

where $E_{ox}\cdot S$ is an oxidized enzyme-substrate complex, E'·S' the purple intermediate, and $E_{red}\cdot P$ the fully reduced enzyme-product complex. By kinetic analysis, the occurrence of $E_{ox}\cdot S$ was first reported by Massey and Gibson (3) by using D-alanine, D-proline and D-methionine as substrate and more clearly demonstrated by Yagi et al. (4) by using D-leucine and D-valine as substrate.

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However, the spectroscopic demonstration has never been successful except for the reaction of this enzyme with an extraordinary substrate, D-lactate (5).

To observe E_{ox}·S easily, the rate constant, k_2, should be slowed down and k_2 must be smaller than k_1. For this purpose, slow reaction method (6) seemed to be useful. Since it is known that k_3 is very small in the reaction of this enzyme with D-α-aminobutyric acid (7), we used this substrate for the observation of the preceding event. To let the rate constant, k_2, small, we decreased the pH and temperature of the reaction from their maxima.

After a systematic investigation we succeeded in observing the absorption spectrum of E_{ox}·S of this enzyme with an ordinary substrate, D-α-aminobutyric acid.

MATERIALS AND METHODS

D-Amino acid oxidase was purified from pig kidney according to Yagi et al. (8,9). D-α-Aminobutyric acid was purchased from Nakarai Chemicals Ltd., Kyoto.

To maintain a fluid state at subzero temperature, we adopted glycerol (35%) as cosolvent. For this experiment, data were available for a precise control of the proton activity of water-glycerol mixture at any temperature (10). The measurement of abosrption spectra was made with an Aminco-DW2 spectrophotometer equipped with a low temperature thermostatic system (11).

RESULTS AND DISCUSSION

Figure 1 shows a typical result. Curve I represents the absorption spectrum of the oxidized enzyme in 100 mM phosphate buffer (pH 6.5) containing 35% glycerol measured at -10°C (pa^{*}_{H} at -10°C : 7). This spectrum is not significantly different from the spectrum of an aqueous solution of the enzyme at +20°C. After anaerobiosis was attained by argon gas flushing, D-α-aminobutyric acid was anaerobically added to its final concentration of 10 mM and mixed rapidly. With a self-recording spectrophotometer, curve I was found to change to curve II and then to curve III. It is noted that the fine structure existing in the second absorption band of the oxidized form disappeared and a new fine structure appeared in the first absorption band. This change in the absorption spectrum is in principle similar to that observed in the formation of the enzyme-benzoate

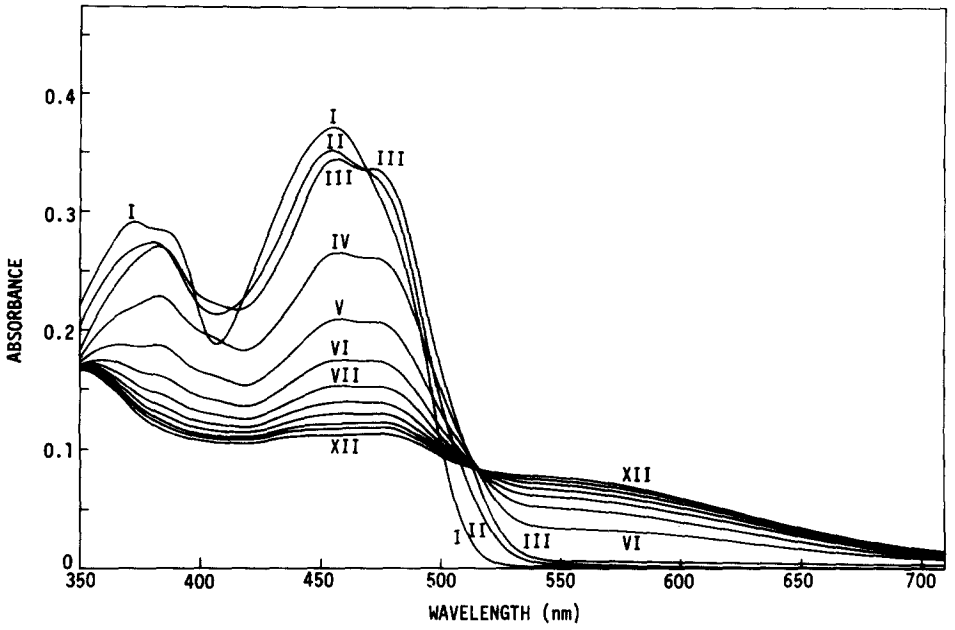

Fig. 1. Spectral change upon complex formation between D-amino acid oxidase and D-α-aminobutyric acid. D-Amino acid oxidase (31 μM) and D-α-aminobutyric acid (10 mM) were mixed anaerobically in 100 mM sodium phosphate buffer (pH 6.5) containing 35% glycerol at -10°C (pa$\overset{\text{H}}{}$ at -10°C : 7). Curve I : the enzyme ; curve II : immediately after the addition of the substrate. Scanning speed of curves II and III is 20 nm/sec and that of curves IV-XII is 5 nm/sec.

complex (12). Since the enzyme-benzoate complex is considered as a model of the enzyme-substrate complex prior to the electron transfer (12), above spectral change can be ascribed to the formation of the enzyme-substrate complex prior to the electron transfer. It is more plausible when we consider the similarity of this change to that in the formation of an initial stage of the enzyme-D-lactate complex (5). Such spectral change as observed for the enzyme-benzoate complex formation indicates the change in the environment surrounding the flavin chromophore of the enzyme (13), and consequently the occurrence of an interaction between the enzyme and the substrate. In curves I, II and III, an isosbestic point appeared at 469 nm, which indicates the occurrence of a stoichiometric equilibrium between the oxidized enzyme and a newly appearing complex. Then, spectrum changed to curve IV and gradually approached to the typical spectrum of the purple intermediate, E'·S'. During this period, an

isosbestic point again appeared at 516 nm. This indicates a stoichiometric equilibrium between the complex represented by curve III and the purple intermediate. All these results indicate that the entity of curve III is $E_{ox} \cdot S$.

Although the same spectral change was observed upon exchanging the phosphate buffer for cacodylate buffer (100 mM), the elevation of pa_H^* up to 8.0 failed in the typical demonstration of the whole spectrum of $E_{ox} \cdot S$. This was also the case for the elevation of the temperature up to +1°C. Therefore, it is clear that the decrease in both pH and temperature resulted in the marked decrease in k_2. When the concentration of the cosolvent was increased up to 50%, the typical spectrum of $E_{ox} \cdot S$ could not be observed. Even in this case, the isosbestic point at 516 nm appeared clearly. These results indicate that k_1 is smaller than k_2 in the presence of 50% glycerol. This could be ascribed to the increased viscosity of the medium.

Although the elucidation of the mechanism of the decrease in k_2 upon decreasing pH and temperature should await further investigation, the shift of the monomer-dimer equilibrium of this enzyme is considered to be involved, since it was found that the rate of formation of the purple intermediate is larger in the dimer than in the monomer (14).

The present work could be generalized to the study of a number of enzyme reactions carried out under physicochemical conditions other than optimum in order to change differentially rate-constants of their elementary steps. Thus, the "slow reaction method" could be used to obtain the "temporal" resolutions of such reactions with subsequent spectroscopic analysis.

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