ISOLATION BY CRYSTALLIZATION OF FULLY REDUCED D-AMINO ACID OXIDASE

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To approach the mechanism of enzyme action, efforts have been concentrated on the isolation of the reaction intermediates appearing during the molecular interaction between an enzyme and its substrate. In the previous work (Yagi and Ozawa, 1964a), isolation by crystallization of the enzyme-substrate complex of D-amino acid oxidase was achieved by withdrawing oxygen, an electron acceptor, and by lowering the temperature and pH from their optima for the enzyme action. The reddish purple crystal thus obtained has an absorption peak at 492 mμ (Yagi and Ozawa, 1964b), and reveals a remarkable ESR signal, the g value being 2.004 (Yagi and Ozawa, 1964a). The results obtained by analysing this crystal indicated that the crystalline complex is composed of the semiquinoid enzyme and the substrate which is easily converted into the product. The crystalline complex was tentatively named as a "Michaelis complex".

On the other hand, it had been known that this enzyme could be fully reduced by an excess amount of its substrate, D-alanine (Yagi and Ozawa, 1962) In this case, the color of the enzyme almost disappears. However, this kind of species has never been crystallized. This paper briefly reports the isolation by crystallization of this species : fully reduced D-amino acid oxidase.

Holoenzyme of D-amino acid oxidase was prepared according to the method of Yagi and Ozawa (Yagi and Ozawa, 1963) : D-amino acid oxidase-benzoate

Fig. 1a Crystals of fully reduced D-amino acid oxidase (x 250). Most of the crystals are hexagonal-shaped.

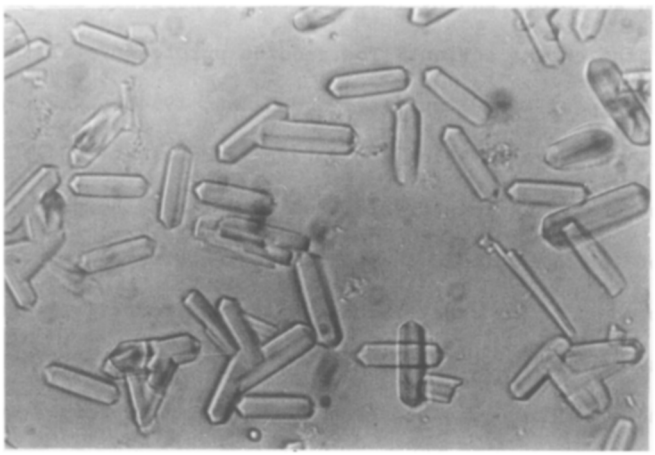

Fig. 1b Crystals of fully reduced D-amino acid oxidase (x 1000). The shape of the crystal is hexagonal prism with bipyramids.

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complex was treated with D-alanine twice and followed by dialysis against pyrophosphate buffer. This holoenzyme was composed of 2 moles of FAD and 1 mole of the apoenzyme per unit of molecular weight 115,000.

Six ml of the transparent yellow solution thus obtained contained 200 mg of the enzyme. To this solution, 160 mg of D-alanine powder was carefully added with stirring. The yellow solution became purple and then changed to pale yellow, indicating that the enzyme was fully reduced. To this pale yellow solution, 168 mg of ammonium sulfate was added with stirring. Gas phase was exchanged for argon. After storing the solution for over a week in a refrigerator at 5°C, a crop of pale yellow crystals was found. Microscopic observation showed that the crystal has hexagonal shape as shown in Fig. 1a. This shape is quite similar to that of the large "Michaelis complex" crystal (Yagi and Ozawa, 1964b). Sometimes, crystals with the shape of a hexagonal prism with bipyramids were obtained as shown in Fig. 1b. We encountered this shape in the case of the "Michaelis complex", too.

The absorption spectrum of the crystal was measured using Olympus micro-spectrophotometer as shown in Fig. 2, Curve I. The absorption spectrum of the mother liquor (Fig. 2, Curve II) was similar to that of the crystal. Neither has an absorption peak in the visible wavelength. They are quite different both from the spectrum of the oxidized holoenzyme and from that of the "Michaelis complex" crystal or its mother liquor (Fig. 2, Curve III). On the contrary, they are rather similar to the spectrum of the enzyme solution just after the reduction by excess D-alanine (Fig. 2, Curve IV) (Yagi and Okamura, 1965).

ESR measurement was performed using model JES-3B X-band instrument of the Japan Electron Optics Laboratory Co. Both the crystals and the mother liquor revealed no ESR signal.

By introducing oxygen into the mother liquor, its pale yellow color was changed, for a moment, to purple and soon returned to pale yellow. Repeating this procedure, the color of the solution became deep yellow, the absorption spectrum of which was identical with that of the enzyme-pyruvate complex as shown in Fig. 2, Curve V (Yagi, 1965). A similar phenomenon was observed in the case of the suspension of the crystals.

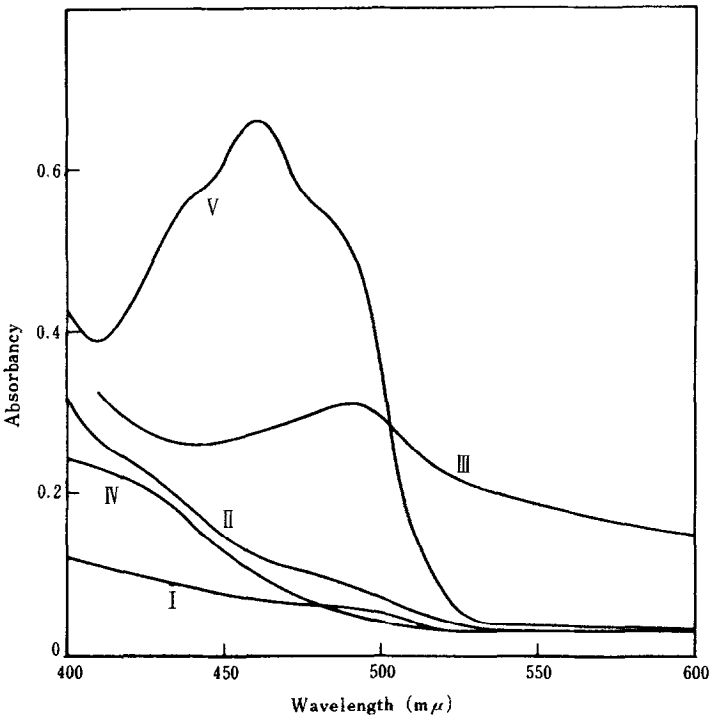

Fig. 2 Absorption spectra of D-amino acid oxidase. I, crystal of fully reduced form; II, the mother liquor of I; III, the "Michaelis complex" solution (Yagi and Ozawa, 1964b); IV, immediately after the addition of D-alanine (3 x 10^{-2}M, final concentration) to the holoenzyme (3.6 x 10^{-5}M, final concentration); V, after the aeration of II.

Then, the analysis of the components of the crystal was performed. Since the present crystal was supposed to be composed of the enzyme and the substrate molecule on the analogy of the "Michaelis complex" crystal, the substrate molecule of the present crystal was carefully examined. Actually, the crystals were analysed as follows : they were washed, under anaerobic conditions, with chilled pyrophosphate buffer (M/60, pH 8.3) which was previously rendered oxygen-free by flushing with argon 5 times at 0°C alternating with exposure to vacuum. After washing 5 times with each 0.5 ml of the buffer, the remaining crystals were dissolved in the same buffer. Then, the 5 washings and the solution of the crystals were treated with trichloroacetic

acid, by which procedure the protein in the solution was precipitated, and the amount of low molecular weight substances liberated in the supernatant was estimated. The analytical results on D-alanine and pyruvic acid in the washings indicated that the molecular ratio of D-alanine to FAD drew nearer to 1.0 with increasing number of washings. A minute amount of pyruvic acid was found, however, the order of its concentration was less than those of FAD and D-alanine. In the dissolved crystals equimolar amounts of D-alanine and FAD were found. Therefore, it might be possible to consider that the crystal was composed of 2 moles of D-alanine, 2 moles of FAD and 1 mole of the apoenzyme based on the molecular weight of 115,000.

The fact that D-alanine was a component of the crystal might be interpreted as follows : The enzyme was reduced by D-alanine, while the substrate was oxidized and then expelled by another molecule of the substrate, D-alanine, thus fully reduced enzyme-D-alanine complex was formed.

The crystal of fully reduced D-amino acid oxidase obtained here may be useful tool to study the mechanism of enzyme action.

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