COMPLEX FORMATION OF APO-ENZYME, COENZYME AND SUBSTRATE OF D-AMINO ACID OXIDASE

III. CRYSTALLIZATION OF ARTIFICIAL MICHAELIS COMPLEXES

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SUMMARY

"Artificial Michaelis complexes" of D-amino acid oxidase and one or other of the "substrate-substitutes" benzoate and *p*-nitrobenzoate have been isolated in crystalline form. The complexes contain equimolecular amounts of apo-enzyme, FAD and substrate-substitute. The substrate-substitutes were driven off by an excess of D-alanine. The properties of the crystalline benzoate complex have been studied in detail.

INTRODUCTION

Because it has been shown that benzoate or its derivatives, such as PNBA, combine with D-amino acid oxidase and are exchangeable with substrate molecules[1], these compounds have been named substrate-substitutes and they have been used for detecting the effect of the binding of a substrate on the absorption spectrum of the enzyme[2,3]. These earlier studies also made it clear that the binding of a substrate molecule to the enzyme must be rather firm. We have now been able to isolate and crystallize two of these "artificial Michaelis complexes".

The results obtained are described in detail, and this paper includes a complete report of the preliminary notes[4,5].

MATERIALS

D-Amino acid oxidase apo-enzyme was prepared by the method described in the previous paper[3], and FAD by the method of YAGI *et al.*[6]. The purity of FAD was more than 92% and it contained no other flavins, nucleic acids or metals. The benzoic acid and PNBA used were chemically synthesized preparations.

METHODS

Determination of homogeneity and molecular weight of the crystalline materials

The degree of homogeneity of the crystalline products was determined by both ultracentrifugation and electrophoresis. The material was dissolved in pyrophosphate

Abbreviation: PNBA, *p*-nitrobenzoic acid.

buffer (*M*/60, pH 8.3) containing 0.1% benzoic acid. A Spinco type E ultracentrifuge was used for the determination of sedimentation coefficient from which the values at infinite dilution were calculated. Electrophoresis and diffusion experiments were carried out in a Spinco type H apparatus. The 3% enzyme solution was dialyzed over night at 3° against the benzoic acid containing pyrophosphate buffer mentioned above. The enzyme concentration in the micro cell was 1.65%. Electrophoresis was performed at 1.9° and 125 V, with a current of 2 mA. Schlieren and interference diagrams were taken at 70-min intervals.

In diffusion experiments, performed in standard cells at 1.9°, the solution contained 2.56 mg of the enzyme per ml. The diffusion coefficients ($D_{20,w}$) were calculated from Rayleigh interference patterns. The partial specific volume, $\bar{V}$, of the enzyme was determined by the method of SCHACHMAN[7].

Determination of enzymic activity

This was performed by the conventional Warburg manometer technique with D-alanine as substrate.

Measurement of absorption spectra

Both a Beckman type DU spectrophotometer and a Hitachi recording spectrophotometer were used. The crystalline material was dissolved in phosphate buffer (0.05 *M*, pH 6.3). The spectrum of reduced oxidase was measured 5 min after the addition of an excess of D-alanine.

RESULTS

Isolation and crystallization of the holo-enzyme –"substrate-substitute" complex

The apo-enzyme was dissolved in pyrophosphate buffer (*M*/60, pH 8.3) to a final concentration of 1% protein. FAD and benzoate were added to final concentrations of $1 \cdot 10^{-3}$ *M*. During continuous stirring 1 *N* acetic acid was added until the pH 5.1 was reached. The solution was then heated at 60° for 3 min. Any precipitate formed was spun down and 114 g $(NH_4)_2SO_4$/l was added to the centrifugate.

After standing at 0° for 30 min the yellow precipitate formed was sedimented by centrifugation and was redissolved in 10 volumes of pyrophosphate buffer (*M*/60, pH 8.3).

This solution was applied to the top of a column of calcium phosphate gel – cellulose powder in 0.05 *M* potassium phosphate buffer (pH 5.1). The complex was eluted with the same buffer, and precipitated from the elute by adding $(NH_4)_2SO_4$ to 0.2 saturation.

The precipitate was dissolved in its own volume of pyrophosphate buffer (*M*/60, pH 8.3) and the solution (pH 6.0, roughly 0.08 saturation of $(NH_4)_2SO_4$) was kept at 20° to 25° for several hours. A crop of needle-shaped crystals gradually formed. In general the crystallization was complete within 24 h.

For recrystallization the product was again dissolved in the pyrophosphate buffer, the pH being adjusted to 5.1 by adding 1 *N* acetic acid and $(NH_4)_2SO_4$ added to 0.2 saturation. The amorphous precipitate obtained was suspended in 2 volumes of pyrophosphate buffer (*M*/60, pH 8.3) and was kept at room temperature. It gradually dissolved and, as Fig. 1 shows, in the course of several hours a crop of crystals was formed.

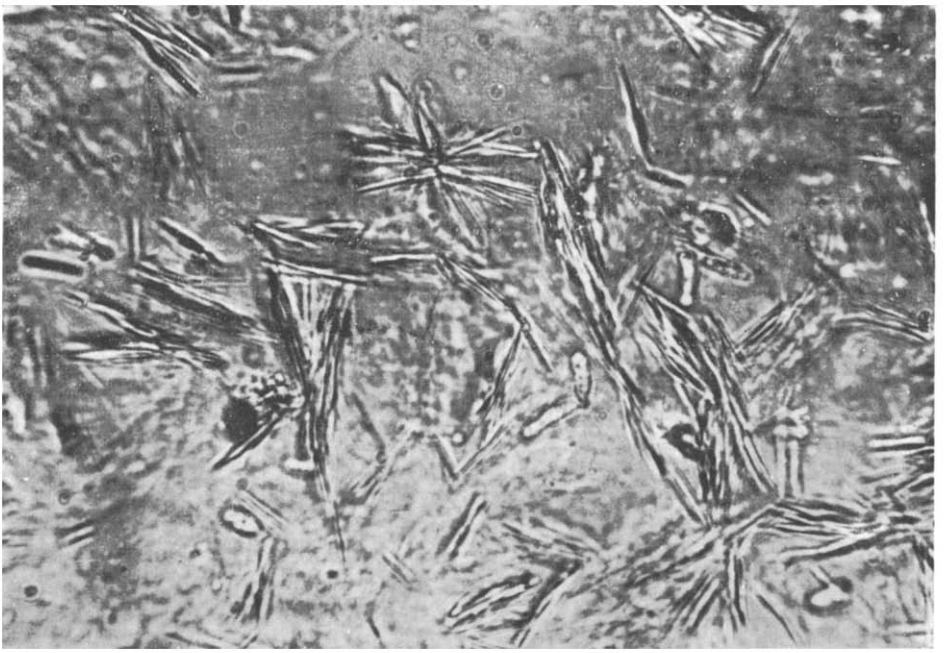

Fig. 1. Crystals of the benzoate complex (1000 times).

Homogeneity and molecular weight of the crystalline material

The sedimentation pattern showed a single, rather narrow, symmetrical peak (Fig. 2); $s_{20,w} = 11.0 \cdot 10^{-13}$ sec. Free boundary electrophoresis also gave, as Fig. 3 shows, a single symmetrical peak in the Schlieren pattern, $\mu = 4.8 \cdot 10^{-5}$ cm²/sec. From the interference pattern in diffusion experiments, it was calculated that $D_{20,w}$ was $7.9 \cdot 10^{-7}$ cm²/sec and that the partial specific volume ($\bar{V}$) of this sample was 0.705 ml/g.

From these results it was concluded that the crystalline product is homogeneous and had a molecular weight of 115000.

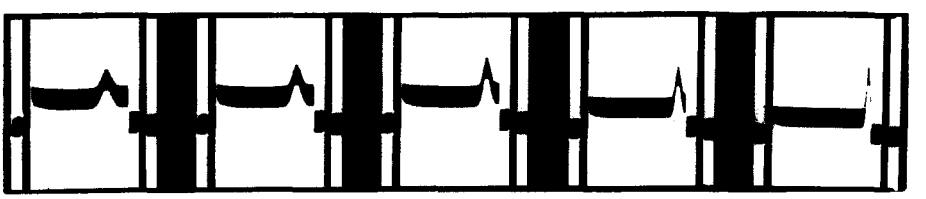

Fig. 2. Representative ultracentrifuge patterns of the benzoate complex. The centrifugal direction is towards the left. Photographs were taken every 8 min. Speed: 59780 rev./min. Protein concentration: 8.25 mg/ml. Schlieren diagram: 70°. Rotor temperature: 15.0°.

Absorption spectrum

The recrystallized sample was washed twice by 10 volumes of cold distilled water, and dissolved in 200 volumes of potassium phosphate buffer (0.05 *M*, pH 6.3). The absorption spectrum is represented by Curve I in Fig. 4. The peaks are situated at

380 mμ and 465 mμ, while a marked shoulder is observed at 490 mμ. These characteristics, as well as the ratios of the absorbancies at these wave-lengths, are the same as those of the spectrum of FAD in the presence of an excess of apo-enzyme and benzoate (see Table I).

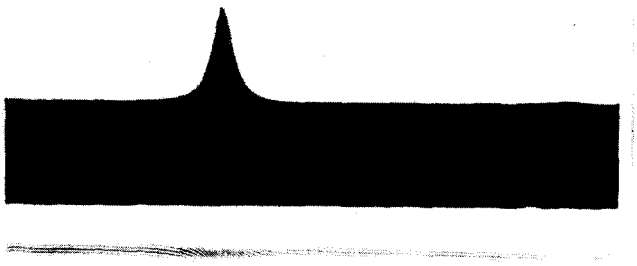

Fig. 3. Typical free boundary electrophoresis diagram of the benzoate complex. Ascending pattern.

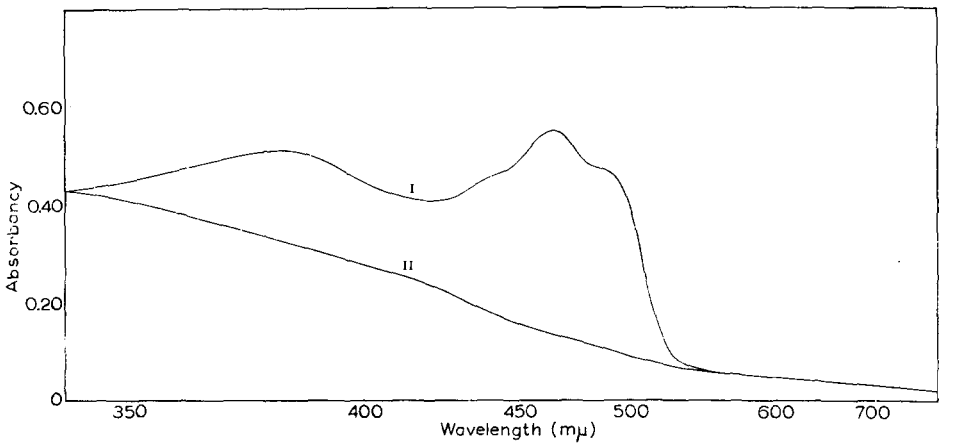

Fig. 4. Absorption spectra of the crystalline benzoate complex dissolved in 0.05 *M* phosphate buffer, pH 6.3. Curve I, not reduced. Curve II, reduced by adding D-alanine.

TABLE I

RATIOS OF ABSORBANCIES OF FAD, SOLUTION OF CRYSTALLINE BENZOATE COMPLEX AND OF MIXTURES OF APO-ENZYME AND FAD OR FAD AND BENZOATE

*Ratio A/B**	*FAD***	*FAD + apo-enzyme***	*FAD + apo-enzyme + benzoate***	*Crystalline complex*
380/465	0.910	0.785	0.735	0.745
380/490	1.715	1.325	0.875	0.860
465/490	1.890	1.700	1.170	1.160

* A and B, absorbancies, at the wave-lengths listed.
** See ref. 3.

Analysis

The apo-enzyme content of the complex was calculated from the absorption in the ultraviolet region by the method of KALCKER[8]. The FAD concentration was calculated from the absorbancy at 465 mμ (molar extinction coefficient $1.13 \cdot 10^4$ (see the previous paper[3])).

For the determination of benzoic acid a solution of the crystalline complex was acidified with diluted hydrochloric acid and was extracted with ethyl ether. From this extract the benzoic acid was re-extracted with 0.01 *N* aqueous potassium hydroxide and determined by measuring the absorption at 225 mμ. The presence of benzoic acid was verified by the reaction given with Folin reagent after oxidation by hydrogen peroxide. As Table II shows, the results demonstrate that the crystalline product contains equimolecular amounts of apo-enzyme, FAD and benzoate.

TABLE II

MOLAR RATIOS OF THE COMPONENTS IN THE CRYSTALLINE COMPLEXES

	Apo-enzyme	*FAD*	*Benzoate*	*PNBA*
Benzoate complex	1.0	1.1	0.9	
PNBA complex	1.1	1.0		1.2

Reaction with substrate

As Curve II, Fig. 4, shows, complete decolorization is caused by adding an excess of D-alanine (final concentration, 0.1 *M*). Obviously the benzoate is replaced on the enzyme by D-alanine and the latter is oxidised.

In an experiment with $1.65 \cdot 10^{-7}$ moles of complex in the mixture, the enzyme was precipitated by $(NH_4)_2SO_4$ at pH 5.1 after the decolorization and the liberated benzoic acid in the supernatant was determined. It appeared to contain $1.56 \cdot 10^{-7}$ moles of this acid, which proved that the D-alanine added had completely expelled the benzoate from the enzyme.

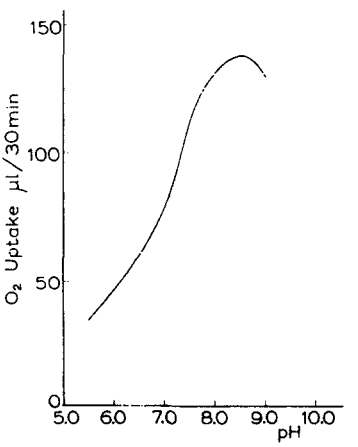

Fig. 5. pH-activity curve of crystalline benzoate complex. Each cup contained $1.3 \cdot 10^{-7}$ *M* of the crystalline product, $2.0 \cdot 10^{-6}$ *M* FAD and $5.0 \cdot 10^{-2}$ *M* D-alanine in a total volume of 1.0 ml. The centre well contained 0.1 ml of 20% NaOH. Gas phase, air. Temperature, 38°.

The pH-activity curve of the complex in the presence of D-alanine is shown in Fig. 5. The optimum is situated between 8.3 and 8.8. From the oxygen uptake, measured at about pH 8.5, it was calculated that the turnover number of the enzyme at the pH optimum was 1550. These values are the same as those found with the apo-enzyme in the presence of an excess of FAD and of D-alanine.

Crystalline complex of apo-enzyme, FAD and PNBA

By the method described above a crystalline product was also obtained with PNBA as a substrate-substitute and the shape of the crystals thus obtained was about the same.

The absorption spectrum in the visual wave-length region is shown by Curve I in Fig. 6. It exhibits the same characteristic features as those found in the apo-enzyme – coenzyme – benzoate complex, the peaks being situated at 380 mμ and 465 mμ with a marked shoulder at 490 mμ.

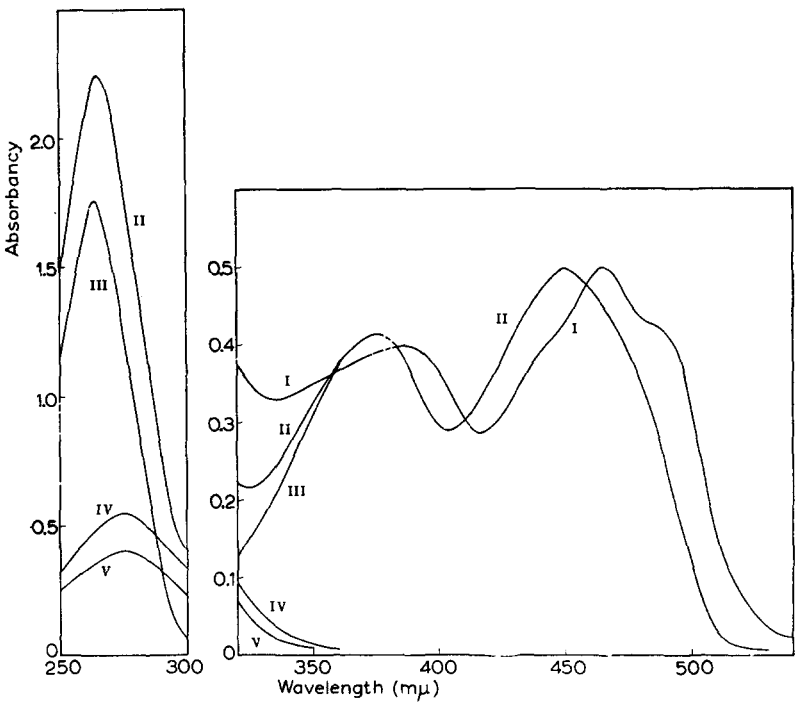

Fig. 6. Absorption spectra of the crystalline PNBA complex and PNBA in pyrophosphate buffer (*M*/60, pH 8.3). I, complex; II, the supernatant obtained after heating complex; III, FAD calculated from the absorbancy of the supernatant at 450 mμ; IV, calculated spectrum of PNBA in the supernatant (II–III); V, PNBA ($4.0 \cdot 10^{-5}$ *M*).

In order to liberate the FAD and the PNBA from the apo-enzyme, the pH of a solution of this complex was adjusted to 5.0. After heating at 90° for 3 min the denatured protein was removed by centrifugation. After the pH of the supernatant had been adjusted to 8.3, its absorption spectrum was measured. It can be presumed that Curve II, Fig. 6 represents the sum of the spectra of FAD and PNBA. As PNBA does not absorb light in the region above 370 mμ, Curve II is identical above this

wavelength with the curve expected for the FAD alone in the same concentration. It was therefore possible to calculate the complete spectrum of FAD (Curve III) from the absorbancy at 450 mμ indicated by Curve II. After this had been done, it was possible to calculate the spectrum of PNBA in the supernatant (Curve IV). This curve has the same shape as that of pure PNBA (Curve V). From the absorbancies at 275 mμ indicated by Curves IV and V, it was possible to calculate the PNBA content of the crystalline material. Table II shows that in this instance also the crystals contain equimolar amounts of apo-enzyme, coenzyme and substrate-substitute.

Experiments similar to those described for the enzyme – benzoate complex demonstrated that the PNBA also is driven off by an excess of D-alanine. The turnover number also appeared to be identical.

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