p-HYDROXYBENZOATE HYDROXYLASE: CONFORMATIONAL CHANGES IN CRYSTALS OF HOLOENZYME VS HOLOENZYME-SUBSTRATE COMPLEX

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Received November 1, 1968

Among the flavoprotein monooxygenases studied, two bacterial hydroxylases for hydroxybenzoates have been purified to homogeneity and investigated in some detail. Salicylate hydroxylase which contains FAD as the prosthetic group and requires NADH as the electron donor (Yamamoto et al., 1965) was shown to form a ternary complex of apoenzyme, FAD and salicylate (Katagiri et al., 1966). The crystalline p-hydroxybenzoate hydroxylase from Pseudomonas putida contains FAD but requires NADPH (Hosokawa et al., 1966). The absorption spectra are slightly changed in the visible region by the substrate addition and were attributed to a conformational change in the protein. Recently, we have prepared two crystalline forms of p-hydroxybenzoate hydroxylase from Pseudomonas desmolytica IAM 1123, namely the holoenzyme and the holoenzyme-substrate complex. Both forms are homogeneous on ultracentrifugation and electrophoresis with a molecular weight of 68,000. The molecule contains 1 mole of FAD and the complex 1 mole of the substrate in addition. Changes in the visible absorption spectrum, a slight shift in sedimentation coefficient and differences in the optical rotatory dispersion in the ultraviolet region suggest a conformational change on the substrate binding.

The enzyme was assayed by the p-hydroxybenzoate-dependent NADPH oxidation followed at 340 mμ. Protein was determined according to Kalcker, 1947. The enzyme was prepared from P. desmolytica IAM 1123 cultured by the method previously reported (Yano et al., 1966). All procedures for the preparation

were done at 0 - 5°C. Cells were disrupted with a sonic oscillator in 0.017 M succinate buffer, pH 6.0, containing 0.1 mM p-hydroxybenzoate and 1 mM mercaptoethanol. The same buffer was used for all steps unless otherwise stated. A clear supernatant fluid obtained by the centrifugation of the sonicate at 100,000 x g for 30 min. was treated with 0.1 volume of protamine sulfate and centrifuged to remove the precipitate. Then it was passed through a DEAE-Sephadex A-25 column, followed by an ammonium sulfate fractionation (0.35 - 0.60 satn.). The precipitate was dissolved in a minimum volume of the buffer, followed by a Sephadex G-100 gel filtration. Active fractions were applied on a DEAE-Sephadex A-50 column. After washing, the enzyme was eluted with the buffer containing 0.15 M sodium chloride, and the eluate was concentrated by precipitation with ammonium sulfate. The enzyme solution, about a 3 % protein concentration, was brought to be a slightly excess saturation of ammonium sulfate in the presence of the substrate. Fine yellow needle-shaped crystals appeared within a few minutes. Recrystallization was done in the same manner. The crystals obtained here were recognized to be the holoenzyme-substrate complex and are shown in Fig. 1. The crystalline holoenzyme-substrate complex was dissolved in a minimum volume of the buffer containing mercaptoethanol and passed through a Sephadex G-25 column to remove the bound substrate. After addition of ammonium sulfate to make a 0.55 saturation, the solution was kept in a desiccator under a slightly reduced pressure for more than 3 days. The crystals of holoenzyme which were yellowish rods appeared with the increase of the concentrations of both ammonium sulfate and the protein (Fig. 2). Both crystalline enzyme forms were homogeneous on ultracentrifugation and electrophoresis. A typical purification procedure is summarized in Table I.

On the assumption that the partial specific volume was 0.75 ml/g, molecular weight was estimated to be 68,000 by the short column method (Yphantis, 1960). Since the maximal specific activity was 40, the molecular activity was calculated to be 2700. The number of FAD molecule was computed

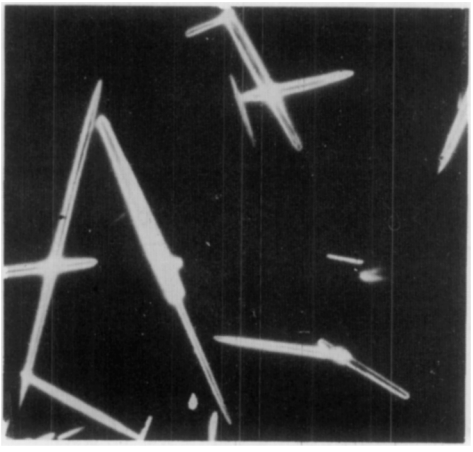

Fig. 1. Crystalline holoenzyme-substrate complex.

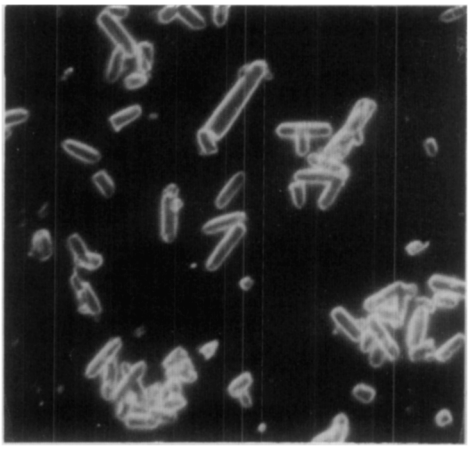

Fig. 2. Crystalline holoenzyme.

to be 0.9 by assuming that the molar extinction of bound FAD is the same as that of free FAD. The Km values were 25 μM for p-hydroxybenzoate and 40 μM for NADPH. Optimun pH was 8.2 to 8.6. The enzyme reacted specifically

Table I. Purification of p-hydroxybenzoate hydroxylase

Treatment	Volume	Total protein	Total activity	Specific activity	Yield
	ml	mg	units	units/ml	%
Crude extract	1,500	40,000	12,000	0.3	100
Protamine sulfate treatment	1,650	29,900	11,850	0.4	98.5
Ammonium sulfate frac.	50	4,210	9,700	2.3	80.5
Sephadex G-100 gel filt.	130	895	7,650	8.5	63.2
DEAE-Sephadex A-50 column	100	160	6,000	37.5	50.1
Holoenzyme-substrate complex					
First crystallization	3	123	4,900	39.8	40.7
Second crystallization	3	97	3,900	40.2	32.3
Holoenzyme					
Second crystallization	2	76	3,060	40.0	25.4

with NADPH and not with NADH, and was also highly specific to p-hydroxybenzoate, however, the concentration of which above 1 mM showed an inhibition. Very low activities were found with 3,4-dihydroxybenzoate (1.4 %) and 2,4-dihydroxybenzoate (0.9 %).

Absorption spectra of both forms are shown in Fig. 3. Shoulders were observed with the spectrum of the complex at 365, 380, 430 and 475 mμ, while

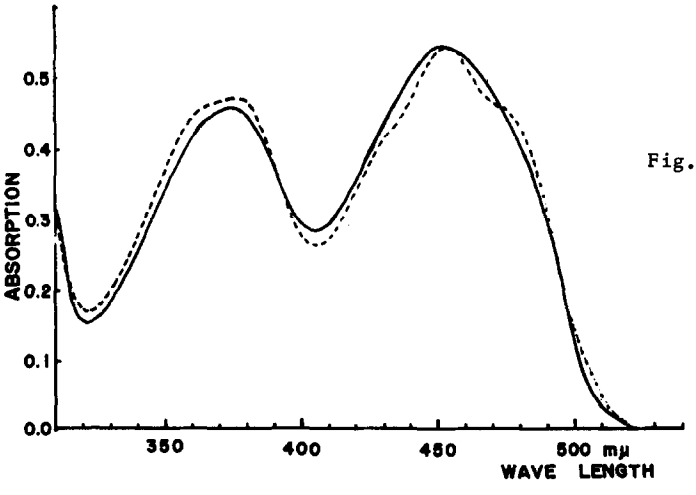

Fig. 3. Visible absorption spectra of both forms. Concentrations of each enzyme solutions were 260 mμmoles (19 mg) in 3 ml, at pH 6.0.

———; the holoenzyme,
-----; the holoenzyme-substrate complex.

Hitachi 124 double beam spectrophotometer.

the spectral curve of the holoenzyme was rather smooth. Spectrophotometric titration of the holoenzyme by p-hydroxybenzoate at 385 mμ showed that the ratio of the holoenzyme : the substrate was 1 : 1.1 at pH 6.0. When the

holoenzyme was excited at 375 mμ, a sharp fluorescence emmision spectrum having a maximum at 520 mμ was observed. On the substrate binding, a considerable quenching of the fluorescence occured, while the substrate itself did not show any emmision at this wave length. Fluorometric titration at 520 mμ showed that the ratio above mentioned was 1 : 0.9. Thus the holoenzyme-substrate complex was specifically formed by an equimolar holoenzyme and substrate.

Sedimentation coefficient, $S_{20,w}$, of the holoenzyme was 5.1 S and that of the complex 5.0 S. To confirm this small difference, experiments were performed in the same run using a Hitachi UCA-1A analytical centrifuge with two cells containing the holoenzyme and the complex, respectively. When the volume of the holoenzyme was slightly larger than that of the complex, two boundaries appeared in Schlieren pattern became closer and finally fused into a single peak. In the reverse case, the distance of two boundaries increased with time elapsed. This indicates that a difference in sedimentation coefficient really exists between the two forms and also demonstrates that a small

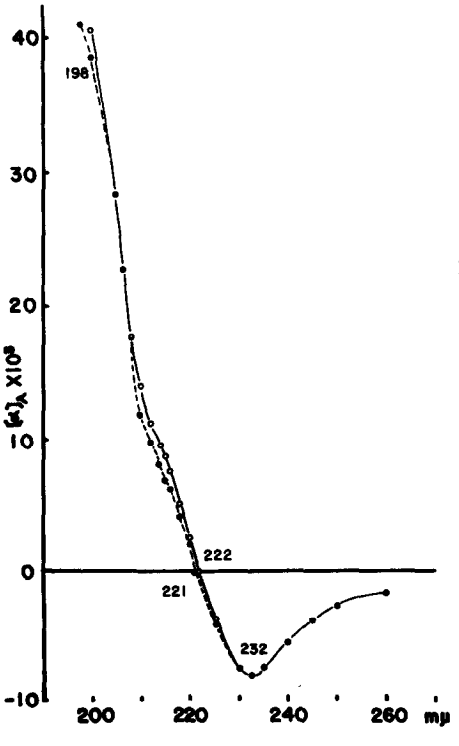

Fig. 4. Ultraviolet optical rotatory dispersion of p-hydroxybenzoate hydroxylase.

Cell chamber was kept an anaerobic condition with nitrogen gas at a slightly positive pressure. The concentration of the enzyme was 0.12 to 0.0022 % at pH 6.0, 22-25°C. The mean residue molecular weight of 115 was employed.

———; the holoenzyme,
------; the holoenzyme- substrate complex.

Japan Spectroscopic model ORD/UV-5 recording polarimeter.

but distinct conformational change of the protein was caused.

An attempt was made to clarify the internal structures of both forms in aqueous solutions by the optical rotatory dispersion in the ultraviolet region. The curve of the holoenzyme shown in Fig. 4 has a trough of Cotton effect at 232 mμ. The positive peak in the shorter wave length region could not be determined, since the strong absorption hindered the measurement. The corrected specific rotation value $[m']_{232}$ was calculated to be -8190 (Fasman, 1963). This value corresponds to a 47.6 % helix content in a protein (Cassim et al., 1967). A slight shift of the curve was observed in the case of the complex from 208 to 225 mμ , however, α-Helix content remained unchanged. The results suggest that a small conformational change might be induced on the substrate binding in the β-structure and/or in the random coil region of the protein. Crystallization of the complex was rather easy as compared with that of the holoenzyme. This might also be due to the difference in the conformations.

Crystallization of an enzyme-substrate complex was first reported with D-amino acid oxidase (Yagi et al., 1964) in the absence of the electron acceptor (molecular oxygen). In the case of p-hydroxybenzoate hydroxylase, the enzyme-substrate complex was easily crystallized even in the presence of oxygen, since it can not react with the substrate without electron donor, NADPH.

A rather large reduction in sedimentation coefficient was reported with D-amino acid oxidase on forming a complex with benzoate, an inhibitor (Yagi et al., 1967). Very small shift was induced by the substrate in the case of phosphoglucomutase (Yankeelov et al., 1965), however, in this case, separate two runs of ultracentrifugation had not drawn a conclusion.

It was found that p-hydroxybenzoate plays a role as an activator of the present enzyme which might be a result of the conformational change of the protein moiety. The details will be described in a subsequent paper.

The authors wish to express their sincere thanks to Prof. T. Kimura, St. Paul's University, Tokyo , Japan for his useful discussion. The

authors also indebted to Dr. E. Ichijima and Mr. K. Hayashi, Central Research Institute, Kikkoman Shoyu Co., Noda, Japan, for their kind help in ultraviolet optical rotatory dispersion measurements.

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