THE REACTION MECHANISM OF p-HYDROXYBENZOATE HYDROXYLASE AND A ROLE OF THE SUBSTRATE AS AN EFFECTOR

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In the previous communication (Yano et al., 1968), it was reported that two crystalline forms of p-hydroxybenzoate hydroxylase, the holoenzyme and the holoenzyme-substrate complex, were obtained from Pseudomonas desmolytica IAM 1123. Both forms contain equimolecular FAD. A slight but distinct conformational change on the substrate binding was demonstrated.

This communication deals with the reaction mechanism of the enzyme and the accelerating effect of the substrate on the anaerobic reduction of enzyme-bound FAD. The reaction catalyzed by the enzyme seems to consist of the following elementary reactions: 1) the binding of p-hydroxybenzoate with the holoenzyme to form the complex, 2) the reduction of the FAD moiety by NADPH, 3) the splitting of the bond between oxygen atoms together with the production of water and protocatechuate by the hydroxylation of the substrate. Under anaerobic conditions, k_{red}, the reduction rate of the FAD moiety by NADPH was increased about 10^4 times by the enzyme's own substrate, p-hydroxybenzoate.

Methods of enzyme assay, protein determination and preparation of the crystalline holoenzyme-substrate complex were as described previously (Yano et al., 1968). The crystalline holoenzyme-substrate complex was dissolved to make about a 2 % protein concentration in 0.05 M tris-maleate buffer, pH 6.0, containing 1 μM FAD and 1 mM mercaptoethanol. For the "flow" experiments, the same buffer with one exception of pH 8.2 was used as the solvent of the complex. The enzyme solution was passed through a Sephadex G-25

column to remove the substrate and the excess FAD. The active fractions were used as the holoenzyme. Protocatechuate was determined enzymatically using crystalline protocatechuate 3,4-oxygenase (Fujisawa et al., 1968) which was kindly supplied by Prof. Hayaishi and Dr. Fujisawa, Kyoto University, Kyoto 606, Japan. To obtain the value of k_{red} of the FAD moiety, "stopped flow" and "rapid flow" experiments were performed under anaerobic conditions using a flow apparatus according to the methods previously reported (S. Nakamura et al., 1968). Each solution of the holoenzyme, the substrate, NADPH and buffer had been deoxygenated previously by repeated evacuation and flushing with oxygen-free nitrogen. The absorption spectrum of the enzyme in the visible region was measured using a Hitach 124 spectrophotometer.

The solutions of both forms showed characteristic absorption spectra in the visible region as shown by the curve a and b in Fig. 1. On the addition of NADPH into the holoenzyme-substrate complex solution in a Thunberg-type cuvette under anaerobic conditions, the absorption spectrum of the enzyme was immediately changed by an amount corresponding to the added amount of NADPH,

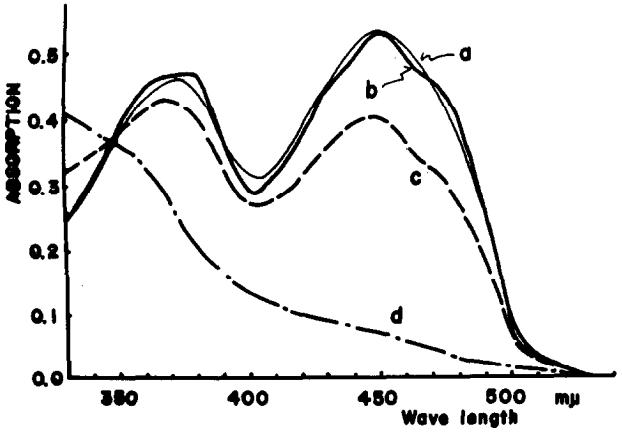

Fig. 1. Spectrophotometric titration of the enzyme.

Thunberg-type cuvette containing 242 mμmoles enzyme protein in 3 ml at pH 6.0. Curve a: holoenzyme; curve b: holoenzyme-substrate complex; curve c: after addition of 72 mμmoles NADPH to b; curve d: after addition of 233 mμmoles NADPH to b.

and finally the yellow color of the enzyme was bleached (curve d). The data of titration thus obtained showed the ratio of the complex to NADPH to be 1 : 1.1, on the assumption that the molar extinctions of the bound FAD and $FADH_2$ in the complex are the same as those of free forms.

The fully reduced form of the complex was rapidly reoxidized by the introduction of oxygen (curve a in Fig. 1). After this treatment, a stoichiometric formation of the product, protocatechuate, was found, as shown in Table I. When the enzyme was inactivated by heating before the introduction of oxygen, no significant amount of the product was observed (Table II). Similar results were obtained when NADH was used as the reductant, although

Table I. Stoichiometry of the reaction with introduction of oxygen.

in mμmoles

Sequence	Enzyme	Reduced enzyme	Reductant	Substrate	Product
Substrate ⟶ NADPH	212	200	212	213	238
NADPH ⟶ Substrate	222	212	222	222	168
NADH ⟶ Substrate	216	181	23*	216	210
$Na_2S_2O_4$ ⟶Substrate	212	197	excess	212	208

The enzyme was anaerobically reduced and mixed with oxygen, followed by heat treatment. The product, protocatechuate, was determined enzymatically in the supernatant obtained by centrifugation of the heated reaction mixture.
* in μmoles.

Table II. Stoichiometry of the reaction without introduction of oxygen.

in mμmoles

Sequence	Enzyme	Reduced enzyme	Reductant	Substrate	Product
Substrate ⟶ NADPH	216	189	189	229	1.9
NADPH ⟶ Substrate	212	183	184	212	0.0
NADH ⟶ Substrate	216	200	15*	229	2.2
$Na_2S_2O_4$ ⟶ Substrate	212	198	excess	212	2.1

The enzyme was anaerobically reduced and then inactivated by heat.
* in μmoles.

the reduction of the enzyme was very slow. The same was true an excess of dithionite (Tables I and II). From these results together with the previous data (Yano et al., 1968), an equimolar relationship between apoenzyme, FAD, substrate, NADPH, oxygen and product was recognized. Although NADPH is known to be the specific electron donor for this enzyme, it was thus found that NADH and dithionite can act as donors, and that the complex reduced by these reductants was also enzymatically active. It is indicated that direct electron donor of the oxygenation reaction is the enzyme-bound $FADH_2$. On the basis of the results obtained, the following reaction sequence can be posturated for the overall reaction of the enzyme.

E-FAD + p-hydroxybenzoate $\longrightarrow$ E-FAD-p-hydroxybenzoate

E-FAD-p-hydroxybenzoate + NADPH $\longrightarrow$ E-$FADH_2$-p-hydroxybenzoate + $NADP^+$

E-$FADH_2$-p-hydroxybenzoate + O_2 $\longrightarrow$ E-FAD + protocatechuate + H_2O

where E denotes the protein moiety of the enzyme. Similar findings were reported in salicylate hydroxylase (Katagiri et al., 1966).

Under anaerobic conditions, the reduction of FAD moiety of the holoenzyme

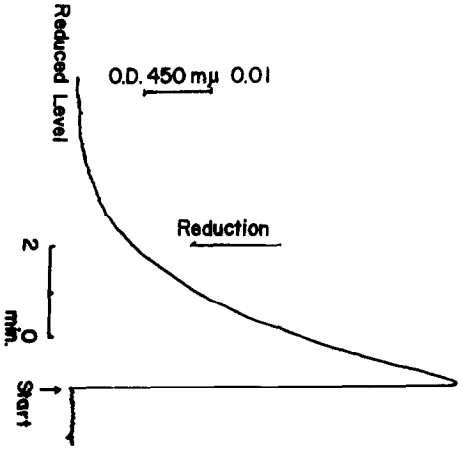

Fig. 2. Trace of change of optical density at 450 mμ obtained in an anaerobic flow experiment.

Holoenzyme: 7.2 μM; NADPH: 235 μM; in 0.05 M Tris-maleate buffer, pH 8.2, at 25°C.

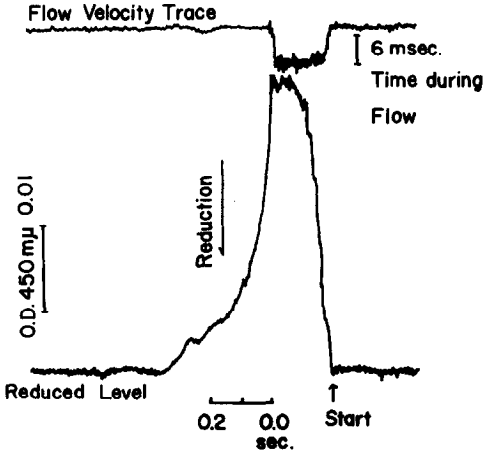

Fig. 3. Trace of change of optical density at 450 mμ obtained in an anaerobic flow experiment.

Holoenzyme: 7.2 μM; substrate: 233 μM; NADPH: 235 μM in 0.05 M Tris-maleate buffer, pH 8.2, at 25°C.

Table III. Anaerobic reduction of FAD moiety in the presence of the substrate.

	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 4.	Expt. 5.
$\underline{k}_{red}$ sec^{-1}	96	96	92	104	91

The mean value of $\underline{k}_{red}$ was calculated to be 96 sec^{-1}. Holoenzyme 7.2 μM, substrate 233 μM and NADPH 235 μM were used.

by NADPH was fairly slow, while that of the holoenzyme-substrate complex was extremely fast and could be measured only by a "flow apparatus". Figs 2 and 3 show the traces of the reductions of the enzyme in the absence and in the presence of the substrate, respectively. Table III shows the $\underline{k}_{red}$ value estimated by "rapid flow" method using the holoenzyme with a sufficient amount of the substrate. The same initial rate of the reduction was also found using the complex. The $\underline{k}_{red}$ values of the holoenzyme and the complex were 0.010 sec^{-1} and 96 sec^{-1}, respectively. Thus the reduction of enzyme-bound FAD was accelerated about 10^4 times by its own substrate. On the other hand, the $\underline{K}_m$ value for NADPH in the absence of the substrate was estimated to be 230 μM, which is about 5 times larger than that in the presence of the substrate (40 μM) (Yano et al., 1968). Both phenomena might be caused by the slight but distinct conformational change of the enzyme protein as stated before. The increased reduction rate is great enough to explain the overall reaction velocity, since the value calculated from the molecular activity was 45 sec^{-1} at 25°C, pH 8.2. The substrate itself was found to have a role as an effector for the reduction of the FAD moiety. It should be mentioned here that p-hydroxybenzoate plays three roles, i.e., the inducer, the effector and the substrate of this enzyme.

The accelerating effects of the substrates have been reported on some other enzymes. In the case of phosphoglucokinase, the relationship between the rate of reaction and the substrate concentration was expressed by a sigmoidal curve (Kemp et al., 1967). p-Hydroxybenzoate hydroxylase, however,

did not show such a relationship. The binding modes of the substrates with these enzymes might be different from each other. Another example is phosphoglucomutase. In this case, the protein structure near the active site is said to be changed by its substrate to take a better conformation for the reaction (Yankeelov et al., 1965). This is known as the "induced-fit" theory. It was also reported with two other hydroxylases that the catalyzed reaction was accelerated by the substrate. The rate of NADH oxidation of salicylate hydroxylase was increased 150 times by the substrate, salicylate, while the K_m value of this enzyme for NADH decreased by a factor of about 1/400 in the presence of the substrate (Katagiri et al., 1965). The reduction of another p-hydroxybenzoate hydroxylase from Pseudomonas putida was accelerated by the substrate but not so significantly as compared with our enzyme (about 130 times) (Hosokawa et al., 1966). In the case of our enzyme, it is evident that similar but more remarkable changes were caused by the substrate in reaction rate as well as in protein conformation.

Further investigation will be published elsewhere.

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