THE PARTICIPATION OF A SULFHYDRYL GROUP IN THE BINDING OF IRON TO PYROCATECHASE

Kenzo Nagami

The Central Research Institute, Suntory Limited, Osaka, Japan

Received April 14, 1972

Summary; Iron-free apopyrocatechase is easily converted to the holoenzyme containing one atom of ferric iron by treatment with ferrous iron under aerobic conditions. The presence of oxygen is essential for the reconstitution reaction and the stoichiometry among apoenzyme, ferrous iron and oxygen is 4 : 4 : 1. A sulfhydryl group of the apoenzyme, which reacts with either PCMB or DTNB, become undetectable in holoenzyme by DTNB. The apoenzyme modified with DTNB, loses its ability to bind iron unless treated with cysteine or β-mercaptoethanol.

These results indicate that one free sulfhydryl group of the apoenzyme is involved in the binding of iron to the enzyme through iron-sulfur bond and show the possibility of the participation of an oxidase-like reaction in the reconstitution of holoenzyme.

Introduction; Pyrocatechase catalyzes the oxidative cleavage of the aromatic ring of catechol to yield cis,cis-muconic acid with the consumption of one mole of oxygen per mole of catechol oxidized. Pyrocatechase, purified from Pseudomonas arvilla C-1 (1) and from Brevibacterium fuscum P-13 (2,3), have non-heme iron as the sole prosthetic group essential for enzyme catalysis (1,2).

The mode of binding of the iron to the enzyme protein is still obscure, although reaction mechanisms have been proposed based on the analyses of the behavior of the enzyme-bound iron with an ESR spectrometer (4,5,6). To solve this problem, it would be helpful to prepare the iron-free apoenzyme and to follow the process of its reconstitution to the holoenzyme.

Recently, Takemori et al. reported the preparation of iron-free apometapyrocatechase by the treatment of the holoenzyme with o-phenanthroline (7). Although the apoenzyme was reconverted to holoenzyme, these authors said nothing about the amino acid residues involved in the binding of iron to the protein.

Brevibacterium pyrocatechase has one atom of ferric iron per mole of enzyme protein. The enzyme-bound ferric iron is easily removed from the holoenzyme by treatment with ethylprotocatechuate to give its apoenzyme (8). The apoenzyme thus prepared is converted

quantitatively into the holoenzyme in the presence of ferrous iron and oxygen.

The present communication reports an analysis of the reconstitution of holoenzyme from apopyrocatechase and the amino acid residues involved in the binding of non-heme iron to the protein moiety of the holoenzyme.

Materials and Methods; Pyrocatechase was purified from Brevibacterium fuscum P-13 according to the method of Kita et al. (5). The iron-free apopyrocatechase was prepared from holopyrocatechase by the treatment with ethyl protocatechuate (8). Oxygen consumption was measured with a Yanagimoto oxygen consumption recorder Model PO-100 with a rotating platinium electrode. Absorption was measured using a 356 Hitachi Two Wavelength Spectrophotometer. The number of free sulfhydryl groups in protein was determined according to the method of Boyer (9) and method of Ellman (10). Protein was determined according to the method of Lowry et al. (11).

Results and Discussion; The stoichiometry of the reconstitution of holopyrocatechase;

The iron-free apopyrocatechase is easily converted to the iron-containing holoenzyme having full enzyme activity by treatment with ferrous ion under aerobic conditions. Simultaneously, the absorption and ESR spectra of the reconstituted enzyme become identical with those of the native enzyme. This reconstitution reaction does not occur following the addition of either ferric ion or ferrous ion under anaerobic conditions. However, the

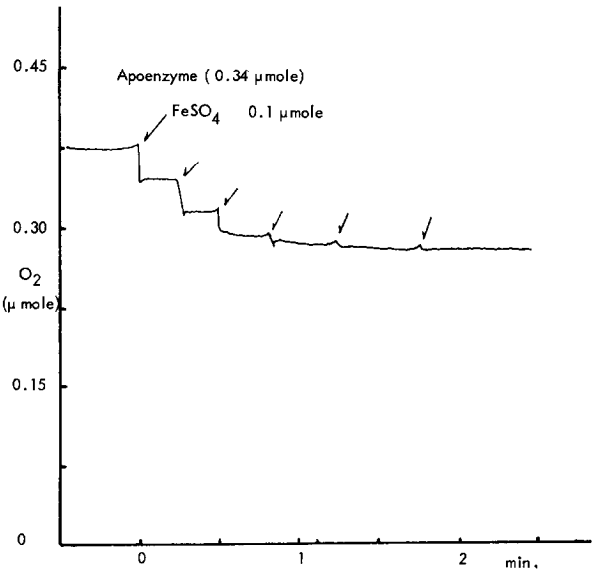

Fig. 1. Apopyrocatechase (0.34 μmole) dissolved in 2 ml of 0.1 M Tris-HCl (pH 7.5) was incubated at 25°C in a reaction cell with a rotating platinium electrode. Each 10 μl of 10 mM ferrous sulfate solution were successively added to the reaction cell.

valence state of the bound-iron, is ferric in active holopyrocatechase (5,6). This reconsti tution process is certainly a kind of oxidase-like reaction that entails the participation of molecular oxygen.

The stoichiometry of this oxidative reconstitution reaction is shown in Figs. 1 and 2. Fig. 1 shows the oxygen consumption by the apoenzyme during titration with ferrous ion, in which there is a constant relationship between the amount of the apoenzyme added and oxygen consumed.

Oxygen consumption was plotted against the amount of ferrous iron added (Fig. 2).

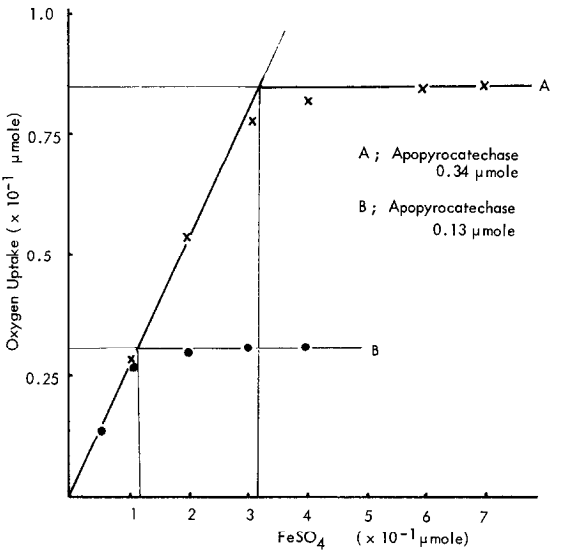

Fig. 2. The experimental conditions were the same as those shown in Fig. 1.

Oxygen consumption increases linearly with increasing concentration of ferrous iron and is saturable. Irrespective of the amount of the apoenzyme used, there is a constant relationship among the amount of the apoenzyme used, oxygen consumed and ferrous ion added at the saturation point. Table 1 shows the relationship among these quantities.

Table 1.

Apopyrocatechase used (µ mole)	Ferrous ion added (µ mole)	Oxygen consumed (µ mole)
0.34	0.33	0.084
0.13	0.13	0.033

Table 1. Stoichiometry of the reconstitution reaction

From these results, it is concluded that the reconstitution of holopyrocatechase involves an oxidation reaction in which molecular oxygen participates and the stoichiometry among apopyrocatechase, ferrous ion and oxygen in the reconstitution reaction is 4 : 4 : 1.

Cysteinyl residue in Pyrocatechase; Modifications with several sulfhydryl reagents have revealed that some dioxygenase-containing non-heme iron enzymes, such as pyrocatechase, purified from Ps. arvilla (1), and 3,4-dihydroxyphenylacetate 2,3-dioxygenase, from Ps. ovalis (12), have cysteinyl residues essential for catalytic activity. But the necessity of cysteinyl residues for enzyme activity remains obscure for the metapyrocatechase purified from Ps. putida (7). For Brevibacterium pyrocatechase, the cysteinyl residue is indispensable for enzyme activity, but its role is unclear.

Table 2.

Reagent	Native Pyrocatechase		Apopyrocatechase	Reconstituted Holopyrocatechase	
	No. of -SH	Inhibition		No. of -SH	Inhibition
PCMB	1.02	85~100%	1.0*	1.0	90~100%
DTNB	0	0	1.03*	0	0

* unable to be converted into holoenzyme

Table 2. Titrations of sulfhydryl residues were performed by the methods of Boyer (9) and Ellman (10).

Table 2 shows the results of quantitative analysis for cysteinyl residues in both holo- and apopyrocatechases with PCMB and DTNB. In general, PCMB is a more reactive reagent than DTNB. PCMB reacts not only with free sulfhydryl groups but also with certain masked ones forming a mercury-sulfur bond, but DTNB reacts only with free sulfhydryl groups, forming a disulfide bond. Both iron-free apopyrocatechase and iron-containing holopyrocatechase have one sulfhydryl group which reacts with PCMB. By treatment with PCMB, the holoenzyme lost its activity and apoenzyme failed to be converted to the holoenzyme following incubation with ferrous ion and oxygen. In contrast, the iron-containing holoenzyme had no DTNB-reactive sulfhydryl groups, although a cysteinyl residue in the iron-free apoenzyme formed a disulfide bond with 4-carboxy-2-nitrothiophenol anion which resulted in loss of the ability of the apoenzyme to be converted into the iron-containing holoenzyme following treatment with both ferrous ion and oxygen. When TNB-bound apoenzyme was treated with large excess of cysteine, the disulfide bond was reduced, and the TNB-depleted apoenzyme was completely reconverted to holoenzyme by treatment with both ferrous ion and oxygen (Fig. 3).

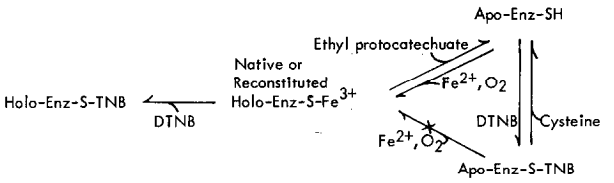

Fig. 3. A proposed reaction scheme for the interconversion of holo- and apopyrocatechase in connection with the oxidation reaction and chemical modification of the sulfhydryl residue with DTNB.

These results show that the iron-containing holopyrocatechase has one iron-sulfur bond (masked sulfhydryl residue) which is essential for enzyme activity, and is exchangeable for Hg cation but not for thiophenol anion, and that the iron-free apopyrocatechase has one free cysteinyl residue that reacts with Hg cation and thiophenol anion.

Conclusion; 1) A cysteinyl residue in the protein moiety of pyrocatechase is involved in the binding of ferric iron and forms an iron-sulfur bond. A cysteinyl sulfhydryl residue is free in the iron-free apoenzyme.

2) The stoichiometry of the reconstitution reaction of holopyrocatechase from the apoenzyme in the presence of ferrous iron and oxygen is consistent with the following scheme:

$$4\ \text{Apo-Enz-SH} + 4\ Fe^{2+} + O_2 \longrightarrow 4\ \text{Holo-Enz-S}\ Fe^{3+} + 2\ H_2O$$

Acknowledgements; The author wishes to thank Dr. Yoshihiro Miyake, Osaka University, and Dr. Siro Senoh and Mrs. Miyoko Ono, Suntory Ltd., for their helpful advice and discussions.

References

1) Y.Kojima, H.Fujisawa, A.Nakazawa, T.Nakazawa, F.Kanetsuna, H.Taniuchi, M.Nozaki and O.Hayaishi, J. Biol. Chem., 242, 3270 (1967).
2) H.Nakazawa, H.Inoue and Y.Takeda, J. Biochemistry (Tokyo), 54, 65 (1963).
3) K.Kawakami, H.Kita and S.Senoh, Seventh Int. Cong. Biochem., Tokyo, Abs. IV, F-85 (1967).
4) T.Nakazawa, M.Nozaki, O.Hayaishi and T.Yamano, J. Biol. Chem., 244, 119 (1969).
5) H.Kita, Y.Miyake, M.Ono(Kamimoto), S.Senoh and T.Yamano, J. Biochemistry, 66, 45 (1969).
6) K.Nagami and Y.Miyake, Biochem. Biophys. Res. Commun., 42, 497 (1971).
7) S.Takemori, T.Komiyama and M.Katagiri, Eur. J. Biochem., 23, 178 (1971).
8) K.Nagami and Y.Miyake, Biochem. Biophys. Res. Commun., 46, 198 (1972).
9) P.D.Boyer, J. Am. Chem. Soc., 76, 4331 (1954).
10) G.L.Ellman, Arch. Biochem. Biophys., 74,4331 (1958).
11) O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J. Biol. Chem., 193, 265 (1951).
12) S.Senoh, H.Kita and M.Ono(Kamimoto), in K.Bloch and O.Hayaishi (Editors), Biological and Chemical Aspects of Oxygenases, Maruzen Company, Ltd., Tokyo, 1966, pp. 378.