THE PARTICIPATION OF A TRYPTOPHAN RESIDUE IN THE BINDING OF FERRIC IRON TO PYROCATECHASE

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Summary: Brevibacterium pyrocatechase has one ferric iron bound to enzyme protein through an iron-sulfur linkage. When this iron-sulfur bond was broken by treatment with mercuric chloride at 0°C and neutral pH, the ferric iron was still bound to enzyme protein although the enzymic activity was almost lost. This result indicates the existence of a second linkage between the iron and another amino acid residue, besides the iron-sulfur bond, which participates in the binding of iron to pyrocatechase. In order to identify this second amino acid residue, chemical modification using N-bromosuccinimide(NBS) and fluorometric studies were performed. After degradation of only one Trp-residue by NBS, the apoenzyme lost its ability to reconvert to the holoenzyme. Both the holo- and apoenzyme showed the Trp-residue fluorescence emission maximum at 342 nm after excitation at 280 nm. The emission intensity at 342 nm was 3-fold higher for the apoenzyme. The quenching of fluorescence of apoenzyme at 342 nm was proportional to the increase of the reconstitution rate from apo- to holoenzyme by the addition of ferrous ion. These results from chemical modification and fluorometric studies on apo- and holopyrocatechase suggest the participation of Trp-residue in the binding of iron to pyrocatechase.

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 Brevibacterium fuscum p-13(2), have non-heme ferric iron as the sole prosthetic group essential for enzyme reaction. Although a reaction mechanism has been proposed based on the ESR changes of bound ferric iron during the enzyme reaction(3,4,5), the structure of the active site and its changes during the enzyme reaction have been unsolved until now.

Brevibacterium pyrocatechase has one g atom of non-heme ferric iron per mole of protein. During the course of our studies on pyrocatechase, it became apparent that the ferric iron binds to the enzyme through the iron-sulfur linkage in the active site of pyrocatechase (6) and that the ferric iron can be easily removed by the reaction of some substrate analogues, for example ethyl protocatechuate, resulting in apoenzyme (7). To elucidate the precise reaction mechanism, the structure and properties of active site containing ferric iron must be studies in detail.

Nakazawa et al (8) have reported a possible existence of interaction between Tyr-residues and the bound-iron in pyrocatechase purified from Pseudomonas arvilla c-1 based on the analysis of C.D. spectra of this enzyme. In our case, though holo- and apopyrocatechase showed different C.D. band in Trp- and Tyr-regions, too and also the holoenzyme was strongly inhibited by NBS accompanying degradation of Trp-residues, but the participations of Trp- and Tyr-residues in the binding of iron to the enzyme protein could not be defined by these observations (9).

The present report concerns the participation of a Trp-residue in the binding of ferric iron to the enzyme protein based on chemical modification and fluorescence spectral studies on holo- and apopyrocatechase.

Materials and Methods : Pyrocatechase was purified from the cells of Brevibacterium fuscum p-13 grown with phenol as the major carbon source according to the method of Kita, et al (10). The iron-free apopyrocatechase was prepared from the holoenzyme by the treatment with ethyl protocatechuate (7). TNB-apoenzyme, thiophenylated apoenzyme with its one free Cys-residue by 2-nitro, 5-sulfhydryl benzoic acid, was obtained from reaction mixture of the apoenzyme and DTNB (Ellman's reagent) through gel filtration using Sephadex G-25 (7).

Fluorescence emission spectra were recorded with a Hitachi Spectrophotometer Model MPF-2A. The exciting light came from a 150 watt xenon lamp. Fluorescence was excited at 280 nm and the emitted light was recorded from 300 to 400 nm. Solutions to be measured had optical density of less than 0.13 at 280 nm. Consequently, emission intensities were proportional to concentration.

Absorption was measured using a 356 Hitachi Two Wavelength Spectrophotometer. Protein was determined according to the method of Lowry, et al (12).

Results and Discussion: Effects of mercuric chloride on pyrocatechase

As previously observed(6), pyrocatechase has a Cys-residue which is involved in the binding of ferric iron in the active site through an iron-sulfur linkage beeing exchangeable with Hg cation.

Pyrocatechase shows a visible absorption maximum at 420 nm due to the iron-sulfur bond (Figure 1, A). Figure 1 shows the changes in the absorption spectrum of pyrocatechase during the reaction with mercuric chloride at pH 7.5. When the reaction was performed in an ice bath, the reaction mixture gave an absorption maximum at 500 nm and a broad absorption between 500 and 700 nm (Figure 1, B) accompanied with loss of enzymic activity. After the reaction was completed, the reaction mixture was treated

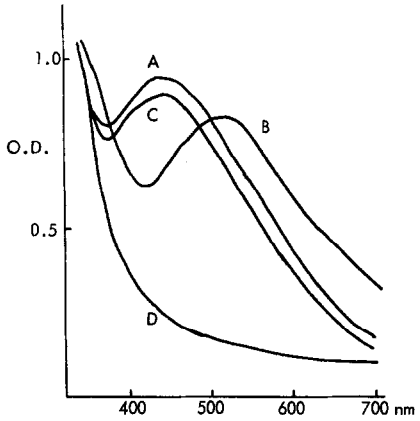

Figure 1. The changes in the absorption spectrum of pyrocatechase caused by treatment with $HgCl_2$. The spectra were taken(A) without treatment, (B) incubation for 45 min. after the addition of $HgCl_2$ on the ice bath, (C) incubation for 20 min. after the addition of cysteine on the ice bath and (D) after incubation at 25°C for 10 min.. A 2.0 ml aliquot of the enzyme solution(18.9 mg/ml, 0.3 mM) in 0.1 M Tris-HCl pH 7.5 was placed in a test tube on the ice bath and was mixed with 0.1 ml of 40 mM $HgCl_2$. After incubation for 45 min., 0.1 ml of 0.1 M cysteine was added on the ice bath or the reaction mixture was incubated at 25°C, respectively.

with a large excess of cysteine to remove the mercuric cation. This procedure restored the absorption spectrum to that of the holoenzyme (Figure 1, C) concomitant with recovery of full enzyme activity without addition of iron. These observations suggest that the bound iron in holopyrocatechase is still bound to the enzyme through the exchange of iron-sulfur linkage to mercuryl-sulfur linkage.

When the above reaction mixture containing pyrocatechase and mercuric chloride was incubated at 25°C for several minutes, it lost its visible absorption spectrum (Figure 1,D) and the ability to reconstitute holoenzyme by the addition of cysteine. This observation suggests that the binding of the iron to the enzyme after the breakage of the iron-sulfur linkage was labile since it could not exist at 25°C.

From these results, it can be concluded that there is the second amino acid residue involved in the binding of ferric iron to pyrocatechase.

Participation of Trp-residues in the binding of iron: In order to identify the second amino acid residue involved in the binding of iron in pyrocatechase, a chemical modification using NBS and fluorometric studies were performed.

Figure 2 shows the results of chemical modification studies on the reconstitution of holoenzyme from apoenzyme using NBS. As previously reported (6), the iron-free apopyrocatechase has a free cysteinyl residue which is used in the binding of iron to the enzyme protein in holoenzyme through an iron-sulfur linkage. NBS reacts with Cys-

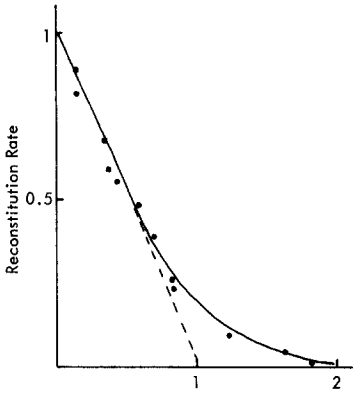

Fig. 2.

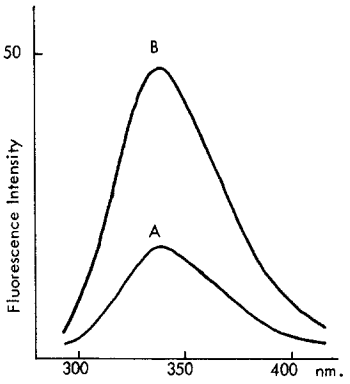

Fig. 3.

Figure 2. Effects of the degradation of Trp-residues by NBS on the reconstitution of holopyrocatechase from TNB-apoenzyme. A 3.0 ml aliquot of TNB-apoenzyme solution(0.44 mg/ml) in 0.1 M phosphate buffer pH 7.0 were placed in test tubes at 25°C and were mixed with 10-50 µl of 1-4 mM NBS solutions. The reaction mixtures were incubated at 25°C for 20 min. and their absorption spectra were measured from 240 to 320 nm. The spectra showed absorption maxima at 282 nm and isosbestic points at 268 and 295 nm. The number of degraded Trp-residues were caliculated using the decrease of absorption at 280 nm according to the equation of Witkop et al (11). The reconstitution of holoenzyme was performed by the addition of ferrous ions under aerobic conditions following preincubation with cysteine to reduce a disulfide bond.

Figure 3. The fluorescence emission spectra of holo- and apopyrocatechase. (A): holopyrocatechase (0.05 mg/ml in 0.025 M Tris-HCl pH 7.5, $O.D._{280\ nm}=0.12$), (B): apopyrocatechase (0.05 mg/ml in 0.025 M Tris-HCl pH 7.5, $O.D._{280\ nm}=0.13$). The excitation wave length was 280 nm.

residues as well as Trp-residues in protein moiety. So, in this experiment, in order to clarify the effect of modification of only Trp-residues by NBS upon the reconstitution reaction, the apoenzyme was changed to the thiophenylated one (TNB-apoenzyme) with its free Cys-residue using Ellman's reagent(DTNB) before treatment with NBS. TNB-apoenzyme was completely reconverted into the iron-containing holoenzyme unless treated with NBS by the addition of ferrous ions under aerobic condition following the reduction of its difulfide bond by a large excess of cysteine.

As shown in Figure 2, as the modification of Trp-residues by NBS proceeded, the reconstitution ability of TNB-apoenzyme directly decreased. When one Trp-residue was modified, TNB-apoenzyme completely lost its ability to reconvert into the holoenzyme by the addition of ferrous ions in the presence of oxygen following the reduction of its disulfide bond.

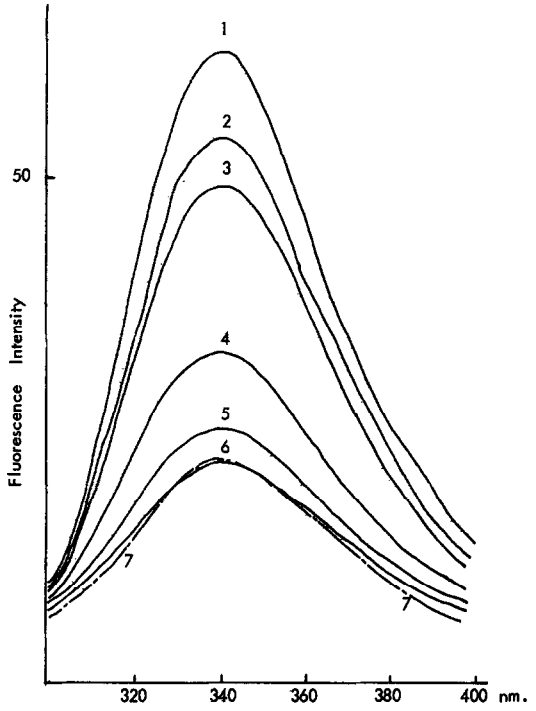

Fig. 4.

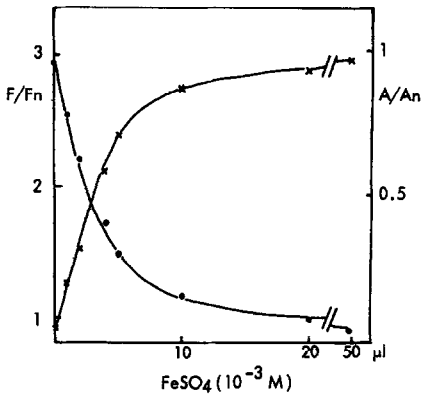

Fig. 5.

Figure 4. The changes of fluorescence emission spectra of apopyrocatechase by treatment with ferrous ions. The spectra were taken(1) without treatment(apoenzyme, 0.05 mg/ml O.D.$_{280\ nm}$=0.13, 3.0 ml), (2) addition of 1 mM ferrous ion solution of 1 µl, (3) 3 µl, (4) 5 µl, (5) 10 µl, (6) 20 µl and (7) holoenzyme (0.05 mg/ml, O.D.$_{280\ nm}$=0.12). The enzyme solution in 0.025 M Tris-HCl pH 7.5 were placed in test tubes at 25°C, the ferrous ion solutions were added and the mixture were incubated at 25°C for 5 min. before measuring the fluorescence spectra. The excitation wavelength was 280 nm.

Figure 5. The decrease of fluorescence at 342 nm and the increase of enzyme activity by the conversion of apo- to holopyrocatechase. The reaction conditions were the same as described in Figure 4. The excitation wavelength was 280 nm. Fn. or An. indicates the fluorescence intensity of holoenzyme or the enzyme activity of holoenzyme.
—— × —— : Enzyme activity —— • —— : Fluorescence

This finding suggests that a Trp-residue in the apoenzyme is essential for its reconstruction to the holoenzyme concerning with the binding of iron to the enzyme protein.

Fluorescence emission spectra of holo- and apopyrocatechase at pH 7.5 after excitation at 280 nm are shown in Figure 3. In general, the fluorescence of organic substances are quenched by the formation of some linkage with some transition metal ions, especially with ferric or cupric ions (13).

Both holo- and apopyrocatechase showed an emission maximum at 342 nm due to Trp-

residues in the protein moiety. But the fluorescence intensity at 342 nm was about 3-fold higher for the iron-free apoenzyme than the iron-containing holoenzyme. The fluorescence of the inactive apoenzyme at 342 nm was rapidly quenched during reconstitution by treatment with ferrous ions under aerobic conditions which coincided with the recovery of enzymic activity(Figure 5).

Figure 6 shows the decrease of the fluorescence intensity at 342 nm and the increase of enzymic activity following the titration of the apoenzyme with ferrous ions, which reveals a direct proportionality to iron added.

From these observations, it became apparent that the fluorescence emission maximum at 342 nm was due to the Trp-residues of pyrocatechase strongly connected with the binding of iron to the enzyme.

The results of chemical modifications with NBS and fluorescence studies on holo- and apopyrocatechase suggest the participation of a Trp-residue in the binding of iron to the enzyme protein.

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