PREPARATION AND PROPERTIES OF APOPYROCATECHASE

Kenzo Nagami and Yoshihiro Miyake *

The Central Research Institute, Suntory Limited, Osaka and *the Department of Biochemistry School of Medicine, Osaka University, Osaka

Received October 22, 1971

Summary; Ethylprotocatechuate removes enzyme-bound ferric iron from pyrocatechase to give apoenzyme without detectably affecting the protein. The apoenzyme is easily reconverted to holoenzyme in the presence of ferrous iron and oxygen, and the reconstituted holoenzyme regains full enzymatic activity. Absorption and circular dichroism spectra in the visible region and ESR signal at g=4.3 of the reconstituted holoenzyme are almost identical with those of the native enzyme, indicating that ferric iron is bound at the active site.

Introduction; Pyrocatechase is a typical dioxygenase which cleaves the aromatic ring of catechol to cis,cis-muconic acid with consumption of 1 mole of oxygen per mole of catechol. Both pyrocatechase purified from Pseudomonas arvilla C-1 (Y. Kojima et al., J. Biol. Chem., 242, 3270) and from Brevibacterium fuscum P-13(1 ,2),have non-heme ferric iron as sole prosthetic group (1,3). From ESR studies, the participation of the non-heme iron in the reaction and valence changes during catalysis have been observed by Nakazawa et al. (4), Kita et al. (5), and by Nagami and Miyake (6). The amino acid residue involved in the binding of the non-heme iron remain obscure. To determine these, it will be very helpful to have apoenzyme and to be able to follow the process of their reconstitution.

Brevibacterium pyrocatechase has one g atom of iron per mole of enzyme protein. During the course of our studies on this enzyme, it became apparent that ethylprotocatechuate, a substrate analogue, interacts with the enzyme bound ferric iron of the

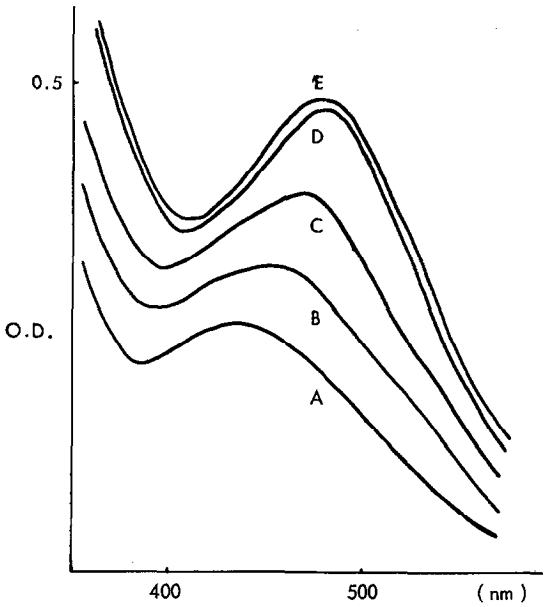

Fig. 1. Changes in the visible absorption spectrum of pyrocatechase induced by the addition of ethylprotocatechuate. (A); without treatment (3.0 mg/ml), (B); at 1 min. after addition of ethylprotocatechuate (10 mM), (C), (D), (E); after incubated at 30°C for 6, 30 and 60 min. respectively.

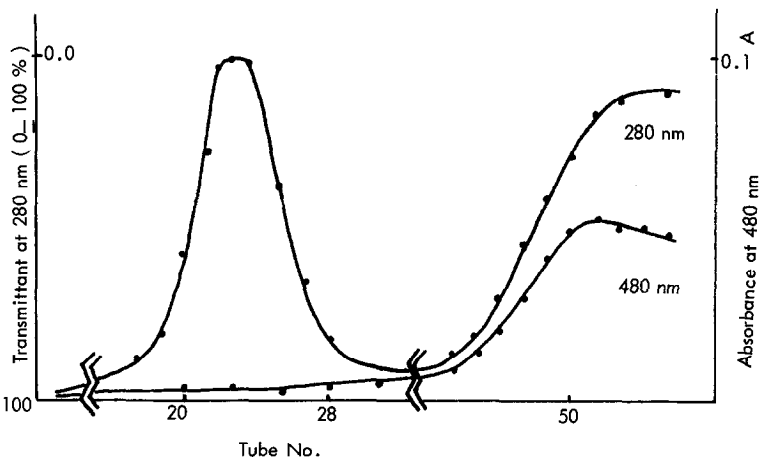

Fig. 2. Chromatography of pyrocatechase reacted with ethylprotocatechuate on Sephadex G-25 (fine). After the enzyme (16 mg/ml, 3.0 ml) was reacted with ethylprotocatechuate (10 mM, 3.0 ml) for 30 min. at 30°C, the reaction products were applied to a 2.2x35 cm column of Sephdex G-25 (fine), buffered with 0.1 M Tris-HCl pH 7.5. Elution was done with the same buffer, and the volume of the fractions collected was 2.0 ml.

pyrocatechase through the formation of an enzyme-pseudosubstrate, and removes the iron from the enzyme to give its apoenzyme at neutral pH (6).

Materials and Methods; Pyrocatechase was purified according to the method of Kita et al. (5). ESR spectra were taken with a Varian V-4500 spectrometer equipped with 100 kc field modulation and a variable temperature accessory. Spectra were measured at incident power of -10 db, at a field modulation amplitude of 15 gauss. A magnetic field of about 500 gauss centered at 1520 gauss was scanned. The temperature in the cavity was very carefully maintained at a temperature of near the boiling point of liquid nitrogen in a series of experiments. The g value was determined by the use of DPPH (1,1- Diphenyl-2-picrylhydrazyl) as a standard. Absorption spectra and circular dichroism spectra were measured using a 356 Hitachi Two Wave Length Spectrophotometer and a JASCO ORD / CD Model-5 Spectrophotometer, respectively.

Results and Discussion; Preparation of Apopyrocatechase; The release of enzyme-bound ferric iron to the medium by ethylprotocatechuate was followed by measuring the increase in the absorption of the reaction mixture at 480 nm (Fig. 1), which is the absorption maximum of the chelate complex between ferric iron and ethylprotocatechuate at pH 7.5.

For the preparation of apopyrocatechase, the holoenzyme was treated with 50 equivalents of ethylprotocatechuate in 0.1 M Tris-HCl. pH 7.5, at 30° C. After the increase in absorbancy at 480 nm has stopped, the reaction mizture was transferred to a Sephadex G-25 (fine) column, which was pre-equilibrated with 0.1 M Tris-HCl, pH 7.5, to separate low molecular weight species from the apoenzyme (Fig. 2). Fractions containing protein were pooled and concentrated using apparatus equipped with either collodion or diaplex membrane.

Apopyrocatechase retained less than 5 % of its original activity and, in contrast to the native enzyme, had no absorption maximum in the visible region (Fig. 3). Furthermore, the ESR signal at g= 4.3 and the Cotton effects in the visible region, disappeared

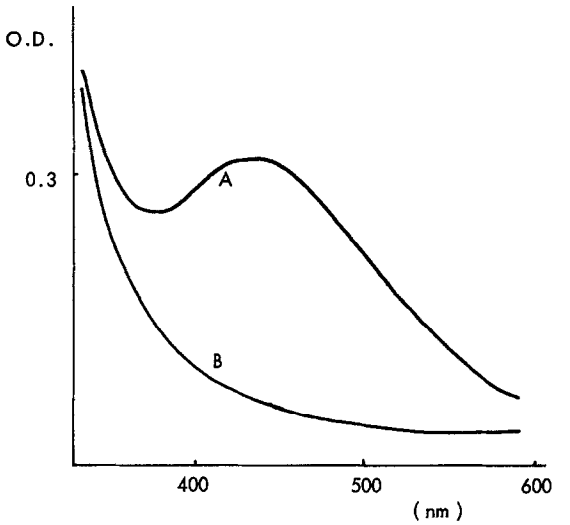

Fig. 3. Visible absorption spectra of apo- and native-pyrocatechase. (A); native enzyme (3.9 mg/ml), (B); apo-enzyme (4.1 mg/ml).

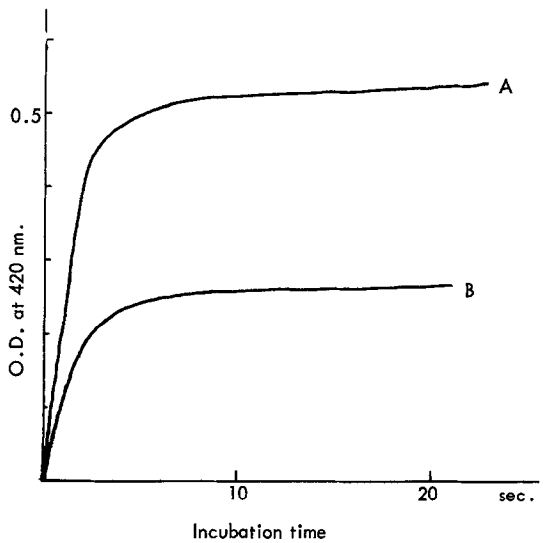

Fig. 4. Time course of the reconstitution of holopyrocatechase. Changes in absorption were measured at 420 nm following the addition of $FeSO_4$ to the apo-enzyme under the following conditions, each 1.2 ml of apo-enzyme (A; 9.4 mg/ml, B; 4.7 mg/ml) were treated with 10 mM $FeSO_4$ (A; 40 µl, B; 30 µl).

as the result of the removed of ferric iron of the holoenzyme.

Reconstitution of holopyrocatechase; For the reconstitution of holopyrocatechase, the

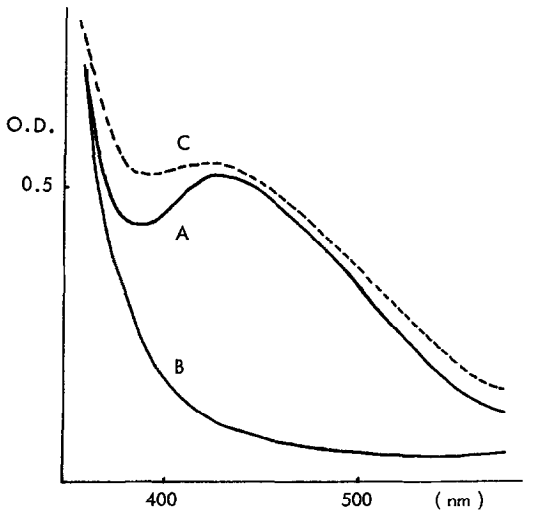

Fig. 5. Visible absorption spectra of native-, apo- and recostituted holo-pyrocatechase. (A); native-enzyme (5.0 mg/ml), (B); apo-enzyme (5.1 mg/ml), (C); reconstituted holo-enzyme (5.0 mg/ml).

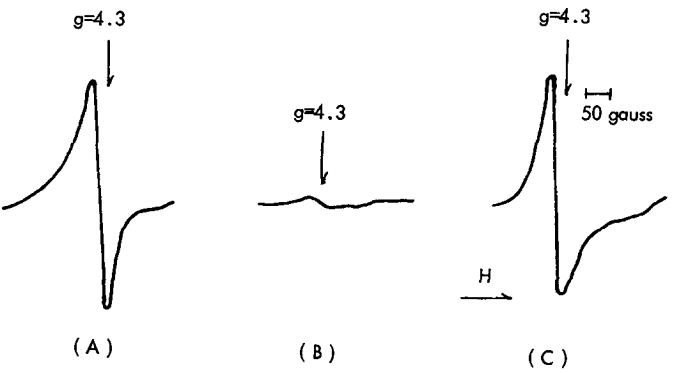

Fig. 6. ESR spectra of native-, apo-and reconstituted holo-pyrocatechase. (A); native-enzyme (3.4 mg/ml), (B); apo-enzyme (3.4 mg/ml), (C); reconstituted holo-enzyme (3.3 mg/ml).

apoenzyme was treated in the presence of oxygen with the stoichiometric amount of ferrous iron in 0.1 M Tris-HCl, pH 7.5, at 30°C. The conversion of the apo- to the holo-enzyme proceeded rapidly. The recovery of absorbancy at 420 nm, absorption maximum of the holoenzyme in the visible region, was stoichiometrically complete within a few seconds (Fig. 4) with full recovery of activity.

Removal of protein-bound metals are carried out by treatment with chelating agents or chelators in combination with reducing agents (7). But, in some case, the removal of metal ions induce profound changes in the protein moieties which result in incomplete recovery of activity or require a long time for reconstitution (8). In the case of our pyrocatechase, the removal of ferric iron from the holoenzyme with ethylprotocatechuate seems to produce no severe changes in the vicinity of the binding site of ferric iron, as the apoenzyme is easily converted into a full active form.

Recovery of activity after treatment of the apoenzyme with an equivalent amount of ferrous iron is a accompanied by the recovery of characteristic absorption and Cotton effect in the visible region, and an ESR signal at g= 4.3 whose patterns and intensities are identical with those of the holoenzyme. As shown in Figures 5, 6 and 7, the enzymatically inactive apopyrocatechase did not show any intrinsic spectroscopic and magnetic properties of native pyrocatechase, such as the absorption maximum at 420 nm, negative Cotton effects at 317 nm and 500 nm, and ESR signal at g= 4.3. These results strongly

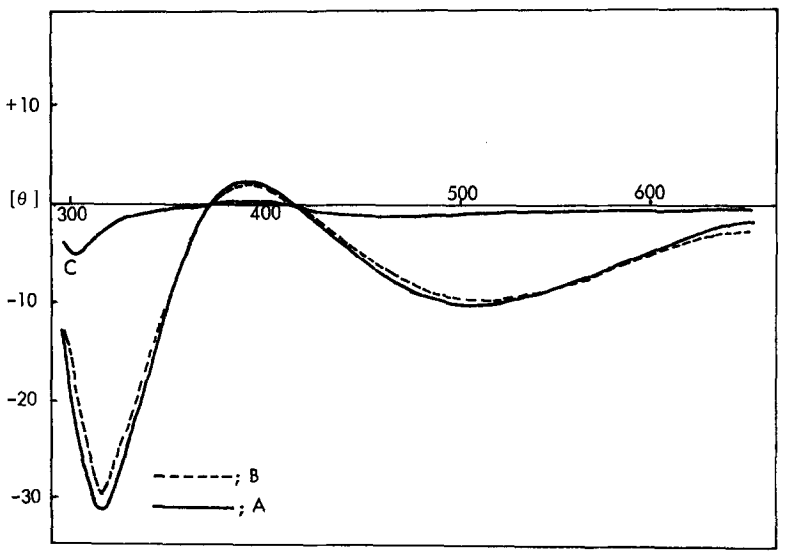

Fig. 7. C.D. spectra of native-, apo- and reconstituted holo-pyrocatechase. (A); native-enzyme (13 mg/ml), (B); apo-enzyme (14.0 mg/ml), (C); reconstituted holo-enzyme (13.2 mg/ml).

indicate that these spectroscopic properties of the holoenzyme are due to the presence of a ferric iron in the enzyme, which is essential for the enzyme reaction.

The stoichiometry of the reconstitution reaction of apoenzyme is shown in Fig 8.

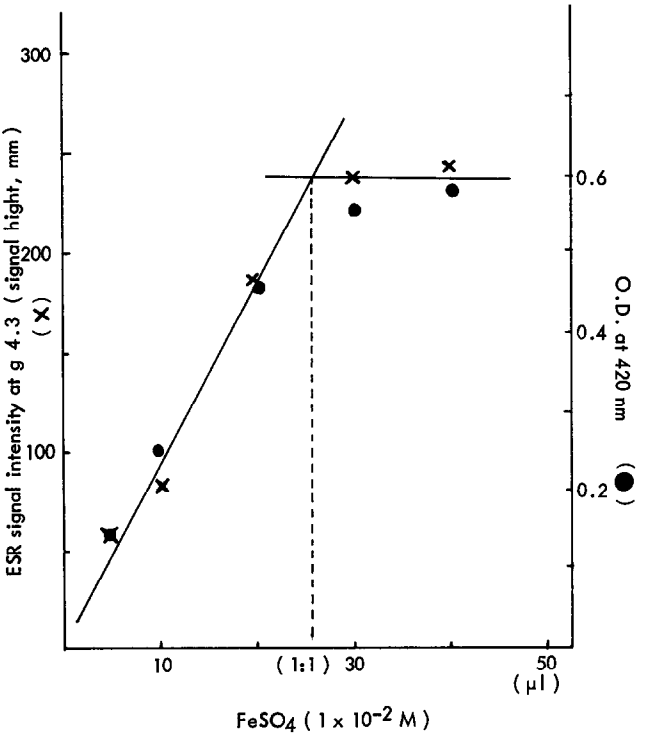

Fig. 8. Stoichiometry of the effect induced in apopyrocatechase by added ferrous iron in the reconstitution reaction. 1.5 ml of apo-enzyme solution (9.8 mg/ml, about 1.5×10^{-4} M) was treated with ferrous iron (10 mM). After incubation at 30°C for 2 min., the absorbancy at 420 nm and ESR signal at g=4.3 were measured.

As the ratio of ferrous iron to the apoenzyme increase stepwise to equivalence, the enzyme activity, optical density at 420 nm, intensity of ESR signal at g= 4.3 and the value of the Cotton effect at 317 nm increased in a manner directly proportional to iron added. At the equivalence point, these showed maximum intensity. These observations suggest that the ferrous iron added is bound to the apoenzyme in a 1:1 ratio and is simultaneously oxidized to the ferric state. The ferric iron in the enzyme participates in the enzyme reaction and is the cause of the intrinsic spectroscopic and magnetic properties of pyrocatechase.

All these results show that ethylprotocatechuate, a substrate analogue, can remove the enzyme bound ferric iron from holopyrocatechase without any irreversible changes in the enzyme protein. The apoenzyme thus prepared, is easily converted to the holoenzyme by the treatment with stoichiometric amount of ferrous iron in the presence of oxygen.

Acknowledgements The authors wish to thank Prof. Toshio Yamano, Osaka University, and Dr. Siro Senoh and Dr. Hiroshi Kita, Suntory Limt., for their helpful advices and discussion.

References

1) H. Nakagawa, H. Inoue, Y. Takeda, J. Biochemistry (Tokyo), 54, 65 (1963)

2) K. Kawakami, H. Kita and S. Senoh, Seventh Int. Cong. Biochem., Tokyo, Abst. IV, F-85 (1967)

3) Y. Kojima, H. Fujisawa, A. Nakazawa, T. Nakazawa, F. Kanetsuna, H. Taniuchi A. Nozaki and O. hayaishi, J. Biol. Chem., 242, 3270 (1967)

4) T. Nakazawa, M. Nozaki, O. Hayaishi and T. Yamano, J. Biol. Chem., 244 , 119 (1969)

5) H. Kita, Y. Miyake, M. Ono (M. Kamimoto), S. Senoh and T. Yamano, J. Biochemistry (Tokyo), 66, 45 (1969)

6) K. Nagami and Y. Miyake, B.B.R.C., 42, 497 (1971)

7) A. Nakazawa, T. Nakazawa, S. Kotani, M. Nozaki and O. Hayaishi, J. Biol. Chem., 244 , 1527 (1969)

8) K. Ando, J. Biochemistry (Tokyo) 68 , 501 (1970)