AN ELECTRON SPIN RESONANCE STUDY ON PYROCATECHASE; THE EFFECTS OF SUBSTRATE ANALOGUES AND CATECHOL

Kenzo Nagami and Yoshihiro Miyake*

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

Received December 21, 1970

Summary: Analogues of catechol (protocatechuic acid and ethyl protocatechuate) as well as catechol itself, combine with the enzyme-bound iron of pyrocatechase and formed complexes with the enzyme. The complexes and the native enzyme showed ESR signals characteristic of a ferric iron complex at g=4.3, but the patterns of the signals were different from that of the native enzyme. After forming the complex, ethyl protocatechuate readily released the bound iron of the enzyme protein to give the apoenzyme. The addition of catechol to the enzyme in the absence of oxygen resulted in a decrease of the ESR signal at g=4.3, but which did not disappear. These results indicate that the bound iron of pyrocatechase in the ES complex is similar to ferric iron in a charge transfer complex.

Introduction: Pyrocatechase is a typical dioxygenase which catalyses the cleavage of the aromatic ring of catechol to cis, cis-muconic acid with the consumption of 1 mole of oxygen per mole of catechol. This enzyme has been purified from cell-free extracts of Brevibacterium fuscum p-13 and has been found to have nonheme iron essential for the enzyme reaction (1, 2). Nakazawa et al. (3) and Kita et al. (2) have independently proposed reaction mechanisms for this enzyme which specify the valency changes of the bound iron during the reaction (Figure 1). However, it has not been confirmed whether the bound iron of pyrocatechase was in ferrous state in the enzyme-catechol complex (ES complex) or not (2, 4, 5). The present communication is concerned with the valence state of the bound iron in the ES complex from ESR studies on catechol analogues complexes of the enzyme (ES' complex) and enzyme-catechol complex.

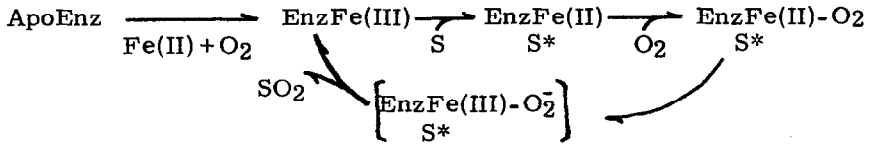

Figure 1.

Materials and Methods: Pyrocatechase was prepared according to the method of Kita et al. (2). ESR spectra were taken with a Varian V-4500 Spectrometer equipped with 100 kc field modulation and a variable temperature attachment. Spectra were measured at an 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 adjusted to be constant at the temperature of liquid nitrogen in a series of experiments. The g value was determined by the use of DPPH as a standard. Absorption spectra were determined with a 356-Hitachi Two Wavelength Spectrophotometer.

Results and Discussion: Changes in the visible absorption spectra of pyrocatechase upon the addition of substrate analogues; From the spectral changes shown in Figure 2, it was clear that pyrocatechase reacted with both protocatechuic acid and ethyl protocatechuate. In both cases, the reaction

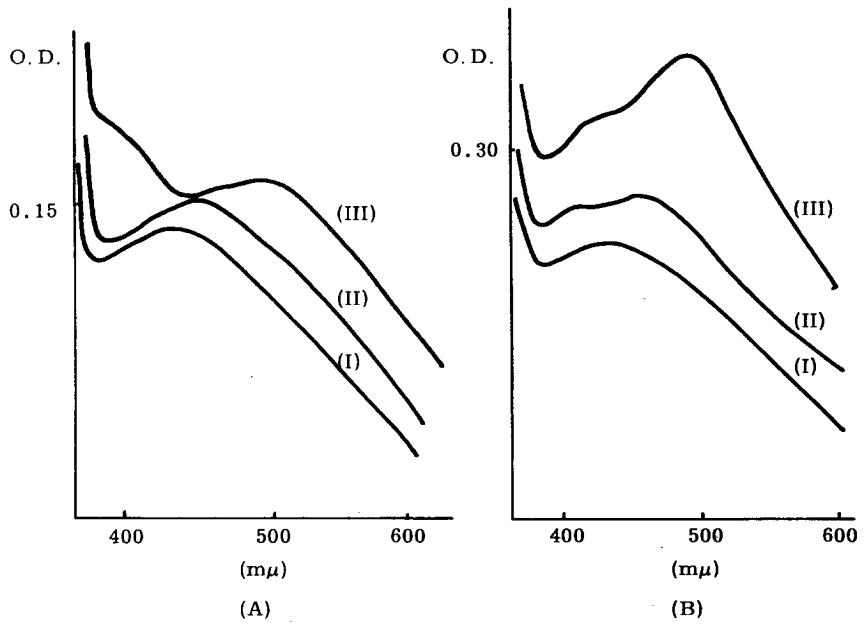

Figure 2. Changes in the visible absorption spectra of pyrocatechase effected by the addition of substrate analogues, (A); protocatechuic acid,(B); ethyl protocatechuate. The spectra were taken (I) without treatment, (II) at 45 sec. after the addition of substrate analogues, and (III) after incubation of solution (II) at 30°C for 30 min. The enzyme solution (A; 3.0 mg/ml, B; 4.0 mg/ml) of 3.0 ml in 0.1 M Tris-HCl, pH. 7.5, was placed in a cuvette and were mixed with either 0.1 ml of 50 mM protocatechuic acid (A) or 0.1 ml of 10 mM ethyl protocatechuate (B), respectively

proceeded in two steps, the rapid and the following slow reactions. The first rapid reaction was due to the complex formation between the enzyme and the substrate analogues (ES' complex). A marked difference in the spectral patterns was observed in the second slow reactions depending on the substrate analogues used. As described later, the release of the bound iron from the enzyme protein had occured upon the addition of ethyl protocatechuate, while protocatechuic acid caused almost no release of iron but modified the ligand field of the bound iron in the enzyme.

Changes in ESR spectra of pyrocatechase upon the addition of substrate analogues: The rapid and the following slow changes in ESR spectra were also observed following the addition of substrate analogues to the enzyme (Figure 3), which correspond to the changes in the absorption spectra, though the nature of the changes differed completely between protocatechuic acid and ethyl protocatechuate. Namely, when protocatechuic acid was used as subst-

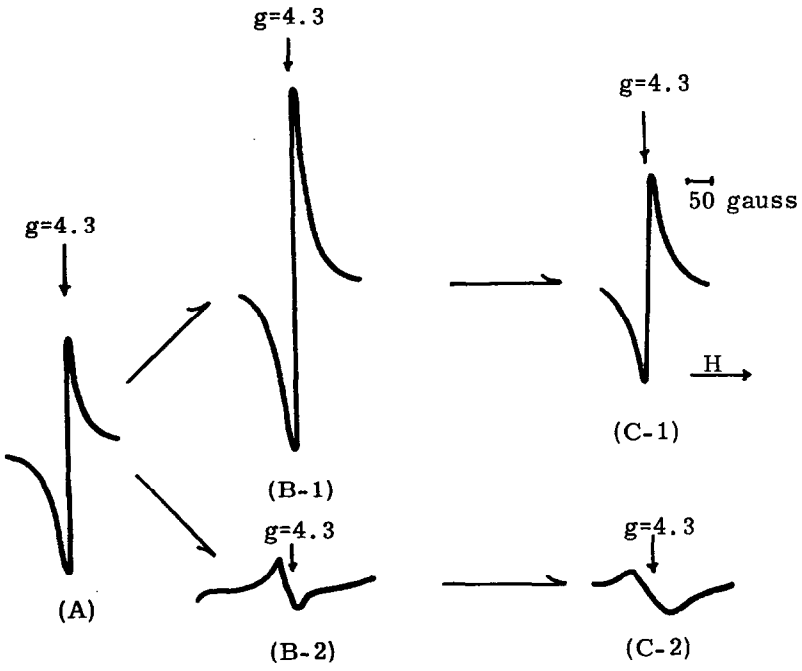

Figure 3. Changes in ESR spectra of pyrocatechase accompanying the addition of substrate analogues. 0.1 ml of the enzyme solution (21.4 mg/ml) in 0.1 M Tris-HCl, pH. 7.5, was placed in a ESR tube and mixed with 0.5 ml of the substrate analogue solution (2 mM). (A); the native enzyme, (B); the reaction mixture at 45 sec. after the addition of substrate analogue, (B-1); protocatechuic acid, (B-2); ethyl protocatechuate, (C); after incubation of solution (B) for 30 min. at 30°C.

rate analogue, the intensity of ESR signal characteristic of a ferric iron complex at g=4.3 increased rapidly and then decreased very slowly. Both the g_m value and the signal width remained identical with those of the native enzyme throughout the reaction. In contrast, ethyl protocatechuate caused a rapid decrease in the intensity of the ESR signal at g=4.3 with the concomitant broadening and asymmetrification. The g_m value also remained unchanged in this case. These results suggest that both substrate analogues bind to the enzyme (formation of ES' complex) and modify the ligand field of the enzyme-bound iron. The degree of modification of the ligand field of the iron is dependent on the chemical nature of the compound used. The bound iron in the ES' complex showed ESR signal of ferric iron complex at g=4.3. It was also shown that the changes in the ESR spectra of the bound iron caused by ethyl protocatechuate following complex formation, was due to the release of the iron from the enzyme protein as shown in the following experiments.

Release of the bound iron from the enzyme protein: At 30 minutes after formation of ES' complex, the reaction mixture of the enzyme with ethyl protocatechuate was filtered through a collodion membrane. As shown in Figure

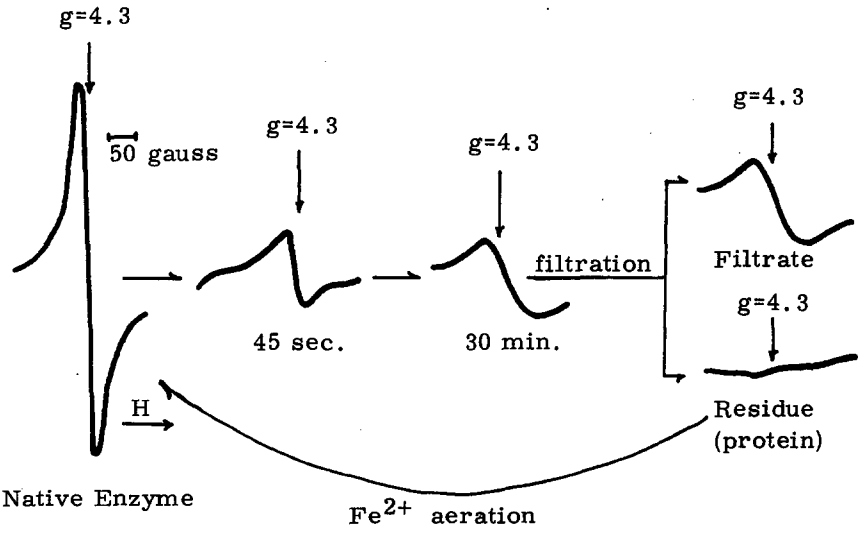

Figure 4. The release of bound iron from pyrocatechase. 3.0 ml of the enzyme solution (6.6 mg/ml) in 0.1 M Tris-HCl, pH. 7.5, was placed in a test tube and mixed with 0.05 ml of ethyl protocatechuate solution (20 mM in 0.1 M Tris-HCl, pH. 7.5). After incubation for 30 min. at 30°C, the reaction mixture was filtered through a collodion membrane. The ferrous iron was added to the residue in the presence of oxygen.

4, the filtrate containing low molecular weight species showed an identical ESR spectrum with that of the reaction mixture prior to the filtration. The enzyme protein remaining on the membrane showed no apparent ESR signal at g=4.3 region. The residue, however, exhibited the typical ESR spectrum of the native enzyme after treatment with ferrous iron in the presence of oxygen. These results show that the ESR signal of the reaction mixture after 30 minutes incubation is caused not by the bound iron of the enzyme but by the iron released from the enzyme. Therefore, the second step of the reaction following the formation of the ES' complex between the enzyme and ethyl protocatechuate is the release of the bound iron from the enzyme protein.

From these spectrophotometric and ESR experiments, it was found that the two substrate analogues, protocatechuic acid and ethyl protocatechuate, were bound to pyrocatechase to form ES' complexes like a catechol. The bound iron of the enzyme in the ES' complex showed an ESR spectrum at g=4.3 whose intensity and signal pattern were different from that of the native enzyme. After complex formation, the ethyl protocatechuate caused the release of the iron from the enzyme to give the apoenzyme and the complex of ferric iron with this compound which showed broad and asymmetric ESR signal at g=4.3. These findings also suggest strongly that the bound ferric iron in the native enzyme still exists as ferric iron in the ES' complex despite the change in the intensity and pattern of the ESR signal at g=4.3.

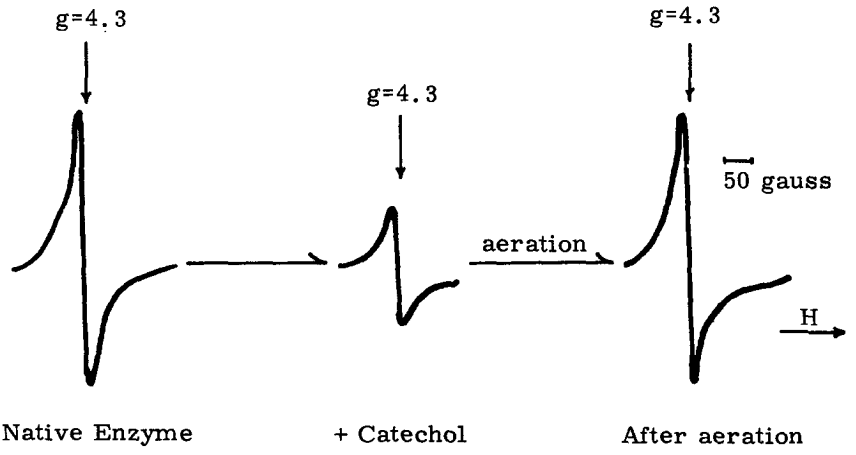

Figure 5. Changes in ESR spectra of pyrocatechase effected by the substrate and oxygen. 0.8 ml of the enzyme solution (4.0 mg/ml) in 0.1 M Tris-HCl, pH. 7.5, was mixed in a ESR tube with 0.1 ml of catechol solution (6 mM) under anaerobic conditions. The reaction mixture was then strongly oxygenated until the added substrate was completly exhausted.

Changes in ESR spectra of pyrocatechase accompanying the addition of catechol: The bound iron of pyrocatechase showed a distinct ESR signal at g=4.3 after addition of catechol under anaerobic conditions. As shown in Figure 5, the signal pattern and g_m value were very similar to those of the native enzyme. However, the pattern was slightly asymmetric and the signal distinctly weaker than that of the native enzyme, though it did not disappear. Considering the results obtained with regard to the ES' complex, this result strongly suggests that this remaining ESR signal is due to the ES complex. When the catechol of the ES complex was completly exhausted by aeration, an ESR signal identical with that exhibited by the native enzyme returned.

Conclusion: The bound iron of pyrocatechase combined with substrate or its analogue to form an ES or ES' complex. Each complex gave a characteristic absorption and ESR spectra in which the bound iron remained in ferric state. This evidence indicates that the bound iron of pyrocatechase in the ES complex is in a state similar to that of ferric iron in a charge transfer complex rather than iron in the ferrous state.

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 discussions.

References

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

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

3) T. Nakazawa, M. Nozaki, O. Hayaishi and T. Yamano, J. Biol. Chem., 240 PC 3224 (1964)

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

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