MÖSSBAUER SPECTRA OF METAPYROCATECHASE

Yoshitaka TATSUNO, Yukikazu SAEKI, Mitsuhiro NOZAKI, Sei OTSUKA[+] and Yutaka MAEDA*

Department of Biochemistry, Shiga University of Medical Science, Ohtsu, Shiga, 520-21, [+]Department of Chemistry, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560 and **Research Reactor Institute, Kyoto University, Kumatori-cho, Sennan-gun, Osaka 590-04, Japan*

Received 17 January 1980

1. Introduction

Metapyrocatechase (catechol:oxygen 2,3-oxidoreductase, EC 1.13.1.2) from *Pseudomonas arvilla* (ATCC 23973) catalyzes the conversion of catechol into α-hydroxy muconic ε-semialdehyde with the insertion of 2 atoms of molecular oxygen (see eq.(1)) [1]:

(1)

The enzyme, an extradiol dioxygenase and the first dioxygenase obtained in a crystalline form [2], contains 4 g atoms iron/molecule (mol. wt 140 000) and consists of 4 identical subunits [3]. Unlike intradiol dioxygenases such as pyrocatechase and protocatechuate 3,4-dioxygenase, metapyrocatechase is colorless, shows no significant signals in ESR spectra and is extremely unstable to air in the absence of 10% acetone [2]. Treatment of the enzyme with H_2O_2 or air results in formation of an inactive form of enzyme which shows a broad signal around $g = 4.2$ in ESR spectra characteristic of Fe^{3+}. Thus, the iron in the active enzyme is likely in the Fe^{2+} form [4]. However, intradiol dioxygenases are wine red in color and contain Fe^{3+} based on ESR and Mössbauer studies [5].

In view of the strong affinity of Fe^{2+} toward molecular oxygen compared to Fe^{3+} which forms a stable complex with catechol, it might be expected for Fe^{2+}-containing dioxygenases that the formation of an enzyme–oxygen complex (EO_2) precedes to the formation of an enzyme–substrate complex (ES). The reverse situation may be expected for Fe^{3+}-containing dioxygenases. Binding and kinetic studies, however, revealed that the both types of enzymes combine first with organic substrate to form an ES complex followed by the addition of oxygen irrespective to the valence state of iron [6–8].

However, another extradiol enzyme, protocatechuate 4,5-dioxygenase, was reported to contain either low spin Fe^{2+} or antiferromagnetically coupled high spin Fe^{3+} based on Mössbauer studies [9]. Thus, the oxidation state of iron embedded at the active center of metapyrocatechase still remains to be elucidated, which is important for understanding of the reaction mechanism of nonheme iron-containing dioxygenases. Here we report Mössbauer data on ^{57}Fe-reconstituted metapyrocatechase which confirm the presence of high spin Fe^{2+}.

2. Materials and methods

Metapyrocatechase was purified and crystallized as in [10], from *Pseudomonas arvilla* grown with benzoate as a carbon source. Crystalline enzyme with spec. act. >240 units/mg was used for preparation of apometapyrocatechase. The apoenzyme and ^{57}Fe-enriched holoenzyme were prepared as in [11]. The apoenzyme had spec. act. <1 unit/mg. Incubation of the apoenzyme with a 10-fold excess of $^{57}FeSO_4$ for 2 h at 4°C afforded a pure reconstituted holoenzyme with spec. act. ~240 units/mg after chromatography on DEAE-cellulose and crystallization. Isotope-enriched ferrous sulfate was prepared from $^{57}Fe_2O_3$ (90.73%

Address correspondence to M. N.

enriched) by reduction with sodium borohydride followed by treatment with an equimolar amount of sulfuric acid, and then crystallization. A suspension of crystalline ^{57}Fe-enriched metapyrocatechase in 50 mM Tris–acetate buffer (pH 7.5) was used as a sample for Mössbauer measurements. ES complex of the enzyme was prepared by mixing ~60 mg enzyme in 0.5 ml 50 mM Tris–acetate buffer (pH 7.5) with a 10-fold excess of catechol under nitrogen. The Mössbauer spectrometer was a constant acceleration type. The source was 30 mCi ^{57}Co in rhodium metal which gave a minimum linewidth of 0.30 mm/s. The velocity scale was calibrated with a metallic iron foil in an independent Mössbauer run, taking the center of this spectrum as velocity zero.

3. Results and discussion

The Mössbauer spectra of native metapyrocatechase were taken at 4.2 K and 77 K. Both consist of a simple quadrupole doublet with slight broadening (0.6 mm/s fullwidth) as shown in fig.1A. Application of magnetic field up to 750 G did not produce any effect on the spectra. The relatively high isomeric shift, δ = 1.31 mm/s and the large quadrupole splitting, ΔE_Q = 3.28 mm/s clearly verify the presence of high spin Fe^{2+} in this enzyme.

The quadrupole splitting and the isomeric shift were independent of temperature. Although values of 0.28–0.35 mm/s for linewidth are reported for other iron-containing proteins [9], a slightly broader absorption was observed with metapyrocatechase. The broadening of the spectra shown in fig.1A may be ascribed to the presence of a small amount of Fe^{2+} contaminated during the reconstitution procedure.

As the isomeric shift and the quadrupole splitting are quite sensitive to the charge and spin state of iron and its coordination environment, comparative studies with other iron-containing proteins should provide valuable information on the active site of this enzyme. The observed values of isomeric shift and quadrupole splitting of metapyrocatechase were found to be very close to those of the reduced form of protocatechuate 3,4-dioxygenase [5] and to reduced hemerythrin [12] but not to that of rubredoxin [13] (table 1). The previous Mössbauer study for protocatechuate 3,4-dioxygenase suggested a more ionic oxygen or nitrogen type environment, and recent resonance Raman studies [14–16] revealed the presence

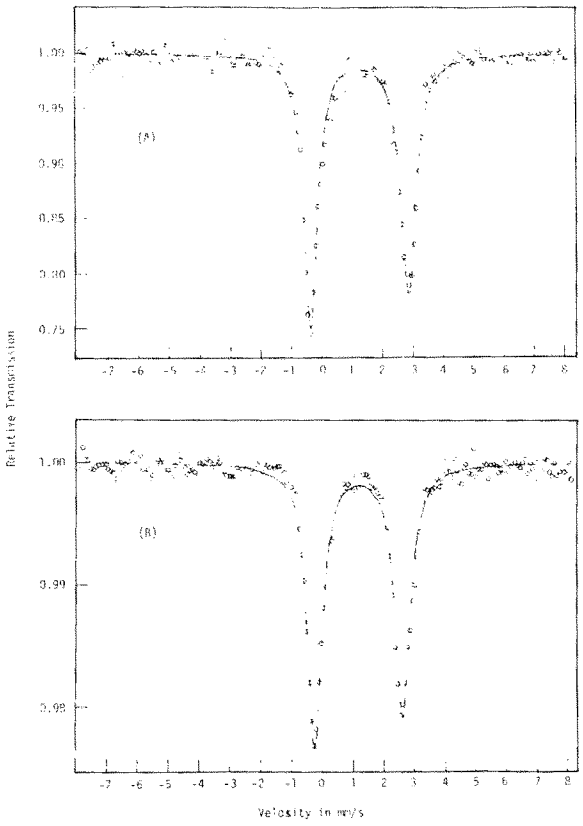

Fig.1. Mössbauer spectra of ^{57}Fe-enriched metapyrocatechase taken at 4.2 K. (A) The crystalline metapyrocatechase; (B) the ES complex of metapyrocatechase with a 10-fold excess of catechol.

Table 1
Mössbauer data of various iron-containing proteins

Iron-containing proteins	T (K)	δ (mm/s)	ΔE_Q (mm/s)	[Ref.]
Metapyrocatechase	4	1.31	3.28	This work
Protocatechuate 3,4-dioxygenase (reduced form)	4	1.21	3.13	[5]
Hemerythrin (reduced form)	4	1.20	2.89	[12]
Rubredoxin (reduced form)	4	0.70	3.25	[13]
Protocatechuate 4,5-dioxygenase	100	0.42 0.60 or 0.49 0.52	0.64 0.63 0.80 0.45	[9]

of tyrosine residue(s), coordinated to Fe^{3+} in the active center. X-ray diffraction studies have suggested that histidine and tyrosine residues participate in the iron coordination in hemerythrin [17], while, cysteine residues coordinate to iron in rubredoxin [18]. Thus, it seems probable that the iron in metapyrochatechase resides in an environment similar to those of protocatechuate 3,4-dioxygenase and hemerythrin, and that a tyrosine residue may coordinate to the iron as a phenolate anion.

The ES complex of metapyrocatechase showed a Mössbauer spectrum essentially identical to that of the native enzyme (fig.2B) and no ESR signals, implying that the substrate changes neither the coordination environment around the Fe^{2+} nor the valence state of the irons upon its binding to the protein. The Mössbauer data of protocatechuate 4,5-dioxygenase [9] are quire different from those of metapyrocatechase, although both enzymes are colorless, ESR silent and catalyze the extradiol cleavage of the catechol ring. The enzyme–inhibitor complex of protocatechuate 4,5-dioxygenase shows ESR and Mössbauer spectra characteristic of high spin Fe^{3+}. Based on these observations, it was suggested that the spin coupling in protocatechuate 4,5-dioxygenase is broken when enzyme–inhibitor (substrate) complex is formed, and that the valence state of irons does not change upon inhibitor (substrate) binding [9].

Whether the different spectroscopic observations in these two extradiol dioxygenases are due to the purity of enzyme, i.e., iron contamination, or to different characteristics of the individual enzymes, needs further experimentation. These data show that metapyrocatechase has a high spin Fe^{2+} and its valence state does not change on forming the ES complex. The second substrate, oxygen, may react with the high-spin Fe^{2+} in the ES complex to form a ternary complex of enzyme–substrate–oxygen as already established for other dioxygenases [19,20].

References

[1] Nozaki, M. (1974) in: Molecular Mechanism of Oxygen Activation (Hayaishi, O. ed) pp. 135–165, Academic Press, New York, London.

[2] Nozaki, M., Kagamiyama, H. and Hayaishi, O. (1963) Biochem. Z. 338, 582–590.

[3] Nozaki, M. (1979) Top. Curr. Chem. 78, 145–186.

[4] Nozaki, M., Ono, K., Nakazawa, T., Kotani, S. and Hayaishi, O. (1968) J. Biol. Chem. 243, 2682–2690.

[5] Que, L. jr, Lipscomb, K. D., Ziemmerman, R., Münck, E., Orme-Johnson, N. R. and Orme-Johnson, W. H. (1976) Biochim. Biophys. Acta 452, 320–334.

[6] Nozaki, M., Nakazawa, T., Fujisawa, H., Kotani, S., Kojima, Y. and Hayaishi, O. (1968) Adv. Chem. ser. 77, 242–251.

[7] Hori, K., Hashimoto, T. and Nozaki, M. (1973) J. Biochem. 74, 375–384.

[8] Fujisawa, H., Uyeda, M., Kojima, Y., Nozaki, M. and Hayaishi, O. (1972) J. Biol. Chem. 247, 4414–4421.

[9] Zabinski, R., Münck, E., Champion, P. M. and Wood, J. M. (1972) Biochemistry 11, 3212–3219.

[10] Nozaki, M. (1970) Methods Enzymol. 17A, 522–525.

[11] Takemori, S., Komiyama, T. and Katagiri, M. (1971) Eur. J. Biochem. 23, 178–184.

[12] Okamura, M. Y., Klotz, I. M., Johnson, C. E., Winter, M. R. C. and Williams, R. J. P. (1969) Biochemistry 8, 1951–1958.

[13] Debrunner, P. G., Münck, E., Que, L. jr and Schulz, C. E. (1976) in: Iron–Sulfur Proteins (Lovenberg, W. ed) vol. 3, pp. 381–417, Academic Press, London, New York.

[14] Tatsuno, Y., Saeki, Y., Iwaki, M., Yagi, T., Nozaki, M., Kitagawa, T. and Otsuka, S. (1978) J. Am. Chem. Soc. 100, 4614–4615.

[15] Keyes, W. E. and Loehr, T. M. (1978) Biochem. Biophys. Res. Commun. 83, 941–945.

[16] Felton, R. H., Cheung, L. D., Phillips, R. S. and May, S. W. (1978) Biochem. Biophys. Res. Commun. 85, 844–850.

[17] Stenkamp, R. E., Sieker, L. C. and Jensen, L. H. (1976) Proc. Natl. Acad. Sci. USA 73, 349–351.

[18] Jensen, L. H. (1973) in: Iron–Sulfur Proteins (Lovenberg, W. ed) vol. 2, pp. 163–193, Academic Press, London, New York.

[19] Ishimura, Y., Nozaki, M., Hayaishi, O., Nakamura, T., Tamura, M. and Yamazaki, I. (1970) J. Biol. Chem. 245, 3593–3602.

[20] Fujisawa, H., Hiromi, K., Uyeda, M., Okuno, S., Nozaki, M. and Hayaishi, O. (1972) J. Biol. Chem. 247, 4422–4428.