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PROTOCATECHUATE 3,4-DIOXYGENASE

IV. PREPARATION AND PROPERTIES OF APO- AND RECONSTITUTED ENZYMES

MOTOKAZU FUJIWARA AND MITSUHIRO NOZAKI
Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto (Japan)

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SUMMARY

Protocatechuate 3,4-dioxygenase is an enzyme containing the ferric form of nonheme iron as the sole cofactor. The iron could be removed from the enzyme by prolonged anaerobic dialysis against a buffer solution containing both *o*-phenanthroline and $Na_2S_2O_4$. The apoenzyme thus obtained was fully reactivated by incubation with Fe^{2+} under anaerobic conditions. The reactivation rate was greatly accelerated by the presence of reducing agents such as $Na_2S_2O_4$. The visible absorption spectrum characteristic of the native enzyme disappeared upon removal of the iron and reappeared upon reactivation. The native enzyme and apoenzyme gave almost identical *s* values (18 S) on ultracentrifugation and both also bound the same maximal amount of substrate (8–10 moles per mole of enzyme). However, the dissociation constant for the apoenzyme was found to be approx. 20-fold higher than that for the native enzyme.

INTRODUCTION

Protocatechuate 3,4-dioxygenase (protocatechuate: oxygen 3,4-oxidoreductase (decyclizing) EC 1.13.11.3) catalyzes the cleavage of the aromatic ring of protocatechuic acid with the incorporation of 2 atoms of molecular oxygen to form β-carboxymuconic acid. The enzyme was first described by Stanier and Ingraham[1] and was obtained in a crystalline form by Fujisawa and Hayaishi[2] from *p*-hydroxybenzoate-induced *Pseudomonas aeruginosa*. Fe^{3+} was reported to be the sole cofactor of the enzyme and to participate actively in the catalysis as an integral part of the reaction[3,4]. However, since the iron is tightly bound to the protein and has not been successfully removed from the protein, the role of iron in the structure and function of the enzyme remains obscure. In order to investigate the role of the iron, attempts were made to prepare the apoenzyme. This paper describes the preparation procedure of the apoenzyme and some of its properties.

Abbreviation: DTNB, 5′,5-dithiobis-(2-nitrobenzoic acid).

MATERIALS AND METHODS

Biogel A-0.5 m (100–200 mesh) was a product of Bio-Rad Laboratories and 4-nitrocatechol was obtained from Sigma. 5′,5-Dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Wako Junyaku Kogyo Co., Japan. All other chemicals, analytical grade, were obtained from commercial sources.

Crystalline protocatechuate 3,4-dioxygenase with a specific activity of 60–70 μmoles/min per mg of protein was prepared from *P. aeruginosa* (ATCC 23975), grown with *p*-hydroxybenzoate as the sole carbon source as previously described[3]. Crystalline metapyrocatechase with a specific activity of 260 μmoles/min per mg of protein was prepared from *P. arvilla*, grown in the presence of benzoate, meat extract and peptone[5,6].

The activity of protocatechuate 3,4-dioxygenase was assayed either polarographically at 24 °C by measuring O_2 consumption or spectrophotometrically at 24 °C by measuring the decrease in absorbance at 290 nm with a Cary 14 recording spectrophotometer as described previously[2]. These two assay methods yielded essentially identical results. One unit of enzyme activity is defined as the amount that produces 1 μmole of the product, β-carboxymuconic acid, per min. The specific activity is expressed as units per mg of protein. Protein concentrations of protocatechuate 3,4-dioxygenase were most frequently determined by measuring the absorbance at 280 nm, taking 13.2 as the absorbance of a 1% solution at pH 8.5 (ref. 2). In the equilibrium dialysis experiment, protein was determined by the biuret method of Gornall *et al.*[7].

The iron determinations were carried out with a Nippon Jarrell-Ash atomic absorption spectrophotometer, Model AA-70 equipped with a total combustion hydrogen–air burner. Solutions of metal iron (2–10 ppm) were used as a standard. The number of free sulfhydryl groups in the protein was determined by the DTNB method of Ellman[8] in 50 mM potassium phosphate buffer (pH 7.5). Ultracentrifugal analyses were performed with a Spinco Model E analytical ultracentrifuge in 50 mM Tris–acetate buffer (pH 8.5), using a protein concentration of approx. 5 mg/ml.

RESULTS

Preparation of apoenzyme

The native enzyme (22.8 mg) was dissolved in 2 ml of 50 mM Tris–acetate buffer (pH 8.5) and dialyzed against 500 ml of 500 mM sodium phosphate buffer (pH 6.5) containing 8 mM *o*-phenanthroline and 10 mM $Na_2S_2O_4$ under anaerobic conditions at room temperature. The dialyzed solution was placed on a Biogel A-0.5 m column (1 cm × 40 cm) which had been equilibrated with 50 mM Tris–acetate buffer (pH 8.5) and the protein was eluted with the same buffer. During this process, the protein was separated from the iron–*o*-phenanthroline complex, sodium dithionite and free *o*-phenanthroline. The protein fractions thus obtained were concentrated with a Diaflo apparatus using Diaflo ultrafilter, XM-100, and insoluble materials were removed by centrifugation. The clear supernatant solution was used as the apoenzyme preparation. The extent of release of enzyme-bound Fe^{3+} was extremely sensitive to the pH value of the dialyzing solution. Above pH 7.5, most of the iron was retained by the enzyme. However, at pH 6.5, 92% of the iron was removed from the protein after

3 days of dialysis and there was a concomitant decrease in enzyme activity to less than 0.2% of the original level (Table I). At pH 6.0, removal of the iron appeared to be accompanied with denaturation of the protein moiety, since insoluble material appeared during the dialysis.

TABLE I

REMOVAL OF IRON AT DIFFERENT TIME INTERVALS

The holoenzyme was dialyzed as described in the text. At the indicated times, an aliquot of the inner solution was removed for the assay and iron determination.

Hours of dialysis	*Spec. act. (units/mg of protein)*	*Iron content (gatoms/mole of enzyme)*
0	66.4	14.1
24	1.6	2.4
48	0.4	1.3
72	0.1	1.1

Preparation of reconstituted enzyme

The apoenzyme prepared at pH 6.5 was fully reactivated when it was incubated with Fe^{2+} and $Na_2S_2O_4$ under anaerobic conditions. Preparation of the reconstituted holoenzyme was performed by incubating the apoenzyme (18 mg) in 2 ml of 50 mM Tris–acetate buffer (pH 8.5), containing 10 mM ferrous ammonium sulfate and 10 mM sodium dithionite under anaerobic conditions. After incubation for 30 min at 24 °C, the mixture was passed through an anaerobic column of Biogel A-0.5 m (1 cm × 40 cm) previously equilibrated with 50 mM Tris–acetate buffer (pH 8.5) saturated with N_2. This treatment separates the protein fraction from excess Fe^{2+} and the reductant. Fractions containing protein were concentrated to 10 mg/ml on a Diaflo apparatus and insoluble material was removed by centrifugation. The reconstructed enzyme thus prepared showed a specific activity of 70–83 with an iron content of 14–16 atoms per mole of enzyme. Prolonged incubation of the apoenzyme with Fe^{2+} alone also brought about significant reactivation, but the presence of a reductant such as $Na_2S_2O_4$ greatly accelerated the reactivation rate (Fig. 1) while the reductant alone

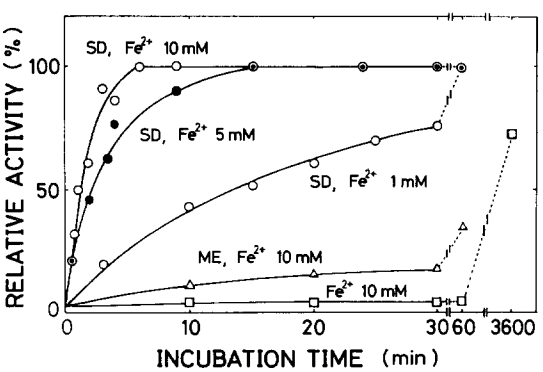

Fig. 1. Time course of the reactivation of apoenzyme. The apoenzyme, 170 μg (spec. act. 2.2), was incubated in 1 ml of 50 mM Tris–acetate buffer (pH 8.5), containing either 10 mM sodium dithionite (SD) or 10 mM β-mercaptoethanol (ME) and/or ferrous ammonium sulfate (Fe^{2+}) as indicated. At the indicated time, an aliquot of the mixture was removed and used for the assay.

showed little effect on the reactivation. The reactivation rate was also dependent on the Fe^{2+} concentration and the maximum rate was obtained at 10 mM Fe^{2+} in the presence of 10 mM $Na_2S_2O_4$. Among other reducing agents tested (β-mercaptoethanol, cysteine, glutathione, dithiothreitol, $NaBH_4$ and ascorbic acid), only β-mercaptoethanol was partially effective in the concentration rang of 5–10 mM. Other metals tested at 10 mM including Cu^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, were unable to replace iron.

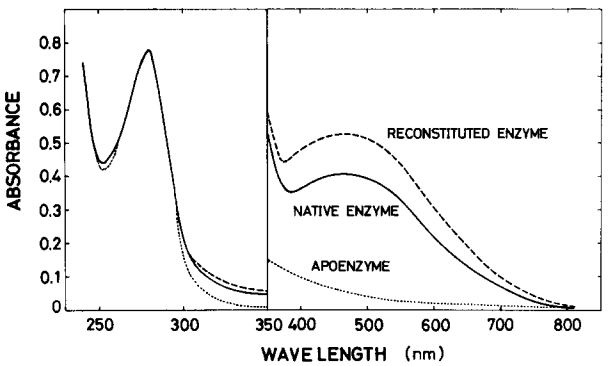

Fig. 2. Absorption spectra of the native, apo-, and reconstituted enzymes. The native, apo- and reconstituted enzymes were prepared as described in the text. Specific activities were 62, 1.1, and 76.5, and the iron contents in gatoms per mole of protein were 14, 1.6 and 16.5, respectively for these preparations. Absorption spectra were measured with protein concentrations of 10 mg/ml for the visible range and 0.6 mg/ml for the ultraviolet range in 50 mM Tris-acetate buffer (pH 8.5). ———, native enzyme; -----, apoenzyme; — — —, reconstituted enzyme.

Absorption spectra

The spectra of the native, apo- and reconstituted enzymes are shown in Fig. 2. As reported previously[3], the native enzyme showed a broad absorption band between 400 and 600 nm. Upon the removal of iron, the absorption peak characteristic to the native enzyme disappeared concomitantly with a decrease in the absorption between 300 to 350 nm. The reconstituted enzyme showed an absorption spectrum similar to that of the native enzyme, but absorbances in the 300–350 nm and 400–600 nm regions were rather higher than those of the native enzyme. However, when the native enzyme was treated in a manner analogous to the reconstitution process, the resulting enzyme showed a higher absorption in these regions than the original one, with a slight increase in specific activity and in iron content as well. These results may indicate that the iron in the native enzyme is partly released from the protein during the purification.

Molecular properties

s values ($s_{20,w}$) of the native, apo- and reconstituted enzymes were calculated to be 17.9, 18.3, and 18.5, respectively. Although the native enzyme has a molecular weight of 700 000 (ref. 2) and consists of 8 identical subunits[3], the removal of iron did not affect the quaternary structure of the enzyme.

Sulfhydryl groups of enzyme

When the sulfhydryl groups of the apo- and reconstituted enzymes were

titrated with DTNB, approx. 19 and 12–13 moles of DTNB reacted per mole of enzyme, respectively. The native enzyme prepared by the method of Fujisawa and Hayaishi[2], which contains 12 cysteine residues that react with *p*-chloromercuribenzoate after prolonged incubation, did not react with DTNB. However, when this native enzyme was treated in a manner analogous to the reconstitution process or when the enzyme was prepared without treatment with β-mercaptoethanol, 12 sulfhydryl groups per mole of enzyme reacted with DTNB.

Number of substrate molecules bound to enzymes

The number of substrate molecules bound to the enzyme was determined by the method of equilibrium dialysis. 4-Nitrocatechol, a nonmetabolizable competitive inhibitor (C. A. Tyson, unpublished data), was used as a substrate analogue. As shown in Fig. 3, the maximum number of substrate molecules bound to the enzyme was

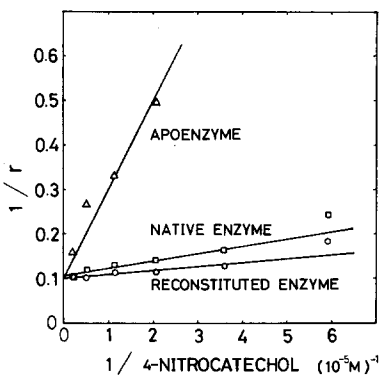

Fig. 3. Substrate binding to the enzymes by equilibrium dialysis. 1 ml each of the enzyme solutions, approx. 5 mg/ml, was dialyzed for 24 h at 24 °C against 200 ml of 50 mM Tris–acetate buffer (pH 7.5), containing 4-nitrocatechol, the concentrations of which were as indicated. After dialysis, concentrations of 4-nitrocatechol and protein in the inner and outer solutions were determined as follows: since the direct spectrophotometric measurement of 4-nitrocatechol was disturbed by the absorption of its complex with the enzyme, 4-nitrocatechol was determined after it was converted to α-hydroxy-γ-nitromuconic semialdehyde by the action of metapyrocatechase, which showed an absorption maximum at 390 nm with a molar extinction coefficient of 20 000. Likewise, since 4-nitrocatechol disturbed the direct spectrophotometric or calorimetric determination of protein, protein was determined by the biuret method after treatment with metapyrocatechase using each outer solution treated in a manner analogous to the inner solution as a blank. r represents 4-nitrocatechol bound to the enzyme (moles/mole of enzyme).

found to be approx. 10 moles per mole of enzyme for the native, apo- and reconstituted enzymes. This value was higher than that obtained from the spectrophotometric titration of the enzyme with protocatechualdehyde, a competitive inhibitor (8 moles per mole of enzyme[3]). Since approx. 1 mole of 4-nitrocatechol can combine with bovine serum albumin under identical conditions, part of the high value obtained by equilibrium dialysis may be due to nonspecific binding. Regardless of this, the dissociation constants of the substrate for native and reconstituted enzymes were almost identical and calculated to be in the range of 0.9–1.5 μM, whereas that for the apoenzyme was 20 μM.

DISCUSSION

Removal of the iron from protocatechuate 3,4-dioxygenase brought about the inactivation and decolorization of the enzyme. Upon reconstition, the enzyme activity as well as the absorption in the visible range was restored to the original level. These results suggest that the iron is an essential cofactor of the enzyme and is responsible for the visible absorption. This view is analogous to that concerning the pyrocatechase from pseudomonads, another Fe^{3+}-containing dioxygenase[9]. The iron in the pyrocatechase can also be removed from the protein by the treatment with both *o*-phenanthroline and $Na_2S_2O_4$ (ref. 10). On the other hand, the iron in another pyrocatechase from *Brevibacterium fuscum* can be easily removed from the protein during the purification[11] or by the incubation with a substrate analogue, ethylprotocatechuate[12]. These results suggest that the mode of iron binding of protocatechuate 3,4-dioxygenase is similar to that of the *Pseudomonas* pyrocatechase but differs somewhat from that of the *Brevibacterium* pyrocatechase. Reconstitution of the *Pseudomonas* pyrocatechase can be partially achieved by the incubation of apoenzyme with ferrous ammonium sulfate and ascorbic acid under aerobic conditions[10] and that of the *Brevibacterium* pyrocatechase by the addition of an equivalent amount of Fe^{2+} in the presence of oxygen[12], while these treatments failed to reconstitute apo-protocatechuate 3,4-dioxygenase. Tokunaga and Sano reported that iron was incorporated into protoporphyrine when it was incubated with Fe^{2+} and $Na_2S_2O_4$ (ref. 13). They suggested that a ferrous–sulfur complex is an effective compound for the incorporation. Although $Na_2S_2O_4$ is the most effective reductant for the reconstitution of protocatechuate 3,4-dioxygenase, FeS alone failed to reconstitute the enzyme, suggesting that the reductant affects the protein moiety of the apoenzyme so as to facilitate the iron incorporation.

Since the enzyme contains approx. 10 gatoms of iron per mole of enzyme, the number of sulfhydryl groups unmasked by the removal of iron (6–7 moles per mole enzyme) is less than one per atom of iron removed. It is far less than the value expected from the results of Peisach *et al.*[14] that all of the ligands of iron are sulfur atoms, arranged in a tetrahedron around the metal.

Although the enzyme consists of 8 subunits[3], its quaternary structure does not seem to be affected by the removal of the iron. This result is not consistent with 3,4-dihydroxyphenylacetate 2,3-dioxygenase in which the removal of the iron brings about the dissociation of the enzyme[15]. On the other hand, the fact that the dissociation constant of the substrate for the apo-protocatechuate 3,4-dioxygenase is much higher than that for the holoenzyme, suggests that the primary binding site of the substrate is in the protein moiety but the iron bound to the enzyme facilitates the binding of the substrate either by inducing a conformational change in the protein moiety or by interacting directly with the substrate.

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