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PURIFICATION AND SOME PROPERTIES OF PROTOCATECHUATE 4,5-DIOXYGENASE*

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SUMMARY

1. Protocatechuate 4,5-dioxygenase (protocatechuate:oxygen 4,5-oxidoreductase, EC 1.13.1.8) was purified from a pseudomonad to an almost homogeneous state. The most purified preparation had a specific activity of 160 μmoles/min per mg of protein at 24° and the molecular weight was estimated to be approx. 150 000. The enzyme contained one atom of iron per mole of the enzyme protein.

2. The enzyme was very unstable and was easily inactivated during storage either at 0° or at room temperature. The inactivation of the enzyme was counteracted by the presence of 10% ethanol. The enzyme was also inactivated in the presence of oxidizing agents such as H_2O_2 even in the presence of 10% ethanol. The inactivated enzyme was partly reactivated by the incubation with both Fe^{2+} and ascorbic acid.

3. Rapid inactivation of the enzyme was observed during the catalysis. The inactivation was found to be due simply to the removal of the iron from the enzyme protein, since the inactivated enzyme was fully reactivated by the addition of Fe^{2+} alone. These results together with the fact that the enzyme was not inactivated by incubation with chelating agents suggest that the interaction between the iron and the enzyme protein is altered during the catalysis.

INTRODUCTION

Most of the dioxygenases are known to contain nonheme iron as a sole cofactor. Among them, the enzymes that contain Fe^{2+} have been reported to be unstable and easily inactivated in the presence of one of the substrates, O_2, whereas Fe^{3+}-containing enzymes are rather stable under these conditions[1-6]. The instability together with the reaction mechanism has been extensively studied with metapyrocatechase, a Fe^{2+}

Abbreviations: PCMB, *p*-chloromercuribenzoate; DOPA, dihydroxyphenylalanine.

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containing enzyme, and the inactivation by O_2 was found to be due to simple oxidation of the Fe^{2+} to the Fe^{3+} form[3,7].

Protocatechuate 4,5-dioxygenase (protocatechuate: oxygen 4,5-oxidoreductase, EC 1.13.1.8), an enzyme catalyzing the cleavage of the aromatic ring of protocatechuic acid with the insertion of two atoms of O_2, was first described by DAGLEY AND PATEL[8] in 1957. The enzyme was also found to be very unstable in the presence of air[9]. For the further elucidation of the instability and reaction mechanism, we have attempted to purify the enzyme from a pseudomonad.

In this paper, we wish to describe the purification procedure, analytical results and some of the properties of the enzyme. The enzyme was easily inactivated during the catalysis. Available evidence indicates that the inactivation is due to removal of the iron from the enzyme protein during the catalysis.

EXPERIMENTAL PROCEDURE

Biological material

The enrichment culture was performed as described previously[10] with *p*-hydroxybenzoic acid or sodium phthalate as a sole carbon source. Among about twenty strains isolated from soil, a strain which was grown on phthalate and tentatively identified as belonging to a Pseudomonas sp. by Dr. Y. KAWATA of the Department of Microbiology, Kyoto University Faculty of Medicine, was selected and used throughout the present work.

Chemicals

Protocatechuic acid used for growth media of bacteria and for enzyme assay was a product of Wako Pure Chemical Industries, Ltd., and of Tokyo Kasei Kogyo Co., Japan, respectively. Acrylamide and *o*-phenanthroline were products of Eastman Organic Chemicals, and α,α'-dipyridyl from Fisher Scientific Co. *p*-Chloromercuribenzoate (PCMB) was a product of Sigma and was recrystallized 3 times by the method of BOYER[11]. Bovine serum albumin, bovine blood hemoglobin, yeast alcohol dehydrogenase, catalase and sodium mersalyl were also products of Sigma. Metapyrocatechase was prepared as previously described[4]. Ethanol was a product of Wako Pure Chemical Industries and DEAE-cellulose of Serva, Germany. Sephadex G-200 was purchases from Pharmacia, Sweden. 3-Methylcatechol, 4-methylcatechol, 2,3-dihydroxyphenylacetic acid, 3,4-dihydroxyacetophenone, 2,3-dihydroxybenzoic acid and dihydroxyphenylalanine (DOPA) were kindly donated by Dr. S. Senoh of the Institute of Food Chemistry, Osaka, Japan. H_2O_2 was obtained from Mitsubishi-Edogawa Chemicals, Ltd. All other chemicals, analytical grade, including ascorbic acid, cysteine, ferrous ammonium sulfate, Tiron (1,2-dihydroxybenzene 3,5-disulfonate), EDTA and diethyldithiocarbamate were purchased from commercial sources.

Enzyme assay, specific activity and determination of protein concentration

The activity of protocatechuate 4,5-dioxygenase was assayed by measuring the decrease in absorbance at 250 mμ with a Shimadzu recording spectrophotometer. The reaction mixture contained in a total volume of 3.0 ml, 1.0 μmole of protocatechuic acid, 150 μmoles of potassium phosphate buffer (pH 7.0) and an adequate

amount of the enzyme in a cuvette with a 1-cm light path. The reaction was started by the addition of 0.1 ml of the enzyme which was dissolved in a buffer solution containing 10% ethanol. The initial rate of decrease of protocatechuic acid was measured spectrophotometrically at 250 mμ at 24°. Molar extinction coefficients of protocatechuic acid and the reaction product at 250 mμ are 8000 and 1940, respectively. Unless otherwise specified, the standard assay method described above was employed. Alternatively, the enzyme activity may be assayed by measuring the increase of the product. In this assay system, Tris–acetate buffer (pH 9.0) was used instead of phosphate buffer (pH 7.0), because the product, α-hydroxy-γ-carboxymuconic semialdehyde shows a yellow color with an absorption maximum at 410 mμ in an alkaline solution[12]. The molar extinction coefficient of the product at pH 9.0 is approx. 11 200. When desired, in some experiments O_2 consumption was measured by an O_2 electrode method[13].

One unit of the enzyme is defined as that amount which degrades 1 μmole of protocatechuic acid per min at 24°. Specific activity was expressed as the number of units/mg of protein under the standard assay conditions at pH 7.0.

Protein concentration was determined by the biuret method[14], using crystalline bovine serum albumin as a protein standard.

Physicochemical and chemical measurements

Ultracentrifugation to examine purity and sedimentation velocity of the enzyme was performed with a Spinco Model E ultracentrifuge apparatus at 4°. The material used for this purpose was dialyzed overnight at 4° against 1 l of 0.05 M potassium phosphate buffer (pH 7.5) containing 10% ethanol. During the dialysis no significant loss of the enzyme activity was observed. Acrylamide gel electrophoresis was carried out in a cold room according to the method of DAVIS[15].

The iron content of the enzyme was determined by using the *o*-phenanthroline method[16] after dry-ashing of the sample at 500° for 20 h. Determination of free sulfhydryl groups was performed according to the method of BOYER[11] by titration with PCMB at 24°. The molecular weight of the enzyme was estimated using Sephadex G-200 by the method of ANDREWS[17] at 4°. For these determinations, all chemical operations, dialysis, *etc.*, were carried out with deionized distilled water.

RESULTS

Purification of protocatechuate 4,5-dioxygenase

Growth conditions. The bacteria were grown for approx. 40 h at 23° with vigorous aeration in a medium containing 0.3% $(NH_4)_2HPO_4$, 0.12% KH_2PO_4, 0.5% NaCl, 0.02% $MgSO_4 \cdot 7\,H_2O$, 0.05% yeast extract and 0.3% protocatechuic acid as the major carbon source. The pH of the medium was adjusted to 7.0 with solid NaOH. Cells were harvested by the aid of a Sharples centrifuge and washed once with 0.85% KCl solution. Approx. 100 g of wet cells were obtained from 15 l of the medium. The cells were stored in a deep freezer with little loss of activity for at least 3 months. All subsequent procedures of extraction and purification were carried out at 0–4°.

Crude extracts. 100 g of the wet cells were suspended in 300 ml of 0.05 M potassium phosphate buffer (pH 7.5) and were disrupted by sonic oscillation in a

batch of 50 ml at 10 kcycles for 10 min with a Raytheon Model DF101 oscillator. The supernatant was separated from the residue by centrifugation at 15 000 × *g* for 15 min. To this supernatant was added ethanol to give 10% at final concentration. The crude extracts thus obtained had a specific activity of about 3.5 μmoles/min per mg of protein. Since the presence of 10% ethanol in the enzyme solution was found to protect the enzyme from inactivation by air as described later, all subsequent purification procedures were carried out in a buffer solution containing 10% ethanol.

Ultracentrifugation. Since the crude extracts were viscous and contained a large quantity of cell debris and particles not sedimented by previous centrifugation, they were centrifuged at 78 000 × *g* for 30 min by a Hitachi ultracentrifuge 40P. Almost all the activity was recovered from the supernatant (ultracentrifugation fraction).

Ethanol fractionation. To the ultracentrifugation fractions was added 1 vol. of cold ethanol and after standing for 10 min the resulting precipitate was removed by centrifugation at 10 000 × *g* for 10 min. A second volume of cold ethanol was added to the supernatant to give a final concentration of 70% ethanol, and the resulting precipitate was collected by centrifugation and suspended in about 150 ml of 0.05 M potassium phosphate buffer (pH 7.5) containing 10% ethanol (hereafter referred to as ethanol buffer). The suspension was dialyzed twice against 1 l each of ethanol buffer overnight at 4°, and the precipitate was discarded after centrifugation to get clear supernatant (ethanol fraction).

Chromatography on DEAE-cellulose. The ethanol fraction was applied to a DEAE-cellulose column, 2.5 cm in diameter and 20 cm in height, previously equilibrated with ethanol buffer. The column was washed thoroughly with ethanol buffer and the enzyme was eluted with 0.2 M potassium phosphate buffer (pH 7.5) containing 10% ethanol and collected in each 20-ml fraction. Active fractions were combined and dialyzed against ethanol buffer overnight at 4°. The DEAE fraction thus obtained with a specific activity of approx. 160 was mainly used for the present studies. When necessary the DEAE fraction was concentrated by precipitating the enzyme protein by the addition of 2 vol. of cold ethanol and dissolving it into a minimum volume of ethanol buffer followed by dialysis. A typical result of the purification is summarized in Table I.

Analytical and catalytic properties of protocatechuate 4,5-dioxygenase

Homogeneity. The ultracentrifugal pattern of the purified enzyme (DEAE

TABLE I

PURIFICATION OF PROTOCATECHUATE 4,5-DIOXYGENASE

Fraction	*Vol. (ml)*	*Activity (units)*	*Protein (mg)*	*Specific activity (units/mg protein)*	*Yield (%)*
Crude extracts	437	71 120	20 233	3.5	100
Ultracentrifugation	380	68 096	10 450	6.4	96
Ethanol fraction	140	43 550	700	62.5	61.5
DEAE-cellulose chromatography	60	18 840	120	157.0	26.5

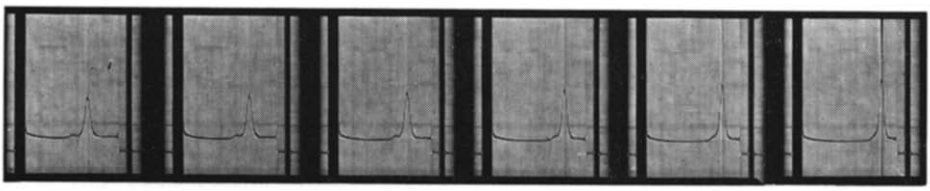


Fig. 1. Ultracentrifugal pattern of protocatechuate 4,5-dioxygenase (DEAE fraction). The protein concentration was approx. 1.3% in 0.05 M potassium phosphate buffer (pH 7.5) containing 10% ethanol. The photographs were taken at 8-min intervals, after reaching 59 780 rev./min at 4°. The progress of ultracentrifugation is shown from right to left.

fraction) showed an almost single peak with a small amount of impurity which ran faster (Fig. 1). The sedimentation constant ($s_{20,w}$) of a 0.6% solution of the enzyme was calculated to be $4.9 \cdot 10^{-13}$ (cm/sec). Acrylamide gel electrophoresis of the enzyme at pH 9.0 resulted in a dense single band with faint bands of impurity. These results together with the fact that the specific activity of the preparation was comparable or rather higher than those of other crystalline dioxygenases, as discussed later, indicated that the enzyme was almost homogeneous.

Molecular weight of the enzyme. Estimation of molecular weight of the enzyme was performed by gel filtration method using a Sephadex G-200 column. The void volume of the column was determined by blue dextran 2000, and authentic proteins used in this experiment and their molecular weights were as follows; bovine hemoglobin (32 000)[17], bovine serum albumin (67 000)[18], yeast alcohol dehydrogenase (150 000)[19], metapyrocatechase (140 000)[4] and liver catalase (251 000)[20]. Protocatechuate 4,5-dioxygenase passed through the column with a single peak of activity, and from the position of the elution volume, the molecular weight of the enzyme was estimated to be approx. 150 000 (Fig. 2).

Spectrum of the enzyme. The absorption spectrum of the purified enzyme (DEAE fraction) showed a peak at 280 mμ with a shoulder at 290 mμ, and there was no significant absorption in the visible range (Fig. 3). The ratio of absorbance of the enzyme at 280 mμ to that at 260 mμ was 1:7.

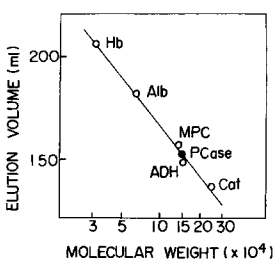
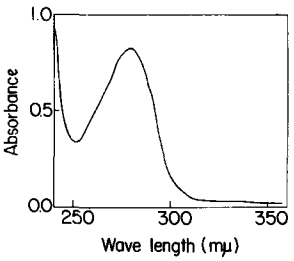


Fig. 2. Determination of molecular weight of protocatechuate 4,5-dioxygenase on a Sephadex G-200 column. Standard curve shows the relationship between the molecular weights of a number of authentic proteins and their elution volumes on a Sephadex G-200 column. Each authentic protein was applied on a column (3.0 cm × 44.0 cm), previously equilibrated with 0.04 M potassium phosphate buffer (pH 7.2) containing 5 mM EDTA, and eluted with the same buffer at a flow rate of 10 ml/h at 4°. Protocatechuate 4,5-di-oxygenase (10 mg) was also treated as above. Abbreviations: Hb, bovine hemoglobin; Alb, bovine serum albumin; MPC, metapyrocatechase; ADH, yeast alcohol dehydrogenase; Cat, liver catalase; PCase, protocatechuate 4,5-dioxygenase.



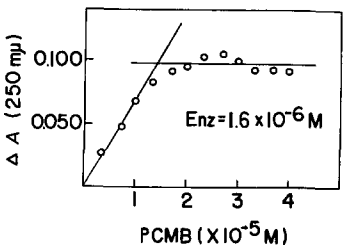


Fig. 3. Absorption spectrum of protocatechuate 4,5-dioxygenase. Absorption spectrum of the enzyme was measured with a Shimadzu recording spectrophotometer RS-27 after dialysis of the enzyme against ethanol buffer, using the dialyzing fluid as a blank. The protein concentration was 0.094%.

Fig. 4. Titration of free sulfhydryl groups in protocatechuate 4,5-dioxygenase with PCMB. Increments in absorbance at 250 mμ as a result of formation of the mercaptide complex were measured approx. 10 min after addition of PCMB into 3 ml of the enzyme solution ($1.6 \cdot 10^{-6}$ M), which contained 4.8 mμmoles of the enzyme, 150 μmoles of potassium phosphate buffer (pH 7.5) and 0.3 ml of ethanol.

Iron content of the enzyme. Iron content of the native enzyme was determined as described under EXPERIMENTAL PROCEDURE. The enzyme preparation with a specific activity of 157 was dialyzed for 24 h against ethanol buffer. No significant loss of the enzyme activity was observed during dialysis. 15.5 mg of the preparation contained 5.3 μg of iron. Assuming that the molecular weight of the enzyme was 150 000, the enzyme contained 0.92 atom of iron per mole of the enzyme.

Sulfhydryl groups. Free sulfhydryl groups of the enzyme were estimated to be 8–9 moles per mole of the enzyme by the titration with PCMB (Fig. 4). No significant change in the number of free sulfhydryl groups was observed when the enzyme was treated with H_2O_2 which brought about almost complete inactivation of the enzyme. All attempts to clarify the relation between the enzyme activity and free sulfhydryl groups were unsuccessful because the enzyme was unstable even without PCMB under these titration conditions.

Stoichiometry of the reaction. The purified enzyme preparation was completely

TABLE II

STOICHIOMETRY OF THE REACTION

Each reaction mixture contained in a total volume of 2.9 ml, 150 μmoles of potassium phosphate buffer (pH 7.0), an appropriate amount of the enzyme and protocatechuic acid as indicated. The reaction was carried out in the reaction cell of an O_2 electrode and started by the addition of substrate. When O_2 consumption was completed, 0.1 ml of 3 M NaOH was added and an aliquot was taken out and diluted with a 0.1 M NaOH solution to measure the absorbance at 410 mμ. The amount of α-hydroxy-γ-carboxymuconic semialdehyde formed was calculated from the molar extinction coefficient at 410 mμ (ε_M = 34 000).

Protocatechuic acid added (μmole)	*O_2 consumed (μmole)*	*Product formed (μmole)*
0.30	0.29	0.32
0.60	0.59	0.68

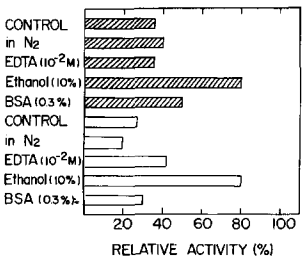


Fig. 5. Effect of various conditions on the stability of protocatechuate 4,5-dioxygenase. Each 1 ml of enzyme solution (0.46 mg/ml) was stored in an open test tube for 3 days at 4° under the conditions indicated in the figure. Hatched area: 0.05 M Tris–acetate buffer (pH 7.5); blank area: 0.05 M potassium phosphate buffer (pH 7.5) .Control, no addition; in N_2, in nitrogen gas; BSA, bovine serum albumin. After incubation the enzyme activity was assayed by measuring the rate of formation of reaction product (λ_{max} = 410 mμ at pH 9.0).

free from the enzyme which catalyzed the following step (NADP-linked α-hydroxy-γ-carboxymuconic semialdehyde dehydrogenase), since the increase in absorbance at 340 mμ was hardly observed upon the addition of NADP to the reaction mixture after completion of the reaction. The stoichiometry of the reaction was determined with the purified enzyme as described in Table II. The results indicated that for each mole of protocatechuic acid added, a stoichiometric amount of the product, α-hydroxy-γ-carboxymuconic semialdehyde, was formed with concomitant consumption of an equivalent amount of O_2.

Stability of the enzyme. Crude preparation of the enzyme was rapidly inactivated either at room temperature or at 0° in the absence of stabilizer. More than 50% of the activity was lost within 24 h under these conditions. Storage of the enzyme in a deep freezer could not prevent the inactivation. Cysteine, glutathione, Fe^{2+}, mercaptoethanol, EDTA, albumin, glycerol, acetone and introduction of nitrogen gas failed to stabilize the enzyme, but only low concentrations of ethanol could prevent inactivation (Fig. 5). Ethanol was required to stabilize the enzyme even after purification. The enzyme was, however, completely inactivated by incubation at 37° for 10 min even in the presence of 10% ethanol. The purified enzyme was found to be most stable at pH 7.5. Alteration of buffer concentration from 0.05 M to 0.5 M phosphate buffer (pH 7.5) showed no significant effect on the stabilization and the enzyme was more unstable at concentrations lower than 0.05 M.

Influence of pH and substrate concentration on the reaction. The optimum pH for the enzyme reaction was found to be 7.0 (Fig. 6). At any pH tested the reaction rate was not proportional to the reaction time but decreased during the reaction, as discussed later. The K_m value for protocatechuic acid determined by a plot according to LINEWEAVER AND BURK[21] was estimated to be $8 \cdot 10^{-5}$ M (Fig. 7). As can be seen from the figure, substrate inhibition was observed with high concentrations of substrate. The K_m value for O_2 was estimated to be $5.4 \cdot 10^{-5}$ M by the polarographic method of LONGMUIR[22].

Effect of inhibitors. Metal-chelating agents such as *o*-phenanthroline, α,α'-dipyridyl, diethyldithiocarbamate, Tiron and EDTA did not show significant inhibitory effect either by their simultaneous addition to the reaction mixture or by preincubation of the enzyme with these agents. Sulfhydryl agents such as PCMB and

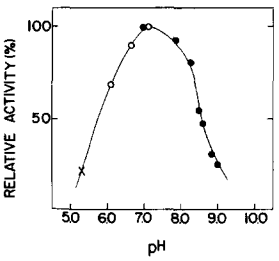


Fig. 6. Effect of pH on the activity of protocatechuate 4,5-dioxygenase. The reaction mixture contained in a total volume of 3.0 ml, 1.0 μmole of protocatechuic acid, 150 μmoles of buffer, and 30 μg of enzyme protein. ×, acetate buffer; ○, potassium phosphate buffer; ●, Tris–acetate buffer. The enzyme activity was assayed by measuring the decrease in absorbance at 250 mμ.

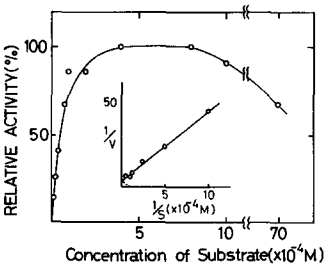


Fig. 7. Effect of substrate concentration on the rate of reaction. The reaction mixture contained in a total volume of 3.0 ml, 150 μmoles of potassium phosphate buffer (pH 7.0); 46 μg of the enzyme protein; and protocatechuic acid as indicated. The rate of the reaction was measured by following the decrease in absorbance at 250 mμ. The broken line in the figure is the mirror image of the extension of the solid line.

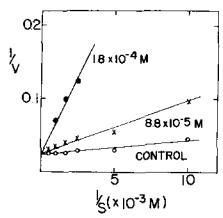
sodium mersalyl inhibited the enzyme activity even when added to the reaction mixture simultaneously with substrate. On the other hand, alkylating agent, such as monoiodoacetate, exerted no inhibitory effect (Table III). KCN showed strong inhibitory effect only when it was preincubated with the enzyme. The enzyme was also inhibited by the presence of H_2O_2 in the reaction mixture, and the type of the inhibition was shown to be competitive with respect to protocatechuic acid (Fig. 8). When preincubated together with H_2O_2, the enzyme was almost instantly inactivated. Ethanol which, as stated before, protects the enzyme from inactivation, also showed

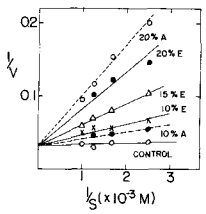
TABLE III

EFFECT OF INHIBITORS

Assay system contained 115 μmoles of potassium phosphate buffer (pH 7.0), 0.7 μmole of protocatechuic acid, 2 μg of the enzyme and an inhibitor as indicated in a total volume of 2.3 ml. The reaction was started by the addition of the substrate solution after the indicated preincubation period at 18°, and O_2 uptake was measured with an O_2 electrode at 18°.

Inhibitor	*Concentration (mM)*	*Inhibition (%)*		
		Preincubation time (min):		
		0	*15*	*30*
None		0	0	0
PCMB	0.1	10.0	15.5	14.5
	0.2	14.5	—	—
	0.5	24.0	—	—
	0.7	56.5	—	—
Sodium mersalyl	1	27.5	57.5	76.0
Monoiodoacetate	1	10.0	7.5	0
Diethyldithiocarbamate	1	10.0	7.5	0
EDTA	1	0	7.5	5.0
α,α′-Dipyridyl	1	5.5	7.5	5.0
o-Phenanthroline	1	0	7.5	9.5
Tiron	1	5.5	0	9.5
KCN	1	0	77.0	86.0





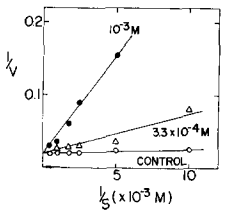


Fig. 8. Competitive type inhibition of protocatechuate 4,5-dioxygenase by H_2O_2. The reaction mixture contained in a final volume of 3.0 ml, 150 μmoles of Tris–acetate buffer (pH 9.0), 20 μg of the enzyme protein, H_2O_2 and protocatechuic acid, the concentrations of which were as indicated. The rate of the reaction was measured at 24° by following the increase in absorbance at 410 mμ at pH 9.0.

Fig. 9. Competitive type inhibition of protocatechuate 4,5-dioxygenase by ethanol and acetone. Reaction mixture contained in a final volume of 3.0 ml, 150 μmoles of Tris–acetate buffer (pH 9.0), 20 μg of the enzyme protein, protocatechuic acid, ethanol and acetone (concentrations were as indicated). Other conditions were the same as in Fig. 8. E, ethanol; A, acetone; and control, no addition.

Fig. 10. Competitive type inhibition of protocatechuate 4,5-dioxygenase by 4-methylcatechol. Reaction mixture contained in a total volume of 3.0 ml, 150 μmoles of Tris–acetate buffer (pH 9.0) 20 μg of the enzyme protein, protocatechuic acid and 4-methylcatechol (concentrations were as indicated). Other conditions were the same as in Fig. 8.

a competitive type of inhibition with respect to the substrate, protocatechuic acid, when it was present in the reaction mixture (Fig. 9). Similar competitive type of inhibition was observed with acetone, although it did not show a protective effect.

Substrate specificity. Substrate specificity of the enzyme was determined polarographically by measuring the rate of O_2 consumption. Among a number of the compounds tested, only protocatechuic acid was found to be oxygenated by the enzyme. The following compounds did not serve as substrate within a experimental limit: catechol, 4-methylcatechol, 3-methylcatechol, protocatechuic aldehyde, pyrogallol, *o*-aminophenol, DOPA, caffeic acid, 3,4-dihydroxyacetophenone, 2,3-

TABLE IV

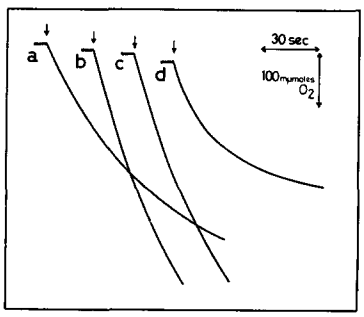
INHIBITORY EFFECT OF SUBSTRATE ANALOGUES

Assay system contained 150 μmoles of Tris–acetate buffer (pH 9.0), 0.33 μmole of protocatechuic acid and the substrate analogue as indicated in the total volume of 3.0 ml. The reaction was started by the addition of 0.1 ml of the enzyme solution and the increase in absorbance at 410 mμ was measured as described in EXPERIMENTAL PROCEDURE.

Substrate analogue	*Concentration (mM)*	*Inhibition (%)*
None		0
Catechol	1	20.5
3-Methylcatechol	0.33	59.5
	1	100
4-Methylcatechol	0.33	37.0
Pyrogallol	1	90.7
3,4-Dihydroxyacetophenone	1	7.5
2,3-Dihydroxybenzoic acid	1	44.5
3,4-Dihydroxymandelic acid	1	26.0
3,4-Dihydroxyphenylacetate	1	18.5

dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, benzoic acid, *p*-hydroxybenzoic acid and phthalic acid.

As shown in Table IV, however, some of the *o*-dihydroxyphenyl compounds including catechol and 3- or 4-substituted catechol inhibited the enzyme activity. The nature of their inhibitions was shown to be competitive with respect to protocatechuic acid. A typical result obtained with 4-methylcatechol as an inhibitor is shown in Fig. 10.



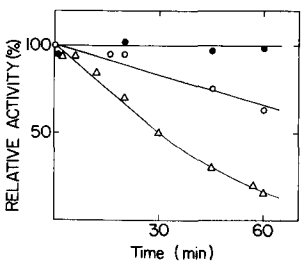


Fig. 11. Time-course of protocatechuate 4,5-dioxygenase reaction at different pH. The reaction mixture contained in a total volume of 2.3 ml, 150 μmoles of potassium phosphate buffer, 1.0 μmole of protocatechuic acid and 2 μg of the enzyme. The reactions were started by the addition of the enzyme as indicated by arrows, and the rate of O_2 consumption was recorded polarographically. pH of the buffer solution was as follows; a, 6.0; b, 7.0; c, 7.5; d, 9.5.

Fig. 12. Inactivation of protocatechuate 4,5-dioxygenase during catalysis. ○—○, control I (without protocatechuic acid); 280 μg of the enzyme protein in 2.0 ml of ethanol buffer containing 2 mg of bovine serum albumin and 80 μg of catalase was kept in an open test tube, and at the incubation period indicated, an aliquot (0.1 ml) of the incubation mixture was taken out and the enzyme activity was measured by the standard method. ●—●, control II (with protocatechuic acid, but under anaerobic conditions); each Thunberg tube contained 140 μg of the enzyme protein, 0.9 ml of ethanol buffer containing 1 mg of bovine serum albumin and 40 μg of catalase in a main chamber, and 0.1 ml of $1 \cdot 10^{-2}$ M protocatechuic acid in a side arm. After being evacuated, they were mixed and incubated. At the incubation period indicated, each one of them was opened and a portion of the incubation mixture (0.1 ml) was taken out and the enzyme activity measured. △—△, complete system; reaction mixture contained in a final volume of 20 ml in a cellophane tube, 1 mmole of potassium phosphate buffer (pH 7.5), 2.8 mg of the enzyme protein, 20 mg of bovine serum albumin, 800 μg of catalase, 2.0 ml of ethanol and 20 μmoles of protocatechuic acid. The reaction was started by the addition of protocatechuic acid into the reaction mixture and the mixture in the cellophane tube was placed in 1 l of ethanol buffer, containing $1 \cdot 10^{-3}$ M protocatechuic acid. During catalysis, a portion of the enzyme solution (0.1 ml) was taken out from the cellophane tube and the enzyme activity was measured. All the incubations and reactions were carried out at 24°, and the enzyme activity was measured by following the decrease in absorbance at 250 mμ.

Inactivation during the reaction. As shown in Fig. 11, reaction rates measured at different pH were not linear but decreased with reaction time. This inactivation during the reaction was minimal at neutral pH and notable at lower and higher pH's. This phenomenon was also observed when, instead of phosphate or Tris buffer, five kinds of Good's buffer which do not chelate metals[23], were used in the reaction mixture. Ethanol, catalase, Fe^{2+}, ascorbate, cysteine, and other protein-stabilizing substances including glycerol and mercaptoethanol could not prevent this inactivation when they were present in the reaction mixture. However, the presence of albumin in the reaction mixture partly prevented the inactivation. Since no

significant inactivation of the enzyme was observed when the enzyme was incubated under the assay conditions prior to the addition of substrate nor was it inhibited in the presence of the product in the reaction mixture, the inactivation was neither due to the denaturation of the enzyme under the assay conditions nor to the product inhibition.

The enzyme was markedly inactivated when it was incubated with both substrate and O_2, but no, or less, inactivation was observed when the enzyme was incubated either with protocatechuic acid under anaerobic conditions or without the substrate under aerobic conditions (Fig. 12). Incubation of the enzyme with catechol, a competitive inhibitor, or salicylic acid, sometimes referred to as perturbator[24] under aerobic conditions, caused no significant inactivation.

Reactivation of the inactivated enzyme. Although 10% ethanol protected the enzyme from inactivation, it was unable to reactivate the enzyme which had been inactivated by H_2O_2 or by catalysis. As shown in Expt. 1 in Table V, however, the inactivated enzyme prepared by treating with H_2O_2 was partly reactivated when it was incubated with reducing agents or Fe^{2+} under aerobic conditions. Among them, ascorbic acid was the most effective and reactivated the inactive enzyme to about 60% of the original activity. Cysteine and glutathione were less effective than ascorbate. The addition of Fe^{2+} gradually reactivated the inactivated enzyme but Fe^{3+} had no effect. Maximal reactivation was observed when both ascorbate and Fe^{2+} were used for reactivation. Essentially similar results were obtained after

TABLE V

REACTIVATION OF H_2O_2-TREATED ENZYME WITH IRON AND REDUCING AGENTS

The inactive enzyme was prepared by incubation of $6.3 \cdot 10^{-6}$ M enzyme with $2.3 \cdot 10^{-4}$ M H_2O_2 in ethanol buffer for 30 min at 0°. Thus prepared inactive enzyme had less than 10% of the original activity. To each 0.4 ml of the inactivated enzyme, before and after dialysis, was added either iron, reducing agents, or both as indicated and 4 μg of catalase to give the total volume of 0.5 ml. After incubated aerobically at 24°, 0.1 ml portion of the incubation mixture was taken out and the enzyme activity was measured by the standard assay method. Ferrous and ferric ammonium sulfate were used as Fe^{2+} and Fe^{3+}, respectively. In Expt. 1, nondialyzed enzyme was used. The inactive enzyme used in Expts. 2 and 3 was dialyzed against ethanol buffer for 12 h, before use.

Expt.	*Addition*	*Concentration (mM)*	*Activity recovered (%)*			
			Preincubation time (min):			
			10	*30*	*60*	*90*
1	None		6	8	—	—
	Fe^{2+}	1	24	38	—	—
	Fe^{3+}	1	4	2	—	—
	Ascorbic acid	1	64	60	—	—
	Glutathione	1	8	10	—	—
	Cysteine	1	28	44	—	—
	Fe^{2+} + ascorbate	1	74	74	—	—
	Fe^{2+} + cysteine	1	64	62	—	—
	Fe^{2+} + glutathione	1	40	56	—	—
2	None		4	4	4	—
	Fe^{2+}	1	18	28	34	34
	Ascorbate	1	40	38	32	—
	Fe^{2+} + ascorbate	1	50	60	62	—
3*	Fe^{2+} + ascorbate	1	—	76	—	—

* Reactivation was carried out under anaerobic conditions.

TABLE VI

REACTIVATION OF THE REACTION-INACTIVATED ENZYME WITH Fe^{2+}

The reaction-inactivated enzyme was prepared as follows: reaction mixture contained in a final volume of 20 ml in a cellophane tube, 1 mmole of potassium phosphate buffer (pH 7.5), 2.8 mg of the enzyme protein, 20 mg of bovine serum albumin, 800 μg of catalase, 2.0 ml of ethanol and 20 μmoles of protocatechuic acid. The reaction was started by the addition of protocatechuic acid and the mixture in a cellophane tube was placed in 1 l of ethanol buffer, containing $1 \cdot 10^{-3}$ M protocatechuic acid. The reaction was carried out at 24°. The enzyme preparations incubated for 30 and 60 min were used for Expts. 1 and 2, respectively. The reactivation of the inactivated enzyme thus obtained was carried out as follows: the reactivation mixture contained in a final volume of 1.0 ml in a Thunberg tube, 0.7 ml of the inactivated enzyme, 0.1 ml of catalase solution (40 μg) in a main chamber and 0.2 ml of Fe^{2+}, ascorbic acid or both as indicated in a side arm. After being evacuated, they were mixed and incubated at 24° for 30 min. A portion of the incubation mixture (0.1 ml) was taken out and the enzyme activity was measured by the decrease in absorbance at 250 mμ.

Expt.	*Addition*	*Concentration (mM)*	*Activity recovered (%)*	
			Before reactivation	*After reactivation*
1	Fe^{2+}	1	44.5	82.0
	Ascorbic acid	1	44.5	34.4
	Fe^{2+} + ascorbic acid	1	44.5	63.5
2	Fe^{2+}	1	16.3	98.5
	Ascorbic acid	1	16.3	36.0
	Fe^{2+} + ascorbic acid	1	16.3	94.0

dialysis of the inactivated enzyme (Expt. 2) and also when the reactivation was carried out under anaerobic conditions (Expt. 3). The native enzyme with a specific activity of about 160, however, could not be further activated with these treatments.

On the other hand, as shown in Table VI, the inactivated enzyme during catalysis was almost fully reactivated by the incubation with Fe^{2+} alone. Ascorbate had essentially no effect on the reactivation either by itself or by the addition with Fe^{2+}. Significant reactivation of the inactivated enzyme was observed by the incubation with Fe^{2+} for 30 min under anaerobic conditions and the extent of the reactivation was gradually increased by prolonging the incubation period up to 90 min, which brought about almost full reactivation. However, no significant reactivation was observed when the inactive enzyme was incubated under aerobic conditions with either Fe^{2+} alone, ascorbic acid alone or both of them. As described in Table VI, inactive enzyme during catalysis was prepared in the presence of bovine serum albumin, catalase and 10% ethanol in the reaction mixture. In the absence of either albumin or ethanol, the inactivated enzyme was only partially reactivated by Fe^{2+} suggesting that these agents protect the enzyme protein from denaturation. In the absence of catalase, not only protocatechuic acid, but also substrate analogue such as catechol caused rapid inactivation of the enzyme presumably due to the generation of H_2O_2. Among a number of metals tested on the reactivation including Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Mg^{2+} (each at 1 mM), only Fe^{2+} was effective.

DISCUSSION

Since protocatechuate 4,5-dioxygenase was first described by DAGLEY AND

PATEL[8] in 1957, there have been a few reports which described some properties of this enzyme[9,12]. Requirement of Fe^{2+} in the reaction was suggested by CAIN[9] with a crude extract of a pseudomonad. Although further purification of the enzyme from *Pseudomonas testosteroni* has been reported by DAGLEY *et al.*[25], the precise nature of the enzyme, including the role of the iron, has remained to be elucidated.

We have succeeded in purifying the enzyme from Pseudomonas sp. to an almost homogeneous state and examined the nature of the enzyme. The specific activity of the present preparation of the enzyme was 160 under the standard assay conditions at pH 7.0. There were no previous reports to which this value was compared, but the activity was comparable to that of crystalline metapyrocatechase (116)[4] and much higher than that of pyrocatechase (29.6)[2] and protocatechuate 3,4-dioxygenase (61.6)[6]. The molecular activity (turnover number) of the enzyme was calculated to be 24 000 moles/min per mole of the enzyme, the molecular weight of which was estimated to be 150 000.

Although the enzyme activity was neither inhibited by metal-chelating agents nor stimulated by the addition of Fe^{2+}, the estimation of iron content of the enzyme revealed that the enzyme contained one gatom of iron per mole of the enzyme protein. From the facts that the inactivated enzyme during catalysis was fully reactivated by incubation with Fe^{2+} and that there was no indication of the existence of heme or flavin in the molecule from the spectral data, it is plausible to assume that the iron is, like other dioxygenases, a sole cofactor of the enzyme and involved in the catalysis. "Extradiol-type" dioxygenases[26], which cleave the bond adjacent to an hydroxyl group of a dihydroxyphenyl compound have so far been reported to be colorless and unstable in the presence of oxidizing agents[3-5], and some of them were established to contain Fe^{2+}, whereas "intradiol-type" dioxygenases which cleave the bond between two hydroxyl groups such as pyrocatechase, protocatechuate 3,4-dioxygenase are red in color and rather resistant to oxidizing agents. These enzymes were established to contain Fe^{3+} (refs. 2, 3, 6). The determination of the valence state of protocatechuate 4,5-dioxygenase has not been performed, but judging from catalytic function, absorption spectrum, and instability against oxidizing agents, the iron in the enzyme is presumably in the divalent state.

The enzyme was extremely unstable and easily inactivated during storage, but the presence of 10% ethanol counteracted the inactivation, as it does with metapyrocatechase. Acetone, which is the most potent stabilizer of metapyrocatechase[4], however, failed to protect protocatechuate 4,5-dioxygenase from the inactivation. On the other hand, these organic solvents including acetone inhibited the enzyme activity when they were present in the reaction mixture, and the nature of the inhibition was found to be competitive with respect to protocatechuic acid. Similar inhibition was also reported with metapyrocatechase[7]. These results may indicate that the organic solvents interact with the enzyme protein at the active area and result in the stabilization of the enzyme.

The enzyme was also inactivated when it was incubated with oxidizing agents such as H_2O_2 even in the presence of a stabilizer, 10% ethanol. Inactivation by H_2O_2 was also reported with metapyrocatechase and was due to the oxidation of Fe^{2+} to Fe^{3+} (ref. 7). The fact that the effect of reducing agents on the reactivation of the inactive enzyme is more pronounced than that of Fe^{2+} may indicate that the inactivation is also due to the oxidation of the Fe^{2+}. No significant change in the

number of free sulfhydryl groups of the enzyme was observed when the enzyme was treated with H_2O_2. Although the enzyme was inhibited by sulfhydryl reagents such as PCMB or mersalyl acid only at high concentration, the role of sulfhydryl groups in the reaction needs further investigation.

It is not entirely unique that enzymes are inactivated during the catalysis (especially with many dioxygenases). Metapyrocatechase was reported to be inactivated during catalysis, especially when substrate analogues were used as a substrate[26]. However, the nature of the inactivation has never been investigated in detail. The rapid inactivation of protocatechuate 4,5-dioxygenase was observed even with physiological substrate, protocatechuic acid. From the following evidences, the inactivation occurred only when the enzyme was functioning: (1) the inactivation was observed only in the presence of both protocatechuate and O_2, (2) substrate analogues such as catechol or a perturbator such as salicylic acid did not inactivate the enzyme when they were incubated with the enzyme under either aerobic or anaerobic conditions and (3) other possibilities that the inactivation is due either to product inhibition, denaturation of the enzyme, chelating action of buffers or to H_2O_2 generated from substrate were ruled out. The inactivated enzyme during catalysis was fully reactivated by incubation with Fe^{2+} under anaerobic conditions, and reducing agents could neither replace nor fortify the effect of Fe^{2+}, suggesting that the inactivation is somewhat different from that by H_2O_2 and due to simple removal of Fe^{2+} during catalysis.

The fact that the enzyme was not inactivated by the prolonged incubation with a chelator such as *o*-phenanthroline indicates that the iron is tightly bound to the enzyme protein. On the other hand, the iron seems to be easily removed from the enzyme protein during catalysis, suggesting that the interaction between the iron and the enzyme protein was greatly affected during catalysis probably being accompanied by dynamic structural changes in protein moiety. These results are consistent with the previous report with pyrocatechase in which ^{59}Fe in the medium exchanged with the iron in the enzyme only when the enzyme was functioning[27]. The elucidation of the detailed nature of the inactivation needs further investigation but may provide a clue for the clarification of the reaction mechanism of dioxygenases.

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