Biochimica et Biophysica Acta, 422 (1976) 280—294

BBA 67712

PURIFICATION, CRYSTALLIZATION AND PROPERTIES OF IRON-CONTAINING SUPEROXIDE DISMUTASE FROM *PSEUDOMONAS OVALIS*

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(Received September 2nd, 1975)

Summary

Three electrophoretically distinct superoxide dismutases (EC 1 15 1 1) were observed in the crude extracts from *Pseudomonas ovalis* One of these was isolated as an iron-containing superoxide dismutase It contained 1 4 gatoms of Fe per mol of enzyme, and had a specific activity of 3900 units per mg of protein A crystallized enzyme contained 1 1 gatoms of Fe per mol of enzyme, and had a specific activity of 3100 units per mg of protein

The results of sedimentation equilibrium and gel filtration indicated a molecular weight of 40 000 $S^0_{20,w}$ was estimated as 3.18 by sedimentation velocity study Sodium dodecyl sulfate gel electrophoresis indicated that the enzyme was composed of two subunits, and had a molecular weight of 19 500

Analysis for sulfhydryl groups showed that there were four such groups per mol of enzyme The spectrum of visible and ultraviolet region, the amino acid composition, the CD spectrum of the enzyme, and the effect of certain compounds on the enzyme, were studied and compared with iron-containing superoxide dismutases isolated from other organisms

Introduction

Superoxide dismutase (EC 1 15 1 1) has been studied extensively in recent years and has been shown to exist in animals [1,2], in microbials [3—7,9,13—15], and in plants [8,10—12] This enzyme catalyzes the dismutation reaction of superoxide free radical ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), formed in many biological oxidations, and has been assumed to play an important role in the protecting of cells against the deleterious action of this radical

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

Three distinct kinds of superoxide dismutase have been isolated from aerobic organisms Superoxide dismutase isolated from eukaryotic sources contains 2 gatoms of Cu and 2 gatoms of Zn per mol of enzyme [1,2,10—12] The Cu,Zn-enzyme was also found in *Photobacterium leiognathi* [9]. Many enzymes isolated from prokaryotes and from eukaryotic mitochondria have 2 g-atoms of Mn per mol of enzyme [2,3,7,8]. In addition, Yost and Fridovich [6] isolated iron-containing superoxide dismutase from *Escherichia coli* This enzyme contains 1 gatom of Fe^{3+} per mol of enzyme

Recently, iron-containing superoxide dismutases were also isolated from *Plectonema boryanum* [13,14] and *Photobacterium leiognathi* and *sepia* [15] These enzymes contained 1 or 2 gatoms of Fe^{3+} per mol of enzyme and had a molecular weight of 40 000 consisting of two subunits. For this reason, characterization and comparison of the iron-containing superoxide dismutase from *Pseudomonas ovalis* was very interesting

In this paper, it is reported that three electrophoretically distinct superoxide dismutases could be detected in the crude extracts from *Ps ovalis* IAM-1002, and that one of these enzymes has been isolated as iron-containing superoxide dismutase Purification, crystallization, and some properties of the Fe-enzyme, such as molecular weight, metal content, absorption spectrum, CD spectrum, and amino acid composition are reported

Materials and Methods

Materials

Xanthine oxidase (EC 1 2.3 2) and cytochrome *c* were products of Boehringer Mannheim. Xanthine, ovalbumin, bovine serum albumin, chymotrypsinogen, and myoglobin were obtained from Sigma DEAE-cellulose (DE-32) was a product of Whatman. All other materials were of the highest grade commercially available

Bacteria and media

Pseudomonas ovalis IAM-1002 was used throughout this study Cells were grown at 30° C in a chemically defined medium the composition of which was, in grams per liter glucose, 2 0, $(NH_4)_2SO_4$, 2 0; $Na_2HPO_4 \cdot 12\ H_2O$, 1 64, KH_2PO_4, 0 15, $MgSO_4$ 7 H_2O, 0 1, $CaCl_2$, 0 002, and $MnSO_4$, 0.007, or $FeSO_4$ 7 H_2O, 0.01. The Fe or Mn content of this medium was minimized by omitting either the $FeSO_4$ 7 H_2O or the $MnSO_4$ In the case of large scale cultivation, a medium was used the composition of which, in grams per liter, was as follows glucose, 4, $(NH_4)_2SO_4$, 4, $Na_2HPO_4 \cdot 12\ H_2O$, 16 4, KH_2PO_4, 1 5, $MgSO_4$ 7 H_2O, 0 2, $CaCl_2$, 0.002, and $FeSO_4$ 7 H_2O, 0 01

Enzyme assay

Superoxide dismutase activity was assayed by the method of McCord and Fridovich [1] For the assay of the crude preparation, the assay mixture contained 5 10^{-4} M cyanate to inhibit cytochrome *c* peroxidase and oxidase Calculation of the enzyme unit was according to the method of Asada et al. [16] For the case of inhibition studies, the enzyme activity was estimated by

the method of Asada et al [16] using the assay system of Nishikimi et al [17]

Determination of protein

Protein was determined by the micro-biuret method of Itzhaki and Gill [18], using bovine serum albumin as standard

Metal analysis

Iron was determined by the method of Massey [19] as the ferrous *o*-phenanthroline complex and by atomic absorption spectrometry using a Nippon Jarrell-Ash atomic absorption spectrometer with a Varian Techtron Model 63 carbon rod atomizer Zinc, copper, and manganese were assayed by atomic absorption spectrometry

Determination of molecular weight

The molecular weight of the enzyme was estimated by gel filtration and by sedimentation equilibrium centrifugation Gel filtration was performed with a column of Sephadex G-100 (3 5 cm × 70 cm) which was eluted with 50 mM Tris HCl buffer (pH 7 8) containing 0 1 M KCl The column was calibrated with the following standards bovine serum albumin, ovalbumin, chymotrypsinogen, and myoglobin Sedimentation velocity and equilibrium experiments were performed at 4° C in a Hitachi UCA-1A analytical ultracentrifuge The molecular weight of the enzyme was estimated by meniscus depletion method as developed by Yphantis [20] The partial specific volume was calculated from the amino acid composition [21]

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed with 6% gel (pH 8 9) at 5° C according to the method of Davis [22] Superoxide dismutase activity on polyacrylamide gel was located by the photochemical method of Beauchamp and Fridovich [23] Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [24]

Amino acid composition

About 300 μg of the superoxide dismutase, denaturated with trichloroacetic acid, was dialyzed against water and hydrolyzed in 6 M HCl for 24, 48, or 72 h at 110° C Amino acids were subsequently determined with a Nihondenshi automatic amino acid analyzer JLC-6AH using a single column Tryptophan was determined by the photometrical method of Edelhoch [25] Half-cystine was determined as cysteic acid after treatment with performic acid by the method of Hirs [26]

Sulfhydryl content

Sulfhydryl content of the enzyme was measured by reaction with Nbs_2 as described by Ellman [27]

CD spectrum

CD spectrum was recorded on a Jasco J-20 automatic recording spectropo-

larimeter In the calculation of the mean residue ellipticity $[\theta]$, the mean residue weight was taken as 110 6

Crystallization

Crystallization of the iron-containing superoxide dismutase was accomplished by the method of Suzuki and Estabrook (unpublished) The purified enzyme solution (34 mg/ml) was poured into a glass tube (0 5 cm diameter), one end of which was covered with dialysis tubing Then the covered end of the glass tube was immersed in 55% saturation of $(NH_4)_2SO_4$ solution at pH 4 5, and after the tube had been allowed to stand for about 1 week at 2°C under reduced pressure, the crystalline enzyme appeared

Results

Superoxide dismutase in the crude extracts from Pseudomonas ovalis

The crude extracts from *Ps ovalis* were subjected to polyacrylamide gel electrophoresis and to staining of the enzymatic activity The activity zones were varied by the metal composition of the chemically defined medium described in Materials and Methods Fig 1a shows the activity zone with the crude extracts from *Ps ovalis* grown in Fe-supplemented medium (R_F 0 79) Fig 1b shows the three activity zones with the crude extracts from *Ps ovalis* grown in Mn-supplemented medium. A major activity zone (R_F 0 71) and other minor activity zones (R_F 0 75, 0 79) were observed (Fig 1b) The activity zone of R_F 0 79 coincided with the activity zone of the purified

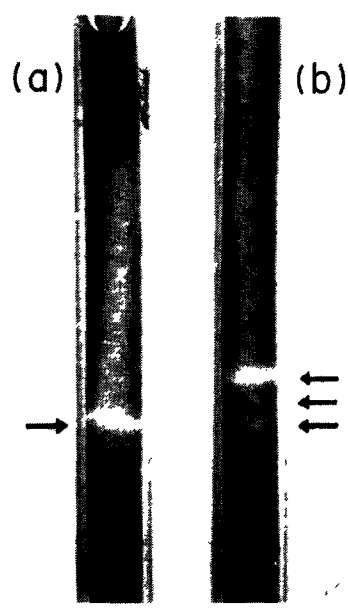


Fig 1 Polyacrylamide gel electrophoresis of crude extracts from *Ps ovalis* 200 μg of the crude extracts were applied to the gel and, after electrophoresis, superoxide dismutase activity was located [23] Achromatic zones denoted by arrows indicate the enzymatic activity (a) crude extracts from *Ps ovalis* grown in Fe-supplemented medium (b) crude extracts from *Ps ovalis* grown in Mn-supplemented medium

iron-containing superoxide dismutase described below, but the other two activity zones were not identified

Purification of iron-containing superoxide dismutase

All the purification procedures were carried out at 0—4° C

(a) Preparation of crude extracts Cells of *Ps ovalis* were harvested by centrifugation in the late log phase Cell extracts were prepared by sonic disruption (10 min) of frozen cells (1 8 Kg) suspended in 9 l of 10 mM potassium phosphate buffer (pH 7 4) The sonicate was centrifuged for 1 h at 15 000 × *g*

(b) DEAE-cellulose batch The supernatant was added to DEAE-cellulose batch (130 g) which had previously been equilibrated with 10 mM potassium phosphate buffer (pH 7 4) The superoxide dismutase was not absorbed under these conditions

(c) $(NH_4)_2SO_4$ fractionation The enzyme solution was brought to 58% $(NH_4)_2SO_4$ saturation The precipitate formed was removed by centrifugation, and the supernatant solution was then brought to 90% $(NH_4)_2SO_4$ saturation The resulting precipitate was recovered and dialyzed for 48 h against 5 mM potassium phosphate buffer (pH 7.8) containing 1 mM β-mercaptoethanol The buffer was exchanged four times every 12 h

(d) First DE-32 column chromatography The dialyzed solution was applied to a column of DE-32 (5 cm × 35 cm), previously equilibrated with 5 mM potassium phosphate buffer (pH 7 8) After the column was washed with the same buffer and 17 mM potassium phosphate buffer (pH 7 8) successively, superoxide dismutase activity was eluted from the column with 31 mM potassium phosphate buffer (pH 7 8) The fractions containing superoxide dismutase activity were pooled and dialyzed against 2 mM potassium phosphate buffer (pH 7 8)

(e) Second DE-32 column chromatography The dialyzed solution was again applied to a column of DE-32 (1 5 cm × 15 cm), previously equilibrated with the dialysis buffer Superoxide dismutase activity was eluted from the column with 23 mM potassium phosphate buffer (pH 7.8) The fractions containing

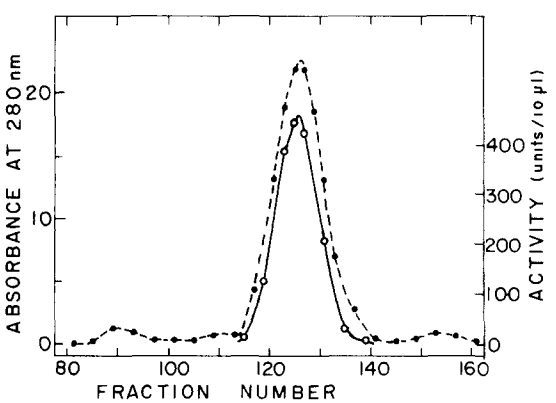


Fig 2 Elution profile of superoxide dismutase from Sephadex G-100 column Details are given in the text ●- - - - -●, absorbance at 280 nm, ———○, activity of the enzyme

TABLE I

PURIFICATION OF IRON-CONTAINING SUPEROXIDE DISMUTASE FROM *PSEUDOMONAS OVALIS*

Purification step	Total protein (mg)	Total units of enzyme (10^{-3} X units)	Specific activity (units/mg)	Recovery (%)
Crude extracts	171 400	2 948	17 2	
DEAE-cellulose batch	133 630	2 673	20 1	91
58—90% $(NH_4)_2SO_4$	10 500	1 922	183	60
1st DE-32	686	1 175	1713	40
2nd DE-32	459	1 055	2 299	36
Sephadex G-100	316	1 036	3 277	35
Hydroxyapatite	220	880	4 000	30

superoxide dismutase activity were pooled and concentrated by ultrafiltration.

(f) Sephadex G-100 gel filtration The concentrated solution was applied to a column of Sephadex G-100 (3 cm X 70 cm), previously equilibrated with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 M KCl, and eluted with the same buffer Fig. 2 shows the elution profile of the enzyme from the Sephadex G-100 column. The active fractions were pooled and dialyzed against 2 mM potassium phosphate buffer (pH 6.1).

(g) Hydroxyapatite column chromatography The dialyzed solution was then applied to a column of hydroxyapatite (1.5 cm X 25 cm), previously equilibrated with the dialysis buffer The column was washed with the same buffer, and superoxide dismutase activity was eluted with a linear gradient (2—150 mM) of this buffer, as a single peak which was congruent with a peak of 280 nm absorbance The results of the purification procedures are summarized in Table I. The preparation in the final step showed an overall 230-fold purification with a recovery of 30% of the original activity.

Criteria of purity

The homogeneity of the purified enzyme was examined by polyacrylamide gel electrophoresis and ultracentrifugation technique

Fig. 3 shows the electrophoretic patterns of the purified enzyme on polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate. A single band was observed in both cases, suggesting that the enzyme preparation was highly homogeneous. The protein band of the purified enzyme coincided with the zone of the enzymatic activity (Fig 3).

The purified enzyme was subjected to ultracentrifugation in 50 mM potassium phosphate buffer (pH 7.8) containing 0 1 M NaCl at 4° C. A single symmetrical boundary was observed (Fig. 4) The sedimentation coefficient in water at 20° C, extrapolated to zero protein concentration ($S^0_{20,w}$), was found to be 3.18 S from six ultracentrifuge runs at protein concentrations of 1, 2, 3, 4, 4 4, and 5 15 mg/ml

Crystallization

The enzyme from the final purification step was crystallized according to the method described in Materials and Methods The enzyme was precipitated as

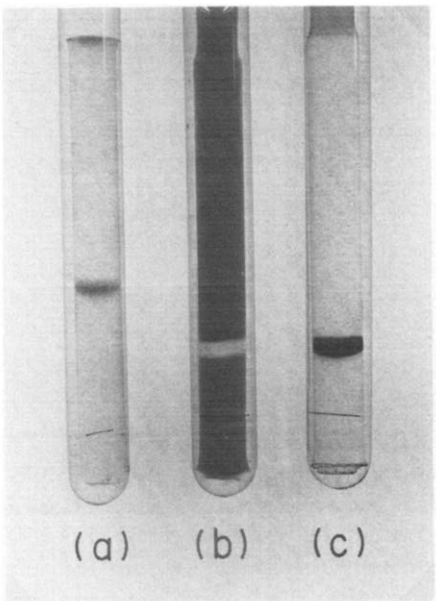


Fig 3 Polyacrylamide gel electrophoresis of purified superoxide dismutase with and without sodium dodecyl sulfate (a) The purified enzyme (7 μg of protein) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis The gel was stained for protein by Coomassie brilliant blue (b)(c) The purified enzymes (10 μg in (b) and 100 μg in (c)) were subjected to polyacrylamide gel electrophoresis at pH 8 9 The gel (c) was stained for protein by Amido black 10B and the achromatic zone indicates the enzymatic activity (b)

light-brown crystals after one week in the covered end of a glass tube (Fig. 5) The specific activity of the crystalline enzyme was about 3100 and its iron content was 1 1 gatoms per 40 000 g of the enzyme (Table II)

Molecular weight

Sedimentation equilibrium experiments were carried out by the meniscus depletion method of Yphantis [20]. When logarithms of fringe displacement

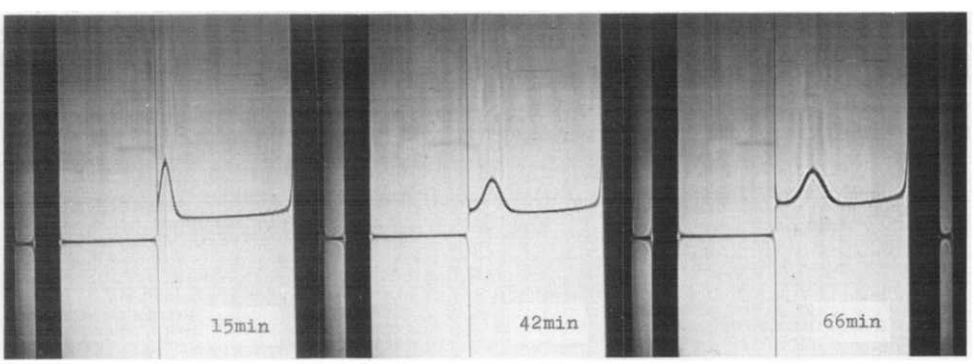


Fig 4 Ultracentrifugal patterns of purified superoxide dismutase The purified enzyme was used at a concentration of 5 15 mg per ml in 50 mM potassium phosphate buffer (pH 7 8) containing 0 1 M NaCl Direction of centrifugation is from left to right Photographs were taken at 15, 42 and 66 min after reaching 60 000 rev /min

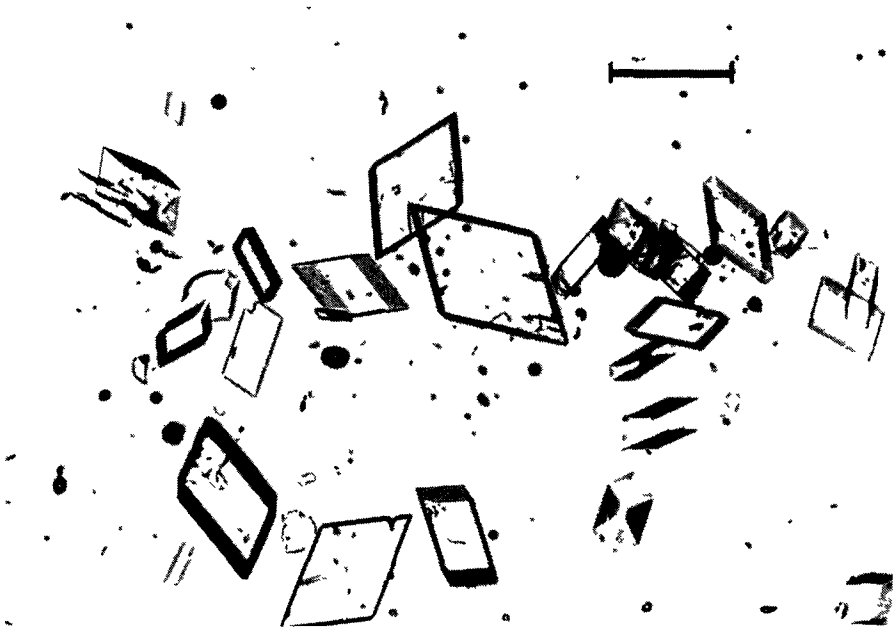


Fig 5 Microphotograph of crystalline superoxide dismutase from *Ps ovalis* Magnification X 160 Length of the scale-bar in the photograph is 0 1 mm

(*J*) were plotted against the square of the distance from the center of rotation (r^2), a straight line was obtained (Fig 6). From the slope of this line and an assumed partial specific volume of 0.727, which was calculated from amino acid composition (Table III), the molecular weight of the enzyme was estimated to be 40 800 ± 1960 (2 determinations)

The molecular weight determined by gel filtration was 40 000 ± 1500 (3 determinations) using a column of Sephadex G-100 (2 6 cm X 70 cm)

The molecular weight of the enzyme subunit was determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis Fig. 7 shows a plot of the logarithms of the molecular weights of several reference proteins versus their relative mobilities. The enzyme preparation migrated as a single band and the molecular weight of the enzyme subunit was estimated to be 19 500 in the

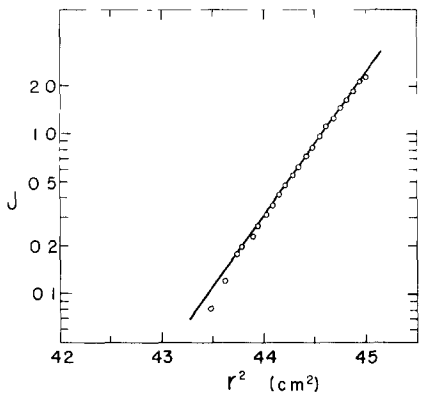
TABLE II

DETERMINATION OF IRON IN SUPEROXIDE DISMUTASE

The atom to mol ratio is calculated on the basis of a molecular weight of 40 000 Values are given as mean ± S D , with the number of determinations in parentheses

Sample	o Phenanthroline method* (gatoms/mol enzyme)	Atomic absorption (gatoms/mol enzyme)	Specific activity* (units/mg protein)
Purified enzyme	1 39 ± 0 013 (4)	1 44 ± 0 02 (2)	3930 ± 87 (5)
pH 3 9 treated enzyme	1 01 ± 0 04 (3)		3050 ± 320 (8)
Crystalline enzyme	1 13 ± 0 05 (4)		3080 ± 150 (12)

* Measured with different preparations



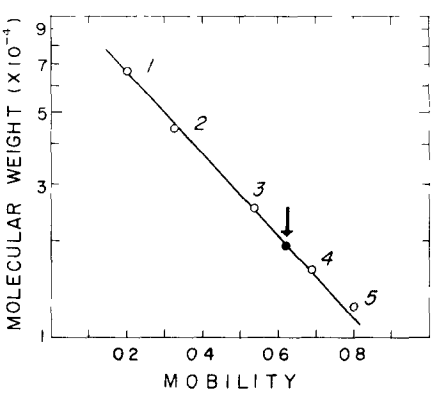


Fig 6 Determination of molecular weight by sedimentation equilibrium The purified superoxide dismutase at a concentration of 1 24 mg per ml in 0 1 M potassium phosphate buffer (pH 7 8) was equilibrated at a rotor speed of 29 500 rev /min The ultracentrifuge was equipped with interference optics, and fringe displacement (J) is plotted against the square of the distance from the center of rotation (r^2)

Fig 7 Determination of the subunit molecular weight of superoxide dismutase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis Samples of the purified superoxide dismutase and standards were denatured by 1% sodium dodecyl sulfate containing 1% β-mercaptoethanol and subjected to electrophoresis in polyacrylamide gel (10%) containing 0 1% sodium dodecyl sulfate The standard proteins used were as follows 1, bovine serum albumin, 2, ovalbumin, 3, chymotrypsinogen, 4, myoglobin, and 5, cytochrome *c* The arrow indicates the mobility of the superoxide dismutase

TABLE III

AMINO ACID COMPOSITION OF IRON-CONTAINING SUPEROXIDE DISMUTASE

The amino acid composition of *Pseudomonas* superoxide dismutase and comparison with the published data for *E coli*, *Photobacterium*, and *Plectonema* superoxide dismutases The number of residues were calculated for 40 000 *g* of *Pseudomonas* enzyme Values are given as residues per molecule of *Pseudomonas* enzyme and as mean of duplicate analysis ± S D

Amino acid	*Pseudomonas* superoxide dismutase	Nearest integer	*E coli* (6)	*Photobacterium* (15)	*Plectonema* (14)
Lysine	19 9 ± 0 1	20	20	20	24
Histidine	13 6 ± 0 5	14	11	12	10
Arginine	3 7 ± 0 2	4	8	4	3
Y [a]	1 4 ± 0 4	1			
Aspartic acid	35 6 ± 0 3	36	45	40	47
Threonine [b]	24 3 ± 0 3	24	26	26	20
Serine [b]	26 8 ± 0 7	27	20	15	18
Glutamic acid	32 3 ± 0 2	32	32	30	32
Proline	18 1 ± 0 9	18	18	17	20
Glycine	35 5 ± 0 8	36	32	25	29
Alanine	33 1 ± 0 6	33	53	40	48
Half-cystine	3 8 ± 0 1 [c]	4	2	—	2
Valine [d]	18 4 ± 0 2	18	22	16	21
Methionine	0 ±	0	0	6	3
Isoleucine [d]	10 8 ± 0 8	11	16	14	5
Leucine [d]	28 3 ± 0 5	28	29	27	34
Tyrosine	12 5 ± 0 4	13	13	11	10
Phenylalanine	21 4 ± 0 3	21	20	20	25
Tryptophan	18 2	18	8	12	11

[a] Unidentified amino acid residue This value was calculated from ninhydrin color of ornithine
[b] Based on extrapolation to zero time of hydrolysis
[c] Corrected for 90% recovery [26]
[d] Based on values obtained after 72 h of hydrolysis

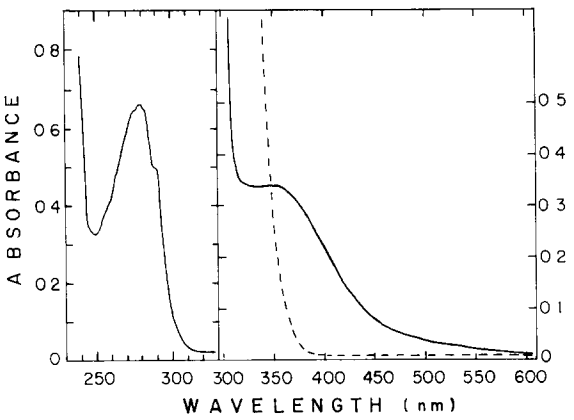


Fig 8 Absorption spectrum of superoxide dismutase from *Ps ovalis* The spectrum in the ultraviolet and visible regions were obtained with solutions containing 0 327 mg per ml and 4 87 mg per ml, respectively, of the enzyme in 50 mM potassium phosphate buffer (pH 7 8) The segmented line indicates the absorption spectrum of the enzyme after reduction with solid $Na_2S_2O_4$

presence of β-mercaptoethanol and 20 000 in its absence. These results indicate that the enzyme from *Ps ovalis* is composed of two subunits of equal size which are not covalently bridged.

Absorption spectrum

Fig 8 shows the optical absorption spectrum of the purified superoxide dismutase In the visible absorption spectrum, a broad absorption in the range from 320 to 600 nm was observed The molar extinction coefficient at 350 nm was 2920 ± 130 M^{-1} cm^{-1} (2 determinations) and at 280 nm it was 80 600 ± 1200 M^{-1} cm^{-1} (4 determinations) based on a molecular weight of 40 000. The visible absorption spectrum was bleached by addition of $Na_2S_2O_4$ to the enzyme solution (Fig 8), and the color was restored by subsequent auto-oxidation of the enzyme The enzymatic activity was not destroyed after this treatment

Metal analysis

Iron determinations of the superoxide dismutases by atomic absorption spectrometry and by spectrophotometry using *o*-phenanthroline are shown in Table II From these data, the purified enzyme contained 1 39 g-atoms of Fe per mol of enzyme However, the enzyme which had been dialyzed against 0 1 M sodium acetate buffer (pH 3.9) containing 1 mM EDTA for 3 days, followed by 2 days dialysis against 50 mM potassium phosphate buffer (pH 7 8), contained 1 01 gatoms of Fe per mol of enzyme The crystallized enzyme also contained 1 13 gatoms of Fe per mol of enzyme Specific activity of the enzymes decreased correspondingly with their iron content (Table II) The content of manganese, copper, and zinc was below detection, or not significant.

Acid-labile sulfide was not detected by the method of Fogo and Popowsky [28]

Amino acid analysis

Table III shows the amino acid composition of the superoxide dismutase A

molecular weight of 39 485 was calculated for the superoxide dismutase from this composition This value is in good agreement with the molecular weight estimated by the gel filtration and the ultracentrifugation

The amino acid composition of the *Ps ovalis* enzyme is apparently similar to the enzyme from *E coli*, *Plectonema boryanum*, and *Photobacterium sepia* (Table III). However, the *Ps ovalis* enzyme had a higher content of serine and a lower content of alanine compared with other enzymes

Amino acid residue, expressed as Y in Table III, was eluted at the same position as ornithine from the amino acid analyzer with two different buffer systems

Sulfhydryl content

Sulfhydryl content was measured using Nbs_2 The enzyme was denatured by addition of 6 M guanidine hydrochloride or 0 4% sodium dodecyl sulfate Excess of Nbs_2 was added and the change in absorbance at 412 nm was monitored In the absence of the denaturation reagents, the reaction was significantly slow and only 0 2 sulfhydryl group had reacted with the reagent after 40 min. In the presence of 6 M guanidine hydrochloride and 0 4% sodium dodecyl sulfate, 1 0 and 4 1 mol of sulfhydryl groups per mol of enzyme were allowed to react with the reagent (Fig 9) Thus the sulfhydryl groups of the enzyme were exposed and had reacted with the reagent only after denaturation The values obtained with Nbs_2 agree with a value of 3 8 half-cystine residues obtained by the amino acid analysis after performic acid oxidation (Table III) These results indicate the absence of any disulfide linkage in the enzyme molecule

Effect of various compounds on the superoxide dismutase

In Method (a), the effect of each of the various compounds in the reaction mixture was studied using the method of Nishikimi et al [17] instead of the xanthine-xanthine oxidase system In Method (b), the enzyme was treated with

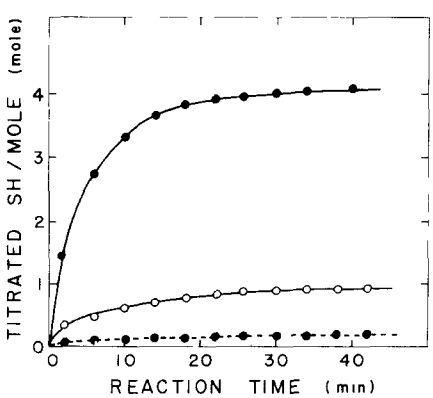


Fig 9 Reaction of native and denatured superoxide dismutase with DTNB The enzyme (0 85 mg) was allowed to react with DTNB (20-fold molar excess) in 50 mM potassium phosphate buffer (pH 7 8) containing 10^{-4} M EDTA The reaction was followed in terms of the absorbance change at 412 nm ●------●, native enzyme, ○———○, denatured enzyme by 6 M guanidine hydrochloride, ●———●, denatured enzyme by 0 4% sodium dodecyl sulfate

TABLE IV

EFFECT OF VARIOUS COMPOUNDS ON SUPEROXIDE DISMUTASE FROM *PSEUDOMONAS OVALIS*

In Method (a), the effect of each compound in the reaction mixture was studied using the assay method of Nishikimi et al [17] In Method (b), the enzyme was treated with each compound for 30 h followed its removal through dialysis and then the enzymatic activity was assayed by xanthine-xanthine oxidase system [1]

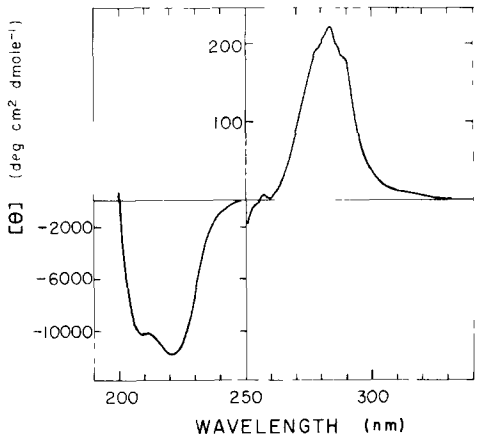
Compound	Remaining activity (%)
Method (a)	
None	100
NaN_3 1 mM	59
NaN_3 10 mM	23
o Phenanthroline 1 mM	117
8-Hydroxyquinoline-5-sulfonate 10 mM	119
α,α'-Dipyridyl 0 25 mM	83
N-Ethylmaleimide 10 mM	100
Method (b)	
None	100
o Phenanthroline 1 mM + β-mercapto-ethanol 4 mM	34
o-Phenanthroline 1 mM + $Na_2S_2O_4$ 1 mM	60
α,α'-Dipyridyl 1 mM + β-mercapto-ethanol 4 mM	85
EDTA 1 mM	100
8-Hydroxyquinoline-5-sulfonate 1 mM	96
1,2-Dihydroxybenzene-3,5-disulfonate 1 mM	100
NaN_3 1 mM	100
$HgCl_2$ 0 1 mM	100

each compound followed by its removal through dialysis and then the enzymatic activity was assayed by xanthine-xanthine oxidase system [1]

The results are summarized in Table IV The superoxide dismutase was insensitive to iron-chelating agents in the two assay methods, (a) and (b) The enzyme was partially inhibited by the presence of 1 mM NaN_3 in the reaction mixture (Method (a)). However the pretreatment of the enzyme with NaN_3 followed by removal of the reagent did not inhibit the enzymatic activity (Method (b)) Treatment of the enzyme with *o*-phenanthroline in the presence of the reducing agent inhibited the enzymatic activity (Method (b))

CD spectrum

CD spectra of the crystalline enzyme from 200 to 340 nm and from 300 to 600 nm are presented in Figs. 10 and 11 The CD profile of the enzyme in the 200—250 nm region exhibits a center at 221 nm and a shoulder near 208 nm (Fig 10) The mean residue ellipticity, $[\theta]$, at 222 nm was calculated to be 11 890 ± 370 (4 determinations). From this value, the enzyme has an α-helical content of 32%, using the approximation method of Chen and Yang [29] for estimation of the α-helical content The CD spectrum in the 250—340 nm region shows a positive band, and a major peak centered at 283 nm with shoulders around 276 and 288 nm The visible region shows a broad and low intensity band centered at 400 nm (Fig 11)



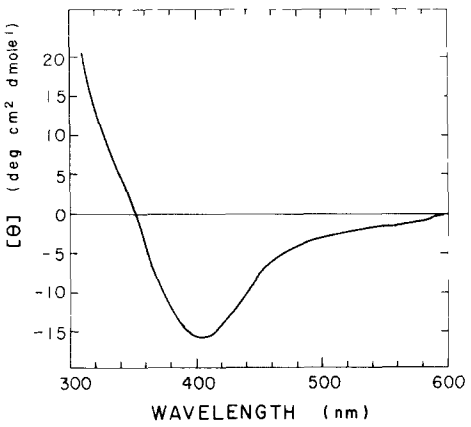


Fig 10 CD spectrum of crystalline superoxide dismutase from 200 nm to 340 nm The measurement of CD spectrum between 200 nm and 250 nm was carried out with a 0 5 mm light-path cell with 0 45 mg per ml of the enzyme in 50 mM potassium phosphate buffer (pH 7 8) In the region of 250 nm to 340 nm, a 5 mm light-path cell was used with 1 35 mg per ml of the enzyme in the same buffer

Fig 11 CD spectrum of crystalline superoxide dismutase from 300 nm to 600 nm The spectrum of the crystalline superoxide dismutase (13 5 mg/ml) in 50 mM potassium phosphate buffer (pH 7 8) was recorded with a 5 mm light-path cell

Discussion

Three electrophoretically distinct superoxide dismutases were observed in the crude extracts from *Ps ovalis* One of these (R_F 0 79) was purified as an iron-containing superoxide dismutase The other two isozymes (R_F 0 75, 0 71) were not isolated These two isozymes resemble manganese-superoxide dismutase since they appeared only in the crude extracts from *Ps ovalis* grown in Mn-supplemented medium and not in Fe-supplemented medium (Fig 1)

The purified preparation of the enzyme was crystallized as light-brown crystals (Fig 5) It contained 1.1 gatoms of Fe per mol of enzyme and had a specific activity of 3100 The shape of the crystals was similar to the crystalline copper, zinc-superoxide dismutase from spinach [10] Preparation of a large single crystal is now under way in this laboratory.

The molecular weight, subunit structure, and absorption spectrum of the iron-containing superoxide dismutase from *Ps ovalis* are similar, though not identical, to those of the *E. coli* enzyme [6], the *Photobacterium* enzyme [15], and the *Plectonema* enzyme [13,14] Similarity is also observed in amino acid composition of the enzymes from *Ps ovalis* and other microorganisms (Table III) However, a difference is observed in the serine, alanine, and half-cystine content of the enzymes. The amino acid residue, expressed as Y in Table III, was eluted at the same position as ornithine from the amino acid analyzer with two different buffer systems The amino acid residue (Y) seems to be ornithine Axcell and Geary [30] showed that an iron-sulfur protein, one component of a benzene-oxidizing system from a strain of *Pseudomonas*, also contained ornithine as its amino acid component Four half-cystine residues of

the *Pseudomonas* enzyme exist as four sulfhydryl groups which are exposed only after denaturation

The metal content of the *Pseudomonas* enzyme is significantly different from that of the *E coli* and *Plectonema* enzymes [6,13] Whereas the *E coli* and *Plectonema* enzymes contain 1 gatom of Fe per mol of enzyme, the *Pseudomonas* enzyme contains 1 4 gatoms of Fe per mol This is similar to the case of *Photobacterium leiognathi* and *sepia*, which contain respectively 1.61 and 1 43 gatoms of Fe per mol of enzyme Very recently, Asada et al [14] showed that *Plectonema* superoxide dismutase contained 2 gatoms of Fe per mol of enzyme The iron content of the *Pseudomonas* enzyme was partially decreased to 1 0 and 1 1 gatoms of Fe per mol of enzyme by the dialysis against 0.1 M sodium acetate buffer (pH 3.9) containing 1 mM EDTA and by the crystallization The specific activity of the enzyme also decreased corresponding to the iron content of the enzyme (Table II) Therefore, the iron removed was closely related to the superoxide dismutase activity It is probable that iron-containing superoxide dismutase from *Ps ovalis* contains 2 gatoms of Fe per mol of enzyme in the native state It seems that one of the two irons in the native enzyme molecule is easily removed but the remaining iron is bound to the enzyme molecule strongly

The activity of the *Pseudomonas* superoxide dismutase was inhibited by *o*-phenanthroline in the presence of the reducing agents followed by removal of the excess reagents. The *Pseudomonas* enzyme was insensitive to iron-chelating agents in aerobic condition (Table IV) These results are similar to those for the enzyme from *Photobacterium sepia* but not to those for the enzyme from *Plectonema boryanum* [15,14].

The CD spectrum of the crystalline enzyme from 200 to 250 nm showed a negative maximum at 221 nm with a shoulder near 208 nm, indicating that the enzyme contained a certain ordered structure such as α-helix and β-structure The negative CD band at 400 nm may be attributed to the asymmetric configuration of the iron-ligand complex of the enzyme The value obtained for $[\theta]_{222}$ showed the α-helical content of the enzyme to be 32% The CD spectrum of copper, zinc-superoxide dismutase from bovine erythrocyte in the wavelength region of 200-250 nm was very different from the iron-containing superoxide dismutase from *Ps ovalis* [31,32] (Fig 10) Bannister et al indicated that the copper, zinc-superoxide dismutase from bovine had a low α-helical content (less that 10%) and consisted mainly of unordered structure [33] Therefore, the secondary structures of these two enzymes must be extremely different from each other

Acknowledgements

The author wishes to express his gratitude to Professor K Suzuki and Mr D Ohmori for many their helpful suggestions and encouragement. He also wishes to thank Dr I. Muramatsu and Mr Y. Motogi of Rikkyo (St Paul's) University for the amino acid analysis, Dr. K. Nakano and Dr. T Takada, also of Rikkyo, for the atomic absorption spectrometry, and Dr T. Ishimori, Dr K Mizumachi, and Mr. S Ujita, also of Rikkyo, for the CD spectrum

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