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A MANGANESE-CONTAINING SUPEROXIDE DISMUTASE FROM *PARACOCCUS DENITRIFICANS*

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Summary

A cyanide-insensitive superoxide dismutase (superoxide: superoxide dismutase, EC 1.15.1.1) has been isolated from *Paracoccus denitrificans*, purified to homogeneity and characterized. It is a soluble, manganese-containing protein with an apparent molecular weight of 41 500 ± 1000. It is composed of two identical subunits (M_r 23 500) not bound by disulfide linkage. It's isoelectric point is 4.5. The amino acid composition shows strong similarities with other dimeric procaryotic and with tetrameric mitochondrial Mn-superoxide dismutases. The fully active enzyme contained from 1.34 to 2 gatom Mn/mol enzyme.

Introduction

Superoxide dismutases (superoxide: superoxide dismutase, EC 1.15.1.1) catalyse the dismutation of the superoxide radical formed by monoelectronic reduction of O_2 and constitute, for the cell, a means of defence against oxygen toxicity. They are very wide-spread in living cells, and are classified into three groups according to the nature of the metal present at the catalytic site which can be either Cu, Fe or Mn. While Cu-containing superoxide dismutase is typically a eucaryotic enzyme present in the cytoplasm of eucaryotic cells, in procaryotes superoxide dismutase activity is due to Fe- or/and Mn-containing enzymes [1,2]. Fe-containing superoxide dismutase is found in anaerobic, in green and in purple sulfur bacteria and seems to be the superoxide dismutase which evolved first. The Mn-containing enzyme is present in procaryotes which evolved later, such as blue-green algae, purple non-sulfur and aerobic bacteria

[2]. Some procaryotes contain both Fe- and Mn-superoxide dismutase [3]. Mn-superoxide dismutase is also present in protozoa, in red algae and in the mitochondria of higher eucaryotes [1,2].

Paracoccus denitrificans is shown in this report to contain two superoxide dismutases. One of them is cyanide-inhibited, a characteristic feature of eucaryotic Cu-containing enzyme. The presence of a Cu-containing superoxide dismutase in *P. denitrificans* has been demonstrated [4] by the NMR assay of Rigo et al. [5], based on the increased nuclear magnetic relaxation of ^{19}F by dismutase and the competitive reversal effect of CN^- in the case of Cu-containing superoxide dismutase. The second superoxide dismutase is cyanide-insensitive. This paper describes the purification and some of the physicochemical properties of that latter enzyme. This superoxide dismutase is of the manganese type; it is a homodimer of 41 500 molecular weight.

Materials and Methods

Materials. Xanthine, nitro-blue tetrazolium, bovine serum albumin, soybean trypsin inhibitor, were obtained from Sigma Chemical Co, St. Louis, MO, U.S.A.: xanthine oxidase and cytochrome *c* from Boehringer, Mannheim, F.R.G.: riboflavin from Hoffman-La Roche, Basel, Switzerland; methionine, Coomassie brillant blue G250 and ovalbumin from Serva, Heidelberg, F.R.G.: CM-52 and DE-52 cellulose from Whatman, Maidstone, U.K.: Sephadex G-75 and electrophoresis calibration kit of standard proteins from Pharmacia, Uppsala, Sweden: ampholines from L.K.B., Bromma, Sweden: acrylamide and *N-N'*-methylene-bisacrylamide from Eastman Kodak Co, Rochester, NY, U.S.A.: Coomassie brillant blue R250 from Merck, Darmstadt, F.R.G.: Chelex 100 from Bio-Rad Lab., Richmond, CA, U.S.A.: KO_2 from Pierce Inorganics, Denvers, MA, U.S.A.

Methods. P. denitrificans, strain 381 DSM 65, was a gift from Professor H.G. Schlegel, Institute of Microbiology, University of Göttingen, F.R.G. It was grown at 28°C in a salt medium described by Burnell et al. [6] either aerobically or anaerobically with nitrate as terminal acceptor. Succinate was used as carbon source. For some experiments the bacteria were grown autotrophically on $O_2 + H_2 + CO_2$ (2 : 7 : 1, by vol.). Cultures were carried out in Erlenmeyer flasks of 10 l or in 15 l fermenters (New Brunswick, NY, U.S.A.). Large scale cultures in 300 l, fermenter were done in the Laboratoire de Chimie Bactérienne (C.N.R.S., Marseille, France). The cells were harvested by centrifugation at 4°C, washed twice with distilled water and stored frozen at —20°C until needed.

The cells were disrupted by sonication, in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) in a Branson sonifier at maximum output (45 s × 4 with 30 s intervals in an iced bath). The suspension was centrifuged at 10 000 × *g* for 10 min to remove intact cells and cell debris. The pellet was treated again by sonication and centrifuged at low speed. The resulting pellet was discarded. The pooled supernatant fluids were centrifuged at 200 000 × *g* for 2 h and the membrane pellet discarded.

Superoxide dismutase assay. Superoxide dismutase was assayed according to Beauchamp and Fridovich [7], using the photochemical system, riboflavin and

methionine with light, to produce $O_2^{\cdot}$, and nitro-blue tetrazolium as superoxide scavenger. Absorbance was read at 560 nm [7] on a PMQ II Zeiss spectrophotometer (Jena, G.D.R.), after the samples had been illuminated for 5 min with a 13 W fluorescent tube. Samples were 0.5 cm far from the tube and absorption at 560 nm of assay reagents without enzyme was about 0.350, after 5 min of illumination at 23°C. 1 unit of superoxide dismutase activity was defined as the amount of enzyme required for a 50% inhibition of A_{560} of nitro-blue tetrazolium in the conditions described [7]. The assay system with KO_2 as $O_2^{\cdot}$ source [8] was used to determine activity in crude cellular extracts.

Proteins. They were determined according to Bradford [9] using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. Gels and buffers were made according to Laemmli [10], omitting stacking gels. For electrophoresis on non-denaturing gels, the procedure of Laemmli was modified as follows: sodium dodecyl sulfate (SDS) and β-mercaptoethanol were omitted from gels and electrode buffer. Samples were applied on to the gels in 20 mM potassium phosphate, pH 7.8, containing one crystal of sucrose and bromophenol blue as tracking dye.

Gels were stained for proteins using Coomassie brillant blue R250. In parallel, superoxide dismutase activity was revealed on another set of gels, from the inhibition of the formation of formazan blue [7]; gels were first soaked in a nitro-blue tetrazolium solution (2 mg/ml) for 20 min in the dark, then rinsed and soaked in the dark in 50 mM potassium phosphate buffer, pH 7.8, containing riboflavin (1.5 mg/ml) and *N*, *N*, *N'*, *N'*-tetramethylethylenediamine (Temed). Then the gels were rinsed and exposed to day light. Achromatic bands appeared where superoxide dismutases were located.

Molecular weight determination. A. Gel filtration. The molecular weights of the purified enzymes were estimated by gel filtration on Sephadex G-75 or on Ultrogel AcA-54 according to Whitaker [11]. A sample of pure enzyme (0.3 mg in 0.1 ml elution buffer) was loaded on a Sephadex G-75 column (1 × 55 cm) or on an Ultrogel AcA-54 column (1 × 50 cm) equilibrated with 100 mM NaCl/20 mM potassium phosphate buffer, pH 7.8, and eluted with the same buffer. The elution volume, V, was determined by localization of either superoxide dismutase activity, or protein peaks assayed according to Bradford [9]. Each protein used as standard was run separately on the column, using for each run Dextran blue for determination of the bed volume V_0 (which remained constant for every run): horse-heart cytochrome *c* (12 500); soybean trypsin inhibitor (20 100); ovalbumin (43 000); bovine serum albumin (67 000), were used as protein standards.

B. Subunits molecular weight determination by gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Fairbanks et al. [12] using 10% (w/v) polyacrylamide gels. Samples of pure enzyme were precipitated from their buffer by 10% (w/v) trichloracetic acid, redissolved in 40 µl 0.1 M Tris-HCl buffer, pH 8.0/1% (w/v) SDS/1% (v/v) β-mercaptoethanol/4, 8 or 12 M urea, and heated at 100°C for 5 min in capped vials before application to gels. A calibration kit of standard proteins was submittted to the same treatment. These molecular weight markers were phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor

(20 100), bovine α-lactalbumin (14 400). Protein samples were applied to the gel with bromophenol blue as tracking dye.

Optical spectrum. Optical spectrum was recorded on a Perkin-Elmer 557 double wavelength double-beam spectrophotometer.

Isoelectric focusing. A. On column. The isoelectric point was determined on an isoelectric focusing LKB column (110 ml), on a pH gradient made with ampholines (pH 3.5—10) and a sucrose gradient. The focusing was performed during 48 h and 1 ml fractions were then collected and assayed for superoxide dismutase activity [7].

B. On slab gel. Isoelectric focusing was also performed on slab gel. The gel was composed of 17.5 g urea (8.38 M), 1.16 ml 1% (v/v) Temed (0.03% v/v), 5.25 ml 33% acrylamide (w/v) and 0.9% *N*, *N'*-methylene-bisacrylamide (w/v) solution (4.9% acrylamide (w/v), 1.75 ml ampholines, pH 4.0—6.0 (4.9% v/v), 13.4 ml H_2O and 1.2 ml 40 mg/l riboflavin (1.35 μg/ml). Polymerization was effected by exposure to ultraviolet light during 3 h. After a prefocusing of 1 h, the samples were focused during 2 h. Proteins were revealed by Coomassie brillant blue R250 (staining solution: 0.1% Coomassie brillant blue/50% trichloroacetic acid in H_2O; destaining solution: 25% ethanol/8% acetic acid in H_2O). Activity was revealed by the method of Beauchamp and Fridovich for electrophoresis gels [7].

Metal analysis. Metal analyses were made by atomic absorption spectrophotometry by the 'Service Central d'Analyse' of the 'Centre National de la Recherche Scientifique' (Solaize, France).

Amino acid analysis. Samples of the enzyme were dialysed against water, dessicated and then hydrolysed under vacuum in 6 M HCl at 110°C for 18 and 48 h. They were then subjected to amino acid analysis in a Rank Hilger autoanalyser. Cysteine and methionine were determined as cysteic acid and methionine sulfone after performate oxidation of the protein [13]. The tryptophan content was estimated from the ultraviolet absorption spectrum according to Edelhoch [14].

Electron paramagnetic resonance. EPR spectra were recorded at liquid N_2 temperature, on a Varian E104 model.

Results

Occurrence and localization of superoxide dismutase in P. denitrificans

The soluble extract obtained by centrifugation (200 000 × *g* for 2 h) of a sonicated cell homogenate (see 'Methods') was submitted to electrophoresis on 6, 8, 10 and 12% polyacrylamide gels. One band having a superoxide dismutase activity insensitive to cyanide was detected on 6 and 8% gels. On 10 and 12% gels, this activity was also detected, but a second one appeared which was inhibited by 2 mM KCN (Fig. 1). These two superoxide dismutase activities were found in extracts from cells grown either aerobically or anaerobically in the presence of nitrate, with glucose or succinate as carbon source and in cells grown autotrophically with hydrogen as energy donor. These results indicate that two superoxide dismutases are contained in the soluble fraction of cells of *P. denitrificans*, independently from the growth condition.

Attempts were made to determine whether any superoxide dismutase

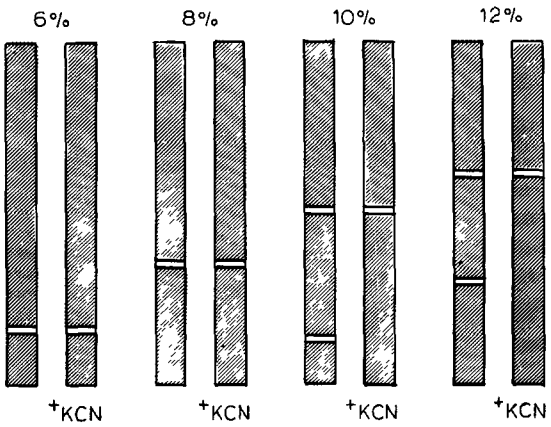


Fig. 1. Detection by polyacrylamide gel electrophoresis of the two superoxide dismutase activities present in the soluble cellular fraction of *P. denitrificans*. Effect of gel concentration on their migration. Gels were stained for activity [7], with or without 2 mM KCN.

activity was contained in the membrane fraction of the cell. In order to avoid interference of crude extracts with the assay system as much as possible, KO_2 was used as O_2^- source and nitro-blue tetrazolium as O_2^- scavenger [8]. However, in the control tubes where homogenates of sonicated cells were incubated in the presence of nitro-blue tetrazolium alone, without added KO_2, there was an increase in absorbance, at 560 nm, indicative of O_2^- production. This superoxide ion production by the cytoplasmic membranes of *P. denitrificans* in the presence of electron donors such as NADH or succinate, has subsequently been demonstrated by Henry and Vignais [15]. It seemed, therefore doubtful that the presence of superoxide dismutase could really be established in a fraction able to produce O_2^- and the study was limited to the enzymes present in the cytoplasm.

Purification of cyanide-insensitive superoxide dismutase

The cyanide-insensitive superoxide dismutase was isolated from cells grown anaerobically on succinate as carbon source and nitrate as electron acceptor. A typical experiment is described and summarised in Table I.

To the supernatant fluid (115 ml) obtained by centrifugation (200 000 × *g* for 2 h) of a sonicated cell homogenate, solid $(NH_4)_2SO_4$ was added up to 60% saturation. The slurry was allowed to stand overnight at 4°C. The precipitated proteins were removed by centrifugation (23 000 × *g* for 30 min). The supernatant was dialysed extensively against a 20 mM potassium phosphate buffer, pH 7.8, so as to reduce the salt concentration by a factor of at least 10^4, and assayed for superoxide dismutate activity. Practically all the activity was found in the supernatant, less than 0.3% of the total activity remained in the proteins precipitated at 60% saturation of $(NH_4)_2SO_4$. The dialysed supernatant (60 mg protein) was applied on to a DE-52 cellulose column (20 ml) equilibrated in 20 mM potassium phosphate buffer, pH 7.8. The column was washed with 100 ml buffer, then with 100 ml of the same buffer containing 60 mM NaCl, which eluted the cyanide-insensitive superoxide dismutase.

TABLE I

PURIFICATION OF THE CYANIDE-INSENSITIVE SUPEROXIDE DISMUTASE OF *P. DENITRIFICANS*

All supernatant fluids and eluates were extensively dialysed before superoxide dismutase assays. The activity of the supernatant after purification by 60% $(NH_4)_2SO_4$ precipitation was taken as the starting reference value.

Fraction	Volume (ml)	Proteins (mg/ml)	Total proteins (mg)	Activity (Units/ml)	Total activity (Units)	Specific activity (Units/ml protein)	Recovery (%)
Soluble extract	115	5.125	589	—	—	—	
60% $(NH_4)_2SO_4$ supernatant	168	0.355	59.6	215	36 120	606	100
First DE-52 cellulose column	64.5	0.105	12.7	244	15 700	1 240	43.5
Second DE-52 cellulose column	6.5	0.220	1.43	2 000	13 000	4 500	37

The column was further washed with 140 mM NaCl/20 mM potassium phosphate buffer, pH 7.8; then a linear gradient from 140—220 mM NaCl in 20 mM potassium phosphate, pH 7.8, could elute a second fraction with superoxide dismutase activity. Analysis by gel electrophoresis showed that it was the cyanide-sensitive enzyme (Fig. 2). Complete purification of both enzymes needs further steps. The fractions containing the cyanide-insensitive superoxide dismutase activity were pooled and dialysed against 20 mM potassium phosphate buffer, pH 7.8. The dialysed fraction (13 mg protein) was then applied on to a DE-52 cellulose column (5 ml) equilibrated in the same buffer. The superoxide dismutase activity was eluted as a single peak by 50 ml of a linear gradient of NaCl (0—100 mM NaCl) in the same buffer (Fig. 3). The tubes 78—92 analysed by gel electrophoresis in non-denaturing conditions contained a single protein band corresponding to a band with superoxide dismutase activity (Fig. 3). A single protein band was also obtained by gel electrophoresis in the presence of SDS. A summary of the purification procedure is given in Table I. The total activity of the soluble extract was always lower than the total activity after precipitation by 60% $(NH_4)_2SO_4$. This may be due to a production of superoxide anions by minute membrane fragments still present in crude extracts and artefactually minimising the real superoxide dismutase activity. To avoid such possible interferences in the evaluation of the degrees of purification and in the recoveries, the activity found in the 60% $(NH_4)_2SO_4$ supernatant was taken as the starting reference value.

Molecular weight determination

The molecular weight of the native enzyme. This was determined by gel filtration according to Whitaker [11], using a Sephadex G-75 column (1 X 55 cm) equilibrated with 100 mM NaCl in 20 mM potassium phosphate buffer, pH 7.8. It was estimated to be 41 500 ± 1000.

The subunit molecular weight. This was determined by gel electrophoresis in the presence of SDS, and urea with and without β-mercaptoethanol, according to Fairbanks et al. [12]. The cyanide-insensitive superoxide dismutase gave a single band of protein after dissociation by SDS, with or without β-mercaptoethanol, corresponding to a molecular weight of 23 500 ± 1000. These results imply that the *P. denitrificans* cyanide-insensitive superoxide dismutase is composed of two subunits of equal size which are not associated by disulfide bridge.

Metal content

The metal content (Mn, Fe, Cu and Zn) was estimated by absorption spectrometry. Metal determinations were made on samples containing from 200—600 μg pure protein/ml. Analysis was carried out either directly on the eluates from the second DE-52 cellulose column or after these eluates had been dialysed against 2 mM potassium phosphate buffer, pH 7.8, previously filtered on Chelex 100 resin. In some cases the superoxide dismutase solution itself was filtered on Chelex 100. The metal content of a dozen superoxide dismutase preparations is given in Table II.

The two first superoxide dismutase preparations had a very high Fe content; this led to the erroneous conclusion that the cyanide-insensitive superoxide

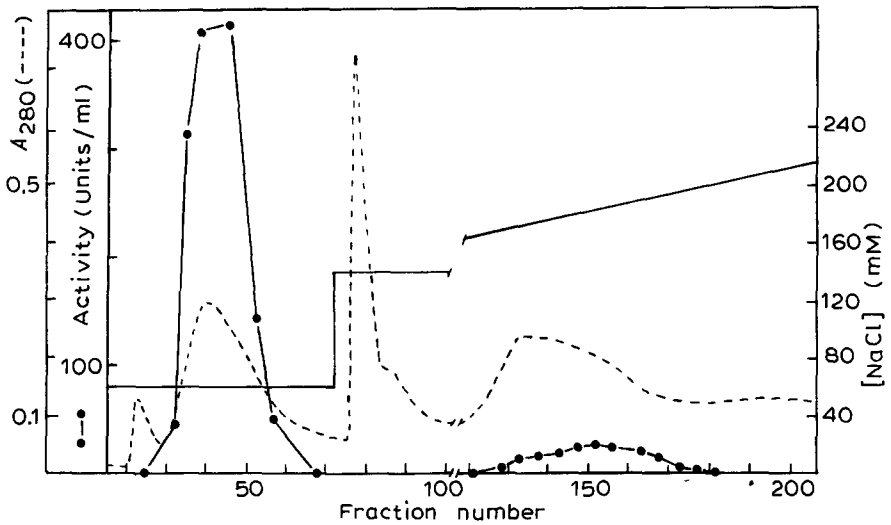


Fig. 2. Separation of the two superoxide dismutase activities by DEAE-cellulose column chromatography. Column volume: 20 ml. Fractions of 2 ml were collected.

dismutase from *P. denitrificans* contained Fe [4]. However, EPR spectra of these preparations, which contained a single protein, later showed a non-characteristic broad feature around $g = 4.3$ in place of the three sharp lines

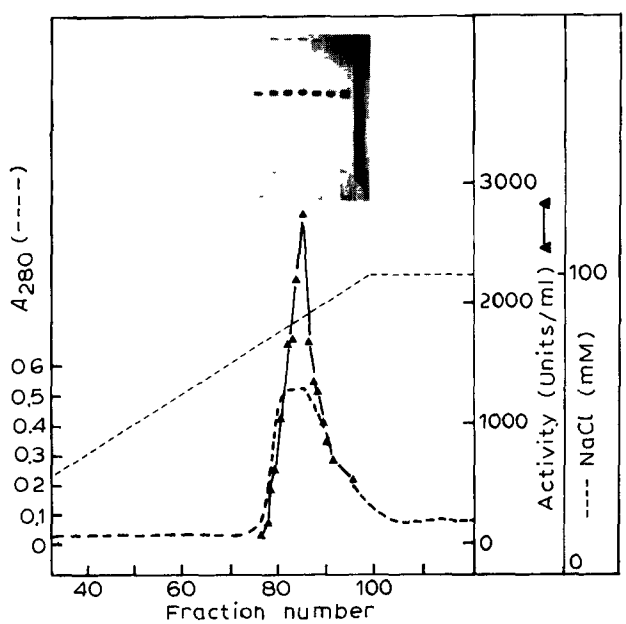


Fig. 3. Purification of the cyanide-insensitive superoxide dismutase from *P. denitrificans* by DEAE-cellulose column chromatography. 0.5 ml fractions were collected. Column volume 5 ml. Analysis of fractions 78–92 by electrophoresis on 12% polyacrylamide gels, stained for activity and proteins, is illustrated in the insert.

TABLE II

METAL CONTENT OF PURIFIED CYANIDE-INSENSITIVE SUPEROXIDE DISMUTASE FROM *P. DENITRIFICANS*

Samples 1—6 were column eluates analysed directly; samples 7—9 were column eluates dialysed against 2 mM potassium buffer, pH 7.8, which had been filtered on Chelex 100; samples 10—12 were samples 7—9, respectively, after filtration through Chelex 100.

Sample	gatom/mol			
	Mn	Fe	Cu	Zn
1	0.44	2.09	0.09	0.06
2	0.46	1.11	0.25	0.46
3	1.88	0.10	0.24	0.15
4	1.42	0.15	0.02	0.07
5	1.30	0.14	0.02	0.09
6	0.34	1.01	0.04	0.20
7	0.80	0.53	0.04	0.56
8	0.41	0.66	0.13	0.84
9	1.34	0.13	0.03	0.42
10	0.66	0.48	0.04	0.75
11	0.24	0.65	0.12	0.97
12	1.13	0.12	0.03	0.18

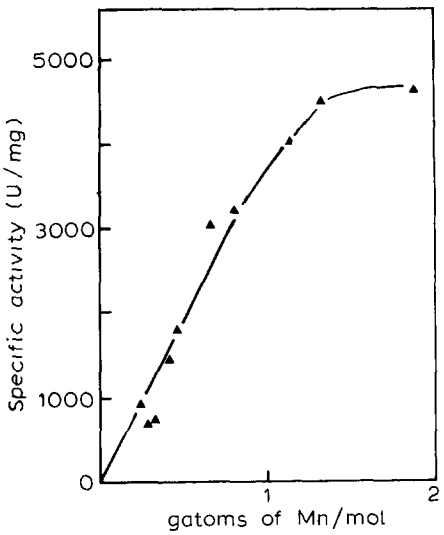
usually observed for a fully active Fe-containing superoxide dismutase [16]. Such a broad line at $g = 4.3$ is known to be produced by Fe unspecifically bound to proteins. Further analysis of other preparations revealed that the amount of Fe and Mn could vary in a wide range. It was noted that the higher the manganese content, the higher the activity. A direct relationship could even be established between Mn content and activity (Fig. 4) independent from the nature of the other metals, such as Fe or Zn, present as contaminants. The curve of Fig. 4 showed a linear relationship up to about 1 gatom Mn/mol enzyme and reached a plateau at about 1.88 gatom Mn/mol, the specific activity remained nearly constant from 1.34 gatom Mn/mol. These results demonstrate that Mn is required for activity and that the cyanide-insensitive superoxide dismutase from *P. denitrificans* is a Mn-containing one. The results suggest that the native and fully-active enzyme contains from 1 to 2 gatom Mn/mol.

Isoelectric point determination

The isoelectric point of the Mn-containing superoxide dismutase was determined by isoelectric focusing on a 110 ml column with an ampholine gradient from pH 3.5 to 10. Focusing was performed during 48 h and the column was then collected as 1 ml fractions. Superoxide dismutase activity was assayed on each fraction. The enzyme focused at pH 4.5 (Fig. 5).

The isoelectric point was also determined by focusing on polyacrylamide slab gel, with ampholines from pH 4.0 to 6.5. This technique gave an isoelectric point of 4.7.

The enzyme activity was directly revealed on the gels by the technique of Beauchamp and Fridovich [7], without treatment with buffer at pH 7.8. This indicates that Mn-superoxide dismutase is, at least in part, still active in 8.38 M



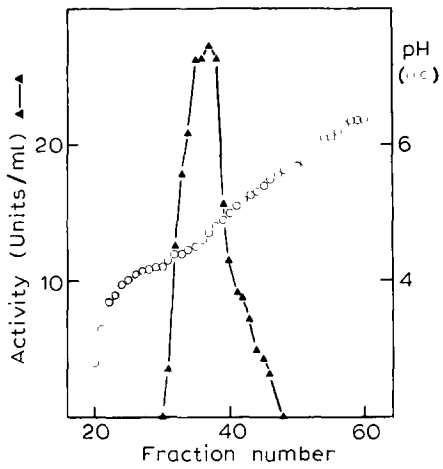


Fig. 4. Relationship between the specific activity of the cyanide-insensitive superoxide dismutase of *P. denitrificans* and the amount of Mn present in the enzyme.

Fig. 5. Determination of the isoelectric point of the cyanide-insensitive superoxide dismutase of *P. denitrificans*. For experimental details, see 'Methods'.

urea and at pH 4.7. Ose and Fridovich [17] have reported that *E. coli* Mn-superoxide dismutase was completely inactivated by treatment with 0.7 M quanidinium chloride at pH 3.2, by loss of all Mn. Apparently, the Mn-dismutase from *P. denitrificans* seems to be able to withstand a high concentration of urea and acidic pH conditions, which usually lead to denaturation, without losing all its activity.

Optical spectrum

The optical spectrum of the pure enzyme is shown in Fig. 6. It shows two shoulders near 470 and 600 nm in the visible spectrum.

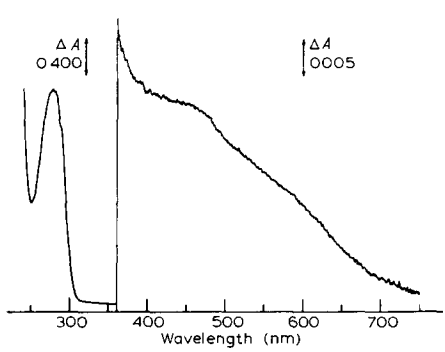


Fig. 6. Optical spectrum of Mn-superoxide dismutase of *P. denitrificans*. It was recorded at room temperature (23° C) on a Perkin-Elmer 557. Sample concentration: 2 mg protein/ml. Specific activity: 3290 units/mg.

TABLE III

AMINO ACID ANALYSIS OF MN-SUPEROXIDE DISMUTASE FROM *P. DENITRIFICANS* AND FROM OTHER SOURCES

Amino acid	Residues per dimer					Residues per subunit	
	P. denitrificans	*S. faecalis* [19]	*E. coli* [17]	*M. lepraemurium* [20]	*R. spheroides* [22]	*P. denitrificans*	Human liver [26]
Lysine	26	22.5	29	23	30	13	15
Histidine	13	17.2	12	19	16	6	9
Arginine	8	7.6	10	6	6	4	5
Aspartic acid	40 *	54.1	42	50	36	20	23
Threonine	18 *	27.8	19	21	16	9	5
Serine	18 *	15.3	22	17	22	9	6
Glutamic acid	40	41.6	37	40	44	20	22
Proline	12	19.6	15	14	14	6	10
Glycine	30	30.9	26	31	38	15	19
Alanine	36	43.1	47	50	36	18	17
Valine	22	23.3	20	20	24	11	11
Cysteic acid	4 **	0	—	6	4	2	3
Methionine	4 **	9.1	3	0	8	2	3
Isoleucine	8	20.0	14	14	10	4	9
Leucine	34	28.7	38	42	30	17	16
Tyrosine	12	17.2	12	21	14	6	7
Phenylalanine	16	15.7	18	18	20	8	5
Tryptophan	12 ***	12	—	13	8	6	5

* Values extrapolated to zero time.

** Determined after performate oxidation [13].

*** Estimated according to Edelhoch [14].

Amino acid analysis

Table III gives the amino acid composition of the Mn-containing superoxide dismutase from *P. denitrificans*. It is apparently similar to dimeric Mn-containing superoxide dismutase from other organisms. It is characterized by a high content of acidic residues (52 Asp, 42 Glu per dimer) with respect to basic residues (22 Lys, 14 His and 6 Arg) as also observed with all other Mn-containing superoxide dismutase (Table III). The enzyme from *P. denitrificans* has a high proportion of aromatic residues (11% of total residues) and a low content of cysteine and methionine.

Discussion

The cyanide-insenstitive superoxide dismutase from *P. denitrificans* has been purified to homogeneity and found to contain Mn. It is a dimer, as a number of Mn-containing superoxide dismutases isolated from procaryotes, e.g. *E. coli* [18], *Bacillus stearothermophilus* [19], *Streptococcus faecalis* [20], *Mycobacterium lepraemurium* [21], *Porphyridium cruentum* [22] or *Rhodopseudomonas spheroides* [23]. As in those organisms, its molecular weight is close to 40 000. Alternatively, in eucaryotes such as *Pleurotus olearius* [24], *Saccharomyces cerevisiae* [25] or in mammalian livers [26—28] the Mn-containing superoxide dismutase exists in a tetrameric form. *Thermus aquaticus* [29] is the only procaryote shown to contain a tetrameric Mn-containing enzyme. In dimers, the Mn stoichiometry is usually of one metal gatom/mol [19,22,23] or a little more for *E. coli* [18] and up to 1.3 for *S. faecalis* [20] or *M. lepraemurium* [21]. In this study a preparation of Mn-containing superoxide dismutase from *P. denitrificans* has been found to contain 1.88 gatom Mn/mol for a specific activity of 4630 units/mg protein; on the other hand, an enzyme containing 1.34 gatom/mol was only slightly less active (4500 units/mg). Ose and Fridovich [17] have shown that, in denaturing conditions, the Mn-containing superoxide dismutase from *E. coli* was able to bind other metallic cations preferentially to Mn^{2+}, when the latter was in a minor concentration in the buffer. A similar exchange may explain the presence of metals, other than Mn, in the Mn-containing superoxide dismutase from *P. denitrificans*. An abnormally low content of Mn leading to low specific activity may also result from a lack Mn during the growth of cells. Further studies on the relationship between the Mn concentration in the culture medium and the Mn content and specific activity are underway.

It has been suggested [30] that aerobic bacteria such as *P. denitrificans* have evolved from a nonsulfur photosynthetic bacterium with loss of its photosynthetic ability. This hypothesis is supported by the presence of cytochrome *c*-550 in *P. denitrificans* which is very similar to cytochrome c_2, the cytochrome *c* contained in photosynthetic bacteria [30,31]. Superoxide dismutases, which have been used for phylogenetic studies [2], can bring a further support to this hypothesis. Indeed the major superoxide dismutase in *Rhodopseudomonas spheroides*, a purple nonsulfur bacterium, has been found to contain Mn [23], as does the cyanide-insensitive dismutase from *P. denitrificans*. Strong analogies exist in the amino acid composition of the various Mn-containing superoxide dismutases (Table III).

It has been observed that *P. denitrificans* has several features in common with mitochondria [32]. According to the endosymbiotic hypothesis, mitochondria arose from a free-living bacterium that established symbiotic relationships with a host cell [33]. John and Whatley [34] proposed that *P. denitrificans* and mitochondria might descend from a common ancestor. The superoxide dismutase contained in mammalian mitochondria is of the Mn type [26—28] similar to that isolated from *P. denitrificans* and described in this paper. Moreover, although mitochondrial Mn-containing superoxide dismutase is a tetramer and the *P. denitrificans* enzyme a dimer, they are both composed of identical subunits. These subunits of molecular weight 23 500, have also been shown to have very similar amino acid composition (Table III). The possible existence of common antigenic sites between the Mn-superoxide dismutase from mitochondria and from *P. denitrificans* was investigated by the radioimmunoassay technique developed by Baret et al. [35] for Mn-superoxide dismutase. By this technique, Baret et al. [35] have shown that rat or bovine liver extracts (i.e., mammalian mitochondrial dismutase) cross-reacted with antibodies against human Mn-superoxide dismutase, indicating that strong similarities exist between the Mn-superoxide dismutase from these species. No cross-reaction was observed however between anti-human Mn-superoxide dismutase antibodies and purified Mn-superoxide dismutase from *P. denitrificans* (Baret, A., unpublished results) indicating that the immunogenic sites were different. As pointed out by Harris et al. [36], it has been shown that in humans [37] and in yeast [38], mitochondrial Mn-superoxide dismutase is encoded in the nucleus. In this case, the genes coding for Mn-superoxide dismutase must have been transposed from the mitochondrion to the nucleus, and the organisms must have then evolved a mechanism to return the synthesised dismutase to the mitochondrion. In this process some immunogenic sites might have been altered. Although analogies have not been further substantiated by immunological assays, the similarities existing between the Mn-superoxide dismutase of *P. denitrificans* and the mitochondrial dismutase entirely support the hypothesis of John and Whatley [34].

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References

1 Fridovich, I. (1975) Ann. Rev. Biochem. 44, 147—159
2 Brock, C.J. and Harris, J.I. (1977) in The Evolution of Metalloenzymes, Metalloproteins and Related Materials (Leigh, G.J., ed.), pp. 85—99, Copyright Symposium Press, London
3 Yost, F.J., Jr. and Fridovich, I. (1973) J. Biol. Chem. 248 (14) 4905—4908

4 Vignais, P.M., Henry, M.-F., Térech, A. and Chabert, J. (1980) in Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase (Bannister, J.V. and Hall, H.A.O., eds.), pp. 154—159, Elsevier/North Holland, Amsterdam
5 Rigo, A., Viglino, P., Argese, E., Terenzi, M. and Rotillio, G. (1979) J. Biol. Chem. 254 (6), 1759—1760
6 Burnell, J.N., John, P. and Whatley, F.R. (1975) Biochem. J. 150, 527—536
7 Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276—287
8 Henry, L.E.A., Halliwell, B. and Hall. D.O. (1976) FEBS Lett. 66, (2) 303—306
9 Bradford, M.M. (1976) Anal. Biochem. 72, 248—254
10 Laemmli, U.K. (1970) Nature 227, 681—685
11 Whitaker, J.R. (1963) Anal. Chem. 35, 1950—1953
12 Fairbanks, G., Stech, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
13 Hirs, C.H.W. (1972) Methods Enzymol. 25, 59—62
14 Edelhoch, H. (1967) Biochemistry 6, 1948—1954
15 Henry, M.-F. and Vignais, P.M. (1980) Arch. Biochem. Biophys. 203, (1) 365—371
16 Slykhouse, T.O and Fee, J.A. (1976) J. Biol. Chem. 251 (18) 5472—5477
17 Ose, D.E. and Fridovich, I. (1979) Arch. Biochem. Biophys. 194 (2) 360—364
18 Keele, B.B., Jr., McCord, J.M. and Fridovich, I. (1970) J. Biol. Chem. 245, 6176—6181
19 Bridgen, J., Harris J.I. and Northrop, F. (1975) FEBS Lett. 49, 392—395
20 Britton, L., Malinowski, D.P. and Fridovich, I. (1978) J. Bacteriol. 134 (1) 229—236
21 Ichihara, K., Kusunose, E., Kusunose, M. and Mori, T. (1977) J. Biochem. 81, 1427—1433
22 Misra, H.P. and Fridovich, I. (1977) J. Biol. Chem. 252 (18) 6421—6423
23 Lumsden, J., Cammack, R. and Hall, D.O. (1976) Biochim. Biophys. Acta 438, 380—392
24 Lavelle, F., Durosay, P. and Michelson, A.M. (1974) Biochimie 56 (3) 451—458
25 Ravindranath, S.D. and Fridovich, I. (1975) J. Biol. Chem. 250, 6107—6112
26 Weisiger, R.A. and Fridovich, I. (1973) J. Biol. Chem.. 248, 3582—3592
27 McCord, J.M., Boyle, J.A. Day, E.D. Jr., Rizzolo, L.J. and Salin, M.L. (1977) in Superoxide and Superoxide Dismutases (Michelson, A.M., McCord, J.M. and Fridovich, I., eds.), pp. 129—138, Academic Press, London.
28 Salin, M.L., Day, E.D., Jr. and Crapo, J.D. (1978) Arch. Biochem. Biophys. 187 (1) 223—228
29 Sato, S. and Harris, J.I. (1977) Eur. J. Biochem. 73, 373—381
30 Dickerson, R.E., Timkovich, R. and Almassy, R.J. (1975) J. Mol. Biol. 100, 473—491
31 Schwartz, R.M. and Dayhoff, M.O. (1978) Science 199, 395—403
32 John, P. and Whatley, F.R. (1977) Biochim. Biophys. Acta 463, 129—153
33 Margulis, L. (1970) Handbook of Genetics (King, R.C., ed.), Vol. I, Yale University Press, New Haven, CT
34 John, P. and Whatley, F.R. (1975) Nature 254, 495—498
35 Baret, A., Schiavi, P., Michel, P., Michelson, A.M. and Puget, K. (1980) FEBS Lett. 112(1) 25—29
36 Harris, J.L., Auffret, A.D., Northrop, F.D. and Walker, J.E. (1980) Eur. J. Biochem. 106, 297—303
37 Creegan, R., Tischfield, J., Ricciuti, F. and Ruddle, F.H. (1973) Hum. Genet. 20, 203—209
38 Weisiger, R.A. and Fridovich, I. (1973) J. Biol. Chem 248, 4793—4796