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An Iron-Containing Superoxide Dismutase from the Chemolithotrophic *Thiobacillus denitrificans* "RT" Strain

Jacques B. Baldensperger*

Laboratoire de Microbiologie des Sols, ORSTOM, Dakar, Sénégal

Abstract. Superoxide dismutase has been purified to homogeneity from aerobically grown *Thiobacillus denitrificans* strain "RT". It has a molecular weight of 43,000, is composed of two identical subunits which are not covalently bound, and contains 1.35 atom of iron per molecule. Absorption spectra and amino acid analysis are similar to those of other Fe-superoxide dismutases from bacteria. Aerobically and anaerobically grown cells contain the same Fe-enzyme with similar levels of activity. Manometric sulfite oxidation measurements suggest for the enzyme a protective function of sulfite against the autooxidation initiated by superoxide free radicals.

Key words: *Thiobacillus denitrificans* — Superoxide dismutase — Sulfite oxidation.

The superoxide free radical (O_2^-) is a common metabolic intermediate in a variety of biological oxidations, and it seems reasonable to expect that it is actually generated intracellularly by organisms exposed to oxygen (Fridovich, 1973). Superoxide dismutase increases about 10^4 fold the spontaneous dismutation rate of the radical ($O_2^- + O_2^- \xrightarrow{2H^+} H_2O_2 + O_2$) (Rabani and Nielsen, 1969). The enzyme was found to be present in all aerobic or facultatively anaerobic microorganisms (McCord et al., 1971) as well as in some anaerobes (Hatchikian and Henry, 1977; Tally et al., 1977; Kanematsu and Asada, 1978), and is assumed to play an important role in the protecting of cells against the deleterious action of superoxide radical (Fridovich, 1975). The involvment of superoxide in the autooxidation of sulfite was also investigated by McCord and Fridovich (1969a) who found that superoxide dismutase could inhibit the chain reaction. Since sulfite is an intermediate in the oxidation of inorganic sulfur compounds by thiobacilli (Suzuki, 1974; Schedel et al., 1975) it was of interest to characterize superoxide dismutase from the facultative anaerobe *Thiobacillus denitrificans*. The present paper reports on the purification and the properties of the Fe-containing superoxide dismutase present in aerobically and anaerobically grown *Thiobacillus denitrificans*, the function of protection of sulfite against autooxidation is discussed.

Non-Standard Abbreviations. DMSO = dimethyl sulfoxide; SDS = sodium dodecyl sulfate; SOD = superoxide dismutase

* *Present address:* Centre d'Ecologie de Camargue, Le Sambuc, 13200, Arles, France

Materials and Methods

Organism and Growth Conditions. Thiobacillus denitrificans strain "RT" (DSM 807, ATCC 29685) was grown using thiosulfate as the electron donor as previously reported (Baldensperger and Garcia, 1975). Aerobic cells were grown using ammonium as nitrogen source, and anaerobic growth was performed with nitrate as electron acceptor and sole nitrogen source.

Assay of Superoxide Dismutase. The activity of superoxide dismutase was measured by the cytochrome reduction assay of McCord and Fridovich (1969b) and enzymatic unit was defined as the amount of enzyme required to inhibit the cytochrome reduction by 50%. Changes in absorbance were followed with a Beckman model 25 spectrophotometer. During the course of purification, column eluates were assayed for superoxide dismutase activity by the procedure described by Elstner and Heupel (1976) which is based upon the inhibition of nitrite formation from hydroxylammonium chloride. Nitrite was determined colorimetrically with a M4QIII Zeiss spectrophotometer.

Assay of Sulfite Oxidase. Enzymic and chemical oxidation of sulfite were measured manometrically using a model V 166 Warburg apparatus. Superoxide radicals were produced in the vessel either by means of a potential difference of 1.5 volt impressed accross sealed in platinium electrodes, or by reaction between sulfite (5mM) and dimethyl sulfoxide (0.25 mM). This concentration in dimethyl sulf-

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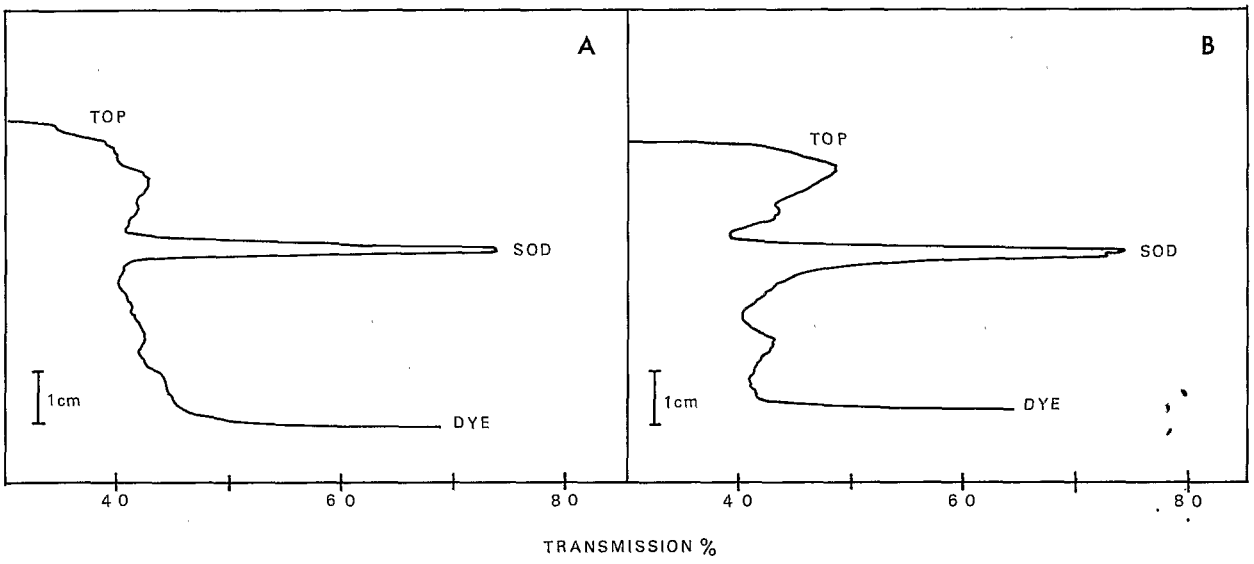


Fig. 1 A and B. Densitometric scans of polyacrylamide gel electrophoregrams stained for SOD activity. Cell free extracts were applied to 7% gels. **A** 0.170 mg protein from anaerobically grown cells ("NO_3" extract). **B** 0.135 mg protein from aerobically grown cells ("O_2" extract)

oxide (DMSO) was found by Puget and Michelson (1974) to have no effect on the superoxide dismutase activity of the Fe enzyme from *Photobacterium leiognathi.*

Determination of Protein. Protein was determined according to Lowry et al. (1951) using bovine serum albumin as the reference.

Absorption Spectra. Visible and ultraviolet absorption spectra were recorded with a Cary model 14 spectrophotometer.

Determination of Molecular Weight. The molecular weight of the purified enzyme was estimated by gel filtration according to Whitaker (1963). Sephadex G-75 column was used with the following standards: bovine serum albumin, 68,000; ovalbumin, 43,000; pepsin, 35,000; soybean trypsin inhibitor, 20,100.

Polyacrylamide Gel Electrophoresis. Analytical gel electrophoresis was carried out with 7% gels (pH 9.5) using the method of Davis (1964). 50 µg of protein were loaded on each gel and protein bands were stained with Coomassie blue. The activity bands were made visible by the photochemical assay of Beauchamp and Fridovich (1971).

The subunit molecular weight was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to Weber and Osborn (1969). Samples were incubated at 37°C for 2 h in 0.010 M phosphate buffer pH 7, 1% in SDS, in the presence or absence of 1% β-mercaptoethanol. Electrophoresis was carried out 3 h with a constant current of 10 mA per gel. The following standards were used: bovine serum albumine, 68,000; ovalbumine, 43,000; chymotrypsinogen A, 25,000; soybean trypsin inhibitor, 20,100 and

Results

Superoxide Dismutase in Crude Extracts from Thiobacillus denitrificans

16 g wet weight of cells were harvested from a 20 l carboy of anaerobic medium "DB" and cell-free extract ("NO_3" extract) was prepared as previously described (Baldensperger and Garcia, 1975). Aerobic growth was conducted in a 20 l fermentor on aerobic medium "A" maintained at pH 6.8 by titrating sterile 10% (w/v) Na_2CO_3. Cells were harvested with similar cell yield and the extract from aerobically grown cells ("O_2" extract) was prepared as for anaerobic cultures. 5 µl (containing about 150 µg of protein) of both extracts were subjected to polycrylamide gel electrophoresis and to staining of superoxide dismutase activity. A sole activity band (RF = 0.40) was observed in both extracts, and corresponded to the activity stained in gels from the purified iron superoxide dismutase described below. Densitometric scans of stained gels from extracts of anaerobically and aerobically grown cells are shown in Fig. 1. Specific activities of "NO_3" extract

aerobically grown cells by centrifugation at 10,000 g for 30 min of washed bacteria, suspended in pH 7.5 0.050 M Tris-HCl buffer (15 g wet wt./30 ml buffer). Sulfite oxidation was assayed manometrically in the presence or absence of KCN 0.001 M. Results are presented in Table 1.

With 1.3 mg of protein from crude extract, 36% inhibition of oxygen uptake was noticed in the presence of 0.001 M KCN. The inhibition rate increased to 75% with 13 mg of proteins, while in the presence of 26 mg of the crude extract proteins it was noticed that oxygen uptake decreased also in the absence of KCN as compared to the uptake with 13 mg.

An attempt was made to produce superoxide radical in the Warburg vessel, either by means of sealed in platinium electrodes according to Fridovich and Handler (1961) or by dimethyl sulfoxide (DMSO) reacting with sulfite (McCord and Fridovich, 1969a). Concentrations in EDTA, sulfite, and DMSO were adjusted as described by the authors to obtain initiation of the chain reaction. In this condition, superoxide dismutase present in crude extract was found to inhibit autooxidation of sulfite, as shown in Tables 2 and 3. Complete inhibition of sulfite oxidation initiated by 1.5 V between the platinium electrodes was obtained by 18 mg of crude extract proteins. In the DMSO sulfite system, a 98% inhibition was measured.

Table 1. Manometric measurement of sulfite oxidation by crude extract from *Thiobacillus denitrificans*

mg protein of crude extract	0	1.3	2.6	13	26
µl O_2 utilized (min^{-1}) minus KCN	5.8	4.7	5.0	18.3	14.3
µl O_2 utilized (min^{-1}) + KCN	5.8	3.0	1.5	4.5	3.0
Inhibition (%)	0	36	70	75	79

Final concentration in Warburg vessels: sulfite, 15 mM; EDTA, 0.015 mM; KCN, 1 mM; phosphate buffer pH 7.6, 70 mM; crude extract, as indicated. The sodium sulfite (freshly prepared) was added from the side arm as solution in EDTA at zero time. Gas phase air. Temperature 37° C

Table 2. Inhibition of electrolytic initiation of sulfite oxidation by crude extract from *Thiobacillus denitrificans*

mg protein of cude extract	0	18
µl O_2 utilized (min^{-1}) 0 Volt	5	14.5
µl O_2 utilized (min^{-1}) 1.5 Volt	8	14.5

Final concentration in Warburg vessels: sulfite, 15 mM; EDTA, 0.23 mM; phosphate buffer pH 7.6, 70 mM; crude extract, as indicated. The sodium sulfite (freshly prepared) was added from the side arm as solution in EDTA at zero time. Gas phase air. Temperature 37° C. Voltage was impressed in the reaction mixture by means of platinium electrodes (0.5 mm diameter, 3 mm length) sealed in the vessel at a distance of 3 mm

Table 3. Inhibition of the DMSO initiated sulfite oxidation by crude extract from *Thiobacillus denitrificans*

mg protein of crude extract	0	22
µl O_2 utilized (min^{-1}) minus DMSO	0.2	8.8
µl O_2 utilized (min^{-1}) + DMSO	9.4	8.9

Final concentration in Warburg vessels: sulfite, 5 mM; EDTA, 0.1 mM; DMSO, 0.25 mM; phosphate buffer pH 7, 100 mM; crude extract, as indicated. The sodium sulfite (freshly prepared) was added from the side arm as solution in EDTA at zero time. Gas phase air. Temperature 37° C

Purification of the Enzyme

All purification procedures were performed at 4° C, and unless otherwise noted phosphate buffer was used at pH 7.8.

a) Preparation of Crude Extract. Cells of *Thiobacillus denitrificans* were harvested by centrifugation during log phase and washed twice in 0.05 M phosphate buffer pH 6.8. The pellet (120 g wet w.) was resuspended in 100 ml 0.05 M phosphate pH 7,8 and homogeneized before passing through a French pressure cell at 20,000 PSI. A few cristals of deoxyribonuclease were added to the preparation which was stirred for 10 min at room temperature before centrifugation 30 min at 30,000 g. The crude extract contained 65.2×10^3 units of superoxide dismutase, its specific activity was 7.5 unit/mg protein.

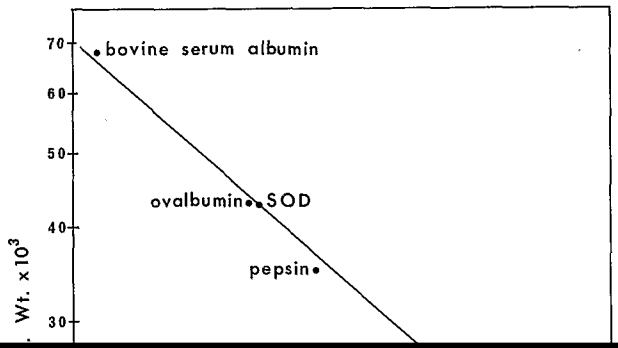
b) Precipitation at 60° C. The crude extract was agitated in a thermostatic bath at 60° C during 5 min and chilled. The precipitate was discarded after centrifugation for 30 min at 30,000 g. The clear supernatant exhibited a specific activity of 12.9 unit/mg protein.

c) Ammonium Sulfate Fractionation. The preparation was brought to 45% of saturation in $(NH_4)_2SO_4$ by addition of a saturated solution and stirred during 5 min at room temperature. The precipitate was discarded after 30 min at 30,000 g. The supernatant was then brought to 80% $(NH_4)_2SO_4$ saturation by addition of the solid salt, and the mixture was centrifuged for 40 min at 20,000 g. The supernatant was free from superoxide dismutase activity.The precipitate was dissolved in a minimum of 0.01 m phosphate buffer pH 7.8, and dialyzed against 2 l of the same buffer, which was exchanged 3 times every 8 h.

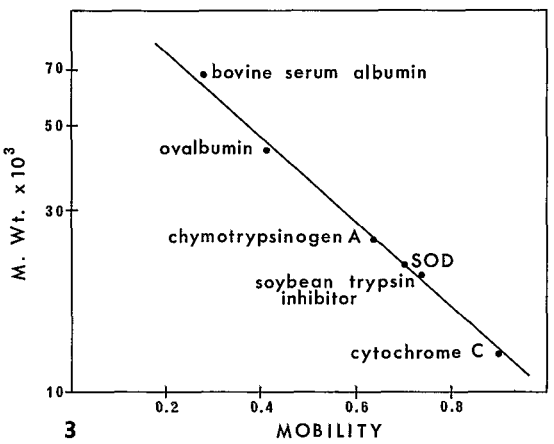
Table 4. Purification of superoxide dismutase from *Thiobacillus denitrificans*

Purification step	Volume (ml)	Total protein (mg)	Total activity (unit) $\times 10^{-3}$	Specific activity (unit/mg protein)	Yield %	Purification fold
Crude extract after centrifugation	150	8,700	65.2	7.5	100	1
Supernatant from heat step	124	4,340	55.9	12.9	86	1.7
50–80% $(NH_4)_2SO_4$	53	1,670	45.3	27.1	69.5	3.6
Sephacryl	140	252	38.4	153	59	20.4
Hydroxylapatite	35	63	26.7	424	41	56.5
DEAE 52	38	12.5	18.1	1,448	36	193
Ultrogel AcA 4/4	29	8.5	16.5	1,940	25	259

d) Sephacryl Gel Filtration. The dialyzed solution (53 ml) was applied to a column of Sephacryl (5 × 84 cm) equilibrated with 0.01 M phosphate buffer pH 7.8. The column was washed with the same buffer at a flow rate of 0.9 ml per min, and the active fractions (140 ml) were concentrated in a 320 ml ultrafiltration cell using a PM-10 membrane filter (Amicon) before dialyzing against 0.002 M phosphate buffer (pH 7.8).



e) Hydroxylapatite Column Chromatography. The



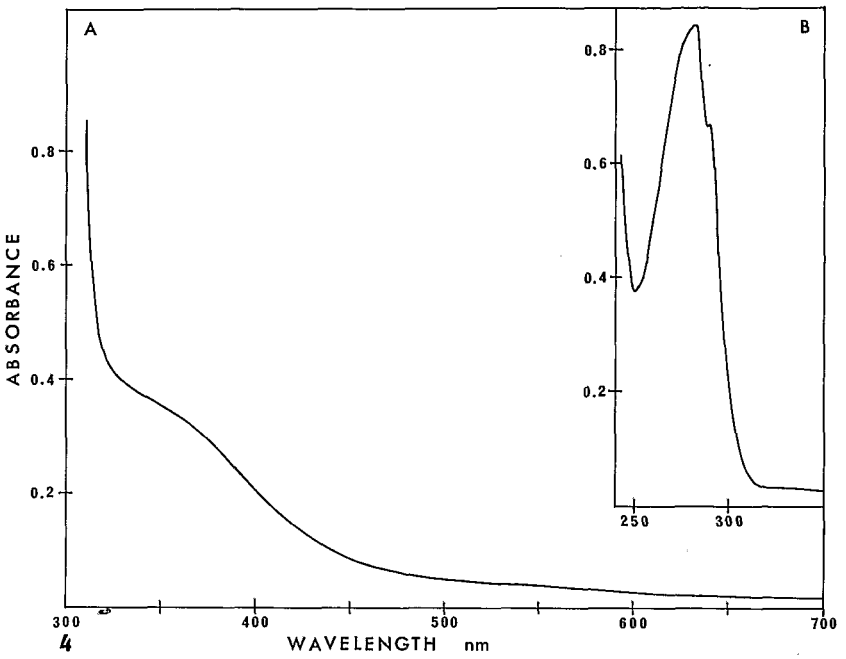


Fig. 3. Determination of the molecular weight of superoxide dismutase subunits from *Thiobacillus denitrificans* by SDS-polyacrylamide gel electrophoresis. 0.8 µg of pure enzyme incubated as described were submitted to SDS-electrophoresis in the presence of 1% β-mercaptoethanol

Fig. 4 A and B. Absorption spectra of superoxide dismutase from *Thiobacillus denitrificans* (1 cm light path). **A** Visible spectrum at 6.83 mg protein/ml in 10 mM potassium phosphate buffer pH 7.6. **B** Ultraviolet spectrum at 0.46 mg protein/ml in the same buffer

absorbance in 13 different fractions which were exam- gave one band both in the presence and in the absence

Table 5. Comparative amino acid analysis of iron containing superoxide dismutases

	Thiobacillus denitrificans[a]	*Desulfovibrio desulfuricans*[b]	*Chromatium vinosum*[c]	*Escherichia coli*[d]
Lysine	14	16	8	10
Histidine	9	5	4	6
Arginine	4	4	2	4
Tryptophan	6	6	6	4
Aspartic acid	22	27	24	22
Threonine	12	12	11	13
Serine	10	7	16	10
Glutamic acid	13	18	20	16
Proline	8	8	7	9
Glycine	15	19	14	16
Alanine	20	22	21	26
$^1/_2$ Cysteine		2	1	1
Valine	9	8	14	11

Densitometric scans of gels stained for superoxide dismutase activity showed that both extracts from aerobically and anaerobically grown cells contained only one superoxide dismutase, which was found to be a Fe-SOD. On the contrary, *Escherichia coli* grown under anaerobic conditions contained only Fe-SOD, but exposure to oxygen induced the synthesis of Mn-SOD and New-SOD (Hassan and Fridovich, 1977; Britton and Fridovich, 1977). The presence of the same enzyme at similar levels of specific activity in aerobically and anaerobically grown *Thiobacillus denitrificans* might indicate that the enzyme has a function other than the dismutation of superoxide radical. A similar statement was made by Kanematsu and Asada (1978) concerning the Fe-SOD from *Chromatium vinosum.* The aerobic autooxidation of sulfite has been shown by Fridovich and coworkers to be a free-radical chain reaction,

reported earlier by Anastasi et al. (1976) concerning the Fe-enzyme from *Bacillus megaterium*. The properties of the *Thiobacillus* enzyme are very similar to the iron-containing superoxide dismutases so far isolated from other sources, with respect to molecular weight, subunit structure, absorption spectra and amino acid composition.

The ultraviolet spectrum shows a shift of the maximum protein absorbance to 282 nm, and a sharp peak at 290 nm as compared to the previously reported Fe-SOD from other sources. The shoulder at 350 nm observed in the visible absorption spectrum is thought to be due to the iron linked to the protein, the molar extinction coefficient at 350 nm reflecting the iron content of the enzyme.

The *Thiobacillus* enzyme contains 1.35 iron atom per molecule of 43,000 daltons. Intermediate values between 1 iron atom in *Escherichia coli* (Yost and Fridovich, 1973) and 2 iron atoms in *Chromatium vinosum* (Kanematsu and Asada, 1978) have been reported. It is unlikely to attribute the deviation to iron lack during cell growth, as the trace metal solution used for the medium (Baldensperger and Garcia, 1975) gave a high yield in other iron proteins, i.e. cytochromes, as demonstrated by the visible absorption spectrum of the crude extract.

As suggested by Yamakura (1976), it seems more probable that one iron atom is bound strongly to the enzyme molecule, the other being removable through the purification procedure. The differences may also be accounted for by difficulties encountered in the estimation of the iron content (Puget and Michelson, 1974) and/or the protein concentration of the preparations (Slykhouse and Fee, 1976).

Amino acid composition of *Thiobacillus denitrificans* superoxide dismutase shows similarities with that of other iron containing enzymes from bacterial sources, with a high content in histidine, tryptophan and tyrosine. It was suggested by Hatchikian and Henry (1977) that these residues could be the ligands of iron. The *Thiobacillus* enzyme contains methionine which is absent from the *Escherichia coli* Fe-enzyme.

From the present data, we can conclude that aerobically and anaerobically grown *Thiobacillus denitrificans* contains a sole Fe-containing superoxide dismutase protecting the cell against the harmfull action of the radical (Fridovich, 1975). A protecting function of superoxide dismutase towards sulfite is suggested.

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