FEBS 14952

FEBS Letters 357 (1995) 79–82

Purification and characterization of an iron superoxide dismutase from the nitrogen-fixing *Azotobacter vinelandii*

Silvia Pagani[a,*], Rita Colnaghi[a], Arianna Palagi[a], Armando Negri[b]

[a]*Dipartimento di Scienze Molecolari Agroalimentari, CISMI, The University of Milano, Via Celoria 2, I-20133 Milano, Italy*
[b]*Istituto di Fisiologia Veterinaria e Biochimica, CISMI, The University of Milano, Milano, Italy*

Received 24 October 1994; revised version received 22 November 1994

Abstract Two electrophoretically distinct forms of superoxide dismutase (SOD; EC 1.15.1.1) which show different inhibition patterns to hydrogen peroxide have been identified in *Azotobacter vinelandii*. The SOD inhibited by hydrogen peroxide was purified to homogeneity, and turned out to be an iron superoxide dismutase. The enzyme is present in only one molecular form with an isoelectric point of 4.1, and it is composed of two identical subunits with an apparent molecular weight of 21,000 Da. Spectroscopic analyses indicated that this enzyme contains ferric iron (1.4–1.6 mol/mol protein) in the typical high-spin form present in other prokaryotic Fe-SODs. N-Terminal sequence alignments (up to the 49th residue) showed that *A. vinelandii* Fe-SOD has high similarity with other prokaryotic Fe-SODs.

Key words: Superoxide dismutase; Prokaryote; Nitrogen-fixation; *Azotobacter vinelandii*

1. Introduction

Superoxide dismutases (SOD; EC 1.15.1.1) are a class of metalloproteins containing either manganese, iron, or copper and zinc as natural prosthetic groups, which catalyze the dismutation of the superoxide anion radical into molecular oxygen and hydrogen peroxide [1].

The phylogenetic distribution of the three types of SOD is considered to be characteristic of the evolutionary state of the organism and of the associated organelles [2,3]. Cu/Zn SOD is the major enzyme in animals, plants and fungi, whereas the prokaryotes (blue-green algae and bacteria) and simple eukaryotes (algae and protozoa) contain iron- or manganese-SOD, with both enzymes sometimes occurring together in the same organism [3–5]. Structural studies [6–9] have shown that the iron- and manganese-SOD types are closely related, but they bear little resemblance to the Cu/Zn-SOD.

The presence of SOD enzymes in aerobic organisms appears to be strictly related to their catalytic property in removing superoxide radicals, thus controlling oxidative risk in the cell [10]. A high level of SOD activity was found in cell-free extracts from *Azotobacter vinelandii* [11], an aerobic nitrogen-fixing organism which has a high respiratory rate. A possible role of SOD in protecting nitrogen fixation from irreversible oxygen damage was claimed [12], but no clear evidence was provided for a specific involvement of this enzyme. This fact prompted us to investigate the presence of SOD enzymes in *A. vinelandii*, grown under nitrogen-fixing conditions.

In this report, we describe the purification and some molecular and spectroscopic properties of a Fe-SOD from *A. vinelandii*.

2. Materials and methods

A. vinelandii strain UW136 (from Nitrogen Fixation Laboratory collection) was grown in Burk's sucrose medium [13] in a 200 l New Brunswick stainless steel fermentor. Aeration rate was 50 l·min^{-1}. Cells were harvested in a Westfalia centrifuge after the culture had reached OD_{600} = 2.00.

Extracts were prepared from 60 g of frozen cells thawed in 200 ml of 0.2 M Tris-HCl, pH 7.5. Cells were disrupted by a repeated cycle of sonication (Uniprep 150, 22 micron, 6 times for 1 min) and treated with lysozyme (50 mg, 30000 U/mg) at 4°C for 60 min with agitation. Cell debris was removed by centrifugation at 18000 × *g* for 90 min and the supernatant was used for the purification of SOD.

2.1. Analytical methods

Proteins were assayed by the microbiuret method [14] or, in the case of partially purified Fe-SOD, from the absorbance at 280 nm by using the extinction coefficient of 2.01 for a solution containing 1.0 mg/ml.

SOD activity was tested by measuring the inhibition of pyrogallol autoxidation [15]. An enzymatic unit was defined as the amount of enzyme required to inhibit pyrogallol autoxidation by 50%.

Non-denaturing PAGE was performed on a 12% polyacrylamide gel at a constant current of 8 mA overnight at 4°C by using 30 mM Tris-acetate buffer, pH 8, as the running buffer.

The activity of SOD was assayed on gels as described by Beauchamp and Fridovich [16]. The presence of different molecular forms of SOD was tested according to their sensitivity to hydrogen peroxide, sodium azide and cyanide [17].

The N-terminal sequence was determined by using an automated protein sequencer (Applied Biosystems 477/A).

The metal content of the purified preparations was determined by ICP analysis at the Center for Metalloenzyme Studies, The University of Georgia, Athens, USA.

EPR spectra were recorded on a Bruker spectrometer at the Center for Metalloenzyme Studies, The University of Georgia, Athens, USA.

2.2. Purification procedure

Step 1: ammonium sulphate precipitation. The extract was made 75% saturated with ammonium sulphate. After centrifugation at 15000 × *g* for 60 min, the precipitate (5–6 g of proteins) was dissolved in 17 mM phosphate buffer, pH 8.0 (150–200 ml). Unless otherwise stated, all procedures were carried out at 4°C.

Step 2: Sephadex G-50 fractionation and ion-exchange chromatography. The redissolved proteins were fractionated on a column of Sephadex G-50 Fine (Pharmacia 9 × 40 cm) equilibrated in 17 mM phosphate buffer, pH 8.0, at a flow rate of 200 ml/h. The fractions containing superoxide dismutase activity were pooled and adsorbed on a column of DEAE Sephacel (Whatman; 6 × 10 cm) equilibrated in 17 mM phosphate buffer, pH 8.0. The column was washed with 1000 ml of the equilibration buffer, and superoxide dismutase activity was eluted with 17 mM phosphate buffer, pH 8.0, containing 0.15 M NaCl. The active material eluted was pooled and concentrated by precipitation with ammonium sulphate (75% saturated) at pH 8.0.

*Corresponding author. Fax: (39) (2) 7063 3062.

Abbreviations: SOD, superoxide dismutase; Fe-SOD, iron-containing superoxide dismutase; Mn-SOD, manganese-containing superoxide dismutase; ICP, ion-coupled plasma emission.

SSDI 0014-5793(94)01339-X

Step 3: Sephacryl S-200 chromatography. The proteins from Step 2 (100–110 mg) were dissolved in 10 ml of 15 mM phosphate buffer, pH 8.0, and loaded on a column of Sephacryl S-200 (Pharmacia; 3.3 × 84 cm) equilibrated with the same buffer. SOD activity was eluted with 15 mM phosphate buffer, pH 8.0, at a flow rate of 25 ml/h. The eluate (10 mg of proteins in 10 ml) was divided in samples of 5 mg protein which were separately chromatographed in a FPLC apparatus.

Step 4: FPLC chromatographies. The active fractions from the Sephacryl S-200 column were then adsorbed on a TSK-DEAE 5PW column (LKB; 0.8 × 7.5 cm) equilibrated in 15 mM phosphate buffer, pH 8.0 (buffer A). Elution of bound proteins was achieved at room temperature with a linear gradient of buffer A to buffer B (50 mM phosphate buffer, pH 8.0) in 30 min. The active material of two FPLC-DEAE elutions (0.3 mg/ml, 7–8 ml) was concentrated about 20-fold in an Amicon ultrafiltration device and loaded on a column of Superdex 75 HR (LKB; 1 × 30 cm) equilibrated with 50 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl. SOD was eluted with the same buffer at a flow rate of 0.5 ml/min. The SOD eluted from the Superdex column (1.5 mg in 7–8 ml) was dialyzed overnight against 1 l of 20 mM Tris-HCl buffer, pH 7.5, and adsorbed to a Mono Q column (Pharmacia, 0.5 × 5 cm). The column was washed with 10 ml of 20 mM Tris-HCl buffer, pH 7.5, and then developed with a 50 ml linear gradient of Tris from 20 to 340 mM. The eluted, homogeneous SOD (about 1 mg) was stored at −20°C.

3. Results and discussion

The crude extract of *A. vinelandii* culture, grown under nitrogen-fixing conditions, contains two electrophoretically distinct forms of SOD which showed different sensitivity to hydrogen peroxide (Fig. 1). Since the lack of inhibition by hydrogen peroxide is considered to be specific for Mn-containing SOD and not for the other metal forms [17,18], this result indicated that one form was Mn-SOD. Analysis of the activity inhibition by either azide or cyanide [19] suggested that the other SOD was probably a Fe-containing SOD.

There is general agreement that Fe-SOD is a constitutive enzyme in prokaryotes, and Mn-SOD is inducible by oxygen [20]. In *Escherichia coli*, which contains both Fe- and Mn-SOD [4,5], the level of Mn-SOD increases upon exposure to oxygen [20–22].

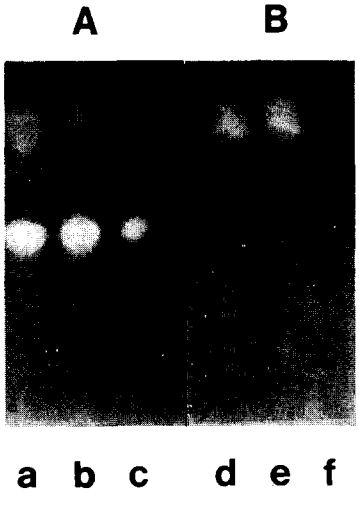

Fig. 1. In situ visualization of superoxide dismutase activity. Analysis of *A. vinelandii* SOD activity at different steps of the purification procedure was carried out in the absence (A) and in the presence of 1 mM H_2O_2 (B). Lanes a,d, *A. vinelandii* crude extract (0.31 enzymatic units of SOD); lanes b,e, SOD active fraction from the Sephadex G-50 column (0.33 enzymatic units); lanes c,f, SOD active fraction from the DEAE Sephacel column (0.20 enzymatic units).

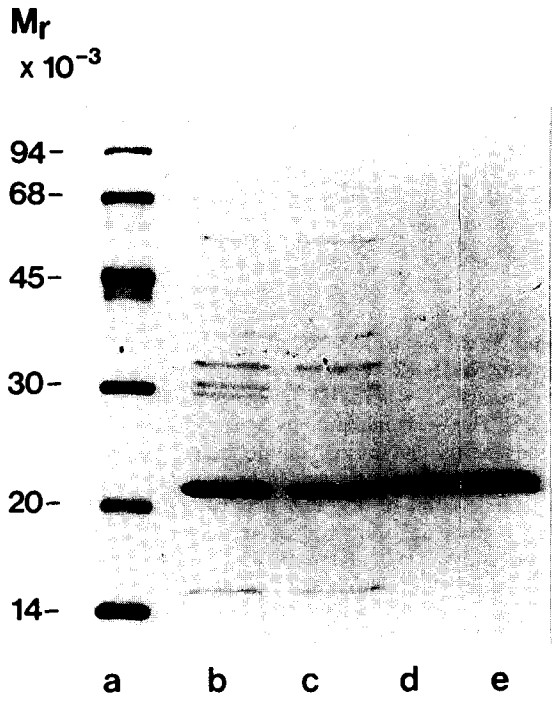

Fig. 2. Electrophoretic analysis of the final enzyme preparation. SDS-PAGE was performed as described by Laemmli [31]. Samples were denatured in the presence of 2% 2-mercaptoethanol and 2% SDS (lanes a–d). Lane a, protein markers (15 μg of a total mixture containing phosphorylase *b*, bovine serum albumin, egg albumin, carbonic anhydrase, trypsin inhibitor and lysozyme); lane b, SOD active peak from the TSK-DEAE 5PW column (6 μg); lane c, SOD from the Superdex column (5 μg); lane d, SOD from the Mono Q column (4.5 μg); lane e, purified Fe-SOD denatured in the absence of 2-mercaptoethanol (5 μg).

In the aerobic nitrogen-fixing organisms, SOD activity could represent a useful tool in protecting nitrogen fixation from oxygen damage. Some free-living nitrogen-fixing bacterial species exhibit high SOD levels [23], and it was reported that

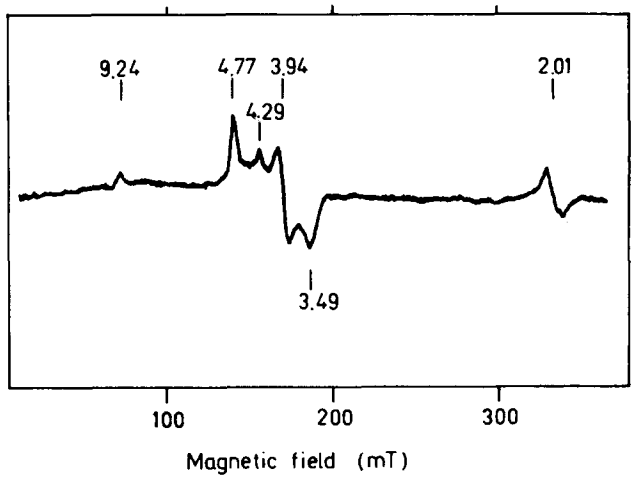

Fig. 3. EPR spectrum of the *A. vinelandii* Fe-SOD. The concentration of the homogeneous enzyme was 11.4 mg/ml (271 μM) in 50 mM sodium phosphate, pH 7.8. The spectrum was recorded at 100 K under the following conditions: modulation amplitude, 0.6 mT; power, 200 mW; and frequency, 9.38 GHz. The g values are indicated on the figure.

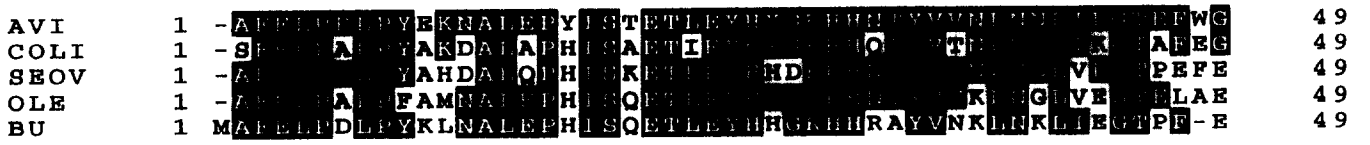

Fig. 4. Multiple N-terminal sequence alignments of Fe-SODs. N-terminal sequence (up to the 49th residue) of *A. vinelandii* Fe-SOD (AVI) is compared with N-terminal Fe-SOD sequences from *Escherichia coli* (COLI), *Pseudomonas ovalis* (SEOV), *Photobacterium leiognathi* (OLE), *Coxiella burnetii* (BU). Amino acid sequence comparisons were carried out with PC/GENE 6.7 software (Intelligenetics, Mountain View, CA). Identical residues are shown as white character on a black background.

Table 1
Purification of Fe-superoxide dismutase from *A. vinelandii*

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification (x-fold)	Yield (%)
Crude extract	8900	64000	7.1	1.0	100.0
Sephadex G50 fine	1300	25600	19.7	2.8	40.0
DEAE-Sephacel	200	21750	109.0	15.3	34.0
Sephacryl FPLC	35	7900	226.0	32.0	12.0
TSK-DEAE	4	1920	480.0	68.0	3.0
Superdex	1.3	1025	790.0	111.0	1.6
MonoQ	0.9	900	1000.0	141.0	1.4

repression of nitrogenase by ammonium was associated with a decrease in SOD activity in *Azospirillum brasilense* [24]. In order to investigate the role of SOD enzymes in the nitrogen fixation process we isolated and characterized SOD from the nitrogen-fixer *A. vinelandii*.

By following the scheme shown in Table 1 we achieved purification of an SOD enzyme that was inhibited by hydrogen peroxide. The Mn-SOD, the activity of which appears to be labile, was still present in the Sephadex G-50 eluate and was lost after the first ion-exchange chromatography (Fig. 1, lane e).

Homogeneous SOD, containing 1.4–1.6 mol iron/mol protein, was obtained after ion-exchange chromatography on the Mono Q column (Fig. 2, lane d). The apparent molecular weight of the *A. vinelandii* Fe-SOD in the presence or absence of 2-mercaptoethanol was about 21,000 Da by SDS-PAGE (Fig. 2). Native *A. vinelandii* Fe-SOD behaved like a protein with a molecular weight of 42,000 Da, as determined on the Superdex column in FPLC. These results indicate that the *A. vinelandii* Fe-SOD is composed of two subunits of equal size not linked by a disulfide bond. These subunits are probably identical, as shown by N-terminal sequence analysis of the homogeneous Fe-SOD (see below).

Purity of *A. vinelandii* Fe-SOD was also checked by isoelectric focusing analysis, and only one form with an apparent isoelectric point of 4.1 was found.

The EPR spectrum of this enzyme is shown in Fig. 3. EPR spectra were recorded at 100, 13 and 4 K, and showed the presence of iron in a high-spin (s = 5/2) ferric form. All the features typical of other Fe-SODs [5,19] were present, including the weak signal at g = 9.2 and a resonance at g = 2.00, which were clearly visible even at 100 K. The weak additional resonance at g = 4.3, that was interpreted to be due to some protein denaturation in Fe-SOD from *E. coli* [5,25] and from *Plectonema boryanum* [19], accounted in our samples for less than 5% of the total signal intensity. Nevertheless, ICP analysis for metal content revealed that the purified *A. vinelandii* Fe-SOD did not contain the expected amount of iron (2 mol iron/mol protein). Evidence for the loss of iron during purification was, however, reported for Fe-SODs from other sources [26,27].

The N-terminal sequence of *A. vinelandii* Fe-SOD up to the 49th residue, along with its sequence alignment to other Fe-SODs, is shown in Fig. 4. Identities ranging from 69 to 79% were found when the N-terminal sequence of *A. vinelandii* SOD was compared with the N-terminal sequences of the other characterized Fe-SODs. With the prokaryotic Mn-SODs from *E. coli* [28], *Bacillus stearothermophilus* [29] and *Thermus aquaticus* [30], the degree of identities (43–53%) is comparable to that found between *E. coli* Fe- and Mn-SOD.

The preliminary structural data presented here suggest a likely structural homology of *A. vinelandii* Fe-SOD with the other bacterial Fe-SODs of known structure [6,7,9].

Acknowledgements: We wish to thank Dr. F. Bonomi for his suggestions during the course of this work, Dr. N. Gupta (Center for Metalloenzyme Studies, The University of Georgia, Athens, USA) for taking EPR spectra and ICP measurements, N. Handerson (AFRC, Nitrogen Fixation Laboratory, Brighton, UK) for growing large-scale cultures, and F. Sessa for skilful technical assistance. The present investigation was supported by grants from Ministero dell'Universita' e della Ricerca Scientifica e Tecnologica, Italy (60%).

References

[1] McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[2] Weisiger, S.N. and Fridovich, I. (1973) J. Biol. Chem. 248, 4793–4796.
[3] Steinman, H.M. (1983) in: Oxy Radicals and Their Scavenger Systems: Molecular Aspects, vol. 1 (Cohen, G. and Greenwald, R.A. eds.) pp. 167–178, Elsevier, New York.
[4] Keele, B.B., McCord, J.M. and Fridovich, I. (1970) J. Biol. Chem. 245, 6176–6181.
[5] Yost, F.J. and Fridovich, I. (1973) J. Biol. Chem. 248, 4905–4908.
[6] Stallings, W.C., Pattridge, K.A., Strong, R.K. and Ludwig, M.L. (1984) J. Biol. Chem. 259, 10695–10699.
[7] Stallings, W.C., Pattridge, K.A., Strong, R.K. and Ludwig, M.L. (1985) J. Biol. Chem. 260, 16424–16432.

[8] Stallings, W.C., Powers, T.B., Pattridge, K.A., Fee, J.A. and Ludwig, M.L. (1983) Proc. Natl. Acad. Sci. USA 80, 3884–3888.
[9] Stoddard, B.L., Howell, P.L., Ringe, D. and Petsko, G.A. (1990) Biochemistry 29, 8885–8893.
[10] Fridovich, I. (1983) Annu. Rev. Pharmacol. Toxicol. 23, 239–257.
[11] Moore, E.R.B., Pinina Norrod, E. and Jurtshuk Jr., P. (1984) FEMS Microbiol. Lett. 24, 261–265.
[12] Puppo, A. and Rigaud, J. (1986) FEBS Lett. 201, 187–189.
[13] Newton, J.W., Wilson, P.W. and Burris, R.H. (1953) J. Biol. Chem. 204, 445–451.
[14] Itzaky, R.F. and Gill, O.M. (1964) Anal. Biochem. 9, 401–410.
[15] Marklund, S. and Marklund, G. (1974) Eur. J. Biochem. 47, 469–474.
[16] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[17] Okada, S., Kanematsu, S. and Asada, K. (1979) FEBS Lett. 103, 106–110.
[18] Beyer Jr., W.F. and Fridovich, I. (1987) Biochemistry 26, 1251–1257.
[19] Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y. and Enmandji, K. (1975) J. Biol. Chem. 250, 2801–2807.
[20] Nettleton, C.J., Bull, C., Baldwin, T.O. and Fee, J.A. (1984) Proc. Natl. Acad. Sci. USA 81, 4970–4973.
[21] Hassan, H.M. and Fridovich, I. (1977) J. Bacteriol. 129, 1574–1583.
[22] Carlioz, A. and Touati, O. (1986) EMBO J. 5, 623–630.
[23] Jurtshuk, P. Jr., Liu, J.K. and Moore, E.R.B. (1984) Appl. Environ. Microbiol. 47, 1183–1187.
[24] Clara, R., W. and Knowles, R. (1984) Can. J. Microbiol. 30, 1222–1228.
[25] Slykhouse, T.O. and Fee, J.A. (1976) J. Biol. Chem. 251, 5472–5477.
[26] Yamakura, F. (1976) Biochim. Biophys. Acta 422, 280–294.
[27] Ringe, D., Petsko, G.A., Yamakura, F., Suzuki, K. and Ohmori, D. (1983) Proc. Natl. Acad. Sci. USA 80, 3879–3883.
[28] Plunkett, G. III, Burland, V.D., Daniels, D.L. and Blatter, F.R. (1993) Nucleic Acids Res. 21, 3391–3398
[29] Bowler, C., Van Kaer, L., Van Camp, W., Van Montagu, M., Inze, D. and Dhaese, P. (1990) J. Bacteriol. 172, 1539–1546.
[30] Sato, S., Nakada, Y. and Nakazawa-Tomizawa, K. (1987) Biochim. Biophys. Acta 912, 178–184.
[31] Laemmli, U.K. (1970) Nature 227, 680–685.