A novel nickel-containing superoxide dismutase from *Streptomyces* spp.

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A novel type of superoxide dismutase (SOD) was purified to apparent homogeneity from the cytosolic fractions of *Streptomyces* sp. IMSNU-1 and *Strep. coelicolor* ATCC 10147 respectively. Both enzymes were composed of four identical subunits of 13.4 kDa, were stable at pH 4.0–8.0 and up to 70 °C, and were inhibited by cyanide and H₂O₂ but little inhibited by azide. The atomic absorption analyses revealed that both enzymes contain 0.74 g-atom of nickel per mol of subunit. Both enzymes were different from iron-containing SOD and manganese-containing SOD from *Escherichia coli*, and copper- and zinc-containing SODs from *Saccharomyces cerevisiae* and bovine

erythrocytes, with respect to amino acid composition, N-terminal amino acid sequence and cross-reactivity against antibody. The absorption spectra of both enzymes were identical, exhibiting maxima at 276 and 378 nm, and a broad peak at 531 nm. The EPR spectra of both enzymes were almost identical with that of Ni^{III} in a tetragonal symmetry of Ni^{III}-oligopeptides especially containing histidine. The apoenzymes, lacking in nickel, had no ability to mediate the conversion of superoxide anion radical to hydrogen peroxide, strongly indicating that Ni^{III} plays a main role in these enzymes.

INTRODUCTION

Superoxide dismutase (SOD), which has been found ubiquitously among oxygen-metabolizing organisms, catalyses the dismutation of superoxide anion radical to molecular oxygen and hydrogen peroxide [1,2]. This enzyme has been proposed to play an important role in protecting living cells from the cytotoxic effects of the superoxide anion radical and of other reactive oxygen species formed from the superoxide anion radical, such as hydrogen peroxide, the hydroxyl radical and singlet oxygen [3]. So far three types of SOD have been classified according to the type of metal in the active site of the enzymes. Copper- and zinc-containing SOD (CuZnSOD) has been purified not only from a wide range of eukaryotes, including mammalian tissues [1] and *Neurospora crassa* [4], but also from some prokaryotes, such as *Caulobacter crescentus* [5] and *Haemophilus influenzae* [6]. Iron-containing SOD (FeSOD) has been found in prokaryotes including *Escherichia coli* [7,8] and *Methanobacterium bryantii* [9]. Manganese-containing SOD (MnSOD) has been found in prokaryotes including *E. coli* [10]. In the course of studying the enzymes involved in scavenging the reactive oxygen species in micro-organisms, we reported the characteristics of catalase–peroxidase found in *Streptomyces* sp. IMSNU-1 [11] and tried to find SOD as one of generators of hydrogen peroxide, a substrate for catalase–peroxidases from *Streptomyces* sp. IMSNU-1 and *Strep. coelicolor* ATCC 10147. Here we report the purification and the remarkable characteristics of a novel type of SOD.

MATERIALS AND METHODS

Chemicals

Anti-mouse IgG (whole molecule), alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate, cytochrome *c*, diethylene-triaminepenta-acetic acid, dimethyl suberimidate, 5,5'-dimethylpyrroline-*N*-oxide (DMPO), guanidinium chloride, Nitro Blue Tetrazolium (NBT), xanthine, xanthine oxidase, molecular mass

marker kit (MW-SDS-17), DEAE-Sephacel and Superose 12 were purchased from Sigma; PICO-TAG for amino acid analysis and Protein-Pak DEAE 5PW from Waters; Chelex-100 resin from Bio-Rad; and CuZnSOD from bovine erythrocytes and molecular mass markers for gel filtration and SDS/PAGE from Boehringer Mannheim. All other reagents used were of the highest quality generally available.

Micro-organisms and culture conditions

The spores of *Streptomyces* sp. IMSNU-1 and *Strep. coelicolor* ATCC 10147 were collected with 20% (v/v) glycerol from plates grown at 28 °C for 5 days in Bennett medium; cells were grown under the same conditions as described previously [11].

The strains used to compare SODs were *E. coli* KCTC 11033, *Saccharomyces cerevisiae* ATCC 26787, *Strep. longisporoflavus* ATCC 19915 and *Strep. coelicolor* ATCC 10147. *E. coli* was cultivated at 37 °C for 18 h and all the other organisms were cultivated and harvested under the same conditions as *Streptomyces* sp. IMSNU-1.

Enzyme assays

The assay for the activity of SOD was performed by the indirect method based on the ability of SOD to inhibit the reduction of cytochrome *c* by scavenging superoxide anion radicals produced by the xanthine/xanthine oxidase system [1]. One unit of SOD activity was defined as the amount of enzyme that gave 50% inhibition of cytochrome *c* reduction. The enzyme activities of column fractions were assayed with NBT instead of cytochrome *c* [12]. Positive staining for SOD on non-denaturing polyacrylamide gel was performed as described previously [13].

Another assay for the activity of SOD was performed by means of a Bruker ESP 300S EPR spectrometer with the spin-trapping agent DMPO [14]: a mixture of 50 µl of 2 mM xanthine,

Abbreviations used: CuZnSOD, copper- and zinc-containing SOD; DMPO, 5,5'-dimethylpyrroline-*N*-oxide; FeSOD, iron-containing SOD; MnSOD, manganese-containing SOD; NBT, Nitro Blue Tetrazolium; NiSOD, nickel-containing SOD; SOD, superoxide dismutase.

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35 μ l of 5.5 mM diethylenetriaminepenta-acetic acid, 15 μ l of DMPO, 50 μ l of xanthine oxidase (0.1 unit/ml) and 10 μ l of purified SOD (3000 units/ml) was placed in a flat EPR cell and DMPO-OH adduct was recorded after 1 min of mixing. The EPR spectra were obtained at room temperature with a microwave power of 4 mW, a modulation amplitude of 0.1 mT, a modulation frequency of 100 kHz and a microwave frequency of 9.76 GHz.

Determination of protein content

The protein content was determined by the method of Bradford [15].

Enzyme purification

The mycelia were harvested by aspiration on filter paper with a Büchner funnel and washed with 0.85% KCl solution. The washed mycelia were suspended in buffer A (20 mM sodium phosphate, pH 7.4, containing 0.1 mM EDTA), and broken with a bead-beater (Biospec Production) for 5 min; the homogenate was centrifuged (4000 *g* for 15 min). Solid ammonium sulphate was added to the supernatant up to 80% saturation. The precipitate was collected by centrifugation (15000 *g* for 20 min) and resuspended in buffer A. The resuspended solution was dialysed against buffer A overnight. The dialysate was loaded on to a DEAE-Sephacel column (4 cm $\times$ 40 cm) previously equilibrated with buffer A and then eluted with a linear gradient of 0–0.5 M NaCl in buffer A. The active fractions were pooled and desalted with an Amicon YM10 membrane. These fractions were purified by a Waters Delta Prep 4000 chromatography system with a Protein-Pak DEAE 5PW column (2.15 cm $\times$ 15 cm) equilibrated with buffer A. After the column had been washed with 0.15 M NaCl in buffer A, the sample was eluted with a linear gradient of 0.15–0.30 M NaCl in buffer A. The active fractions were again pooled and desalted with an Amicon YM10 membrane. These fractions were further purified by preparative electrophoresis with Bio-Rad Model 491 Prep Cell apparatus. The active fractions were mixed with 0.1 M Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol and 0.002% Bromophenol Blue; this mixture was eluted with 25 mM Tris/HCl, pH 8.8, at 180 V on a 7.5% non-denaturing polyacrylamide gel (3.7 cm $\times$ 11 cm). The active fractions of SOD were pooled and buffer-exchanged with buffer A, then stored at -70°C .

Molecular mass determination

The molecular mass of purified enzyme was determined by gel-filtration chromatography on preparative grade Superose 12 (2 cm $\times$ 60 cm) calibrated with catalase (240 kDa), aldolase (158 kDa), albumin (68 kDa) and chymotrypsinogen (25 kDa). To determine the number of subunits in SOD, dimethyl suberimide was used as a cross-linking agent [16] and the cross-linked proteins were subjected to SDS/PAGE with a linear gel concentration gradient of 5–20% (w/v) [17]. As standard markers, α_2 -macroglobulin (170 kDa), β -galactosidase (116.4 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa) were used. Proteins were revealed by silver staining [18]. A molecular mass marker kit (MW-SDS-17) was used for low-molecular-mass markers.

Determination of metal contents

Purified SOD was dialysed against demetallized Milli-Q grade water for 24 h. The contents of each metal (Fe, Mn, Cu, Zn and

Ni) were measured with an AA-880 mark II atomic absorption spectrometer from Jarrell Ash.

Preparation and reconstitution of the apoenzyme

Apoenzyme was prepared by the procedure proposed by Privalle et al. [19]: purified enzyme was dialysed against 10 mM 8-hydroxyquinoline, 5 M guanidinium chloride, 0.1 mM EDTA and 5 mM Tris, pH 3.8, at 4°C for 18 h. For the reconstitution, apoenzyme solution was dialysed against several changes of 50 mM Tris buffer, pH 7.8, containing 1 mM metal salts for 24 h. Excess amounts of metal salts were removed by further dialysis against the same buffer. Denatured proteins were discarded by centrifugation at 12000 *g* for 15 min and then the solution was exchanged with 50 mM sodium phosphate buffer, pH 7.4, through an Amicon ultrafiltration system. All the buffers used in this study were demetallized by passing through Chelex-100 resin.

Amino acid analysis

Proteins were hydrolysed *in vacuo* in 6 M HCl at 110°C for 24 h, and amino acids were analysed by reverse-phase chromatography on a PICO-TAG column (8.5 mm $\times$ 300 mm; pore size, 5 μ m) after phenylisothiocyanate derivatization. Methionine and cysteine were oxidized to methionine sulphone and cysteic acid respectively by performic acid [20]. For the analysis of tryptophan, proteins were hydrolysed in alkaline conditions as described previously [21].

N-Terminal amino acid sequence analysis

For analysing N-terminal amino acid sequences, enzymes were subjected to Tricine/SDS/PAGE. After electrophoresis, electrotransfer of proteins was performed by the method proposed by Towbin et al. [22]. The N-terminal amino acid sequence of SOD was determined with a MilliGen/Biosearch 6600 Prosequencer protein-sequencing system from Millipore.

Preparation of SOD antiserum

Purified SOD was subjected to 10% Tricine/SDS/PAGE [23]. After electrophoresis, the major polypeptide was cut out from the gel and pulverized with a homogenizer. This pulverized preparation was used as an immunogen. Each of three mice was initially boosted subcutaneously with approx. 10 μ g of SOD emulsified in Freund's complete adjuvant. Approx. 10 μ g of SOD was subsequently injected for 8 weeks at 2-week intervals.

Immunoblot analysis

SDS/PAGE was performed on the same gel as described above. Electrotransfer of proteins was performed by the method proposed by Towbin et al. [22]. The blotted membrane was incubated with a 1:100 dilution of SOD antiserum and then with a 1:10000 dilution of anti-mouse IgG (whole molecule) alkaline phosphatase conjugate. The alkaline phosphatase was detected with 5-bromo-4-chloro-3-indolyl phosphate by the method proposed by Blake et al. [24].

Spectroscopic measurements

The absorption spectra of all enzyme preparations were measured at 25°C in 50 mM sodium phosphate buffer, pH 7.4, in a 1 cm quartz cuvette with a Shimadzu UV-265 spectrophotometer.

The EPR spectra were measured with 100 μ M of SOD in

sodium phosphate buffer, pH 7.4, with a Bruker ESP 300S EPR spectrometer at 77 K with a microwave frequency of 9.44 GHz, a microwave power of 1 mW and a modulation amplitude of 1.0 mT.

Preparation of protoplasts

Protoplasts were prepared as described previously [25] with some modifications: mycelia were incubated with 5 ml of lysozyme solution (2 mg/ml in PBS) at 30 °C for 1 h, filtered through glass wool, sedimented by centrifugation (3000 *g* for 5 min); the precipitated cells were then dissolved in PBS and used as protoplasts.

RESULTS AND DISCUSSION

Detection and purification of SOD

A single band with SOD activity was detected from the crude extract of *Streptomyces* sp. IMSNU-1 (Figure 1A). Some bacteria contain two types of SOD, namely MnSOD and FeSOD, whereas other bacteria contain only one kind of SOD. For example, *Streptococcus sanguis* contains MnSOD and *Bacillus cereus* contains FeSOD [26]. As outlined in Table 1, SOD from *Streptomyces* sp. IMSNU-1 was purified 20-fold relative to

ammonium sulphate fractionation with a recovery of 20.3 %. The purity was confirmed on 7.5 % non-denaturing polyacrylamide gel (Figure 1B) and SDS/polyacrylamide gel (results not shown). The rate of decrease of DMPO-OH production by this enzyme was also confirmed by means of EPR spectroscopy. The superoxide anion radical was allowed to react with a spin-trapping agent, DMPO, to produce a long-lived radical (DMPO-OH, $a_N = a_H = 1.49$ mT). The addition of SOD led to a decrease in the production rate of DMPO-OH, providing strong evidence that SOD purified from *Streptomyces* sp. IMSNU-1 can scavenge the superoxide anion radical produced by the xanthine/xanthine oxidase system (results not shown).

Molecular properties

The molecular mass of SOD was determined as 13.4 kDa by SDS/PAGE. To gain an insight into the enzyme's substructure, SOD was incubated with a cross-linking agent, dimethyl suberimidate, before denaturation and electrophoresis. Four bands (13.4, 26.9, 41.7 and 53.7 kDa) were found after SDS/PAGE [16] and the apparent molecular mass of the native enzyme was estimated to be approx. 60 kDa by gel filtration chromatography on Superose 12, indicating that the enzyme consists of four subunits. In general, the subunits of bacterial SODs have molecular masses in the range 18.0–22.0 kDa [7,10,27]. The molecular mass of *Streptomyces* SOD was much smaller than that of any other bacterial SOD. Most SODs are usually dimeric forms, although tetrameric forms were reported in FeSOD from *Methanobacterium bryanthii* [9] and MnSODs from higher organisms [26].

Stability to pH and temperature

This enzyme was stable up to 70 °C. At 75 °C, half of the activity was lost, and 20 % of the enzyme activity remained at 80 °C. In general, SODs have been reported to be resistant to thermal inactivation except for the CuZnSOD discovered recently in *E. coli* [28]. SOD from *Streptomyces* was also thermostable, like other SODs. More than 80 % of the enzyme activity was maintained over a broad pH range of 4.0–8.0, but at highly alkaline pHs (more than 9.0) the enzyme activity was rapidly lost (Figure 2).

Inhibition pattern

It is known that CuZnSOD is sensitive to H₂O₂ and cyanide [3], whereas FeSOD is sensitive to H₂O₂ but not to cyanide. MnSOD,

Figure 1 PAGE of *Streptomyces* SOD

(A) SOD activity was revealed with *o*-dianisidine and riboflavin as its substrates on 7.5 % non-denaturing polyacrylamide gel as described in [13]: lane a, crude extracts of *Streptomyces* sp. IMSNU-1 (80 µg); lane b, purified *Streptomyces* SOD (10 µg). (B) Protein was stained with Coomassie Blue on 7.5 % non-denaturing polyacrylamide gel: lane a, crude extracts of *Streptomyces* sp. IMSNU-1 (80 µg); lane b, purified *Streptomyces* SOD (10 µg).

Table 1 Purification of SOD from *Streptomyces* sp. IMSNU-1

Protein concentration was determined by the method proposed by Bradford [15]. One unit of SOD activity is defined as the amount of enzyme that could give 50 % inhibition of NBT reduction.

Step	Total protein (mg)	Total activity (10 ³ units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	1894.3	—	—	—	—
Ammonium sulphate precipitation	814.8	122.2	150.2	100	1
DEAE-Sephacel chromatography	225.3	90.6	401.4	73.9	2.7
DEAE 5PW chromatography	96.2	76.6	796.3	62.7	5.3
Preparative cell electrophoresis	8.2	24.8	3030.3	20.3	20.2

Figure 2 Effect of pH on the activity of *Streptomyces* SOD

Purified SOD was incubated for 30 min over a range of pH values. The buffer system used contained 0.2 M boric acid, 0.05 M citrate and 0.1 M trisodium phosphate.

Table 2 The inhibition of *Streptomyces* SOD by KCN, NaN₃ and H₂O₂

Aliquots of SOD dissolved in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 min with various concentrations of chemicals. SOD activity was assayed as described in the Materials and methods section.

Inhibitor	Concentration (mM)	Inhibition (%)
KCN	1	3.4
	5	70.0
	10	100.0
NaN ₃	1	0
	5	7.1
	10	15.0
H ₂ O ₂	1	16.8
	5	56.8
	10	73.4

in contrast, is inhibited by azide but not by cyanide or H₂O₂ [29]. The SODs were completely inhibited after the incubation with 10 mM KCN for 30 min (Table 2). The addition of 5 mM KCN inhibited 70 % of the SOD activity (Table 2). Also, the addition of H₂O₂ at a concentration of 5 or 10 mM inhibited 56.8 % and 73.4 % of the SOD activity respectively. However, only 15 % of the enzyme activity was inhibited by the addition of 10 mM

NaN₃ after 30 min of incubation (Table 2). The inhibition pattern of *Streptomyces* SOD by specific metal-associated inhibitors was similar to that of CuZnSOD.

Metal at the active site

It has been reported that SODs from various sources contain iron, manganese, copper and zinc in their active sites. As shown in Table 3, the atomic absorption analyses for metals contained in SODs from both *Streptomyces* sp. IMSNU-1 and *Strep. coelicolor* ATCC 10147 demonstrated the occurrence of 0.74 g-atom of nickel per mol of subunit. Small amounts of iron and zinc were detected only in the enzyme preparation from *Streptomyces* sp. IMSNU-1, and copper and manganese were not detected at all in the sample preparations (Table 3), even if the culture medium used in this work contained nickel, iron, zinc, manganese and copper. Therefore SODs purified from both *Streptomyces* sp. IMSNU-1 and *Strep. coelicolor* ATCC 10147 are considered to take up nickel specifically as the metal at the active site, and not iron, manganese, zinc or copper. The specific activity per metal of native nickel-containing SOD (NiSOD) was 50.9 units/ng-atom of Ni, which is closely similar to that of CuZnSOD (55.0 units/ng-atom of Cu) from bovine erythrocyte (Table 4). In contrast, the specific activity on a protein basis (units/mg of enzyme) of apo-NiSOD was only 7 % of the specific activity of the native enzyme on a protein basis. These results indicate that nickel in *Streptomyces* SOD might play a pivotal role in enzymic action, as does copper in CuZnSOD from bovine erythrocytes. It has been reported that nickel-oligopeptide with a histidine residue in the third position has the SOD-like activity of quenching the superoxide anion radical [30].

The specificity of metal replacement in the denatured apo-enzyme was investigated by the procedures described in experiments to reconstitute MnSOD from *E. coli* [19] and FeSOD and MnSOD from *Bacteroides thetaiotaomicron* [31]. In the present study, after dialysis in buffer containing nickel salts 12.5 % of the SOD activity was recovered, after dialysis in buffer containing iron salts 2.1 % of the enzymic activity was restored, and after dialysis in buffer containing manganese salts 4.6 % of the enzymic activity was restored (results not shown). Among metal salts, nickel salts were more specific than iron or manganese salts, although they did not completely restore the enzymic activity. It has been reported that urease could be partly activated *in vitro* by providing nickel in the presence of carbon monoxide, but complete activation *in vivo* required a series of protein components and a nickel-binding protein [32]. Hydrogenase has also been reported to require *hypB* gene product, a GTP-binding protein, to incorporate nickel into protein [33]. Therefore in NiSOD from *Streptomyces*, the low recovery of the enzymic

Table 3 Metal contents of SODs from *Streptomyces* sp. IMSNU-1 and *Strep. coelicolor* ATCC 10147

Metal contents were determined by means of atomic absorption spectroscopy as described in the Materials and methods section. The molarity of the subunit was calculated on the basis of a molecular mass of 13.4 kDa. Each metal content of culture medium was determined before inoculation. The mean values of two different analyses from two different sample preparations are given.

Metal	SOD from <i>Streptomyces</i> sp. IMSNU-1		SOD from <i>Strep. coelicolor</i> ATCC 10147		Culture medium (μM)
	(μg/mg of enzyme)	(g-atom metal/mol of subunit)	(μg/mg of enzyme)	(g-atom metal/mol of subunit)	
Nickel	3.31	0.74	3.23	0.74	0.29
Iron	0.38	0.09	—	—	4.96
Zinc	0.24	0.05	—	—	1.50
Manganese	—	—	—	—	0.15
Copper	—	—	—	—	0.42

Table 4 Comparison of SOD activities between native NiSOD and apo-NiSOD

SOD activity was assayed by an indirect method as described in [1]. The reaction mixture contained 0.1 mM EDTA, 50 μ M xanthine and 10 μ M cytochrome *c* in 50 mM potassium phosphate buffer, pH 7.8. The reduction of cytochrome *c* was followed at 550 nm after a suitable amount of xanthine oxidase, giving an absorbance rate between 0.02 and 0.04 min⁻¹, had been added to the reaction mixture. The Fridovich unit of SOD activity was the amount of enzyme that gave 50% inhibition of cytochrome *c* reduction.

Source	Metal content (ng-atom metal/mg of enzyme)	Specific activity	
		(units/mg of enzyme)	(units/ng-atom of metal)
NiSOD	66.4 Ni	3380	50.9 (Ni)
Apo-NiSOD	11.2 Ni	240	21.4 (Ni)
CuZnSOD	61.6 Cu	3390	55.0 (Cu)
	62.7 Zn	—	54.1 (Zn)

Table 5 Amino acid compositions of SODs from various sources

Abbreviation: n.d., not detected.

Amino acid	NiSOD, <i>Streptomyces</i> sp. IMSNU-1*	FeSOD, <i>E. coli</i> [7]	MnSOD, <i>E. coli</i> [10]	CuZnSOD, <i>C. crescentus</i> [5]
	(mol/mol of subunit)	(mol/mol of subunit)	(mol/mol of subunit)	(mol/mol of subunit)
Cys†	1.4	1.1	n.d.	1.8
Asp + Asn	8.4	22.4	21.0	15.9
Glu + Gln	11.4	15.8	18.4	6.2
Ser	15.3	9.8	10.8	6.9
Gly	43.5	15.9	13.2	19.2
His	1.8	5.7	6.2	8.0
Arg	3.5	4.0	5.2	2.1
Thr	3.8	12.9	9.3	10.9
Ala	8.2	26.4	23.6	25.9
Pro	2.8	8.8	7.7	8.2
Tyr	1.4	6.4	5.9	0.9
Val	4.4	11.0	10.2	12.2
Met	0.8	0.0	1.6	< 0.05
Ile	3.9	8.2	6.9	4.9
Leu	6.6	14.5	18.6	11.9
Phe	4.1	9.9	9.0	2.9
Trp‡	0.3	3.9	n.d.	0.8
Lys	3.5	10.2	14.6	8.7
Total	125.1	186.9	182.2	147.5

* Calculations were based on a molecular mass of 13.4 kDa.

† 6 M HCl hydrolysis after oxidation with performic acid as described in [20].

‡ Alkaline hydrolysis instead of 6 M HCl hydrolysis, as described in [21].

activity *in vitro* might be related to the occurrence of chaperones to make the mature enzyme form *in vivo*.

Amino acid composition

The amino acid composition of the NiSOD from *Streptomyces* was compared with those of other prokaryotic SODs with different metals (Table 5). Remarkable differences were found between these enzymes. Specifically, NiSOD from *Streptomyces* sp. IMSNU-1 contains a relatively large amount of glycine, similar amounts of serine and glutamic acid + glutamine, and relatively small amounts of the other amino acids.

N-terminal amino acid sequence

The N-terminal amino acid sequences of NiSODs purified from *Streptomyces* sp. IMSNU-1 and *Strep. coelicolor* ATCC 10147 are shown in Figure 3, together with those of other SODs known previously. The N-terminal amino acid sequences of the two NiSODs from *Streptomyces* were identical apart from the residues

Micro-organism	N-Terminal amino acid sequence
<i>Streptomyces</i> sp. IMSNU-1 NiSOD	X G D L P G X V Y D P A Q A
<i>Streptomyces coelicolor</i> NiSOD	H G D L P G G V Y D P A Q A
<i>Escherichia coli</i> MnSOD	S Y T L P S L P Y A Y D A L E
<i>Escherichia coli</i> FeSOD	S F E L P A L P Y A K D A L A
<i>Bacillus stearothermophilus</i> MnSOD	P F E L P A L P Y P Y D A L E
<i>Pseudomonas ovalis</i> FeSOD	A F E L P P L P Y A H D A L Q
<i>Saccharomyces cerevisiae</i> MnSOD	K V T L P D L K W D F G A L E

Figure 3 N-terminal amino acid sequences of SODs from various sources

The N-terminal amino acid sequences of SOD from *Streptomyces* sp. IMSNU-1 and *Strep. coelicolor* ATCC 10147 were compared with those of *E. coli* MnSOD [34], *E. coli* FeSOD [35], *B. stearothermophilus* MnSOD [36], *P. ovalis* FeSOD [37], and *S. cerevisiae* MnSOD [38]. X stands for a non-determined amino acid residue.

Figure 4 Immunoblot analysis of SODs from various sources

Soluble proteins from various sources were subjected to 10% Tricine-SDS/PAGE [23] and incubated, after electrotransfer to nitrocellulose, with a 1:100 dilution of the anti-SOD serum: lane a, cell extract of *Streptomyces* sp. IMSNU-1; lane b, that of *Strept. coelicolor* ATCC 10147; lane c, that of *Strept. longisporoflavus* ATCC 19915; lane d, that of *E. coli* KCTC 11033; lane e, that of *S. cerevisiae* ATCC 26787; lane f, bovine CuZnSOD; lane g, SOD purified from *Streptomyces* sp. IMSNU-1. The protein content loaded was 40 µg per slot, except for commercially purchased bovine CuZnSOD (5 µg), and purified SOD (10 µg).

Figure 5 Absorption spectra of *Streptomyces* SOD

Approx. 24 µM protein was used: curve A, native SOD; curve B, apoenzyme. The apoenzyme was prepared as described in the Materials and methods section.

that were not determined. However, we can find no sequence similarity to the other types of SODs except for the leucine and proline residues at positions 4 and 5.

Comparison of SODs by immunoblotting

To explore the immunological cross-reactivity with other SODs, crude extracts from several *Streptomyces* species, *E. coli* and *S. cerevisiae* as well as commercially available CuZnSOD from bovine erythrocytes were subjected to SDS/PAGE and immunoblotted with the antibody raised against SOD from *Streptomyces* sp. IMSNU-1 (Figure 4). This antibody detected only one band with a similar molecular mass within the genus *Streptomyces*. In

Figure 6 X-band EPR spectra of *Streptomyces* SOD

(A) Native SOD; (B) apoenzyme. Native enzyme and apoenzyme (each approx. 100 µM) dissolved in sodium phosphate buffer, pH 7.4, was frozen in liquid nitrogen. The EPR spectra were obtained at 77 K with a microwave frequency of 9.43 GHz, a microwave power of 1 mW and a modulation amplitude of 1.0 mT. The g values are indicated.

contrast, cross-reactivities were not detected with the crude extracts of *E. coli* and *S. cerevisiae*, nor with CuZnSOD from bovine erythrocytes.

Spectral properties

As shown in Figure 5, curve A, *Streptomyces* SOD exhibited absorption maxima at 276 and 378 nm, and a broad peak at 531 nm. These absorption maxima were distinct from those of CuZnSOD, MnSOD and FeSOD. The absorption bands at 276 and 378 nm originated from the apoprotein and Ni^{III} respectively, as shown in the spectrum in Figure 5, curve B. The absorption spectrum of FeSOD exhibited a broad featureless shoulder in the region 320–500 nm [7], that of MnSOD gave an absorption maximum at 473 nm [10], and that of CuZnSOD exhibited an absorption maximum at 660 nm and lacked the maximum peak at 280 nm [4].

As shown in Figure 6A, *Streptomyces* SOD exhibited a remarkable EPR spectrum with unique g values of 2.304, 2.248 and 2.012 at liquid nitrogen temperature, which was quite distinct from those of other SODs reported previously: the g values of the EPR spectrum of CuZnSOD were $g_{\text{m}} = 2.073$ and $g_{\text{L}} = 2.260$ [4] and those of FeSOD were $g_z = 4.90$, $g_x = 3.88$ and $g_y = 3.53$ [7], whereas the EPR spectrum of native MnSOD was not detected [10]. However, in the EPR spectrum of the apoenzyme all signals had disappeared (Figure 6B); also the saturation pattern of the microwave power for $g = 2.304$ was proportional to that for $g = 2.012$ on increasing the microwave power (Figure 7). All of the g values were therefore considered to originate from the same species.

The EPR spectrum of NiSOD was almost identical with that of Ni^{III} with a tetragonal symmetry of Ni^{III} -oligopeptides especially containing histidine, which have distinct g -values ($g_{xx} = 2.170$ – 2.340 , $g_{yy} = 2.163$ – 2.295 and $g_{zz} = 2.006$ – 2.025) [39,40]: in particular the three-line hyperfine splitting with 2 mT of splitting constant (a_3) in the region of $g = 2.012$ (Figure 6A) in NiSOD is quite similar to that in the g_{zz} region of the histidine-containing Ni^{III} -oligopeptides, strongly suggesting a species containing a single nitrogen nucleus (^{14}N , $I = 1$) bound in an axial position. It was similar to a slow relaxing rhombic EPR signal of periplasmic hydrogenase from *Desulfovibrio gigas* with $g_1 = 2.31$, $g_2 = 2.23$ and $g_3 = 2.02$ [41]. In the *D. gigas* hydrogenase no hyperfine splitting at $g = 2.02$ due to nitrogen coordination has been

Figure 7 The saturation of microwave power for the EPR signal of *Streptomyces* SOD

Approx. 100 μ M native enzyme dissolved in sodium phosphate buffer, pH 7.4, was frozen in liquid nitrogen (77 K). The EPR spectrum was obtained by an increase in microwave power (1–100 mW) with a microwave frequency of 9.43 GHz and a modulation amplitude of 1.0 mT.

observed, because the Ni^{III} coordination sphere is dominated by sulphur atoms [42].

Enzyme localization

It has been reported that some SODs are secreted extracellularly [6,43]. To investigate the localization of *Streptomyces* SOD, the enzyme activity in crude extract of mycelia was compared with that in protoplasts by using NBT as a scavenger of superoxide anion radicals produced by xanthine oxidase. Both preparations showed similar SOD activities, indicating that *Streptomyces* SOD is located in the cytoplasm.

Conclusion

Until now, SOD has not been purified and characterized from *Streptomyces*, which is widely used to study cellular differentiation as a member of the actinomycetes. Surprisingly, SOD purified from *Streptomyces* spp. contained nickel and thus it can be designated as NiSOD.

Recently it was reported that cobalt could be incorporated into the 'cambialistic' FeSOD of *Propionibacterium shermanii* [44]; also, silver could replace the copper site of CuZnSOD from *Saccharomyces cerevisiae* [45]. However, these modified SODs had much lower activities than the native ones. Nevertheless NiSOD from *Streptomyces* showed a similar specific activity per metal to that of bovine erythrocyte CuZnSOD. In addition, NiSOD from *Streptomyces* was unique in N-terminal amino acid sequence, amino acid composition and cross-reactivity against antibodies. With all these points taken into consideration, it is believed that SOD from *Streptomyces* is a novel type. Additionally, NiSOD was purified from *Strep. griseus* and *Strep. lavendulae* in our laboratory (results not shown). This NiSOD might therefore be universally distributed in the genus *Streptomyces*.

A unique form of SOD containing both Fe and Mn as active metals was isolated from *Nocardia asteroides*, another member of the actinomycetes, which secreted that SOD to the outer cell wall of the organism [43]. The secretion of this SOD seemed to afford *Nocardia* resistance to the attacks of neutrophils and macrophages [46]. In contrast, SOD activity was not detected in the culture filtrate of *Streptomyces*. That may be attributed to the fact that, unlike *N. asteroides*, most *Streptomyces* are non-pathogenic, even though both *Nocardia* and *Streptomyces* belong to the actinomycetes.

So far little understanding has been gained about nickel-proteins because of their scarcity in biological systems. Hydro-

genase, CO dehydrogenase, urease and methyl cofactor M reductase of methanogenic bacteria are known to contain nickel [41,47–49]. Thus NiSOD, as another type of nickel-containing protein, can contribute to an understanding of the role of nickel in biological systems.

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