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In vivo incorporation of cobalt into *Propionibacterium shermanii* superoxide dismutase

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Abstract

Propionibacterium shermanii, an aerotolerant anaerobic bacterium, has already been shown to incorporate, depending on the metal supplementation to the medium, either iron or manganese or copper into the same superoxide dismutase protein. The in vivo incorporation of cobalt in the same superoxide dismutase was obtained in an iron-, manganese- and copper-depleted medium. The protein was isolated and characterized by NMR which offers the possibility to identify the amino acid residues at the active site exploiting isotropically shifted proton resonance.

Key words: Superoxide dismutase; *Propionibacterium shermanii*

1. Introduction

Superoxide dismutases (SOD) are regarded as one of the defense mechanisms against oxidative stress. All SODs are metalloproteins, containing Cu^{2+}–Zn^{2+}, or Fe^{3+}, or Mn^{3+} as active metal cofactor. The Fe- and Mn-SODs exhibit high structural homology, but activity can only be reconstituted with the metal present in the native enzyme, with the exception of four groups of bacteria, building SODs which are active with either iron or manganese: *P. shermanii* [1,2], *Bacteroides fragilis* [3], *Bacteroides thetaiotaomicron* [4] *Bacteroides gingivalis* [5], *Streptococcus mutants* [6] and *Methylomonas J* [7]. SODs that are active with either iron or manganese incorporated into the same protein moiety are distinguished from other Fe- or Mn-SODs showing strict metal specifity, and are referred to as 'cambialistic' SODs. Recently, it has been shown that also bacteria which developed SODs showing activity only with iron or manganese can incorporate other metals in vivo if the 'native' metal is not available [8]. All these observations suggest that the group of Fe-/Mn-SODs is not restricted to a specific metal. In fact, we have shown that, in a medium lacking iron and manganese an enzymatically inactive Cu-SOD was build by *P. shermanii* with the same protein enzymatically active with iron or manganese [2]. Thus the question arises: can a substitution by other 'non-physiological' metals take place if iron-, manganese- and copper- are absent in the medium?

In the present study we show that in vivo incorporation of cobalt into the 'cambialistic' Fe-SOD of *P. shermanii* in an iron, manganese and copper depleted medium actually occurs.

2. Materials and methods

Propionibacterium freudenreichii sp. shermanii PZ3, obtained from the Deutsch Sammlung of Mikroorganisms (DSM, Braunschweig), was grown on a complex medium to obtain an Fe-SOD or on an iron, manganese and copper free synthetic medium containing only $CoCl_2$ and $ZnCl_2$ as transition metal ions. The SODs were isolated as described [1] SOD activity was determined and units were defined by the cytochrome *c*–xanthine oxidase method [9].

Electrophoresis was performed on polyacrylamide gels as described by Davis [10] and stained for SOD activity by the NBT assay [11] and for protein with Amido black [12]. Cobalt was visualized on gels by formation of the light blue thiocyanate complex as follows: the gels were boiled for 30 min before staining with a solution of sodium pyrophosphate (2%) and ammonium thiocyanate (6%) in water/acetone (1:10, v/v) until a blue band was observable.

The protein concentration was estimated colorimetrically [13].

Metals were determined by atomic absorption spectrometry with a Perkin-Elmer spectrophotometer.

The in vitro substituted Fe- and Co-SODs were obtained from the Fe-SOD as previously described [1], using $FeCl_2$, $CoCl_2$ or sodium hexanitrocobaltate (III) for the reconstitution.

X-band ESR-spectra were recorded on a Bruker ESR-320 spectrometer. ESR parameters were: frequency = 9.4302 GHz, temperature = 4 to 10 K, microwave power = 20 mW, modulation = 10 G. Protein concentration was 5.9 g/l in potassium phosphate buffer (50 mmol/l, pH 7.8).

NMR-spectra were recorded on a Bruker AM 400 spectrometer. The spectra were obtained by using a modified driven equilibrium Fourier transform (DEFT) pulse sequence to suppress water and bulk protein signals [14]. A delay time of 20 ms was applied to select the short relaxing resonances and suppress the long relaxing ones. A repetition

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Abbreviations: SOD, superoxide dismutase; Fe-SOD, iron containing superoxide dismutase; Mn-SOD, manganese containing superoxide dismutase; Co-SOD, cobalt containing superoxide dismutase; Cu-SOD, copper containing superoxide dismutase; *P. shermanii*, *Propionibacterium freudenreichii sp. shermanii*; *E. coli*, *Escherichia coli*.

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time of 40–50 ms was used to ensure the complete relaxation of the paramagnetic components of the spectrum. Numbers of scans ranged from 10000 to 40000 in order to obtain a satisfactory S/N ratio. The water signal assumed at 4.76 ppm, was used as reference of the ppm scale.

Optical spectra were recorded on a Kontron Uvikon 930 spectrophotometer in a 1 cm cuvette and CD spectra on a Jobin Yvon CD spectrophotometer model Mark III in a 0.1 cm cuvette in potassium phosphate buffer (50 mmol/l, pH 7.8).

Antibodies against the Fe-SOD were raised in rabbits by injection 1 ml SOD-solution (0.7 g/l SOD in potassium phosphate buffer, 50 mmol/l, pH 7.8 supplemented with Freund's adjuvant) intracutaneously twice with an interval of 10 days and then with decreasing amounts of Fe- SOD (1 ml, 0.7 ml, 0.5 ml, 0.3 ml subcutan) each in intervals of 10 days. 40 ml blood was removed from the ear of rabbits. Serum was received after coagulation of the blood by centrifugation and the antibody-titer was controlled by ELISA-tests. Immunoblotting was performed on nitrocellulose membranes (Schleicher and Schüll) and the SOD-antibody reaction was visualized using the 5-bromo-4-chloro-3-indolyl phosphate (BCIP)-alkali phosphatase assay by standard methods.

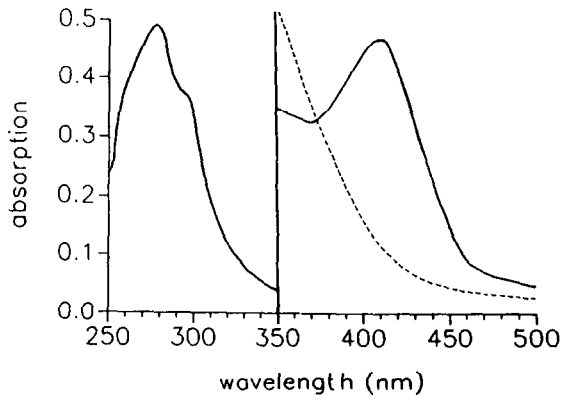

Fig. 2. Optical spectra of the Co- and Fe-SOD from *P. shermanii* Protein concentration for the Fe- and Co-SOD were 0.25 g/l in the UV and 7 g/l for the Fe-SOD and 3.6 g/l for the Co-SOD in the visible range of the spectrum. Spectra were recorded in 50 mmol/l phosphate buffer, pH 7.8 in a 1 cm quartz cell. —, Co-SOD; ---, Fe-SOD.

3. Results

Co-SOD was purified from bacteria grown on an iron, manganese and copper depleted medium by a similar procedure described for the purification of the Fe- or Mn-SOD from *P. shermanii* [1]. The same elution volumes were measured for the Fe-and Co-SOD in size-exclusion and ion-exchange chromatography.

The purified SOD moved as a single band on polyacrylamide and SDS-polyacrylamide gels showing comparable mobility to the Fe-SOD (Fig. 1). Cobalt in the SOD was visualized on gels with thiocyanate (Fig. 1).

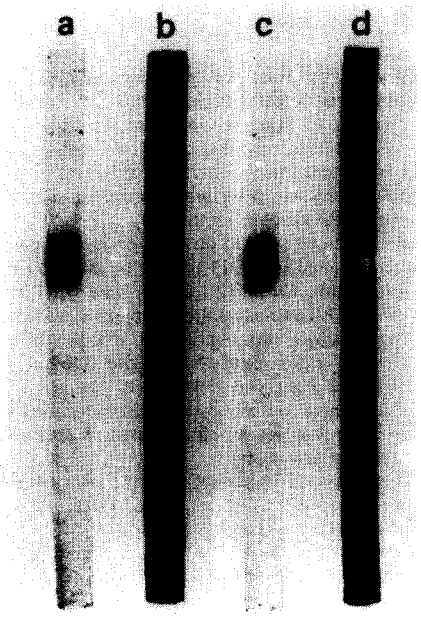

Fig. 1. Protein and cobalt stained polyacrylamide gel electrophoresis of the purified Co-SOD 35 μg purified Co-SOD was separated by electrophoresis on a 7.5% polyacrylamide gel and stained for protein (a), activity (b) and cobalt with thiocyanate (c). As comparison 35 μg of Fe-SOD were separated under the same conditions and stained for activity (d).

The purified Co-SOD contained 3.4 mol cobalt per mol SOD using the molecular weight determined by sequence of the Fe-SOD (22.637 kDa per subunit and 90.548 kDa per tetramer) [2]. The Co-SOD had less than 10% activity in comparison to the Fe-SOD.

The Co-SOD showed a positive reaction with antibodies raised against the Fe-SOD, indicating identical epitopes on the protein surface.

The Co-SOD had a weak blue-green color and the optical spectrum of the Co-SOD showed a stronger absorption in the visible region compared to the Fe-SOD. The UV spectrum was identical to that of Fe-SOD (Fig. 2).

The CD spectrum in the UV range of the Co-SOD was only slightly different from the Fe-SOD and showed a typical alpha-structure (Fig. 3).

No ESR-signals typical of cobalt signals were observed in EPR-spectra in the range of 4 to 10 K.

Co-SOD samples show isotropically shifted resonances in the NMR-spectum (Fig. 4) in contrast to Fe-SOD. Signals were observable in the range of 70 to −35 ppm. In the lowfield region (60 to 25 ppm) and in the highfield one (−0.5 to −35 ppm) broad bands are clearly visible.

An in vitro substitution of the apo-SOD with cobalt led to a lower extent of substitution than the one obtained in vivo (Table 1). Like the in vivo substituted form of SODs the in vitro incorporation of cobalt did not restore activity. Only Co^{2+} was able to bind to apo-SOD, while no incorporation of Co^{3+} was observable.

4. Discussion

The superoxide dismutase of *P. shermanii* can incorporate in vivo a much wider variety of trace metals than

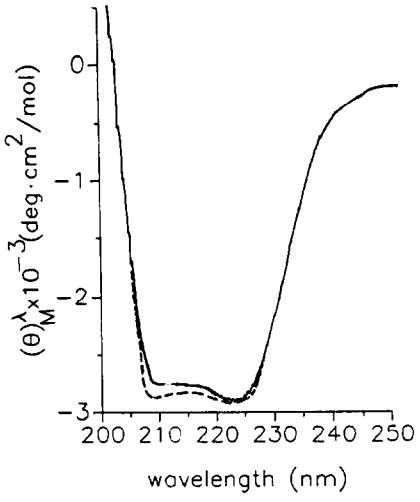

Fig. 3. CD-spectra of the Co- and Fe-SOD from *P. shermanii* Protein concentration was 2 g/l. Spectra were recorded in 50 mmol/l phosphate buffer, pH 7.8 in a 0.1 cm quarz cell. —, Co-SOD; ---, Fe-SOD.

previously recognized. As a function of metal supplementation to the medium iron, manganese, copper and cobalt display binding by the same SOD protein moiety. This excludes a strict metal specifity of SODs as the type of metal incorporated into the active center is strongly dependent on growth conditions including the metal availability. Growth on a complex medium supplemented with a large variety of metals might also induce the in vivo incorporation of these metals beside iron or manganese in different amounts depending on the growth conditions. This hypothesis can explain on the one hand why only half of the metal to protein ratio was detected in the Fe-SOD isolated from bacteria grown on a complex medium in comparison to the Mn-, Cu- and Co-SODs grown on a synthetic medium [1,2]. A non stochiometric incorporation of metals as well as the incorporation of traces of other metals beside the 'native' metal was also detected in several other SODs beside those showing activity with either iron or manganese. Differences up to 100% were reported p.e. for the Fe- and Mn-SOD from *E. coli* [15–18], and the Fe-SODs from *Plectonema boryanum* [19,20], *Desulfovibrio desulfuricans* [21], *Photobacterium leiognathi* [22–24] and *Pseudomonas ovalis* [25].

Table 1
In vitro metal exchange of the Fe-SOD from *P. shermanii*

Sample	Metal content	Activity
Holoprotein	1.95 ± 0.4	100 ± 0.5
Apo-protein	0.57 ± 0.1	31.5 ± 0.5
Apo-protein + Fe^{2+}	1.79 ± 0.2	85.7 ± 3.5
Apo-protein + Co^{2+}	0.89 ± 0.2	31.3 ± 0.3
Apo-protein + Co^{3+}	0.00	31.0 ± 0.8

The in vivo incorporation of cobalt, described here for the first time, into the Fe-SOD offers a new tool in order to investigate the active center of the enzyme by NMR-spectroscopy. The chemical shifts and broad linewidths observed in the NMR-spectrum of the Co-SOD are diagnostic of the presence of a coordinated paramagnetic metal ion. In particular, such resonances have typical isotropic shifts due to interaction, via molecular orbitals or dipolarly through space, of unpaired electrons with the magnetization of the nuclear spins. In our samples, protons giving resonances of this type should belong to residues directly coordinated to the cobalt. The values of the chemical shifts of the lowfield shifted resonances can be considered as indicative of a tetrahedrically coordinated cobalt. In fact a shift of 60–50 ppm is found in cobalt-substituted carbonic anhydrase. In the case of pentacoordinated cobalt isotropic shifts of lesser extent have been observed, as in the case of cobalt substituted alkaline phosphatase [26].

The nature of coordinating residues can not be identified at the present stage. However, if the ligands were histidines, as often is the case of protein-bound metal ions, the resonances in the lowfield region could belong to the imidazole protons with the exclusion if the CE1 protons, which generally are not visible due to their extremely large linewidths.

In the case of Cu(I)Co(II) superoxide dismutase, where cobalt ion replaces Zn(II) in the tetrahedrical coordination site, the imidazole proton of the three liganding histidines resonate in the range between 65 and 30 ppm [27,28].

The resonance in the region between 16 and −15 ppm could be due to the beta protons. The broad band at −40 ppm can be assigned either to beta protons of the coordinating residues or to groups not belonging to the coordination sphere but very near to the paramagnetic metal ion. This has already been found in the case of methyl groups of a Thr residues very close to cobalt in the cobalt-substituted carbonic anhydrase [26].

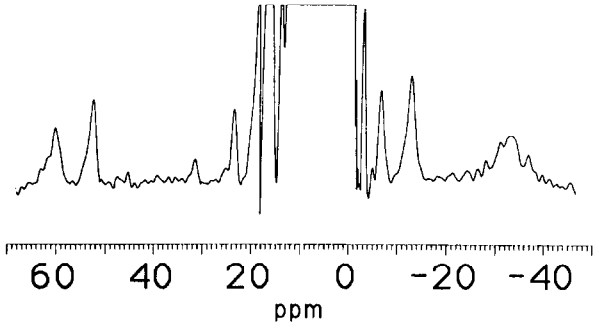

Fig. 4. MNR-spectra of the Co-SOD from *P. shermanii* Protein concentration was 5.9 g/l. Spectra were recorded in 50 mmol/l phosphate buffer, pH 7.8 with 10% D_2O. The spectrum was obtained as reported in section 2. The usual range for protein resonance is 15 to −4 ppm. The resonances observable are due to the effects of the unpaired spin of the coordinated metal.

Work is in progress in our laboratory to obtain larger amounts of protein in order to perform 2D NMR experiments that could lead to precise assignments of resonances, from which structural parameters about ligands and geometry of the metal coordination site can be obtained.

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