Regulation of an *in vivo* metal-exchangable superoxide dismutase from *Propionibacterium shermanii* exhibiting activity with different metal cofactors

Anja P. SEHN and Beate MEIER*

Chemisches Institut der Tierärztlichen Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany

The anaerobic, but aerotolerant *Propionibacterium freudenreichii* sp. *shermanii* contains a single superoxide dismutase [EC 1.15.1.1.] exhibiting comparable activity with iron or manganese as metal cofactor. The formation of superoxide dismutase is not depending on the supplementation of iron or manganese to the culture medium. Even in the absence of these metals the protein is built in comparable amounts. Bacteria grown in the absence of iron and manganese synthesize a superoxide dismutase with very low activity which had incorporated copper. If the medium was also depleted of copper, cobalt was incorporated, leading to an enzymically inactive form. In the absence of cobalt

an enzymically inactive superoxide dismutase was built with unknown metal contents. Upon aeration the amount of superoxide dismutase activity increased continuously up to 9 h, due to a *de novo* synthesis of the protein. This superoxide dismutase had incorporated iron into the active centre. The superoxide dismutase of *Propionibacterium shermanii* is able to form a much wider variety of complexes with trace metal ions *in vivo* than previously recognized, leading to the hypothesis that the original function of these proteins was the binding of cytoplasmic trace metals present in excess.

INTRODUCTION

Superoxide dismutases (SODs) (EC 1.15.1.1.) are metalloproteins belonging to a family of antioxidative enzymes which provide the cell with a major defence against oxidative damage. They contain either Cu²⁺, Fe³⁺, or Mn³⁺ as active metal cofactors. Function and distribution of the various forms of SOD have been reviewed [1].

Bacteria usually have one or two SODs with iron or manganese as the active metal cofactor. Fe- and Mn-SODs exhibit high structural similarity, but activity could only be reconstituted with the metal present in the native enzyme [2–6]. Up to now four groups of bacteria are known, which show activity with iron or manganese: *Propionibacterium shermanii* [7], *Bacteroides fragilis* [8], *Bacteroides thetaiotaomicron* [9], *Bacteroides gingivalis*, [10], *Streptococcus mutans* [11] and *Methylobacterium J* [12]. SODs active with either iron or manganese have been termed cambialistic SODs [11].

Also bacteria which developed SODs showing activity only with iron or manganese can incorporate the other metal *in vivo*, if the native metal is not available. This was extensively studied in *Escherichia coli* possessing a constitutive Fe-SOD and an inducible Mn-SOD [13–15]. Despite a high structural similarity and comparable catalytic constants the genes of the Mn- and Fe-SOD respond to environmental signals quite differently. The Mn-SOD transcription is not only regulated by oxygen or redox-active drugs but also by iron in a complicated way, as reviewed recently [16]. In contrast, the Fe-SOD activity responds to none of these factors. Therefore Fe-SOD is generally regarded as constitutive and Mn-SOD as inducible, especially with respect to oxidative stress.

Furthermore the eukaryotic Cu,Zn-SOD turned out to be regulated by copper being the active metal cofactor [17]. As in *E.*

coli, post-transcriptional regulation was also observed, as in the absence of copper an inactive metal-free (apo) form of SOD was synthesized [18].

Up to now it was not known whether transcription of the ‘cambialistic’ SODs was regulated by iron or manganese, or whether metal-free apo form was synthesized in the absence of these metals. Therefore one aim of this study was to investigate whether the SOD of *P. shermanii* is regulated by transition metals.

First of all, oxygen and superoxide are potent substances for inducing the formation of SOD even in anaerobic bacteria. Bacteria possessing SOD activities with iron or manganese synthesize an Fe-SOD in anaerobic conditions, whereas oxygenation triggers the incorporation of manganese into the protein moiety [8–11]. *P. shermanii* can grow only if the oxygen supplementation is lower than the respiratory capacity, but under these conditions the bacterial growth is stimulated in comparison with strict anaerobic conditions. As soon as oxygen is detectable in the medium it is toxic to the bacteria and the cell yield is reduced drastically. Equally SOD activity first increased after supplementation of oxygen in low amounts to a continuous culture and decreased drastically with increasing oxygen concentrations [19]. So we decided to re-investigate the effects of oxygen and superoxide radicals on the induction and metal-incorporation of SOD, by aerating cultures in the late logarithmic state.

MATERIAL AND METHODS

Propionibacterium freudenreichii sp. *shermanii* PZ3 was obtained from the Deutsche Sammlung von Mikroorganismen (DSM) and grown in complex medium to produce an Fe-SOD, or in a

Abbreviations used: SOD, superoxide dismutase; Cu,Zn-SOD, copper,zinc-containing SOD; Fe-SOD, iron-containing SOD; Mn-SOD, manganese-containing SOD; *P. shermanii*, *Propionibacterium freudenreichii* sp. *shermanii*

* To whom correspondence should be addressed.

synthetic medium substituted with $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (2.24 mg/l), CuSO_4 (0.25 mg/l), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.5 mg/l) and ZnCl_2 (2.9 mg/l) as transition-metal ions [7]. To study the regulation of SOD protein by metals the medium was subsequently depleted of manganese, copper and cobalt. This was done by passaging the bacteria at least six times by diluting them 1:10 with medium lacking these metals. Bacteria were harvested and cytoplasm was obtained as described [7].

Electron-transporting particles were isolated from the cytoplasm by ultracentrifugation (105000 *g* for 1 h) and stored in liquid nitrogen. Inside-out particles were prepared by sonication for 60 s, with cycles of 2 s of sonication and 2 s intervals using a Braun ultrasonicator (Melsungen) at 100 W.

Fe-SOD was isolated as previously described [7].

SOD activity was determined by the cytochrome *c*-xanthine oxidase method [20]. To test the possibility of reconstituting SOD activity *in vitro* by adding Fe and/or Mn salts to the cytosol FeCl_2 , FeCl_3 or MnCl_2 (each 1 mmol/l) respectively, or a combination of these salts, were added to the cytoplasm of bacteria showing an active SOD protein for 1 h at room temperature. Afterwards the samples were dialysed three times against 1 litre of potassium phosphate buffer (50 mmol/l, pH 7.0), each time for 8 h, at 4 °C followed by determination of SOD activity.

The production of superoxide radicals by inverted electron-transporting particles was followed by determination of the SOD-inhibited part of the cytochrome *c* reduction at 550 nm or Nitroblue Tetrazolium reduction at 430 nm using a Kontron Uvikon 930 spectrophotometer (Hannover) in a 1 cm cuvette. NADH (1 mmol/l) in phosphate-buffered saline (potassium phosphate buffer, 50 mmol/l, pH 7.0, supplemented with 150 mmol/l NaCl) was used as substrate.

Aeration of *P. shermanii* was performed in late logarithmic state by bubbling air through a vigorously stirred culture. Aliquots were removed at the times indicated and bacteria were sedimented by centrifugation (10 min at 10000 *g*). The bacteria were stored frozen at -20 °C. Preparation of cytoplasm and activity tests were performed as described [7].

Electrophoresis was carried out on polyacrylamide gels as described by Davis [21] and were stained for SOD activity by the Nitroblue Tetrazolium assay [22] and for protein with Amido Black [23]. The protein concentration was estimated colorimetrically [24].

To visualize copper or cobalt on gels, sensitive colorimetric methods established for selective and quantitative determinations of traces of copper or cobalt were modified for staining on gels [25]. Staining of Cu^{2+} with salicylic acid as a red, stable complex turned out to be the most useful and sensitive method, while Co^{2+} could be determined with ammonium thiocyanate as a light-blue complex which faded slowly within some weeks.

To stain for copper the gels were boiled for 45 min in a mixture of 200 μl of potassium nitrate (2%) and 200 μl of acetic acid added to 3 ml of 0.5% salicylic acid in ethanol/water (1:10, v/v). After cooling to room temperature the colour developed slowly within some hours.

To stain for cobalt the gels were boiled for 30 min in water and, after cooling to room temperature, incubated in a solution of sodium pyrophosphate (2%) and ammonium thiocyanate (6%) dissolved in water/acetone (1:10, v/v). A light-blue colour was observable within some hours.

E.s.r. spectra were recorded on a Bruker ECS-1-6 spectrophotometer (Karlsruhe) in the X-band with the following parameters: frequency, 9.4302 GHz; temperature, 6 K; microwave power, 40 mW, modulation, 1.5×10^{-3} T; amplification, 10^5 . Bacteria were measured as a wet pellet obtained after centrifugation in a quartz tube opened at both sides. The purified

SOD was measured in a protein concentration of 17.5 mg/ml in potassium phosphate buffer (50 mmol/l, pH 7.8).

Antibodies raised against the Fe-SOD were prepared in rabbits by intracutaneous injection of 1 ml of SOD solution (0.7 g/l SOD in potassium phosphate buffer 50 mmol/l, pH 7.8, supplemented with Freund's adjuvant) 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) delivered subcutaneously each at intervals of 10 days. No more than 40 ml of blood was removed from the ears of the rabbits. Serum was obtained after coagulation of the blood by centrifugation. The antibody titre was determined by e.l.i.s.a. Immunoblotting was performed on nitrocellulose membranes (Schleicher and Schüll) and the SOD-antibody reaction was visualized using the 5-bromo-4-chloro-3-indyl phosphate/alkaline phosphatase/Nitroblue Tetrazolium assay by standard methods [26].

RESULTS

Depletion of the culture medium of different trace metal ions drastically decreased the growth rates and SOD activities of *P. shermanii* (Table 1). When the medium was depleted of iron, manganese was incorporated into the active centre without loss of activity [7]. When the medium was lacking iron and manganese a low SOD activity, which was not removable by dialysis, was observed when copper was added to the medium. When copper was also absent in the medium, no SOD activity was observable after dialysis. This is in accordance with the results obtained from staining for activity after separation of the proteins by gel electrophoresis (Figure 1). By supplementation of the medium with iron or manganese a high SOD activity was observed, whereas bacteria grown on a medium without these metals only showed a weak activity band when the medium was supplemented with copper.

Thus the following question had to be answered: is an SOD synthesized in the absence of manganese and iron? A polyclonal antibody was raised against the Fe-SOD, and cytoplasm of bacteria grown on media subsequently depleted of trace metals

Table 1 Influence of metal supplementation to the culture medium on the growth rates and SOD activities of *P. shermanii*

1 g wet weight of bacteria corresponds to 175 ± 8.35 mg of protein. Mean values and S.D.s were obtained from four independent experiments.

Trace element in material	Growth rate (g wet weight/l)	SOD activity (units/mg)	SOD activity after dialysis (units/mg)
Fe, Mn, Cu, Co, Zn	9.3 ± 0.2	$3.3 \pm 1.0^\dagger$	4.1 ± 0.5
Mn, Cu, Co, Zn	4.5 ± 0.3	$5.4 \pm 1.3^\dagger$	3.9 ± 0.5
Cu, Co, Zn	4.3 ± 0.3	2.1 ± 0.7	0.95 ± 0.5
Cu, Co	3.6 ± 0.5	2.7 ± 0.5	0.72 ± 0.3
Cu, Zn	4.2 ± 0.5	2.0 ± 0.3	0.72 ± 0.2
Co, Zn	3.3 ± 0.5	2.7 ± 1.4	$< 23\%$
Co	1.8 ± 0.6	2.1 ± 1.2	$< 10\%*$
Zn	1.5 ± 0.6	0.00	0.00
—	1.1 ± 0.3	0.00	0.00

* A 50% inhibition to define 1 unit was not reached by adding cytoplasm (10%, v:v) to the reaction mixture.

† The difference in the SOD activity detectable in the crude extract between the culture in the presence of iron or absence of iron is significant with $P > 0.99$ using the chi-square analysis. However, a lower SOD activity in the cytoplasm of bacteria grown in the presence of iron is often observed, and is possibly due to the production of O_2^- by membrane fragments still present, and artificially minimizing the real SOD activity [7].

Figure 1 Activity-stained PAGE

Cytoplasm of *P. shermanii* grown in the presence of different trace metals were separated by electrophoresis on a 10% polyacrylamide gel and stained for activity using the Nitroblue Tetrazolium assay. The lanes were loaded with samples of cytoplasm from bacteria grown in the presence of (lanes 1), Fe^{2+} , Mn^{2+} , Cu^{2+} , Co^{2+} , and Zn^{2+} ; (lanes 2) Mn^{2+} , Cu^{2+} , Co^{2+} , and Zn^{2+} ; (lanes 3) Cu^{2+} , Co^{2+} , and Zn^{2+} ; (lanes 4), Co^{2+} and Zn^{2+} ; (lanes 5), Zn^{2+} only. To each lane 200 μg or 20 μg (+) of protein (cytoplasm) was applied.

Figure 2 Staining with antibodies for SOD protein

Cytoplasm of *P. shermanii* grown in the presence of different trace metals were separated by electrophoresis on a 10% polyacrylamide gel and stained with antibodies directed against the Fe-SOD for the presence of SOD protein as described in the Materials and methods section. To lane (1) 10 μg of Fe-SOD was applied. The other lanes (2–6) were loaded with 200 μg of protein (cytoplasm) from bacteria grown in the presence of (lane 2, Fe^{2+} , Mn^{2+} , Cu^{2+} , Co^{2+} , Zn^{2+} ; lane 3, Mn^{2+} , Cu^{2+} , Co^{2+} , Zn^{2+} ; lane 4, Cu^{2+} , Co^{2+} , Zn^{2+} ; lane 5, Co^{2+} , Zn^{2+} ; lane 6, Zn^{2+}).

were separated on acrylamide gels and stained for the SOD protein with this antibody (Figure 2). In all samples the SOD was detectable. The reaction was completely prevented by adding purified SOD to the antibody, which indicated a specific reaction. These results prove that the SOD protein was still synthesized in the absence of iron and manganese.

A further question arose from these observations: is a metal-free apo protein synthesized in the absence of iron or manganese, or are other trace metals which are still available in the medium incorporated into the protein moiety? To answer this question specific sensitive methods for staining copper and cobalt were developed as described above. If no iron and manganese supplementations were added to the medium, copper was incorporated

Figure 3 Staining for copper on gel electrophoresis with salicylate

Cytoplasm of *P. shermanii* grown in the presence of different trace metals were separated by electrophoresis on a 10% polyacrylamide gel and stained for the presence of copper as described in the Materials and methods section. The lanes were loaded with 200 μg of protein (cytoplasm) from bacteria grown in the presence of (1) Fe^{2+} , Mn^{2+} , Cu^{2+} , Co^{2+} , Zn^{2+} ; (2) Mn^{2+} , Cu^{2+} , Co^{2+} , Zn^{2+} ; (3) Cu^{2+} , Co^{2+} , Zn^{2+} ; (4) Co^{2+} , Zn^{2+} ; (5) Zn^{2+} .

Figure 4 Staining for cobalt on gel electrophoresis with thiocyanate

(a) Cytoplasm of *P. shermanii* grown in the presence of Co^{2+} and Zn^{2+} (500 μg of protein) and (b) purified Fe-SOD (17.5 μg of protein) were separated by electrophoresis on a 10% polyacrylamide gel and stained with thiocyanate in order to demonstrate the presence of cobalt and SOD-activity as described in the Materials and methods section.

into the SOD, as shown in Figure 3. The staining for cobalt was negative. If the medium was furthermore depleted of copper, cobalt was incorporated (Figure 4) leading to an inactive SOD. No cobalt was detectable if the bacteria were grown in the presence of copper, iron or manganese.

It was not possible to reconstitute SOD activity upon addition

Figure 5 Effect of oxygen on the SOD-activity of *P. shermanii*

P. shermanii grown in the presence (a) or absence (b) of iron were aerated in the late logarithmic state and SOD activities were determined in the cytoplasm by the cytochrome *c* test. The inhibition of cytochrome *c* reduction upon addition of 20 µg of protein (cytoplasm) from bacterial culture was determined. Bacteria were aerated: without any addition (●); with 2 mmol/l methyl viologen (○); with 0.1 mmol/l chloramphenicol (□); or with 2 mmol/l methyl viologen and 0.1 mmol/l chloramphenicol (△). Mean values and S.D. were calculated from three different experiments. Induction of SOD activities were different in the presence and absence of methyl viologen by $P > 0.99$ using the chi-square analysis, whereas no differences were observable between these treatments in the presence of chloramphenicol. Also the differences observable between the bacteria grown in the presence or absence of iron were significant by $P > 0.99$.

of Fe^{2+} , Fe^{3+} or Mn^{2+} to the cytoplasm of bacteria, showing an inactive SOD protein.

When bacteria, grown on an iron-supplemented or iron-depleted medium, were aerated a subsequent increase of SOD activity was determined, which was completely abolished by chloramphenicol (Figures 5a and 5b). Thus the alteration was due to a *de novo* synthesis of SOD and not to activation of an inactive form. Bacteria grown on an iron-supplemented medium showed a stronger induction in comparison with bacteria grown on iron-depleted medium. This is in correlation to the superoxide release by electron-transporting particles which was 1.7 nmol/min per mg of protein in particles obtained from bacteria grown on an iron-supplemented medium and 0.8 nmol/min per mg of protein in the case of an iron-depleted medium. Methyl viologen (0.1–2 mmol/l), which can potentiate O_2^- release when it is reduced by the electron-transport chain, further induced superoxide release in bacteria grown on an iron-supplemented medium

Figure 6 E.p.r. spectra of *P. shermanii*

E.p.r. spectra were recorded as described in the Materials and methods section. *P. shermanii* in the late logarithmic state was measured without aeration (a) and after aeration for 3 h in the presence of methyl viologen (b).

but not in those grown on an iron-depleted medium (Figures 5a and 5b).

In accordance with this observation, methyl viologen (0.1–2 mmol/l) triggered an increased O_2^- release by a factor of 1.8 in electron-transporting particles (inside-out) isolated from bacteria grown in the presence of iron, but not in particles from bacteria grown in the absence of this metal.

By heating the cytoplasm for 1 min to 60 °C SOD activity increased by a factor of 2 in all cases, indicating interference of heat-labile compounds with the test system as already observed previously [7].

In order to test whether the induced SOD had incorporated iron or manganese as active cofactor e.p.r. spectra of the bacteria were recorded and quantified. Bacteria in the late logarithmic state showed an iron signal in the $g = 4$ region which is caused only by Fe-SOD and corresponds to the spectrum of the isolated enzyme at pH 5.5–6.0 (A. P. Sehn and B. Meier, unpublished work). If manganese is incorporated in the SOD no e.p.r. signals were observed. The amount of iron incorporated into the SOD corresponds directly to the spin concentration obtained by double integration of the spectrum. After aerating the bacteria grown on an iron-supplemented medium in the presence or absence of methyl viologen the spin concentration of the iron signal increased with time, indicating that in the presence of oxygen iron was still incorporated into SOD.

DISCUSSION

P. shermanii produces a single constitutive SOD which is active with either iron or manganese as metal cofactor incorporated into an identical protein moiety [7,27]. If the medium was depleted of iron and manganese then copper or cobalt were incorporated into the same protein *in vivo* depending on the trace-metal supplementation, as could be proven for the Cu-SOD by sequence analysis [27]. The Co-SOD showed an identical molecular mass and electrophoretic mobility as well as identical

u.v.- and c.d.-spectra in comparison with the other SOD forms, suggesting the formation of an identical protein moiety (A. P. Sehn and B. Meier, unpublished work). Copper and cobalt were incorporated in nearly stoichiometric amounts: 3.2 mol of Cu/mol of protein tetramer or 3.4 mol of Co/mol of protein tetramer ([27]; A. P. Sehn and B. Meier, unpublished work). Thus the SODs may show a much wider ability to form complexes with trace-metal ions *in vivo* than previously recognized. On the other hand full activity was only obtained by incorporation of iron or manganese, even if a very low activity was still detected in the presence of copper. Remarkable is the metal specificity: i.e. if the medium is depleted of iron, manganese is incorporated; if the medium is depleted of manganese, copper is incorporated; and if copper also is depleted, cobalt is incorporated into the same protein with high preference to each metal. In each case the other trace metals also present in the medium are only bound in minor amounts, a phenomenon whose reason is unknown. It might be speculated that the formation of a protein-metal complex is mainly affected by the cytoplasmic concentration of the trace metal, because a lack of iron and manganese in the environment is affected by an increase of intracellular copper (Figure 3) or cobalt (Figure 4), which is mainly bound to the SOD.

P. shermanii synthesized the SOD protein independent from supplementation of trace metals in the medium. Because of this a transcriptional regulation by metals, as observed for the Mn-SODs in *E. coli* or the Cu,Zn-SODs in eukaryotic organisms, is not probable [13–17].

In *E. coli* the *fur* (fur uptake regulation) protein not only represses the transcription of the *sodA* gene (Mn-SOD) [28,29], but also the genes involved in iron assimilation [30–32]. By iron depletion siderophores are induced, showing a high affinity towards iron and thus maintaining their minimum level. However, they prevent the enrichment of 'free' iron, which would be toxic, by catalysing the Fenton reaction. In fact mutants showing a permanent derepression of iron assimilation exhibit an increased oxidative stress [29]. So oxygenation may lead to a decrease of 'free' iron being available for the bacteria and thus gives preference to the formation of Mn-SOD. Oxygen does not only induce the Mn-SOD by the *fur* gene but also by an *arc* (aerobic respiratory control) gene and other genes responding to redox-active compounds. The Fe-SOD on the other hand is not affected [17]. Hassan and Fridovich [33] and Nettleton et al. [34] showed that the Fe-SOD levels, in contrast with Mn-SOD actually decreased about 2-fold upon oxygenation of the cell cultures. Moreover, methyl viologen [35] and other redox-active aromatic drugs [36] trigger the induction of Mn-SOD, whereas no effect on the Fe-SOD was observed. A similar effect of methyl viologen was detected in *Photobacterium leiognathi*, the activity of the Cu,Zn-SOD (bacteriocuprein) increased upon addition of this drug whereas the Fe-SOD activity was not altered [37].

Also bacteria possessing SODs being active with iron or manganese synthesized an Fe-SOD in anaerobic conditions, whereas oxygenation triggered the incorporation of manganese into the protein moiety [8–11]. Iron seemed to be still available and was incorporated into other proteins, as could be proven with radiolabelling in *Streptococcus mutans* [10]. *P. shermanii* behaves differently, as oxygen not only stimulates the synthesis of SOD but also the incorporation of iron into the protein. *P. shermanii* has a special metabolism leading to the formation of propionic acid and the bacteria are relative insensitive to acidification of the culture medium down to pH values of 5–6. Under these conditions iron is not precipitated as its hydroxide and so is still available to the bacteria in large amounts. Additionally the redox potential of $\text{Fe}^{2+}/\text{Fe}^{3+}$ is strongly pH-

dependent and Fe^{2+} is not spontaneously oxidized under these conditions. The redox potential of the medium is additionally decreased upon addition of dithiothreitol, which has to be added to support the growth of the bacteria. All these factors make iron available under aerobic conditions and could be a reason for the formation of Fe-SOD.

Regulation of SOD transcription by metals was not observed in *P. shermanii* in contrast with *E. coli* and eukaryotic organisms up to mammals. *E. coli* belongs to the group of enterobacteria and is phylogenetically much 'younger' than the four bacterial groups exhibiting SODs which show activity with different metal cofactors incorporated into the same protein moiety. So these SODs may be regarded as the prototypes of the Fe- and Mn-SODs which later developed metal specificity due to special environmental conditions. In evolutionary terms, regulation of SOD isoenzymes by metals is only advantageous if it prevents the formation of inactive SOD-forms. Thus a response of genes, regulating the transcription of SODs, to metals has to be regarded as a phylogenetic late achievement.

Since the discovery of SODs in anaerobic bacteria in comparable amounts to aerobic organisms (in the anaerobic *Methanobacterium bryantii* SOD runs up to 0.4% [38] and in *P. shermanii* up to more than 1% [7,27] of the cytoplasmic proteins) doubts about the enzymic function of the SOD and questions about another 'activity' have arisen. The observation that SOD of *P. shermanii* incorporates a variety of metals *in vivo* suggests the hypothesis that this protein originally functioned to form complexes with trace metals present in excess, which were toxic to the organisms. This might still be a reason why SOD is synthesized by anaerobic bacteria. With increasing concentrations of oxygen in the environment, the dismutation of superoxide radicals probably became a major function in aerobic organisms.

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