Comparative stability studies on the iron and manganese forms of the cambialistic superoxide dismutase from *Propionibacterium shermanii*

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Abstract The superoxide dismutase of *Propionibacterium shermanii* shows similar activity with iron and manganese bound at the active site of the protein. On the other hand, the iron form, in comparison to the manganese form, exhibits higher stability towards thermal- and pH-dependent inactivation. Upon inactivation the metal ions are released from the active site. Thus, in comparison to the manganese form, a higher stability of the iron–protein complex might be the triggering reason for this behavior.

Key words: Superoxide dismutase; pH stability; Thermal stability; (*Propionibacterium shermanii*)

1. Introduction

Superoxide dismutases (SOD) are metalloproteins containing Cu^{2+}, or Fe^{3+}, Mn^{3+} or Ni^{3+} as active metal cofactor. They are regarded as one of the defending enzymes against oxygen toxicity, although Fe- and Mn-SODs are also found in large amounts in anaerobic organisms. SODs catalyze the dismutation of O_2^- by successive oxidation and reduction of the transition metal ion at the active site in a 'ping-pong' type mechanism with remarkable high reaction rates.

Cu,Zn-SODs, and probably Ni-SOD, are structurally distinct from Fe- and Mn-SODs which show homology and possess identical metal chelating amino acid groups at the active site. Despite this high homology, Fe-SODs possess higher pH stability and thus the activity of Mn-SODs could not be determined by polarography in the alkaline range [1,2].

It is not known exactly if these differences in stability are caused by little differences in the protein structures or by the metal bound to the active site because most Fe- and Mn-SODs show strict metal specificity. Up to now only five groups of bacteria are known to possess enzymes which are active with either iron or manganese incorporated into the same protein moiety [3–10]. To distinguish SODs being active with iron and manganese from those showing strict metal specificity, the former ones are referred at 'cambialistic' SODs. *P. shermanii*, an anaerobic bacterium which tolerates low amounts of oxygen, belongs to these groups of bacteria and builds a single SOD being active with iron or manganese. This SOD offers the possibility to study the influence of the transition metal in comparison to the protein moiety on the thermal and pH-dependent stability of Fe- and Mn-SODs.

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

2. Materials and methods

Chemicals were obtained from the following companies: yeast extract and tryptone (Difco) and xanthine oxidase (grade IV) and cytochrome *c* (Sigma). All other chemicals used were the highest purity available from Merck.

Propionibacterium freudenreichii sp. *shermanii PZ3* (*P. shermanii*) was obtained from the Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig-Stöcken) and grown on a complex medium to produce a Fe-SOD or on an iron-free synthetic medium containing $MnCl_2$ to produce a Mn-SOD, as well as $CuSO_4$, $CoCl_2$ and $ZnCl_2$ as transition metal ions to support the growth conditions of the bacteria. SODs were isolated as described [3]. SOD activity was determined and units were defined by the cytochrome *c*–xanthine oxidase method [10].

The protein concentration was estimated colorimetrically [11]. The pH stabilities of both enzymes were determined by incubation in 50 mmol/l acetate buffer (pH <6), phosphate buffer (pH 6–9) or borate buffer (pH >9) for 10 min at 30°C. Afterwards the remaining activity was determined by the cytochrome *c*–xanthine oxidase method. Thus all stability tests were determined by the remaining activity of the SOD at pH 7.8.

The thermal stability was determined by incubation of the SODs in phosphate buffer (50 mmol/l, pH 7.8) at different temperatures and the remaining activity was measured by the cytochrome *c*–xanthine oxidase test in small aliquots similar to the pH stability tests.

Optical and CD spectra were recorded in quartz cuvettes of 1 cm (optical spectra) and 0.2 cm (CD spectra) on a Beckmann 5230 spectrophotometer and a Jasco J-500 A CD spectrometer in 50 mmol/l phosphate buffer, pH 7.8.

EPR spectra were recorded in the X-band on an EPR spectrometer (Type B-ER 420, with an automatic frequency counter and a nuclear magnetic resonance oscillator; Bruker, Karlsruhe) with the following conditions: microwave frequency = 9.2320 GHz, temperature = 77 K, microwave power = 20 mW, field modulation = 10 G, and a protein concentration of 5 g/l in potassium phosphate buffer (50 mmol/l) at different pH. Mn(III) bound in the SOD is EPR-silent while Mn(II) complexes not bound to proteins show a characteristic signal of six lines in the $g = 2$ range. By comparing the signal amplitudes with a solution of known concentrations it is possible to calculate the amount of manganese released from the protein.

Concentration of iron released from the SOD was determined with *o*-phenantroline in the presence of ascorbate as reducing agent (both 1 mmol/l) in potassium phosphate buffer, pH 6.5, by colorimetry.

3. Results

The SOD of *P. shermanii* incorporated iron or manganese into the same protein moiety [2,4] and both forms show similar activities. Differences were noticed, however, in the stability of the two metal forms which was determined by measuring the remaining activity after the different treatments in a standardized assay at pH 7.8. Fe-SOD is stable in the pH range 5–10 for 10 min at 30°C, whereas Mn-SOD is inactivated below pH 6 and above pH 8 using the same treatment (Fig. 1).

Also in respect to thermal denaturation, the Fe form shows a higher stability in comparison to Mn-SOD (Fig. 2). At 60°C Fe-SOD is not inactivated within 60 min while Mn-SOD is

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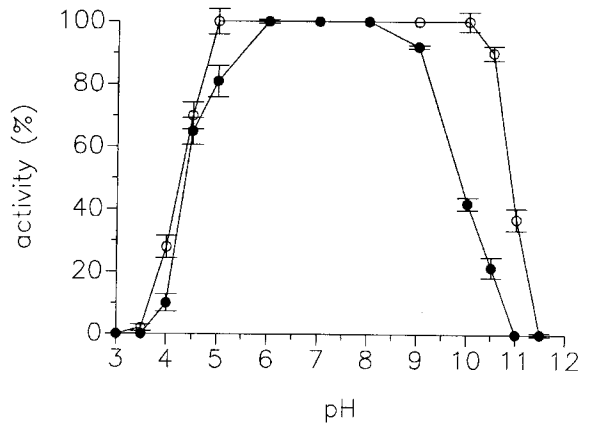

Fig. 1. pH stability of Fe- or Mn-SOD. Fe-SOD (○) or Mn-SOD (●); each sample (1 μmol/l) was incubated for 10 min in acetate, phosphate or borate buffer (50 mmol/l) at 30°C at the pH indicated. The remaining activity was determined by the cytochrome *c* test at pH 7.8 to which all tests were standardized. Standard deviation and mean values were determined from 5 independent experiments.

slowly inactivated and possesses only a rest activity of about 15% after 30 min. At 80°C both forms were inactivated rapidly. The thermal stability of both forms is remarkably increased in the presence of salts like KCl or ammonium sulfate.

During the isolation of the enzymes, a heat denaturation step to remove contaminating proteins was performed by heating the SOD to 70°C for 1.5 min and an addition of KCl up to a final concentration of 0.1 mol/l is necessary to avoid inactivation [12].

If ammonium sulfate is added up to a concentration of 1 mol/l, the thermal stability of Mn-SOD is increased up to 75°C and at a concentration of 2 mol/l up to 80°C. Fe-SOD is not inactivated at all up to 90°C in the presence of 1 and 2 mol/l ammonium sulfate (Fig. 2). Even at 95°C an inactivation was not observable within 15 min.

Inactivation of the SOD by changing the pH values is related to a release of the metal from the active site as determined by EPR-spectroscopy (Mn) or colorimetry (Fe). The secondary structure of the protein is not altered significantly because the optical and CD spectra in the UV range are similar before and after treatment of the enzyme. However, below pH 4.5 the protein is denaturated leading to an irreversible precipitation.

Optical as well as EPR spectra were not altered by adding KCl or ammonium sulfate to the SOD, suggesting that they are not bound to the metal ion at the active site.

4. Discussion

Like metal-specific Fe- and Mn-SODs, where Fe-SODs exhibit higher stabilities in comparison to Mn-SODs, this cambialistic SOD of *P. shermanii* also shows a higher stability of the Fe form in respect to pH as well as thermal inactivation. This inactivation might be triggered by a denaturation starting at the protein moiety, a release of the metal ion which is essential for the catalytic activity from the protein with remaining structure or a combination of both. As both metals are bound to the same protein moiety and both forms exhibit similar structure [13], the effects observed are likely to be triggered by a release of the metal from the active site. This interpretation is supported by the fact that the general secondary conformation is not altered significantly. The iron ion seems to form a stronger complex with the protein than the manganese ion. The ions seem to migrate from the active site, making rebinding impossible when the pH is altered towards pH 7.8 where the activity determinations were performed. However, alterations of the protein conformation, specially at the active site, cannot be excluded.

Both forms of this cambialistic SOD are stabilized by addition of salts leading to a remarkably high thermal stability. Because the spectra of the SODs are not altered in the presence of these salts, binding to the metal ion at the active site is not likely. Salts as well as molecules with positive and negative charges are well known to stabilize proteins in general by destabilising the denaturated form [14,15]. As release of the metal ion from the active site seems to be the triggering step for inactivation, this part might be influenced by the protein and thus salts stabilizing the structure would also stabilize binding of the metal.

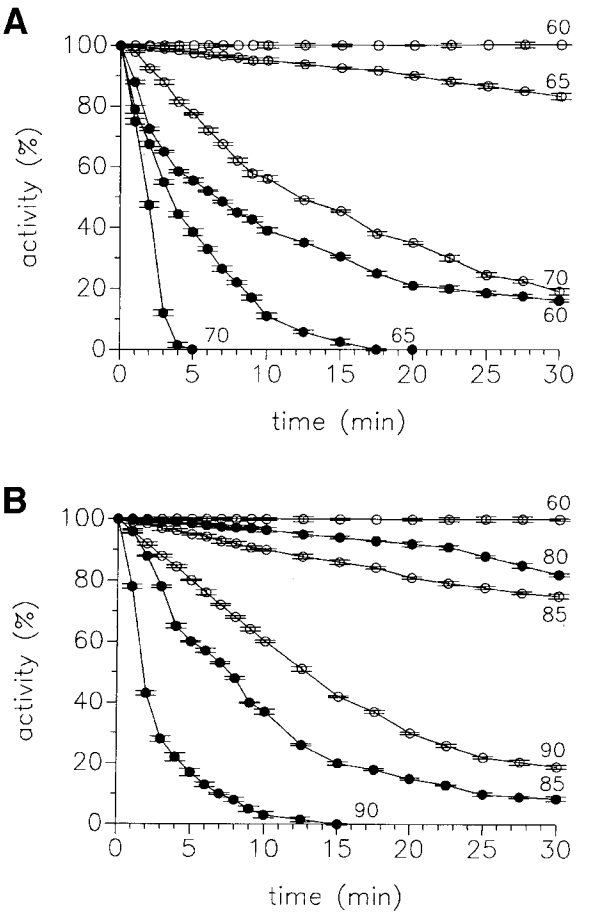

Fig. 2. Thermal stability of Fe- or Mn-SOD. A: Fe-SOD (○) or Mn-SOD (●); each sample 1 μmol/l was incubated in phosphate buffer (50 mmol/l, pH 7.8) at different temperatures. The remaining activity was determined by the cytochrome *c* test at pH 7.8 to which all tests were standardized. B: Mn-SOD with test conditions like (a) but in the presence of 1 mol/l ammonium sulfate (○) or 2 mol/l ammonium sulfate (●). Standard deviation and mean values were determined from 5 independent experiments.

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