pH-dependent inhibition by azide and fluoride of the iron superoxide dismutase from *Propionibacterium shermanii*

Beate MEIER¹, Christoph SCHERK, Marius SCHMIDT and Fritz PARAK

Fakultät für Physik E17, Technische Universität München, James Franck Strasse, D-85747 Garching, Federal Republic of Germany

The iron-containing superoxide dismutase from *Propionibacterium shermanii* shows, in contrast with other iron superoxide dismutases, only a minor inhibition by azide or fluoride (10–100 mM) of up to 23 % at pH 7.8. The activity of the protein with Mn bound to the active site was not diminished under the same conditions. The binding constant between azide and the Fe³⁺ ion was determined as approx. 2 mM and for fluoride approx. 2.3 mM; they are so far comparable to those known for other iron superoxide dismutases. This seems to be a discrepancy because all other iron superoxide dismutases so far known are described as being inhibited by 50–70 % by 10 mM azide. However, towards lower pH there was a drastically increased

inhibition by both anions. At pH 6.8 about 80 % inhibition was exhibited by azide or fluoride at a concentration of 10 mM or higher. In contrast, on increasing the pH, azide or fluoride still bound to the Fe³⁺ at the active site but their inhibition capacity decreased. This observation implies that both anions bind to the metal at a position that is empty at low pH, whereas at higher pH water or a negatively charged hydroxyl anion is bound. It is likely that the superoxide anion binds to the same position and has to replace the sixth ligand, leading to a diminished catalytic activity of the superoxide dismutase owing to steric and/or electrostatic inhibition of the ligand.

INTRODUCTION

Superoxide dismutases (SODs) are regarded as one of the defending enzymes against oxygen toxicity and have been isolated from a variety of organisms including strictly anaerobic bacteria. They are metalloproteins, containing Cu²⁺, Ni³⁺, Fe³⁺ or Mn³⁺ as active metal cofactor; they catalyse the dismutation of O₂^{•−} by successive oxidation and reduction of the transition metal ion at the active site in a Ping Pong type mechanism with remarkably high reaction rates.

Cu- and probably Ni-SODs are structurally distinct from Fe- and Mn-SODs, which show homology and possess identical metal-chelating residues at the active site. All types of SOD bind azide and fluoride, but distinct differences have been noted in the susceptibilities of Fe-, Mn- or Cu-SODs towards inhibition by azide anions [1]. Thus at pH 7.8, 50 % inhibition of the SOD activity was caused by azide at a concentration of approx. 5 mM in Fe-SODs and approx. 20 mM in Mn-SODs; 10 mM azide inhibited Fe-SODs by approx. 73 %, Mn-SOD approx. 30 % and Cu-SODs only approx. 10 %. The sensitivity to inhibition by azide seems to be dependent on the metal at the active site and not on the organism from which the enzyme was isolated. For this reason inhibition by azide was used as a test system to distinguish between the different SOD types in the cytoplasm without purification of the enzymes [1].

However, an unusual discrepancy between the affinity of the Fe³⁺ ion for azide or fluoride and the ability of these anions to inhibit dismutase activity has been observed by another group [2,3]. Whereas the dissociation constants (*K_d*) for the Fe³⁺ complexes with fluoride or azide were determined as 1–2 mM, concentrations of 30–59 mM fluoride and approx. 10 mM azide were necessary to cause 50 % inhibition of the dismutation reaction. This led to the conclusion that an anion-binding pocket

exists near the active centre; thus anions, in addition to co-ordinating the iron directly, might also bind near, but not directly to, the iron [3].

This hypothesis was not supported by X-ray structure analysis of the Fe-SOD–azide complex, in which azide was observable only as a ligand of the iron centre [4].

The Fe-SOD of *Propionibacterium freudenreichii* sp. *shermanii* PZ3 (*P. shermanii*) exhibits equal activity with Fe³⁺ or Mn³⁺ bound to the same protein moiety, and is therefore called a ‘cambialistic’ SOD [5,6]. Recently we determined the X-ray structure of the Fe- and Mn-SOD, and confirmed a similar three-dimensional structure [7]. However, the Fe form of this SOD is only inhibited to a small extent (approx. 18 %) by 5 mM azide; even concentrations of 10–100 mM led to a final inhibition of only approx. 23 %. In contrast the patterns of inhibition by azide and fluoride showed a strong pH dependence. Here we give a tentative explanation for this unusual behaviour.

MATERIALS AND METHODS

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

P. shermanii was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig-Stöcken, Germany) and the growth of the bacteria as well as the isolation of the Fe-SOD and the Mn-SOD were performed as described [5]. SOD activity was determined and units were defined by the cytochrome *c*–xanthine oxidase method [8] at different pH values.

Protein concentration was estimated colorimetrically [9].

Metal contents were determined by atomic absorption in an analytical laboratory (Pascher, Bonn, Germany).

EPR spectra were recorded on an EPR spectrophotometer (B-ER 420, Bruker, Karlsruhe, Germany) in the X-band under the following conditions: microwave frequency 9.4302 GHz, temperature 77 K, microwave power 25 mW, field modulation 0.8 mT, and iron concentration 420 μM (protein concentration of 18 g/l = 196 μM) in potassium phosphate buffer (50 mM, pH 7.8).

CD spectra were recorded on a spectropolarimeter (Jasco J-500A, Perkin Elmer, Überlingen, Germany) in a 1 cm quartz cell at 25 °C and at an iron concentration of 450 μM (protein concentration of 19.3 g/l = 210 μM) in potassium phosphate buffer (50 mM, pH 7.8).

RESULTS

The Fe-SOD from *P. shermanii* showed only minor inhibition by azide or fluoride at pH 7.8 (Figure 1), whereas the activity of the Mn-SOD was not decreased by these anions. The inhibition pattern of both anions depended on the pH, increasing towards acid and decreased towards alkaline pH. Higher concentrations of azide or fluoride (up to 100 mM above pH 7.5 or up to 30 mM below this pH) did not cause further inhibition. The final concentrations of anions used at different pH values were chosen to prevent a disturbance of the test system [1,2]. Higher con-

centrations led to a decreased reduction rate of cytochrome *c* by xanthine oxidase. This was pH-dependent and increased when the pH was decreased; this was taken into account in calculating the inhibition rates.

To exclude the possibility that the observed resistance of the Fe-SOD towards inhibition by azide was not caused by a contamination of Mn^{3+} bound to the active site, the metal content of the isolated SOD was determined as follows (in mol of metal per mol of SOD tetramer): Fe, 2.14; Mn, 0.29; Ni, 0.19; Cu, 0.18; Zn, 0.05; Cr, 0.03; Co, below 4×10^{-4} . This proved that the Fe^{3+} content was high enough in comparison with that of Mn^{3+} and that it was the inhibition of the Fe form that we were measuring.

The optical spectrum of the *P. shermanii* Fe-SOD [5] shows a much smaller absorption coefficient (ϵ_{350} 295 $\text{M}^{-1} \cdot \text{cm}^{-1}$) than that of *Escherichia coli* (ϵ_{350} 1850 $\text{M}^{-1} \cdot \text{cm}^{-1}$ [2], ϵ_{350} 1675 $\text{M}^{-1} \cdot \text{cm}^{-1}$ [10]), making an optical determination of the binding pattern of these anions difficult. Characteristic alterations on the binding of azide or fluoride to the Fe-SOD of *P. shermanii* were observable in the EPR and CD spectra.

The EPR spectrum of the Fe-SOD at pH 7.8 consists of two overlapping spectral species, one with rhombic symmetry ($g_1 = 4.95$; $g_2 = 3.85$; $g_3 = 3.50$) and one with a more axial symmetry ($g_1 = 4.32$; $g_2 = 4.18$) and a weak signal in the $g = 9$ region [5,11] (Figure 2a). On binding of azide or fluoride, both the rhombic species and the axial species interconvert into a single species more similar to the axial one (Figures 2b and 2c). Because the rhombic signal has an equilibrium with the axial one in the 'native' SOD and converts into another species on the addition of azide or fluoride, the decrease in the $g_1 = 4.95$ amplitude of the untreated Fe-SOD in relation to an increase in the $g_1 = 4.36$ signal in the anion-ligated Fe-SOD makes it possible to determine the dissociation constant between these anions and the Fe^{3+} ion at the active centre. Whereas the EPR spectra of the 'native' SOD showed a strong pH dependence [12], the spectra of the anion-ligated forms were independent of pH in the range 6.5–8.5. The dissociation constant for azide was determined as approx. 2.0 mM and that of fluoride approx. 2.3 mM, independent of pH (Figures 2d and 2e) in the range between 6.5–8.5.

The CD spectra of Fe-SOD from *P. shermanii* also showed characteristic alterations on binding of azide and fluoride to the active site, making a calculation of the binding constants possible. The azide- Fe^{3+} complex showed a decreased negative ellipticity and a bathochromic shift in the absorption from 380 to 387 nm (Figure 3, upper panel). On plotting the ellipticity as a function of the concentration of azide a dissociation constant of 2 mM was determined (Figure 3, lower panel), which is in accordance with those determined by EPR spectroscopy. Fluoride also formed a complex with the Fe^{3+} ion at the active centre triggering increased negative dichroism and a hypsochromic shift from 380 to 370 nm (Figure 4, upper panel). A binding constant of 2.1 mM was determined for the fluoride- Fe^{3+} complex (Figure 4, lower panel), also in agreement with that obtained by EPR spectroscopy.

DISCUSSION

The Fe-SOD of *P. shermanii* showed a remarkable resistance towards inhibition by azide in the alkaline pH range. At pH 7.8 the dismutase activity was diminished only to a small extent (5 mM azide caused 18% inhibition; 10–100 mM caused 23% inhibition), whereas in most other Fe-SODs 50% inhibition was caused at approx. 1–10 mM azide [1–3,13,14]. The only exception known so far is the Fe-SOD of *Methanobacterium bryantii*, in which a concentration of 15 mM azide was needed to cause 50%

Figure 1 pH-dependent inhibition of the Fe-SOD from *P. shermanii* by azide and fluoride

Upper panel: the activity of the Fe-SOD from *P. shermanii* was determined in the presence of 5 mM azide (●) or 10 mM azide (○) as a function of pH and calculated as percentage inhibition with the xanthine oxidase–cytochrome *c* test (left ordinate). The pH dependence of the reaction rate (□) (right ordinate) is shown, as described in [6]. Lower panel: the activity of the Fe-SOD from *P. shermanii* was determined in the presence of 5 mM fluoride (●) or 10 mM fluoride (○) under the same conditions as for the upper panel (left ordinate). The pH dependence of the reaction rate (□) (right ordinate) is shown, as described in [6].

Figure 2 Determination of the binding constants of the Fe^{3+} -azide and Fe^{3+} -fluoride complexes by EPR spectra

EPR spectra of the native (a), azide-ligated (b) or fluoride-ligated (c) SOD from *P. shermanii* [5]. The binding constants of the azide- Fe^{3+} complex (d) and the fluoride- Fe^{3+} complex (e) of the SOD from *P. shermanii* were determined by plotting the relative amplitude of the signal at $g_1 = 4.95$ (○, native SOD) in relation to the signal at $g_1 = 4.36$ (●, anion-ligated form). The signal at $g_1 = 4.36$ was corrected to zero.

inhibition [15]. However, taking into account the pH dependence of azide inhibition, on decreasing the pH to 6.8 the inhibition patterns of approx. 80% diminished activity are similar to those observed for 'typical' Fe-SODs.

The EPR spectra prove that the anions are bound to the SOD [11] independently of pH. The 'native' enzyme exhibits two different spectral species that are interconverted on changing the pH: a low-pH species, here denoted as rhombic on account of the g factors ($g_1 = 4.8$, $g_2 = 3.8$, $g_3 = 3.5$) with a lower symmetry,

probably five-fold co-ordinated, and a high-pH species described as axial with $g_1 = 4.32$ and $g_2 = 4.18$, which is probably six-fold co-ordinated [6]. The sixth ligand might be a water molecule or hydroxyl anion. The dismutase activity of the 'native' SODs from *P. shermanii* is also strongly dependent on the pH and decreases gradually towards alkaline pH [6]. These observations lead to the following hypothesis: azide or fluoride binds to the sixth co-ordination position of the Fe^{3+} iron at the active site, which is empty at low pH and is occupied at high pH by a water

Figure 3 CD spectra of the Fe-SOD from *P. shermanii* as function of increasing azide concentration and calculation of the binding constant

Upper panel: CD spectra of the Fe-SOD from *P. shermanii* as function of increasing azide concentration were measured as described in the Materials and methods section. Concentration of azide: solid line, 0 mM; dotted line, 0.9 mM; dot-dashed line, 2.25 mM; broken line, 3.6 mM; long-short dashes, 4.5 mM. Lower panel: the binding constant of the azide-Fe³⁺ complex was determined by plotting the alteration of ellipticity (minimum of each signal amplitude) as a function of the concentration of azide.

molecule or a negatively charged hydroxyl anion. The superoxide anion binds to the same position and has to replace the sixth ligand at alkaline pH, which is azide or fluoride at any pH. In this way the catalytic rate decreases owing to steric and/or electrostatic effects independently of the kind of ligand bound at the sixth position. In fact, if the 'absolute catalytic activity', which means the difference between activity and inhibition at each pH, in the presence of azide or fluoride is calculated this is nearly independent of pH and tends to approximately the value obtained for the 'native' Fe-SOD at pH 9–10, where only the sixfold co-ordinated species is observable.

The dissociation constant for the Fe³⁺-ligand complex was calculated as approx. 2 mM for azide and approx. 2.3 mM for fluoride, which is comparable with those of other Fe-SODs [1–3]. The apparent K_m for the superoxide anion was determined as 0.54 mM and is also in the range of those determined for other SODs [16,17]. Thus the affinity of the substrate for the Fe³⁺ ion is nearly 5-fold those of the ligands, making a substitution possible; this is necessary for the catalytic cycle.

This explanation is supported by an experiment performed by

Figure 4 CD spectra of the Fe-SOD from *P. shermanii* as function of increasing fluoride concentration and calculation of the binding constant

Upper panel: CD spectra of the Fe-SOD from *P. shermanii* as function of increasing fluoride concentration were measured as described in the Materials and methods section. Fluoride concentrations were as described for azide in the legend to Figure 3. Lower panel: the binding constant of the fluoride-Fe³⁺ complex was determined by plotting the alteration of ellipticity (minimum of each signal amplitude) as a function of the concentration of fluoride.

Yamakura et al. [18], who replaced Mn³⁺ in the Mn-specific SOD of *Serratia marcescens* with Fe³⁺. At pH 8.1 this Fe-SOD exhibited only approx. 1% activity in comparison with the 'native' Mn-SOD at the same pH. However, the rate constant increased with decreasing pH to approx. 10% of the Mn-SOD at this pH, with a p*K* of approx. 7. Similarly the affinity of the azide ion as an analogue of the superoxide for Fe-SODs increased with decreasing pH from 0.9 mM at pH 8.2 to 0.1 mM at pH 6.2, also with a p*K* of 7.0, whereas the binding constant between azide and Mn³⁺ in the 'native' SOD was independent of pH between pH 8.2 and pH 6.2. In addition the inhibition by azide of the Fe-substituted Mn-SOD of *S. marcescens* showed a similar pH dependence to that of the Fe-SOD from *P. shermanii*. To cause a 50% inhibition at pH 8.2, 3 mM azide was necessary, whereas at pH 6.2 the same effect was caused with only 0.07 mM azide. Obviously in this Fe-substituted Mn-SOD the pH-dependent changes in activity are caused by the same mechanism as in the cambialistic Fe-SOD of *P. shermanii*, the binding of an additional ligand to the five-fold co-ordinated Fe³⁺ at the active site. Comparing the p*K* values (approx. 7) of the Fe-SODs from *S.*

marcescens and *P. shermanii* [6] with that (approx. 9) of the Fe-SOD from *E. coli* [3], determined by similar pH-dependent alterations in the spectroscopic and activity properties as well as by azide binding, it is likely that the differences in behaviour between these SODs are caused by a diversity in pK values, probably caused by a hydroxyl anion bound to the Fe^{3+} . In this respect the Fe-SOD of *P. shermanii* behaves like a Mn-SOD.

The Fe-SOD of *P. shermanii* is one of the few 'cambialistic' SODs that show similar activities with Fe^{3+} or Mn^{3+} bound to the same protein moiety [5]: the question arises whether the resistance towards inhibition by azide is characteristic of these SODs. The cambialistic Fe-SODs of some *Bacteroides* species are far more sensitive to inhibition by azide than are most of the other Fe-SODs. A concentration of only 0.2 mM azide is necessary to cause a 50 % inhibition of Fe-SODs from *Bacteroides fragilis* [19] or *B. thetaiotaomicron* [20]. In addition the Mn-SODs of these species are more sensitive to inhibition by azide than are other Mn-SODs [1], as only 10 mM azide diminished the activity by approx. 60 % [20]. The Fe-SOD of *B. gingivalis* behaved more similarly to other Fe-SODs and was inhibited by 80 % by 5 mM azide, whereas the Mn-SOD was only inhibited by 26 % by this concentration [21]. In addition the cambialistic Fe-SOD of *Methylobacterium J* showed a similar inhibition pattern to that of others: 1 mM azide caused 40 %, and 20 mM azide 70 %, inhibition of the Fe-SOD, whereas the Mn-SOD was only inhibited by 10 % by 2 mM azide and 40 % by 20 mM azide [22]. In this respect the resistance to inhibition by azide of the Fe-SOD from *P. shermanii* in the usual cytochrome *c*-xanthine oxidase test system at pH 7.8 [8] is unique among the 'cambialistic' SODs.

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