Kinetic and spectroscopic studies on a superoxide dismutase from *Propionibacterium shermanii* that is active with iron or manganese: pH-dependence

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Kinetic studies were performed on the superoxide dismutases isolated from the anaerobic bacterium *Propionibacterium shermanii* as active enzymes with either iron or manganese, which were naturally incorporated into the same molecule depending on the metal supply. Both the Fe- and Mn- forms showed decreasing activity with increasing pH. This suggests the protonation of some groups near the metal, possibly a metal-bound water molecule. Thus the kinetic behaviour of this superoxide dismutase is much more dependent on the protein structure than on the metal incorporated into the active site. The secondary structures of both forms were not influenced by variations in pH, whereas the EPR spectra of the Fe-superoxide dismutase changed

as a function of pH. The EPR spectra apparently consist of two overlapping species. Steady-state experiments proved that all iron-containing species show catalytic activity, but the species predominating in the alkaline pH range displays a lower reaction rate. The Michaelis constant and maximal turnover number for the Fe-superoxide dismutase were determined polarographically as $K_m = 0.54$ mmol/l and $V_{max} = 2000$ mol · s⁻¹ at pH 9.5. These data indicate that, in anaerobic bacteria under physiological conditions, the superoxide dismutase is not saturable with O₂⁻ and the catalytic activity is similar to that of metal-specific Fe- or Mn-superoxide dismutases from aerobic organisms.

INTRODUCTION

Superoxide dismutases (SODs) are metalloproteins containing Cu²⁺, Fe³⁺ or Mn³⁺ as the active metal cofactor. They are regarded as one of the enzymes that defend the organism against oxygen toxicity, although Fe- and Mn-SODs are found in large amounts in anaerobic organisms. SODs catalyse the dismutation of O₂⁻ into O₂ and H₂O₂ by successive reduction and oxidation of the transition metal ion at the active site by the substrate in a Ping-Pong type mechanism with remarkably high reaction rates of about 2.6×10^9 M⁻¹ · s⁻¹ for the Cu,Zn-SODs [1–5] and $(0.5–2) \times 10^9$ M⁻¹ · s⁻¹ for Fe- or Mn-SODs [6–14]. However, up to now only SODs obtained from aerobic organisms have been investigated with respect to kinetic parameters.

Cu,Zn-SODs are structurally distinct from Fe- and Mn-SODs, which show a high degree of structural similarity and contain identical metal chelating amino acid groups at the active site [15]. Despite this structural identity most of them show strict metal specificity, being enzymically active with only iron or manganese. To date only four groups of bacteria are known to possess enzymes which are active with either iron or manganese incorporated into the same protein moiety [16–22]. To distinguish SODs that are active with either iron or manganese from those showing strict metal specificity, the former are referred to as ‘cambialistic’ SODs. The SOD of *Propionibacterium shermanii*, an anaerobic bacterium which tolerates low amounts of oxygen, belongs to this family. This SOD offers the possibility to study the influence of the transition metal on the kinetic parameters of the enzyme activity.

Because of the instability of the O₂⁻ radical at physiological

pH in aqueous solution, with a dismutation rate of $k_1 = 5 \times 10^5$ M⁻¹ · s⁻¹ [23,24], kinetic studies have usually been performed by indirect methods under steady-state conditions. In such methods O₂⁻ is generated chemically or enzymically and the SOD activities are determined by competition for a detector molecule that reacts with O₂⁻ to form a product with high molecular absorbance such as cytochrome *c* [25] or NitroBlue Tetrazolium (NBT) [26]. The kinetic constants for the reduction of cytochrome *c* with O₂⁻ are pH-dependent and are strongly influenced by transition metals that are present as contaminants in most of the chemicals and enzyme preparations that are commercially available [27,28]. The other commonly used indirect method to determine O₂⁻ is the reduction of NBT to the insoluble Blue Formazan. The spectrum and the reaction rate are pH-independent over a wide range when the concentration of NBT is high with respect to that of O₂⁻ [28]. Indirect methods have the disadvantage that many environmental conditions can influence the rate of the reaction between O₂⁻ and the detector molecule and thus may lead to erroneous calculations of kinetic parameters.

Among direct methods, the pulse radiolysis technique offers the possibility of determining kinetic parameters over a wide pH range [29]. Due to the high concentrations of O₂⁻ that can be generated, the dismutation reaction can be monitored photometrically by observing the decline in absorption of O₂⁻ at 260 nm. The two other direct methods which have been developed are limited to alkaline pH values, where spontaneous dismutation of O₂⁻ is slowed down. Photometrically detectable amounts of O₂⁻ can be generated by adding solid KO₂ to aqueous solutions at pH 9.5 and the SOD-catalysed decline in absorption can be followed at 250 nm [30]. KO₂ dissolved in dry DMSO in the

Abbreviations used: SOD, superoxide dismutase; Fe-SOD, iron-containing SOD; Mn-SOD, manganese-containing SOD; Cu,Zn-SOD, copper- and zinc-containing SOD; NBT, NitroBlue Tetrazolium.

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absence or presence of crown ether also serves as a source of $O_2^{\cdot-}$ [31]. Another possible means of directly generating $O_2^{\cdot-}$ radicals is by electrochemical one-electron transfer to oxygen at a mercury electrode covered with triphenylphosphine oxide [32]. The kinetic parameters of SODs can be calculated from the increase in the limiting current.

To date, detailed kinetic analyses have been performed on Fe- or Mn-SODs which show strict metal specificity and which were isolated from aerobic organisms [6–14]. In the present study we investigate the kinetic parameters of a 'cambialistic' SOD from the anaerobic bacterium *P. shermanii* with respect to pH-dependence. The results obtained show that the activity of both forms decreases steeply above pH 6 and suggest that these SODs may have some physiological functions when the pH of the culture medium becomes acidic.

MATERIALS AND METHODS

Materials

Chemicals were obtained from the following companies: yeast extract and tryptone (Difco); xanthine oxidase (grade IV) and cytochrome *c* (Sigma); NBT (Serva); O_2 and N_2 gas (Linde). All other chemicals used were from Merck and were of the highest purity available. Cu,Zn-SOD was a gift from Grünenthal G.m.b.H. (Aachen, Germany).

Propionibacterium freudenreichii sp. *shermanii* PZ3 (*P. shermanii*) was obtained from the Deutsche Sammlung von Mikroorganismen (DSM; Braunschweig-Stöcken, Germany) and grown on a complex medium to produce Fe-SOD or on an iron-free synthetic medium containing $MnCl_2$, $CuSO_4$, $CoCl_2$ and $ZnCl_2$ as transition metal ions to produce Mn-SOD. The SODs were isolated as described [16]. The protein concentration was estimated colorimetrically [33]. SOD activity was determined and units were defined by the cytochrome *c*/xanthine oxidase method [25].

Measurement of enzyme activity and kinetic parameters

Enzyme activity was determined using the cytochrome *c* [25] or NBT [26] assays. The xanthine/xanthine oxidase system was used as enzymic source for the generation of $O_2^{\cdot-}$ in both systems. As an additional direct source of $O_2^{\cdot-}$, KO_2 dissolved in dry DMSO in the presence of crown ether (dicyclohexyl-18-crown-6) was used [31]. The final concentration of $O_2^{\cdot-}$ was 1 mmol/l and this solution was pumped continuously in low amounts via a glass capillary to the vigorously stirred reaction mixture. Purified bovine Cu,Zn-SOD with a catalytic-centre activity of $2.6 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ was used as internal standard.

Polarographic determinations of the kinetic parameters were performed with a dropping mercury electrode covered by triphenylphosphine oxide (1 mmol/l in ethanol; final concentration 5%, v/v) in phosphate or borate buffer (100 mmol/l) with different pH values as indicated, or in phosphate buffer (100 mmol/l, pH 9.5) to determine the Michaelis constant [32]. The experiments were performed at 25 °C with a Polarecord E 261 R (Methrom, Herisau, Germany). The reaction mixtures were fluxed with mixtures of O_2 and N_2 until constant $O_2^{\cdot-}$ concentrations were reached. The actual O_2 concentrations were determined from the mean limiting currents in the polarographic assay according to the equation:

$$I_d = 60.7nc_{O_2}D^{1/2}m^{2/3}t^{1/6}(1 + 34D^{1/2}m^{-1/3}t^{1/6} + Dm^{-2/3}t^{1/3})$$

where I_d is the mean current (A); n is the number of electrons; c_{O_2} is the concentration of O_2 (mol/l); D_{O_2} is the diffusion coefficient

of O_2 , with $D_{O_2} = 2.15 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ [34]; m is the amount of mercury streaming out of the capillary ($\text{g} \cdot \text{s}^{-1}$); and t is the time taken for the formation of a mercury drop (s)

At pH 9.5 only the reduction of oxygen to superoxide was observable, while at lower pH two electrons were also transferred to oxygen, leading to hydrogen peroxide as a side reaction.

The accuracy of this calculation was checked using Cd^{2+} , which has a similar redox potential, as an internal standard. If both O_2 and Cd^{2+} are present at the same concentration, the following equation is applicable:

$$I_{dO_2}/I_{dCd^{2+}} = nD_{O_2}/nD_{Cd^{2+}}$$

Determination of the calibration line was performed in 0.1 mol/l KCl and calculated using a diffusion coefficient of $7.15 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$ for Cd^{2+} [35,36]. The results were in good agreement with those obtained by calculating the oxygen concentration from the Ilcovic equation adjusted by Koutecky for spherical expansion [37].

The oxygen concentrations were additionally measured using a colorimetric assay based on the oxidation of Fe^{2+} to Fe^{3+} by oxygen at alkaline pH [38]. Samples (x ml) were taken with a gas-tight syringe and added to a solution of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ in 0.1 ml of NaOH (30%) mixed with 2.5x ml of water which had previously been flushed with N_2 for 5 min to remove oxygen. The mixture was allowed to react for 5 min at room temperature before 0.5 ml of a KSCN solution (10% in water), previously flushed with N_2 , was added. The red iron complex was measured at 546 nm and determined quantitatively by using $FeCl_3$ as standard.

The Michaelis constant and the maximal turnover number were determined polarographically for Fe-SOD. The straight line in the Lineweaver–Burk diagram was fitted to the experimental data by the method of the least squares. The reaction showed linear dependence on the enzyme concentration in the range 2–10 nmol/l and was independent of the dropping time of the electrode within the same range. The Michaelis constant of Mn-SOD was not determined by this method because of the instability of this enzyme at alkaline pH.

Pulse radiolysis experiments were performed as described in detail elsewhere [29]. $O_2^{\cdot-}$ was generated using a Febetron 705 accelerator delivering 100 ns pulses of 1.8 MeV electrons. The concentration of $O_2^{\cdot-}$ was determined photometrically based on a molar absorption coefficient of $2300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 260 nm. Spectra were recorded with an array of 15 photomultipliers attached to a spectrograph. All tests were performed in 50 mmol/l phosphate buffers of the pH indicated with or without supplementation with 10 mmol/l formate to transform $OH^{\cdot}$ radicals to $O_2^{\cdot-}$.

EPR spectroscopy

X-band EPR spectra were recorded with an EPR spectrometer (ESP-300; Bruker, Karlsruhe, Germany) working at a microwave frequency of 9.4302 GHz, at 77 K, using a microwave power of 20 mW and a field modulation of 10 G. Protein concentration was 25 g/l in potassium phosphate buffer (50 mmol/l) at different pH values as indicated. Experiments in the steady state were performed with a protein concentration of 80 g/l in order to bring all molecules into the steady state. $O_2^{\cdot-}$ was generated by addition of xanthine oxidase (10 μ l to 1 ml) to a solution of SOD in potassium phosphate buffer (50 mmol/l, pH 7.8) saturated with xanthine at room temperature. The mixture was stirred vigorously and aliquots were taken at the times indicated and rapidly frozen in liquid nitrogen. EPR spectra of SOD in the steady state were recorded with a microwave power of 100 mW.

RESULTS AND DISCUSSION

The SOD of *P. shermanii* incorporates iron or manganese into the same protein moiety [16,17]. Differences were noticed, however, in the stability of the two metal forms. Fe-SOD was stable in the range pH 5–10 for 10 min at 30 °C, whereas Mn-SOD was inactivated below pH 6 and above pH 9 by the same treatment. The reaction rates of Fe- and Mn-SODs of *P. shermanii* show a strong pH-dependence and their activities decrease with increasing pH (Figure 1). Formate added in the pulse radiolysis experiments did not alter the reaction rate of either enzyme.

The data obtained by the different methods were in good agreement when the indirect methods were corrected using Cu,Zn-SOD as internal standard; otherwise the values were about 10–30% lower than those obtained by the direct methods. This was also observed by other groups and attributed to contamination with trace metals or re-oxidation of the dyes by oxygen [28,29].

$^2\text{H}_2\text{O}$ did not decrease the reaction rate of either SOD form from *P. shermanii*. In contrast, the SODs from *Thermus thermophilus* [9] and *Escherichia coli* [39] showed a 2.5-fold decrease in reaction rate in the presence of $^2\text{H}_2\text{O}$, suggesting that proton transfer is the limiting step in the reaction. Moreover, from the pH-dependence of these activities, which are independent of pH below pH 8.5, it was concluded that H_2O and not H_3O^+ may serve as proton donor in these bacteria [12]. On the other hand, the SOD of *P. shermanii* shows a strong pH-dependence also in the acid pH range (Figure 1). In this respect it is more similar to the Mn-SOD of *Paracoccus denitrificans*, which also shows an increase in the reaction rate at acidic pH values. At pH 6.5 the Fe- and Mn-SODs of *P. shermanii* have comparable activities to human and bovine Cu,Zn-SODs. This demonstrates that Fe- and Mn-SODs are not generally less active under any conditions than Cu,Zn-SODs [28]. However, the activities of the SODs from *P. shermanii* decrease gradually with increasing pH, whereas the Cu,Zn-SODs show a steep decrease in activity only above pH 10.

The question arises as to whether structural changes could be

Figure 1 Reaction rates of the Fe- and Mn-SODs of *P. shermanii* as function of pH

The reaction rates of the Fe- and Mn-SODs from *P. shermanii* were determined by direct and indirect methods as described in the Material and methods section: $\square$, Fe- and $\blacksquare$, Mn-SOD by pulse radiolysis; $\circ$, Fe- and $\bullet$, Mn-SOD by the cytochrome *c* assay; $\diamond$, Fe- and $\blacklozenge$, Mn-SOD by the NBT assay; $\triangle$, Fe-SOD by polarography. Mean values $\pm$ S.D. were obtained from at least three independent experiments.

observed as a function of pH. Whereas the CD spectra of the Fe- and Mn-SODs did not change in the pH range 6–10 (results not shown), we could determine alterations in the environment of the active site of the Fe-SOD by EPR spectroscopy (Figure 2a). The variation in the spectra with pH from pH 4.5–10 as shown in Figure 2(a) can be explained by the existence of two different species which contribute variably to the spectra. One species is present in nearly pure form at pH 10; it can be denoted axial on account of its *g*-factors ($g_1 = 4.32$, $g_2 = 4.18$). The other, a rhombic species ($g_1 = 4.8$, $g_2 = 3.8$, $g_3 = 3.5$) shown in Figure 2(b), is obtained by subtraction of the spectrum at alkaline pH from that at acid pH. Since the double integration of the EPR spectra shows an increase in spin concentration up to pH 8 (Figure 2c), it appears that the rhombic signal increases with increasing pH and converts, beyond pH 8, into the axial one. However, the apparent spin concentration is also influenced by relaxation parameters which have yet to be measured systematically. Normally in EPR spectra the species are characterized by their *g*-values. The complete description of the total signal is very seldom given because many influences, e.g. proton relaxation, are rarely taken into account. However, it should be mentioned that such a complete simulation of the spectral shape is in progress using the complete Hamiltonian, as has been done for the azide-ligated SOD [40]; a detailed discussion and simulation will be given elsewhere. The amino acid sequence [17] gives no hint of the existence of differences in the metal chelation residues at the active site in the SOD of *P. shermanii* with respect to other Fe- or Mn-SODs. Assuming, therefore, a structure similar to other Fe- or Mn-SODs, the metal at the active site should be pentagonally co-ordinated by three histidines, one aspartate and a water molecule, with a distorted trigonal pyramidal geometry [41,42]. The conversions of different iron sites observed by EPR spectroscopy in the *P. shermanii* Fe-SOD are thus probably due to changes at the fifth position depending on whether H_2O or OH^- is bound or the position is free. The transition of $[\text{Fe}^{3+}\text{-OH}_2]$ to $[\text{Fe}^{3+}\text{-OH}] + \text{H}^+$ at high pH was also postulated for the metal binding site of the Fe-SOD of *E. coli* [9,42]. Another possible explanation for the alterations observed may be the presence of an ionizable amino acid group near the active site in the SOD of *P. shermanii*, as postulated from kinetic experiments for the SOD of *E. coli* [9].

Because the environment of the metal at the active site is influenced by an alteration of the pH, the decreasing reaction rate observed in the alkaline pH range might be due either to an increase in a catalytically inactive iron species or to a species with a lower reaction rate. Thus we brought the Fe-SOD into a steady state at pH 7.8, where both species were observable. The amplitude of the EPR signals decreased, but no alteration in the spectral shape was observed (Figure 3). This suggests that all of the putative iron centres are involved in the catalytic turnover and that the two iron species must possess similar thermodynamic constants in the equilibrium state. However, the major SOD form in the alkaline region must have a lower kinetic reaction rate than that predominating in the acidic range. A maximal decrease in the EPR signals was observed after 3 min, followed by an increase. This was probably due to the formation of hydrogen peroxide, which re-oxidized Fe^{2+} to Fe^{3+} at the active site of the SOD, after limitation of the turnover by the substrate xanthine.

The NADH/phenazinemethosulphate system described by Fried [43] for the reduction of the Cu,Zn-SOD was unable to bring the Fe-SOD into the steady state. Phenazinemethosulphate rapidly re-oxidizes the reduced form of SOD in the pH range 6.0–8.5.

Mutants of Cu,Zn-SOD with different water-binding proper-

Figure 2 EPR spectra of the Fe-SOD from *P. shermanii* as function of pH

Spectra were recorded as described in the Materials and methods section. (a) EPR spectra of the Fe-SOD from *P. shermanii* at different pH values; (b) difference spectra of Fe-SOD at pH 7.8 minus pH 5.0; (c) integration of the spin concentration over the whole signal as a function of pH.

ties were tested for the pH-dependence of the reaction rate by pulse radiolysis, but no substantial alteration of activity was observed [44]. It was concluded that the redox reaction between the metal and $O_2^{\cdot -}$ is not mediated by water at the active site. A similar behaviour was postulated for the SOD of *P. shermanii* because the alterations seen by EPR spectroscopy at the active site could not be correlated with a distinct species showing lower catalytic activity. Thus in the SODs of *P. shermanii*, as in Cu,Zn-SOD, a negatively charged surface guiding the $O_2^{\cdot -}$ anion to the positively charged metal centre might be the factor that determines the dismutation rate, rather than proton transfer at the active site. This hypothesis is in agreement with the pH-

dependence of the reaction rate and with the observation that the reaction rate shows no isotope effect.

The Michaelis constant and maximal turnover number for the Fe-SOD were determined polarographically as $K_m = 0.54$ mmol/l and $V_{max} = 2000$ mol $\cdot$ s $^{-1}$ at pH 9.5 (Figure 4). Detailed kinetic studies have been described for the Fe-SOD of *E. coli*, showing that the enzyme is saturable by $O_2^{\cdot -}$. At about 0.1 mM $O_2^{\cdot -}$ the catalytic-centre activity is 26000 s $^{-1}$ and is independent of pH below 8.5 [12]. The limiting step under saturation conditions is the transfer of a proton from a general acid (H_2O) to a basic centre that develops during catalysis. The Mn-SOD of *Paracoccus denitrificans* was also studied by pulse

Figure 3 EPR spectra of the Fe-SOD from *P. shermanii* in the steady state

SOD (8×10^{-5} mol/l) was brought into the steady state by generating $O_2^{\cdot -}$ using the xanthine/xanthine oxidase system. The samples were frozen at the times indicated after the addition of $10 \mu\text{l}$ of xanthine oxidase, and EPR spectra were recorded as described in the Materials and methods section.

Figure 4 Determination of the Michaelis-Menten kinetics of the Fe-SOD from *P. shermanii* as a function of pH

The kinetic parameters of the Fe-SOD from *P. shermanii* were determined polarographically as described in the Materials and methods section. A Lineweaver-Burk diagram was used for the determination of the Michaelis constant for $O_2^{\cdot -}$ at pH 9.5.

radiolysis and a K_m of 0.1 mM with a reaction rate of 1260 s^{-1} [11] was observed. In contrast to the Fe-SOD of *E. coli*, the reaction rate increased in the acid pH range. The Fe-SOD of *P. shermanii* is comparable with the *Paracoccus* SODs with respect to reaction rate, K_m and pH-dependence. In this context, it should be recalled that *P. shermanii* decreases the pH of the culture medium during growth by forming large amounts of acids. Thus, under physiological conditions, the SOD of this anaerobic bacterium can be expected to show full activity. Moreover, the enzyme is not saturable by the substrate under physiological conditions. Additionally, *P. shermanii* contains comparable amounts of SOD to *E. coli* grown under anaerobic conditions, and the SOD of *P. shermanii* is inducible by oxygen [45,46]. However, *P. shermanii*, which tolerates low amounts of

oxygen, is not able to grow under aerobic conditions. Thus differences in the levels or activity of SODs between aerobic and anaerobic organisms cannot be regarded as the main reason for oxygen toxicity.

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