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INVOLVEMENT OF THE COPPER IN THE INHIBITION OF Cu,Zn SUPEROXIDE DISMUTASE ACTIVITY AT HIGH pH

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The alkaline spectroscopic transition of the copper at the active site of Cu,Zn superoxide dismutase has been reexamined by room temperature EPR, in order to correlate it with the inhibition of the enzyme activity at high pH. The EPR transition is governed by a single prototropic equilibrium, with pK values of 11.3 and 11.1 for ox and shark superoxide dismutase, respectively. This result suggests possible contributions of changes of the copper environment to the higher pK of the activity/pH curve.

When Arg141 was chemically modified by phenylglyoxal treatment of the ox protein, a lower pK value (10.8) was obtained, indicating that Arg141 is involved in the observed modifications of the EPR spectra.

KEY WORDS:

INTRODUCTION

A current scheme for the mechanism of action of Cu,Zn superoxide dismutase (SOD) involves alternate reduction and oxidation of the copper by O_2^- in a ping pong reaction giving O_2 and H_2O_2 respectively.[1] Among the various intriguing aspects of Cu,Zn SOD catalysis, the nearly diffusion controlled catalytic constant of the enzyme (in the order of 10^9 $M^{-1}s^{-1}$) and the identical value of the separate constants of reduction and oxidation of the copper by O_2^-[2] are the object of continuing investigation. The reversible inhibition of the enzyme activity by pH values between 10 and 12 and by salts that are not able to coordinate to the copper (ClO_4^-, phosphate etc.) have led to an "electrostatic theory" of the rate limiting step of the Cu,Zn SOD mechanism.[3] The rate of the enzyme would be controlled by ionization of positively charged surface side chains, which are located in such a way as to selectively guide O_2^- to the copper site. In particular two *pK* values, approximately 10 and 11, have been calculated for the alkaline titration of the enzyme activity in several Cu,Zn SODs.[4] This idea is in line with results obtained from chemical modifications of basic amino acid residues on the enzyme,[5] with the refined crystallographic model of the bovine protein,[6] and with electrostatic calculations on differently charged natural mutants of the enzyme.[7] The identification of the group(s) responsible for the alkaline activity decay is however a challenging task. Lys 120 and 134 have been proposed for the bovine enzyme,[3] being positioned not far from the copper (12 Å) at the edge of the active site channel shown by X-ray analyses.[6] However, there are Cu,Zn SODs where

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these two residues are not simultaneously present and which show nonetheless activity/pH curves essentially identical to that of the bovine enzyme.[4,8] Arg 141 is much closer to the copper (5 Å), is conserved in all cases, and is essential to full activity. However, it seems not to be the best candidate for sensing salt effects its *pK* being expected to be higher than the pH range where Cu,Zn SODs are inhibited by ionic strength.[3] In this context, it is interesting to recall that long before the activity parameters were established, it was shown that bovine Cu,Zn SOD undergoes an alkaline transition of spectroscopic properties of the copper as well. The frozen solution EPR spectrum[9] and the water proton relaxation rate[10,11] are affected by pH in a way which roughly parallels the activity inhibition. A number of questions arise: i) how much superimpossible are these phenomena; ii) how much comparable are frozen and liquid solution spectra; iii) how much the first coordination sphere and the nearest environment of the copper, probed by the EPR spectrum, are involved in the alkaline titration of the activity. The present report is the investigation of the alkaline transition of Cu,Zn SOD as followed by room temperature EPR spectra. It is in fact established that freezing of Cu,Zn SOD solution induces effects on the EPR lineshape which are not detected at room temperature.[12] The results obtained permit for the first time a careful comparison of activity and spectroscopic studies and suggest that copper is involved, at least in part, in the alkaline activity transition, although they are not in conflict with the electrostatic theory of the copper zinc superoxide dismutase mechanism of action.

MATERIALS AND METHODS

SODs from ox and shark were prepared and purified as previously described.[13,14] Modification of the bovine protein by phenylglyoxal (PHG) was carried out according to Borders and Fridovich.[15] The reaction of bovine SOD with butanedione was performed at pH 8 in 50 mM borate buffer, after addition of 20 mM butanedione to 0.33 mM SOD. In spectrophotometric experiments an equal amount of the reagent was added in the buffer of the reference cuvette. The spectra were recorded until no further changes were noticeable (after approx. 60 minutes). Activity measurements on native and modified SODs were performed by the pyrogallol assay.[16]

Optical spectra were recorded with a Perkin Elmer 330 spectrophotometer equipped with a Haake Mod. G temperature control unit. Room temperature X-band EPR spectra were recorded with a Varian E9 spectrometer, interfaced to a Stelar Prometheus Data System for computer analysis and handling of the spectra. Curve fitting procedures were performed as previously described.[4] During titration experiments, the pH of the samples was raised by addition of small aliquots of 0.1 or 1 M NaOH.

RESULTS

The effect of alkaline pH on Cu,Zn superoxide dismutase from various sources has been investigated through EPR spectroscopy at room temperature. Figure 1 (upper set) shows EPR spectra of bovine Cu,Zn superoxide dismutase recorded at various pH values (pH 7–12). Raising the pH from 7 to 10 had no effect on the EPR spectrum of the enzyme, while at higher pH values the spectrum became more axial with an

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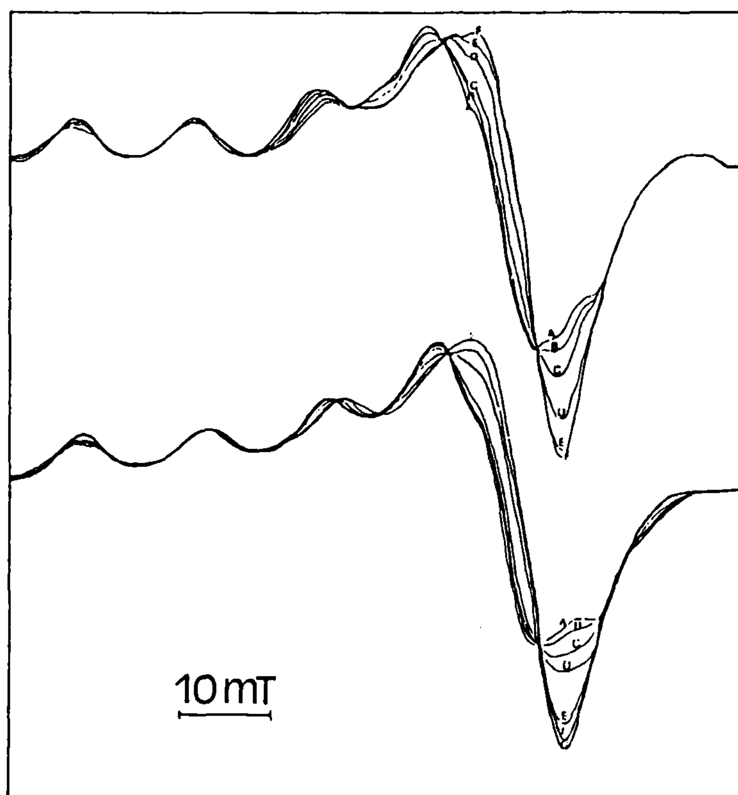

FIGURE 1 Effect of alkaline pH on the room temperature X-band EPR spectrum of ox (upper set) and shark (lower set) Cu,Zn SOD. The pH values were: 10.0 (A); 10.5 (B); 11.0 (C); 11.5 (D); 11.9 (E); 12.2 (F) for ox SOD and 9.25 (A); 9.65 (B); 10.1 (C); 10.7 (D); 11.4 (E); 11.67 (F); 12.2 (G) for shark SOD. Protein concentration was ~ 1 mM for both proteins. Experimental settings were: microwave frequency, 9.53 GHz; power, 150 mW; modulation amplitude, 1 mT; temperature, 300 K.

increased value of the hyperfine coupling constant $A_{\parallel}$ (128 G at neutral pH, 138 G at pH 12.2), and a lower value of $g_{\perp}$ (2.073 at neutral pH, 2.056 at pH 12.2). As already reported for the experiments done at liquid nitrogen temperature,[9] all the modifications observed below pH 12 are fully reversible when the pH was lowered back to pH 7, while irreversible denaturation of the protein occurred above pH 12. The same behaviour was observed, with no significant differences, with shark Cu,Zn SOD, which has been shown to have an Arg residue in the place of the Lys 134 present in the bovine sequence (Figure 1, lower set). A transition in the room temperature EPR spectrum at high pH values was also obtained on the bovine SOD derivative chemically modified with phenylglyoxal (Figure 2). In this PHG-SOD derivative, the selective modification of the conserved Arg 141 results in a nearly complete enzyme inactivation.[15] The room temperature EPR spectrum of the derivative, retaining ~ 10 % of the activity as assayed with the pyrogallol method, was not very much altered at neutral pH with respect to the native protein, at variance with previous observations at low temperatures.[17] It has a slightly lower $g_{\perp}$ value (2.071), and a slightly higher value of $A_{\parallel}$ (134 G), Figure 2, curve a. Raising the pH above 10 caused

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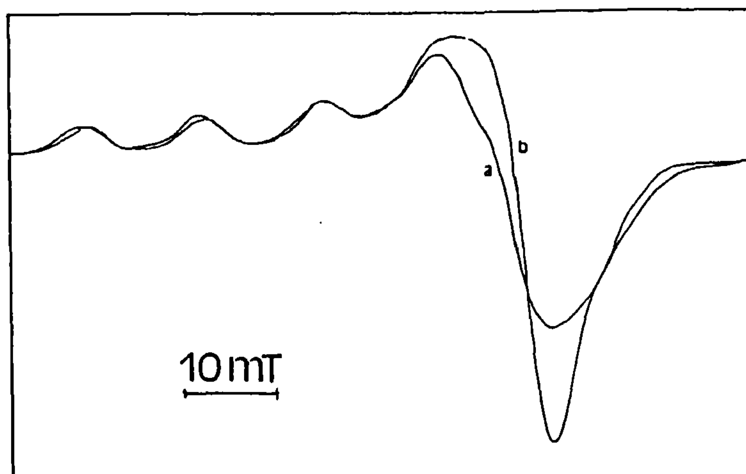

FIGURE 2 Effect of alkaline pH on the room temperature X-band EPR spectrum of PHG-modified ox SOD. The pH values were 8.0 (a) and 12.2 (b). Protein concentration was 1 mM. Experimental settings as in Figure 1.

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further changes of the spectrum, which at pH 12.2 had values of $g_\perp$ and $A_\parallel$ of 2.056 and 142 G, respectively (Figure 2, curve b). For a quantitative analysis of the transition monitored by the modifications of the spectroscopic properties of the three enzyme samples at alkaline pH, $g_\perp$ values were plotted versus pH (Figure 3). Curve fitting procedures, based on the pH dependence of the spectroscopic parameter governed by one or two prototropic equilibria, were performed to obtain best fits of the experimental data. For both bovine Cu,Zn SOD and shark Cu,Zn SOD best fits were obtained for a single prototropic equilibrium with *pK*s of 11.3 ± 0.1 and 11.1 ± 0.1 respectively, while a slightly lower value was obtained for the PHG derivative, *pK* 10.8 ± 0.1.

The changes of the electronic absorbtion spectrum of the bovine enzyme in the alkaline region are reported in Figure 4. The spectrum remained essentially unaltered between pH 6 and 9, while above pH 9 a ~ 20 nm blue shift of the main absorbtion

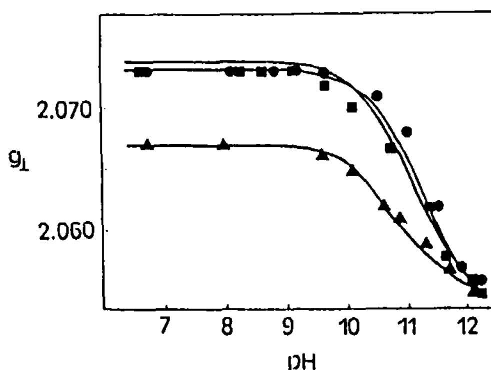

FIGURE 3 pH dependance of the $g_\perp$ value of Cu,Zn SOD from ox (●), shark (■) and PHG-modified ox SOD (▲). Continuous lines are theorical curves obtained by best fittings of the experimental data.

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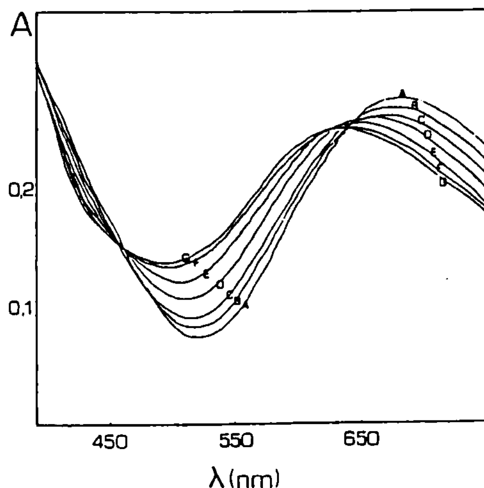

FIGURE 4 Effect of alkaline pH on the optical absorbtion spectrum of bovine Cu,Zn SOD. Protein concentration was 0.93 mM. The pH values were: 9 (A); 10.4 (B); 11 (C); 11.7 (D); 12.1 (E); 12.4 (F); 12.5 (G).

band (with a maximum at 680 nm) occurred with a concomitant 20 % decrease of the molar extinction coefficient. A parallel decrease was also observed for the intensity of the 420 nm shoulder, with an isosbestic point around 450 nm. Similar changes were also noticeable upon addiction of butanedione to the bovine enzyme at pH 8. This treatment results in a modification of Arg 141 leading to enzyme inactivation[18] as in the case of phenylglyoxal with the advantage that the modification by butanedione is reversible. Figure 5 shows difference optical spectra for the bovine enzyme either butanedione-treated (upper curve) or at high pH (lower curve). In both cases the variations around 400 nm are clearly due to the disappearance of a band centered at ~ 420 nm. The butanedione adduct, however, resulted to be not suitable for spectroscopic observations at pH values above 9, as irreversible changes of both the copper visible optical and EPR spectrum occurred, leading to a yellowish product formation.

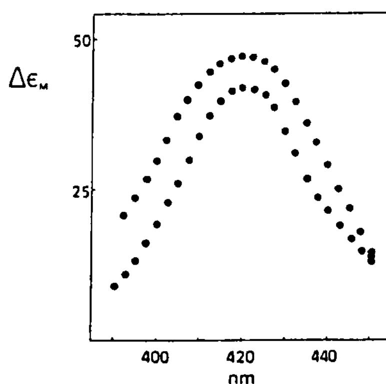

FIGURE 5 Difference optical spectra of bovine SOD at pH 12.2 (lower curve) and of the bovine enzyme in the presence of 20 mM butanedione in 50 mM borate buffer (upper curve).

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DISCUSSION

The "electrostatic theory" of the guidance of O_2^- into the active site channel of Cu,Zn SOD is in line with the assumption that, in the pH range in which the reversible decrease of the enzymatic activity is observed, the surface charge of the enzyme is locally altered by deprotonation of residues in the vicinity of the active site channel.[3,4] The relevant changes of the copper chemistry, reflected in the spectroscopic changes of the EPR spectrum, would not be involved in the control of activity. In fact results obtained with ^{19}F as NMR probe at pH 11.5[3] indicate that even when the copper site is modified, at the steady state, the rate constant of the reduction of the copper is identical to that of oxidation. Thus the activity appears to be governed by the rate of diffusion of the O_2^- into the active site channel and not by the oxidation state of the copper. The results reported here confirm this assumption only for the pH region below pH 11. The best fits of the experimental data (Figure 3) show that the EPR transition is governed by only one prototropic equilibrium with a $pK = 11.3$ for the bovine enzyme, 11.1 for the shark enzyme, and 10.8 for the phenylglyoxal-modified enzyme. This implies that the reversible decrease of the activity observed at alkaline pH is independent of the spectroscopic transition as far as the first prototropic equilibrium ($pK \leqslant 10$) is concerned.

It should also be noticed that the EPR transition is, like the activity transition, nearly identical in the bovine and the shark enzymes.[8] Thus it seems not to be affected by the substitution of a surface residue, hypothetically involved in the electrostatic facilitation of the catalysis, such as Lys 134 (substituted by Arg 134 in the shark enzyme). On the contrary substitution of Arg 141 by an Arg-phenylglyoxal complex in the bovine enzyme causes a lowering by approx. 0.5 pH units of the pK of the spectroscopic transition.

The modifications of the optical spectrum of bovine Cu,Zn SOD at alkaline pH may provide additional information on the mechanism of the spectroscopic transition. The blue shift of the spectrum observed above pH 9 and the disappearance of the band centered at 420 nm can be interpreted in terms of detachment of an imidazole ligand from copper, since absorbtion at this wavelength has been attributed to a LMCT from a ligand to the copper atom.[19] The transition ovserved in the EPR spectra can be explained in the same way, the modifications being similar to those already observed in the case of N_3^- binding to the copper[20] in which detachment of a ligand from the copper is involved. Also with reagents that neutralize Arg 141, such as butanedione, the shoulder at 420 nm in the electronic absorbtion spectrum of bovine Cu,Zn SOD vanishes (Figure 5). This result is in line with recent NMR evidence[21] which demonstrate detachment of an imidazole ligand upon modification of Arg 141 by phenylglyoxal or butanedione. Thus the alkaline transition would be a particular case of anion binding. As recently suggested for other anions,[21] OH^- would interact with Arg 141, leading to a rearrangement of the copper coordination sphere monitored by the "detachment" of a histidine. This detachment would facilitate coordination of OH^- in the copper equatorial plane, resulting in an axial EPR spectrum. This process is likely to be governed by the pK of Arg 141, which being hydrogen bonded to water in the protonated state,[22] could also affect the pK of the copper bound H_2O.

It is plausible that this pK is involved in the higher pK of the activity/pH curve. First of all, this pK, at variance with the lower one, is unaffected by ionic strength[3] in line with its association with the copper environment. Secondly, it is much less sensitive

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to species variation affecting the surface lysine than the lower pK.[3,4] Furthermore, the spectroscopic transition pK is nearly identical to the activity transition pK_2, surprisingly enough with respect to previous EPR data at low temperature.[9]

However, how involvement of the copper can be reconciled with the finding of identical rates of oxidation and reduction of the enzyme at pH 11.5 is still an open question.

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