Biochem. J. (1988) 251, 41–46 (Printed in Great Britain)

The effects of pH and various salts upon the activity of a series of superoxide dismutases

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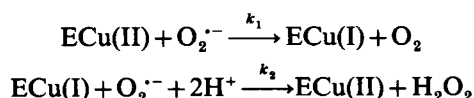
The CuZn superoxide dismutases (SODs) from ox, sheep, pig and yeast were investigated by pulse radiolysis in order to evaluate the role of electrostatic interactions between $O_2^{\cdot -}$ and SOD proteins in the mechanism of action of the SOD enzymes. The protein net charge in this series varies, as evaluated by the protein pI values spanning over a large range of pH: 8.0 (sheep), 6.5 (pig), 5.2 (ox) and 4.6 (yeast). The amino acid sequences are largely conserved, with the three mammalian proteins being highly homologous and the yeast protein having some distinct variations in the region surrounding the active site. At pH 8.0 the activities of the SODs from various sources are similar, though the minor differences observed suggest that in the highly homologous mammalian series the most acidic protein is the most enzymically efficient one. The pH-dependences of the various activities in the pH range 7–12 are similar, and the related curves are best fitted by two p*K* values, which are approx. 9.2 and 11.0 for the mammalian enzymes and 9.4 and 11.4 for the yeast enzyme. The activities of the proteins at *I* 0.1 are decreased by approx. 20% when compared with the activity at *I* 0.02 at pH 8.5, whereas at pH above 10 the pH-dependence of the activity approaches that determined at *I* 0.02 and at pH 11.9 the activity is essentially independent of ionic strength. The dependence upon ionic strength also depends on the salt used, with perchlorate being more effective than phosphate or borate or Mops and still effective at pH above 10.5, where the effect of other salts becomes negligible. The dual and concerted dependence of the activities of different SODs on pH and salt concentration is explained with the encounter of $O_2^{\cdot -}$ with the active-site copper being governed by the protonation of two positively charged groups in the vicinity of the active site. The gradient between these localized charges and the rest of the protein may explain the different activities of the mammalian proteins at lower pH. On the basis of the sequence variation of the SODs examined it is not possible to definitely identify these groups. Likely candidates are conserved basic amino acid side chains in the vicinity ($\leqslant$ 1.2 nm) of the active site, i.e. Lys-134 and Arg-141, but co-ordination of OH^- in the first copper co-ordination sphere may be an additional factor accounting for the higher p*K*. The effects of various ions may reflect their ability to approach and electrostatically shield these centres to different extents.

INTRODUCTION

The structure and the catalytic mechanism of CuZn superoxide dismutase (SOD) are known in detail from studies of the bovine enzyme (for review see Fielden & Rotilio, 1984).

This enzyme consists of two identical subunits of 16 kDa, each containing a catalytically active copper ion and a zinc ion, which share a common ligand, the imidazole of His-61 in the linear sequence of the bovine enzyme. It has been demonstrated experimentally (Klug-Roth *et al.*, 1973; Fielden *et al.*, 1974) that the enzyme catalyses the dismutation of $O_2^{\cdot -}$ to O_2 and H_2O_2 by a cyclic oxidation–reduction mechanism involving the copper ion:



$$ECu(II) + O_2^{\cdot -} \xrightarrow{k_1} ECu(I) + O_2$$
$$ECu(I) + O_2^{\cdot -} + 2H^+ \xrightarrow{k_2} ECu(II) + H_2O_2$$

The reactions between $O_2^{\cdot -}$ and either reduced or oxidized forms of the enzyme are diffusion-limited and occur at the same rate of approx. $2 \times 10^9\ M^{-1} \cdot s^{-1}$ for each copper site. Detailed steps of this mechanism appear to involve displacement by $O_2^{\cdot -}$ of a copper-bound water molecule followed by electron exchange between $O_2^{\cdot -}$ and the copper (Rotilio *et al.*, 1978). The proton source for the formation of the peroxide product in the oxidation step may be the imidazolate nucleus of His-61 bridging the copper and the zinc ions, which is released from the copper and protonated in the reduction step (McAdam *et al.*, 1977). These details fit in very well with a refined crystallographic model (Tainer *et al.*, 1983). However, some peculiar features of the enzyme reaction are not easily explained with a model based only on the chemical properties of the active-site copper. Firstly, why is $O_2^{\cdot -}$ so selective in driving the redox cycle? Secondly, why does $k_1 = k_2$ although the chemistry of the copper is different in the oxidized and in the reduced enzyme? In particular,

Abbreviation used: SOD, CuZn superoxide dismutase.
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the latter argument would exclude any participation in steady-state catalysis of changes occurring in the first copper co-ordination sphere whenever the condition $k_1 = k_2$ proved to hold. Essentially in view of this restriction, electrostatic interaction of $O_2^{\cdot-}$ with positively charged amino acid residues near the active site have been suggested to play an important role in explaining these aspects of bovine SOD catalysis, under the conditions of current kinetic studies, i.e. $[O_2^{\cdot-}] \ll K_m$, when the limiting process is the binding of substrate to the enzyme.

Getzoff *et al.* (1983) showed that the surface of the bovine enzyme in the proximity of the active site forms a sort of channel, which is markedly positive because of the presence of side chains of Arg-141, Lys-120 and Lys-134 at 0.5, 1.2 and 1.3 nm from the copper respectively. Chemical modification of these residues causes a drastic fall in the enzyme activity. The residual activity of enzyme samples having all lysine residues modified was unaffected or was increased by raising ionic strength depending on whether the lysine charge was neutralized or inverted respectively (Cocco *et al.*, 1982; Cudd & Fridovich, 1982). On the other hand the residual activity of the protein in which only the side chain of Arg-141 was modified showed an inhibition by increasing ionic strength that was similar to that observed with the native enzyme (Cudd & Fridovich, 1982). On the basis of these results Argese *et al.* (1987) investigated the pH- and ionic-strength-dependences of the native enzyme in a more quantitative fashion and concluded that two residues with pK approx. 10 and pK approx. 11 were rate-determining in the bovine SOD under the limiting condition $[O_2^{\cdot-}] \ll K_m$.

The present paper was aimed at a comparative study of this problem. In fact, several SODs have been sequenced in recent years. In all cases the primary structure showed a high degree of homology as compared with the bovine enzyme, in particular with regard to those residues that are expected to be activity-linked on the basis of the crystallographic model of the bovine enzyme (Tainer *et al.*, 1983; Getzoff *et al.*, 1983). However, although Arg-141 is present in all the sequenced enzymes, not all SODs contain both lysine residues corresponding to Lys-120 and Lys-134 in the bovine enzyme. Furthermore some sequenced SODs have different protein net charge, as reflected by their pI values, and this may alter the electrostatic gradient between the positively charged active-site channel and the rest of the protein surface. This gradient may be important in making more effective the encounter between $O_2^{\cdot-}$ and the protein molecule, as the substrate will be attracted by the active site and repelled by negative charges elsewhere (Koppenol, 1981). The most interesting enzymes in this respect, of which sequence was already available, were selected for an extensive pulse-radiolysis study of the pH- and ionic-strength-dependences of their activities. This study is the object of the present paper.

EXPERIMENTAL

The SODS from ox, pig, sheep and yeast were prepared and purified as previously described (McCord & Fridovich, 1969; Marmocchi *et al.*, 1983; Schininá *et al.*, 1986; Goscin & Fridovich, 1972). The properties and sequence data of these proteins have previously been reported (Steinman *et al.*, 1974; Schininá *et al.*, 1985, 1986; Steinman, 1980). The copper content of the proteins was determined by the procedure of Brumby & Massey (1967). All concentrations of SOD are expressed in terms of protein concentration as determined by the method of Lowry *et al.* (1951). pI values were determined by isoelectric focusing on 4% polyacrylamide gels containing 3% Ampholines spanning ranges pH 3.5–9.5 and pH 4–6. All solutions containing protein were assayed for activity, based upon the first-order rate of loss of $O_2^{\cdot-}$ at 250 nm (Fielden *et al.*, 1974). The pulse radiolysis and data storage and evaluation have been described previously (O'Neill & Chapman, 1985). All solutions were prepared in water that had been purified by using a Millipore Milli-Q system and contained 0.1–0.3 M-ethanol and 0.2 mM-EDTA. In order to maintain the required ionic strength of 0.02 or 0.1, the following buffers were used: Tris/Mes, up to pH 7; Tris/Mops, pH 8–9; borate, pH 9 and above. The pH of the samples was measured before and after the irradiation and found to be unchanged. Before irradiation, the solutions were saturated with O_2 so that in this system the radiolytically produced primary species of water radiolysis ($e^-_{aq.}$, $^{\cdot}OH$, $H^{\cdot}$) are all converted into $O_2^{\cdot-}$, the concentration of which was determined by assuming $\epsilon_{250} = 2000\ M^{-1}\cdot cm^{-1}$ (Rabani & Nielsen, 1969).

Solutions contained in an optical cell of pathlength 0.07 dm were irradiated with electron pulses of 1.6 μs duration. The $[O_2^{\cdot-}]$/pulse was approx. 17 μM, which represents a considerable concentration excess compared with that of SOD.

In order to study the effect of different salts upon the SOD activity, $NaClO_4$ or phosphate was used to adjust I and all solutions were buffered with Tris/Mes or Tris/Mops or borate buffer, the concentration of which gave an I of 0.005. The ionic strength was further adjusted using either ClO_4^- or phosphate (pH 6.0 and 8.0).

Curve-fitting procedures were performed to obtain best fits of the data based upon pH-dependences of the activity being governed by two prototropic equilibria, K_1 and K_2, the contributions of which are defined as A and B respectively, with $A+B=1$. The following equation was used to obtain the best fits (Table 2) with the enzyme activity, $k_{pH}/k_{8.0\text{–}8.5}$, (E) at pH 8.0 and 8.5 being set to 1:

$$(E) = A\left(1-\frac{K_1}{K_1+[H^+]}\right)+B\left(1-\frac{K_2}{K_2+[H^+]}\right)$$

RESULTS

The activities of a series of SODs at pH 8.0 (Tris/Mops buffer, I 0.02) were determined from the dependence of the first-order rate of decay of $O_2^{\cdot-}$ on [SOD] (0.2–1.0 μM) under turnover conditions ($[O_2^{\cdot-}] \gg$ [SOD]) and are presented in Table 1. At pH 8.0 the activities of these proteins from various sources are similar, even though there are minor changes that may be related to the different pI values of the proteins (Table 1).

The pH-dependences of the activities of pig, sheep and ox SODs in the pH range 7–12 are shown in Figs. 1 and 2 and were determined at I 0.02 and 0.1. The activities of the proteins at I 0.1 (Fig. 2) are decreased by 25±4% when compared at pH 8.5, and this decreased activity is consistent with previous reports on the bovine enzyme activity (Cudd & Fridovich, 1982; Argese *et al.*, 1987).

Table 1. Bimolecular rate constants for the interaction of $O_2^{\cdot-}$ with SODs having different pI values, determined under turnover conditions at pH 8.0 and *I* 0.02 (Tris/Mops)

SOD	$10^{-9} \times k$ ($M^{-1} \cdot s^{-1}$)	pI
Ox	3.8	5.2
Pig	3.2	6.5
Sheep	3.3	8.0
Yeast	3.4	4.6

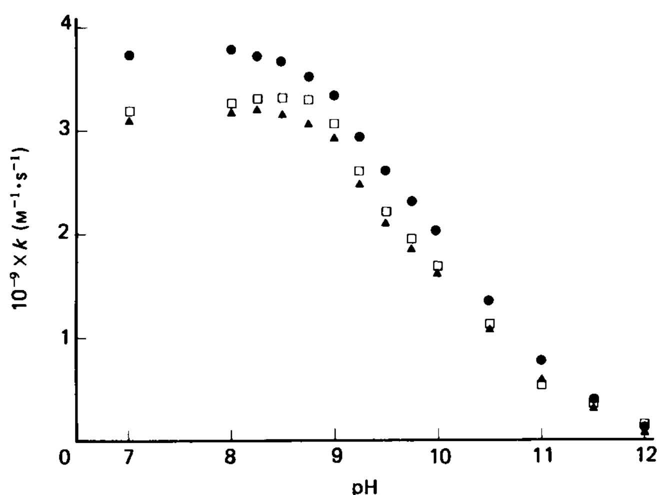

Fig. 1. Variation with pH of the bimolecular rate constants for interaction of $O_2^{\cdot-}$ with ox, sheep and pig SODs at *I* 0.02

Buffers were changed in the different pH ranges as follows: pH 7.0, Tris/Mes buffer; pH 8.0–9.0, Tris/Mops buffer; pH 9.0 and above, borate buffer. The protein concentration was 0.6 μM. ●, Ox SOD; □, sheep SOD; △, pig SOD.

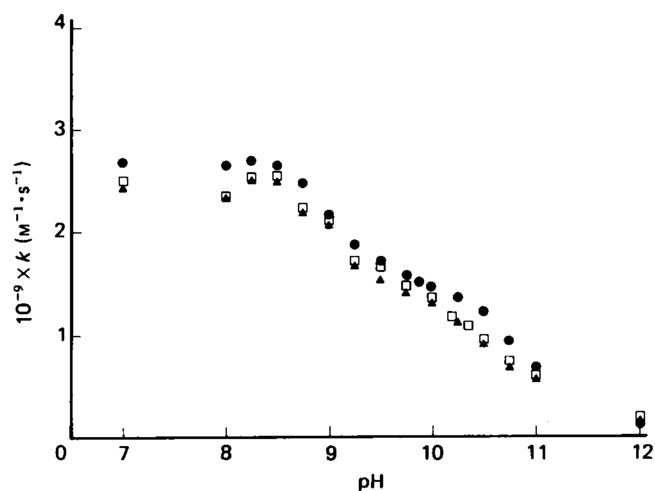

Fig. 2. Variation with pH of the bimolecular rate constants for interaction of $O_2^{\cdot-}$ with ox, sheep and pig SODs at *I* 0.1

Experimental conditions were as given in Fig. 1 legend. ●, Ox SOD; □, sheep SOD; ▲, pig SOD.

Table 2. Values of pK_1, pK_2 and (in parentheses) their respective contributions to the pH-dependence of the activities of various SOD proteins

The values were obtained by the fitting procedure described in the text from the data shown in Figs. 2 and 3.

SOD	*I*	pK_1	pK_2
Ox	0.1	9.30±0.15 (53%)	11.00±0.05 (47%)
Pig	0.1	9.20±0.20 (50%)	11.00±0.10 (50%)
Sheep	0.1	9.30±0.20 (52%)	11.00±0.20 (48%)
Yeast	0.1	9.35±0.15 (61%)	11.40±0.20 (39%)
Yeast	0.02	9.50±0.20 (53%)	11.30±0.20 (47%)

At *I* 0.02 (Fig. 1) the pH-dependences for all these proteins are similar, within experimental error, with an 'apparent' pK_a value of 10.0±0.2. It should be noted that the pH-dependences of the activities are not satisfactorily fitted with the use of only one pK_a value. The pH–activity profile for bovine SOD is similar to that previously described (Argese *et al.*, 1987). In the case of pig SOD, the activity of the protein is independent of pH in the range 7–9 at *I* 0.02, an observation that is at variance with the reported decrease of activity at pH above 7.5 as determined polarographically (Argese *et al.*, 1984).

The pH–activity profiles for the SODs at *I* 0.1 are similar for all proteins and show a different dependence to that determined at *I* 0.02. From closer examination of these dependences it is apparent that at pH values above 9.5 a point of inflexion is present (Fig. 2). Such an effect would be consistent with the pH-dependence being controlled by more than one factor. At pH above 10.0 the pH-dependence of the activity approaches that determined at the lower ionic strength. Curve-fitting of these dependences in terms of two pK_a values (see the Experimental section) was performed, and the calculated values are presented in Table 2 together with their percentage contribution to the dependences. For ox, pig and sheep SODs the pK_a values are similar, with equal contribution from both pK_a values.

The pH-dependences of the activity of yeast SOD at *I* 0.02 and 0.1 are shown in Fig. 3. Both dependences show at least two obvious components, in contrast with the observations with the other proteins, especially at the lower ionic strength, where an inflexion is witnessed. The calculated pK_a values and their respective contributions based upon these curve-fitting procedures are presented in Table 2. It should be noted that the yeast protein contains only one lysine residue (Lys-134) in the vicinity of the active channel compared with two lysine residues with the other proteins used. The second pK_a calculated with the yeast protein is approx. 0.4 p*K* unit in excess of those for the other proteins.

The effect of ionic strength at pH 8.5, adjusted with different salts, on the activity of SOD from yeast, ox and sheep are shown in Fig. 4. The ionic strength was adjusted with Mops (*I* 0.005–0.1) or perchlorate ($I_{ClO_4^-}$ = 0.005–0.195 plus I_{MOPS} = 0.005). The rate constant for interaction of $O_2^{\cdot-}$ with these proteins decreases with increasing ionic strength. Over this range of ionic strength, the plot of log*k* versus $I^{\frac{1}{2}}$ appears linear and the gradients so obtained are shown in Table 3. The

ionic-strength-dependence of the SOD activity with perchlorate was also determined at pH 10.7 with the bovine and yeast proteins and at pH 11.9 with the bovine protein. The slopes determined from the linear dependence of log$k$ upon $I^{\frac{1}{2}}$ are also presented in Table 3. The effect of ionic strength with perchlorate at pH 10.7, a pH value intermediate between the calculated pK_1 and pK_2 (Table 2), is about half that at pH 7–8.5. At pH 11.9, where pH > pK_2, the activity is essentially independent of ionic strength. These findings with perchlorate are consistent with those whereby the pH-dependence of the SOD activity in the presence of perchlorate was determined polarographically (Argese *et al.*, 1987). It should be noted that the dependence upon ionic strength for these proteins is greater with perchlorate. This point is emphasized by the smaller effect of ionic strength at pH 8.0 (I 0.02–0.2) with phosphate (slope = −1.0). It is also apparent that the effect of ionic strength is dependent upon the state of protonation of the protein (pH) and also upon the salt used to adjust I. Indeed, the effect of I upon the activity of these proteins, with the use of borate to adjust I, becomes negligible at pH above 10.5 (see Figs. 1–2), in contrast with the observations made when perchlorate was used to adjust I. Preliminary observations of the pH-dependence of the activity of bovine SOD at I 0.1 adjusted with perchlorate indicate that an inflexion is not apparent as witnessed with borate, and that the activity only becomes independent of I at pH above 11.5.

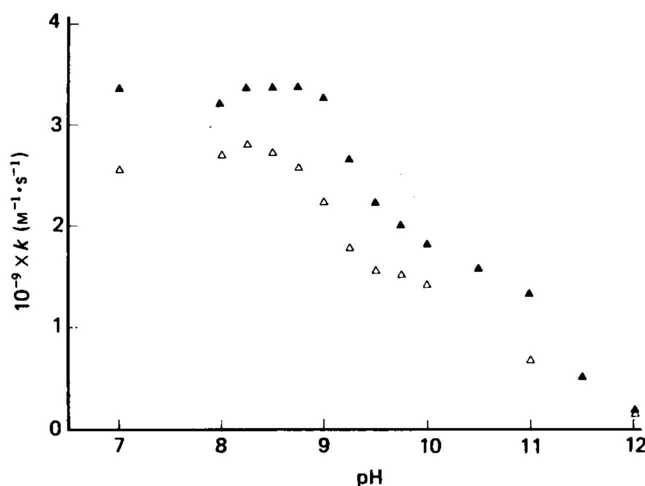

Fig. 3. Variation with pH of yeast SOD activity at two different ionic strengths

Experimental conditions were as given in Fig. 1 legend. ▲, I 0.02; △, I 0.1.

DISCUSSION

The pH-dependences and ionic-strength effects (at pH below 9.0) on the activity of bovine SOD are broadly in keeping with previous observations, especially at low I (Argese *et al.*, 1987). For all the SOD proteins studied the activities and their pH-dependences are similar, with yeast SOD showing greater differences. These pH-dependences are suggested to be governed by group(s) with different pK_a values (Table 2). It has previously been suggested with bovine SOD that two positively charged

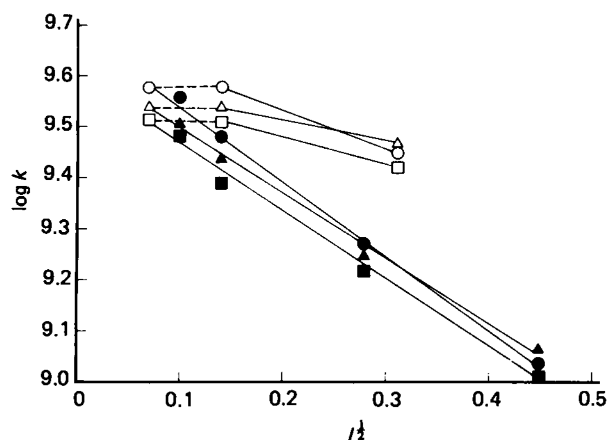

Fig. 4. Influence of ionic strength on the bimolecular rate constant for interaction of $O_2^{\cdot-}$ with ox, sheep and yeast SODs at pH 8.5

○, Ox SOD (Tris/Mops); □, sheep SOD (Tris/Mops); △, yeast SOD (Tris/Mops); ●, ox SOD [perchlorate plus Tris/Mops (I 0.005)]; ■, sheep SOD [perchlorate plus Tris/Mops (I 0.005)]; ▲, yeast SOD [perchlorate plus Tris/Mops (I 0.005)]. Other experimental conditions were as given in Fig. 1 legend.

Table 3. Dependence on ionic strength at various pH values of the bimolecular rate constants for the interaction of $O_2^{\cdot-}$ with ox, sheep and yeast SODs

The dependences are expressed as the slopes of linear plots of log$k$ versus, $I^{\frac{1}{2}}$. The slopes for Mops were obtained from only two experimental points (I 0.02 and I 0.1; see Fig. 4). For the experimental details see the Experimental section. Abbreviation: N.D., not determined.

		Slope		
pH	Salt	Ox SOD	Yeast SOD	Sheep SOD
6.5, 8.5	Perchlorate	−1.5	−1.2	−1.3
10.7	Perchlorate	−0.7	−0.6	N.D.
11.9	Perchlorate	Independent	N.D.	N.D.
8.5	Mops	−0.8	−0.4	−0.5
6.0, 8.0	Phosphate	−1.0	N.D.	N.D.

groups with pK_a 10.1 and 10.8 are involved in the control of activity with about equal contribution of each to the overall activity (Argese *et al.*, 1987). In fact, from the present study the involvement of groups with pK_a values of approx. 9.0–9.5 and approx. 11.0–11.5 become more apparent at the higher ionic strength, even though at the lower ionic strength there is an indication from curve-fitting of pK_a that the dependence is not solely governed by a group(s) with pK of approx. 10.0. Whether the effect of I upon pK_a values of the various residues is responsible for changes in the shape of these dependences is as yet not quantified. The activity of all the proteins becomes less dependent upon ionic strength with increasing pH. Indeed, the effects of ionic strength at various pH values (Table 3) clearly indicate a decreased effect of ionic strength upon activity whereby the activity is essentially independent of I at pH 11.5 and above, consistent with the finding by Argese *et al.* (1987) with bovine SOD only. All these observations are in support of more than one charged group assisting in the guidance and interaction of $O_2^{\cdot -}$ with the copper centre, and that the electrostatic effect of these groups is governed by pH (i.e. their state of protonation) and the ions involved in modifying I. With the yeast protein the pH-dependence of the activities shows two distinct portions even at the lower ionic strength (Fig. 3). For all the proteins with the exception of the yeast protein, the sequence of the charged residues in the fragment 110–150, which form the active-site channel, is essentially conserved. Indeed, at pH below 9 the activities of all the proteins are similar and independent of pH in the range 7–9. This independence does not therefore reflect differences in the overall charge (pI) of the proteins, as shown in Table 1. It is also consistent with the effect of ionic strength on the activity (McAdam, 1977; Cudd & Fridovich, 1982) whereby the activity should increase, and not decrease as observed, for interaction of $O_2^{\cdot -}$ with proteins of overall negative charge. Therefore the decrease in the activity of these proteins with ionic strength (at pH below 9) is consistent with localized electrostatic shielding of positively charged centres and not with the overall charge of the proteins. The pH-dependence of the activity is consistent with decreasing this electrostatic effect upon charge neutralization of the group involved (deprotonation).

The identification of the centres responsible for this pH-dependent behaviour of SOD in the pH range 9–12 is not straightforward. Previous work has essentially been conducted along the following two lines. (*a*) Elimination by chemical modification of the charges on lysine residues or Arg-141 (Cudd & Fridovich, 1982; Cocco *et al.*, 1982; Argese *et al.*, 1984) had the same effect on activity as did raising the pH above pH 9 or increasing the ionic strength; the latter conclusion only pertains for lysine modification. (*b*) Important spectroscopic transitions that involve changes in the first co-ordination sphere have been reported to occur in SOD within the same pH range (Rotilio *et al.*, 1971; Terenzi *et al.*, 1974; Boden *et al.*, 1979; Bertini *et al.*, 1981, 1985). All these reports agree in considering co-ordination of OH^- to the copper as the most likely event that may account for the experimental observations.

With these considerations in mind and taking into account the species variation of SOD amino acid sequence, the conserved Lys-134 and Arg-141 are the best candidates among the amino acid residues to explain the determined pK values, and OH^- co-ordination cannot be ruled out as possible source of the higher pK. It should, however, be kept in mind that Argese *et al.* (1987) observed that the condition $k_1 = k_2$ is still valid at pH 11.5, and this, in our present opinion, would not favour the idea that changes of the first copper co-ordination sphere, which were established to occur in this pH range, can be rate-determining under the conditions of the kinetic assay used in this work.

The other major finding from these studies is that the dependence of the SOD activity on ionic strength reflects a dependence on the salt used. With perchlorate the effect of I is apparent over the pH range studied whereby at pH above 11.5 the activity becomes independent of I. On the other hand borate and phosphate modify the activity of the SOD at pH above 10.5 with the exception of the yeast protein. This finding implies that the electrostatic shielding by these latter ions is only important with the residue(s) with pK_a approx. 9.3, whereas perchlorate may also participate in shielding of the centre(s) with pK_a approx. 11.0. The effects of various ions upon the activity of the proteins need to be studied in more detail concerning ease of access of different ions to the various sites. This ease of access is particularly pronounced with the yeast protein. Indeed electrostatic shielding of Arg-141 will depend upon access by the ions to the solvent channel.

It is concluded that the interaction of $O_2^{\cdot -}$ with these proteins is not strongly influenced by the overall charge of the protein as reflected by the pI values. It should, however, be pointed out that the slight difference of rate constants between the bovine and the two other mammalian SODs (the yeast enzyme being much less homologous for such a comparison) is roughly in line with the most acidic protein having a higher probability of effective collisions of $O_2^{\cdot -}$ with the localized charged centres that are responsible for guiding the substrate to the copper site. Whether the effects of various ions used to adjust I reflect their ability to approach and electrostatically shield these centres to different extents is, as yet, not known.

This work was supported in part by the Progetto Strategico Biotecnologie of C.N.R., Rome, Italy. We are grateful to D. Papworth for performing the curve-fitting procedures.

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Received 13 April 1987/25 September 1987; accepted 19 November 1987