# Modulation of the Catalytic Rate of Cu,Zn Superoxide Dismutase in Single and Double Mutants of Conserved Positively and Negatively Charged Residues†

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*Received August 4, 1994; Revised Manuscript Received December 1, 1994*⊗

ABSTRACT: The catalytic rate of four single and three double mutants of *Xenopus laevis* Cu,Zn superoxide dismutase B, neutralized at Lys120, Asp130, Glu131, and Lys134, has been determined by pulse radiolysis as a function of ionic strength. Neutralization of Glu131 increases the catalytic rate by 80% at low ionic strength, but the effect is reduced to 50% at physiological ionic strength. The rate is unperturbed upon neutralization of Asp130, while neutralization of either of the two lysines drastically decreases the enzyme activity. The Lys120Leu-Lys134Thr and Lys134Thr-Asp130Gln double mutations have an additive and a compensative effect, respectively, on the activity values, while neutralization of the Glu131-Lys134 pair, which also has a compensative effect, gives rise to a faster enzyme at any ionic strength value. The effects observed in the single Asp130Gln and Lys120Leu mutants differ from those reported on human or bovine enzymes [Getzoff et al. (1992) *Nature (London) 358*, 347−351; Sines et al. (1990) *Biochemistry 29*, 9403−9412], indicating that some residues occupying the same position in the linear sequence of different Cu,Zn superoxide dismutases have a different functional weight. Our results also suggest that the strategy of multiple charge mutation may be a promising approach in order to increase the catalytic rate of Cu,Zn SODs independently of ionic strength.

Cu,Zn superoxide dismutases (Cu,Zn SODs) are a class of metalloenzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide by alternate reduction and oxidation of a copper ion which constitutes the active redox center (Bannister et al., 1987). In nonsaturating conditions the rate-limiting step for the overall reaction is the binding of substrate; *i.e.*, the chemical step involving the redox reaction is much faster than the encounter rate (Fielden et al., 1974). The diffusion of the negatively charged substrate toward the active site may be modulated by the distribution of the electrostatic potential around the protein (Getzoff et al., 1983; Klapper et al., 1986), which has been suggested to be constant in the evolution of this protein (Desideri et al., 1992). In line with this hypothesis, selective chemical modification of positively charged residues surrounding the active site (Malinowski & Fridovich, 1979; Cocco et al., 1982; Cudd & Fridovich, 1982), alkaline pH, and high ionic strength values (Argese et al., 1987; O'Neill et al., 1988) have an inhibitory effect on the enzymatic activity. Evaluation of the role of the charged amino acid residues carried out by Brownian dynamics simulation (Sines et al., 1990) indicated that alteration of single charges substantially affects the calculated reaction rates only in the case of three residues, namely, Arg141, Lys134, and Glu131.

The crucial role of Arg141 for the catalytic activity in providing both an electrostatic sink and a terminal docking site for $O_2^-$ along the catalytic pathway has been recently demonstrated by site-directed mutagenesis (Fisher et al., 1994). Reversing the charge of Glu131 was predicted to increase the enzyme−substrate association rate and thus the catalytic rate (Getzoff et al., 1983; Sharp et al., 1987; Sines et al., 1990). This prediction was recently confirmed by site-directed mutagenesis on the human enzyme (Getzoff et al., 1992). Finally, a major role of Lys134 in electrostatically steering superoxide toward the Cu,Zn SOD active site has been demonstrated by comparing the catalytic properties of the bovine and the shark SODs in which all lysine residues have been neutralized by carbamoylation (Polticelli et al., 1994). In the latter enzyme, in fact, Lys134 is substituted by an arginine residue which is insensitive to the chemical treatment, allowing to quantitate as 20% the contribution of the charge in position 134 to the facilitated diffusion of the substrate.

The design of more efficient superoxide dismutases by increasing positive electrostatic field around the active site may be relevant not only to the general theory of electrostatic effects in diffusion-controlled enzyme catalysis but also to biotechnological applications, in view of the possible use of superoxide dismutases as antioxidant agents in human therapy (Omar et al., 1992). However, a simple increase of the positive charge in the proximity of the active site has also the effect of increasing the ionic strength dependence of the catalytic rate, making the difference of activity between

† This work was supported by the CNR Center for Molecular Biology and by the EC program "Human Capital and Mobility". F.P. is a Human Capital and Mobility fellowship holder.

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1995.

0006-2960/95/0434-6043$09.00/0 

the native and the mutant enzymes smaller at physiological ($\mu^{1/2} = 0.38$) than at low ionic strengths (Getzoff et al., 1992). Mutant enzymes having an increased catalytic rate without suffering for an increased ionic strength dependence could be obtained by a multiple mutagenesis approach involving both positively and negatively charged residues.

In our laboratory, the isoenzyme B of the Cu,Zn SOD from *Xenopus laevis* (XSODB) has been recently expressed (Battistoni et al., 1992), and its structure, partly described in this study, has been resolved at 1.5 Å resolution (Djinovic et al., unpublished results).

In this work we have investigated by pulse radiolysis, the only method which allows a direct determination of the superoxide dismutase activity (O'Neill & Chapman, 1985), the effect of ionic strength on the catalytic properties of four single mutants of XSODB in which the charged residues nearest to the copper active site, besides the catalytically crucial Arg141, were neutralized: Asp130 → Gln (D130Q), Glu131 → Gln (E131Q), Lys120 → Leu (K120L), and Lys134 → Thr (K134T). Three double mutants, constructed from combinations of the single mutants, were also studied, *i.e.*, Asp130-Lys134 → Gln-Thr (D130Q-K134T), Glu131-Lys134 → Gln-Thr (E131Q-K134T), and Lys120-Lys134 → Leu-Thr (K120L-K134T). Comparison with previous experimental or simulated results obtained on the human (Getzoff et al., 1992) and the bovine enzyme (Sines et al., 1990), respectively, indicates that residues occupying the same position in the linear sequence have different catalytic relevance in different Cu,Zn SOD variants depending on even minor differences in 3D structure. Moreover, catalytic measurements of the activity of the double mutants indicate that the effects of double mutation are not simply additive but considerably more complicated.

## MATERIALS AND METHODS

All reagents used were of the highest grade available. Protein concentration was determined by the absorption at 258 nm ($\epsilon_{mM} = 10.3\ M^{-1}\ cm^{-1}$; Rotilio et al., 1972). Copper content was determined by double integration of the EPR spectra using a Cu−EDTA solution as a standard (Aasa & Vanngard, 1975).

*Site-Directed Mutagenesis.* Standard DNA manipulations were carried out as described by Sambrook et al. (1989). Molecular mutants of the recombinant Cu,Zn superoxide dismutase from *X. laevis*, isoenzyme B (XSODB), were prepared by site-directed mutagenesis according to Kunkel et al. (1990). The single-stranded DNA template for mutagenesis was generated using plasmid pXLSODBcDNA1 containing the full-length nucleotide sequence coding for XSODB inserted in pEMBL 8(+) (Carrì et al., 1990). Mutagenesis reactions were performed as follows: single-stranded DNA was mixed with the mutagenic oligonucleotide in 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 50 mM NaCl, incubated for 2 min at 80 °C, and allowed to cool slowly at room temperature. This hybridization mixture was diluted 1:4 with 20 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 12.5 mM $MgCl_2$, 62.5 $\mu$M each of dATP, dTTP, dCTP, and dGTP, and 0.05 mM ATP, and the *in vitro* DNA polymerization was started by the addition of native T7 DNA polymerase and T4 DNA ligase. The reaction mixtures were incubated for 1 h at 37 °C and then terminated by the addition of EDTA to a final concentration of 15 mM. The amounts of double-stranded DNA products obtained with the *in vitro* DNA synthesis reactions were compared to those obtained in control reactions performed in the absence of the mutagenic oligonucleotides, by subjecting to electrophoresis 20 $\mu$L of each reaction mixture in a 1% agarose gel (Kunkel et al., 1990). Competent BMH 71/18 mutS cells (Kramer et al., 1984) were transformed with the mutagenesis mixtures, inoculated in 10 mL of LB containing 100 $\mu$g/mL ampicillin, and grown overnight at 37 °C. In order to obtain segregation of mutant and wild-type plasmids, DNA was extracted and transformed in BMH 71/18 cells (Messing et al., 1977) that were plated on LB−ampicillin. Colonies containing the mutated plasmid were identified by colony hybridization, using the radiolabeled mutagenic oligonucleotides as hybridization probes. Nucleotide sequence analysis was carried out by the chain termination method.

*Expression and Purification of Wild-Type and Mutant XSODBs.* Plasmid pKB overexpressing XSODB under control of the trc promoter has been described previously (Battistoni et al., 1992). Plasmids obtained in mutagenesis experiments were digested with *Eco*RI, treated with Klenow polymerase, and then restricted with *Nco*I. The 600 bp DNA fragment containing the entire cDNA was inserted in plasmid pKK233-2 (Amann & Brosius, 1988) previously cut with *Hin*dIII, treated with the Klenow enzyme, and finally digested with *Nco*I. In order to avoid possible contaminations of bacterial Mn− and Fe−SODs, wild-type and mutant XSODs were constitutively produced in the *Escherichia coli* strain QC871 which is defective in the bacterial enzymes.

*E. coli* cells were disrupted as described by Marston (1987). Cell debris and insoluble proteins were removed by centrifugation, and supernatants were subjected to $(NH_4)_2SO_4$ fractionation. The enzyme activity was found in the 60−100% saturation fractions, which, after removal of ammonium sulfate, were heated for 30 min at 60 °C and centrifuged. The supernatants were subjected to gradient elution on a Whatman DE52 column with 5−50 mM phosphate buffer, pH 7.4. Fractions containing XSODBs were pooled and purified to >98% homogeneity, as judged by SDS−PAGE, by one or more steps on a Mono Q 5/5 FPLC column (Pharmacia).

EPR spectra were recorded at X-band and liquid nitrogen temperature with a Bruker ESP 300 spectrometer. The integrity of the copper site of mutant enzymes was confirmed by EPR spectroscopy.

*X-ray Diffraction.* Orthorhombic crystals of recombinant *X. laevis* Cu,Zn SOD (space group $P2_12_12_1$, one enzyme dimer per asymmetric unit) suitable for X-ray diffraction experiments were grown as described previously (Djinovic et al., 1993). A single crystal was used to collect three-dimensional diffraction data to 1.49 Å using the EMBL beam line X31 at the DORIS storage ring, DESY, Hamburg ($\lambda$ = 0.91 Å). The data were recorded at 4 °C by employing a locally developed image plate system as detector. The refinement of orientation parameters and integration of intensities were performed using the MOSFLM suite of programs (Leslie, 1991). A total of 245 181 intensities were merged to 42 209 unique data ($R_{merge} = 0.078$) with the CCP4 suite (CCP4, 1979).

*Catalytic Measurements.* Activity assays were carried out by the pulse radiolysis method (O'Neill & Chapman, 1985) which is based on the first-order rate of loss of $O_2^-$ at 250 nm (Fielden et al., 1974). To avoid binding of anions like

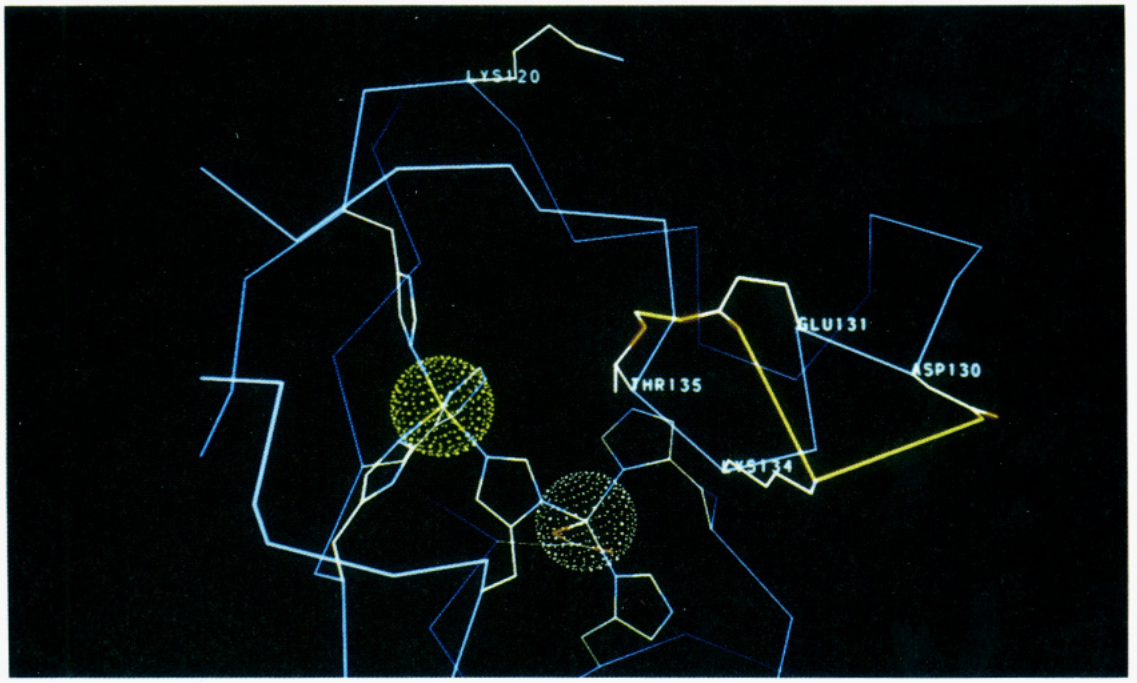


FIGURE 1: Three-dimensional view of the active site region of *X. laevis* Cu,Zn superoxide dismutase B pointing out the residues involved in the long-range electrostatic recognition of the $O_2^-$ substrate. The protein backbone is represented by a blue line connecting all the Cα atoms; the copper and zinc ions are represented by a green and a white dotted sphere, respectively. Yellow lines represent the network of hydrogen bonds and salt bridges connecting Thr135, Glu131, Lys134, and Asp130.

phosphate or chloride to the copper, enzymes were assayed in Tris−Mops buffer, pH 7.4, containing 0.1 M ethanol and 0.1 mM EDTA, $\mu = 0.02$ M, and the ionic strength was increased by adding $NaClO_4$ to the sample buffer. Solutions placed in a 0.7-cm path-length optical cell were irradiated with a 1.6-$\mu$s electron pulse, giving a concentration of $O_2^-$ equal to 15 $\mu$M. Since the protein concentration for each sample varied in the range 0.3−1.2 $\mu$M, activity assays were carried out with $[O_2^-] \gg [SOD]$, under turnover conditions.

## RESULTS

*Active Site Structure of XSODB.* The three-dimensional model (Djinovic et al., 1993) of *X. laevis* SOD was initially refined using the simulated annealing protocols of X-PLOR (Brunger, 1990). The refinement was continued with the PROLSQ program suite (Konnert & Henderickson, 1980) in combination with the ARP package (Lamzin & Wilson, 1993) alternated with inspection of electron density maps using the FRODO program suite (Jones, 1978). When refinement of the model reached convergence, hydrogen atoms were added at standard positions and included in the refinement as a fixed-atom contribution. Finally, the SHELXL93 program package (kindly provided by Dr. G. Scheldrick, University of Gottingen, Gottingen, Germany) was applied, allowing restrained refinement of atomic positional and anisotropic thermal parameters. The conventional *R*-factor converged to the value of 0.103 for 2160 protein atoms and 353 ordered solvent molecules bound to the protein surface.

The active site coordination environment is displayed in Figure 1. As expected, the stereochemistry of the copper center is substantially conserved, when compared with the other available structures (Tainer et al., 1982; Kitagawa et al., 1991; Djinovic et al., 1992; Parge et al., 1992), as far as the location of R141 and the copper-coordinating histidyl residues are concerned. The backbone as well as the side-chain conformations of the residues mutated in this work, i.e. 120, 130, 131, and 134, are also conserved. Residues 130, 131, 134, and 135 build up the upper rim of the active site channel and are characterized by a hydrogen-bonding network which is also observed in human (Parge et al., 1992) and in yeast SOD (Djinovic et al., 1992). This network connects T135 to E131 and keeps the latter in the most appropriate position to form a salt bridge with K134, which electrostatically interacts with D130. In the active site region the side chain of D122 acts as a bridge between H44 (copper ligand) and H69 (zinc ligand), forming hydrogen bonds with histidyl side chains of each of these two metal ligand residues, defining their correct orientation. In detail the D122 OD1 atom is surrounded by three hydrogen atoms, H69 NE2, D123 N, and L124 N atoms, at distances of 2.78, 3.10, and 2.80 Å, respectively, while D122 OD2 interacts with H44 NE2, H69 NE2, and a water molecule at distances of 2.68, 3.10, and 2.79 Å, respectively. These interactions, which are relevant for the active site structural organization, are conserved in the other known SOD three-dimensional structures (Bordo et al., 1994).

The EPR spectra of the wild-type enzyme and of XSODB mutants are identical (data not shown), indicating that no perturbation of the copper site occurs upon neutralization of the charged residues.

*Modification of Negatively Charged Residues.* The activity of the native enzyme and single mutants E131Q and D130Q was measured by pulse radiolysis at pH 7.5 as a function of the ionic strength (Figure 2A). Neutralization of E131 markedly increases the catalytic rate value, which is 80% higher than that of the wild type, reaching the value of $6.5 \times 10^9$ $M^{-1}$ $s^{-1}$ at $\mu = 0.02$ M, very close to the limit of the pure diffusion rate. A similar result was already observed when the same residue was neutralized in the human enzyme (Getzoff et al., 1992). The ionic strength dependence of the activity of E131Q is more pronounced

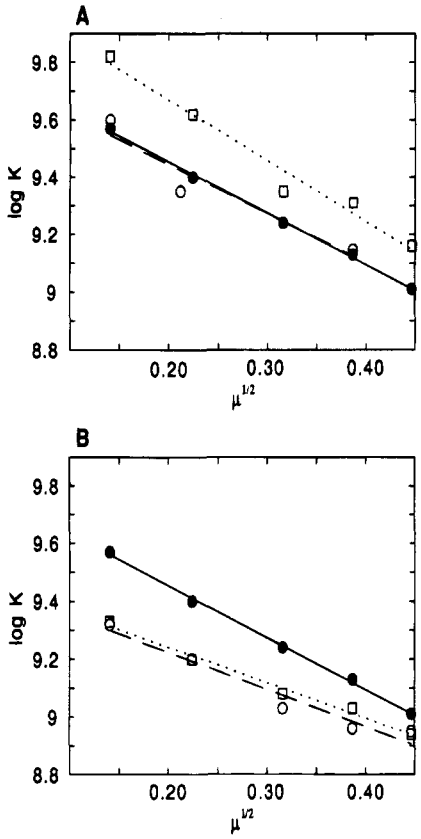


FIGURE 2: Ionic strength dependence of the activity of wild-type and single mutant XSODBs: (A) wild type (●), D130Q (○), and E131Q (□); (B) wild type (●), K120L (○), and K134T (□). The straight lines represent least-squares fittings of the experimental data. The assays were carried out at room temperature in Tris–Mops buffer, pH 7.4, and $\mu$ = 20 mM; the ionic strength was increased by addition of $NaClO_4$.

than that of the wild type, so that its catalytic rate at physiological ionic strength ($\mu^{1/2}$ = 0.38) is reduced to 2.0 × $10^9$ $M^{-1}$ $s^{-1}$, a value that, however, is still 50% higher than that of the wild-type enzyme. The apparent charge felt by the incoming $O_2^-$ substrate was evaluated by the slope of the ionic strength dependence of the association rate constant (Argese et al., 1987). Although the interpretation in terms of apparent charge of such plots is widely used in the literature (Argese et al., 1987; O'Neill et al., 1988; Getzoff et al., 1992), it is an oversimplification of the Bronsted–Debye–Huckel theory (Koppenol, 1980), since straight lines may be expected only for ions with a radius of about 0.3 nm (Alberty & Hammes, 1958, Koppenol & Margoliash, 1992). With this caution in mind, this approach has been used only to qualitatively compare the results obtained on different mutants.

A similar slope of the activity *vs* ionic strength plot is obtained for the wild-type enzyme and the E131Q mutant (apparent charge of 1.8 and 2.1, respectively). The sensitivity of the activity to ionic strength suggests that the effect of the charge in position 131 is not localized within the channel but it extends into the solvent, where the diffusion process occurs as already pointed out by the simulation of the association rate by the Brownian dynamics approach (McCammon et al., 1985; Sharp et al., 1987).

The D130Q mutant has a catalytic rate comparable to that of the native enzyme at every ionic strength value, indicating that the incoming substrate feels a very similar apparent charge in the two cases. An explanation for this result may be that neutralization of D130 is compensated by the remaining E131 residue, which, in the absence of the negative charge of D130, can interact with the positive charge of K134 more strongly than it does in the wild-type enzyme, reinforcing its negative contribution. This is confirmed by the apparent charge felt by superoxide (1.5 e), a value similar to that displayed by the wild-type enzyme.

*Modification of Positively Charged Residues.* Neutralization of K134, which is located at the lip of the active site channel at about 12 Å from the copper, reduces the activity of the wild-type enzyme by 40% at low ionic strength (Figure 2B). This result confirms the prediction, made on the bovine enzyme by Brownian dynamics simulations (Sines et al., 1990; Polticelli et al., 1994), that this residue plays a major role in the electrostatic guidance of superoxide into the channel. The ionic strength dependence of the activity of the K134T mutant is much less pronounced than that of the wild type, as the apparent charge felt by the superoxide was only 1.1 e. This result indicates the strong effect of the positive charge of K134 that extends into the solvent and is not purely confined to the active site channel region. Due to the different salt dependence of the activity of the wild-type enzyme and of the K134T mutant, their association rate values are very similar at physiological ionic strength (Figure 2B), again demonstrating that salt concentration has a crucial role in the modulation of the catalytic rate.

Neutralization of K120 produces a decrease in activity similar to that observed in the K134T mutant (40% at $\mu$ = 0.02 M, Figure 2B). This result is quite surprising, since Brownian dynamics simulation carried out on the bovine enzyme did not indicate any specific role for this residue in the electrostatic steering mechanism (Sines et al., 1990). Note that while in the bovine enzyme K134 is much closer to the copper site (approximately 9 Å; Tainer et al., 1982) than K120, in the *X. laevis* protein the two residues are practically at the same distance from the metal (approximately 12 Å for both residues). The salt dependence of the activity of the K120L mutant is comparable to that of the K134T one (apparent charge 1.2 e) and results in a similar value for the activity at physiological ionic strength with respect to the wild-type enzyme (Figure 2B).

*Double Mutants.* The ionic strength dependencies of the activities of the mutants doubly neutralized at residues 130–134 (D130Q-K134T), 131–134 (E131Q-K134T), and 120–134 (K120L-K134T) are reported in Figure 3. The mutants displaying the neutralization of both a positive and a negative residue have an ionic strength dependence similar to that of the native enzyme (apparent charge 1.5 e and 1.7 e for D130Q-K134T and E131Q-K134T, respectively). The catalytic rate of the D130Q-K134T mutant is lower (20% lower at $\mu^{1/2}$ = 0.1) than that of the wild-type enzyme while that of the E131Q-K134T mutant is higher (30% higher at $\mu^{1/2}$ = 0.1). This is in line with the larger effect on activity displayed by modification of Glu131 as compared to Asp130. The mutant neutralized at two positive residues (K120L-K134T) has a markedly low activity (31% residual activity at $\mu$ = 0.02M), but as its salt dependence is lower than that of the wild type, the activity decrease is reduced to 50% at physiological ionic strength (Figure 3).

In Figure 3 the activity of the double mutants is also compared to that predicted by a simple multiplicative model based on component single mutations (Sines et al., 1992).

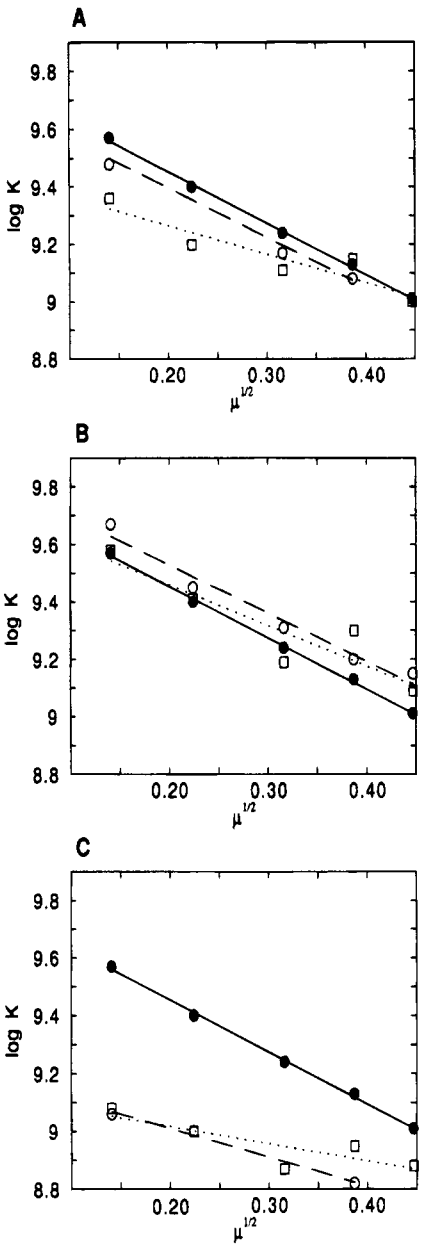


FIGURE 3: Ionic strength dependence of the activity of wild-type (●) and double mutant XSODBs: (A) D130Q-K134T, (B) E131Q-K134T, and (C) K130L-K134T. Symbols: experimental ionic strength dependence (○); ionic strength dependence calculated by a multiplicative model based on component single mutations (□) (see Results); dashed lines, experimental data fitting; dotted lines, multiplicative data fitting. Assay conditions are as in Figure 2.

In this model the catalytic constant of a double mutant modified in two sites, A and B ($K_{AB}$), is given by

$$K_{AB}/K_{wt} = K_A/K_{wt} + K_B/K_{wt}$$

where $K_A$, $K_B$, and $K_{wt}$ are the association rate constants for the enzymes modified at sites A and B and for the wild type, respectively. The physical meaning of this model is that the substrate has a preferential pathway along the active site channel and that the crossing of a free energy barrier is the rate-determining step for its diffusion down into the channel. Deviation from the multiplicative model may imply either a shift in the location of the energy barrier or the formation of alternative pathways when the enzyme's charge distribution is altered. Inspection of Figure 3 indicates that this model partly fits the experimental values for the E131Q-K134T (experimental slope = −1.7, calculated slope = −1.4), while it substantially deviates from the experimental values in the case of the K120L-K134T (experimental slope = −1.0, calculated slope = −0.6) and the D130Q-K134T (experimental slope = −1.5, calculated slope = −1.0) mutants at high and low ionic strength, respectively.

## DISCUSSION

The results reported in Figure 2 indicate that neutralization of one of the two negative (D130 or E131) or one of the two positive K120 or K134) charges has the effect of increasing or decreasing, respectively, the ability of the enzyme to attract the negatively charged substrate. Among the mutants neutralized at negative residues, the E131Q is catalytically more efficient than D130Q, as the activity of this latter mutant is nearly identical to that of the wild type. This result is in line with Brownian dynamics simulations carried out on the bovine enzyme (Sines et al., 1990). However, in the human enzyme, neutralization produces an increase of the catalytic activity in both E131 and D130, despite the fact that the hydrogen-bonding network in which the two residues are involved (Figure 1), as well as their distances from the copper atom, is nearly identical in both the human [$d_{OG1(Glu133)-Cu}$ = 10.9 Å, $d_{OD1(Glu132)-Cu}$ = 17.9 Å (Parge et al., 1992)] and the *X. laevis* enzymes ($d_{OG1(Glu131)-Cu}$ = 10.8 Å, $d_{OD1(Asp130)-Cu}$ = 17.7 Å). This shows that although Cu,Zn SODs, as a class, are very conserved, subtle changes may occur in different species, giving rise to a different effect after mutation of equivalent residues. This may explain why, although the key charged residues involved in the substrate attraction are conserved in both enzymes, the activity of the wild-type human enzyme (Getzoff et al., 1992) is almost half that of XSODB.

Differences between SOD species are also observed for the mutants in which single positively charged residues are neutralized. In this case, no experimental results concerning the catalytic rate of single mutants neutralized at positively charged residues are available; comparisons are then carried out only with simulated rates obtained by Brownian dynamics on the bovine enzyme (Sines et al., 1990). In XSODB, neutralization of K134 or K120 markedly decreases the activity, while simulation carried out on the bovine enzyme (Sines et al., 1990) predicted a strong decrease for neutralization of K134 but not for K120 residues. Comparison of the 3D structure of the XSODB with that of the bovine enzyme (Tainer et al., 1982) indicates that also in this case the distance of K120 from the copper atom is virtually the same in the two enzymes (approximately 12.0 Å). This result, together with that observed for E130 (Getzoff et al., 1992), indicates that residues in the same position within the linear sequence of Cu,Zn SODs from different species may have a different contribution to the facilitated diffusion of the substrate, although the differences observed at the level of the K120 residue need an experimental confirmation in the human and/or in the bovine enzymes. It is important to stress that the changes of the activity observed in the mutant enzymes with respect to the wild type must be attributed only to neutralization of the charges of the mutated residues since the coordination geometry of the copper active site is identical to that of the wild type, as demonstrated by the EPR spectra.

A common feature of all "electrostatic" single mutants studied up to now is that the activity difference between the wild-type enzyme and the mutants is reduced with increasing ionic strength, resulting in similar catalytic rates at physiological ionic strength (Figure 2). This is true for both the human (Getzoff et al., 1992) and the XSODB enzymes and is due to the fact that neutralization of a negative or a positive charge in the surrounding of the active site increases or reduces, respectively, the ionic strength dependence of the catalytic rate. The only exception is the D130Q mutant of XSODB, which displays an ionic strength dependence very similar to that of the wild type. However, in this case no appreciable increase of the activity with respect to the wild type, even at low ionic strength, was observed. The absence of any effect of the neutralization of D130 on the catalytic activity of XSODB is quite surprising, since a negative charge in position 130 is conserved in all cytoplasmic Cu,Zn SODs (Bordo et al., 1994). It may be suggested therefore that in XSODB the function of this residue is to ensure correct positioning for the E131-K134 pair, as confirmed by the large deviation from multiplicative behavior of the D130Q-K134T double mutant. Another interesting question concerning the D130Q-K134T mutant is why the effect of K134 mutation is dampened so much by neutralization of D130 if the mutation of the latter residue has virtually no effect on the catalytic rate. A possible explanation is that a different network of interacting charges occurs in the D130Q-K134T mutant, possibly due to the higher mobility of the 131 residue because of the lack of the interaction with the positively charged K134, resulting in a lower repulsion of the negatively charged substrate. Further work is needed to clarify this point, but the large deviation between the calculated and the experimental rate (40% at $\mu^{1/2} = 0.1$) indicates that the effect of double mutation in this case is not simply additive but considerably more complicated.

The double mutant E131Q-K134T displays a salt dependence of activity virtually identical to that of wild type, with a consistently higher rate constant. In this case the multiplicative model fits the experimental data only at high ionic strength, where the higher activity of E131Q-K134T, with respect to the wild type, can be predicted by simply taking into account the activity of the single mutants, as the increase due to the effect of negative charge neutralization in E131 is greater than the decrease due to positive charge neutralization in K134. Natural Cu,Zn SOD variants with neutral 131 and 134 residues have been purified from plants (Kwiatowski et al., 1991), but in these cases neutralization of such charges is accompanied by a large rearrangement of the charge network around the active site, such as introduction of a histidine in position 129 and, in some cases, deletion of the positive charge of K120 and insertion of a lysine at position 133 (Kitagawa et al., 1991). In the case of the K120L-K134T mutant, the multiplicative model strongly deviates from the experimental data, at least at high ionic strength, since the decrease in the activity of the double mutant is higher than the sum of the decreases observed in the two single ones.

In conclusion, this work shows that (a) some residues in the same position within the linear sequence of Cu,Zn SODs from different species have a different functional weight in the process of steering the anionic substrate toward the active site; (b) mutants in which charged residues are neutralized display large differences (either positive or negative) in the catalytic rate with respect to the wild type, at low ionic strength, but these differences are quite reduced at physiological ionic strength ($\mu^{1/2} = 0.38$); and (c) the strategy of multiple charge mutation may be a promising approach in order to increase the catalytic rate of Cu,Zn SODs independently of ionic strength, although in the present study the single E131Q mutant is the fastest enzyme, even at physiological ionic strength. Moreover, utilization of such an approach requires some caution as the catalytic rate of the double mutants is not simply additive, possibly because a rearrangement of the charge network around the active site occurs in the double mutants.

## ACKNOWLEDGMENT

The authors thank Dr. F. Polizio for recording the EPR spectra. The *E. coli* strain QC871 was a kind gift of Dr. D. Touati.

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BI941795N