Identification of the residues responsible for the alkaline inhibition of Cu,Zn superoxide dismutase: A site-directed mutagenesis approach

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Abstract

The catalytic rate of wild type, two single (Lys 120 → Leu, Lys 134 → Thr), and one double (Lys 120 → Leu-Lys 134 → Thr) mutants of *Xenopus laevis* B Cu,Zn superoxide dismutase has been studied by pulse radiolysis as a function of pH. The pH dependence curve of the activity of the wild-type enzyme can be deconvoluted by two deprotonation equilibria, at pH 9.3 (pK_1) and at pH 11.3 (pK_2). Catalytic rate measurements on single and double mutants indicate that pK_1 is mainly due to the deprotonation of Lys 120 and Lys 134, with only a minor contribution from other surface basic residues, whereas pK_2 is due to titration of the invariant Arg 141, likely coupled to deprotonation of the copper-bound water molecule. Accordingly, Brownian dynamics simulations carried out as a function of pH reproduce well the pH dependence of the catalytic rate, when the experimentally determined pK s are assigned to Lys 120, Lys 134, and Arg 141.

Keywords: alkaline inhibition; Brownian dynamics; electrostatics; pulse radiolysis; site-directed mutagenesis

Cu,Zn superoxide dismutases (SODs) are a class of dimeric metalloenzymes that catalyze the dismutation of the superoxide anion into oxygen and hydrogen peroxide through a cyclic oxidation–reduction reaction of the catalytically active copper ion (Bannister et al., 1987). The active site is made up of a metal cluster containing one copper ion and one zinc ion, bridged by the common ligand His 61. The copper ion is coordinated by three more histidyl residues and by one water molecule, hydrogen bonded to the guanidinium group of the invariant Arg 141 (Fig. 1; Tainer et al., 1982). Alkaline pH, high salt concentration (Argese et al., 1987; O'Neill et al., 1988), and neutralization of basic residues by chemical reagents (Malinowski & Fridovich, 1979; Cocco et al., 1982; Cudd & Fridovich, 1982) have an inhibitory effect on enzyme activity. These findings suggest that diffusion of the negatively charged substrate toward the catalytic metal is driven by the positive electrostatic potential generated by the metals and the basic residues surrounding the active site channel (Getzoff et al., 1983; Klapper et al., 1986); the magnitude and distribution of such potential are conserved in the evolution of Cu,Zn SODs (Desideri et al., 1992). In agreement with this hypothesis, increase of the positive electrostatic

potential around the active site, neutralizing the negatively charged 130 and 131 residues by site-directed mutagenesis, results in a faster enzyme both in human (Getzoff et al., 1992) and in *Xenopus laevis* Cu,Zn SODs (Polticelli et al., 1995a). For the latter enzyme, it was also shown that Lys 120 and Lys 134, which are approximately 12 Å distant from the copper ion (Djinovic-Carugo et al., 1995; Fig. 1), significantly contribute, at neutral pH, to the steering of the superoxide substrate toward the catalytic metal (Polticelli et al., 1994, 1995a). On the other hand, the role of Arg 141, which is only 6 Å away from the copper ion, is rather that of correctly docking the substrate near the active metal (Djinovic-Carugo et al., 1994) more than long-range steering. Accordingly, its neutralization, by chemical reagents (Malinowski & Fridovich, 1979) or site-directed mutagenesis (Fisher et al., 1994), results in a drastic decrease (approximately 90%) of the catalytic activity of the enzyme.

The alkaline pH dependence of the activity of Cu,Zn SOD has been studied in detail in several natural variants to identify the residues responsible for the reversible decrease of the activity observed between pH 9 and 12 (Argese et al., 1987; O'Neill et al., 1988; Calabrese et al., 1989). In all cases, the pH dependence of the activity was best fitted by two pK values: $pK_1 = 9.5$ and $pK_2 = 11$. pK_1 was suggested to be due to deprotonation of Lys 120 (Argese et al., 1987; Polticelli et al., 1995b) and/or Lys 134 (O'Neill et al., 1988; Polticelli et al., 1995b). pK_2 is

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Fig. 1. Stereo view of the active site of XSODB generated using the program Sybyl (Tripos Associates). The only side chains shown are those of the charged residues located in the electrostatic loop (Lys 120, Asp 130, Glu 131, Lys 134, and Arg 141) and of the copper ligands. The solvent-accessible surface of the active site has been obtained using Connolly's program (Connolly, 1983).

likely due to Arg 141, because its mutation into lysine in the human enzyme (Fisher et al., 1994) causes an unusual drop of the catalytic rate as the pH is increased above pH 7, indicating that Arg 141 is responsible for keeping a strong positive charge near the active site up to pH 10, whereas deprotonating at higher pH values. In this context, it should be noted that a pK of 11.3 has also been determined spectroscopically following the EPR spectrum of the copper ion in the bovine enzyme (Calabrese et al., 1991). It can be reasonably assumed that the change of the copper EPR spectrum in this pH region reflects the prototropic equilibrium of the arginine, hydrogen bonded to the water molecule coordinated to the copper ion.

In our laboratory, the isoenzyme B of the Cu,Zn SOD from *X. laevis* (XSODB) has been expressed recently (Battistoni et al., 1992) and its structure has been resolved at 1.5 Å resolution (Djinovic-Carugo et al., 1995).

In order to identify the residues responsible for the alkaline pH dependence of Cu,Zn SOD and their relative weight in this process, we have investigated by pulse radiolysis the pH dependence of the catalytic properties of two single mutants and one double mutant of XSODB in which the charged residues nearest to the copper site, besides the catalytically crucial Arg 141, were neutralized: Lys 120 → Leu (K120L), Lys 134 → Thr (K134T), and Lys 120 → Leu–Lys 134 → Thr (K120L–K134T).

The results indicate that the pH dependence of wild-type XSODB activity, at pH values lower than 10, is governed mainly by Lys 120 and Lys 134, both contributing approximately 30% to a $pK_1 = 9.3$, whereas Arg 141 is responsible for $pK_2 = 11.3$ with a contribution of 25% to the steering process, in line with the mainly stereochemical role attributed to the latter residue on the basis of the study of site-directed mutants of the human en-

zyme (Fisher et al., 1994). In agreement, Brownian dynamics simulations, carried out considering the five charges nearest to the copper site (i.e., Lys 120, Asp 130, Glu 131, Lys 134, and Arg 141) plus the copper and zinc ions, reproduce well the pH dependence of the experimental catalytic rate.

Results and discussion

Figure 2 shows the alkaline pH dependence of the catalytic rate of wild-type XSODB at 0.02 M ionic strength. As already observed in Cu,Zn SODs variants studied so far (O'Neill et al., 1988; Calabrese et al., 1989), the activity of the enzyme decreases above pH 9. Best fit of the experimental data (Table 1) is obtained with two prototropic equilibria, $pK_1 = 9.3$ and $pK_2 = 11.3$.

To investigate the contribution of basic residues near to the copper site to the two pK s, the pH dependence of the catalytic activity was studied at $\mu = 0.02$ M on site-specific mutants of XSODB in which K120 and/or K134 had been substituted by neutral residues. Figure 3A shows the pH dependence of the activity of the K120L mutant. The activity of the mutant is 65% that of the native enzyme, at neutral pH, confirming previously reported data (Polticelli et al., 1995a). Best fit of the experimental data is obtained with two pK s, $pK_1 = 9.1$ and $pK_2 = 11.2$ (Table 1). The contribution of pK_1 is reduced to 50% of the total activity compared to 75% in the native enzyme, whereas, above pH 10, the pH curve of the mutant is superimposable with that of the native enzyme. These results demonstrate that K120 in the wild-type enzyme contributes only to the first part (pH < 10.5) of the pH-dependent decrease of the activity.

Fig. 2. Plot of the catalytic activity of wild-type XSODB as a function of pH. Continuous line represents best fit of the experimental data. Activity was determined by pulse radiolysis at room temperature and $\mu = 0.02$ M.

The activity of the K134T mutant at neutral pH is slightly lower than that of the K120L mutant (approximately 60% that of the wild type at pH 7.4; Fig. 3B) and, in the pH range 10.5–12, it is nearly superimposable with that of the wild type; best fit of the experimental catalytic rate data gives two pK s, $pK_1 = 9.0$ and $pK_2 = 11.2$ (Table 1). This finding, together with the observation that the contribution of pK_1 to the total activity is reduced to 45% in the K134T mutant, indicates that K134 as well is efficient in the substrate steering process only for pH values lower than 10.5.

The pH dependence of the K120L–K134T mutant is shown in Figure 3C. The catalytic rate of the double-mutated enzyme at neutral pH is only 40% that of the wild-type enzyme. The experimental data are best fitted by two protonation equilibria, $pK_1 = 8.8$ and $pK_2 = 11.3$ (Table 1) and a further decrease of the contribution of pK_1 to the total activity of the double mutant (40%) is observed with respect to the single mutated enzymes. Interestingly, the pH-activity curve of the double mutant parallels those of the wild type and of the single mutants above pH 10, confirming that neither Lys 120 nor Lys 134 play any role in the substrate steering above pH 10.5.

Results on single mutants indicate that the contribution of Lys 120 and Lys 134 to the activity of the wild-type enzyme is 25 and 35%, respectively, as evaluated on the basis of the contribution of pK_1 to the activity of K134T and K120L mutants,

Table 1. pK values of the pH dependence of the activity of wild-type and site-specific mutants of *Xenopus laevis* B Cu,Zn SOD

	% pK_1	pK_1	% pK_2	pK_2	R^2
Wild-type	75	9.3 ± 0.2	25	11.3 ± 0.3	0.99
K120L	50	9.1 ± 0.3	50	11.2 ± 0.2	0.98
K134T	45	9.0 ± 0.3	55	11.2 ± 0.2	0.98
K120L–K134T	40	8.8 ± 0.3	60	11.3 ± 0.3	0.94

Fig. 3. Plot of the catalytic activity of (A) K120L, (B) K134T, and (C) K120L–K134T mutant XSODBs as a function of pH. The pH dependence of the activity of wild type (circles) is reported in each panel for comparison. Continuous lines represent best fit of the experimental data. Assay conditions as in Figure 1.

respectively (see Equations 1A and 1B in the Materials and methods). In the case of the double mutant, the same equations allow us to evaluate as 17% the contribution of the surface basic residues to the total activity of the wild-type enzyme. The sum

of the three contributions (77%) matches well the relative weight of pK_1 (75%, Table 1) to the pH dependence of the activity of the wild-type enzyme, leading to the conclusion that effects of mutation of Lys 120 and Lys 134 are independent and additive with respect to each other. To verify further the mutual independence of the effects of the two mutations, the activity of the double mutant was compared to that predicted by a simple multiplicative model based on the component single mutations (Sines et al., 1992), in which the catalytic rate of the double mutant K_{AB} was related to that of the single mutants by the following equation:

$$K_{AB}/K_{wt} = K_A/K_{wt} \times K_B/K_{wt}.$$

It can be seen from Figure 4 that, in the pH range in which deprotonation of Lys 120 and Lys 134 occurs (7–10), catalytic rate values calculated by the multiplicative model match well the experimental values of the double mutant, confirming that mutation of each of the two lysines does not affect the properties of the other one. As a consequence, pK_1 of the K134T mutant (9.0, Table 1) is to be assigned to Lys 120 and pK_1 of the K120L mutant (9.1, Table 1) to Lys 134.

The identity of the activity pH dependence at pH values higher than 10, in all the samples investigated, suggests that the same basic residue is responsible for pK_2 both in the wild type and in the mutant enzymes. A pK around 11 has been already identified in the pH dependence of the activity of several natural and chemically modified variants of Cu,Zn superoxide dismutase (O'Neill et al., 1988; Calabrese et al., 1989; Polticelli et al., 1995b). Site-directed mutagenesis experiments conducted on the human enzyme have shown that Arg 141 is responsible for keeping a strong, stable positive charge near the active site up to pH 10, while deprotonating at higher pH values (Fisher et al., 1994). Because of the invariance of the Arg 141 residue, pK_2 can be assigned to this residue also in XSODB.

The low contribution (25%) of the pK attributed to Arg 141 (pK_2) to the total activity of wild-type XSODB deserves further comment. In fact, neutralization of Arg 141 by chemical mod-

ification (Malinowski & Fridovich, 1979) or site-directed mutagenesis (Fisher et al., 1994) resulted in both cases in a 90% drop in the catalytic rate. However, the electrostatically conservative substitution of Arg 141 with lysine also causes a drastic decrease of the catalytic rate (approximately 60%, Fisher et al., 1994), even at pH values in which deprotonation of both residues is expected to occur. This finding suggests a stereochemical, more than a long-range steering, role for Arg 141, thus explaining the low contribution of pK_2 to the activity of wild-type XSODB. In fact, such a role is expected to be relatively independent of the protonation state of Arg 141, because it relies on its side-chain conformation, which is unique in its ability to bind the superoxide anion near the copper atom (Djinovic-Carugo et al., 1994).

The above reported results indicate that, in XSODB, the pH profile of the catalytic activity is essentially modulated by the three basic residues nearest to the copper site, i.e., Arg 141, Lys 120, and Lys 134, confirming their dominant role in the interaction of the superoxide anion with the active site (Fisher et al., 1994; Polticelli et al., 1994, 1995a, 1995b). A major role in the electrostatic diffusion pathway of the substrate has been shown to be played also by the negative residues in position 130 and 131, because their neutralization increases the catalytic rate of the enzyme (Getzoff et al., 1992; Polticelli et al., 1995a). It may be then hypothesized that the enzyme-substrate encounter is governed mainly by the five charged residues nearest to the copper site, i.e., Arg 141, Lys 120, Asp 130, Glu 131, and Lys 134. To test this hypothesis, Brownian dynamics simulations were conducted on wild-type XSODB, as a function of pH, considering as charged only the above mentioned residues plus the copper and zinc ions. In the simulation, Asp 130 and Glu 131 were considered fully deprotonated all over the pH range investigated, whereas Arg 141, Lys 120, and Lys 134 were assigned the experimentally determined pK s 11.3, 9.0, and 9.1 (Table 1), respectively. Figure 5, in which the relative simulated association rate is reported in comparison with the relative experimental rate, shows that the pH profile of the activity of the wild-type enzyme is well reproduced, taking into account only the five charged residues nearest to the active site. The largest deviation, which occurs at pH 9, might be due to the contribution of surface basic residues far from the copper site that are not included in the simulation. However, the contribution of the latter type of residues never exceeds 20%, as evaluated by the study of the double mutant, confirming that the pH dependence of the catalytic rate, in the alkaline pH range, is mainly governed by the three basic residues nearest to the copper site.

Materials and methods

All reagents used were of the highest grade available. Protein concentration was determined by the optical absorption at 258 nm ($\epsilon_{mM} = 10.3 \text{ M}^{-1} \text{ cm}^{-1}$, Rotilio et al., 1972). Copper content was determined by double integration of the EPR spectra using a Cu^{2+} -EDTA solution as a standard.

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 accord-

Fig. 4. Plot of the catalytic activity of K120L-K134T XSODB (open circles) compared to the relative activity calculated on the basis of a multiplicative model based on the component single mutations (full circles).

Fig. 5. Plot of the relative catalytic rate versus pH of wild-type XSODB (circles) compared to the simulated association rate for a model XSODB (squares) in which only the five charged residues nearest to the copper site plus the metals were considered in the calculation of electrostatic forces. For each Brownian dynamics simulation, 10,000 trajectories were run. Trajectories were started randomly at 61.5 Å from the center of the protein and pursued until exit (from a 150-Å radius sphere) or reaction (defined by approach of the superoxide within 4 Å of either copper) occurred (Sergi et al., 1994).

ing to Kunkel et al. (1990), as described previously (Polticelli et al., 1995a).

Expression and purification of wild-type and mutant XSODs B

Plasmid pKB overexpressing XSODB under control of the trc promoter has been described previously (Battistoni et al., 1992). In order to avoid possible contamination 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 $(\text{NH}_4)_2\text{SO}_4$ 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 XSODs 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).

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

Activity assays

Activity assays were conducted by the pulse radiolysis method (O'Neill & Chapman, 1985), which is based on the first-order rate of loss of O_2^- , monitored spectrophotometrically at 250 nm (Fielden et al., 1974). Interference arising from binding of anions

like phosphate or chloride to the copper ion was avoided by assaying the enzymes in Tris-MOPS buffer, in the pH range 7.0–9.0, and in borate buffer, in the pH range 9.5–12. In both cases, the assay mixture contained 0.1 M ethanol and 0.1 mM EDTA. Solutions contained in a 0.7-cm-pathlength optical cell were irradiated with a 1.6-μs electron pulse giving $[\text{O}_2^-] = 15 \mu\text{M}$. Because protein concentration for each sample varied in the range 0.3, 1.2 μM, activity assays were carried out with $[\text{O}_2^-] \gg [\text{SOD}]$ under turnover conditions.

Fitting the experimental data

Best fit of the experimental data was carried out by the steepest descent method, using the following equation to simulate a two-pK dissociation equilibrium:

$$A = A_{\max} (\%pK_1 ([\text{H}^+]/(K_1 + [\text{H}^+])) + \%pK_2 ([\text{H}^+]/(K_2 + [\text{H}^+]))), \quad (1A)$$

where A is the catalytic activity of the enzyme, $A_{\max}$ the catalytic constant at neutral pH, K_1 and K_2 are the equilibrium dissociation constants, and $\%pK_1$ and $\%pK_2$ are the relative contributions of each pK to the activity curve.

The contribution of each residue (A_{res}) to the activity of the wild-type enzyme was determined by multiplying the $\%pK$ attributed to the residue by the activity of the mutant (A_{mut}) relative to that of the wild-type enzyme (A_{wt}), at neutral pH:

$$A_{\text{res}} = \%pK \times A_{\text{mut}}/A_{\text{wt}}. \quad (1b)$$

Brownian dynamics simulations

Brownian dynamics simulations of the diffusion of superoxide under the influence of the protein-generated electrostatic field were conducted on a digital DEC Alpha 3000/300x, with a modification of a previously described method (Sharp et al., 1987; Sines et al., 1990). The simulation method and parameters have been described in detail elsewhere (Sergi et al., 1994). Electrostatic potential was calculated with the program DelPhi (Biosym, Inc.) using the focusing procedure (Gilson et al., 1987). Values of 4 and 78.5 were assigned to the dielectric constant of the protein interior and solvent, respectively (Gilson & Honig, 1986). The ion exclusion radius (Gilson et al., 1987) was set to 2 Å. Net charges, calculated considering the experimentally determined pKs, were assigned to the Nε atoms of lysine and arginine residues. Aspartate and glutamate residues were considered fully deprotonated in all the pH range investigated (pH 7–12).

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