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Mutation of Lys-120 and Lys-134 drastically reduces the catalytic rate of Cu,Zn superoxide dismutase

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Abstract Lys-120 and Lys-134, located at the edge of the active site channel in most Cu,Zn superoxide dismutases, have been suggested to play a major role in steering the anionic substrate towards the catalytic copper ion. In this study, mutants of *Xenopus laevis* Cu,Zn superoxide dismutase have been engineered, with Lys-120 and Lys-134 changed into leucine and threonine, respectively, and their catalytic properties have been investigated by pulse radiolysis. Results obtained demonstrate that both residues decrease the catalytic rate by about 40%, in partial disagreement with previous brownian dynamics calculations, carried out on bovine Cu,Zn superoxide dismutase.

Key words: Superoxide dismutase; Site-directed mutagenesis; Pulse radiolysis; Brownian dynamics; Electrostatic interaction

1. Introduction

Superoxide dismutases (SODs) are ubiquitous metalloenzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide by alternate reduction and oxidation of a copper ion which constitutes the catalytically active redox center [1]. The diffusion of the anionic substrates toward the active site is modulated by the distribution of the electrostatic potential around the protein [2,3] which has been suggested to be constant in the evolution of this protein [4]. Brownian dynamics simulation has been used in order to evaluate the role of electrically charged amino acid residues in contributing to such an electrostatic field around the copper [5] in the best characterized enzyme from bovine erythrocytes (BSOD) [6]. That study indicated that alteration of single charges substantially affects the calculated reaction rates only in the case of three residues, namely Arg-141, Lys-134 and Glu-131. The role of Arg-141, as directly demonstrated by site-directed mutagenesis [7], is predominantly that of correctly docking the substrate near the copper ion, this residue being situated at the bottom of the active site channel, very close to the catalytic metal (approx. 5 Å). Neutralization by site directed mutagenesis of the negatively charged residues Glu-130 and Glu-131, placed at the entrance of the active site channel, on the protein surface, caused a twofold increase of the catalytic rate with respect to the wild type, in the human enzyme [8]. On the other hand, a quantitative evaluation of the specific role of the positively charged surface side chains of Lys-134 and Lys-120 which are located at the same edge of the active site channel, as the glutamate residues [6], has not yet been reported. A major role of Lys-134 in the electrostatic steering of superoxide towards the active site, predicted by brownian dynamics simulation on the bovine enzyme [5], has been evidenced only indirectly by comparing the activity of BSOD with all lysine residues neutralized by a specific chemical reagent, with that of a similarly treated shark enzyme, where position 134 is occupied by an arginine residue. This latter residue is insensitive to the chemical treatment used [9].

In this study we have investigated for the first time the catalytic properties of single and double mutants, neutralized at sites 120 and 134, of the Cu,Zn SOD from the amphibian *Xenopus laevis* whose structure has been recently resolved at 1.5 Å resolution (Djinovic et al., manuscript in preparation). The enzyme activities were assayed by pulse radiolysis [10], the only method that allows a direct measurement of the catalytic rates at neutral pH values. The results obtained show that both lysine residues contribute equally (40%, μ = 0.02 M) to steering the superoxide anion towards the copper site. Further, the role of conserved, activity-linked, charged residues may be different in Cu,Zn SOD from different species.

2. Materials and methods

All materials used were reagent grade and were used without further purification. Protein concentration was determined by the absorption at 258 nm (ε_{mM} = 10.3 $M^{-1} \cdot cm^{-1}$ [11]). Copper content was determined by EPR spectroscopy using a Cu-EDTA solution as a standard. EPR spectra were recorded at X-band and liquid nitrogen temperature with a Bruker ESP 300 spectrometer.

2.1. Preparation of mutants

Molecular mutants of the recombinant Cu,Zn superoxide dismutase from *Xenopus laevis*, isoenzyme B (XSODB), overexpressed from plasmid pKB under control of the *trc* promoter [12], were prepared by site-directed mutagenesis according to Kunkel and coworkers [13]. The single-stranded DNA template for mutagenesis was generated using plasmid pXLSODBcDNA1 [14]. 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 [15] 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 (kind gift of D. Touati).

E. coli cells were disrupted as described by Marston [16]. Cell debris and insoluble proteins were removed by centrifugation and supernatants were subjected to $(NH_4)_2SO_4$ fractionation. The enzyme activity

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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 DE-52 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).

2.2. Catalytic measurements

Activity assays were carried out by the pulse radiolysis method [10] which is based on the first-order rate of loss of O_2^- at 250 nm [17]. To avoid binding of anions like phosphate or chloride to the copper, enzymes were assayed in Tris-MOPS buffer, pH 7.5, 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 contained in a 0.7 cm pathlength optical cell were irradiated with a 1.6 μs electron pulse giving a concentration of O_2^- equal to 15 μM. Since protein concentration for each sample varied in the range 0.3–1.2 μM, activity assays were carried out with $[O_2^-] \gg [SOD]$, under turnover conditions.

2.3. Brownian dynamics

Brownian dynamics simulations of the diffusion of superoxide under the influence of the protein-generated electrostatic field were carried out with a modification of a previously described method [5,18]. Electrostatic forces were calculated with the program DelPhi by Biosym Inc. using the focussing procedure [19]. The simulation method and parameters will be described in detail elsewhere [20].

3. Results and discussion

The EPR spectra of the wild type and the K120L, K134T and K120L-K134T mutants are reported in Fig. 1. The lineshape of the spectra of the mutants is nearly identical to that displayed by the native enzyme indicating that the active site geometry is not perturbed upon neutralization of these residues.

The catalytic rate constants for wild type and mutant XSODB at low (0.02 M) and physiological ionic strength (0.145 M) are reported in Table 1. Neutralization of K134 lowers the catalytic rate constant of the wild type by approximately 40% at low ionic strength. However, the difference in the catalytic rate between the mutant and the wild type is only 20% at physiological ionic strength, indicating that K134 in the wild type enzyme is strongly shielded by solvent ions, in line with the solvent-exposed nature of this residue. The decrease of catalytic rate observed upon neutralization of K134 is in agreement with the prediction made by brownian dynamics on the bovine enzyme [5] and confirms the results obtained by a comparative study on carbamoylated forms of bovine and shark Cu,Zn SODs [9]. This latter approach quantified the contribution of this residue to the electrostatic guidance of superoxide into the active site channel as approximately 20%.

The catalytic rate constant of the K120L mutant, measured at 0.02 M ionic strength, is 40% lower than that of the wild type. This value is reduced to 20% at physiological ionic strength indicating that, also in this case, solvent ions have an higher

Table 1
Catalytic rate constant of wild type and mutants of *Xenopus laevis* Cu,Zn SOD at two different ionic strength values

	$K_2 \times 10^{-9}$ $M^{-1} \cdot s^{-1}$	
μ(M)	0.02	0.145
Wild type	3.7 (100%)	1.3 (100%)
K120L	2.1 (57%)	1.0 (77%)
K134T	2.1 (57%)	1.0 (77%)
K120L-K134T	1.1 (31%)	0.7 (54%)

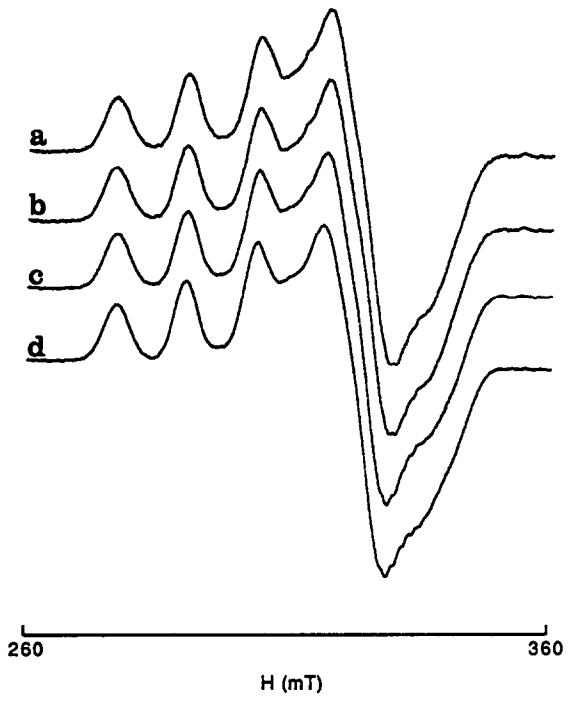

Fig. 1. EPR spectra of wild type XSODB (a) and K120L (b), K134T (c) K120L-K134T (d) mutants in 10 mM phosphate buffer, pH 7.4. Spectra conditions: microwave power = 20 mW, $\nu = 9.43$ GHz and $T = 100$K.

screening effect on the wild type with respect to the mutant enzyme. Neutralization of both the K120 and the K134 residues has a synergic effect on the decrease of the catalytic rate constant. In fact, the catalytic rate of the K120L-K134T mutant is 70% and 50% lower than that displayed by the wild type, at low and high ionic strength respectively (Table 1). This result further indicates that both K120 and K134 are involved in facilitating the encounter of the substrate with the active site, and that this lysine pair on the protein surface plays a major role, together with Arg-141 at the bottom of the active site channel, in the electrostatic guidance of the substrate in Cu,Zn superoxide dismutases.

The finding that neutralization of the K120 residue has the same effect as the neutralization of K134 is in contrast with brownian dynamics simulations on BSOD, which did not indicate any specific role for the former residue in the electrostatic steering mechanism [5]. For this reason, simulations of 10^4 trajectories of incoming substrate under the influence of brownian motion and of the enzyme electrostatic field were carried out on wild type and mutants XSODB (Fig. 2). The results obtained are in good agreement with the experimental data, confirming that brownian dynamics simulations is a powerful tool in the prediction of the catalytic properties and in the design of charge mutants of Cu,Zn SOD. The difference with the brownian dynamics prediction made on BSOD may be due to subtle structural differences in Cu,Zn SODs from different species, which however are not reflected in changes of the electrostatic potential distribution around the active site. Apparently this distribution is evolutionarily conserved and results in an almost identical catalytic rate for all the wild type Cu,Zn superoxide dismutase assayed up to date [4]. More specifically, a residue in the same position within the linear sequence of

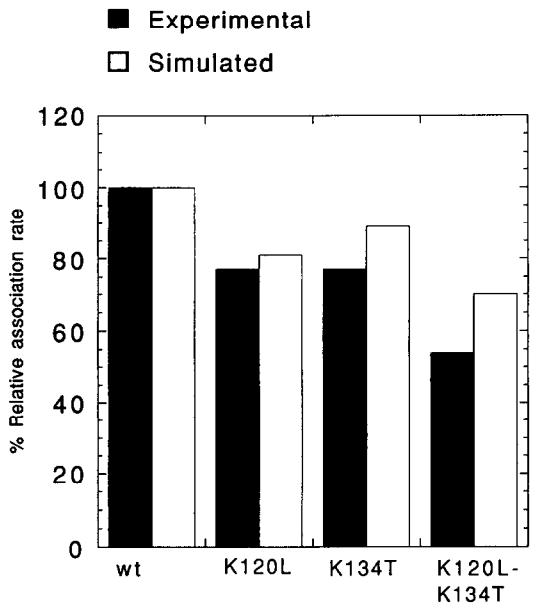

Fig. 2. Catalytic rate determined by pulse radiolysis at $\mu = 0.02$ M (shaded bars) and simulated association rate calculated by brownian dynamics (white bars), of wild type and mutants XSODBs. For comparison experimental and simulated values are given relative to the wild type enzyme. Depending on the distance from the protein center, five different $81 \times 81 \times 81$ grids with 1, 1.5, 2, 4 and 8 Å spacing, respectively, were used in the simulations. 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. Time step for simulations increased with distance from the protein center: 0.125 within 40 Å, 0.187 between 40 and 60 Å, 0.250 between 60 and 90 Å, 0.500 between 90 and 160 Å and 1.000 ps beyond 160 Å. For each simulation 10,000 trajectories were run (Sergi et al., 1994). The structure of the wild type XSODB (kindly provided by Dr. K. Djinovic-Carugo) has been used for the simulations in the case of both wild type enzyme and mutants; point mutations were obtained by neutralizing the mutated charged residues.

Cu,Zn SODs from different species (for instance, Lys-120 in the bovine and *Xenopus laevis* Cu,Zn SODs) may be shown to have a different contribution to the facilitated diffusion of the substrate. This finding should be taken as a caution when extrapolating a general electrostatic theory for the Cu,Zn SOD substrate steering mechanism on the basis of data obtained on mutants of a single natural variant.

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