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Crystallographic Study of Mutant Lys120Leu *Xenopus laevis* Cu,Zn Superoxide Dismutase*

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Theoretical calculations and experimental measurements on the *Xenopus laevis* Cu,Zn superoxide dismutase (XSODB) wild-type protein and on some of its engineered mutants showed that the electrostatic arrangement around the active site channel plays a fundamental role in determining the catalytic properties of the enzyme. Lys120, which lies on the lip of the active site channel, about 11 Å from the catalytic copper ion, influences the enzyme electrostatic environment and binding selectivity. Neutralization of this residue has the effect of decreasing the activity of the enzyme *versus* the negatively charged substrate. In order to get precise information about the mutated residue and its effects on the structure

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* Dedicated to Professor Boris Kamenar on the occasion of his 70th birthday.

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of the engineered protein, the crystal structure of single site Lys120Leu mutant XSODB was determined at 2.0 Å resolution, and refined to an *R*-factor value of 0.181. The structure of Lys120Leu mutant XSODB is little affected by the amino-acid substitution, suggesting that the main effect of the mutation is perturbation of the electrostatic properties of the SOD catalytic center.

Key words: superoxide dismutase, enzyme, protein crystallography.

INTRODUCTION

Cu,Zn superoxide dismutases (Cu,Zn SODs) are a class of metalloenzymes that catalyze the superoxide radical dismutation reaction into molecular oxygen and hydrogen peroxide.[1] The catalytically active Cu^{2+} ion is cyclically reduced and oxidized by successive encounters with the superoxide substrate during the enzymatic reaction. The arrangement of electrostatic charges in Cu,Zn SODs promotes productive enzyme-substrate interaction through substrate guidance and charge complementarity, particularly in the protein region surrounding the active site. In Cu,Zn SODs isolated from eukariotes, the conserved basic residues Arg141, Lys120 and Lys134, as well as two negatively charged residues Asp130 and Glu131, create an extended electrostatic field that steers the negatively charged superoxide substrate to the highly positive catalytic binding site, at the bottom of the active site channel.[2] Alkaline pH, high salt concentration,[3] and neutralization of basic residues by chemical reagents[4,5] have an inhibitory effect on the enzyme activity. These findings suggest that diffusion of the $O_2^{\cdot -}$ substrate toward the catalytic metal is driven by the positive electrostatic potential generated by the metals and by the basic residues surrounding the active site channel.[6]

Increase of the positive electrostatic potential around the active site, obtained by neutralizing the negatively charged residues Asp130 and Glu131 through the site-directed mutagenesis, results in a more active enzyme both in human[7] and in *X. laevis* SODs.[8] On the other hand, neutralization of two positively charged residues Lys120 and Lys134 decreases the ability of the enzyme to attract the negatively charged substrate. The design of more efficient superoxide dismutases by increasing the 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.[9] An 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 in activity

between the native and the mutant enzymes smaller at physiological than at low ionic strengths.[7] Mutant enzymes having an increased catalytic rate, without suffering from increased ionic strength dependence, could be obtained by a combinatorial approach involving the use of both positively and negatively charged residues.[8] In this context, we have determined the three-dimensional structure of *X. laevis* Cu,Zn SOD, bearing the Lys120Leu mutation, by X-ray crystallography, in order to shed light on the electrostatic control of substrate diffusion to the active site in this enzyme family.

EXPERIMENTAL

Crystals of the Lys120Leu mutant of *X. laevis* superoxide dismutase (XSODB) were grown by the hanging drop vapour diffusion method under conditions similar to those used to obtain crystals of the wild-type.[10] Briefly, the protein stock solution (at a concentration of 10 mg/ml) was added to an equal volume of crystallization medium containing 18% (w/v) PEG 4000, 0.05 M sodium phosphate (pH = 6). Crystals with dimensions $0.5 \times 0.2 \times 0.1$ mm^3 grew in about 3 months, at 28 °C. Lys120Leu XSODB crystals are isomorphous to those of the wild-type enzyme, and belong to the orthorhombic space group $P2_12_12_1$, with unit cell dimensions a = 73.4 Å, b = 68.9 Å, c = 58.8 Å. The calculated packing density parameter[11] V_M is 2.34 Å^3/Da, assuming one dimer (32 000 Da) per asymmetric unit.

The X-ray diffraction data of the mutant Lys120Leu XSODB were collected to 2.0 Å resolution on a Rigaku RAXIS II image plate system using Cu-Kα (λ = 1.5418 Å) radiation. Diffracted intensities were evaluated and integrated using the MOSFLM program.[12] Merging of the observed intensities were into a unique data set of reflections was carried out using ROTAVATA/AGROVATA programs from the CCP4 suite[13] and the intensities converted to structure factor amplitudes using the TRUNCATE program.[14] A total of 19056 independent reflections were collected up to 2.0 Å resolution with an overall R_{merge} of 0.067.

The three-dimensional structure of the mutated enzyme was refined by means of restrained refinement methods using the PROLSQ program suite,[15] in combination with ARP[16] for automatic search of solvent molecules in the model, and alternated with inspections of model/electron density maps with 'O'.[17] The starting atomic coordinates were those of wild-type XSODB,[18] omitting all water molecules and the side chain of residue 120. During refinement, geometrical constraints were applied to the copper and zinc coordination spheres, adopting metal–ligand distances observed in the coordination chemistry of model compounds.[19] The initial R value of the model was 0.405; after 30 cycles of refinement, it was reduced to 0.235, at 2.0 Å resolution. In the next stage, automatic search of solvent molecules in the difference electron density map was included and 186 water molecules were located. The model was refined to an R-factor of 0.181, with an overall r.m.s. deviation from ideal geometry of 0.018 Å for bond lengths and 0.028 Å for angle distances.

Connolly's surfaces for the molecules were generated by the SYBYL[20] program package. Electrostatic point charges on the atoms were calculated using the empirical method of Pullman and Berthod.[21]

RESULTS AND DISCUSSION

Lys120 lies at the beginning of the extended electrostatic loop[18] of XSODB, which includes residues in the sequence range 119 to 140. This residue is positioned on the lip of the active site channel, 10–12 Å away from the catalytic Cu^{2+} ion. Lys120 is highly exposed to solvent and forms a salt bridge to the neighbouring Glu119 residue in the wild-type protein. The side chain conformation of the mutated residue Leu120 generally follows that of the wild-type Lys120, despite the absence of the charged interaction with Glu119.

Inspection of the refined three-dimensional structure of the mutated XSODB shows that the overall structure is, as expected, just slightly perturbed by the amino acid substitution: the overall flattened eight-stranded β-barrel fold, the metal-site ligands and geometry, as well as the dimer interface match well those of the wild type enzyme[18] (Figure 1). The least-squares superposition of two Cα backbones of the mutated XSODB and the wild-type enzyme (the latter structure determined at 277 K) shows a r.m.s. deviation of 0.197 Å.

The overall electrostatic potential field surrounding the enzyme active sites is visually presented by colour mapping onto the molecular surfaces of the XSODB wild-type (Figure 2a) and Lys120Leu mutant structures (Figure 2b). The molecular surface of both protein structures is, as previously

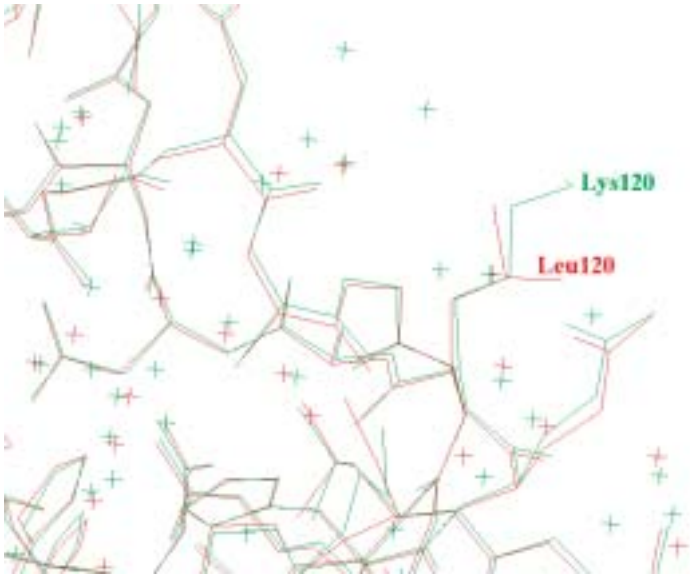
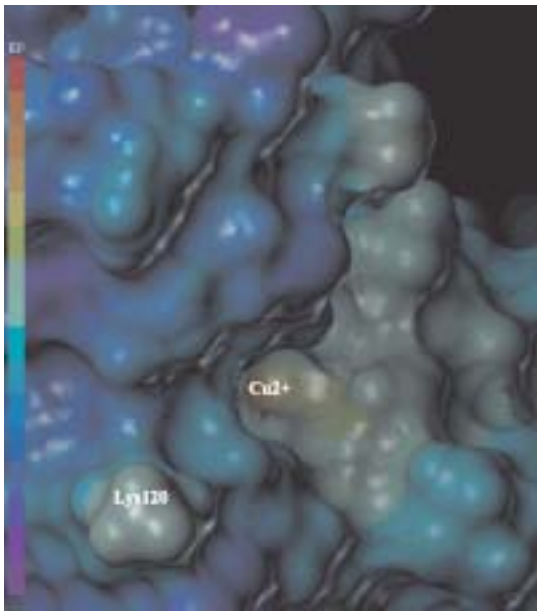


Figure 1. Overlap diagram of some residues near the active site of the wild-type XSODB (green) and Lys120Leu mutant (red).

(a)



(b)

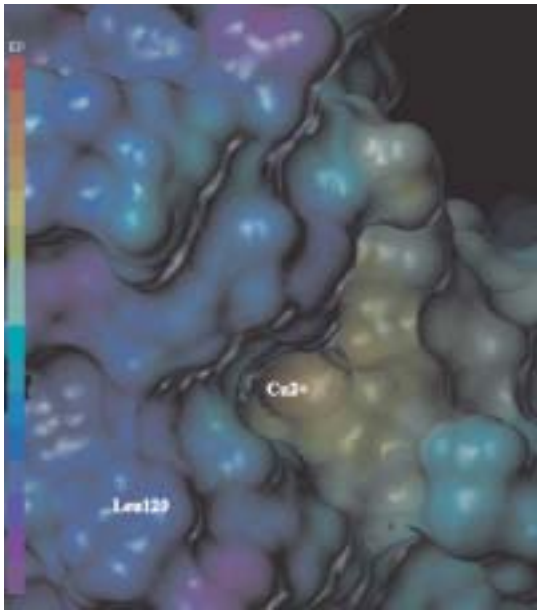


Figure 2: View of the active site of the XSODB wild-type (a) and Lys120Leu mutant of XSODB (b) generated using the SYBYL program package.[20] The only labeled residues are Lys120 in XSODB wild-type and Leu120 in mutated XSODB. The molecular surface has been obtained using Connolly's program,[22] as red colored positive and blue negative potential.

shown,[2] surrounded by negative values, except for two restricted regions around the active centers. The only evident difference of the potential distribution is, as expected, around position 120.

Previous measurement results the catalytic rate of two single mutants of XSODB, replacing Lys120 or Lys134 with neutral residues, have shown that neutralization of either one of the two lysines drastically decreases the enzyme activity. These results, obtained for the Lys120Leu XSODB mutant, differ from those reported for the human or for the bovine enzymes that predict a strong decrease of the activity for neutralization of Lys134 but not for Lys120 residues,[7,23] indicating that some residues occupying the same positions in the linear sequence of different Cu,Zn SODs have a different functional weight.[8] In further work, the catalytic rate of the wild-type XSODB and two single mutants Lys120Leu and Lys134Thr were studied by pulse radiolysis as a function of pH.[24] The pH dependence of the activity of mutant proteins was compared to that of the wild-type enzyme,[24,25] showing that the activity of the mutant decreased by around 30%, compared to the wild-type enzyme, at pH = 7. At pH > 10, the curve of the mutant can be superimposed to that of the wild-type enzyme. Thus, in the wild-type enzyme, Lys120 contributes only to the first part (pH < 10.5) of the pH-dependent activity decrease.[24,25]

We have determined the 3D structure of the XSODB Lys120Leu mutant at atomic resolution in order to investigate further the role of this residue. The absence of major structural perturbations in the mutant enzyme structure allows us to draw two main conclusions. On the one side, from a phenomenological viewpoint, it is sufficient to remove the charge at residue 120 to affect significantly the electrostatic properties of the active site (30% drop in activity). On the other side, mechanistically, it may be proposed that the removal of Lys120 positive charge alters the static/dynamic properties of the protein surface water molecules surrounding the electrostatic loop, thereby playing an indirect role in affecting superoxide diffusion to the catalytic centre.

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SAŽETAK

Kristalografsko istraživanje mutanta Lys120Leu *Xenopus laevis* Cu,Zn-superoksid-dismutaze

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Teorijski proračuni i eksperimentalna mjerenja na divljem tipu Cu,Zn-superoksid-dismutaze iz *Xenopus laevis* (XSODB) i njenim dizajniranim mutanatima pokazali su da elektrostatska raspodjela u okolici aktivnog mjesta igra ključnu ulogu u definiranju katalitičkih svojstava enzima. Lys120 leži na rubu kanala aktivnog mjesta, oko 11 Å od katalitičkog bakrova iona te utječe na elektrostatsko okruženje enzima i njegovu selektivnost. Neutralizacija tog aminokiselinskog ogranka smanjuje aktivnost enzima prema negativno nabijenom substratu. U cilju dobivanja preciznih informacija o mutiranom ogranku, kao i o njegovu utjecaju na strukturu dizajniranog proteina, kristalna struktura jednostruko zamijenjenog Lys120Leu mutanta od XSODB određena je pri rezoluciji od 2,0 Å i utočnjena do R vrijednosti 0,181. Struktura Lys120Leu mutanta XSODB neznatno je narušena zamjenom jedne aminokiseline, što znači da je glavna posljedica mutacije promjena elektronskih svojstava katalitičkog centra SOD.