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Structural and denaturation studies of two mutants of a cold adapted superoxide dismutase point to the importance of electrostatic interactions in protein stability

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ABSTRACT

A peculiar feature of the psychrophilic iron superoxide dismutase from *Pseudoalteromonas haloplanktis* (*Ph*SOD) is the presence in its amino acid sequence of a reactive cysteine (Cys57). To define the role of this residue, a structural characterization of the effect of two *Ph*SOD mutations, C57S and C57R, was performed. Thermal and denaturant-induced unfolding of wild type and mutant *Ph*SOD followed by circular dichroism and fluorescence studies revealed that C→R substitution alters the thermal stability and the resistance against denaturants of the enzyme, whereas C57S only alters the stability of the protein against urea. The crystallographic data on the C57R mutation suggest an involvement of the Arg side chain in the formation of salt bridges on protein surface. These findings support the hypothesis that the thermal resistance of *Ph*SOD relies on optimization of charge–charge interactions on its surface. Our study contributes to a deeper understanding of the denaturation mechanism of superoxide dismutases, suggesting the presence of a structural dimeric intermediate between the native state and the unfolded state. This hypothesis is supported by the crystalline and solution data on the reduced form of the enzyme.

1. Introduction

Life at low temperatures requires a vast array of adaptation at molecular and physiological levels. Psychrophilic enzymes are proteins from organisms that inhabit areas with temperatures lower than 20 °C [1–4]. They have to adapt their structure and function to cope with slow reaction rate at low temperatures. The molecular mechanisms of cold adaptation in psychrophilic enzymes are still partially unknown. However, some general features of cold-adapted proteins have been suggested [5–7]. These molecules are endowed with a high conformational flexibility that is often coupled to a reduced thermal stability. The iron superoxide dismutase from *Pseudoalteromonas haloplanktis* (*Ph*SOD) represents an exception to this general rule [8,9]. Although this enzyme comes from an organism living in a hostile habitat (5–15 °C), it presents an unusual thermal stability, which is comparable with that of its mesophilic counterpart from *Escherichia coli* (*Ec*SOD) [8]. Both *Ph*SOD and *Ec*SOD are dimeric metalloenzymes that catalyze the dismutation of superoxide anion radicals into molecular oxygen and hydrogen peroxide [9]. The elucidation of the three-dimensional structure of *Ph*SOD suggested that a major role in its unusually high thermal stability could be played by electrostatic interactions on the protein surface [8]. Recent reviews summarized the structural and functional features of the different families of SODs [10,11].

An interesting feature of *Ph*SOD is the presence in its sequence of a highly reactive cysteine residue (in position 57) that undergoes endogenous glutathionylation upon induction of oxidative stress in *P. haloplanktis* cell cultures [12]. This post-translational modification significantly reduces the inactivation of the enzyme by peroxynitrite, suggesting that this reaction could represent a strategy to improve the antioxidant cellular defense mechanism in the psychrophilic organism. Cys57 belongs to a segment of Fe- and Mn-SODs with a highly variable sequence [9]. To investigate the role of Cys57 in the functional regulation of *Ph*SOD, the mutants C57S and C57R, where the cysteine on the protein surface (Fig. 1) is replaced by serine or arginine, were produced and characterized. Furthermore, the adducts formed by reaction with β-mercaptoethanol [9] and glutathione [12] were analyzed. Substitution of the sulfhydryl group with an oxydryl one did not affect the *Ph*SOD enzymatic activity, whereas the substitution of Cys with Arg causes a slight reduction of the activity [12].

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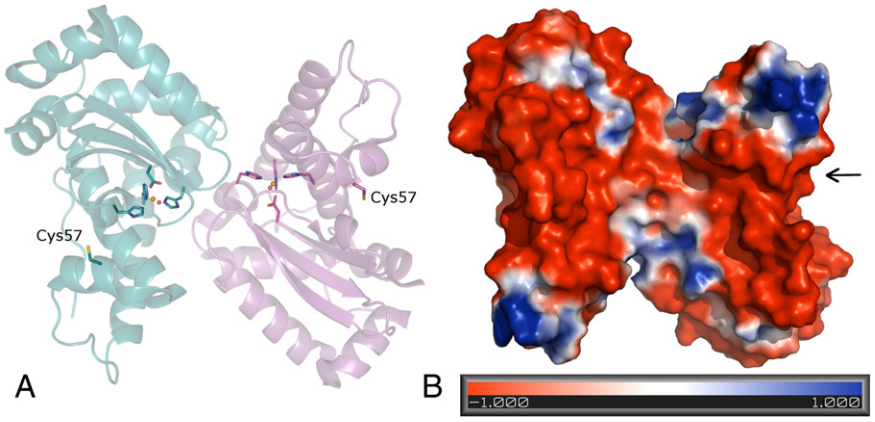

Fig. 1. (A) Overall structure of *Ph*SOD. The two subunits are colored cyan and magenta respectively, active site residues and cysteine 57 are also shown as sticks, iron atoms and the coordinated water molecules are shown as spheres. (B) *Ph*SOD electrostatic surface. Position of Cys57 in one of the two subunits is indicated by an arrow.

Previous results on SODs have suggested a major role for Cys residues in irreversible protein aggregation [13,14]. To complete the characterization of *Ph*SOD, we have now performed a combined structural and biochemical study, in solution and in the crystal state, of the two mutants. Moreover we solved the structure of the reduced form of the enzyme (Fe(II)*Ph*SOD). No significant variation in the structural features of the protein has been found in the Fe(II) form. CD data confirm that *Ph*SOD thermally unfolds through a three-step mechanism and show that the mutation C57R enhances the stability of the native dimer, but destabilizes the intermediate partially unfolded dimer. This finding has been interpreted on the basis of the crystal structure of the mutants.

2. Methods

2.1. Crystallization and data collection

Wild type *Ph*SOD and its mutants containing the replacement of Cys57 with Arg or Ser, hereafter named C57R and C57S, were prepared as previously described [8,9]. Crystals of *Ph*SOD and C57S were obtained by hanging drop vapor diffusion method [15], by using the procedure previously reported [16]. Briefly, crystals were grown in 1.8–2.0 M ammonium sulfate, 1.0–1.2 M NaCl, 100 mM Hepes pH 7.2–7.5. Crystals of C57R were grown by hanging drop vapor diffusion method in the same crystallization conditions (C57R-I) and also in 23% PEG8K, 0.2 M ammonium sulfate, 0.1 M sodium cacodylate pH 7.0 (C57R-II). The reduced form (Fe(II)*Ph*SOD) was obtained by soaking *Ph*SOD crystals in a holding solution containing an excess of dithionite, according to the procedure described in a previous paper [17].

Diffraction data on C57S, C57R-I and Fe(II)*Ph*SOD were collected on a Saturn944 CCD detector at the Istituto di Biostrutture e Bioimmagini (CNR, Naples, Italy) with Cu$K\alpha$ X-ray radiation from a Rigaku Micromax 007 HF generator, whereas those on C57R-II were collected at the Elettra synchrotron, Trieste (Italy). All the crystals were mounted in nylon loops and flash-frozen at 100 K in a nitrogen gas produced by an Oxford Cryosystems Cryostream and maintained at 100 K during the data collection. Crystals were flash-cooled after the addition of 400 mg/ml trehalose to the harvesting solution, with the only exception of crystals of C57R-II that were flash-cooled after the addition of 25% glycerol. Data were processed by HKL2000 [18]. Statistics describing the other crystallographic data are reported in Table 1.

2.2. Structure solution and refinement

Given the strict isomorphism with the wild type protein, the phase determination was carried out by the difference Fourier method. The refined coordinates of *Ph*SOD (PDB IDs: 3LIO and 3LJF, [8]) were used as starting models. The refinement was carried out with CNS [19] and Refmac5 [20] programs. Several alternating cycles of positional refinement, energy minimization, individual temperature factor refinement and manual model building were performed. Model building was done using "O" [21] and "Coot" [22]. Water molecules and trehalose molecules were located in difference Fourier maps and added to models. PROCHECK [23] was used to analyze the quality of the final structures. The refinement statistics are presented in Table 2. The coordinates of C57S, C57R-I, C57R-II and Fe(II)*Ph*SOD were deposited in the Protein Data Bank (PDB IDs: 4L2B, 4L2C, 4L2A, and 4L2D, respectively). C57R-I and Fe(II)*Ph*SOD crystals contain two dimers in the asymmetric unit, whereas the others contain a single dimer in the asymmetric unit.

The figures were drawn using PyMOL (http://pymol.org).

2.3. Guanidine hydrochloride- and urea-induced denaturation studies

Protein concentration has been determined spectrophotometrically from absorbance at 280 nm and using a molar extinction coefficient $\varepsilon = 2.561$ ml/(mg·cm). UV spectra were recorded on a Jasco V-560 spectrophotometer.

Protein samples (0.1 mg/ml in 10 mM Tris/HCl buffer at pH 7.8) were incubated overnight with increasing concentration of guanidine hydrochloride (GdnHCl) (0–5.6 M) or urea (0–7.8 M) at 20 °C.

A non-linear least-square analysis was used to fit the unfolded protein fraction versus the denaturant concentration curves and to

Table 1
X-ray diffraction data-collection and processing statistics.

	C57S (4L2B)	C57R-I (4L2C)	C57R-II (4L2A)	Fe(II)*Ph*SOD (4L2D)
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Cell parameters				
a (Å)	46.72	50.49	45.62	50.30
b (Å)	103.44	103.77	103.74	103.62
c (Å)	50.53	89.83	50.24	89.34
α (°)	90	90	90	90
β (°)	108.0	103.6	108.3	103.7
γ (°)	90	90	90	90
Resolution range(Å)	30.0–1.97 (2.04–1.97)	50.0–1.66 (1.72–1.66)	50.0–2.06 (2.13–2.06)	30.0–2.07 (2.04–2.07)
Observations	107,691	261,688	96,935	268,327
Unique reflections	30,651	95,529	27,167	53,308
Completeness (%)	95.1 (90.0)	90.1 (76.7)	98.8 (90.4)	98.2 (85.6)
I/σ (I)	9 (3)	8 (3)	13 (4)	9 (4)
Redundancy	3.5 (2.8)	2.7 (2.3)	4.0 (3.2)	5.0 (3.7)
R_{merge} (%)	9.9 (26.9)	9.6 (31.4)	7.4 (23.5)	10.8 (20.5)
Mosaicity	0.8	0.7	0.7	1.2
Molecules in the asymmetric unit	1 dimer	2 dimers	1 dimer	2 dimers