Isolation of an active and heat-stable monomeric form of Cu,Zn superoxide dismutase from the periplasmic space of *Escherichia coli*

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Abstract **We have purified the Cu,Zn superoxide dismutase (CuZnSOD) from the periplasmic space of an *Escherichia coli* strain unable to synthesize MnSOD and FeSOD. Gel filtration chromatography evidenced that under all the experimental conditions tested the enzyme was monomeric. The catalytic activity of this CuZnSOD was comparable to that of other well characterized dimeric eukaryotic isoenzymes, indicating that a dimeric structure is not essential to ensure enzymatic efficiency. Furthermore the purified enzyme proved to be highly heat-stable and, uniquely among CuZnSODs, protease-sensitive. The latter property may explain the previously described lability of this protein in cell extracts.**

Key words: Cu,Zn superoxide dismutase; Monomer–dimer equilibrium; Heat stability; Protease sensitivity; *Escherichia coli*

1. Introduction

Cu,Zn superoxide dismutases (Cu,ZnSODs), the key enzymes in primary defence of the aerobic cell against oxygen toxicity, are a class of highly conserved homodimeric enzymes that are extremely stable to physical treatments (heating, freezing etc.) and attack by proteolytic enzymes [1]. CuZnSODs are ubiquitous among eukaryotes and have also been identified in a few distantly related bacterial species [2–7], although their presence in prokaryotes is still considered an exception rather than a rule.

Little is known about the function-structure relationships of bacterial CuZnSODs (frequently referred to as bacteriocupreins). In all the cases tested so far they are localized in the periplasmic space, suggesting that they may serve to protect the cell against extracellular oxidative stress. This hypothesis is supported by the observed decrease of survival of CuZnSOD deletion mutants of *Brucella abortus* in mice [8]. Furthermore, it has been demonstrated that the absence of CuZnSOD in *Caulobacter crescentus* affects neither cell viability nor the integrity of the cell envelope, but protects a periplasmic or membrane-associated function requiring magnesium or calcium [9]. A preliminary crystallographic structure of the *Photobacterium leiognathi* CuZnSOD has been obtained [10], but no detailed information on the three-dimensional structure of bacterial Cu,ZnSODs is yet available. The alignment of their amino acid sequences has evidenced several peculiar features that suggest that the structure of bacterial CuZnSODs, while maintaining a general similarity in the arrangement of secondary structures, must significantly differ from that of the eukaryotic enzymes [11]. For example, insertion and deletion in the loop regions connecting β-strands can be observed, as well as several substitutions in the amino acids involved in the subunit interaction. Furthermore, in the enzyme from *Caulobacter crescentus* a zinc ligand (His-78) is substituted by an Asp residue [12] and in *Haemophilus influenzae* [6] a copper ligand (His-46) is replaced with a tyrosyl residue, giving rise to an inactive enzyme. Besides the lack of activity [6] there might be other factors that make bacteriocupreins difficult to detect. In particular, it was found that in *E. coli* cell extracts CuZnSOD activity was very labile and so sensitive to alkaline pH to prevent its visualization on polyacrylamide gels by standard activity staining [7].

In the light of these data we have purified the Cu,ZnSOD from *Escherichia coli* in order to gain a deeper insight into its stability features. Surprisingly, we have found that a fully active enzyme can be purified in a heat-stable monomeric form, which is, however, highly sensitive to the treatment with proteases.

2. Materials and methods

2.1. CuZnSOD purification

CuZnSOD was purified from the periplasmic space of the *sodAsodB Escherichia coli* deficient strain QC871 [13]. Cells were grown at 37°C in LB supplemented with 0.5 mM $CuSO_4$ to an A_{600} value of 1.2 and cell fractionation was performed using a lysozyme–EDTA method, as already described [14]. Proteins contained in the periplasmic fraction were concentrated by ultrafiltration through an Amicon YM10 membrane or, as an alternative, by ammonium sulphate precipitation. Proteins were extensively dialysed to remove salts and sucrose and subsequently subjected to fractionation with ammonium sulphate. The 60–95% saturation fraction that contained the SOD activity was dialysed against 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl and then injected onto a HiLoad 16/60 Superdex 75 FPLC gel filtration column (Pharmacia) and eluted with the same buffer. The major peak of activity (roughly corresponding to the 90% of total SOD activity) was concentrated, dialysed against 20 mM Tris-HCl, pH 7.5 and eluted with a 0–0.1 M NaCl linear gradient on a Mono Q 5/5 FPLC column (Pharmacia). Active fractions were further subjected to a second ionic exchange chromatography on the same column, but at pH 7.8. At this stage CuZnSOD was more than 95% pure, as judged by SDS-PAGE analysis [15]. Copper content was evaluated with a Perkin Elmer 3030 atomic absorption spectrometer equipped with a graphite furnace. *Xenopus laevis* CuZnSOD B and bovine CuZnSOD were purified by standard procedures [16,17].

2.2. SOD activity assays

SOD activity was assayed at pH 8.2 using the pyrogallol method [18]. Activity assays were also performed by staining 10% native continuous polyacrylamide gels according to standard procedures [19]. Gels were prepared with 50 mM Tris-acetate, pH 7.8 both in the gel and in the buffer reservoirs.

2.3. Thermal inactivation assays

Heat inactivation experiments have been performed at 70°C in sealed glass vials containing 100 μl of enzyme in 10 mM potassium phosphate, pH 7.4. Aliquots were withdrawn at the indicated times, cooled on ice-water and assayed for residual superoxide dismutase activity.

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2.4. Proteinase K treatment

Proteins at a concentration of 100 U/mg were incubated at 37 or 43°C in 20 mM Tris-HCl, pH 7.5 in the presence of 2 μg/ml proteinase K. At the indicated times aliquots were withdrawn and immediately assayed for residual SOD activity.

3. Results

3.1. Isolation of a monomeric form of CuZnSOD

When the periplasmic fraction (200–500 SOD U/ml) was subjected to fractionation by gel filtration chromatography, SOD activity eluted with an apparent molecular weight roughly corresponding to that of a monomer (Fig. 1). As it has been suggested that the CuZnSOD from *Pseudomonas maltophilia* may dissociate into monomers at alkaline pH [4], we have subjected the periplasmic fraction to gel filtration in different pH conditions (ranging from pH 6.8 to pH 8.9). In all the cases CuZnSOD activity displayed an identical elution profile and the monomer peak eluted with an apparent molecular weight of 16.5 kDa at pH 6.8, 17 kDa at pH 7.5 and 8.2 and 17.5 kDa at pH 8.9. These data, together with a slow but significant decrease in activity at pH 8.9 indicate that the monomeric enzyme undergoes minor conformational changes depending on pH. The same elution pattern was also observed performing gel filtration on a Superose 6 HR 10/30 column (Pharmacia) in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl. To test the effect of protein concentration on the elution of *E. coli* CuZnSOD, the enzyme was concentrated up to 3600 U/ml (more than 500 μg/ml) and newly subjected to gel filtration at pH 7.5. Also in this case the enzyme eluted as a single peak of 17 kDa protein. It is worth noting that in the same experimental conditions the CuZnSOD B from *Xenopus laevis* and the bovine enzyme eluted as a single peak of 32 kDa (see Fig. 1) at a protein concentration as low as 10 μg/ml.

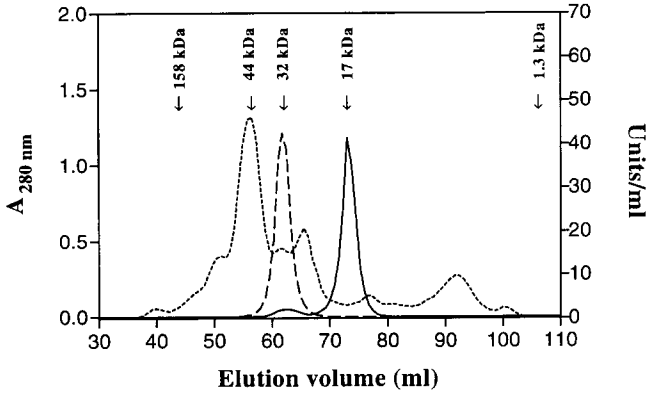

Fig. 1. Gel filtration chromatography of CuZnSOD on HiLoad 16/60 Superdex 75 FPLC column operating at 1.5 ml/min. The periplasmic proteins from a 2 liter culture were fractionated by ammonium sulphate precipitation and concentrated to 0.5 ml. The sample, containing approximately 200 U of *E. coli* CuZnSOD, was applied to the column and eluted with 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl. Fractions of 0.75 ml were collected and assayed for superoxide dismutase activity by the pyrogallol method. The column was calibrated with bovine gamma globulin (158,000 Da), chicken ovoalbumin (44,000 Da), equine myoglobin (17,500 Da) and vitamine B12 (1,350 Da). Dotted line, A_{280} of *E. coli* periplasmic proteins; solid line, *E. coli* CuZnSOD activity; dashed line, elution profile of activity of purified bovine CuZnSOD (25 μg/ml sample).

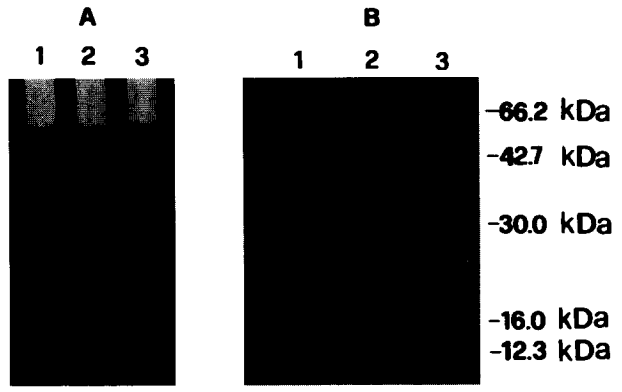

Fig. 2. (A) Activity staining of a 10% native continuous polyacrylamide gel at pH 7.8: (1) 1 μg *X. laevis* CuZnSOD, (2) 1 μg bovine CuZnSOD, (3) 1 μg *E. coli* CuZnSOD. (B) 12.5% discontinuous SDS-PAGE of purified *E. coli* CuZnSOD: (1) 1 μg bovine CuZnSOD, (2) molecular weight markers, (3) 1 μg *E. coli* CuZnSOD.

3.2. Characterization of E. coli *CuZnSOD*

As previously described [7] attempts to visualize the *E. coli* Cu,ZnSOD on standard (pH 8.8) native polyacrylamide gels using the nitro blue tetrazolium staining [19] were unsuccessful. However, when electrophoresis was performed in a continuous system at pH 7.8 an SOD activity band, migrating more slowly than the bovine enzyme, was clearly identified (Fig. 2A). The band corresponded unequivocally to a CuZnSOD since this activity was completely inhibited by 2 mM KCN [20] or by submerging the gel for 30 min in 2 mM diethyldithiocarbamate [21] prior to activity staining (not shown). The apparent molecular weight of the bacteriocuprein evaluated by SDS-PAGE was 18.5 kDa (Fig. 2B). Under the same gel conditions the bovine and the *Xenopus laevis* enzymes displayed an identical molecular weight of 16 kDa [22]. The identity of the CuZnSOD was also confirmed by amino-terminal sequencing (R. Petruzzelli, unpublished observation). The activity of the bacterial enzyme at pH 8.2 proved to be 70% that of the bovine enzyme (these values were based on the copper content of the enzymes evaluated by atomic absorption).

3.3. Heat stability of E. coli *CuZnSOD*

When the purified monomeric bacteriocuprein was incubated at 70°C the enzyme exhibited a thermal stability comparable to that of the bovine enzyme and significantly higher than that of the *Xenopus laevis* CuZnSOD B (Fig. 3). As previously described [22] the *Xenopus laevis* CuZnSOD was less resistant to the heat treatment than the bovine enzyme and both the eukaryotic enzymes showed a concentration-dependent inactivation rate, suggesting that denaturation of the enzymes at high temperatures occurred upon monomerization. This hypothesis is supported by previous fluorescence studies on human CuZnSOD demonstrating that monomerization of CuZnSODs is favoured by dilution [23] and that the unfolding intermediate is a monomer that displays a molten globule state [24]. Furthermore, dissociation of bovine CuZnSOD as a function of protein concentration has also been demonstrated using gel filtration chromatography [25]. Conversely, the *E. coli* CuZnSOD was only marginally affected by protein concentration, as expected for a monomeric protein.

3.4. Protease sensitivity of CuZnSOD

CuZnSOD of *Escherichia coli* has been reported to be a very labile enzyme, which is readily inactivated by sonication, freezing and thawing and short incubations at temperatures above 40°C [7]. These findings are in sharp contrast with the heat stability of the purified enzyme. A possible explanation for this contradiction is that some protease present in the periplasmic extracts may be responsible for the inactivation of the enzyme. To test this hypothesis we have incubated the bacteriocuprein at two different temperatures in the presence of proteinase K, a protease with very low substrate specificity which preferentially cleaves peptide bonds at the carboxylic side of aliphatic and aromatic residues. The results reported in Fig. 4 indicate that, unlike the bovine CuZnSOD that is completely resistant to the treatment with proteinase K, the bacterial enzyme is rapidly inactivated upon incubation with protease.

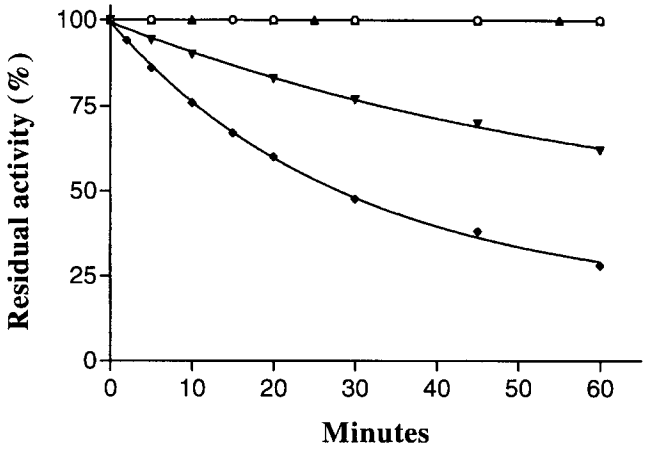

Fig. 4. Proteinase K sensitivity of *E. coli* CuZnSOD at 37 (▼) and 43°C (◆) and of bovine CuZnSOD at 37°C (○) and 43°C (▲). At each time the percent residual activity was evaluated from the ratio between the amount of enzyme producing 50% decrease of the rate of pyrogallol autoxidation before and after the treatment with proteinase K.

4. Discussion

Eukaryotic CuZnSODs are characterized by a very stable dimeric structure that is resistant to the treatment with 8 M urea, as demonstrated by sedimentation equilibrium analysis of the bovine enzyme [26]. Monomerization of CuZnSODs by treatment with SDS [27] or by site-directed mutagenesis [28] heavily affects the enzymatic activity and produces large modifications in the active site geometry as shown by EPR and NMR spectroscopy, respectively. Therefore the isolation of a monomeric CuZnSOD from the periplasmic space of *Escherichia coli* has important implications for the understanding of the role of the dimeric structure in this class of enzymes. In fact, this bacterial SOD is highly heat-stable and displays a catalytic activity comparable to that of other eukaryotic CuZnSODs. Our results are reminiscent of those obtained with the CuZnSOD IV from rice, the only monomeric CuZnSOD so far isolated [29]. These findings strongly suggest that the dimeric structure is not essential either for the high catalytic activity of CuZnSODs or for their structural stability.

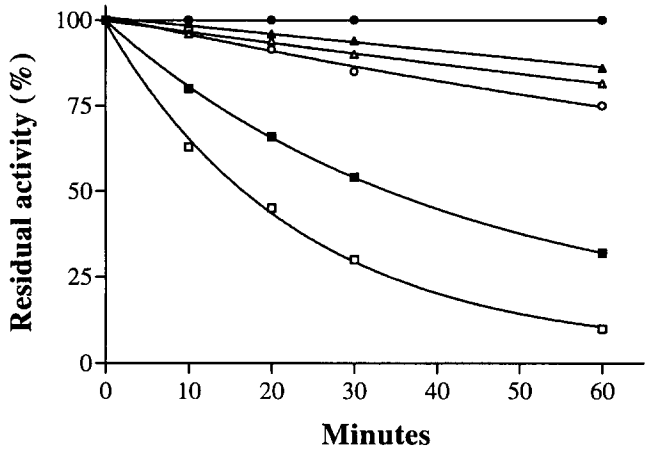

Fig. 3. Heat stability of CuZnSODs at 70°C. ▲ = *E. coli* CuZnSOD (32 μg/ml), △ = *E. coli* CuZnSOD (6.4 μg/ml), ● = Bovine CuZnSOD (32 μg/ml), ○ = Bovine CuZnSOD (6.4 μg/ml), ■ = *X. laevis* CuZnSOD (32 μg/ml), □ = *X. laevis* CuZnSOD (6.4 μg/ml). Specific activities were 8330 U/mg for bovine CuZnSOD, 8700 U/mg for *X. laevis* CuZnSOD and 6100 U/mg for *E. coli* CuZnSOD, where 1 unit of enzyme activity represents the amount of enzyme which produces 50% inhibition of the rate of pyrogallol autoxidation. The percent residual activity values were obtained from the ratio between the amount of enzyme required to obtain 50% inhibition of pyrogallol autoxidation before and after the incubation at 70°C. Each data point is the mean of at least three independent measures.

It is worth noting that, although the other characterized bacteriocupreins are dimeric, amino acid alignment indicates that the residues involved in subunit interactions in the known Cu,ZnSOD structures display substantial amino acid substitutions with respect to the eukaryotic enzymes [11] and that a pH dependent monomer–dimer equilibrium has been evidentiated in the case of the CuZnSOD of *P. maltophilia* [4]. Therefore we can not exclude that under conditions different from those used in this work the *E. coli* CuZnSOD may form a dimeric structure.

In conclusion, we demonstrate that purified *E. coli* CuZnSOD is as stable as the bovine enzyme in vitro and suggest that the previously reported lability of this enzyme [7] may be related to its unusual protease sensitivity. Eukaryotic holo-CuZnSODs are known to be completely resistant to the treatment with proteases in vitro [30] and in vivo [31]. We suggest that the amino acid insertions observed in the loop regions connecting β-strands in all the bacterial CuZnSODs may represent a target for proteases in vivo. This protease sensitivity can have a role in modulating the enzyme concentration in response to the needs of the bacterial cell.

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