In vivo formation of Cu,Zn superoxide dismutase disulfide bond in *Escherichia coli*

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Abstract We have found that the in vivo folding of periplasmic *Escherichia coli* Cu,Zn superoxide dismutase is assisted by DsbA, which catalyzes the efficient formation of its single disulfide bond, whose integrity is essential to ensure full catalytic activity to the enzyme. In line with these findings, we also report that the production of recombinant *Xenopus laevis* Cu,Zn superoxide dismutase is enhanced when the enzyme is exported in the periplasmic space or is expressed in thioredoxin reductase mutant strains. Our data show that inefficient disulfide bond oxidation in the bacterial cytoplasm inhibits Cu,Zn superoxide dismutase folding in this cellular compartment.

Key words: Cu,Zn superoxide dismutase; DsbA; Disulfide bond; Thioredoxin reductase; Redox potential

1. Introduction

Superoxide dismutases constitute a class of enzymes that play a key role in the defense machinery of the aerobic cell against oxygen toxicity [1]. *Escherichia coli* expresses two highly homologous cytoplasmic superoxide dismutases containing manganese and iron, respectively (MnSOD, FeSOD) and a structurally unrelated periplasmic superoxide dismutase containing copper and zinc (Cu,ZnSOD) [2,3]. Little is known about the function of the periplasmic enzyme in *E. coli*, but its specific location, common to all the Cu,ZnSODs so far isolated from Gram-negative bacteria [4], suggests that it should play a role in the protection of the cell against superoxide generated in the periplasm itself or extracellularly [5]. This hypothesis has obtained support by the recent observation that overexpression of the enzyme significantly enhances the ability of *E. coli* to survive macrophage killing [6]. Cu,ZnSODs are characterized by an intrasubunit disulfide bond [7,8], which contributes to their exceptionally high structural stability and is considered of extreme importance for the enzyme functionality as it stabilizes a loop that forms part of the active site [8]. With respect to the eukaryotic Cu,ZnSODs, bacterial variants of the enzyme show a modified connectivity of the intrasubunit disulfide bond due to a seven amino acid insertion in the disulfide bond containing loop which makes this protein region highly flexible and solvent accessible [9–11]. The presence of the disulfide bond could explain the general absence of Cu,ZnSODs in the cytoplasm of bacteria. In fact, the bacterial cytoplasm is a very reducing environment preventing the oxidation of disulfide bonds [12], while the periplasmic space represents an oxidizing cellular compartment containing a battery of enzymes, called Dsb proteins, which mediate disulfide bond formation [13].

In this paper we demonstrate that DsbA, the main enzyme assisting disulfide bond formation in *E. coli* [14], catalyzes the formation of the disulfide bond in bacterial Cu,ZnSOD. In the light of this finding, we reconsidered our previous data showing that folding of eukaryotic Cu,ZnSODs is heavily impaired in the bacterial cytoplasm [15,16]. We provide evidence that supports the hypothesis that the critical step in the process of eukaryotic Cu,ZnSOD folding in this cellular compartment is the formation of the disulfide bond.

2. Materials and methods

2.1. Bacterial strains and plasmids

Formation of the disulfide bond in *E. coli* Cu,ZnSOD was studied in MC1000 [17] and JCB773 [18] *E. coli* cells. Periplasmic and cytoplasmic expression of *Xenopus laevis* Cu,ZnSOD B (XSODB) was carried out in 71/18 cells [19], while the effect of thioredoxin (*trxA*) and thioredoxin reductase (*trxB*) mutations on XSODB production was studied using the isogenic strains K38 (wt), A179 (*trxA*), A304 (*trxB*) and A306 (*trxA*, *trxB*) described by Russell and Model [20]. Plasmids pPSEcSOD1 and pKB overexpressing periplasmic *E. coli* Cu,ZnSOD and cytoplasmic XSODB, respectively, have been previously described [21,15]. To overexpress XSODB in the periplasmic space of *E. coli*, the XSODB coding sequence was amplified by polymerase chain ration with the primers 5′-CACCTGGCCGTGAAGGCAGTGTGTGTA and 5′-CTGAATTCACGGACTATAACCGCC, using plasmid pKB as a template. The amplified DNA was restricted with *Nco*I and *Eco*RI and ligated into plasmid pHEN-1 [22], thus obtaining plasmid pPXSODB. In this plasmid the XSODB sequence is fused to the leader sequence of the *pelB* gene from *Erwinia carotovora*, under the control of the *lacZ* promoter.

2.2. Influence of dithiothreitol (DTT) on E. coli Cu,ZnSOD disulfide bond formation

Cells were grown in LB medium [23] containing 50 μM $CuSO_4$ (required to impart enzymatic activity to the overexpressed enzyme) and Cu,ZnSOD synthesis was induced in mid-log phase by the addition of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). At this stage DTT was added at different concentrations and cells were further grown for 2.5 h at 37°C. Cells were harvested by centrifugation, washed in LB and resuspended in LB containing 10 mM iodoacetamide to trap free cysteines. After a 10 min incubation on ice the periplasmic fraction was extracted by a lysozyme-EDTA method [21] and Cu,ZnSOD activity was determined as previously described [3]. The oxidized and reduced forms of the enzyme were separated on a 15% polyacrylamide gel containing 0.1% SDS under non-reducing conditions. Samples (each one corresponding to the periplasmic extracts from 2×10^8 cells) were boiled in the presence of 2% SDS before loading.

2.3. Kinetics of disulfide bond formation

The overexpressed *E. coli* Cu,ZnSOD was pulse-labeled with [^{35}S]methionine according to described procedures [14], growing the cells in M9 minimal medium [23]. The enzyme was labeled for 40 s with 50 μCi/ml [^{35}S]methionine and then chased with an excess of cold methionine. At different times after the chase 10 mM iodoacetamide was added to the culture and, after extraction of the periplasmic

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fraction, samples were boiled in 2% SDS and the reduced and oxidized forms were separated by SDS-PAGE. After electrophoresis the labeled proteins were electroblotted on a nitrocellulose membrane and evidenced by autoradiography.

2.4. XSODB periplasmic and cytoplasmic expression

To compare the efficiency of periplasmic and cytoplasmic production of XSODB, experiments were carried out as previously described [15,21]. Single colonies of freshly plated 71/18 cells bearing plasmid pPXSODB or pKB were used to inoculate 5 ml overnight cultures. These cultures were diluted 1:40 into LB medium containing 100 μg/ml ampicillin grown at 30 or 37°C to an A_{600} value of 0.5 and induced by the addition of 0.1 mM IPTG and 50 μM $CuSO_4$. After 3 h of growth cells were harvested by centrifugation and the periplasmic and cytoplasmic fractions were extracted [21]. Cytoplasmic expression of XSODB was analyzed in *E. coli* K38 and *trxA*, *trxB* isogenic mutants harboring pKB. After an overnight growth at 30 or 37°C in LB medium containing 0.5 mM $CuSO_4$ and 10 μM $ZnSO_4$, cells were centrifuged, resuspended in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and lysed by sonication. Cu,ZnSOD activity was determined polarographically, as already described [16].

3. Results and discussion

We have tested the effective importance of an oxidative environment in the *E. coli* Cu,ZnSOD folding process following disulfide bond formation of the enzyme overexpressed from pPSEcSOD1 in the periplasm of the *E. coli* strain JCB773, which lacks DsbA, and of the isogenic wild type strain MC1000. We have initially measured the effect of the addition of DTT to the culture medium on the recovery of Cu,ZnSOD activity from the periplasmic space of both strains. Table 1 shows that the recovery of Cu,ZnSOD activity from MC1000 cells was not affected by the addition of up to 1 mM DTT, and a significant decrease of activity was observed only at a high DTT concentration (2.5 mM). The activity decrease was paralleled by the appearance of an electrophoretic band migrating as the reduced enzyme in a non-reducing SDS gel (Fig. 1, lane 5), which may be used to distinguish the oxidized and reduced forms of the enzyme [21]. On the other hand, the activity of Cu,ZnSOD expressed in JCB773 cells was heavily affected by DTT also at very low

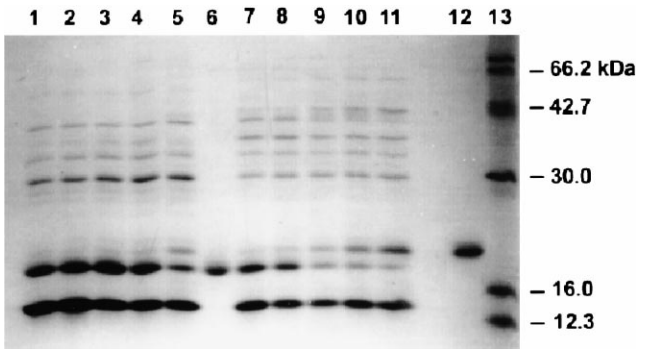

Fig. 1. Effect of DTT on Cu,ZnSOD disulfide bond formation. Periplasmic extracts from MC1000 (lanes 1–5) and JCB773 (lanes 7–11) *E. coli* cells overexpressing Cu,ZnSOD were obtained from cells grown in LB medium in the presence of increasing amounts of DTT. DTT concentrations (mM): 0 (lanes 1 and 7), 0.2 (lanes 2 and 8), 0.5 (lanes 3 and 9), 1 (lanes 4 and 10), 2.5 (lanes 5 and 11). Markers: oxidized Cu,ZnSOD (lane 6), reduced Cu,ZnSOD (lane 12). Full reduction of purified Cu,ZnSOD and of molecular weight markers (lane 13) was obtained by boiling the proteins in the presence of 5% mercaptoethanol. The gel was stained with Coomassie blue.

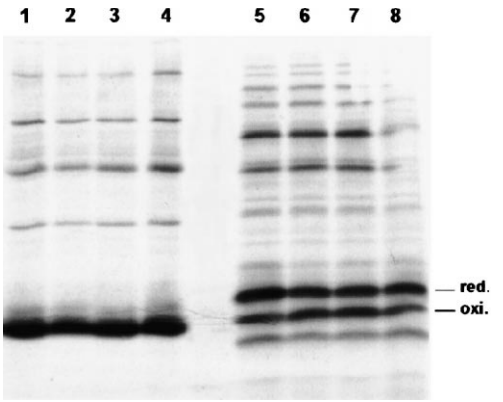

Fig. 2. Kinetics of Cu,ZnSOD disulfide bond formation in MC1000 (lanes 1–4) and JCB773 (lanes 5–8) *E. coli* cells. The positions of the reduced (red.) and oxidized (oxi.) forms of the enzymes shown on the autoradiogram were determined in the same gel using purified *E. coli* Cu,ZnSOD standards stained with Coomassie blue. After the chase with cold methionine, aliquots of the cultures were withdrawn and treated with iodoacetamide at the following times: 0 min (lanes 1 and 5), 15 min (lanes 2 and 6), 30 min (lanes 3 and 7), 45 min (lanes 4 and 8).

reductant concentrations, and a 50% decrease of catalytic activity was observed at a 0.5 mM DTT concentration. Moreover, a band migrating as reduced Cu,ZnSOD could be detected also in periplasmic extracts from *dsbA* mutant cells grown in the absence of DTT (Fig. 1, lane 7), suggesting that oxidation of the disulfide bond in the absence of DsbA is slow and inefficient. It should be also noticed that the effective importance of DsbA in assisting Cu,ZnSOD folding could be partially masked by the addition of copper to the culture medium and by the possible presence of oxidants (such as oxidized glutathione) in the LB rich medium, as they could have an effect opposite to that of DTT and favor the formation of the enzyme disulfide bond. SDS-PAGE analysis showed that, although the percentage of reduced Cu,ZnSOD increased as a function of DTT concentration, paralleling the decrease of enzyme activity, the amount of Cu,ZnSOD produced in the *dsbA* mutant was not significantly affected by the addition of DTT to the culture medium, thus indicating that formation of the disulfide bond is necessary to impart full catalytic activity to the enzyme.

To further demonstrate that DsbA mediates Cu,ZnSOD folding in vivo, we have followed the kinetics of disulfide bond formation in M9 minimal medium by pulse labeling

Table 1
DTT effect on the recovery of Cu,ZnSOD activity

DTT concentration (mM)	Cu,Zn residual activity (%)	
	MC1000	JCB773
0	100	100
0.2	100	64.5
0.5	100	51.6
1	98.5	23.4
2.5	51.2	10.7

Cu,ZnSOD activity was determined as previously described [3]. Percent residual Cu,ZnSOD activity values were obtained from the ratio between the enzyme activity at the specific DTT concentration and the activity obtained from cells grown in the absence of the reducing agent. The activity values reported here refer to the samples loaded in the gel of Fig. 1. The experiment was repeated three times essentially with the same results.

Table 2
Cytoplasmic and periplasmic production of XSODB

Plasmid	XSODB production (μg/mg)	
	30°C	37°C
pKB	1.94 ± 0.32	0.83 ± 0.38
pPXSODB	2.34 ± 0.48	9.82 ± 0.48

Cu,ZnSOD production was determined measuring the catalytic activity of the enzyme in the cell extracts by a polarographic method, as previously described [16]. Purified XSODB was used as a standard. Values are expressed as mean ± S.D. evaluated on five different experiments.

the enzyme with [^{35}S]methionine. Fig. 2 shows that immediately after the chase *E. coli* Cu,ZnSOD was completely oxidized in the wild type strain, while in the *dsbA* null mutant it was found to be mostly in the reduced state and only a fraction of the reduced form of the enzyme underwent spontaneous (or catalyzed by other periplasmic oxidoreductases) oxidation during 45 min of chase. This behavior is in contrast to what is observed for other proteins such as alkaline phosphatase and OmpA whose disulfide bond slowly oxidized also in the absence of DsbA [14], and suggests that fast oxidation of the disulfide bond is required for productive folding of *E. coli* Cu,ZnSOD. Taken together, our results show that DsbA is necessary for fast and efficient oxidation of the *E. coli* Cu,ZnSOD disulfide bond and are the first evidence that Cu,ZnSOD may require assistance by a folding catalyst to acquire its native conformation in vivo. As the presence of the disulfide bond is required for Cu,ZnSOD to exert its enzymatic activity, our findings provide an explanation for the specific subcellular location of this enzyme and suggest that Cu,ZnSOD will be hardly found in the cytoplasm of other bacteria.

These findings prompted us to reconsider our previous data showing that eukaryotic Cu,ZnSODs folding in the cytoplasm of *E. coli* is heavily impaired [15,16]. In fact, we have shown that the production of XSODB in *E. coli* is inefficient and markedly temperature sensitive [15], a behavior that is frequently observed with proteins that fold improperly in the bacterial cytoplasmic environment. This hypothesis was further sustained by the observation that co-overexpression of *E. coli* chaperonins GroEL and GroES reduced proteolytic degradation of the newly synthesized enzyme and enhanced the production of different eukaryotic Cu,ZnSODs [16]. The observation that *E. coli* Cu,ZnSOD requires the assistance of DsbA to efficiently fold in its native conformation suggested to us that the critical step in the process of eukaryotic Cu,ZnSOD folding could be the oxidation of the disulfide bond in the reducing cytoplasmic environment. Two alternative strategies have been adopted to test this hypothesis: export in the periplasmic space and expression of the enzyme in mutant strains characterized by a less reducing cytoplasmic environment.

To evaluate the effect of cytoplasmic and periplasmic environments on XSODB production, we compared the enzyme yields in 71/18 cells containing plasmid pKB or pPXSODB. We observed a 10-fold increase in the enzyme production at 37°C when XSODB was exported in the periplasmic space (Table 2), in spite of the use of an expression vector characterized by a slightly less efficient promoter (*LacZ*) than that used for cytoplasmic expression (*trc*). Periplasmic production was greater at 37 than at 30°C, probably due to differences in the rate of XSODB synthesis and/or to differences in the efficiency of the export apparatus at the two temperatures. Although we have not attempted to measure the kinetics of XSODB disulfide bond formation in *dsbA* mutants, we have also observed that the production of XSODB in the periplasmic space is scarcely affected by the lack of DsbA (not shown). Therefore, unlike the bacterial enzyme, the eukaryotic Cu,ZnSOD disulfide bond may form efficiently also in the absence of this catalyst.

Different factors are known to be responsible for the reduction of disulfide bonds in the bacterial cytoplasm, including the high intracellular level of glutathione and several thiol-disulfide oxidoreductases that actively reduce disulfide bonds via their redox active disulfide [24]. *E. coli* mutants missing thioredoxin reductase allow substantial formation of cytoplasmic disulfide bonds in different proteins [25–27]. In support of the hypothesis that inefficient formation of the Cu,ZnSOD disulfide bond is responsible for the enzyme folding impairment, we found that expression of XSODB in the cytoplasm of a thioredoxin reductase mutant (A304) significantly increases the enzyme production (Table 3), while mutation in the thioredoxin gene had no significant effects on the enzyme yield, as previously reported for bacterial alkaline phosphatase [24,25]. It is worth noting, however, that the temperature dependent pattern of expression of XSODB was not abolished in *trxB* mutants (Table 3) indicating that also in the absence of thioredoxin reductase other systems operate to maintain reduced disulfide bonds of cytoplasmic proteins [24]. The XSODB behavior parallels that of an antibody single chain fragment expressed in a *trxB* mutant strain [27], whose folding in the native conformation (stabilized by a disulfide bond) was favored by low temperatures of growth. As many proteins cannot be expressed at high levels in the periplasm [28,29] these findings may be of general usefulness to set up suitable strategies for the expression of proteins containing disulfide bonds.

Finally, this study raises a question concerning the oxidation of the disulfide bond in eukaryotic Cu,ZnSODs, which fold in the reducing cytoplasmic compartment that, being

Table 3
XSODB production in *trxA* and *trxB E. coli* mutants

Bacterial strain (relevant genotype)	XSODB production (μg/mg)	
	30°C	37°C
K38	2.05 ± 0.44	0.92 ± 0.36
A179 (*trxA*)	2.18 ± 0.41	0.94 ± 0.29
A304 (*trxB*)	8.35 ± 1.88	3.32 ± 1.3
A306 (*trxA,trxB*)	6.65 ± 1.62	2.80 ± 1.2

XSODB production was evaluated as described in the legend to Table 2. Values are the mean ± S.D. of four different experiments. Comparable results were obtained by determining the enzyme catalytic activity from cell extracts obtained by disrupting the cell in the presence of 10 mM iodoacetamide to prevent air dependent oxidation of the disulfide bond.

characterized by a very high ratio of reduced glutathione to the disulfide form, usually prevents disulfide bond formation [13,30]. The possibility to recover active XSODB from the cytoplasm of wild type *E. coli* and previous observations showing that eukaryotic Cu,ZnSOD intracellular expression may functionally complement *E. coli* strains lacking MnSOD and FeSOD [31,32] indicate that at least a fraction of the enzyme is able to fold in a disulfide bond containing conformation in the *E. coli* cytoplasm. These findings and the ability of the enzyme to fold in the periplasmic space in the absence of DsbA suggest that eukaryotic Cu,ZnSOD disulfide bond could be characterized by a redox potential significantly lower with respect to other characterized eukaryotic proteins containing structural disulfide bonds [30], which may form only in the endoplasmic reticulum. As the cytoplasm of eukaryotic cell is less reducing than that of bacteria [30], this feature could account for the ability of eukaryotic Cu,ZnSOD to fold in a stable, disulfide bond-containing form in this cellular compartment.

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