Periplasmic competition for zinc uptake between the metallochaperone ZnuA and Cu,Zn superoxide dismutase

Giovanni Berducci, Anna Paola Mazzetti, Giuseppe Rotilio, Andrea Battistoni*

Dipartimento di Biologia, Università di Roma Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy

Received 27 April 2004; revised 2 June 2004; accepted 2 June 2004

Available online 15 June 2004

Edited by Hans Eklund

Abstract **We have investigated the availability of zinc in the periplasmic space of *Escherichia coli* using a mutant Cu,Zn superoxide dismutase whose dimerization is triggered by zinc binding. This mutant enzyme accumulates in the monomeric form when wild type cells are grown in minimal medium, but assembles in the dimeric form when it is produced in the same medium by a mutant strain lacking the periplasmic zinc metallochaperone ZnuA. These results indicate that periplasmic zinc-containing proteins compete for metal binding when bacteria grow in environments where this element is present in traces. The effective ZnuA ability to sequester the available zinc ions from the periplasm suggests that zinc-containing cytoplasmic proteins are more important for bacterial viability than the periplasmic ones.**

Keywords: Cu,Zn superoxide dismutase; ZnuA; Zinc uptake; Monomer–dimer equilibrium; Quaternary structure; Metallochaperone

1. Introduction

Zinc is an essential cofactor for a large number of proteins where it plays important catalytic and/or structural roles [1]. Therefore, all organisms have evolved mechanisms to obtain adequate amounts of zinc, while avoiding the unnecessary and potentially toxic intracellular accumulation of this metal ion. Recent studies have focused on the mechanisms of metal uptake of zinc in bacteria [2,3]. The ability of several bacteria to survive and multiply in environments containing low concentrations of available zinc ions is critically dependent on the activation of the ZnuABC high affinity Zn^{2+} uptake system, which was initially described in *Escherichia coli* [4]. This system is highly homologous to other members of the family of ATP-binding cassette (ABC) transporters and is constituted by three proteins: ZnuA, ZnuB and ZnuC [4,5]. ZnuB is an integral membrane protein, ZnuC is the ATPase component of the transporter, whereas ZnuA is a soluble periplasmic metallochaperone which captures zinc in this cellular compartment and then delivers the metal to the transmembrane component of the transporter. Expression of *znuABC* is under transcriptional control of Zur [4,6], a metalloregulatory DNA-binding protein with femtomolar sensitivity to free intracellular zinc [7]. Interestingly, the inactivation of ZnuA or other members of the ZnuABC Zn-uptake system dramatically affects the ability of bacteria to grow in synthetic media deprived of zinc or to survive and cause disease in infected animals [4,8–11].

In Gram-negative bacteria the majority of proteins using zinc as a cofactor are cytoplasmic but a few zinc-containing proteins are also present in the periplasmic space. One of these proteins is Cu,Zn superoxide dismutase (Cu,ZnSOD), an antioxidant enzyme which plays an important role in protecting bacteria from extracellular sources of superoxide [12]. In Cu,ZnSOD zinc has a pivotal role in conferring structural stability to the enzyme [13], but also finely tunes the redox properties of the Cu ion constituting the redox center of the enzyme [14]. It is possible to hypothesize that under conditions of low metal availability Cu,ZnSOD should compete for metal binding with the other periplasmic proteins involved in metal homeostasis, including the high-affinity zinc metallochaperone ZnuA. To test this hypothesis, we have investigated the monomer–dimer equilibrium of a *Photobacterium leiognathi* mutant Cu,ZnSOD, whose dimerization is induced by zinc binding at the active site [15]. Our results suggest that under conditions of low zinc availability Cu,ZnSOD does not bind the zinc ion and that this is largely due to competition with ZnuA.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* strain 71/18 [16] was from our laboratory collection, while the *E. coli* strains MC4100 [17] and SP488 (isogenic to MC4100, but *znuA*::MudX) [4] were generously provided by Klaus Hantke.

Plasmid pPlSODTrp83Phe expressing the Trp83Phe mutant *P. leiognathi* Cu,ZnSODs has been previously described [15]. This plasmid was used as template for PCR amplifications carried out using the oligonucleotides EcoPHOTO (5′-acGAATTCcatgaacaaggcaaaaac) and XbaPhoto (5′-gaTCTAGAttattggatcacaccaca) and the high fidelity polymerase Expand™ (Roche Diagnostics). The amplified DNA was digested with *Eco*RI and *Xba*I and cloned in the corresponding sites of pUC18, previously digested with the same enzymes. This plasmid was made kanamycin resistant by the introduction of a kanamycin resistance GenBlock cassette (Pharmacia Biotech) into the *Pst*I site. Such plasmid, denominated pUCKanPlT83FSOD, was introduced into the *E. coli* strains MC4100 and SP488.

2.2. Protein expression and purification

The Trp83Phe PlSOD mutant was purified from *E. coli* 71/18 cells harbouring plasmid pPlSODTrp83Phe. Bacteria were grown at 37 °C in LB broth containing 100 μg/ml ampicillin, 0.25 mM $CuSO_4$ and 50 μM $ZnSO_4$ until the culture reached an A_{600} value of 0.5 and then

* Corresponding author. Fax: +39-0672594311.
E-mail address: andrea.battistoni@uniroma2.it (A. Battistoni).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.06.008

Cu,ZnSOD production was induced by the addition of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown for other 18 h at 37 °C, harvested by centrifugation and resuspended in 1/10 volume of a solution containing 20% sucrose, 30 mM Tris–HCl, pH 8.0, 1 mM EDTA and 1 mg/ml lysozyme. After a 10-min incubation on ice, cells were centrifuged for 5 min at $17\,000 \times g$ and the supernatant, containing the periplasmic proteins, was removed. Then, the periplasmic extract was concentrated by ultrafiltration and loaded onto a gel filtration FPLC column previously equilibrated with 20 mM Tris–HCl, pH 7.0, 150 mM NaCl, and 100 μM EDTA, in order to separate the monomeric and dimeric forms of the enzyme. Further purification of the dimeric form of the enzyme has been carried out by ion exchange chromatography, accordingly to previously described procedures [18]. Proteins were purified to 98%, as judged by SDS–PAGE, and their concentration was evaluated by the method of Lowry [19].

2.3. Preparation of apo-superoxide dismutase

Metal-free Trp83Phe mutant Cu,ZnSOD (devoid of both copper and zinc ions) was prepared by extensive dialysis against 50 mM sodium acetate buffer and 2 mM EDTA, pH 3.8, followed by a further dialysis against 50 mM sodium acetate and 0.1 M NaCl, pH 3.8, to remove excess EDTA, as previously described [13]. The metal devoid protein was dialyzed twice against 50 mM Na acetate buffer, pH 5.0, and then against 100 mM potassium phosphate buffer, pH 6.5. The metal content of the apo-enzyme was evaluated by atomic absorption using a Perkin–Elmer spectrometer AAnalyst 300 equipped with the graphite furnace HGA-800. The zinc and copper content of the demetallated protein was below 0.2%.

2.4. Analysis of Trp83Phe dimerization in vitro

The effects of metal ions on the dimerization of Tpr83Phe mutant Cu,ZnSOD were analyzed by gel filtration chromatography, following a procedure similar to that previously described [15]. To minimize contaminations with metals, all solutions were treated with Chelex-100 (Bio-Rad) and the gel filtration column and the FPLC apparatus were extensively washed for 24 h with 1 mM EDTA and then equilibrated with 0.15 M NaCl, 20 mM Tris–HCl, and 100 μM EDTA, pH 7.0.

3.17×10^{-8} mol of enzyme subunits were diluted in 50 mM sodium acetate, pH 6.0, in the presence or absence of various amounts of zinc, copper or both the metals and incubated for 1 h at 25 °C. Subsequently, EDTA was added at 100 μM concentration and, after a further incubation of 1 h, Cu,ZnSOD was injected onto a High Load 16/60 Superdex 75 gel filtration FPLC column (Amersham Biosciences) to separate the monomeric and dimeric forms of the enzyme. Fractions of 1 ml were collected and analyzed by SDS–PAGE. The peaks corresponding to the dimeric and monomeric forms of the enzyme were obtained with elution volumes of 70 and 80 ml, respectively.

2.5. Analysis of Trp83Phe dimerization in vivo

To analyze the ability of mutant Cu,ZnSOD to capture the zinc ion in vivo, *E. coli* MC4100 and SP488 cells bearing plasmid pUCKanPlT83FSOD were grown in M9 minimal medium [20]. To remove contaminating metals, the glassware used to grow bacteria and prepare the medium was pre-treated with diluted nitric acid and subsequently extensively washed with doubly distilled water pre-treated with Chelex-100. To further minimize metal contaminations in the M9 medium, salts were dissolved in bi-distilled water (prepared with a Millipore Milli-Q plus apparatus) pre-treated with Chelex-100.

Overnight cultures were diluted in M9 containing 50 μg/ml kanamycin and grown for 18 h at 37 °C. When required the medium was supplemented with zinc, copper or both the metal ions. Periplasmic proteins were isolated as described above and immediately injected onto the High Load 16/60 Superdex 75 gel filtration FPLC column. Fractions containing Cu,ZnSOD were analyzed by SDS–PAGE and the relative amount of monomeric and dimeric forms of the enzyme was evaluated by the densitometric analysis of gels.

3. Results and discussion

The residue Trp83 is central to the largest hydrophobic cluster located at the dimer interface of *P. leiognathi* Cu,ZnSOD and is conserved in several other bacterial Cu,ZnSODs known to be dimeric [21]. Previous studies have shown that the substitution of this residue with phenylalanine or tyrosine has little effect on the three-dimensional structure of the enzyme probed by X-ray crystallography, but influences the accessibility of the active site and the reactivity of the copper ion [18]. Moreover, dimerization of these mutants is triggered by zinc binding [15]. When these mutants are expressed in *E. coli*, they can be purified in two distinct forms, one of which is dimeric and fully active, and the second one is monomeric and devoid of metals. The partitioning of the enzyme in these two forms can be modulated by metal supplementation to the culture medium [15], thus suggesting that these proteins can be used as tools to investigate the metal availability in the periplasmic space.

To evaluate this possibility, we have further investigated the stability of metal binding in the Trp83Phe *P. leiognathi* Cu,ZnSOD mutant. Samples of the demetallated enzyme were incubated in Na–acetate buffer, pH 6.0, containing various amounts of zinc, copper or both the metals and then incubated for 1 h in the presence of EDTA. After these treatments, the protein samples were subjected to gel filtration chromatography to analyze the partitioning between the monomeric and dimeric forms. Fig. 1 shows the SDS–PAGE analysis of fractions eluted from the gel filtration column. After incubation in the absence of metals, the apo-enzyme eluted essentially as a

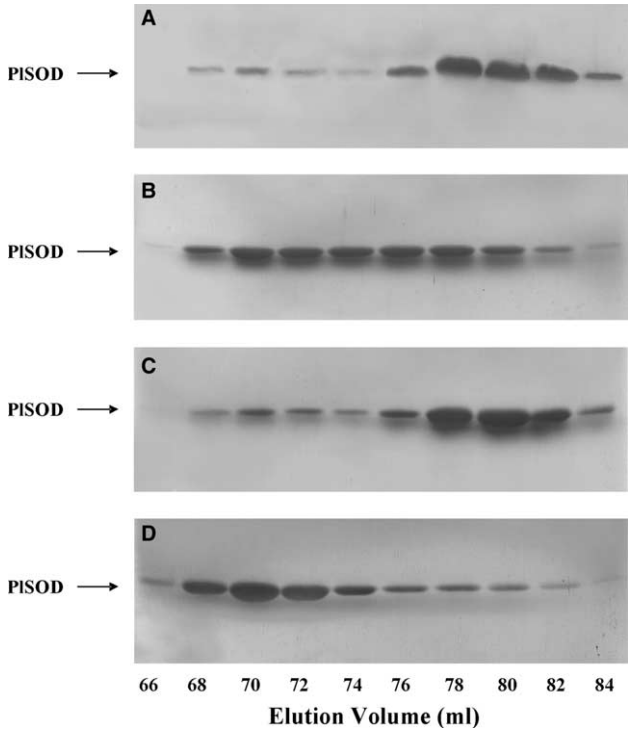

Fig. 1. Effect of metal ions on Trp83Phe dimerization. SDS–PAGE analysis of Trp83Phe *P. leiognathi* CuZnSOD eluted from a HiLoad™ 16/60 Superdex™ 75 gel filtration FPLC column. Before being subjected to gel filtration, protein samples were incubated with metals and then with EDTA as described in Section 2. The gel shows the fractions corresponding to the dimeric (eluting after 70 ml) and monomeric (eluting after 80 ml) forms of the enzyme. (A) apo-Trp83Phe mutant incubated in the absence of metals; (B) apo-Trp83Phe mutant incubated with one equivalent of zinc; (C) apo-Trp83Phe incubated with one equivalent of copper; and (D) apo-Trp83Phe mutant incubated with one equivalent of copper and one equivalent of zinc.

monomer (Fig. 1, panel A). The small amount of dimer present in the sample is probably due to traces of metals present in the incubation buffer. The elution profile of the enzyme incubated with an equimolar amount of copper was very similar to that of the apo-enzyme, indicating that copper is not stably bound by the enzyme in the absence of zinc (Fig. 1, panel C). The demetallated enzyme incubated with an equimolar amount of zinc showed a very broad elution profile (Fig. 1, panel B). The same elution was obtained when the enzyme was incubated with two equivalents of zinc (not shown). This behavior is probably due to metal loss during the incubation with EDTA and/or during the chromatographic elution. In fact, when the zinc-reconstituted enzyme was eluted from a column equilibrated with a buffer lacking EDTA, Trp83Phe mutant eluted as a dimer (data not shown). Therefore, zinc is sufficient to trigger subunit assembly, but its binding to the enzyme appears to be weak and sensitive to chelating agents. In contrast, when the apo-enzyme was reconstituted with equimolar amounts of copper and zinc, the protein eluted as a dimer (Fig. 1, panel D). This result indicates that the presence of both the metals is required to obtain their stable binding in the active site. It should be noted that this property is probably common to other bacterial Cu,ZnSODs, as previous studies have indicated that *E. coli* Cu,ZnSOD can be purified in the holo- or apo-forms, but, unlike eukaryotic Cu,ZnSODs, not in the form containing only zinc in the active site [13]. These observations suggest that experiments aiming at evaluating the efficiency of zinc binding in vivo should be carried out in the presence of copper, in order to stably trap the zinc bound in the active site.

To test whether under conditions of zinc deprivation ZnuA and Cu,ZnSOD compete for zinc binding, we examined the influence of ZnuA on the monomer–dimer equilibrium of the Trp83Phe mutant protein expressed in the periplasm of two *E. coli* strains differing in their ability to produce ZnuA. To find out suitable conditions for testing the effect of ZnuA expression on the assembly of the Cu,ZnSOD dimer, we compared the growth of *E. coli* MC4100 (wild type) and SP488 (*znuA* mutant) cells in LB and M9 minimal medium (prepared with water treated with Chelex-100 to remove traces of metals) and their ability to grow in agar plates containing EDTA. We observed that the two strains grow equally well in LB medium, indicating that zinc availability is not limiting for bacterial growth in such medium (data not shown). In contrast, the rate of growth of the SP488 strain is severely impaired in M9 medium, while supplementation of this medium with 3 μM $ZnSO_4$ restores a growth rate comparable to that of the wild type strain (Fig. 2). This observation suggests that ZnuA is required to ensure the efficient uptake of zinc in minimal salt medium. This was confirmed also by the analysis of bacterial growth in agar plates containing EDTA. The wild type *E. coli* strain MC4100 can form colonies of normal size in LB agar plates containing up to 2 mM EDTA, while the growth of the SP488 strain is inhibited at concentrations of EDTA exceeding 0.4 mM (not shown). The effect of EDTA on the growth of the SP488 *E. coli* strain is much more dramatic in minimal medium agar plates. In fact, while 100 μM EDTA does not inhibit the growth of the MC4100 *E. coli* strain, the growth of the SP488 strain is inhibited by 5 μM EDTA (not shown). On the one hand, these experiments indicate that the affinity of ZnuA for zinc is so high that it can compete with EDTA for metal binding. On the other hand they show that zinc availability in minimal medium is limited and that ZnuA is required to enable

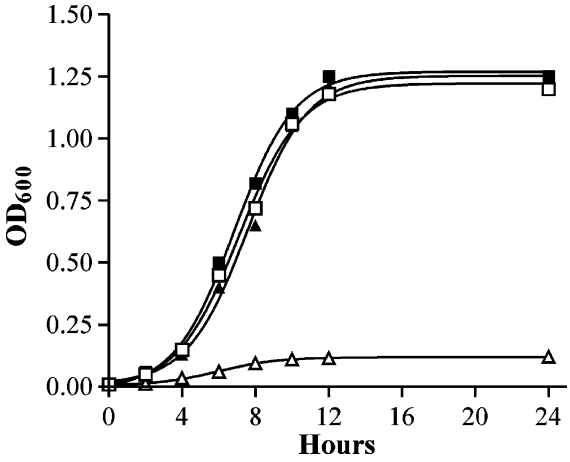

Fig. 2. Phenotype of the *E. coli znuA* mutant strain SP488. Growth curves of the *E. coli* strains MC4100 (squares) and SP488 (triangles) in M9 medium prepared with Chelex 100-treated water. Strains were grown either in standard M9 medium (open symbols) or in M9 supplemented with 3 μM $ZnSO_4$ (filled symbols).

bacteria to efficiently obtain zinc in this medium. On this ground, we decided to analyze dimerization of Trp83Phe mutant Cu,ZnSOD in M9 medium.

Fig. 3 shows the SDS–PAGE analysis of the *E. coli* periplasmic proteins fractionated according to their size by gel filtration chromatography. When bacteria were grown in standard M9 medium nearly all the mutant proteins eluted as a monomer (Fig. 1, panel A). No reproducible differences could be detected between extracts from MC4100 and SP488 cells (not shown). This was possibly due to the above described low stability of zinc binding in the active site in the absence of the copper ion. To test this possibility, bacteria were grown in M9 supplemented with 3 μM $CuSO_4$. This condition did not appreciably affect the elution profile of Trp83Phe Cu,ZnSOD produced in MC4100 cells, but induced significant protein dimerization in cells lacking ZnuA (Fig. 3, panels B and B′), indicating that, when bacteria grow in environments containing low zinc concentrations, ZnuA sequesters the available periplasmic zinc and effectively prevents the binding of this metal by mutant Cu,ZnSOD. In agreement with this hypothesis, we have observed that the enzyme produced in cells grown in M9 supplemented with 3 μM CuSO4 and 3 μM ZnSO4, i.e., a condition where ZnuA is not required to ensure normal growth of bacteria, mostly eluted as a dimer either from MC4100 or SP488 *E. coli* cells (Fig. 3, panels C and C′). Noticeably, under these conditions hardly no monomeric Cu,ZnSOD could be detected in the extract from SP488 cells, while a fraction of the mutant enzyme produced in MC4100 cells still eluted as a monomer.

Recent studies have demonstrated that several tightly regulated metal-trafficking systems control the homeostasis of transition metals within all cells, in order to deliver such essential cofactors to metalloproteins and prevent metal toxicity [21–24]. Our observations fit in this scenario and also suggest that, at least under conditions of zinc shortage, the periplasmic space is a highly competitive environment. In fact, although the outer membrane of Gram-negative bacteria is highly permeable to metal ions, periplasmic zinc-binding proteins compete for metal binding when zinc availability is low. The

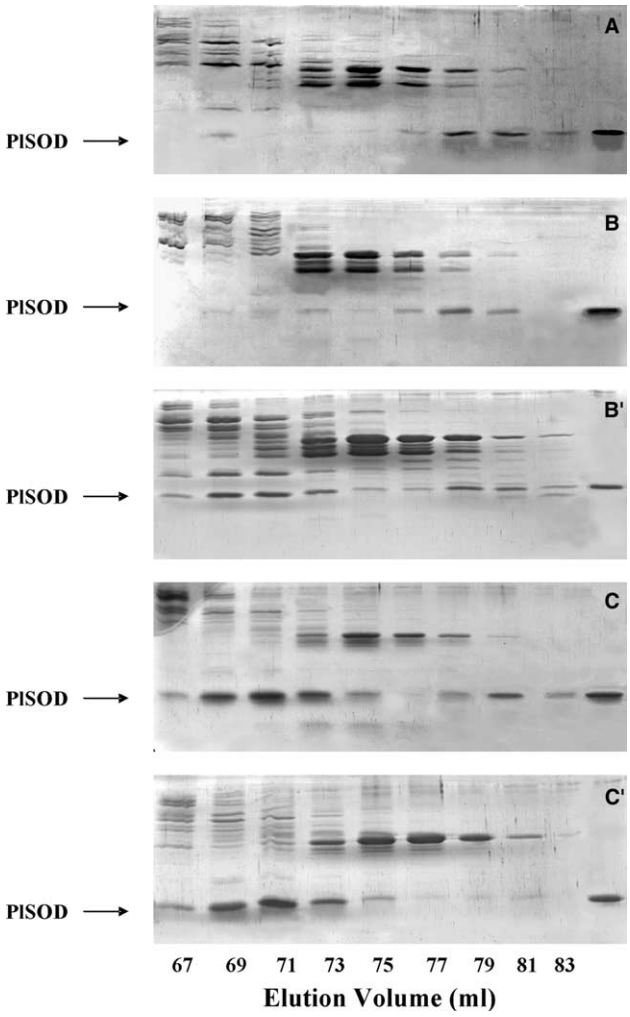

Fig. 3. Effect of ZnuA expression on the monomer–dimer partitioning of the Trp83Phe mutant Cu,ZnSOD. SDS–PAGE analysis of periplasmic proteins from *E. coli* cells expressing Trp83Phe *P. leiognathi* mutant CuZnSOD eluted from a HiLoad^{TM} 16/60 Superdex^{TM} 75 gel filtration FPLC column. The gel shows the fractions corresponding to the dimeric (showing an elution peak centered between 69 and 71 ml) and monomeric (showing an elution peak centered between 79 and 81 ml) forms of the enzyme. Proteins were obtained from wild type MC4100 cells (panels A, B, C) or *znuA* mutant SP488 cells (panels B′ and C′) grown in standard M9 medium (panel A) or in the same medium supplemented with 3 μM $CuSO_4$ (panels B and B′) or with 3 μM $CuSO_4$ and 3 μM $ZnSO_4$ (panels C and C′). Last lane of each panel: purified *P. leiognathi* Cu,ZnSOD.

ZnuABC system appears to have a central role in this process as our data indicate that ZnuA expression prevents metal binding by Cu,ZnSOD. These findings suggest that there is a hierarchy in the importance of the zinc binding proteins located in the periplasm and in the cytoplasm, the latter being much more important for bacterial survival under metal starvation conditions. This hypothesis is in agreement with the observation that the characterized periplasmic zinc-containing proteins (including, besides Cu,ZnSOD, the alkaline phosphatase encoded by *phoA* [25] and the 5′-nucleotidase encoded by *ushA* [26]) are dispensable for *E. coli* growth, whereas zinc is a cofactor of several cytoplasmic proteins involved in housekeeping functions [27].

Acknowledgements: We are indebted to Prof. Klaus Hantke (University of Tübingen) for providing us the *E. coli* strains used in this work. This work was partially supported by a MURST PRIN grant.

References

[1] Coleman, J.E. (1998) Curr. Opin. Chem. Biol. 2, 222–234.
[2] Hantke, K. (2001) Biometals 14, 239–249.
[3] Blencowe, D.K. and Morby, A.P. (2003) FEMS Microbiol. Rev. 27, 291–311.
[4] Patzer, S.I. and Hantke, K. (1998) Mol. Microbiol. 28, 1199–1210.
[5] Claverys, J.P. (2001) Res. Microbiol. 152, 231–243.
[6] Patzer, S.I. and Hantke, K. (2000) J. Biol. Chem. 275, 24321–24332.
[7] Outten, J.E. and O'Halloran, T.V. (2001) Science 292, 2488–2492.
[8] Lu, D., Boyd, B. and Lingwood, C.A. (1997) J. Biol. Chem. 272, 29033–29038.
[9] Lewis, D.A., Klesney-Tait, J., Lumbley, S.R., Ward, C.K., Latimer, J.L., Ison, C.A. and Hansen, E.J. (1999) Infect. Immun. 67, 5060–5068.
[10] Campoy, S., Jara, M., Busquets, N., Perez De Rozas, A.M., Badiola, I. and Barbe, J. (2002) Infect. Immun. 70, 4721–4725.
[11] Cheng-Yen, C. and Morse, S.A. (2001) FEMS Lett. 202, 67–71.
[12] Battistoni, A. (2003) Biochem. Soc. Trans. 31, 1326–1329.
[13] Battistoni, A., Folcarelli, S., Cervone, L., Polizio, F., Desideri, A., Giartosio, A.. and Rotilio, G. (1998) J. Biol. Chem. 273, 5655–5661.
[14] Ellerby, L.M., Cabelli, D.F., Graden, J.A. and Valentine, J.S. (1996) J. Am. Chem. Soc. 118, 6556–6561.
[15] D'Orazio, M., Battistoni, A., Stroppolo, M.E. and Desideri, A. (2000) Biochem. Biophys. Res. Commun. 272, 81–83.
[16] Messing, J., Gronenborn, B., Mueller-Hill, B. and Hofschneider, P.H. (1977) Proc. Natl. Acad. Sci. USA 74, 3642–3646.
[17] Casadaban, M.J., Chou, J. and Cohen, S.N. (1980) J. Bacteriol. 143, 971–980.
[18] Stroppolo, M.E., Pesce, A., D'Orazio, M., O'Neill, P., Bordo, D., Rosano, C., Milani, M., Battistoni, A., Bolognesi, M. and Desideri, A. (2001) J. Mol. Biol. 308, 555–563.
[19] Lowry, H.O., Rosebrough, N.J., Farr, L.A. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[20] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[21] Bordo, D., Matak, D., Djinovic-Carugo, K., Rosano, C., Pesce, A., Bolognesi, M., Stroppolo, M.E., Falconi, M., Battistoni, A. and Desideri, A. (1999) J. Mol. Biol. 285, 283–296.
[22] O'Halloran, T.V. and Culotta, V.C. (2000) J. Biol. Chem. 275, 25057–25060.
[23] Cavet, J.S., Borrelly, G.P.M. and Robinson, N.J. (2003) FEMS Microbiol. Rev. 27, 165–181.
[24] Luk, E., Jensen, L.T. and Culotta, V.C. (2003) J. Biol. Inorg. Chem. 8, 803–809.
[25] Stec, B., Holtz, K.M. and Kantrowitz, E.R. (2000) J. Mol. Biol. 299, 1303–1311.
[26] Knofel, T. and Strater, N. (1999) Nat. Struct. Biol. 6, 448–453.
[27] Katayama, A., Tsujii, A., Wada, A., Nishino, T. and Ishihama, A. (2002) Eur. J. Biochem. 269, 2403–2413.