RESEARCH COMMUNICATION

The Cu,Zn superoxide dismutase from *Escherichia coli* retains monomeric structure at high protein concentration

Evidence for altered subunit interaction in all the bacteriocupreins

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Gel-filtration chromatography experiments performed at high protein concentrations demonstrate that the Cu,Zn superoxide dismutase from *Escherichia coli* is monomeric irrespective of the buffer and of ionic strength. The catalytic activity of the recombinant enzyme is comparable with that of eukaryotic isoenzymes, indicating that the dimeric structure commonly found in Cu,Zn superoxide dismutases is not necessary to ensure efficient catalysis. The analysis of the amino acid sequences suggests that an altered interaction between subunits occurs in all bacterial Cu,Zn superoxide dismutases. The substitution of hydrophobic residues with charged ones at positions located at the dimer interface of all known Cu,Zn superoxide dismutases could be specifically responsible for the monomeric structure of the *E. coli* enzyme.

INTRODUCTION

Eukaryotic cytoplasmic Cu,Zn superoxide dismutases (SODs) are homodimeric metalloenzymes involved in the mechanisms of cellular defence against oxidative damage [1]. Comparisons of the amino acid sequences of a large number of isoenzymes and of the available high-resolution crystal structures have revealed that the enzyme structure has been preserved throughout evolution [2,3] and that all the Cu,ZnSODs possess a nearly identical three-dimensional fold, based on a flattened Greek-key eight-stranded β-barrel. Although Cu,ZnSOD has long been considered as almost exclusively an eukaryotic enzyme, Cu,ZnSODs (frequently referred to as bacteriocupreins) have been isolated also from some bacteria [4–9], and there is evidence suggesting that this enzyme may be widespread among prokaryotes [10]. Very little information is available about the structure of bacterial Cu,ZnSODs. The enzymes from *Photobacterium leiognathi* and *Caulobacter crescentus* have been characterized in some detail. Both these Cu,ZnSODs were shown to be homodimers of about 32 kDa [4,11] and to possess catalytic properties comparable with those of other Cu,ZnSODs [12].

Recently, we purified an active and heat-stable monomeric form of Cu,ZnSOD from the periplasmic space of *Escherichia coli* [13], an exceptional finding in that all the Cu,ZnSODs so far characterized are dimeric proteins. Monomerization at low protein concentrations has been observed for some Cu,ZnSODs [14,15], but the paucity of Cu,ZnSOD in *E. coli* extracts prevented an accurate analysis of the effects of protein concentration on a possible monomer–dimer equilibrium of this enzyme. We report here that the overexpressed enzyme is monomeric also at high protein concentrations, irrespective of pH and ionic strength. The amino acid sequences of the *E. coli* enzyme and of the other bacteriocupreins provide significant evidence of an altered subunit interaction in prokaryotic with respect to the eukaryotic SODs.

EXPERIMENTAL

Determination of the N-terminal sequence

Native Cu,ZnSOD was isolated from the periplasmic space of *E. coli* [13] and was subjected to 40 cycles of automated Edman degradation performed with an Applied Biosystems model 473 A pulsed liquid sequencer.

Isolation of the nucleotide sequence coding for mature Cu,ZnSOD

The following degenerate primers were used to amplify the nucleotide sequence of mature (lacking the leader peptide sequence) *E. coli* Cu,ZnSOD: Cod2 5′-TACCATGGA(A/G)-AA(A/G)GT(A/G)GA(G/A)ATGAA(C/T)(C/T)T and Ant1 5′-TCGAATTC(T/C)T(T/C/G)(T/A/G)ATIACICC(A/G) CA(C/A/T/G)GC. PCR mixtures contained 1 μg of genomic DNA, 100 pmol of each primer, 100 μM dNTPs and 2 units of *Taq* polymerase. Reactions were performed in 20 mM Tris/HCl (pH 8.6)/50 mM KCl/1.5 mM $MgCl_2$. The PCR consisted of a first cycle of 5 min of denaturation at 94 °C, a 1.5 min annealing period at 42 °C and a 1.5 min extension period at 72 °C; the next five cycles were of 1.5 min at 95 °C, 1.5 min at 42 °C and 1.5 min at 72 °C; the last 30 cycles were of 1.5 min at 94 °C, 1.5 min at 55 °C and 1.5 min at 4 °C. PCR products were digested with *Nco*I and *Eco*RI and were ligated into pHEN-1 [16]. The deduced amino acid sequence matches that of the recently reported *sodC* gene [17].

Cu,ZnSOD expression and purification

The nucleotide sequence was amplified using the primers Per1 (5′-TTCCATGGCCGCTTCTGAAAAAGTAGAGATG) and Per2 (5′-CTGAATTCTCATTTGATGACGCCGCATG), which restore the complete N-terminal sequence and introduce a stop codon respectively. The amplified DNA was restricted with *Nco*I and *Eco*RI and was ligated into plasmid pHEN-1, thus providing

Abbreviation used: SOD, superoxide dismutase.

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The nucleotide sequence coding for *E. coli* Cu,ZnSOD is available from the EMBL Nucleotide Sequence Database under accession number X97766.

pPEcSOD81A. To improve the level of protein expression by the use of a stronger promoter, pPEcSOD81A was digested with *Nco*I, treated with the Klenow polymerase and finally digested with *Eco*RI. The 600 bp DNA fragment containing the SOD sequence was cloned in pSE420 (Invitrogen), previously treated with *Nco*I, Klenow and *Eco*RI, providing plasmid pPSEcSOD1. Overexpression of periplasmic Cu,ZnSOD was carried out in Luria broth supplemented with 0.25 mM $CuSO_4$ and 2.5 μM $ZnSO_4$. Periplasmic recombinant Cu,ZnSOD was purified as previously described [13]. The purified enzyme contained 0.95 mol of Cu per mol of polypeptide.

Biochemical characterization

Apparent molecular mass was determined by SDS/PAGE carried out by two methods [18,19]. Isoelectrofocusing on precast polyacrylamide gels was performed using the Multiphor II apparatus from Pharmacia LKB. pI was determined using a Pharmacia calibration kit. Copper content was evaluated by atomic absorption [13]. Activity assays at pH 8.2 were performed by the pyrogallol method [20]. The catalytic rate constants were determined polarographically [21].

RESULTS AND DISCUSSION

Periplasmic overproduction of *E. coli* Cu,ZnSOD and characterization of the recombinant enzyme

It has been shown previously that the Cu,ZnSOD activity accounts for only 2% of the total SOD activity in *E. coli* extracts [9,17], and, in agreement with this observation, only very limited amounts of protein (2–5 μg/l of culture) can be purified from the periplasmic space of a *sodAsodB E. coli* strain grown to the early stationary phase [13]. Therefore, we decided to isolate by PCR the sequence coding for mature Cu,ZnSOD and to overproduce the enzyme in *E. coli*. The sequence was successfully isolated using two degenerate primers that were deduced from the N-terminal sequence of the native protein (Cod2) and from the highly conserved C-terminal sequence of all the known bacterial Cu,ZnSODs (Ant1) (Figure 1). The amplified nucleotide sequence was fused to the leader sequence of the *Erwinia carotovora pel*B gene, under control of the *lac* promoter, in pHEN-1, and under control of the stronger *trc* promoter in pSE420. SDS/PAGE analysis of periplasmic fractions showed that a protein of 18.5 kDa accumulated in the periplasm of cells bearing pPEcSOD81A or pPSEcSOD1 (Figure 2). Activity assays showed that when overproduced in standard Luria broth the enzyme had a low catalytic activity, but the addition of 0.25 mM $CuSO_4$ to the culture resulted in the production of a fully active enzyme. Yields of purified protein ranged from 5 to 10 mg/l of cultures of cells bearing plasmid pPSEcSOD1. Electrophoretic analysis in the presence of SDS and isoelectrofocusing demonstrated that recombinant Cu,ZnSOD has the same apparent molecular mass and pI (5.6) of the previously purified native enzyme. Native and recombinant enzymes displayed identical EPR spectra and the same elution profile when loaded on a HiLoad 16/60 ion-exchange FPLC column (Pharmacia). Finally, the catalytic activity of recombinant SOD (standardized on the copper content of the samples) at pH 8.2 is identical with that previously found for the native enzyme [13]. These results demonstrate that the physicochemical properties of the recombinant enzyme are identical with those of the protein naturally produced by *E. coli*.

The *E. coli* Cu,ZnSOD is monomeric at high protein concentration

Cu,ZnSODs are characterized by a highly stable subunit association that is mainly due to hydrophobic interactions. The dimeric structure and the catalytic activity of bovine Cu,ZnSOD are unaffected by treatment with 8 M urea or 4% SDS [22]. We have observed that, in addition, the catalytic activity of the Cu,ZnSOD from *E. coli* is not reduced after a 30 min incubation at 37 °C with 2% SDS. Interestingly, the electrophoretic behaviour of the bovine and *E. coli* Cu,ZnSODs in the presence of SDS is indicative of a different quaternary organization of the two enzymes (Figure 3). In the absence of reducing agents, the bovine enzyme is not dissociated into subunits and migrates with an apparent molecular mass of 65 kDa [22], whereas the enzyme from *E. coli* always migrates as a single band of approx. 18 kDa.

To probe the monomericity of the enzyme at high protein concentration, a 25 mg/ml solution of the purified enzyme was injected on to an FPLC gel-filtration column. As depicted in Figure 4, under these conditions the enzyme eluted as a single peak of 17 kDa, demonstrating that the enzyme is monomeric in solution also at high protein concentration. It is worth mentioning that we have previously observed [13] that under the same experimental conditions bovine Cu,ZnSOD and *Xenopus laevis* Cu,ZnSOD B elute with an apparent molecular mass of 32 kDa even when protein concentration is kept 2500 times lower. To rule out the possibility of a salt-induced monomerization of the enzyme, we have also performed gel filtration with lower ionic strength buffers or without NaCl: in all the cases the enzyme eluted as a monomer.

The catalytic activity of the *E. coli* Cu,ZnSOD is comparable with that of dimeric eukaryotic enzymes

The homodimeric structure found in all the Cu,ZnSODs has raised a debate about the importance of subunit–subunit interactions in determining or modulating the catalytic activity and protein stability of these enzymes [1]. It is generally assumed, on the basis of experiments carried out with hybrids of native and chemically modified subunits, that each enzyme subunit acts independently in catalysis [23]. However, several observations have supported the possibility of communication between subunits [24], a hypothesis that has been further substantiated by the recent identification of subunit-destabilizing mutations in the Cu,ZnSOD from *Drosophila melanogaster* [25]. To shed some light on the importance of the dimeric structure, a few studies have been carried out on monomeric derivatives obtained by SDS treatment [26] or by genetic engineering [27]. In all the cases monomerization was followed by an abrupt decrease in the catalytic activity of the enzyme and by large changes in copper geometry, reflecting alterations in the tertiary structure due to extensive solvation and/or distortion of the newly exposed dimer interface. Now, the discovery of a monomeric Cu,ZnSOD in *E. coli* and the set up of an efficient expression system to overproduce the enzyme provide a key for the understanding of the role of the dimeric structure in Cu,ZnSODs. We have found that the catalytic rate constant of purified *E. coli* Cu,ZnSOD determined polarographically and normalized with respect to the bovine enzyme (assumed to have a $k = 2.0 \times 10^9\ M^{-1} \cdot s^{-1}$) is 1.35×10^9 $M^{-1} \cdot s^{-1}$. This activity value is significantly higher than that which we have previously found for recombinant human Cu,ZnSOD ($1 \times 10^9\ M^{-1} \cdot s^{-1}$) [28]. Therefore, a first conclusion that can be drawn from our data is that the activity of the monomeric enzyme is comparable with the activity of eukaryotic isoenzymes and that communication between subunits is not necessary to ensure efficient catalytic activity. It is worth noting that both the polarographic and the pyrogallol methods [13] indicate a 30% decrease in the activity of the *E. coli* Cu,ZnSOD with respect to the bovine enzyme. As the polarographic technique is at least ten times more sensitive than the pyrogallol

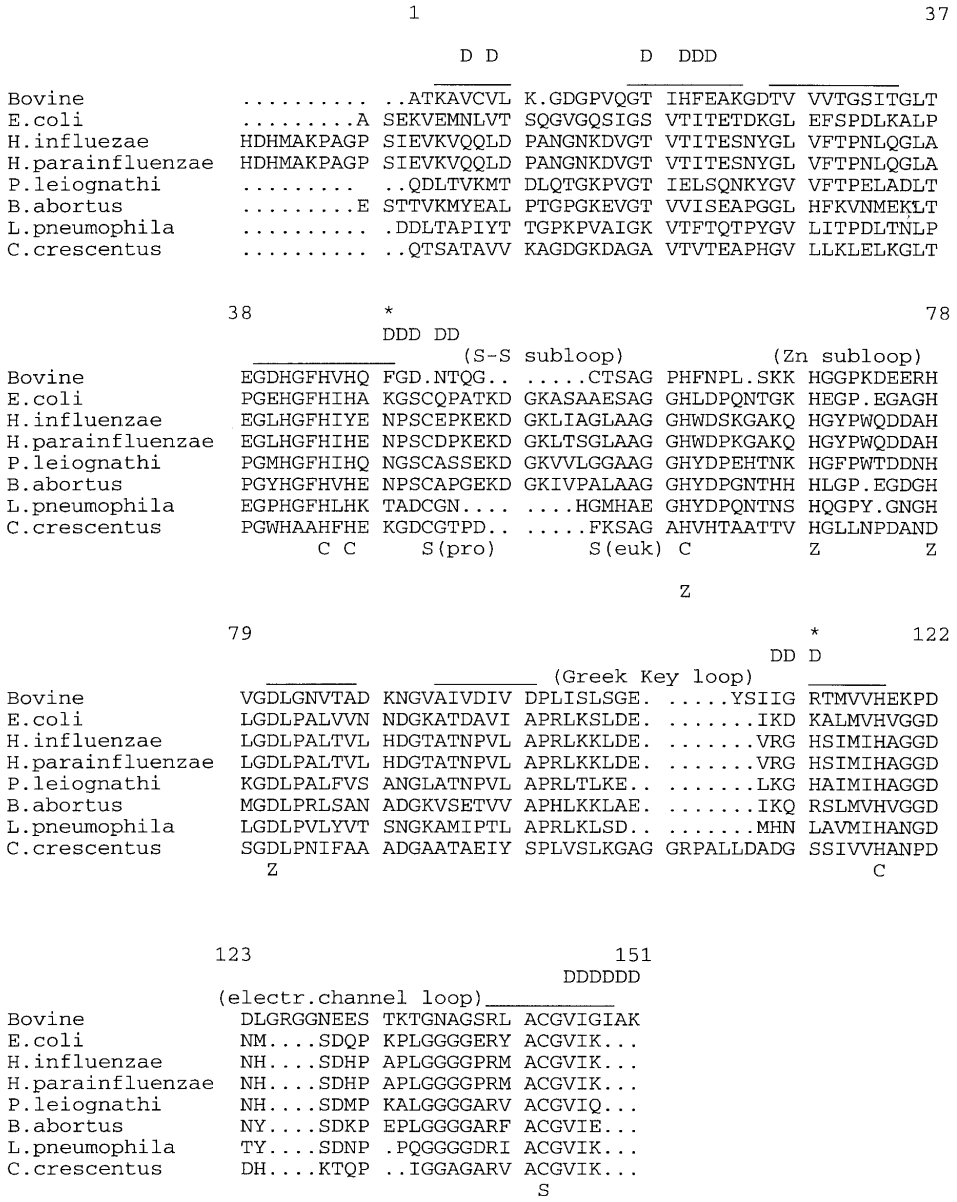

Figure 1 Amino acid alignment of prokaryotic Cu,ZnSODs versus the bovine enzyme

Amino acid sequence alignments were performed using the GCG PileUp program [29]. Optimization of the sequences alignment was based on the results of a previously published approach based on the combination of sequence alignments with structural superimposition of crystal structures [3]. The amino acid numbering refers to the bovine enzyme. D, Residues known to form contacts between subunits in all the available eukaryotic three-dimensional structures; asterisks indicate residues proposed to play a critical role in monomerization of the *E. coli* enzyme. C, Copper ligand; Z, zinc ligand. S, Cysteine residues involved in the disulphide-bridge formation in eukaryotes (euk) and in prokaryotes (pro). Overlines indicate β-strands. Electr., electrostatic.

method, the relative activity of the two enzymes is independent of their concentration.

Modified subunit interaction in bacteriocupreins

Figure 1 shows the alignment of the amino acid sequence of the *E. coli* Cu,ZnSOD with the other published bacteriocupreins and the bovine enzyme. Although the secondary structures forming the β-barrel are probably conserved also in the bacterial Cu,ZnSODs [2,3,17], the striking differences that can be observed in the major loops suggest that large differences in the three-dimensional organization of prokaryotic and eukaryotic enzymes are to be expected. In particular, inspection of the aligned amino acid sequences suggests a modified dimer interface in all bacteriocupreins. In fact these enzymes, comprising the well-characterized dimeric enzymes from *P. leiognathi* and *C. crescentus*, show a large number of amino acid substitutions (frequently from hydrophobic to polar or charged amino acids) in residues known to be involved in the subunit–subunit contacts in the eukaryotic SODs, as well as the insertion of eight residues in the S–S subloop (involving significant differences in the disulphide bond environment) and the deletion of the C-terminal residues. Significantly, the *E. coli* Cu,ZnSOD, which is characterized by the presence of charged residues at positions 48, 113–115 and 148, is the bacteriocuprein with the largest number of charged residues at positions expected to play a role in dimer formation. The

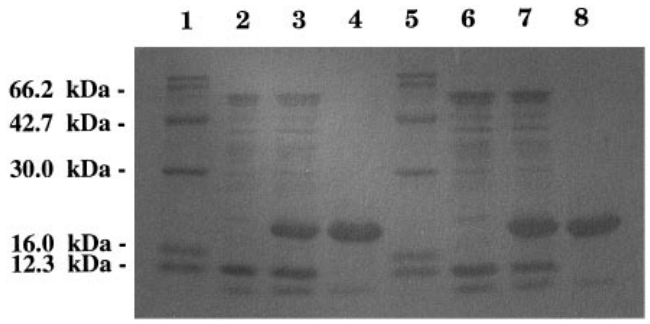

Figure 2 SDS/PAGE analysis of periplasmic fractions

Expression experiments were carried out in the *E. coli* strain 71/18 to ensure control of the inducible promoter. Cells were grown at 37 °C to an A_{600} value of 0.5 and were induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside. After 3 h of growth, cells were harvested by centrifugation and periplasmic extracts were prepared by a lysozyme/EDTA method [13]. Lanes 1 and 5, molecular-mass markers; lanes 4 and 8, 5 μg of purified *E. coli* Cu,ZnSOD. The cell extracts correspond to the periplasmic proteins from 5×10^8 cells harbouring the following plasmids: lane 2, pUC19; lane 3, pPEcSOD81A; lane 6, pSE420; and lane 7, pPSEcSOD1.

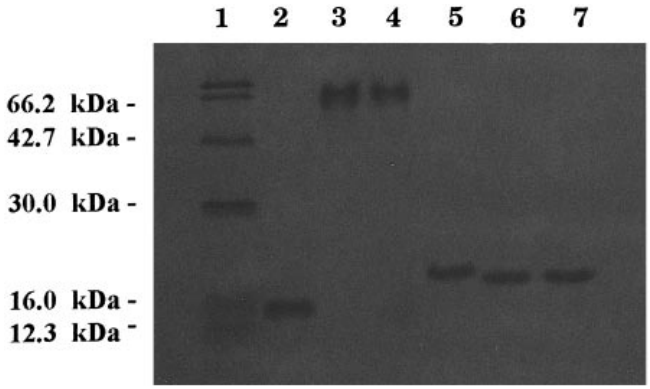

Figure 3 SDS/PAGE analysis of bovine and *E. coli* Cu,ZnSODs

Samples of purified enzymes at a concentration of 1 mg/ml were incubated for 20 min at 37 °C or 60 °C in the presence of 2% SDS. The electrophoretic migration of these protein samples (which maintain most of their initial activities) was compared with that of fully denatured enzymes boiled in the presence of 5% β-mercaptoethanol. Lane 1, molecular-mass markers; lane 2, denatured bovine Cu,ZnSOD; lane 3, bovine Cu,ZnSOD incubated at 37 °C; lane 4, bovine Cu,ZnSOD incubated at 60 °C; lane 5, denatured *E. coli* Cu,ZnSOD; lane 6, *E. coli* Cu,ZnSOD incubated at 37 °C; and lane 7, *E. coli* Cu,ZnSOD incubated at 60 °C. It should be noted that reduced (lanes 4) and oxidized (lanes 5 and 6) forms of the *E. coli* enzyme display slightly different electrophoretic migration.

enzyme from *E. coli* shows at least two potentially critical substitutions (at positions 48 and 114) with respect to the dimeric enzyme from *P. leiognathi*. The presence of a lysine residue at position 48 is not unique to the *E. coli* SOD, as it is also found in the dimeric protein from *C. crescentus*, which is, however, characterized by a different organization of the S–S subloop and of the Greek-key loop. Furthermore, it is worth noting that monomerization of human Cu,ZnSOD has been obtained by the insertion of charged residues at positions 48 and 49 [27]. In contrast, the insertion of a charged residue at position 114 has not been observed in other Cu,ZnSODs, and is expected to affect significantly the protein fold and the dimer interaction.

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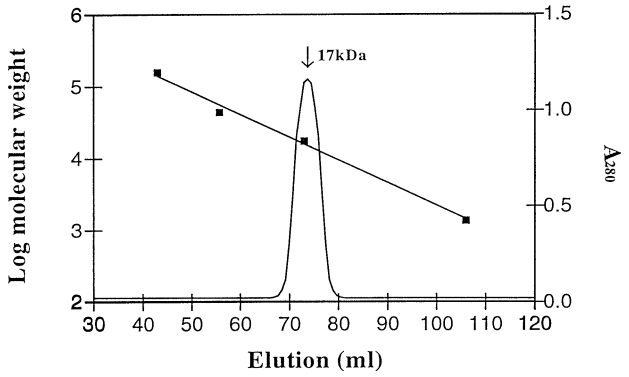

Figure 4 Elution profile of purified *E. coli* Cu,ZnSOD

E. coli Cu,ZnSOD was injected on to a HiLoad™ 16/60 Superdex™ 75 gel-filtration FPLC column (Pharmacia) operating at 1.5 ml/min. The sample, at a concentration of 25 mg/ml, was applied to the column and eluted with 20 mM Tris/HCl (pH 7.5)/0.15 M NaCl. Identical elution profiles were obtained using buffers at pH 6.8 and 8.5, in the absence of NaCl or with phosphate buffers. The column was calibrated with bovine gamma globulin (158 kDa), chicken ovoalbumin (44 kDa), equine myoglobin (17.5 kDa) and vitamin B12 (1.35 kDa).

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