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Chaperonins dependent increase of Cu,Zn superoxide dismutase production in *Escherichia coli*

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Over-expression of the chaperonins GroEL and GroES significantly suppressed the temperature-dependent pattern of expression of Cu,Zn superoxide dismutases in *Escherichia coli* and increased the yield of active enzyme. The results obtained indicate that chaperonins prevent degradation of metal-deficient enzyme molecules. GroEL was shown to form a complex with unfolded Cu,Zn superoxide dismutase in vitro, confirming that GroEL can interact with β-stranded proteins.

Cu,Zn superoxide dismutase; Chaperonin; Protein folding

1. INTRODUCTION

The ability of several polypeptides to fold into ordered three-dimensional structures in vivo is dependent on the action of a class of proteins, collectively known as 'molecular chaperones', that specifically interact with polypeptide chains in non-native conformations [1]. Chaperones binding to partially folded molecules prevent aggregation and promote their isomerization to the native structure. It has been proposed that alterations in the cellular concentration of bacterial chaperones could be of help in the production of functional recombinant proteins, the folding of which is inefficient in the bacterial cytoplasmic environment [2]. However, up to now very few examples of the feasibility of such a strategy have been reported [3–6].

In this study we report that the over-expression of the bacterial chaperonins GroEL and GroES increases the production of the two Cu,Zn superoxide dismutases (SODs) from *Xenopus laevis* (XSODA and XSODB) and of the human isoenzyme (HSOD). It is proposed that chaperonins increase the stability of partially folded, metal-deprived CuZnSODs. The in vitro formation of a complex between GroEL and denatured Cu,ZnSODs was demonstrated.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

Plasmids pKA, pKB (over-expressing XSODA and XSODB) and pGroESL have been previously described [3,7], while plasmid pKH (over-expressing HSOD) was obtained by inserting a *Nco*I restriction site at the 5′ end of the HSOD cDNA coding region of plasmid PS61-10 [8], and subsequent subcloning of the *Nco*I–*Pst*I fragment into plasmid pKK-233-2 [9]. The 2.3 kb *Eco*RI–*Hin*dIII fragment from pGroESL was inserted into the corresponding sites of pKK-233-2 obtaining plasmid pKG. Plasmid pKGB (over-expressing both XSODB and the GroE operon gene products) was constructed by inserting the *Eco*RI fragment from pKB (comprising the whole XSODB cDNA sequence and the *trc* promoter) into the *Eco*RI site of pKG. The *E.coli* strains 71/18 and SG4044 were used for expression experiments [7].

2.2. *Cu,ZnSODsexpression*

Cells were grown at different temperatures in LB medium containing 1 mM $CuSO_4$, 10 μM $ZnSO_4$ to an optical density of 1.2 (measured at 600 nm) as previously described [7]. Cells were harvested by centrifugation, resuspended in 50 mM potassium phosphate, pH 7.8, and disrupted by sonication.

2.3. *Analysis of XSODB intracellular degradation*

SG4044 cells bearing plasmid pKB or pKGB were grown at 30°C to a cell density of 1.0 OD_{600}. After a 10 min incubation at 42°C, protein synthesis was blocked by the addition of 400 μg/ml chloramphenicol and aliquots of each culture were incubated at 30, 37 and 42°C. Samples were collected after 1 and 3 h and CuZnSOD activity in the extracts was assayed.

2.4. *Protein purification and GroEL-CuZnSOD complex identification*

Cu,ZnSOD from bovine erythrocytes (BSOD), recombinant HSOD, XSODA and XSODB were purified as previously described [10–12]. GroEL was purified to > 95% from the *E. coli* strain SG4044 bearing plasmid pGroESL according to a previously described procedure [13]. CuZnSODs, which are higly stable β-barrel proteins, were denatured by incubation in 6 M guanidine-HCl, 20 mM DTT, 50 mM Tris-HCl, pH 7.5, for 60 min at 25°C. 2 μl (100 pmol) of the denatured XSODB was added to 200 μl of buffer containing 50 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, pH 7.5, and 5 μM GroEL. After incubation for 10 min at 10°C the mixture was injected onto a gel filtration column. Under these experimental conditions GdCl-denatured rhodanese (a kind gift of Prof. C. Cannella, Rome) quantitatively co-eluted with GroEL.

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2.5. *Assays*

CuZnSOD activity was measured by a polarographic method at pH 9.6 [14] using a model 466 Amel polarographic unit. The presence of metal-deficient SOD was determined as the extent of reconstitutable apoenzyme upon Cu addition [15]. Protein was determined according to Lowry [16].

3. RESULTS AND DISCUSSION

3.1. *Enhancement of CuZnSODs production by chaperonin over-expression*

The production of XSODA and XSODB in *E. coli* is inefficient and markedly depressed at high temperatures [7], a behaviour that is frequently observed with proteins for which folding is impaired in the bacterial cell [17]. Table I shows that over-expression of bacterial chaperonins in *E. coli* enhanced XSODB yield up to three times at 30°C and nearly twice at 37 and 42°C. Such an increase in XSODB recovery is due neither to modification of mRNA stability nor to changes in plasmid copy number, as assayed by standard Northern and Southern blot analysis, respectively; it was confirmed also by SDS analysis of total cellular proteins (not shown).

A comparable GroE-dependent increase in Cu,ZnSOD production was also observed for XSODA and HSOD (not shown), suggesting that chaperonin-sensitive expression is a general feature for Cu,ZnSOD proteins.

3.2. *GroESL over-expression is associated with a different cellular content of metal-deficient CuZnSOD*

It is known that the thermal stability of CuZnSODs is heavily affected by metal depletion [18] and that, unlike holo-CuZnSOD, apo-SOD is highly sensitive to proteolytic degradation [19]. Production of active Cu,ZnSOD in *E. coli* requires the addition of adequate amounts of copper and zinc to the the culture media [7,11,20]. However, it has been suggested that copper uptake in bacteria could be inefficient, thus preventing recombinant SOD reaching the correct Cu/protein stoichiometry inside the cell [20]. Table II shows that apo-XSODB is actually present both in cells containing a wild-type content of chaperonins and in those over-expressing GroEL and GroES. The increment in XSODB activity upon reconstitution shows a different temperature dependence in the two cellular environments, which is particularly evident at 42°C, where we observed a 6-fold increase in XSODB yield. The low content of copper-free XSODB at 37 and 42°C in cells containing wild-type levels of chaperonins is highly suggestive of a selective degradation of metal-deficient XSODB. In fact, we observed that the degradation rate of apo-XSODB is reduced in GroE over-producing cells (Fig. 1), while the degradation of holo-XSODB is not significantly affected by the intracellular concentration of chaperonins (not shown). These results suggest that the GroE proteins exert their effect on XSODB production by modulating the stability of metal-deficient XSODB species in a temperature-dependent manner, thus favouring metal uptake by the newly synthesized protein.

Table I

Increase in XSODB activity upon chaperonins co-expression

Plasmid	XSODB activity (μg/mg of total proteins)		
	30°C	37°C	42°C
(A) pKB	1.99 ± 0.44	0.83 ± 0.38	0.50 ± 0.16
(B) pKB + pGroESL	5.50 ± 1.88	1.55 ± 0.27	0.94 ± 0.33
A/B	2.72 ± 0.23	2.05 ± 0.45	1.87 ± 0.62

XSODB activity in cell extracts from *E. coli* SG4044 grown at different temperatures and harbouring either pKB plasmid or both pKB and pGroESL plasmids. Values are expressed as mean±S.D. evaluated on six independent experiments. A/B ratio was evaluated for each experiment; mean±S.D. of A/B ratios is reported. Under the assay conditions the activity of the *E. coli* SODs (MnSOD and FeSOD) amounted to 2–10% of the total SOD activity in the extracts. Their contribution was determined by adding KCN to 2 mM to the buffer in order to inhibit Cu,ZnSOD activity. Essentially identical results were obtained using pKGB instead of the combination of pKB and pGroESL. Comparable results were obtained using *E. coli* 71/18, where the *trc* promoter was induced by IPTG addition.

3.3. *Isolation of a GroEL-Cu,ZnSOD binary complex*

When isolated GdCl-denatured SODs were incubated

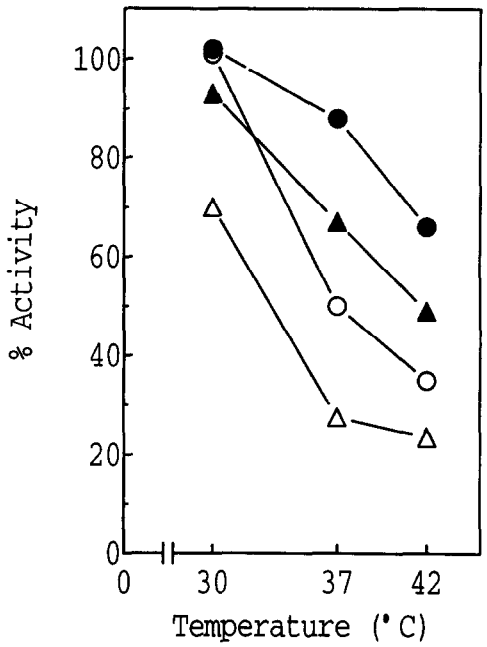

Fig. 1. Intracellular degradation pattern of apo-XSODB in *E. coli* SG4044 over-expressing XSODB (pKB-bearing cells) or XSODB and both the GroE proteins (pKGB-bearing cells). Apo-XSODB was evaluated upon reconstitution with copper (see section 2.5.) and expressed as a percentage of apo-XSODB before incubation at the indicated temperatures. (○) pKB after 1 h incubation; (△) pKB after 3 h; (●) pKGB after 1 h; (▲) pKGB after 3 h.

Table II

Effect of chaperonins on the recovery of apo- and holo-Cu,Zn SOD

Plasmid	Temperature (°C)	XSODB activity		Increment (%)
		No copper addition	Copper addition	
pKB	30	1.69	4.38	159
pKB	37	0.52	0.84	62
pKB	42	0.34	0.52	52
pKB + pGroESL	30	4.33	9.27	114
pKB + pGroESL	37	1.34	3.28	144
pKB + pGroESL	42	0.97	3.05	214

Increase of XSODB activity recovered from cells grown at different temperatures upon Cu addition to extracts. Values are the average of two separate experiments; XSODB activity is given in μg/mg of total proteins in the extracts; increments after Cu addition were calculated assuming XSODB activity before addition = 100%.

with a 10-fold molar excess of purified GroEL, a fraction of enzymatically active XSODB, or of immunoreactive BSOD or HSOD co-eluted with the chaperonin upon subsequent size-exclusion chromatography (Fig. 2). Binding of SODs was specific as it was not observed either in the presence of 5 μM GroES and 5 mM ATP, which cause release of GroEL-bound substrates, or in the presence of 50 μM casein, which competes with unfolded proteins for the GroEL 14-mer binding site [21]. On the other hand, the amount of GroEL-bound SOD was increased in the presence of millimolar amounts of Zn, which inhibits productive refolding [22]. The broad peak of elution of the GroEL-bound Cu,ZnSOD is probably due to an ATP-independent release of the protein during chromatography. These data demonstrate that GroEL can interact with β-stranded proteins, as previously reported only for a F_{ab} fragment and phage P22 tailspike protein ([23] and references therein). However, the binding of GroEL to β-sheets is characterized by a spontaneous release of the unfolded substrates, suggesting that, while sufficient to promote productive refolding, the interaction between GroEL and β-strands is generally weaker than with other structural elements.

Work is in progress in our laboratory to further analyze factors affecting Cu,ZnSODs binding to GroEL.

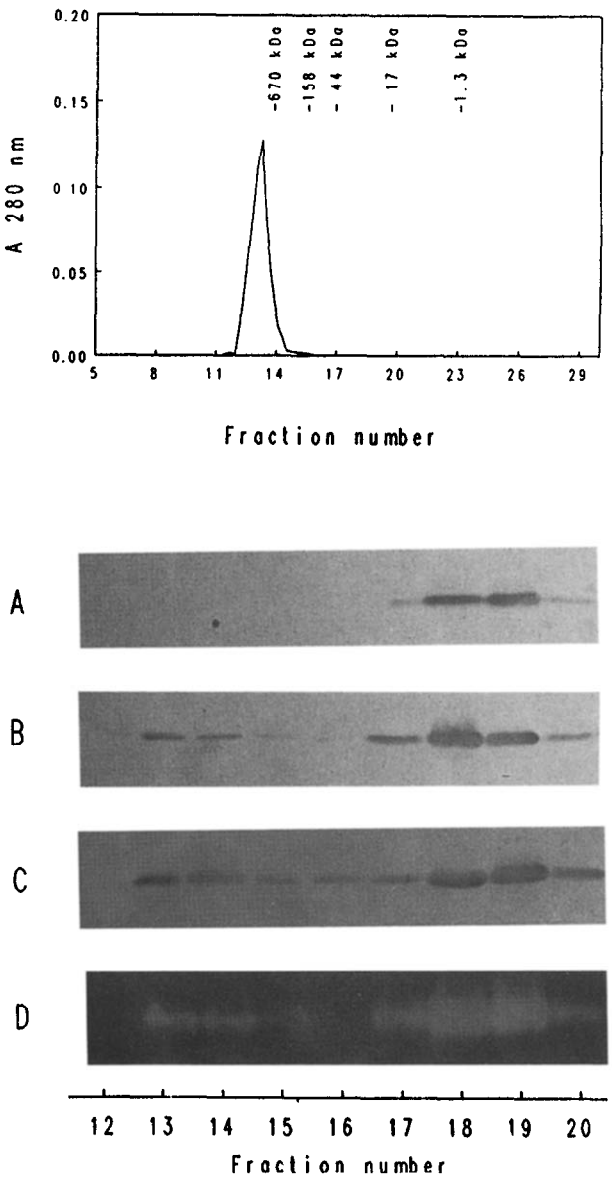

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Fig. 2. (Top) Gel filtration of the GroEL–Cu,ZnSOD complex on Superose 6 HR 10/30 FPLC column operating at 0.5 ml/min. 1 ml fractions were collected. The peak corresponding to unbound Cu,ZnSOD was below detection limits. Molecular weight marker (Bio-Rad) elution positions are indicated. (Bottom) Western blot analysis (A–C) and activity staining on PAGE (D) of fractions from gel filtration of GroEL-CuZnSOD complexes formed as described in section 2. (A) BSOD; (B) BSOD+GroEL; (C) BSOD+GroEL+1 mM $ZnCl_2$; (D) XSODB+GroEL. Identical results were obtained with HSOD.

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