Escherichia coli heat-shock proteins IbpA/B are involved in resistance to oxidative stress induced by copper

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The small heat-shock proteins IbpA/B are molecular chaperones that bind denatured proteins and facilitate their subsequent refolding by the ATP-dependent chaperones DnaK, DnaJ, GrpE and ClpB. In this report, we demonstrate that IbpA/B participate in the defence against copper-induced stress under aerobic conditions. In the presence of oxygen, Δ i**bpA/B cells exhibit increased sensitivity to copper ions and accumulate elevated amounts of oxidized proteins, while under oxygen depletion, the Δ i**bpA/B mutation has no effect on copper tolerance. This indicates that IbpA/B protect *Escherichia coli* cells from oxidative damage caused by copper. We show that AdhE, one of the proteins exposed to oxidation, is protected by IbpA/B against copper-mediated inactivation both *in vivo* and *in vitro*.****

INTRODUCTION

IbpA and IbpB proteins were identified for the first time as components of inclusion bodies formed in *Escherichia coli* cells overproducing heterologous proteins (Allen *et al.*, 1992). It was also demonstrated that during heat stress, IbpA/B localize to the fraction of denatured and aggregated *E. coli* proteins (Laskowska *et al.*, 1996). IbpA and IbpB share about 50 % amino acid homology and belong to the ubiquitous family of small heat-shock proteins (sHsps) or α -heat-shock proteins (α -Hsps) characterized by low molecular mass (12–30 kDa) and a conserved C-terminal ‘ α -crystallin’ domain (Narberhaus, 2002; Haslbeck *et al.*, 2005). α -Crystallin is a mammalian eye lens sHsp which prevents protein aggregation and is responsible for maintaining transparency of the lens. sHsps form oligomers comprising between 9 and 50 subunits, depending on the particular sHsp. Some sHsps have a defined oligomeric structure with a fixed number of subunits; others, like α -crystallins and IbpA/B, form polydisperse oligomers (Haslbeck *et al.*, 2005; Nakamoto & Vigh, 2007). sHsps bind denatured proteins and facilitate their refolding by the ATP-dependent molecular chaperones of the Hsp70 family (Haslbeck *et al.*, 2005). In *E. coli* cells, the substrates bound to IbpA/B are refolded by DnaK and its co-chaperones DnaJ and GrpE in cooperation with the AAA⁺ protein ClpB (Veinger *et al.*, 1998; Mogk *et al.*, 2003a, b; Matuszewska *et al.*, 2005). The lack of IbpA/B proteins results in increased aggregation of heat-denatured proteins

in *E. coli* cells. However, the phenotype of Δ i**bpA/B mutation is only observed under severe heat stress (50 °C) (Kuczyńska-Wiśnik *et al.*, 2002) or at lower heat-shock temperature (45 °C) in the double mutant Δ i**bpA/B Δ clpB, and in Δ i**bpA/B cells with downregulated DnaK/DnaJ levels (Mogk *et al.*, 2003a). It was also reported that removal of aggregated proteins at a recovery temperature of 30 °C was delayed in the absence of IbpA/B (Mogk *et al.*, 2003a; Jiao *et al.*, 2005). Both IbpA and IbpB are required during substrate inactivation *in vitro*, to efficiently stabilize denatured protein in a folding-competent state (Matuszewska *et al.*, 2005). It is known that IbpA and IbpB interact *in vitro*; however, the nature of cooperation between IbpA and IbpB is not fully understood. Some *in vivo* and *in vitro* data suggest that IbpA may be responsible for targeting IbpB to denatured and aggregated proteins (Kuczyńska-Wiśnik *et al.*, 2002; Matuszewska *et al.*, 2005).******

IbpA/B have been suggested to participate in the defence of *E. coli* cells against oxidative stress. It was demonstrated that bacteria overproducing IbpA/B proteins acquired resistance to superoxide stress (Kitagawa *et al.*, 2000); moreover IbpA/B suppressed inactivation of selected enzymes by hydrogen peroxide and potassium superoxide *in vitro* (Kitagawa *et al.*, 2002). It was also found that an i**bpA/B-deficient strain showed increased sensitivity to superoxide radicals generated by tellurite (Pérez *et al.*, 2007).**

In this study we investigated the role of IbpA/B proteins in protection of *E. coli* against oxidative stress induced by copper ions. Copper is an essential transition metal required as a cofactor for numerous respiratory and

Abbreviations: DNP, 2,4-dinitrophenol; DNPH, 2,4-dinitrophenylhydrazine; Hsp, heat-shock protein; MCO, metal-catalysed oxidation; ROS, reactive oxygen species; sHsp, small heat-shock protein.

metabolic enzymes, mainly those that utilize dioxygen or reactive oxygen species (ROS); however, copper is toxic even at low concentrations (Silver & Phung, 2005; Kershaw *et al.*, 2005). At present, knowledge on the toxicity of copper is limited. Excess copper may compete with other essential transition metals for binding to the active sites of metalloproteins and catalyse formation of non-native disulfide bonds in proteins, resulting in perturbation of protein function (Hiniker *et al.*, 2005). Copper changes the permeability and fluidity of membranes and affects conductance of ionic channels (Suwalsky *et al.*, 1998; Avery *et al.*, 1996). The mechanism of copper cytotoxicity also includes generation of highly toxic hydroxyl radicals (Kershaw *et al.*, 2005) and other ROS (Tree *et al.*, 2005; Macomber *et al.*, 2007). In this process, Cu^{2+} and Cu^+ ions react with superoxide anion and hydrogen peroxide, which are byproducts of aerobic metabolism that are present at low, non-toxic concentrations in *E. coli* cells (Storz & Imlay, 1999). The level of superoxide anions may be increased via reaction of Cu^+ ions with molecular oxygen:

ROS generated by copper may damage proteins, DNA and lipids. It should be noted that copper toxicity increases under anaerobic conditions, when ROS are not produced; this may result from the increased reduction of Cu^{2+} to more toxic Cu^+ ions (Outten *et al.*, 2001).

In this paper we demonstrate that IbpA/B contribute to the tolerance of *E. coli* to copper under aerobic conditions. We report evidence that IbpA/B protect proteins from copper-mediated oxidation both *in vivo* and *in vitro*.

METHODS

Bacterial strains and growth conditions. *E. coli* MC4100 [*araD139* Δ (*lacI*POZYA *argF*)U169 *fla* *relA* *rpsL*] used as a wild-type (WT) strain, *E. coli* MC4100 Δ *ibpA/B*::*cm* (Kuczyńska-Wisnik *et al.*, 2002), *E. coli* MC4100 Δ *clpB*::*kan* (Geuskens *et al.*, 1992) and *E. coli* MC4100 Δ *dnaK52*::*cat* (Mogk *et al.*, 1999) were grown at 30 °C in Luria broth (LB) supplemented with 0.2 % glucose, aerobically, in flasks with shaking (200 r.p.m.) or anaerobically, without shaking, in bottles filled to the top.

Purification of proteins for *in vitro* tests. After prolonged incubation of *E. coli* under oxygen depletion, AdhE forms large active oligomers which can be isolated from bacterial cell extracts by ultracentrifugation (Matayoshi *et al.*, 1989; Kessler *et al.*, 1992); therefore, we applied a one-step AdhE purification method. After testing different growth conditions, we found that almost homogeneous enzyme could be isolated from *E. coli* cells growing microaerobically at 25 °C, in M9 minimal medium with 0.2 % glucose. AdhE was purified from MC4100 *ibpA/B* cultures (250 ml), incubated without shaking in 250 ml Erlenmeyer flasks for 68 h. Bacteria were collected, resuspended in 0.2 M Tris/HCl pH 8.0, converted to spheroplasts and sonicated as described previously (Kucharczyk *et al.*, 1991). Cell lysates (6 ml) were incubated with 2 % Triton X-100 at room temperature for 15 min and loaded on a two-step sucrose gradient (1 ml 55 %, w/w, sucrose and 5 ml 17 %, w/w, sucrose in

3 mM EDTA pH 8.0). AdhE oligomers were pelleted by ultracentrifugation at 200 000 g, for 1.5 h. The pellet was resuspended in 10 mM Tris/HCl pH 8.0 and immediately used for the experiments. IbpA and IbpB proteins were purified as described previously (Matuszewska *et al.*, 2005). Protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

Detection of protein-bound carbonyl groups. The bacteria were collected, washed in 10 mM Tris/HCl pH 8.0, resuspended in buffer A (0.5 M Tris/HCl pH 6.8, 6 % SDS, 10 mM EDTA) and lysed at 95 °C for 5 min. Aliquots of extracts containing equal amounts of protein were derivatized with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl for 30 min at room temperature. After neutralization with 2 M NaOH, proteins were dissolved in Laemmli (1970) lysis buffer, separated by SDS-PAGE and transferred to a nitrocellulose membrane. Protein-bound 2,4-dinitrophenylhydrazones were visualized using anti-2,4-dinitrophenol (DNP) antibodies (Sigma) and ECL Western blotting detection reagents (Pierce Biotechnology).

AdhE oxidation *in vitro* and AdhE activity assay. Oxidation of AdhE (0.4 μM) *in vitro* was performed at room temperature in 0.15 mM potassium phosphate buffer pH 7.4 containing 0.2 mM CuCl_2 and 2 mM ascorbate (Stadtman, 1991). AdhE activity was measured spectrophotometrically at 340 nm in a reaction mixture containing 0.66 mM NADH, 1.6 M ethanol and 0.3 M potassium carbonate buffer pH 10. A unit of enzyme activity is defined as 1 nmol of NADH produced min^{-1} (Echave *et al.*, 2002). To determine AdhE activity in cell extracts, bacteria were pelleted, resuspended in 10 mM Tris/HCl pH 7.5 and disrupted by sonication.

SDS-PAGE and immunoblotting. Gel electrophoresis was performed according to standard protocols (Laemmli, 1970). Immunodetection of proteins was carried out using antisera specific for protein-bound dinitrophenylhydrazones (Sigma), IbpA/B or AdhE as primary antibodies (Kuczyńska-Wisnik *et al.*, 2002), with anti-rabbit IgG horseradish peroxidase conjugate (Sigma) and ECL detection reagents (Pierce Biotechnology). Membranes were scanned and analysed with the 1DScan EX program (Scanalytics).

RESULTS

Copper tolerance in the *E. coli* Δ *ibpA/B* strain is decreased under aerobic conditions

To investigate whether IbpA/B participate in the protection of *E. coli* cells against the toxic effects of copper, we first compared the sensitivity of the WT and Δ *ibpA/B* strains to Cu^{2+} and analysed the level of IbpA/B proteins in *E. coli* cells exposed to copper. We found that indeed Δ *ibpA/B* cells showed increased sensitivity to Cu^{2+} (Fig. 1a). As expected, the amount of IbpA/B proteins increased (10-fold) and reached the maximum after 1–2 h of Cu^{2+} treatment (Fig. 1d). The 2D-PAGE used for separation of the IbpA from IbpB revealed that the IbpA:IbpB molar ratio was 1.6:1 (data not shown). Further incubation of the WT strain in the presence of Cu^{2+} resulted in a gradual decline in the amount of IbpA/B to the basal level at the end of the experiment. The described phenotype of the Δ *ibpA/B* mutation was observed only under aerobic conditions. In the absence of oxygen, the toxicity of copper increased significantly (Fig. 1b) in agreement with

Fig. 1. Lack of IbpA/B chaperones increases sensitivity of aerobically growing *E. coli* to Cu²⁺. MC4100 WT (grey bars) and MC4100 Δ IbpA/B (white bars) were grown aerobically (a) or anaerobically (b, c) at 30 °C. The anaerobic cultures shown in (b) were grown in the presence of the indicated amounts of CuCl₂ from inoculation. Other cultures (a and c) were supplemented with CuCl₂ at an OD₅₉₅ of 0.2. Simultaneously, anaerobically growing cells (c) were transferred to flasks for aerobic incubation. Final optical density was measured after 18 h of incubation. The data correspond to the means $\pm$ SD of three independent experiments. (d) Cu²⁺ induces production of IbpA and IbpB proteins in *E. coli* cells. IbpA and IbpB were detected in the samples taken from the WT cultures treated with 4 mM CuCl₂ at the indicated time points. Proteins were separated by SDS-PAGE and immunodetected using anti-IbpA/B serum and ECL reagents. Both IbpA and IbpB migrated in the polyacrylamide gel as one band.

published results (Outten *et al.*, 2001). However, we did not observe any remarkable differences in growth between the WT and Δ IbpA/B strains (Fig. 1b). These results suggest that IbpA/B may suppress toxicity of copper by protection of cells against ROS whose production is induced by Cu²⁺. Further data indirectly supported this assumption: a more severe phenotype of the Δ IbpA/B mutation was observed when copper treatment in the presence of oxygen was preceded by anaerobic incubation of the cultures (Fig. 1c). In this experiment, the effect of copper treatment was enhanced by oxidative stress initiated by the shift of the cultures from anaerobic to aerobic conditions.

One of the types of damage caused by ROS in cells is oxidation of proteins, resulting in the formation of carbonyl groups in some amino acid residues. To determine whether IbpA/B protect *E. coli* proteins from oxidation by ROS, we compared the degree of protein carbonylation in the WT and Δ IbpA/B strains exposed to copper stress (Fig. 2). After 30 min of Cu²⁺ treatment, the level of oxidized proteins increased 2-fold and 2.5-fold in aerobically growing WT and Δ IbpA/B cells, respectively (Fig. 2a). During further incubation in the presence of copper, the levels of carbonylated proteins decreased to the initial amounts. This was in agreement with the observation that oxidized proteins are rapidly removed from the cell by proteolysis (Nyström, 2005). Therefore after prolonged treatment with Cu²⁺, removal of oxidized proteins proceeded faster than protein carbonylation. Elevated protein oxidation was also detected in bacteria untreated with Cu²⁺ that were shifted from anaerobic to aerobic conditions (Fig. 2b). In this case, the level of carbonylated proteins was comparable in both the WT and Δ IbpA/B strains. At the end of the experiment, bacteria entered the stationary phase, in which accumulation of an increased amount of oxidized proteins has been noted previously (Dukan & Nyström, 1998). Copper treatment after the shift from anaerobic to aerobic conditions caused a further enhancement of protein oxidation. After 30 min, the amount of carbonylated proteins in Δ IbpA/B cells was only slightly higher compared to the WT strain. However, in the absence of IbpA/B the level of carbonylated proteins decreased at a slower rate (Fig. 2c). The above results taken together indicate that IbpA/B inhibit protein oxidation and/or facilitate degradation of oxidatively damaged proteins under copper stress.

IbpA/B protect AdhE from metal-catalysed oxidation *in vivo* and *in vitro*

One of the oxidized proteins, visible as a dominant band immunostained with anti-DNP antibodies (Fig. 2d), was further identified as AdhE by immunodetection (data not shown). AdhE is a Fe²⁺-dependent protein with the activity of alcohol dehydrogenase and acetaldehyde-CoA dehydrogenase (Membrillo-Hernández *et al.*, 2000; Nnyepi *et al.*, 2007). AdhE converts acetyl-CoA to acetaldehyde, and then to ethanol in NADH-dependent reactions. The

Fig. 2. Protein oxidation induced by Cu²⁺ is increased in the absence of IbpA/B. *E. coli* WT (●) and $\Delta ibpA/B$ (○) strains were grown aerobically (a) or anaerobically at 30 °C to an OD₅₉₅ of 0.2. The anaerobically growing bacteria were transferred to flasks and split into two samples (b and c). One of the samples was treated with 4 mM CuCl₂ (c). CuCl₂ (4 mM) was also added to the aerobic cultures (a). At the indicated time points, bacteria were harvested and proteins were derivatized with DNPH (see Methods). Samples containing equal amounts of protein were resolved by SDS-PAGE and analysed by immunoblotting using anti-DNP antibody. The level of carbonylated proteins was estimated by densitometry (a–c). (d) Western blotting results showing the protein carbonylation pattern in selected samples. AdhE protein is indicated by an arrow. The data are representative and similar results were obtained in two other experiments.

adhE gene is very highly expressed under anaerobic conditions. After the shift of bacteria to aerobic conditions, expression of the gene is inhibited and AdhE is inactivated by metal-catalysed oxidation (MCO). In this process, Fe²⁺ ions react with H₂O₂ to generate hydroxyl radicals, which covalently attack the amino acid residues near the metal-binding site (Cecarini *et al.*, 2007). Our result showing that AdhE is one of the main proteins oxidized in Cu²⁺-treated cells (Fig. 2d) is in good agreement with the observation that AdhE is one of the major targets of hydrogen peroxide stress (Tamarit *et al.*, 1998).

We investigated the effect of IbpA/B on *in vivo* inactivation of AdhE after the shift of cultures to aerobic conditions (Fig. 3). It was found previously that in the presence of

oxygen, AdhE is protected against MCO by DnaK, but not ClpB chaperone (Echave *et al.*, 2002); therefore, in our experiment $\Delta dnaK$ and $\Delta clpB$ strains were used as controls. Since a decrease of AdhE activity might result from both MCO and degradation of the enzyme, the amount of AdhE protein in bacteria was also monitored by Western blotting using anti-AdhE antibodies with serial dilutions of purified AdhE as standards (data not shown). We determined AdhE concentration and its activity and calculated specific AdhE activity expressed as units of AdhE mg⁻¹. This allowed comparison of the degree of AdhE inactivation in different strains regardless of the rate of AdhE degradation. In non-stressed anaerobic WT and $\Delta ibpA/B$ cultures, 7500 ± 200 U AdhE mg⁻¹ was detected and set to 100 %. After 1 h of aerobic growth, 90 % of AdhE specific activity was detected

in WT and $\Delta clpB$, whereas in the absence of IbpA/B and DnaK the specific activity of AdhE decreased to 20 % and to 10 %, respectively (Fig. 3). Thus, IbpA/B, similarly to DnaK chaperone, inhibited aerobic inactivation of AdhE. However, the levels of carbonylated AdhE immunodetected in the WT and $\Delta ibpA/B$ cultures shifted to aerobic conditions were comparable (Fig. 2d). This may indicate that oxidized and inactivated AdhE is removed faster in $\Delta ibpA/B$ than in WT cells. Indeed, the amount of total AdhE declined to approximately 40 % in WT and to 30 % in $\Delta ibpA/B$ cells (data not shown).

To confirm that IbpA/B proteins prevent oxidation of AdhE we compared activities of the enzyme in the WT and $\Delta ibpA/B$ strains submitted to copper stress. In subsequent experiments, we used cultures that were incubated anaerobically and then exposed to copper, because a high initial level of AdhE was necessary for reliable activity measurements. The presence of Cu^{2+} ions during aerobic incubation resulted in accelerated inactivation of AdhE (Fig. 4a) in comparison with the previous experiment (Fig. 3). After 1 h incubation in the presence of copper, activity of AdhE dropped to 48 % and 9 % in WT and $\Delta ibpA/B$ strains, respectively. Again, the levels of carbonylated AdhE were comparable in both strains (Fig. 2d), indicating that damaged AdhE was removed faster in the absence of IbpA/B. Indeed, the amount of total AdhE decreased in WT and $\Delta ibpA/B$ by 20 % and 30 % respectively (data not shown). Prolonged incubation (2 h) of WT and $\Delta ibpA/B$ bacteria in the presence of Cu^{2+} resulted in further proportional decrease of AdhE activities (Fig. 4a). The amounts of total

Fig. 3. IbpA/B protect AdhE from inactivation after a shift from anaerobic to aerobic growth conditions. *E. coli* WT (grey bars), $\Delta ibpA/B$ (white bars), $\Delta dnaK$ (black bars) and $\Delta clpB$ (striped bars) strains were grown anaerobically at 30 °C to an OD_{595} of 0.3. The activity of AdhE was measured at the indicated time points after transferring bacterial cultures to flasks for aerobic incubation; 100 % corresponds to AdhE activity before the shift (7500 ± 200 U AdhE mg^{-1}). Means $\pm$ SD of three independent experiments are shown.

Fig. 4. IbpA/B participate in protection of cellular AdhE against Cu^{2+} -induced inactivation and carbonylation. (a) MC4100 WT (grey bars) and $\Delta ibpA/B$ cells (white bars) were cultured as described in the legend to Fig. 3. Immediately after the shift of the cultures to aerobic growth conditions, $CuCl_2$ (4 mM) was added. AdhE activity was measured at the indicated times; 100 % corresponds to AdhE activity before the shift (7500 ± 200 U AdhE mg^{-1}). (b) Relative amounts of carbonylated AdhE in the WT and $\Delta ibpA/B$ cells estimated from Fig. 2(d). The data correspond to the means $\pm$ SD of three independent experiments.

AdhE did not change (not shown) in either strain but the fraction of carbonylated AdhE was apparently higher in the $\Delta ibpA/B$ strain (Fig. 4b). In summary, presented data demonstrated that IbpA/B inhibited inactivation of AdhE under oxidative stress induced by copper ions. It should be noted that inactivation of AdhE *in vivo* may result not only from the replacement of Fe^{2+} by Cu^{+} and copper-catalysed oxidation, but also from direct modifications of amino acids by ROS produced in the presence of copper (Cecarini *et al.*, 2007). Moreover, copper treatment may also increase the rate of H_2O_2 generation, which can accelerate iron-mediated AdhE inactivation. In order to confirm that IbpA/B prevent copper-catalysed oxidation of AdhE, we performed *in vitro* experiments with purified AdhE (Fig. 5a) and IbpA/B proteins. AdhE was oxidized *in vitro* by an ascorbate/ Cu^{2+} mixture (Stadtman, 1991) in the presence of IbpA, IbpB or both IbpA/B proteins (used at 1.6 : 1 molar ratio, as estimated in copper-stressed cells). After 3 h incubation at 25 °C, AdhE activity was measured (Fig. 5b) and carbonylation of AdhE was analysed by

Fig. 5. IbpA/B protect purified AdhE against Cu^{2+} -induced inactivation and carbonylation. (a) Purified AdhE protein. Whole-cell extract (lane 1) obtained from a microaerobic $\Delta\text{ibpA/B}$ culture (see Methods) and purified AdhE (lane 2) were separated by SDS-PAGE and stained with Coomassie brilliant blue. (b) AdhE (0.4 μM) was oxidized at room temperature in 0.15 M potassium phosphate buffer pH 7.4 containing 0.2 mM CuCl_2 and 2 mM ascorbate, in the absence or presence of 1 μM IbpA (+), 1 μM IbpB (+), 2 μM IbpA (++) , 2 μM IbpB (++) or both chaperones (1.2 μM IbpA and 0.8 μM IbpB). AdhE activity was measured after 3 h exposure of the enzyme to Cu^{2+} /ascorbate. (c) Carbonylation of AdhE and IbpA/B was analysed in duplicate samples by immunodetection, as described in the legend to Fig. 2. (d) AdhE and IbpA/B were resolved in a control polyacrylamide gel and stained with Coomassie brilliant blue. All experiments shown in b–d were repeated three times and representative results are shown.

Western blotting (Fig. 5c). In the absence of chaperones, AdhE was almost completely inactivated and only 5 % of the control AdhE activity was detected. The best efficiency of AdhE protection (30 % of the AdhE initial activity) was achieved in the samples containing fivefold molar excess of IbpA or IbpB, or both IbpA/B proteins (1.2 μM IbpA and 0.8 μM IbpB) (Fig. 5b). Neither higher IbpA and IbpB concentrations, nor different IbpA:IbpB molar ratios improved the efficiency of protection (data not shown). Results obtained after estimation of AdhE carbonylation (Fig. 5c) were consistent with the activity assay. In the presence of optimal IbpA/B amounts, the level of

carbonylated AdhE was fivefold lower than in the sample oxidized in the absence of the chaperones. The comparison of the intensities of immunodetected (Fig. 5c) and Coomassie-stained bands (Fig. 5d) indicated that the extent of IbpA/B carbonylation was much lower than that of AdhE. Taken together, these results showed that purified AdhE is protected from Cu^{2+} -catalysed oxidation by IbpA and IbpB proteins.

DISCUSSION

The transition metal copper is an important cofactor for many enzymes. However, even moderately increased levels of copper may be highly toxic; therefore copper concentrations need to be regulated within narrow limits. Copper homeostasis in *E. coli* is controlled mainly by the efflux of excess copper out of the cell. The P-type ATPase CopA, embedded in the inner membrane, translocates Cu^+ from the cytoplasm and multicopper oxidase CueO converts periplasmic Cu^+ into less-toxic Cu^{2+} . During anaerobic growth, copper toxicity increases and another efflux system encoded by the *cusCFBA* operon is necessary for full copper tolerance (Grass & Rensing, 2001; Outten *et al.*, 2001; Silver & Phung, 2005). In the presence of high copper concentrations, when the efflux complexes are overloaded, *E. coli* cells activate general stress responses governed by the sigma factor RpoE (Egler *et al.*, 2005) and the two-component signal transduction system CpxRA (Kershaw *et al.*, 2005; Yamamoto & Ishihama, 2005). Both pathways respond to environmental stresses and control expression of genes required for repairing of damaged proteins in the envelope (DiGiuseppe & Silhavy, 2003). Recently, it was found that activity of the disulfide isomerase DsbC (Hiniker *et al.*, 2005) and increased expression of the porin OmpC (Egler *et al.*, 2005) are required for copper tolerance. DsbC rearranges non-native disulfide bonds formed in periplasmic proteins damaged by copper; the underlying mechanism of OmpC function remains unclear. DNA microarray experiments revealed that in response to superoxide stress as a secondary effect of excess copper, *E. coli* cells increase transcription of the SoxRS regulon (Kershaw *et al.*, 2005; Yamamoto & Ishihama, 2005). The above information indicates that *E. coli* copes with copper stress using several different mechanisms. In this report we present evidence that the sHsps IbpA/B constitute an additional element of the defence against copper toxicity. We demonstrated that the lack of IbpA/B proteins increased sensitivity of aerobically growing *E. coli* to Cu^{2+} ions (Fig. 1a–c) and found that IbpA/B were induced by copper (Fig. 1d). In previous reports (Kershaw *et al.*, 2005; Yamamoto & Ishihama, 2005), the *ibpA/B* operon was not found among copper-induced genes, probably because less harsh conditions (lower copper concentration or shorter time of exposure to copper) were applied compared to our experiments. IbpA/B may be the second line of defence, triggered in response to protein damage caused by copper in the cytoplasm.

IbpA/B proteins are markers of inclusion bodies and protein aggregates produced under heat stress or in trimethoprim-treated cells (Allen *et al.*, 1992; Laskowska *et al.*, 1996, 2003); thus it could be expected that similar protein aggregates were formed in the copper-stressed cells. This assumption is supported by the fact that introduction of carbonyl groups into proteins may induce intra- and intermolecular cross-links or conformational changes leading to formation of large aggregates resistant to proteolysis (Nyström, 2005; Cecarini *et al.*, 2007). Such protein aggregates were not found either in WT or in Δ i**bpA/B** cells submitted to copper stress (data not shown). It is possible, however, that proteins damaged by copper formed smaller complexes which were soluble or could not be separated from the membranes by ultracentrifugation in a sucrose gradient. Indeed, we found that AdhE inactivated and oxidized by copper did not change its position in the sucrose gradient when compared to the native enzyme. Moreover, in the copper-treated WT cells, IbpA/B proteins were localized in the same fractions as AdhE (data not shown). It was demonstrated that *in vitro* solubility of sHsp complexes with substrate depends on the ratio of sHsp to substrate. The size of sHsp/substrate complexes decreases as the ratio of sHsp to substrate increases (Mogk *et al.*, 2003b). In other words, insoluble aggregates are formed when sHsps are overloaded with non-native substrates (Jiao *et al.*, 2005). Apparently, this is not the case in the copper-treated cells. By contrast, protein aggregates containing IbpA/B are formed in cells subjected to heat stress (Laskowska *et al.*, 1996).

We showed that a possible mechanism of Δ i**bpA/B** copper sensitivity involves copper-induced carbonylation of proteins (Fig. 2). *In vivo* (Figs 3 and 4) and *in vitro* experiments (Fig. 5) revealed that IbpA/B decreased oxidative inactivation and carbonylation of AdhE. The mechanism of protein protection by IbpA/B remains to be elucidated. It can be expected that the overall ability of IbpA/B to protect cells from copper-induced damage may result from the metal binding and direct interaction with protected proteins, since a similar activity has been previously proposed for the mammalian sHsp homologue, α -crystallin (Moschini *et al.*, 2006). In addition, Ganadu *et al.* (2004) have shown that Cu^{2+} stably binds to α B-crystallin and elevates its chaperone-like activity. IbpA and IbpB were equally effective in protection of AdhE against copper-induced oxidation *in vitro* (Fig. 5). One must keep in mind that IbpA and IbpB may act in a different manner, by binding and protecting substrate or sequestering copper ions. This is possible, since it has been established that IbpA and IbpB exhibit different affinity for unfolded proteins (Kuczyńska-Wisnik *et al.*, 2002; Matuszewska *et al.*, 2005).

Several papers on the role of Hsp in prevention of protein carbonylation have been published recently. Echave *et al.* (2002) found that DnaK protects AdhE against MCO. Fredriksson *et al.* (2005) reported that the molecular chaperones DnaK/DnaJ, GroEL/GroES and heat-shock proteases Lon and HslVU counteract protein carbonylation

induced by stasis. Winter *et al.* (2005) demonstrated that the redox-regulated holdase Hsp33 protects proteins, including AdhE, against irreversible aggregation during oxidative heat stress. The authors found that DnaK is reversibly inactivated under oxidative heat stress due to a massive drop in intracellular ATP level caused by ROS. In consequence, ATP-independent Hsp33 is activated and takes over the protective role of DnaK. It is therefore conceivable that IbpA/B, which, like Hsp33, are ATP-independent chaperones, have a similar function.

AdhE is active as a dehydrogenase under anaerobic conditions. In the presence of oxygen, transcription of the gene is reduced and AdhE is irreversibly inactivated by MCO. The physiological significance of the protection of AdhE by IbpA/B chaperones against MCO is a question which needs to be addressed. One can imagine that maintenance of active AdhE may be an advantage for fast adaptation of aerobically growing cells to new, anoxic conditions. IbpA/B may not only decrease protein oxidation but also prevent other toxic effects of copper. It was found that the Δ i**bpA/B** mutation causes increased membrane fluidity and permeability under heat-stress conditions (Nakamoto & Vigh, 2007). Therefore, it is possible that IbpA/B stabilize and protect the inner membrane in cells exposed to copper.

In summary, we have gained new insights into the role of IbpA/B in protection of *E. coli* cells against oxidative stress induced by copper. We have demonstrated that IbpA/B are molecular chaperones preventing inactivation of a fermentative enzyme, AdhE, by copper-mediated oxidation. Further studies are necessary to explain the mechanism of AdhE protection by IbpA/B against metal-catalysed oxidation.

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