

Role of *Escherichia coli* heat shock proteins IbpA and IbpB in protection of alcohol dehydrogenase AdhE against heat inactivation in the presence of oxygen

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Escherichia coli small heat shock proteins IbpA and IbpB are molecular chaperones that bind denatured proteins and facilitate their subsequent refolding by the ATP-dependent chaperones DnaK/DnaJ/GrpE and ClpB. *In vivo*, the lack of IbpA and IbpB proteins results in increased protein aggregation under severe heat stress or delayed removal of aggregated proteins at recovery temperatures. In this report we followed the appearance and removal of aggregated alcohol dehydrogenase, AdhE, in *E. coli* submitted to heat stress in the presence of oxygen. During prolonged incubation of cells at 50°C, when AdhE was progressively inactivated, we initially observed aggregation of AdhE and thereafter removal of aggregated AdhE. In contrast to previous studies, the lack of IbpA and IbpB did not influence the formation and removal of AdhE aggregates. However, in $\Delta ibpAB$ cells AdhE was inactivated and oxidized faster than in wild type strain. Our results demonstrate that IbpA and IbpB protected AdhE against thermal and oxidative inactivation, providing that the enzyme remained soluble. IbpA and IbpB were dispensable for the processing of irreversibly damaged and aggregated AdhE.

Keywords: small heat shock proteins IbpA and IbpB, protein aggregation, protein oxidation, AdhE

INTRODUCTION

Escherichia coli IbpA and IbpB proteins belong to the ubiquitous family of small heat shock proteins (sHsps) whose members are involved in many cellular processes: cell development, carcinogenesis, regulation of apoptosis, rearrangement of the cytoskeleton and protection against various stresses including heat shock (reviewed in Haslbeck *et al.*, 2005; Sun & MacRae, 2005; Nakamoto & Vigh, 2007). The common features of sHsps are: low molecular mass, oligomeric structure and the presence of a conserved C-terminal “ α -crystallin” domain. sHsps form stable complexes with damaged proteins and facilitate refolding of the substrates in cooperation with the ATP-dependent molecular chaperones belonging to the Hsp70 fam-

ily (Haslbeck *et al.*, 2005; Nakamoto & Vigh, 2007). When sHsps are overloaded with non-native substrates *in vitro*, the sHsp-substrate complexes form insoluble aggregates (Mogk *et al.*, 2003b; Jiao *et al.*, 2005). In *E. coli* cells submitted to heat stress IbpAB are present mainly in aggregates of denatured endogenous proteins (Laskowska *et al.*, 1996). *In vitro*, proteins bound to IbpA and IbpB are refolded by the ATP-dependent DnaK chaperone and its cochaperones DnaJ and GrpE. ClpB, the AAA⁺ family member, which cooperates with the DnaK machinery, increases the efficiency of refolding of polypeptides bound to IbpA and/or IbpB (Veinger *et al.*, 1998; Mogk *et al.*, 2003a; 2003b; Matuszewska *et al.*, 2005). IbpA and IbpB share 50% amino-acid homology and interact *in vitro*, but the nature of their cooperation in the refolding remains elusive

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Abbreviations: DNP, 2,4-dinitrophenol; Hsps, heat shock proteins; MCO, metal-catalyzed oxidation; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; wt, wild type.

(Matuszewska *et al.*, 2005). It has been suggested that IbpA and IbpB may exhibit different substrate specificity and the binding of IbpB to unfolded substrates is IbpA-dependent (Kuczyńska-Wiśnik *et al.*, 2002; Matuszewska *et al.*, 2005).

The lack of IbpA and IbpB proteins results in increased aggregation of denatured proteins in *E. coli* cells under severe heat stress (50°C) (Kuczyńska-Wiśnik *et al.*, 2002) or at a lower heat shock temperature (45°C) in the double mutant $\Delta ibpAB\Delta clpB$, and in $\Delta ibpAB$ cells with down-regulated DnaK/DnaJ levels (Mogk *et al.*, 2003a). It was also reported that in the $\Delta ibpAB$ strain removal of aggregated proteins is inhibited after a shift from high to recovery temperatures (Kuczyńska-Wiśnik, *et al.*, 2002; Mogk *et al.*, 2003a; Jiao *et al.*, 2005). In this paper we extended *in vivo* studies focusing on a single natural IbpA and IbpB substrate. We investigated the influence of IbpA and IbpB on inactivation and aggregation of alcohol dehydrogenase (AdhE) promoted by heat shock in the presence of oxygen. AdhE is an Fe²⁺ dependent protein produced at a high level under anaerobic conditions. After a shift of bacteria from anaerobic to aerobic conditions transcription of the *adhE* gene is significantly reduced and AdhE is irreversibly inactivated by metal-catalyzed oxidation (MCO). In this reaction, Fe²⁺ reacts with H₂O₂ and generates hydroxyl radicals which covalently attack the amino-acid residues near the metal binding site, which results in formation of carbonyl derivatives (Membrillo-Hernandez *et al.*, 2000; Cecarini *et al.*, 2007). Recently, we have demonstrated that IbpA and IbpB protect AdhE against MCO *in vivo* and *in vitro* during oxidative stress induced by copper ions (Matuszewska *et al.*, 2008).

MATERIALS AND METHODS

Bacterial strains and growth conditions.

E. coli MC4100 [*araD139* Δ (*lacI*POZYA *argF*) *U169 fla relA rpsL*] and its derivative MC4100 $\Delta ibpAB::cm$ (Kuczyńska-Wiśnik *et al.*, 2002) were grown anaerobically in plastic tubes filled to the top at 30°C (without agitation) in Luria broth (LB) supplemented with 0.2% glucose. It should be noted that such conditions were not strictly anaerobic, although the availability of oxygen was significantly limited (Eraso & Weinstock, 1992). At the exponential phase bacteria were exposed to heat stress under aerobic conditions, i.e. the cultures were transferred to flasks and incubated at 50°C in a water bath with shaking (200 r.p.m.).

Isolation of protein aggregates. Insoluble protein aggregates were isolated as described previously, with modifications (Kuczyńska-Wiśnik *et al.*, 2002). Briefly, cells were harvested, converted into spheroplasts, lysed by sonication and fractionated by ultracentrifugation in a sucrose density gradient

(1 ml of 60%, 1.5 ml of 55%, 1.5 ml of 50% 1 ml of 45%, 1 ml of 40%, 1 ml of 35% and 0.6 ml of 30%, w/w, sucrose) to separate insoluble protein aggregates (buoyant density of 1.24–1.26 g/ml) from membranes and soluble proteins. Twenty-eight subfractions were collected from the bottom of a tube and analysed for protein concentration according to the method of Bradford (1976).

Detection of protein-bound carbonyl groups.

Bacteria were collected, resuspended in 0.5 M Tris/HCl buffer (pH 6.8) containing 6% SDS, 10 mM EDTA and lysed at 95°C for 5 min. Aliquots of extracts containing equal amounts of total protein were subjected to derivatization with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl for 30 min at room temperature. The samples were neutralized with 2 M NaOH, separated by SDS/PAGE and transferred to a nitrocellulose membrane. Protein-bound 2,4-dinitrophenylhydrazones were detected using anti-2,4-dinitrophenol (DNP) antibodies (Sigma) and ECL Western blotting reagents (Pierce Biotechnology).

AdhE inactivation and refolding assay.

AdhE, IbpA, IbpB, DnaK, DnaJ and GrpE proteins were purified as described previously (Matuszewska *et al.*, 2005; 2008). AdhE (0.4 μ M) was inactivated in buffer C (50 mM Tris/HCl, pH 7.4, 150 mM KCl, 2 mM dithiothreitol, 20 mM MgCl₂) at 50°C for 15 min. The refolding was performed in buffer C supplemented with ATP (5 mM), ATP regeneration system (10 mM phosphocreatine and 100 μ g/ml phosphocreatine kinase) and chaperone proteins (1.5 μ M DnaK, 0.12 μ M DnaJ, 0.15 μ M GrpE) at 25°C. AdhE activity was measured spectrophotometrically at 340 nm in a reaction mixture containing 0.66 mM NAD⁺, 1.6 M ethanol and 0.3 M potassium carbonate buffer pH 10. A unit of enzyme activity is defined as the amount producing one nanomole of NADH per minute (Membrillo-Hernandez *et al.*, 2000).

SDS/PAGE and immunoblotting. Gel electrophoresis and Western blotting were performed according to standard protocols (Sambrook *et al.*, 1989). Immunodetection of proteins was carried out using polyclonal antibodies against AdhE (Matuszewska *et al.*, 2008), anti-rabbit IgG horseradish peroxidase-conjugate (Sigma) and ECL detection reagents (Pierce Biotechnology). Membranes were scanned and analyzed with the 1DScan EX program (Scanalytics).

RESULTS

IbpA and IbpB inhibit inactivation of AdhE during heat shock *in vivo*

To study the role of IbpA and IbpB in AdhE protection, we first compared the activity of AdhE

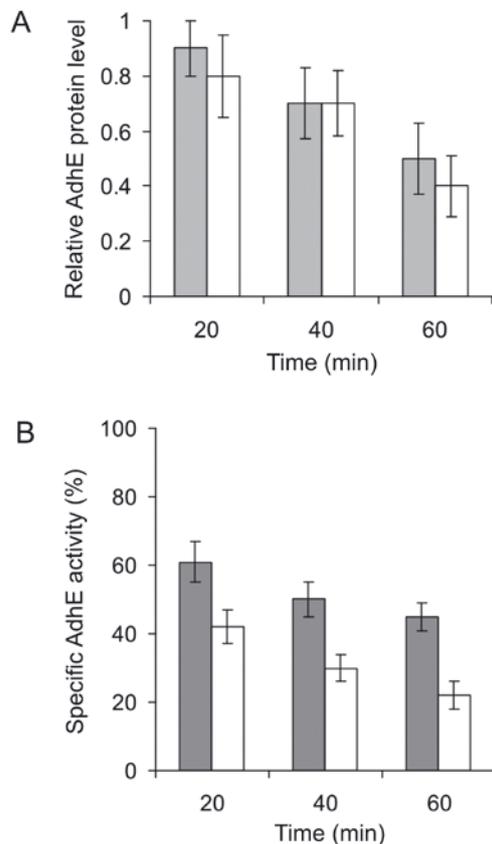


Figure 1. IbpA and IbpB proteins protect AdhE against thermal inactivation *in vivo*.

A. *E. coli* wt and $\Delta ibpAB$ strains were grown anaerobically at 30°C to an OD₅₉₅ of 0.3 and then shifted to 50°C. At the indicated time points, samples containing equal amounts of bacteria were collected, resolved by SDS/PAGE and analyzed by immunoblotting using anti-AdhE antibody. The level of AdhE was estimated by densitometry (wt, grey bars; $\Delta ibpAB$, white bars). **B.** Specific AdhE activity in wt and $\Delta ibpAB$ cell submitted to heat shock. The data correspond to the means \pm S.D. of three independent experiments.

in wild type (wt) and $\Delta ibpAB$ *E. coli* strains submitted to heat stress. To induce high-level synthesis of AdhE, the cells were grown anaerobically at 30°C. At the mid-exponential phase the cells were shifted to 50°C and incubated under aerobic conditions for 1 h. Since a decrease of AdhE activity might result from denaturation as well as degradation of the enzyme the amount of AdhE protein in bacteria was monitored by Western blotting. After 1 h at 50°C the level of AdhE protein decreased to 50% and 40% in wt and in $\Delta ibpAB$ cells, respectively (Fig. 1A). At various time points of the experiment we determined AdhE concentration by Western blotting using serial dilutions of purified AdhE as standards and anti-AdhE antibodies (not shown), measured its activity and calculated specific AdhE activity. This approach enabled us to compare the degree of AdhE inactivation in the wt and $\Delta ibpAB$ strains regardless of the rate of AdhE degradation. In non-stressed anaerobic

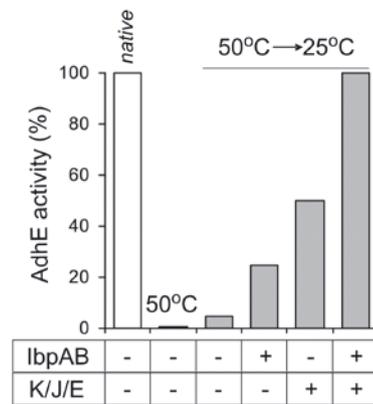


Figure 2. AdhE reactivation by the IbpA-IbpB-DnaK chaperone system.

AdhE (0.4 μ M) was inactivated in the presence of 2 μ M IbpA and 7.8 μ M IbpB or alone at 50°C for 15 min. After inactivation, the reaction mixtures were supplemented with DnaK (1.5 μ M), DnaJ (0.12 μ M) and GrpE (0.15 μ M). AdhE activity was measured in the samples after 1 h incubation at 25°C. Representative results of three independent experiments are shown.

wt and $\Delta ibpAB$ cultures 7500 \pm 300 U/mg AdhE was detected and set to 100%. We found that after 1 h of growth at 50°C specific AdhE activity decreased to 45% and 22% in wt and $\Delta ibpAB$ cells, respectively (Fig. 1B), indicating that IbpA and/or IbpB participate in the protection of AdhE against heat-inactivation.

IbpA and IbpB cooperate in the stress management with the ATP-dependent chaperones, therefore we performed *in vitro* assay to confirm that AdhE can be reactivated by the IbpA-IbpB-DnaK-DnaJ-GrpE system (Fig. 2). The amounts of IbpA (2 μ M), IbpB (7.8 μ M), DnaK (1.5 μ M), DnaJ (0.12 μ M), GrpE (0.11 μ M) optimal for reactivation, were used according to published data (Matuszewska *et al.*, 2005). AdhE was denatured at 50°C for 15 min in the absence or presence of IbpA and IbpB and subsequently the ATP-dependent DnaK/DnaJ/GrpE system was added or not. The efficiency of protection/refolding was measured after 1 h incubation at 25°C. In the presence of IbpA and IbpB alone 25% of AdhE activity was restored. When DnaK/DnaJ/GrpE were added after denaturation of AdhE in the absence of IbpA and IbpB, only 50% of the enzyme was refolded. Complete refolding was observed when the DnaK system was added to AdhE preincubated with IbpA and IbpB (Fig. 2). Therefore, IbpA and IbpB increase the efficiency of reactivation of heat-denatured AdhE by the DnaK system.

Lack of IbpA and IbpB proteins does not affect the level of aggregated AdhE under heat stress

To further confirm participation of IbpA and IbpB in AdhE protection, we analyzed heat-in-

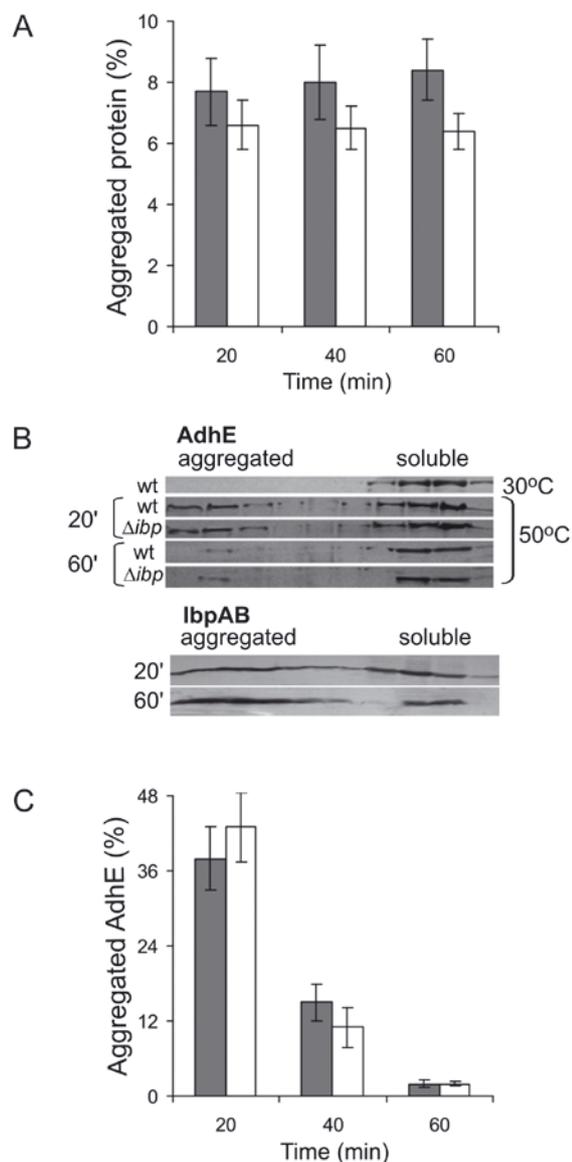


Figure 3. Aggregation of AdhE in wt and $\Delta ibpAB$ cells exposed to heat stress.

Wt and $\Delta ibpAB$ strains were grown as described in Fig. 1. To isolate aggregated proteins, the cells were harvested and fractionated by centrifugation in 30–60% sucrose gradient (see Materials and Methods) **A**. The amount of aggregated *E. coli* proteins was calculated in relation to total protein (100%). **B**. AdhE, IbpA and IbpB were detected by immunoblotting in every second fraction collected from the gradient. The panel shows localization of AdhE, IbpA and IbpB after 20' and 60' at 50°C (wt and $\Delta ibpAB$ strains). IbpA and IbpB were detected in fractions obtained from wt cells (20' and 60'). Equal volumes of each fraction were loaded on the gel. **C**. The amount of aggregated AdhE was estimated by densitometry in relation to the total amount of AdhE loaded on the gradient (100%). Wt, grey bars; $\Delta ibpAB$, white bars. Means \pm S.D. of three independent experiments are shown.

duced aggregation of AdhE by ultracentrifugation of whole cell extracts in a sucrose gradient. Since the specific AdhE activity was significantly decreased

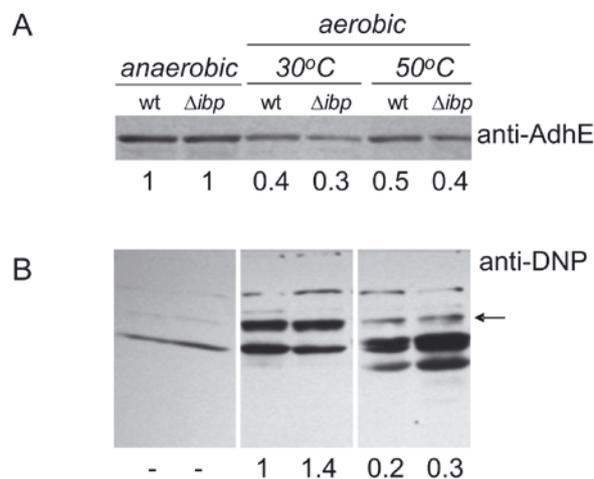


Figure 4. Carbonylation of AdhE is inhibited by IbpA and IbpB.

A. *E. coli* wt and $\Delta ibpAB$ strains were grown anaerobically at 30°C to an OD_{595} of 0.2 and then incubated aerobically at 30°C or 50°C. After 1 h the bacteria were collected and submitted to DNP derivatization. Samples containing equal amounts of protein were resolved by SDS/PAGE and analyzed by immunoblotting using anti-AdhE (**A**) or anti-DNP antibody (**B**). The levels of total AdhE (numbers below blots in panel **A**) and carbonylated AdhE (not shown) were estimated by densitometry. Subsequently, the relative levels of carbonylated AdhE were normalized to total AdhE amounts (numbers below blots in panel **B**). Representative results of three independent experiments are shown. The arrow indicates AdhE.

after heat shock in the absence of IbpA and IbpB, we expected that $\Delta ibpAB$ cells would contain a higher level of aggregated AdhE than wt cells. After 20 min at 50°C, about 8% and 6% of total cellular proteins was aggregated in wt and $\Delta ibpAB$ strains, respectively (Fig. 3A). The aggregates contained approx. 38% and 43% of total AdhE in wt and $\Delta ibpAB$ strains, respectively (Fig. 3B, C). According to previously published results (Kuczyńska-Wiśnik *et al.*, 2002), the level of aggregated cellular proteins was slightly lower in $\Delta ibpAB$ than in wt cells, and did not change significantly during 1 h incubation at 50°C in the wt or in $\Delta ibpAB$ strain (Fig. 3A). However, the amount of IbpA and IbpB proteins in the aggregates increased from about 50% (at 20th min) to 70% (at 60th min) of total IbpA and IbpB level in wt cells (Fig. 3B). Interestingly, we observed disappearance of aggregated AdhE in both strains — at the end of the experiment the aggregates contained only approx. 2% of total AdhE protein (Fig. 3C). These results indicated that, the more rapid inactivation of AdhE in cells lacking IbpA and IbpB (Fig. 1B) was not accompanied by increased aggregation of AdhE (Fig. 3C). We estimated that after 20 min at 50°C inactive AdhE was mainly sequestered in the aggregates, whereas soluble fractions contained active enzyme. For example, in wt cells the specific

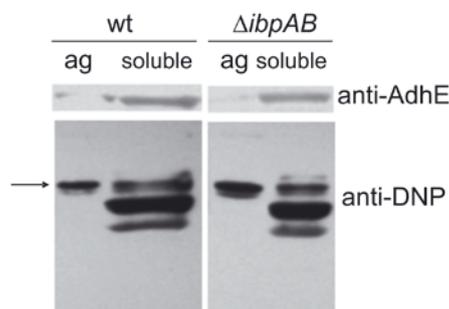


Figure 5. Carbonylation of aggregated and soluble AdhE in *E. coli* wt and $\Delta ibpAB$ cells.

The cultures were grown as described in Fig. 1. After 1 h incubation at 50°C cell extracts were prepared and subjected to fractionation (see Materials and Methods). Insoluble (ag) and soluble fractions, each containing 20 μ g protein, were resolved by 12% SDS/PAGE, subjected to immunodetection using anti-AdhE and anti-DNP sera. The data are representative of three experiments. The arrow indicates AdhE.

AdhE activity was decreased by approx. 39% (Fig. 1B) and concomitantly approx. 38% of total AdhE was aggregated (Fig. 3C). At the end of the experiment (60 min) the vast majority of heat-inactivated enzyme in wt cells was soluble: the specific AdhE activity was reduced by approx. 55% (Fig. 1B) but only approx. 2% of the enzyme was detected in the aggregates (Fig. 3C).

IbpA and IbpB inhibit oxidation of AdhE under heat stress

In *E. coli* cells exposed to heat stress in the presence of oxygen, inactivation of AdhE results from heat-denaturation and from MCO. Since we have previously found that IbpA and IbpB protect AdhE against MCO under oxidative stress induced by copper ions (Matuszewska *et al.*, 2008), we examined the influence of IbpA and IbpB on AdhE oxidation at 50°C. Oxidation of AdhE in wt and $\Delta ibpAB$ cells was analyzed by immunodetection of carbonyl derivatives (see Materials and Methods). The intensities of bands corresponding to carbonylated AdhE in wt and $\Delta ibpAB$ cells were comparable (Fig. 4B). However, the level of total AdhE in the $\Delta ibpAB$ mutant was lower than that in wt cells (Fig. 4A), therefore the relative levels of carbonylated AdhE at 30°C and 50°C were higher in $\Delta ibpAB$ cells. In both strains, the relative levels of oxidized AdhE at 50°C were apparently lower than those detected at 30°C (Fig. 3B). It might result from the protection of AdhE against carbonylation by IbpA, IbpB and DnaK (Echave *et al.*, 2002; Matuszewska *et al.*, 2008), whose overproduction was induced by heat shock. Next, we compared the degree of carbonylation of aggregated and soluble AdhE (Fig. 5). After

60 min at 50°C, both fractions, insoluble and soluble, contained carbonylated AdhE. We observed a strong signal corresponding to oxidized AdhE in the aggregates, although the fraction of aggregated proteins contained only trace amounts of AdhE (Fig. 5). Therefore, the aggregates were enriched in oxidized AdhE. In $\Delta ibpAB$ cells, the level of carbonylated AdhE detected in the aggregates was at least 2-fold higher than in the wt strain. These results confirmed that IbpA and IbpB inhibited oxidation of AdhE.

DISCUSSION

Results presented in this report provide new information on the role of IbpA and IbpB in protection of *E. coli* against heat and oxidative stress. We followed the fate of AdhE, a natural IbpA and IbpB substrate, in *E. coli* cells submitted to 50°C in the presence of oxygen. One can imagine that protein aggregates formed under heat stress are dynamic structures: unfolded proteins are constantly incorporated into aggregates but other denatured polypeptides are extracted from the aggregates and refolded or degraded. Therefore, an almost constant level of aggregated cellular proteins may be detected under certain conditions (Fig. 1A) but the composition of the aggregates can be variable. We found that heat-inactivated AdhE was initially sequestered in the aggregates and almost completely removed from them during further incubation at high temperature (Fig. 3). It should be noted that newly synthesized proteins are prone to aggregation, whereas those with native conformation are resistant (Mitraki & King, 1989). Therefore, it seems that just after the shift from 30°C (anaerobic conditions) to 50°C (aerobic conditions) newly synthesized, but not native AdhE was denatured and sequestered into aggregates. Since AdhE synthesis is inhibited after a shift from anaerobic to aerobic conditions (Clark & Cronan, 1980) we assume that further incubation at 50°C (40–60') did not cause significant incorporation of additional AdhE molecules into aggregates. We observed, however, progressive degradation (Fig. 1A) and inactivation of AdhE (Fig. 1B) which remained in a soluble form. It is tempting to speculate that during prolonged incubation at 50°C inactive but still soluble AdhE is immediately degraded. It is also possible that unfolded AdhE is maintained in a soluble form by molecular chaperones which are ineffective in protecting or reactivating AdhE due to the severity of the stress and overloading of the chaperone systems with unfolded substrates.

We are not able to distinguish whether aggregated AdhE was renatured or removed by proteolytic degradation. The aggregates contained very high amounts of irreversibly carbonylated AdhE when

compared to the soluble fraction (Fig. 5). This result is consistent with the observation that misfolded proteins are more susceptible to carbonylation than their native counterparts (Nyström, 2005). It is also proposed that carbonyl groups signal that the protein is irreversibly damaged and mark the protein for degradation (Dalle-Donne *et al.*, 2006). Therefore, we suppose that removal of aggregated AdhE relied mainly on proteolytic degradation. Consistently, aggregated non-carbonylated AdhE, which was denatured at 50°C under anaerobic conditions, was more stable than that denatured and aggregated in the presence of oxygen (J. Kwiatkowska, unpublished).

In cells lacking IbpA and IbpB, inactivation of AdhE proceeded faster (Fig. 1B) but was not accompanied by increased aggregation of the enzyme (Fig. 3C), as could be expected from earlier studies (Kuczyńska-Wiśnik *et al.*, 2002). Moreover, the $\Delta ibpAB$ mutation did not affect the removal of aggregated AdhE. This observation seems contradictory to previous results demonstrating that IbpA and/or IbpB are necessary for efficient removal of unfolded proteins from aggregates (Kuczyńska-Wiśnik *et al.*, 2002; Mogk *et al.*, 2003a, Jiao *et al.*, 2005). However, in those studies the disappearance of aggregated proteins was observed at recovery temperatures (25°C, 37°C), whereas in our experiments aggregated AdhE was removed in *E. coli* cells exposed to 50°C. It is possible that IbpA and/or IbpB are not required for the extraction of aggregated substrates subjected to irreversible oxidative damage and tagged for degradation. Nevertheless, IbpA and IbpB sequestered in the aggregates may cooperate with the DnaK system and facilitate refolding of aggregated but non-oxidized proteins. It is worth noting that AdhE was the only carbonylated protein detected in the aggregates (Fig. 5). Our results indicate that IbpA and IbpB protect AdhE against heat inactivation, providing that the enzyme remains soluble. We found that during prolonged heat stress the IbpA and IbpB proteins are relocated in wt cells from the soluble to insoluble fraction (Fig. 3B) and a deficiency of IbpA and/or IbpB in the soluble fraction may result in increased inactivation of AdhE.

This work provides further evidence that IbpA and IbpB protect proteins against thermal oxidation (Figs. 4 and 5). These results are consistent with previous reports demonstrating that IbpA and IbpB enhance *E. coli* resistance to superoxide stress and suppress inactivation of selected enzymes by hydrogen peroxide and potassium superoxide *in vitro* (Kitagawa *et al.*, 2000; 2002). It was also found that the $\Delta ibpAB$ strain exhibits increased sensitivity to superoxide radicals generated by tellurite (Perez *et al.*, 2007). In our previous work we demonstrated that IbpA and IbpB protect *E. coli* cells against oxidative stress induced by Cu²⁺ (Matuszewska *et al.*,

2008). The role of other Hsps under oxidative stress has also been documented. Echave and coworkers (2002) found that DnaK prevents MCO of AdhE. It was also reported that overproduction of Hsps reduced stasis-induced carbonylation of mistranslated proteins (Fredriksson *et al.*, 2005; 2007). Cells exposed to high temperature in the presence of oxygen are also challenged by oxidative stress (Benov & Fridovich, 1995), it is therefore reasonable that Hsps possess additional anti-oxidant activities.

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