Acta Biochimica Polonica

Vol. 51 No. 4/2004

925–931

QUARTERLY

Escherichia coli small heat shock proteins IbpA/B enhance activity of enzymes sequestered in inclusion bodies✪

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Received: 09 July, 2004; revised: 15 October, 2004; accepted: 24 October, 2004

Key words: IbpA/B, inclusion bodies, protein aggregation

Escherichia coli **small heat shock proteins, IbpA/B, function as molecular chaperones and protect misfolded proteins against irreversible aggregation. IbpA/B are induced during overproduction of recombinant proteins and bind to inclusion bodies in *E. coli* cells. We investigated the effect of Δ*ibpA/B* mutation on formation of inclusion bodies and biological activity of enzymes sequestered in the aggregates in *E. coli* cells. Using three different recombinant proteins: Cro-β-galactosidase, β-lactamase and rat rHtrA1 we demonstrated that deletion of the *ibpA/B* operon did not affect the level of produced inclusion bodies. However, in aggregates containing IbpA/B a higher enzymatic activity was detected than in the IbpA/B-deficient inclusion bodies. These results confirm that IbpA/B protect misfolded proteins from inactivation *in vivo*.**

The IbpA and IbpB proteins were first identified as a component of inclusion bodies (IBs) formed in *E. coli* cells overproducing heterologous proteins (Allen *et al.*, 1992). Later, it was found that IbpA/B coaggregated with endogenous *E. coli* proteins, heat denatured (Laskowska *et al.*, 1996) or misfolded upon folate stress (Laskowska *et al.*, 2003). IbpA and IbpB show 50% homology of amino acid sequence and belong to the family of small heat shock proteins (sHsp) widely distributed among prokaryotes and eukaryotes. The sHsp are characterized by their low molecular mass (12–35 kDa), oligomeric structure and conserved COOH-terminal "α-crystallin" domain. α-Crystallins are vertebrate

✪This work was supported by grant No. 0461/P04/2001/20 from the State Committee for Scientific Research.

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Abbreviations: Cro-β-gal, Cro-β-galactosidase; IBs, inclusion bodies; Ibp, inclusion bodies protein; IPTG, isopropyl β-D-thiogalactoside; sHsp, small heat shock protein.

sHsps which prevent formation of cataract caused by protein aggregation in the eye lens (Narberhaus, 2000). Several *in vitro* experiments have revealed that sHsp function as ATP-independent molecular chaperones and protect unfolded proteins from irreversible aggregation. Substrates bound to sHsp preserve their "folding-competent state" and are delivered for subsequent refolding to the Hsp70/Hsp40 ATP-dependent chaperones (Ehrnsperger *et al.,* 1997; Veinger *et al.,* 1998; Lee & Vierling, 2000; Kitagawa *et al.*, 2002; Mogk *et al.*, 2003b). In *E. coli* cells IbpA/B cooperate with the ClpB-DnaK/DnaJ/GrpE bi-chaperone system in reversing protein aggregation. *In vitro* the DnaKJE system can dissociate soluble sHsp–substrate complexes, whereas ClpB is additionaly required for disaggregation of large, insoluble complexes (Mogk *et al.*, 2003a). According to a model of IbpA/B cooperation with DnaKJE, overproduction of IbpA and/or IbpB in *E. coli* cells stabilizes aggregates of denatured proteins (Kuczyńska-Wiśnik *et al.*, 2002). *In vivo* experiments have revealed that IbpA/B are dispensable during mild stress conditions (45°C) but are required for removal of misfolded proteins upon extreme, long-term heat conditions (50°C). Deletion of the *ibpA/B* operon results in a twofold increase of aggregated proteins and a 10-fold decrease of cell viability under the extreme conditions (Kuczyńska-Wiśnik *et al.*, 2002).

Upon high level production, recombinant proteins may aggregate and form inclusion bodies in *E. coli* cells. IB formation results from a limiting availability of molecular chaperones, which are required for protein folding. Proteins aggregate because hydrophobic patches exposed by folding intermediates are not sufficiently protected by chaperones against nonspecific interactions (Carrió & Villaverde, 2002). The formation of IBs can be an advantage during protein purification, as the IBs contain mainly the recombinant protein and are easily isolated by centrifugation. On the other hand, to recover biologically active proteins from IB, additional steps in purification procedure are required: solubilization of IBs by strong denaturants (6 M guanidinum hydrochloride, GuHCl, or 6–8 M urea) and subsequent refolding of desired proteins. It is possible to avoid IBs formation and increase the level of soluble recombinant protein by co-overproduction of the molecular chaperone systems DnaK-DnaJ or GroEL-GroES (Baneyx, 1999). However, it is important to point out that the formation of IBs and the effect of chaperones' coexpression on recombinant protein solubility depends on the nature of the overproduced protein. IBs have a dynamic structure: protein release and refolding occur simultaneously with protein aggregation (Carrió & Villaverde, 2002). Polypeptide chains forming IBs have various conformations, including entirely misfolded proteins and proteins with a high level of secondary structure. Moreover, even properly folded polypeptides can be trapped in IBs. Thus, in IBs formed by enzymes, biological activity can be detected (Carrió & Villaverde, 2002).

In this paper we investigated the influence of IbpA/B on the formation of IB in *E. coli* cells overproducing recombinant proteins. We were particularly interested whether the presence of IbpA/B affects the activity of enzymes in IBs.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. For Cro-β-galactosidase (Cro-β-gal) production, MC4100 [*araD139* Δ*(lacIPOZYA argF)U169 fla relA rpsL*] and MC4100 Δ*ibpA/B* strains (Kuczyńska-Wiśnik *et al.*, 2002) were transformed with pUEX3 plasmid. The plasmid contains a λ *cro-E. coli lacZ* gene fusion under control of the λ P_R promoter (Bressan & Stanley, 1987). RB791 (*flbB5301, deoC1, ptsF25, rbsR*) and RB791 Δ*ibpA/B* cells transformed with pGB1 or pJG108 were used for cytoplasmic β-lactamase or peri-

plasmic OmpA-β-lactamase synthesis, respectively (Bowden *et al.*, 1991). Recombinant rHtrA1 was overproduced in the BL21 (DE3) and BL21(DE3) Δ*ibpA/B* strains containing pET-rHtrA1 plasmid. The bacteria were grown at 30°C in LB medium supplemented with appropriate antibiotics: 100 μg/ ml ampicillin (strains transformed with pUEX3 or pJG108), 50 μg/ml neomycin (pGB1) or 30 μg/ml kanamycin (pET- rHtrA1). To induce overproduction of Cro-β-galactosidase bacteria were shifted to 45°C at exponential phase. For overproduction of β-lactamase and rHtrA1, isopropyl β-D-thiogalactoside (IPTG) (1 mM) was added to the exponentially growing cultures.

Construction of the pET-rHtrA1 plasmid. Total RNA was extracted from kidney of Wistar rats using RNeasy Isolation kit (Qiagen). RNA was reverse transcribed using MuLV Reverse Transcriptase, according to the supplier's protocol (Fermentas). cDNA encoding rat *HtrA1* gene fragment was amplified in polymerase chain reactions using REDTaq Polymerase (Sigma-Aldrich). The primers were based on the nucleotide sequence of *ratHtrA1* (GenBank^{TM} Accession No. AF179370): 5′cggaattcatatgaatggagcgacttatgaagcc 3′, 5′caggatcctcctgcctctgcctaggg 3′. The PCR product flanked by sequences containing *Nde*I and *Bam*HI restriction sites was inserted into pET28b+ vector (Novagen) cut with *Nde*I and *Bam*HI. The resulting pET-rHtrA1 plasmid contains most of the *rHtrA1* gene (1041 bp) and codes for a fusion protein which consist of a vector-encoded fragment (20 amino acids) with a hexahistidine tag and of 337 amino-acids from the COOH-terminal part of rHtrA1.

Purification of inclusion bodies. IB with Cro-β-gal were purified according to Sambrook *et al.* (1989). Briefly, *E. coli* cells were collected by centrifugation and lysed by sonication in the presence of lysozyme, 0.5% deoxycholic acid and 1% Triton X-100. After incubation of the extract with DNase I, IBs were pelleted and washed with 1% Triton X-100. β-Lactamase aggregates were isolated by a method similar to that described in Bowden *et al.* (1991). IBs containing rHtrA1 were isolated with CellLytic B — bacterial cell lysis extraction reagent (Sigma) according to the supplied protocol. For the determination of enzymatic activities the IBs were suspended in 50 mM Tris/HCl, pH 8.0.

Analytical methods. Protein was determined according to Bradford (1976), with bovine serum albumin as a standard. SDS/PAGE and Western blotting were performed according to Sambrook *et al.* (1989). Polyclonal rabbit antiserum against IbpA/B, anti-rabbit peroxidase conjugate (Sigma) and substrates: 4-chloro-1-naphtol and H_2O_2 (Sigma) were used for IbpA/B immunodetection. The activities of Cro-β-gal and β-lactamase were determined spectrophotometrically using 2-nitrophenyl β-D-galactopyranoside and penicillin G as substrates, respectively (Sambrook *et al.*, 1989; Meerman & Georgiou, 1994). To determine rHtrA1 proteolytic activity β-casein was used as a substrate. Products of β-casein digestion were resolved by SDS/12% PAGE and their amounts were estimated by densitometry (Sigma Gel program) after Coomassie staining.

RESULTS AND DISCUSSION

The Δ*ibpA/B* mutation does not influence the level of IBs formed in *E. coli* cells

The level of IbpA/B induced by IBs and IbpA/B distribution between IBs and the soluble fraction depend on the nature of recombinant protein (Rinas, 1996; Hoffmann & Rinas, 2000; Jürgen *et al.*, 2000). To obtain more general conclusions on the IbpA/B function we used in our experiments three different systems producing cytoplasmic IBs: Cro-β-gal, β-lactamase and rHtrA1. Cro-β-gal (116 kDa) overexpression was induced by heat shock (45°C) from a plasmid containing the λ-cro-*E. coli lacZ* gene fusion (Bressan &

Stanley, 1987). β-Lactamase (29 kDa) used in this study was devoid of the signal sequence and formed cytoplasmic IBs (Bowden *et al.*, 1991). In rat HtrA1 protein the N-terminal sequence containing 143 amino acids has been replaced by a vector-encoded fragment of 20 amino acids with a hexahistidine tag (see Materials and Methods). Similarly to the *E. coli* HtrA homolog, the rat HtrA fusion protein retained autoproteolytic activity, hence a shorter polypeptide (about 35 kDa) was present in the IBs apart from the full-length fusion protein (40 kDa) (Fig. 1C). IBs containing β-lactamase or rHtrA1 were produced at 30°C, after IPTG induction (Fig. 1B, C). The Δ*ibpA/B* mutation did not affect the synthesis of the recombinant proteins, since we could not observe any significant differences between the amounts of overproduced Cro-β-gal, β-lactamase or rHtrA1 in the wild type (wt) and Δ*ibpA/B* strains (Fig. 1A, B, C). Assuming that IbpA/B protect recombinant proteins from aggregation, we expected that increased amounts of IBs would be detected in the Δ*ibpA/B* mutant. However, when we isolated IBs from *E. coli* wt and Δ*ibpA/B* cells (Fig. 2A) we found that the level of IBs was similar in both strains. Only after prolonged production of the recombinant proteins was a slightly increased accumulation of IBs evident in the Δ*ibpA/B* mutant (Fig. 2A). For instance, the rHtrA1 IB constituted 19% and 23% of total protein in wt and Δ*ibpA/B* cells, respectively. Thus, the influence of the IbpA/B proteins on IBs formation was minor. However, we cannot exclude that in the case of other recombinant proteins more severe effects of the Δ*ibpA/B* mutation might be observed. Carrió and Villaverde (2002) found that in Δ*ibpA/B* cells a lower amount of β-galactosidase fusion protein VP1LAC was accumulated in IB comparing to a wt strain. They suggested that in the absence of IbpA/B VP1LAC was degraded before its aggregation.

Overproduction of Cro-β-gal, β-lactamase or rHtrA1 induced synthesis of IbpA/B which coaggregated with the IBs (Fig. 3). Since the synthesis of Cro-β-gal was induced by heat shock, the amount of IbpA/B bound to Cro-β-gal was higher than in the other IBs and was visible on gels stained with Coomassie (Fig. 2B). In the case of each recombinant protein the level of IbpA/B in *E. coli* cells increased in good correlation with the growth of IBs during the course of the experiment (not shown). Only a very low amount of IbpA/B could be detected in cells overproducing OmpA'-β-lactamase, a fusion protein with the leader peptide of the outer membrane pro-

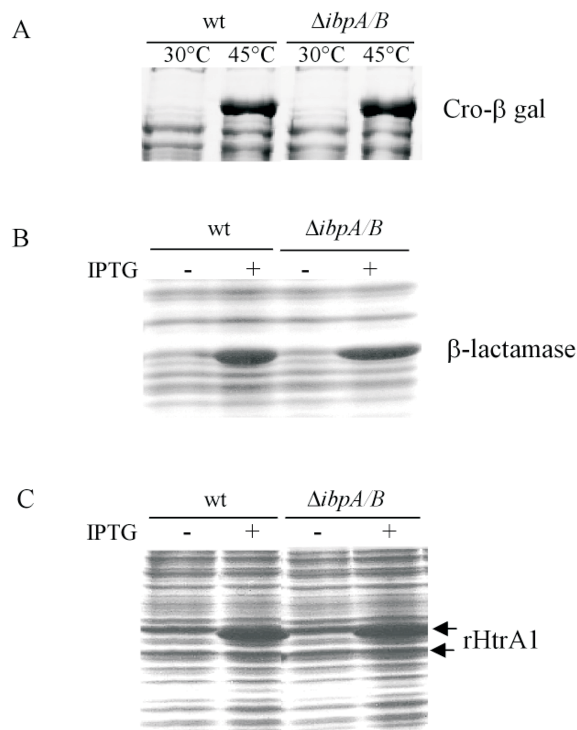

Figure 1. The Δ*ibpA/B* mutation does not affect levels of overproduced recombinant proteins in *E. coli* cells.

Whole cell extracts of *E. coli* overproducing the recombinant proteins: Cro-β-gal, β-lactamase or rHtrA1 were analyzed by SDS/12% PAGE. Samples corresponding to the same amount of bacteria were taken from exponentially growing cultures before and 30 min after the temperature shift from 30°C to 45°C (panel A) and 2 h or 1 h after IPTG (1 mM) was added (panel B and C, respectively). The gels were stained with Coomassie brilliant blue.

tein (OmpA) instead of its native signal sequence (results not shown). It is known that OmpA'-β-lactamase does not aggregate in the cytoplasm but forms IBs in the periplasmic space (Bowden *et al.,* 1991). Therefore, in

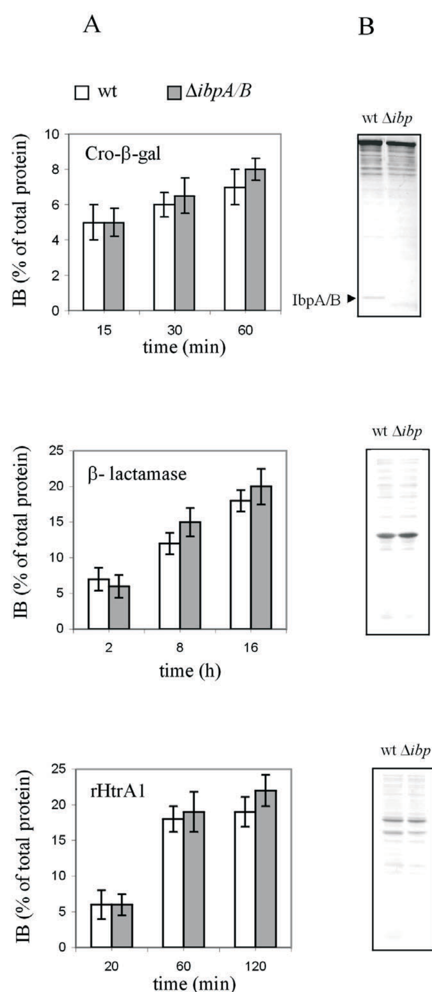

Figure 2. Production of IBs in wt and Δ*ibpA/B* cells.

A. IBs were isolated from *E. coli* cells after induction of recombinant protein synthesis by heat shock or 1 mM IPTG (see Materials and Methods) at the times indicated on the Figure. The amounts of the IBs were calculated in relation to the total protein in bacterial extracts (100%). Error bars represent the standard deviation of three values. **B.** Purified IBs (5 μg) were resolved in SDS/12% polyacrylamide gels and stained with Coomassie brilliant blue.

E. coli cells producing the recombinant protein IbpA/B were not induced, providing that the overproduced protein was soluble in the cytoplasm. Our results are in good agreement with the data presented by Hoffmann and Rinas (2000) who analyzed the kinetics of the heat shock response in *E. coli* cells producing human basic fibroblast growth factor (hFGF-2). They found that just after induction hFGF-2 formed IBs and then IbpA/B were remarkably induced but during further synthesis a soluble recombinant protein accumulated and the IbpA/B proteins disappeared from the cells.

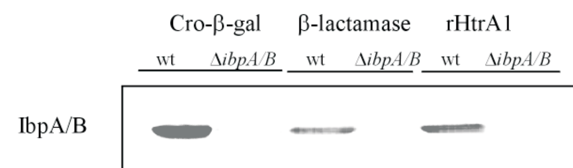

Figure 3. The IbpA/B proteins coaggregate with IBs.

IBs (5 μg) purified from *E. coli* wt and Δ*ibpA/B* strains overproducing: Cro-β-gal, β-lactamase or rHtrA1 were subjected to SDS/15% PAGE and Western blotting with anti-IbpA/B serum.

IbpA/B proteins enhance enzymatic activity of recombinant proteins in IBs

Since IbpA/B protected denatured proteins from irreversible aggregation and inactivation *in vitro* (Ehrnsperger *et al.,* 1997; Veinger *et al.,* 1998; Lee & Vierling, 2000; Kitagawa *et al.*, 2002), we supposed that more active polypeptide chains could be present in IBs isolated from wt cells compared to the IBs from the Δ*ibpA/B* mutant. We did not detect any activity of β-galactosidase in Cro-β-gal IBs. We believe that the long polypeptide chains (116 kDa) which aggregated upon heat stress could not maintain any native structure. We were able to detect enzymatic activities of both β-lactamase (29 kDa) and rHtrA1 (40 kDa) in the IBs formed at low temperature (30°C). The activities were very low — in the case of rHtrA1 overnight incubation of IBs (10 μg) with β-casein (20 μg) at 37°C was necessary to observe degradation of the substrate. Nevertheless, the differences in the enzymatic activity of the IBs from wt and mu-

tant cells were remarkable (Fig. 4). In IbpA/B-deficient aggregates the activity of β-lactamase or rHtrA1 was about 40% or about 43% lower, respectively, than in IBs containing IbpA/B. In a control experiment, after incubation of IBs in a reaction buffer without substrates and IBs re-separation, we did not detect any protein or enzymatic activity in the supernatant (not shown). Thus, the active polypeptides were present only in the

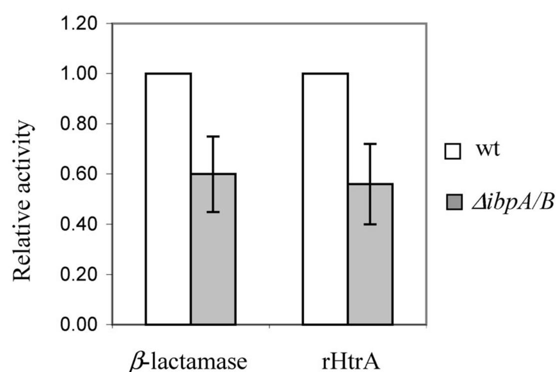

Figure 4. Enzymatic activity in IBs is decreased in the absence of IbpA/B.

β-Lactamase activity was measured with Penicillin G as a substrate in IBs isolated from *E. coli* cells 2 h after IPTG induction of the recombinant protein. β-Casein was used as rHtrA1 substrate and the rHtrA1 proteolytic activity was determined in IBs isolated 1 h after IPTG induction. Error bars represent the standard deviation of three values.

IBs and not released into solution. These results support our hypothesis that the IbpA/B proteins inhibit inactivation of enzymes sequestered in IBs. Most of the evidence proving that IbpA/B protect proteins against irreversible aggregation and facilitate refolding of bound enzymes by the ATP-dependent system DnaK/DnaJ/GrpE was provided by experiments in which model substrates were denatured and formed complexes with sHsp *in vitro*. In this paper we have demonstrated, for the first time, that the protective function of IbpA/B can be observed in IbpA/B-substrate complexes formed *in vivo*. Interestingly, IbpA/B-deficient IBs were more efficiently solubilized in 8 M urea or 6 M GuHCl than wt IBs (not shown). This suggests that in the absence of IbpA/B the interactions between polypeptides in IBs are weaker and more easily disrupted by denaturants. These data are in agreement with the results presented by Mogk *et al.* (2003b) who proved that sHsp-substrate complexes are very stable and do not release polypeptides spontaneously. It is tempting to speculate that IbpA/B bound to IBs fulfill a dual function by (1) protecting misfolded proteins from irreversible inactivation and facilitating their refolding; (2) preventing unspecific interactions between misfolded proteins (captured in IBs) with properly folded ones in *E. coli* cells.

We are grateful to Dr. G. Georgiou (University of Texas, U.S.A.) for the pGB2 and pJG108 plasmids.

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