Chaperone Properties of the Bacterial Periplasmic Substrate-binding Proteins*

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Bacterial periplasmic substrate-binding proteins are initial receptors in the process of active transport across cell membranes and/or chemotaxis. Each of them binds a specific substrate (e.g. sugar, amino acid, or ion) with high affinity. For transport, each binding protein interacts with a cognate membrane complex consisting of two hydrophobic proteins and two subunits of a hydrophilic ATPase. For chemotaxis, binding proteins interact with specific membrane chemotaxis receptors.

We report, herewith, that the oligopeptide-binding protein MglB of *Escherichia coli*, the maltose-binding protein MalE of *E. coli*, and the galactose-binding protein MglB of *Salmonella typhimurium* interact with unfolded and denatured proteins, such as the molecular chaperones that are involved in protein folding and protein renaturation after stress. These periplasmic substrate-binding proteins promote the functional folding of citrate synthase and α-glucosidase after urea denaturation. They prevent the aggregation of citrate synthase under heat shock conditions, and they form stable complexes with several unfolded proteins, such as reduced carboxymethyl α-lactalbumin and unfolded bovine pancreatic trypsin inhibitor. These chaperone-like functions are displayed by both the liganded and ligand-free forms of binding proteins, and they occur at binding protein concentrations that are 10–100-fold lower than their periplasmic concentration. These results suggest that bacterial periplasmic substrate-binding proteins, in addition to their function in transport and chemotaxis, might be implicated in protein folding and protection from stress in the periplasm.

The periplasmic binding proteins of Gram-negative bacteria constitute a set of at least 30 proteins that are involved in the transport of, and chemotaxis toward, substrates (reviewed in Refs. 1–3). These proteins bind a variety of ligands, such as sugars, amino acids, peptides, ions, and vitamins with submimolar dissociation constants (1–3). For transport, each periplasmic binding protein interacts with, and delivers its ligand to, a membrane complex comprising one or two integral inner membrane proteins (4–6) and one or two subunits of an energy-transducing ATPase (7–10). For chemotaxis, each binding protein interacts with an inner membrane receptor that transmits the chemotactic signal to the flagellar apparatus via a cAMP receptor protein. For transport, each binding protein interacts with a cognate membrane complex consisting of two hydrophobic proteins and two subunits of a hydrophilic ATPase. For chemotaxis, binding proteins interact with specific membrane chemotaxis receptors.

The periplasmic binding proteins of *E. coli* contain maltose-binding protein MalE and maltose receptor Trg, and with a transport complex consisting of the MglC inner membrane protein and the MglA ATPase (13). The polypeptide chain of most binding proteins folds in two globular domains (14). Their specific ligand binds in a cleft between the two globular domains, which are connected by a movable hinge, and upon ligand binding, the two lobes move close to each other and enclose the ligand (15). Interaction of binding proteins with transport and chemotaxis membrane proteins probably involves the rim of the substrate-binding cleft, opposite the hinge region (16, 17). Some of the binding proteins interact with the porins of the outer membrane (18). The amount of each binding protein in the periplasm exceeds by approximately 50-fold that of cognate membrane components (reaching a concentration of 1 mM for MglB or OppA) and seems to be in excess of the amount required for maximal transport and chemotaxis activities (1).

Molecular chaperones form a class of polypeptide-binding proteins that are implicated in protein folding, protein targeting to membranes, protein renaturation or degradation after stress, and control of protein-protein interactions. They can distinguish native proteins from their non-native forms, owing to the specificity of their peptide binding site, and they catalyze protein folding and renaturation in vitro (reviewed in Refs. 19–21). The major classes of bacterial chaperones comprise DnaK/Hsp70 (and its assistant proteins DnaJ and GrpE), GroEL/Hsp60 (and its assistant protein GroES), HtpG/Hsp90, and the small heat shock proteins (19–21). Most of the known bacterial molecular chaperones are located in the cytoplasm. The bacterial periplasm contains specialized chaperones implicated in pilus biogenesis, such as PapD (22), but little is known about the existence of general chaperones in this compartment (23). In the present study, we show that the periplasmic substrate-binding proteins, in a manner similar to that of molecular chaperones, increase the refolding of unfolded proteins, protect proteins against thermal denaturation, and form complexes with unfolded proteins. We propose that, in addition to their role in transport and chemotaxis, these overabundant proteins might help in protein folding and renaturation in the periplasm.

EXPERIMENTAL PROCEDURES

Purification of MglB, MalE, OppA, DnaK, and DnaJ—MglB and MalE were purified as described previously (24). OppA was prepared from *E. coli* strain RH 7742 grown overnight in rich medium. The spheroplast supernatant from 10 g of bacteria was prepared as described by Higgins and Staros (25), loaded onto a DE52-Sephacel column (20-ml bed volume, Pharmacia Biotech Inc.) equilibrated in 20 mM Tris-HCl, pH 8, 1 mM EDTA, and eluted with a linear gradient of 0 to 0.3 M NaCl in the same buffer. The active fractions were loaded onto a hydroxyapatite column (6-ml bed volume, Bio-Rad) in the same buffer, and the column was eluted with a linear gradient of 0 to 0.3 M sodium phosphate, pH 8, in the same buffer. OppA was identified by its amino-terminal sequence (26). Binding proteins were deprived of their endogenous bound ligand as described previously (24). DnaK and DnaJ were prepared, as described previously (27–29), from an overproducing
strain of E. coli bearing plasmid pLNA2 derived from plasmid pDM38 (30) (a gift from Dr. O. Fayet, Microbiologie et Génétique Microbienne CNRS, Toulouse, France).

Refolding of Citrate Synthase and α-Glucosidase—Citrate synthase was denatured at a concentration of 20 μM in 8 M urea, 50 mM Tris-HCl, 2 mM EDTA, 20 mM dithiorthiolethyl, pH 8.0, at 20 °C. Renaturation was initiated by a 50-fold dilution in 40 mM Hpes, 20 mM KOH, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM potassium acetate, pH 8.0, at 30 °C. The enzymatic activity of citrate synthase was measured as described by Jakob et al. (31). α-Glucosidase was denatured at a concentration of 15 μM in 8 M urea, 0.1 M potassium phosphate, 2 mM EDTA, 20 mM dithiorthiolethyl, pH 7.0, at 20 °C. Renaturation was initiated by a 50-fold dilution in 40 mM Hpes-KOH, pH 7.5, at 20 °C. The enzymatic activity of α-glucosidase was measured as described by Jakob et al. (31).

Thermal Aggregation of Citrate Synthase—The native enzyme (80 μM) was diluted 100-fold in 40 mM Hpes, 20 mM KOH, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM potassium acetate, pH 8.0, at 43 °C, and its aggregation was monitored by measuring the absorbance at 650 nm as described by Jakob et al. (31).

Size Exclusion Chromatography—For binding assays, gel permeation columns (Bio-Gel P-200 from Bio-Rad, or Sephadex G-75 from Pharmacia, 300-m bed volume) were equilibrated with 50 mM Tris-HCl pH 8, 50 mM KCl, 1 mM dithiorthiolethiol, 100 μM/ml bovine serum albumin. Reaction mixtures containing binding protein (OppA, MglB, and MalE) or chaperone (DnaK and DnaJ) and radiolabeled unfolded BPTI1 or radiolabeled R-CMLA at indicated concentrations were incubated for 30 min at 23 °C in the same buffer, without serum albumin, and applied to the column at room temperature. Fractions were collected at a flow rate of 1 drop/fraction/15 s, and counted for radioactivity. DnaK was incubated for 3 h at 37 °C before use. Unfolded BPTI was prepared as described previously from native BPTI obtained from Sigma (32), and R-CMLA was obtained from Sigma. Unfolded BPTI, native BPTI, and R-CMLA were 1H-labeled by reductive methylation (33).

Radiolabeling, Immunoprecipitation, and Electrophoresis—One-milliliter samples of cells (E. coli K-12, strain C600 [leuB6 thi-1 thr-1 supE44]) growing exponentially at 30 °C in glyceral medium and then transferred to 43 °C, were pulse-labeled with 60 μCi/ml (32)methionine (Amersham Corp., 1000 Ci/mmol), chased with nonradioactive l-methionine (200 μg/ml), and precipitated with trichloroacetic acid as described previously (34). Each binding protein was immunoprecipitated with its specific antiseraum, resolved by electrophoresis on 12% SDS-polyacrylamide, detected, and quantified with a Molecular Dynamics PhosphorImager. Antiserum against MglB, MalE, and OppA were prepared in rabbits by immunization with 200 μg of purified protein in Freund’s complete adjuvant, followed by a booster immunization containing 100 μg of protein in Freund’s incomplete adjuvant 3 weeks later; bleeding was performed 10 days after the last injection.

Materials—Citrate synthase, α-glucosidase, BPTI, R-CMLA, bovine serum albumin, ovalbumin, lysozyme, and all other chemicals were from Sigma and were reagent grade. The tripeptides Ala₃, Ser₃, and Lys₃ were from Bachem Feinchemikalien AG.

RESULTS

Periplasmic Binding Proteins Increase the Amount of Correctly Folded Citrate Synthase and α-Glucosidase—We first investigated whether OppA, MglB, and MalE act as molecular chaperones in the folding of proteins. Citrate synthase and α-glucosidase, whose refolding is facilitated by several chaperones, such as GroEL, Hsp90, and small Hsps (31, 35, 36), were chosen as substrates for this reaction. They were unfolded in the presence of 8 M urea and allowed to refold upon dilution of the denaturant, in the absence of added proteins or in the presence of the periplasmic binding proteins OppA, MglB, and MalE (protein folding in the presence of DnaK, DnaJ, bovine serum albumin, ovalbumin, and lysozyme was comparatively studied). Under our experimental conditions, the refolding yield of 0.4 μM citrate synthase was increased from 4%, in the absence of added proteins, to 12%, in the presence of 4 μM OppA, and 22%, in the presence of 4 μM DnaK (Fig. 1A). The dependence of citrate synthase reactivation on the concentrations of added proteins is shown in Fig. 1B (OppA, MglB, and MalE), 1C (DnaK and DnaJ), and 1D (bovine serum albumin, lysozyme, and ovalbumin). Maximal recovery of enzyme activity was obtained with DnaK (22%); serum albumin (20%); OppA, MglB, and DnaJ (around 13%); followed by ovalbumin (7%); lysozyme (5%); and buffer (5%) (the positive effect of serum albumin on protein folding has already been reported by others (37)). Half-maximal reactivation of citrate synthase occurred at relatively similar concentrations of DnaK, DnaJ, OppA, MglB, MalE, or serum albumin (1–5 μM). These concentrations are slightly higher than that of citrate synthase (0.4 μM), and they are 100-fold lower than the concentrations of substrate-binding proteins in the periplasm (up to 1 mM).

As shown in Fig. 2, the refolding of 0.3 μM α-glucosidase is increased from 5–6% in the absence of added protein (or in the presence of ovalbumin or lysozyme) to 11–20% in the presence of chaperones (DnaK and DnaJ), binding proteins (OppA, MglB, and MalE) or bovine serum albumin (all proteins were added at 5 μM). These results suggest that, like molecular chaperones, the periplasmic substrate-binding proteins interact with unfolded proteins, and increase their productive folding.

Periplasmic Binding Proteins Protect Citrate Synthase from Irreversible Aggregation during Thermal Stress—We investigated the function of periplasmic binding proteins under heat

1 The abbreviations used are: BPTI, bovine pancreatic trypsin inhibitor; R-CMLA, reduced carboxymethyl l-lactalbumin; Hsp, heat shock protein.
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...chaperones, including DnaK (38). Complex formation between 3 \( \mu \)M DnaK and 35 \( \mu \)M MglB suppressed citrate synthase aggregation (Fig. 3A (MglB), SB (OppA), and 3C (DnaK)). In contrast, the addition of up to 35 \( \mu \)M bovine serum albumin (BSA), 35 \( \mu \)M ovalbumin, or 35 \( \mu \)M lysozyme (not shown) does not protect citrate synthase against thermal aggregation. Although the periplasmic substrate-binding proteins are somewhat less efficient than DnaK (5 \( \mu \)M DnaK as efficient as 35 \( \mu \)M MglB) and other chaperones (31, 35) in protecting citrate synthase from thermal denaturation, they are much more efficient than the other proteins tested, such as bovine serum albumin, ovalbumin, or lysozyme. Furthermore, the concentration of binding protein required for the thermal protection of citrate synthase is approximately 30-fold lower than its periplasmic concentration.

Interaction between Periplasmic Binding Proteins and Unfolded Proteins—One characteristic of molecular chaperones is their preferential interaction with unfolded proteins (19–21). R-CMLA, a permanently unfolded protein that maintains an extended conformation without any stable secondary structure in the absence of denaturant, strongly interacts with several chaperones, including DnaK (38). Complex formation between R-CMLA (20,000 Da) and OppA (54,000 Da) was analyzed by gel filtration on a Bio-Gel P-200 column. When R-CMLA (3 \( \mu \)M) is filtered in the presence of OppA (5 \( \mu \)M), 56% of R-CMLA fractionates as a complex with a higher molecular mass than R-CMLA alone (Fig. 4A). Similarly, as previously reported, when 3 \( \mu \)M R-CMLA is added to 5 \( \mu \)M DnaK, 53% of R-CMLA fractionates as a high molecular mass complex (Fig. 4B). In the presence of 2 \( \mu \)M OppA or 2 \( \mu \)M DnaK, these amounts are, respectively, 30 and 32% (Fig. 4A and B). Thus OppA seems to interact strongly with R-CMLA, in a manner similar to that of DnaK.

Unfolded BPTI is known to interact with chaperones, including DnaK (39). Complex formation between 3 \( \mu \)M unfolded BPTI (6,000 Da) and 30 \( \mu \)M MglB (33,000 Da) was studied by gel filtration on a Sephadex G-75 column (Fig. 4C). A significant percentage of unfolded BPTI fractionates with the higher molecular weight than unfolded BPTI alone. In contrast, when 3 \( \mu \)M native BPTI and 30 \( \mu \)M MglB were loaded on the gel permeation column, native BPTI did not elute as a high molecular weight complex (not shown). DnaK interacts more strongly with unfolded BPTI than MglB, since 5 \( \mu \)M DnaK retained as much unfolded BPTI as 30 \( \mu \)M MglB (not shown), in accordance with Liberek et al. (39). When similar experiments were carried out with bovine serum albumin (30 \( \mu \)M) or ovalbumin (30 \( \mu \)M), unfolded BPTI did not elute as a high molecular weight complex (not shown). Thus, periplasmic substrate-binding proteins, like molecular chaperones, interact preferentially with unfolded proteins.

Behavior of the Liganded and Ligand-free Forms of Periplasmic Binding Proteins—The periplasmic binding proteins of Gram-negative bacteria serve as chemoreceptors for substrate transport and chemotaxis. Upon binding their specific ligand, they shift from an unliganded form with a wide open cleft between the two globular domains, to a “closed” form with the substrate bound in the cleft and completely entrapped between the two globular domains (14, 15). We ran tests to see whether the chaperone properties of binding proteins are affected by their specific ligand. The binding proteins were deprived of endogenous bound ligand, as described under “Experimental Procedures,” and assayed in the absence or presence of ligand. The OppA-dependent renaturation of citrate synthase was not significantly affected by 60 \( \mu \)M of Ala3, Ser3, or Lys3, which are, respectively, excellent (micromolar affinity), good, and feeble binders of OppA (40) (Fig. 5). The MglB-dependent renaturation of citrate synthase was unaffected by the presence of 60 \( \mu \)M galactose (the MglB-galactose dissociation constant is 0.5

![Fig. 2. Influence of periplasmic binding proteins on the refolding of urea-denatured \( \alpha \)-glucosidase. \( \alpha \)-Glucosidase was denatured in urea and subsequently refactored for 70 min by dilution of the denaturant as described under “Experimental Procedures,” at a concentration of 0.3 \( \mu \)M in the presence of 5 \( \mu \)M additional protein.](image)

![Fig. 3. Thermal aggregation of citrate synthase in the presence of binding proteins. The kinetics of citrate synthase aggregation was determined by light scattering at 650 nm. Native citrate synthase was diluted to a final concentration of 0.8 \( \mu \)M at 43 °C, as described under “Experimental Procedures,” in the absence of additional protein (○), or in the presence of A, 5 \( \mu \)M MglB (■), 35 \( \mu \)M MglB (■); B, 5 \( \mu \)M OppA (○), 35 \( \mu \)M OppA (●); C, 2 \( \mu \)M DnaK (■), 5 \( \mu \)M DnaK (■); D, 5 \( \mu \)M bovine serum albumin (○), 35 \( \mu \)M bovine serum albumin (●).](image)
mM) (Fig. 5). Similarly, the interaction between MglB and unfolded BPTI, measured as described above, was not significantly altered by the presence of galactose (not shown). These results suggest that the substrate-induced conformational change of periplasmic binding proteins does not influence their interaction with unfolded proteins, and that their ligand binding site (including the peptide binding site of OppA) is not involved in this interaction.

**Expression of OppA, MglB, and MalE during Heat Shock**—MglB and MalE are inducible by galactose and maltose, respectively (1–3). OppA is constitutively expressed regardless of the composition of the growth medium and is overexpressed during the stationary phase of growth (41). Since binding proteins appear to possess several of the properties of molecular chaperones, we determined their expression during heat shock. We measured by immunoprecipitation the amount of pulse-labeled OppA, MglB, and MalE, before and several minutes after transfer of cells from 30 to 43 °C. We found that OppA, MglB, and MalE are synthesized at similar rates before and several minutes immediately after heat shock (Fig. 6). Thus, in contrast to heat shock proteins, the synthesis of binding proteins is not transiently increased after heat shock. However, their synthesis remains constant immediately after heat shock, in contrast with many cellular proteins whose synthesis is transiently decreased after heat shock (42).

**DISCUSSION**

We present biochemical evidence suggesting that periplasmic substrate-binding proteins have a chaperone-like function in protein folding, protection against thermal denaturation, and interaction with unfolded proteins. All three binding proteins tested (OppA, MalE, and MglB) increase approximately 3-fold the yield of active citrate synthase and α-glucosidase renaturation as do molecular chaperones. The stimulation factors of protein renaturation are not much lower than those obtained with DnaK, DnaJ, Hsp90, or small Hsps (this study) (31, 36, 37). Half-maximal reactivation of citrate synthase occurs in the presence of micromolar binding protein concentrations, similar to those of DnaK and DnaJ, and somewhat higher than those of Hsp90 or small Hsp (however, the concentration of small Hsp was expressed in 30-mer in Jakob et al. (31), and the concentration in monomers is consequently 30-fold higher (31, 35)). Furthermore, these concentrations are of the same order of magnitude as that of unfolded citrate synthase in the reaction mixture, which reflects a strong interaction between binding proteins and unfolded proteins. Notably, the binding protein concentrations used in this study (within the micromolar range) are at least 100-fold lower than
their estimated concentration in the periplasmic space (up to 1 µM).

Periplasmic substrate-binding proteins protect citrate synthase from thermal denaturation. This protection requires higher concentrations of binding proteins than those used in protein folding. These concentrations (around 30 µM) are 5-fold higher than the concentrations of DnaK (this study) and of small Hsp (expressed as monomers) required for a similar protection. Binding protein concentrations required for an efficient protection of citrate synthase are more than 30-fold lower than their periplasmic concentration. Furthermore, the other proteins tested (bovine serum albumin, ovalbumin, and lysozyme) do not protect citrate synthase efficiently.

Periplasmic substrate binding proteins form stable complexes with unfolded proteins. The OppA-R-CMLA and DnaK-R-CMLA complexes display a similar stability (this study) (38). Like DnaK (39), binding proteins interact with unfolded BPTI, but not with native BPTI, and thus appear to discriminate between unfolded and native proteins.

Periplasmic substrate binding proteins possess a binding site for a small molecular weight ligand and protein binding sites that are involved in their interaction with a transport complex, a chemoreceptor, and among themselves (in multimerization) (1–3). The binding protein-dependent refolding of citrate synthase is not affected by the presence of their specific low molecular weight ligand. This suggests that neither the ligand binding site of binding proteins nor their ligand-induced conformational change is involved in their protein renaturation activity. While the ligand-induced conformational change of binding proteins has been implicated in many of their functions (1), it has been recently shown that liganded and unliganded binding proteins interact with equal affinity with their cognate membrane complex (43). Our results suggest that there is relatively strong binding of non-native proteins to a potential hydrophobic site on periplasmic binding proteins. While periplasmic binding proteins are considered to be remarkably hydrophobic molecules (1), mutational studies suggest that several hydrophobic amino acids are directly involved in their interaction with membrane transport components (44) (reviewed in Ref. 45). These amino acids might constitute hydrophobic patches responsible for a nonspecific and strong interaction between binding proteins and unfolded proteins. The huge amount of binding protein molecules in the periplasm might constitute a reservoir of chaperone-like molecules serving as a buffer in preventing the aggregation of non-native proteins. Binding proteins might help in the folding of translocated proteins, and during heat shock, they might bind an impressive amount of denatured proteins until permissive renaturation conditions are restored (binding proteins are heat-stable (1), and their huge higher amount over their membrane partners leaves most of them free to interact with non-native proteins, more especially since both their liganded and ligand-free forms possess chaperone-like properties).

Several molecular chaperones (small Hsps (31) and PapD (22)) appear to function without the help of any ATPase activity, in contrast to chaperones (such as DnaK or GroEL) that possess a peptide-dependent ATPase (19–21) or chaperones (such as SecB or Hsp90), which can recruit an assistant ATPase (SecA or Hsp70, respectively) (19–21, 46). The periplasmic binding proteins do not possess any ATPase activity, and probably function as a chaperone buffer, as suggested above. Nevertheless, it cannot be entirely excluded that a binding protein conformational change linked with their activity in transport (which is energized by an ATPase) is involved in their protein binding function. The ATP-binding cassette transporters (which comprise the binding protein-dependent transport systems) have already been implicated in the regulation of heterologous membrane proteins (47).

All three binding proteins tested appear to possess similar efficiencies for protein renaturation and protection against thermal denaturation. This suggests that other binding proteins should possess similar properties, including the substrate-binding lipoproteins of Gram-positive bacteria, which are the counterparts of the periplasmic substrate-binding proteins of Gram-negative bacteria (48). While most binding proteins are inducible, with a low level of expression in the absence of their specific inducer, the oligopeptide-binding protein OppA is constitutively expressed in large amounts, and could play de facto a prominent chaperone role. In addition to playing an essential role in the uptake of peptides as nutrients, the peptide transport systems of Gram-positive bacteria are involved in sporulation, competence, and adherence to eukaryotic cells (49–52). Although, in some cases, these additional functions can be explained by the peptide binding/transport properties of these permeases (49), some functions should perhaps be reconsidered in the light of a possible chaperone function of oligopeptide binding proteins. The fact that many binding proteins appear to possess a chaperone-like activity might explain why binding protein-deficient single mutants are not impaired in the expression of periplasmic or outer membrane proteins. Interestingly, a prlB mutant (prl stands for protein localization defect), which can phenotypically suppress lamB signal sequence mutations, was identified as a mutant harboring a small deletion in the ribose binding protein gene rbsB (53, 54).

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