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The Folding of Bovine Pancreatic Trypsin Inhibitor in the *Escherichia coli* Periplasm*

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PreOmpA-bovine pancreatic trypsin inhibitor (BPTI) (Goldenberg, D. P. (1988) *Biochemistry* 27, 2481–2489) was expressed in *Escherichia coli*, and the folding pathway of the mature protein in the periplasmic space was analyzed by pulse-chase experiments. Folding intermediates were trapped with iodoacetamide, immunoprecipitated with antisera specific for either the reduced or the native protein, and resolved by electrophoresis. *In vivo*, native BPTI formed with a half-life of 7 min which is 3-fold faster than the optimal *in vitro* folding rate in growth media supplemented with low molecular weight disulfides. The measured *in vivo* half-life includes the time required for translocation and processing by leader peptidase and therefore represents the lower limit for the actual folding rate in the cell. In addition to the native species, two-disulfide intermediates accumulated in the cell at appreciable levels and did not chase to the native species for at least 20 min. We found that the folding of BPTI in *E. coli* was absolutely dependent on DsbA, a protein which accelerates the formation of disulfide bonds in the periplasm. In a *dsbA* mutant strain, trace amounts of oxidized BPTI could be detected only in cultures grown under strongly oxidizing conditions. In wild-type cells, the addition of different concentrations of GSH/GSSG or oxidized dithiothreitol did not affect the kinetics of BPTI oxidation by DsbA. Finally, even though the folding of BPTI *in vitro* decreases by almost 10-fold/unit pH decrease, folding in cells grown at pH 6.0 was only marginally slower than folding in cells grown at neutral pH, despite the fact that the pH of the periplasmic space varies in response to the extracellular fluid.

A central question in protein folding is to what extent *in vitro* experiments reflect the physiological folding pathway (Chun *et al.*, 1993). In the cell, newly synthesized polypeptides can interact with a variety of chaperones and foldases and, quite possibly, with other cellular components such as membranes, ligand or substrate molecules, and low molecular weight solutes. Furthermore, because of the vectorial nature of ribosomal synthesis, and of the export apparatus in the case of secreted proteins, the initial state from which folding commences is non-random. The denatured state of the protein can exert a significant influence on the folding pathway. Unfortunately, determining the folding pathway *in vivo* is no simple matter because of the paucity of methods for isolating partially folded proteins. Progress in the elucidation of *in vivo* folding processes has been possible only for proteins which form exceedingly stable intermediates, such as the P22 endoramnosidase (Goldenberg and King, 1982; Mitraki *et al.*, 1991) and for those where partially folded molecules can be trapped by blocking the formation of disulfide bonds. Elegant studies by the groups of Helenius and Ruddon have employed chemical modification of free thiols to quench folding and dissect the pathway of disulfide bond formation in influenza hemagglutin and human corionic gonadotropin, respectively (Braakman *et. al.* 1992; Bedows *et al.*, 1992). However, only for the latter protein is the *in vitro* folding pathway sufficiently well characterized to allow a direct comparison with results of *in vivo* studies (Huth *et al.*, 1993). Furthermore, the folding of *H. influenza* hemagglutin and human gonadotropin has been studied in mammalian cells in which the use of genetic techniques for dissecting the role of cellular factors is technically difficult.

One of the best characterized folding pathways *in vitro* is that of bovine pancreatic trypsin inhibitor (BPTI).[1] The folding pathway has been elucidated through the isolation and characterization of the one- and two-disulfide intermediates that occur during folding (Creighton and Goldenberg, 1984; Weissman and Kim, 1991; Goldenberg, 1992). The main steps in this pathway are shown below:

$$\mathrm{R} \leftrightarrow \mathrm{I} \leftrightarrow \mathrm{II} \leftrightarrow \mathrm{N}^{\mathrm{SH,SH}} \leftrightarrow \mathrm{N}$$
$$\searrow \swarrow$$
$$\mathrm{N}^*$$

SCHEME 1

where R is the fully reduced protein, I represents the various one disulfide species, II represents the native like intermediate N′(30–51, 14–38) as well as two-disulfide species with one non-native and one native disulfide, N* represents the kinetic trap (5–55, 14–38), $\mathrm{N}^{\mathrm{SH,\ SH}}$ is the native-like species (30–51, 5–55) and N is the native protein with three disulfides. The rate-limiting step in folding is the conversion of II to $\mathrm{N}^{\mathrm{SH,\ SH}}$ which is then rapidly oxidized to the native protein. The structure of all major folding intermediates has been extensively characterized using ^{1}H and ^{15}N NMR (van Mierlo *et al.*, 1993). N*, and to a lesser extent N′, have a native-like conformation and therefore the activation energy for the conformational change required to position the two thiols in close proximity is high and thus the rate of rearrangement to $\mathrm{N}^{\mathrm{SH,\ SH}}$ is slow. The unpaired cysteines of N* are buried within the interior of the protein. Consequently, N* is exceedingly slow to rearrange to other species and is described as a kinetic trap in the folding pathway (Creighton and Goldenberg, 1984).

Protein disulfide isomerase is an endoplasmic reticulum enzyme that catalyzes cysteine oxidation and disulfide bond rearrangement and is essential for cell viability in *Saccharomy-*

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[1] The abbreviations used are: BPTI, bovine pancreactic trypsin inhibitor; PDI, protein disulfide isomerase; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-tris(hydroxymethyl)methylglycine.

ces cerevisiae (Freedman, 1989; Noiva *et al.*, 1991). *In vitro*, PDI has been shown to catalyze all steps in the BPTI folding pathway (Creighton, *et al.*, 1980) including the rearrangement of N′ and N* to the labile intermediate $N^{SH, SH}$ under conditions that resemble those of the endoplasmic reticulum (Weissman and Kim, 1993). The folding of BPTI is accelerated by the presence of total protein from the endoplasmic reticulum, a phenomenon which has been shown to be totally accounted for by the activity of protein disulfide isomerase (Zapun *et al.*, 1992). Creighton and co-workers (1993) recently studied the folding of proBPTI produced by an *in vitro* translation system and imported into microsomes. ProBPTI consists of the mature protein with a 13-residue extension on the N-terminal and 7 residue extension at the C-terminal. Its folding was found to be dependent on the presence of GSSG. The formation of native protein was found to be substantially higher than *in vitro* with complete folding occurring within 1 min under strongly oxidizing conditions (10 mM GSSG) and within 2 min with 4 mM GSSG. It was postulated that the higher rate of folding in microsomes was due to PDI which was shown to catalyze both disulfide bond formation and rearrangement in proBPTI (Creighton *et al.*, 1993).

The periplasmic space of *E. coli* is topologically equivalent to the endoplasmic reticulum. It is maintained at a redox state favoring the formation of disulfide bonds, a process which is accelerated by at least two interacting proteins, DsbA and DsbB (Bardwell and Beckwith, 1993; Bardwell *et al.*, 1993). The three-dimensional structure of DsbA was solved recently and shown to consist of a thioredoxin-like domain joined to a second domain which may be responsible for substrate specificity (Martin *et al.*, 1993). Similarly, PDI has also been predicted to contain thioredoxin-like domains. Given the analogies between the endoplasmic reticulum and the bacterial periplasmic space, it is not surprising that native BPTI can form in *E. coli* provided it is expressed with a bacterial leader peptide (Marks *et al.*, 1986; Goldenberg, 1988).

In this article we have analyzed the BPTI folding pathway in bacteria using pulse-chase techniques coupled with chemical quenching of disulfide bond formation and separation of folding intermediates. We show that folding is substantially faster than *in vitro* under conditions analogous to those of the periplasmic space, and it is absolutely dependent on the action of DsbA. In *dsbA*$^+$ cells, folding is not affected by the presence of exogenous oxidants even though small solutes are capable of equilibrating through the outer membrane. Similarly, the extracellular pH was found to have a minor effect on folding despite the fact that the pH of the periplasm strongly reflects that of the extracellular environment.

EXPERIMENTAL PROCEDURE

Bacterial Strains and Plasmids—The *E. coli* K12 strains used in this study were JCB502((λ*D69*), *lacZ::Tn10* (tet^s by fusaric acid)) and JCB572 (JCB502 *dsbA::kan1*) (Bardwell *et al.*, 1991) containing F′(*proAB*, *lacIq*, *lacZ ΔM15*, *Tn*). Since preOmpA-BPTI is expressed from the strong *lpp-lac* promoter, an episomal *lacIq* gene was transferred to both strains by mating with XL1Blue(*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, *lac*, F′(*proAB*, *lacIq*, *lacZ ΔM15*, *Tn*)) and selected for tetracycline resistance. The cells were transformed with the plasmid pTI103 containing the OmpA leader-BPTI gene fusion under the control of the *lpp-lac* promoter (Goldenberg, 1988).

Growth Conditions—Unless otherwise specified, cells were grown in M9 minimal salts media adjusted to pH 7.0 and supplemented with 0.2% glucose, 50 μg/ml ampicillin, and 50 μg/ml L-amino acids (except cysteine). The synthesis of BPTI was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside at an OD_{600} of 0.3–0.35. For some experiments, cultures were also grown in modified pH 6.0 M9 salts containing 62 mM KH_2PO_4, 12 mM Na_2PO_4, 19 mM NH_4Cl, and 9 mM NaCl or in pH 8.0 Tris media containing 23 mM KH_2PO_4, 50 mM Tris, 19 mM NH_4Cl, and 9 mM NaCl. Both the pH 6.0 M9 salts and the pH 8.0 Tris media were supplemented with ampicillin and L-amino acids as above. All experiments were conducted at 37 °C.

Pulse-Chase Experiments and Immunoprecipitation—Approximately one generation after induction with isopropyl-1-thio-β-D-galactopyranoside (0.65–0.7 OD_{600} units), cultures were pulse labeled for 1 min with 100 μCi/ml L-[^{35}S]cysteine (1300 Ci/mmol, SJ.15232: Amersham Corp.). Incorporation of the label was terminated by the addition of 8 mM cysteine. Where indicated, sodium azide, glutathione, or dithiothreitol was added 1 min before the pulse. At different times after the addition of the chase solution, 1-ml samples were removed and added to 250 μl of disulfide blocking solution (500 mM iodoacetamide, 25 mM EDTA, 250 mM Tris-HCl, pH 6.8) After incubation at room temperature for 4 min, the samples were rapidly frozen in ethanol-dry ice and stored at −70 °C. BPTI samples were thawed to 4 °C, and all subsequent procedures were carried out at this temperature. Cells were first lysed by sonication (three 30-s pulses). BPTI species in cell lysates were immunoprecipitated by adding 5 μl each of the anti-R and anti-N antisera and 30 μl of protein A-Sepharose CL-4B beads. After 90 min of gentle agitation, the protein A-Sepharose beads were recovered by centrifugation for 1 min at 300 × *g* and washed twice with 10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 0.14 M NaCl, and 0.025% NaN_3, once in the same buffer without Triton and finally with 10 mM Tris-HCl, pH 6.8.

Electrophoresis—Following immunoprecipitation, samples were either resuspended in 40 μl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% β-mercaptoethanol, 1% sodium dodecyl sulfate, 10% glycerol, and 0.0005% bromphenol blue) and resolved by SDS-PAGE or in 50 μl of running buffer (88 mM β-alanine, 0.2% acetic acid) with or without 8 M urea as necessary and resolved by non-denaturing electrophoresis. SDS-PAGE samples were boiled for 5 min and then loaded on precast 16% acrylamide Tricine gels (Novex, San Diego CA). For non-denaturing electrophoresis without urea, the samples were boiled for 5 min and then loaded on 15% acrylamide gels (Reisfeld system) as described (Creighton and Goldenberg, 1984). Samples which were resolved in 15% acrylamide, 8 M urea Reisfeld gels were incubated for 1 h at 4 °C prior to electrophoresis. The gels were run at a constant current (10 mA) until all dye fronts had entered the resolving gel and then at 20 mA until the first dye front was 1 cm from the end of the gel. The gels were dried on a SE 1160 gel dryer (Hoefer Scientific Instruments, San Francisco, CA) and exposed to Hyperfilm βmax film (Amersham Corp.) for 1–2 weeks. Densitometry was performed on a LKB 2202 ultra scan laser densitometer.

In Vitro Folding—Purified BPTI (Boehringer Mannheim) was reduced and unfolded with 100 mM reduced dithiothreitol in 6 M guanidine HCl and 0.2 M Tris-HCl, pH 8.7, overnight. The reduced protein was desalted through a Sephadex G-25 column in 10 mM HCl immediately prior to refolding experiments. Folding was initiated by the addition of reduced BPTI to M9 salts buffer containing the indicated oxidizing agent or redox couple. The concentration of BPTI during refolding was 27 μM. All refolding experiments were performed at 37 °C. Samples were withdrawn at the indicated times and mixed with the quenching solution used in *in vivo* experiments to a final concentration of 100 mM iodoacetamide, 5 mM EDTA, and 50 mM Tris-HCl, pH 6.8. After a 2-min incubation at room temperature, the samples were placed on ice until they were resolved by electrophoresis in the Reisfeld gel systems as described above. The maximum time between the end of the refolding experiment and the start of electrophoresis was 1 h.

RESULTS

When BPTI is expressed with an OmpA leader peptide to direct export to the periplasmic space of *E. coli*, small amounts (~1 mg/liter) of native protein are formed (Goldenberg, 1988). To analyze the folding of BPTI *in vivo*, cultures were grown in minimal media and pulse-labeled with L-[^{35}S]cysteine. The incorporation of the radiolabel was terminated by the addition of an excess of unlabeled cysteine. We confirmed that the presence of free cysteine at a final concentration between 8 μM and 8 mM did not have any effect on the kinetics of BPTI folding *in vivo*. Furthermore, when reduced BPTI was refolded with 150 μM oxidized glutathione in the presence of 8 mM cysteine the pattern of folding intermediates was not affected, although the formation of the native protein seemed to be slightly accelerated.[2]

After the addition of the chase solution, samples were collected at different times and incubated with 100 mM iodoacet-

[2] M. Ostermeier and G. Georgiou, unpublished observations.

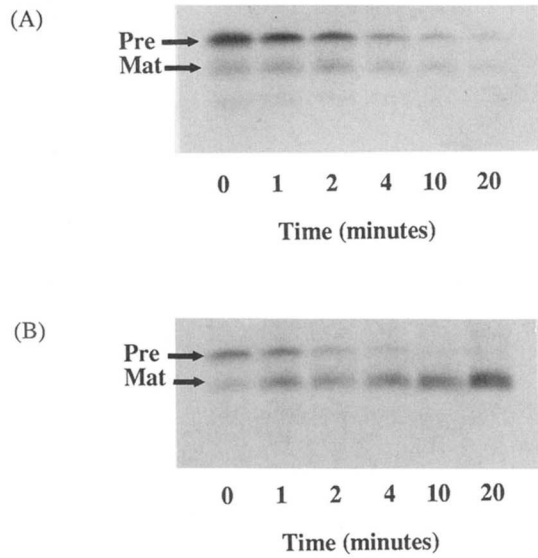

FIG. 1. **Folding of BPTI *in vivo* as monitored by reducing SDS-PAGE electrophoresis.** Cells were labeled for 1 min, and the BPTI species were immunoprecipitated with antisera against reduced BPTI (*A*) and native BPTI (*B*). Times indicated are minutes after chase. Symbols: *Pre* = preOmpA-BPTI, *Mat* = mature BPTI.

amide in the presence of EDTA so as to increase the permeability of the outer membrane and thus facilitate the diffusion of the blocking agent into the periplasmic space. Lysed cells were immunoprecipitated with antisera and resolved by gel electrophoresis. Fig. 1 shows autoradiograms of proteins immunoprecipitated with anti-R or anti-N and resolved by SDS-PAGE. The lower band (the fastest migrating species) had the same electrophoretic mobility as the band corresponding to purified BPTI and corresponds to the mature protein after cleavage of the leader peptide. The upper band was identified as preOmpA-BPTI because it migrated at the same position as the precursor band in cells which had been treated with 12 mM sodium azide. Sodium azide inhibits the activity of SecA (Oliver *et al.*, 1990) resulting in the accumulation of unprocessed precursor protein in the cytoplasm. No protein bands could be detected in samples immunoprecipitated from cells that did not contain the plasmid. In Fig. 1 a very faint band that migrated faster than the mature protein was detectable and presumably corresponds to a proteolytic degradation product.

The level of mature BPTI immunoprecipitated with anti-R was low with the exception of very early time points and decreased to barely detectable levels after 20 min (Fig. 1*A*). The decrease in mature BPTI with reduced epitopes was accompanied by an increase in the mature BPTI precipitated by anti-N antibodies. Fig. 1*B* shows that mature BPTI recognized by anti-N is detectable immediately after the chase and continues to accumulate throughout the course of the experiment.

The preOmpA-BPTI band decreased exponentially throughout the course of the experiment with a small amount detectable even 20 min after the chase. This indicates that transport of the radiolabeled protein across the cytoplasmic membrane is not very efficient. The disappearance of the precursor is both due to processing of the leader peptide and proteolytic degradation in the cytoplasm. The latter was evident in cultures where transport had been blocked with sodium azide. When sodium azide was added 2 min before the onset of the chase, the half-life of the precursor was 3 min. This was only marginally higher than the half-life of the precursor in the absence of sodium azide. Thus, a substantial fraction of the precursor molecules is degraded in the cytoplasm before translocation.

A portion of preOmpA-BPTI is processed rapidly as indicated by the presence of mature BPTI even at the time of addition of the chase solution. An estimate of the fraction of preOmpA-BPTI that is processed rapidly can be deduced from the accumulation of native-like molecules following inhibition of transport with sodium azide (Fig. 2). About 50% of the BPTI that eventually reaches the native-like state is exported from the cytoplasm and is processed in less than 4 min after the addition of the chase.

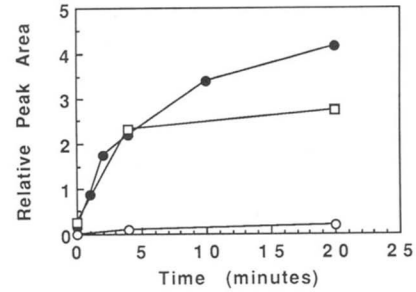

FIG. 2. **Effect of 12 mM sodium azide on the appearance of N + N*.** Cells were labeled for 1 min, and the BPTI species were isolated by immunoprecipitation and resolved by non-reducing gel electrophoresis. Relative levels were determined by densitometry of autoradiograms. Times indicated are minutes after chase. Symbols: ●, control cells (no sodium azide); ○, 12 mM sodium azide 1 min before the pulse; □, 12 mM sodium azide 1 min after the chase.

SDS-PAGE resolves the precursor and mature BPTI but cannot be used to detect the formation of folding intermediates. This can be accomplished by electrophoresis under non-reducing conditions using the Reisfeld gel system (Reisfeld *et al.*, 1962) as shown in Fig. 3. The presence of 8 M urea is required to separate the native protein from the native-like intermediate N* (Creighton and Goldenberg 1984). The bands corresponding to the precursor, reduced mature protein, and N were identified by comparison with the electrophoretic mobility of preOmpA-BPTI in sodium azide-treated cells, reduced carboxymethylated BPTI, and native protein, respectively. No bands with electrophoretic mobility similar to that of any BPTI species were detected in cells without the plasmid. From Fig. 3 it can be deduced that the half-life for the formation of native BPTI is approximately 7 min. Since this number accounts for folding, translocation, and processing, it represents an upper estimate of the time required for the reduced mature BPTI to reach the native conformation. Thus, the actual half-life for folding must be less than 7 min. It is very difficult to obtain more precise kinetic data since (i) the rate of export and precursor processing cannot be readily discerned in this case and (ii) proteolysis is a competing process.

In non-reducing urea gels, a distinct band with the same electrophoretic mobility as *in vitro* prepared two-disulfide intermediates is evident and is designated as *. This band appears to be stable and does not chase to the native protein much like in *in vitro* experiments in which N* has been also shown to accumulate and only very slowly convert to other species. The fraction of N* in the * band cannot be ascertained. This is because brief boiling or treatment with 8 M urea is required to fully dissociate the antigen-antibody complexes prior to electrophoresis. These treatments do not appear to change the electrophoretic mobility of intermediates in which all thiols are either carboxymethylated or part of a disulfide.[2] However, because the free thiols of N* are deeply buried and not carboxymethylated during quenching (Creighton and Goldenberg, 1984) this intermediate may rearrange to other two-disulfide species. In any case, even though the identity of the two-disulfide intermediates in the cell cannot be clearly determined, the rearrangement of N* does not alter the amount of native BPTI, reduced BPTI, or the one disulfide intermediates.

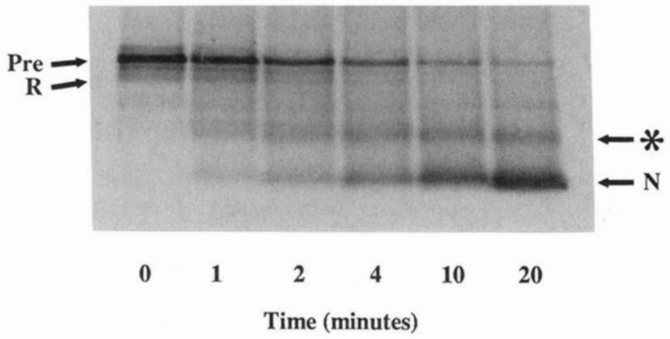

FIG. 3. **Folding and disulfide formation of BPTI in *E. coli* as monitored by non-reducing electrophoresis.** Proteins immunoprecipitated from cultures labeled with L-[^{35}S]cysteine for 1 min and quenched with 100 mM iodoacetamide were resolved in non-reducing gels containing 8 M urea. Times indicated are minutes after chase. Symbols: *Pre* = preOmpA-BPTI, *R* = reduced carboxymethylated BPTI, *N* = native BPTI, and * = putative two-disulfide intermediates which includes N*.

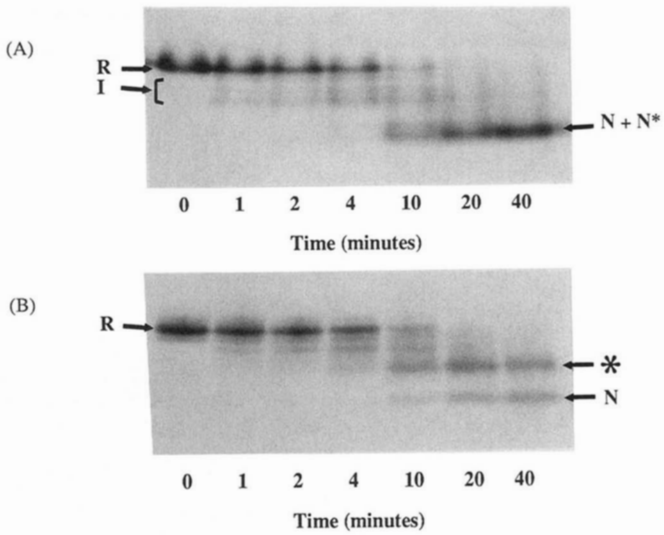

FIG. 4. **Refolding of BPTI in M9 salts with 150 μM GSSG.** Samples were electrophoresed through non-reducing gels (*A*) and non-reducing gels containing 8 M urea (*B*). Symbols: *R* = reduced carboxymethylated BPTI, *I* = one disulfide intermediates, *N* = native BPTI, *N** = native like intermediate (5–55, 14–38), and * = putative two-disulfide intermediates which includes N*.

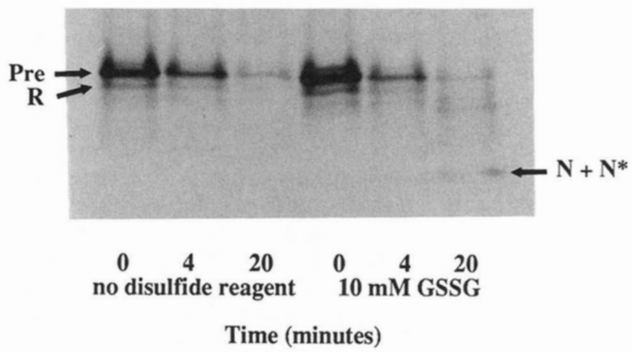

FIG. 5. **Effect of *dsbA* mutation on BPTI folding as monitored by non-reducing gel electrophoresis.** Cells were labeled for 5 min and the BPTI species isolated by immunoprecipitation. *Lanes 1–3*, expression of BPTI in cells lacking functional DsbA. *Lanes 4–6*, same cells with 10 mM GSSG added 1 min before the pulse. Times indicated are minutes after chase. Symbols: *Pre* = preOmpA-BPTI, *R* = reduced carboxymethylated BPTI, *N* = native BPTI, and *N** = native-like intermediate (5–55, 14–38).

The kinetics of formation of native BPTI were compared with *in vitro* refolding under conditions representative of those in the periplasmic space. Because the periplasmic space is permeable to ions and hydrophilic low molecular weight solutes found in the growth medium, refolding experiments were conducted in M9 buffer at pH 7.0 to better approximate the *in vivo* conditions. Folding of reduced BPTI was initiated by the addition of 150 μM oxidized glutathione, and intermediates were trapped with 100 mM iodoacetamide as above. The conditions used to initiate folding are the same as those employed in previous *in vitro* studies and result in the most efficient formation of native protein without the accumulation of mixed disulfides (Creighton, 1980; Weissman and Kim, 1991). Folding intermediates were separated in Reisfeld gels with and without 8 M urea. In the absence of urea, N and N* migrate as one band, whereas with urea, N is resolved from N* and other two-disulfide species. Under the conditions we used, N+N* was not detectable before 10 min (Fig. 4, *A* and *B*), and the formation of N occurred with a half-life of at least 20 min, three times higher than the upper estimate for the *in vivo* half-life. Furthermore, the amount of two-disulfide intermediates continued to increase throughout the experiment in contrast to our *in vivo* results.

The higher rate of folding observed in *E. coli* suggests strongly that the formation of native BPTI in the cell must be catalyzed by cellular factors. We examined whether DsbA, the *E. coli* equivalent of PDI, is involved in the folding of BPTI in the periplasmic space. Indeed, in the *dsbA* mutant strain JCB572, an isogenic derivative of JCB502 used in the experiments discussed above, no trace of native BPTI nor any folding intermediates could be detected (Fig. 5). Thus, the oxidation of BPTI in *E. coli* is absolutely dependent on the presence of a functional DsbA enzyme. To suppress the effect of the *dsbA* lesion, various oxidants and redox buffers were added to the growth medium prior to labeling. High levels of oxidants can mediate the formation of disulfide bonds in urokinase and other proteins expressed in *dsbA* mutants (Bardwell *et al.*, 1993). However, neither the presence of oxidized dithiothreitol at concentrations up to 20 mM or oxidized glutathione at 150 μM, 400 μM, or 2 mM nor a mixture of 2 mM GSH and 0.5 mM GSSG resulted in the formation of any oxidized BPTI whatsoever. Very small amounts of native BPTI and traces of folding intermediates could be detected only under highly oxidizing conditions (10 mM glutathione). Higher concentrations of oxidized glutathione or oxidized dithiothreitol could not be used because they interfere with cell growth and protein synthesis. Identical results were obtained in cells that had been incubated in redox buffers for 30 min prior to labeling.

The dependence of the redox state of DsbA *in vitro* on the relative levels of GSH to GSSG has been clearly established (Zapun *et al.*, 1993; Wunderlich and Glockshuber, 1993a). However, no noticeable effect on the rate of formation of native BPTI was observed in wild-type cells grown in the presence of 150 μM, 2 mM, or 10 mM GSSG or in 2 mM GSH/0.5 mM GSSG (data not shown). The amount of two-disulfide intermediates (the * band) which accumulated in the cells was also not affected, consistent with the fact that DsbA is unable to catalyze the formation of the final disulfide in N* *in vitro*.[3] Also, addition of GSH at concentrations as high as 10 mM did not have any effect in the folding of BPTI *in vivo* even though addition of reduced glutathione was shown recently to improve the formation of correctly folded *Ragi* bifunctional α-amylase/trypsin inhibitor (RBI) in the *E. coli* periplasm (Wunderlich and Glockshuber, 1993b).

The degree of ionization of sulfhydryls has a strong effect on the kinetics of disulfide bond formation *in vitro*. Even though normal levels of preOmpA-BPTI were synthesized in cells grown in pH 8.0 media, the amount of native BPTI formed was

[3] A. Zapun and T. E. Creighton, personal communication.

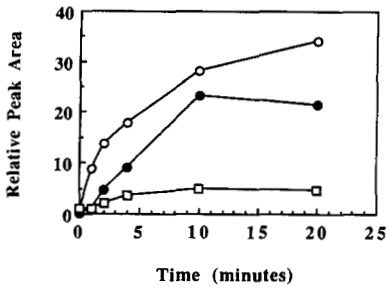

FIG. 6. **Effect of media pH on BPTI folding in the *E. coli* periplasm.** Experimental conditions as in Fig. 3. Samples immunoprecipitated with anti-N were resolved by non-reducing gels containing 8 M urea and the amount of native BPTI determined by densitometric scans of the autoradiogram. Times indicated are minutes after chase. Symbols: ●, pH 6.0; ○, pH 7.0; and □, pH 8.0.

severely reduced (Fig. 6). Under these conditions unfolded BPTI is subjected to extensive degradation in the periplasmic space. BPTI folding was successful in cells grown in pH 6.0 media, though it occurred at a slightly slower rate than at pH 7.0. In contrast, the folding rate of BPTI *in vitro* decreases by about 10-fold/unit decrease in pH (Creighton, 1980).

DISCUSSION

In this study we have investigated the folding of BPTI exported to the periplasmic space of *E. coli* by trapping radiolabeled intermediates with iodoacetamide. The carboxymethylation of free cysteines by iodoacetamide is a fast reaction that proceeds with a half-time of less than 2 s *in vitro* (Creighton, 1978). While it is not possible to ascertain the rate of the quenching step *in vivo*, we have verified that the addition of iodoacetamide with the chase solution completely prevents the formation of either native protein or folding intermediates.[2] Thus, the time scale for the completion of the quenching step should be of the order of 1 min.

Following quenching, BPTI was immunoprecipitated with two different rabbit antisera raised against the native (anti-N) and the reduced, carboxymethylated protein (anti-R). The two antisera have nearly equal titers and their reactivity toward chromatographically purified folding intermediates has been characterized by Creighton and co-workers (1978). Because the carboxyl-methylated, reduced BPTI is a poor immunogen, the anti-R sera consists of two low affinity immunoglobulin populations with apparent affinity constants of 3×10^6 M^{-1} and less than 10^5 M^{-1}. The anti-N sera have a high affinity for the native protein (10^9 M^{-1}) and bind tightly to one- and two-disulfide intermediates. Furthermore, as it is common for conformation-specific antibodies, the anti-N sera also exhibit a low degree of cross-reactivity toward the reduced protein. The cross-reactivity of the antisera does not affect the results reported here because folding species were identified based on their electrophoretic mobilities and not on their recognition by the antisera. For all experiments except those in Fig. 1, BPTI was immunoprecipitated using both anti-R and anti-N at a fixed ratio. Identical results, except for noticeably lower levels of preOmpA-BPTI and reduced BPTI were obtained following immunoprecipitation with anti-N antibodies alone. The anti-R sera was used only to better visualize and monitor the fate of the reduced species. Separate immunoprecipitations with anti-R and anti-N antibodies were employed only for the analysis of the processing of the signal sequence of pre-OmpA-BPTI (Fig. 1) solely to monitor the disappearance of reduced epitopes and the appearance of native epitopes in the mature protein.

In this case, a band corresponding to preOmpA-BPTI was evident in samples immunoprecipitated with anti-N antibodies. It is conceivable that one or more disulfides might have been formed in the precursor with oxidation occurring either in the cytoplasm or in the periplasm prior to processing by leader peptidase. Even though disulfides do not form in the cytoplasm in some proteins such as alkaline phosphatase (Derman and Beckwith, 1991), they may be capable of forming in other proteins if the redox potential of the relevant cysteines is sufficiently low and their formation is kinetically favored. *In vitro*, BPTI will form disulfides and fold stably in cytosolic-like conditions of 10 mM GSH and 0.1 mM GSSG[4] and disulfide formation in BPTI expressed as a fusion protein with a two-domain derivative of staphylococcal protein A in the cytoplasm has been reported (Nilsson *et al.*, 1991). However, in the case of preOmpA-BPTI two lines of evidence argue against it being oxidized prior to the cleavage of the leader peptide. First, preOmpA-BPTI immunoprecipitated with either anti-R or anti-N antisera has the same electrophoretic mobility in non-reducing urea and Tricine gels (data not shown) suggesting that they are the same species. Second, the kinetics of disappearance of preOmpA-BPTI precipitated with anti-R and anti-N antibodies were identical. If the precursor can exist in both an unfolded and a partially folded conformation, one would expect that its kinetics of disappearance should be different for the two conformers. Thus, the precipitation of preOmpA-BPTI by anti-N antibodies is likely to be a consequence of the low cross-reactivity of the sera for the reduced protein coupled with the high sensitivity of the immunoprecipitation technique.

We found folding *in vivo* to be faster than *in vitro* in the presence of molecular disulfides. The accelerated folding of BPTI in the periplasmic space is apparently due to the actions of DsbA. Similarly, the folding of proBPTI in microsomes has been found to be very efficient because of the presence of PDI (Creighton *et al.*, 1993). However, in contrast to the *E. coli* periplasm, folding within microsomes was found to depend strongly on the presence of GSSG. It is not clear why GSSG and oxidized dithiothreitol have no effect on the folding of BPTI in *E. coli*, particularly since some of the higher concentrations we used result in the rapid formation of a wide spectrum of intermediates and mixed disulfides *in vitro*. This difference in the folding of BPTI in microsomes and in the *E. coli* periplasm may be related to the differences in the sulfhydryl oxidation machinery of eukaryotic and prokaryotic cells. Alternatively, the mature BPTI may be sequestered in a periplasmic compartment which is inaccessible to GSSG, GSH, and other similar low molecular weight solutes. However, there is no conclusive evidence for such compartments in Gram-negative bacteria, and therefore we believe that this possibility is unlikely.

The fact that high concentrations of GSSG failed to increase the folding of BPTI indicates that DsbA in the periplasmic space is predominantly present in the oxidized form and therefore folding proceeds at the maximum possible rate. Very recently, Wunderlich and Glockshuber (1993b) reported that the addition of GSH and/or GSSG to the growth media affects the formation of correctly folded *Ragi* bifunctional α-amylase/trypsin inhibitor (RBI) expressed in the *E. coli* periplasm. They found that the formation of native RBI is increased by coexpressing DsbA and growing the cells in media containing GSH. This result suggests that rate of folding of RBI is limited by the reduction of incorrect disulfides, a process which is accelerated by the addition of GSH. In contrast, non-native disulfide intermediates do not accumulate to a great extent during the folding of BPTI. Therefore, unlike RBI, the reduction of incorrect disulfides by exogenous GSH did not have any effect on the folding of BPTI in the cell. Thus, it appears that the manipulation of the redox state of the growth medium can only facilitate the

[4] T. E. Creighton, personal communication.

folding of proteins which require extensive rearrangement of disulfide bonds. For proteins such as BPTI where non-native intermediates rearrange relatively rapidly, the predominant function of DsbA is to catalyze cysteine oxidation.

Preliminary experiments have shown that stoichiometric amounts of DsbA are very efficient in oxidizing reduced BPTI *in vitro* (Zapun *et al.*, 1993), and we now showed that DsbA is essential for folding *in vivo*. Not even one-disulfide-containing species could be detected in *dsbA*$^-$ mutants. One-disulfide intermediates are stable enough to be detected in *dsbA*$^+$ cells and have an appreciable degree of native structure (van Mierlo *et al.*, 1993) and thus should be less susceptible to proteolysis than the reduced protein. The absence of one-disulfide intermediates indicates that the reduced protein in *dsbA* mutants is degraded extremely rapidly. Thus, DsbA is required for the rapid oxidation of reduced BPTI which otherwise is susceptible to proteolytic degradation. We have constructed strains deficient in all loci that affect the stability of secreted proteins (*degP, ptr, prc, ompT, rpoH*, Baneyx and Georgiou, 1992),[5] and on-going studies are underway to determine whether they increase the stability of folding intermediates.

The *E. coli* outer membrane is permeable to hydrogen ions and thus the pH of the periplasmic space varies according to the pH of the growth medium. However, it is not equal to the external pH because of a Donnan equilibrium across the outer membrane (Stock *et al.*, 1977). We found that the formation of native BPTI is hindered in cells grown in pH 8.0 media. Growth at pH 8.0 results in enhanced proteolytic degradation of periplasmic proteins (Baneyx and Georgiou, 1992, Georgiou *et al.*, 1988), and this is the most likely explanation for the reduced level of BPTI in cells grown at pH 8.0. In growth media adjusted to pH 6.0, the rate of formation of native BPTI was only slightly lower than at pH 7.0. *In vitro*, both the rates of cysteine oxidation and BPTI folding decrease by about 10-fold/pH unit decrease (Creigthon, 1980). The relative insensitivity of folding to a decrease in pH *in vivo* stems from the catalytic role of DsbA which has been shown to catalyze the formation of disulfides in hirudin even at pH 4 whereas molecular disulfides are ineffective at or below pH 6 (Wunderlich *et al.*, 1993).

In conclusion, BPTI becomes oxidized relatively fast when expressed in the periplasm of wild type *E. coli*, with appreciable formation of two-disulfide intermediates occurring within 1 min. Two-disulfide intermediates were found to accumulate in the cell and possibly consist of the kinetic trap N*. These intermediates did not chase to the native protein even after 20 min. Folding was found to be faster than *in vitro* because of the catalytic role of DsbA. Indeed, the formation of native BPTI was completely dependent on the action of DsbA and could only be marginally rescued by the addition of very high concentrations of GSSG.

[5] H. Meerman and G. Georgiou, unpublished results.

Our results show that BPTI represents a valuable model for studying the pathway of protein folding and disulfide formation in the bacterial periplasmic space. The elucidation of the effects of other environmental and physiological factors on folding in the periplasmic space are underway as are efforts to characterize the pattern of cysteine bonding in the *in vivo* folding intermediates.

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