In Vivo Control of Redox Potential during Protein Folding Catalyzed by Bacterial Protein Disulfide-isomerase (DsbA)*

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The formation of disulfide bonds in Escherichia coli is catalyzed by periplasmic protein disulfide-isomerase (DsbA). When the α-amylase/trypsin inhibitor from Ragi, a protein containing five intramolecular disulfide bridges, is secreted into the periplasm of E. coli, large amounts of misfolded inhibitor with incomplete or incorrect disulfides are accumulated. Folding of the inhibitor in the periplasm is not improved when DsbA is coexpressed and cosecreted. However, an up to 14-fold increase in correctly folded inhibitor is observed by coexpression of DsbA in conjunction with the addition of reduced glutathione to the growth medium. This peptide acts as a disulfide-shuffling reagent and can pass the outer membrane of E. coli. Since the influence of DsbA on the folding yield of the inhibitor is reduced in the presence of oxidized glutathione, the in vivo function of DsbA appears to be dependent on the ratio between oxidizing and reducing thiol equivalents in the periplasm. The high stability of thiol reagents against air oxidation during growth of E. coli allows the investigation of oxidative protein folding in vivo under controlled, thiol-dependent redox conditions.

Disulfide bonds are a typical feature of secretory proteins and are considered to contribute significantly to their overall stability (Goldenberg, 1993; Matsumura et al., 1989). The formation of a disulfide bridge is a posttranslational protein modification and involves a redox reaction that is catalyzed by protein disulfide-isomerase (PDI) in vivo (Freedman, 1992; Noiva and Lennarz, 1992). Disulfide formation in eukaryotic cells takes place in the lumen of the endoplasmic reticulum (ER). In this cellular compartment, the ratio between oxidized and reduced glutathione (GSSG/GSH) determines the thiol-dependent redox conditions. Since the GSSG/GSH ratio in the ER is about 100 times higher than in the cytoplasm, GSSG is likely to provide the oxidizing equivalents for the formation of protein disulfides in the ER (Hwang et al., 1992).

Numerous in vitro experiments have shown that the oxidation of a folding polypeptide chain in redox buffers containing GSH and GSSG occurs spontaneously via disulfide exchange reactions between GSH/GSSG and the folding polypeptide (Saxena and Wetlaufer, 1970; Jaenicke and Rudolph, 1989). However, although oxidative protein folding does not necessarily have to be catalyzed by PDI, the enzyme has turned out to be essential for effective disulfide formation in the ER (Bulleid and Freedman, 1986). Eukaryotic PDI is a well-characterized, multifunctional disulfide oxidoreductase and constitutes one of the most abundant proteins in the ER lumen. It is a strong oxidant with an intrinsic redox potential of -0.11 V (Freedman, 1992; Hawkins et al., 1991).

In contrast to eukaryotic cells, little is known about the process of disulfide formation and its necessity in bacteria. In Escherichia coli, disulfide formation takes place in the periplasmic space. Recently, the dsbA gene product was identified as the first protein required for effective disulfide bond formation in E. coli. The DsbA protein consists of 189 residues and contains an active disulfide (Cys-Pro-His-Cys) very similar to the catalytic disulfides present in eukaryotic PDI and other disulfide oxidoreductases like thioredoxins and glutaredoxins (Bardwell et al., 1991; Kamitani et al., 1992). This monomeric protein is a strong oxidant similar to eukaryotic PDI with an intrinsic redox potential of -0.089 V (Wunderlich and Glockshuber, 1993; Zapun et al., 1993; Wunderlich et al., 1993).

Unlike in the ER lumen, the molecular species providing the oxidizing equivalents for disulfide formation in the periplasm of E. coli is unknown. However, it seems likely that different dithiols and molecular oxygen may be the real oxidants of cysteines in the periplasm, since the outer membrane of E. coli is permeable for molecules smaller than about 500 Da (Payne and Gilvarg, 1968; Decad and Nikaido, 1976). Consequently, the oxidant responsible for disulfide bond formation in E. coli may essentially depend on the growth conditions and the composition of the surrounding medium.

The permeability of the outer E. coli membrane for small peptides prompted us to use the periplasm of E. coli as "reaction vessel" and to mimic the situation in the ER by adding GSH and GSSG to the growth medium. To analyze the influence of DsbA on disulfide formation at different concentrations of GSH and GSSG, DsbA was overexpressed in conjunction with the addition of GSH/GSSG to the medium.

As a measure for the efficiency of disulfide formation, we analyzed the heterologous periplasmic expression of the bifunctional α-amylase/trypsin-inhibitor (RBI) from Ragi (Eleusine coracana Gaertneri), since the yield of functionally expressed RBI in E. coli is strongly hampered by insufficient disulfide formation. RBI belongs to the family of plant α-amylase/trypsin inhibitors (Laskowski, 1986) and contains 122 residues with five intramolecular disulfides that are essential for its activity (Shivaraj and Pattabiraman, 1981; Campos and Richardson, 1983). In this report, we show that the addition of reduced glutathione in combination with coexpression of DsbA leads to an up to 14-fold increase in the yield of native RBI, whereas the addition of oxidized glutathione and the overproduction of DsbA alone have no influence on the yield of correctly folded inhibitor in the periplasm. The results point out the importance of the reduction of incorrect disulfides during oxidative protein
folding in vivo and are discussed in terms of a general strategy to improve the yield of recombinant secretory proteins expressed in E. coli.

**EXPERIMENTAL PROCEDURES**

**Materials**—5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and reduced and oxidized glutathione (GSH and GSSG) were from Sigma. Benzoyl-L-arginine-p-nitroanilide was purchased from Boehringer (Mannheim, Germany). Bacto-tryptone and Bacto-yeast extract were from Difco Laboratories (Detroit, MI). All other chemicals including trypsin were from Sigma. (pRBI) were induced with IPTG, and the expression of RBI was analyzed by SDS-PAGE. Densitometric scans of Coomassie-stained gels revealed that about 95% of the expressed and correctly processed protein were accumulated in insoluble periplasmic aggregates, while only about 5% of processed RBI were obtained as soluble, native inhibitor. Immunoblot analysis using reducing and nonreducing SDS-PAGE and antibodies that preferably recognize the correct disulfide-bridged conformation of the inhibitor revealed that native RBI is only present in the soluble fraction (Fig. 1A). When the aggregated material was solubilized by reducing agents and denaturants, native RBI could be renatured in redbottomed containing GSH and GSSG (Jaenicke and Rudolph, 1989). Therefore, as a result of the complex cystine pattern of RBI (Fig. 1B), it is likely that the formation of native disulfides is rate-limiting for folding of RBI in vivo and competes with the aggregation of nonnative molecules (Kiefhaber et al., 1991).

**RESULTS AND DISCUSSION**

For the functional expression of the Ragi α-amylase/trypsin inhibitor (RBI) in E. coli, a synthetic gene coding for the known peptide sequence (Campos and Richardson, 1983) was fused to the bacterial OmpA signal sequence (Inouye et al., 1982). This allows the transport of the inhibitor into the periplasmic space where disulfide bridges are formed. The OmpA/RBI fusion was cloned into the expression plasmid pASK40 (Skerra et al., 1991), where it is controlled by the lac promoter/operator. Cells of E. coli JM83 harboring the resulting expression plasmid (pRBI) were induced with IPTG, and the expression of RBI was analyzed by SDS-PAGE. Densitometric scans of Coomassie-stained gels revealed that about 95% of the expressed and correctly processed protein were accumulated in insoluble periplasmic aggregates, while only about 5% of processed RBI were obtained as soluble, native inhibitor. Immunoblot analysis using reducing and nonreducing SDS-PAGE and antibodies that preferably recognize the correct disulfide-bridged conformation of the inhibitor revealed that native RBI is only present in the soluble fraction (Fig. 1A). When the aggregated material was solubilized by reducing agents and denaturants, native RBI could be renatured in redbottomed containing GSH and GSSG (Jaenicke and Rudolph, 1989). Therefore, as a result of the complex cystine pattern of RBI (Fig. 1B), it is likely that the formation of native disulfides is rate-limiting for folding of RBI in vivo and competes with the aggregation of nonnative molecules (Kiefhaber et al., 1991).

**Construction of Expression Plasmids**—The plasmid pRBI for functional periplasmic expression of RBI was constructed by cloning a synthetic gene coding for the OmpA/RBI fusion (Campos and Richardson, 1983; Inouye et al., 1982) into the plasmid pASK40 (Skerra et al., 1991) via the XbaI and HindIII restriction sites. The dsaA gene, including the natural ribosomal binding site and the natural signal sequence, was amplified from the genome of the E. coli K12 wild type strain W3110 (Bachmann, 1972) by the polymerase chain reaction using oligonucleotide primers based on the published dsaA sequence (Bardwell et al., 1991) as described (Wunderlich and Glockshuber, 1993). The amplified dsaA gene was cloned into pRBI directly at the 3′ end of the RBI gene via the HindIII and BamHI sites.

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**Growth of Bacteria**—Cells of E. coli JM83 (Yanisch-Perron et al., 1985) harboring the plasmids pRBI and pRBI-PDI were grown in LB medium containing ampicillin (100 μg/ml) at 26 °C to an optical density at 550 nm of 1.0 and were induced with IPTG (final concentration: 1 mM). At the time of induction, different amounts of GSH/GSSG were added (see Table 1). The cultures were shaken for 16 h, and the cells were harvested by centrifugation and suspended in lysis buffer (100 mM Tris/HCl, pH 7.5, 20 mM EDTA) to an identical optical density. The cells were disrupted in a French press cell, and the lysate was centrifuged (48,000 g, 30 min, 4 °C). The supernatant (soluble fraction) was removed, and the insoluble fraction was suspended in the original volume.

**Determination of Inhibitor Concentration**—The concentration of functional RBI in the soluble fractions of crude extracts was determined by trypsin inhibition assays. Trypsin (0.2 μg) was incubated with increasing amounts of the extracts of E. coli JM83/pRBI or JM83/pRBI-PDI for 30 min in 100 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 mM CaCl₂, 0.05% (w/v) Triton X-100 at 25 °C. The activity of free trypsin was determined by adding benzoyl-L-arginine-p-nitroanilide to a final concentration of 0.2 mM and recording the increase in absorbance at 405 nm. Since the dissociation constant of the trypsin/RBI complex is more than 2 orders of magnitude below that of trypsin in the test, the concentration of RBI was calculated by linear extrapolation of the titration curves to 100% inhibition assuming a 1:1 stoichiometry of the complex (Shivaraj and Pattabiraman, 1981; Bieth, 1974). Control experiments with identical extracts of E. coli JM83 proved that trypsin was not inhibited by other soluble E. coli proteins.

The amount of RBI present in the insoluble fractions was evaluated by densitometric scans of Coomassie-stained SDS gels, where known amounts of purified RBI were applied simultaneously as a standard for the quantitative analysis. Gel scans were performed at 546 nm using a Hirschmann Elscript 400 densitometer.

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2 M. Wunderlich and R. Glockshuber, unpublished results.
GSSG were added to the culture medium assuming identical redox conditions in the medium and in the periplasm. Based on the observation that oxidative folding of RBI in vitro was strongly dependent on the GSH/GSSG ratio (data not shown), we expected a significant effect of the added thiol reagents on the folding of RBI in vivo. The yield of native RBI in the periplasm could indeed be increased by adding GSH and GSSG in varying concentrations at the time of induction without coexpression of DsbA. A more than 5-fold increase in functional RBI was found at a GSH concentration of 5 mM in the medium (Table I). In contrast to coexpression of DsbA in the absence of GSH, coexpression of DsbA in the presence of 5 mM GSH further increased the yield of native inhibitor almost 3-fold leading to an overall, 14-fold improvement of periplasmic folding of RBI (Table I and Fig. 2B). However, the analysis of the insoluble cellular protein by Coomassie-stained SDS gels revealed that the amount of insoluble RBI is practically independent of DsbA coexpression and presence of thiols in the medium (Fig. 2C). This demonstrates that improved folding of RBI in the periplasm mainly prevents the inhibitor from being proteolytically degraded, but does not decrease the overall amount of aggregated RBI significantly. Therefore, DsbA apparently does not act as an “aggregation inhibitor” in a chaperone-like way (Buchner et al., 1990).

The catalytic activity of coexpressed DsbA was strongly influenced by the GSH/GSSG ratio. DsbA activity was reduced when the relative amount of GSSG was increased and was tightly correlated with the GSH/GSSG-dependent yields of RBI without coexpression of DsbA (Table I).

The intracellular concentrations of native RBI in E. coli JM83 cells induced for 16 h (given in mg-liter⁻¹·OD₅₆₀ nm⁻¹) were determined by trypsin inhibition assays as described under "Experimental Procedures."

![Fig. 2. Coexpression of DsbA in the presence and in the absence of GSH.](image)

![Fig. 3. Determination of the yield of functionally expressed RBI and the stability of GSH in the growth medium.](image)

| Addition to the medium | Yield of native RBI | Relative increase | Yield of native RBI | Relative increase |
|------------------------|---------------------|------------------|---------------------|------------------|
|                        | mg-liter⁻¹·OD₅₆₀    |                  | mg-liter⁻¹·OD₅₆₀    |                  |
|                        | RBI                 |                  |                      |                  |
| 1 mM GSH               | 0.07                | 1.0              | 0.07                 | 1.0              |
| 5 mM GSH               | 0.05                | 0.7              | 0.42                 | 6.0              |
| 10 mM GSH              | 0.34                | 4.9              | 0.54                 | 7.7              |
| 1 mM GSSG              | 0.06                | 0.9              | 0.10                 | 1.4              |
| 1 mM GSH + 1 mM GSSG  | 0.09                | 1.3              | 0.38                 | 5.4              |
| 5 mM GSH + 1 mM GSSG  | 0.25                | 3.6              | 0.60                 | 8.6              |
| 10 mM GSH + 1 mM GSSG | 0.27                | 3.9              | 0.46                 | 6.6              |
be rescued when induced cells of E. coli JM83/pRBI were grown in
the absence of disulfide shuffling reagents and were suspended
in LB medium containing GSH/GSSG after harvest. Therefore, GSH and GSSG added to the medium at the time of
induction are directly involved in the oxidative folding of RBI in
vivo.

The fact that the yield of native RBI in the periplasm of E.
coli is diminished by GSSG and increased with GSH up to
concentrations of 10 mM with and without coexpression of DsbA
suggests that the reduction of incorrect disulfides limits the rate
and yield of folding of RBI in vivo. Due to the high cysteine
content of RBI (10 out of 122 residues) it appears likely that
folding intermediates with nonnative disulfides are signific-
antly populated during oxidative folding of the inhibitor.

From the known equilibrium constant between DsbA and
glutathione at pH 7 (1.2 × 10⁻⁴ M; Wunderlich and Glockshuber,
1993), it becomes clear that less than 1% of all DsbA molecules
were oxidized at the conditions providing maximal yields of
functional inhibitor (5 mM GSH and 5 mM GSH/1 mM GSSG).
Therefore, the almost fully reduced state of DsbA at optimal
conditions for folding of RBI further supports the view that
DsbA is mainly involved in the breakage of nonnative disulfides
during folding of RBI. Similar results were also reported for
eukaryotic PDI during the oxidative folding of RNase A in vitro
(Lyles and Gilbert, 1991). Thus, it seems likely that misfolded
proteins with nonnative disulfides occur during protein folding
in vivo. However, the almost fully reduced state of DsbA at
optimal conditions for folding of RBI does not necessarily ex-
clude a significant contribution of DsbA in the formation of
disulfides, since the oxidation of a folding polypeptide by DsbA
is in the order of 10³ to 10⁴ times faster than its reduction
(Wunderlich and Glockshuber, 1993). Therefore, high relative
amounts of reduced DsbA may indeed be required to guarantee
DsbA-catalyzed reduction of nonnative disulfides.

It appears likely that the optimal redox conditions for folding
of other secretory proteins in the periplasm of E. coli may be
different from the outlined conditions for maximal folding yield
of RBI. However, the best growth conditions for optimal yields
in the periplasm (in conjugation with coexpression of DsbA) can
easily be evaluated by varying the concentrations of GSH and
GSSG in the medium.

Unlike oxidative folding in the ER (Bulled and Freedman,
1988; Braakman et al., 1992), folding in the periplasm of E. coli is
probably not assisted by ATP-dependent chaperones like BiP
(Haas, 1991) due to the lack of an ATP-specific transport sys-
tem and the presence of alkaline phosphatase that hydrolyzes
ATP (Heppel et al., 1962). The absence of ATP-dependent chaper-
one that are known to prevent aggregation (Buchner et al.,
1991) (for a review, see Gething and Sambrook (1992)) is con-
sistent with the observation that periplasmic aggregates of RBI
have no effect on the bacterial growth. Since DsbA and pept-
dyprolyl cis,trans-isomerase (Liu and Walsh, 1990) are the
only known periplasmic components of E. coli promoting pro-
tein folding and may act synergistically (Schonbrunner and
Schmid, 1992), oxidative folding in the bacterial periplasm ap-
pears to be less complex than in the ER lumen. Recently, an-
other E. coli protein (termed DsbB) was found to be involved in
disulfide formation in E. coli. It was suggested that DsbB,
which is a component of the cytoplasmic membrane, may be
required to transfer oxidizing equivalents from the cytoplasm
to DsbA that in turn oxidizes folding polypeptide chains
(Bardwell et al., 1993). However, our results indicate that fold-
ing of RBI and probably also the redox state of DsbA is deter-
mined by the thiols and disulfides present in the medium.
Thus, DsbB may become essential for disulfide formation in E.
coli, when oxidants are rare in the medium (e.g. under anaer-
obic conditions), but may play a secondary role when oxidizing
equivalents are abundant. This is consistent with the finding
that the lack of DsbB (in dsb⁻ strains) can be complemented by
adding GSSG or cystine to the growth medium (Bardwell et al.,
1993) and with our finding that the thiol-dependent redox con-
ditions in the periplasm of E. coli can indeed be controlled by
thiol reagents in the medium. We have found that other thiol
reagents such as N-acetylcyesteine exhibit improvements of the
yield of functional RBI very similar to GSH when used at con-
centrations of 5 mM in the medium. Since N-acetylcyesteine is
less expensive than GSH, it may be more convenient for tech-
nological applications.

In conclusion, we believe that the possibility to control the
thiol-dependent periplasmic redox potential and the demon-
stration of the thiol-dependent activity of DsbA in vivo may be
useful for the investigation of oxidative protein folding in bac-
teria and may greatly facilitate the heterologous expression of
secretory proteins in E. coli.

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References—A list of references is provided in the
end of the document.