Protein Folding Activities of *Escherichia coli* Protein Disulfide Isomerase

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ABSTRACT: DsbA is an *Escherichia coli* periplasmic protein that mediates disulfide bond formation in newly secreted proteins *in vivo*. Addition of thiol reagents to purified dsbA reduces its disulfide bond and yields disulfide isomerase activity after removal of the thiol reagent. DsbA can catalyze the conversion of a stable misfolded protein, misfolded IGF-I (mis-IGF-I), to its correctly folded conformation under physiological conditions. This conversion is the result of breaking and re-forming two disulfide bonds. The uncatalyzed rate of this reaction is undetectable. Kinetic analysis of the reaction yielded a K_m of 43 μM and a k_{cat} of 0.2 min^{-1}. The oxidized form of dsbA stimulates the oxidative folding of completely reduced IGF-I at pH 7.0. Thus, dsbA has two possible functions depending on its redox state. The reduced form of the protein is a disulfide isomerase while the oxidized protein can assist formation of disulfide bonds in reduced substrates under physiological conditions.

The folding of polypeptides after secretion can be aided by the presence of chaperones, prolyl isomerases and disulfide isomerases. This last class of enzymes plays an important role in the correct pairing of cysteine residues in the secreted protein. In the eukaryotic endoplasmic reticulum (ER),[1] this function is performed by protein disulfied isomerase (PDI) (Goldberger et al., 1963; Lambert & Freedman, 1985). In *Escherichia coli*, a PDI homolog, dsbA, was identified through genetic screens (Bardwell et al., 1991; Kamitani et al., 1992). In strains lacking dsbA function, disulfide bond formation was slowed significantly. Normally a secreted protein is oxidized seconds after signal peptide cleavage (Pollitt & Zalkin, 1983) while in dsbA mutant strains oxidation can take minutes (Bardwell et al., 1991). The dsbA protein has homology with PDI and other disulfide oxidoreductases, but only at the proposed active-site region. This active site contains two cysteines spearated by two amino acids. These intervening residues usually include a glycine or proline or both, resulting in a turn or bend structure at the active site. The recently reported crystal structure of oxidized dsbA does reveal this bend structure (Martin et al., 1993a). The crystal structure also showed that the tertiary structure of dsbA resembled the tertiary structure of thioredoxin even though primary sequence homology outside the active site was low.

For a disulfide oxidoreductase to be an effective oxidizer of newly secreted proteins, a desirable property would be for the reduced form of the enzyme to be more stable than the oxidized form. This is indeed the case as reported in several recent *in vitro* studies (Zapun et al., 1993; Wunderlich & Glockshuber, 1993; Wunderlich et al., 1993). PDI and dsbA present rare examples where the disulfide bonds are destabilizing for the overall conformation of the protein. Normally disulfide bonds are effective stabilizers of protein structure (Goldenberg, 1985; Creighton, 1988). A crystal structure of the reduced form of dsbA will be interesting to compare with the oxidized form.

Previous protein folding studies using dsbA *in vitro* have shown mixed results. As an oxidant, dsbA was effective when present in a large molar excess over the reduced substrate alkaline phosphatase (Akiyama et al., 1992). Additionally, when present in a large molar excess, purified dsbA could convert scrambled ribonuclease (RNase) to enzymatically active RNase (Akiyama et al., 1992). Periplasmic fluid preparations containing wild-type or an increased level of dsbA can also isomerize scrambled RNase (Barth et al., 1988; Yu et al., 1993). A previous publication notes similar oxidative folding results with reduced bovine pancreatic trypsin inhibitor (BPTI), but no disulfide isomerase activity or rearrangement of incorrectly disulfide-bonded BPTI (Zapun et al., 1993). We decided to investigate the ability of dsbA to interact with a stable yet improperly disulfide-bonded protein. Human recombinant insulin-like growth factor I (IGF-I) can refold *in vitro*, forming two isomers differing in their disulfide bonding patterns (Raschdorf et al., 1988; Meng et al., 1989). IGF-I is a member of a family of proteins with three disulfide bonds that are homologous with insulin (Blundell et al., 1978). In the misfolded isomer of IGF-I (mis-IGF-I), two of the three disulfide bonds are incorrectly paired (Figure 1). Both of these disulfide bonds involve conserved adjacent cysteines. The misfolded isomer and the correctly folded IGF-I isomer (cor-IGF-I) are stable structures under physiological conditions in the absence of thiol reagents. At 4 °C, both isomers are stable indefinitely (Miller et al., 1993).

In the present study, we show that the disulfide isomerase activity of dsbA requires the reduced form of dsbA and that this activity is catalytic and not stoichiometric. Reduced dsbA can convert mis-IGF-I to cor-IGF-I without the aid of reducing agents. Additionally we show that the oxidized form of dsbA greatly stimulates the refolding of reduced IGF-I under physiological conditions.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human IGF-I was produced in *E. coli* and recovered at Genentech. The protein was expressed

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[1] Abbreviations: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; RNase, ribonuclease; BPTI, bovine pancreatic trypsin inhibitor; IGF-I, insulin-like growth factor I; mis-IGF-I; misfolded isomer of IGF-I; cor-IGF-I, correctly folded isomer of IGF-I, DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; IEF, isoelectric focusing.

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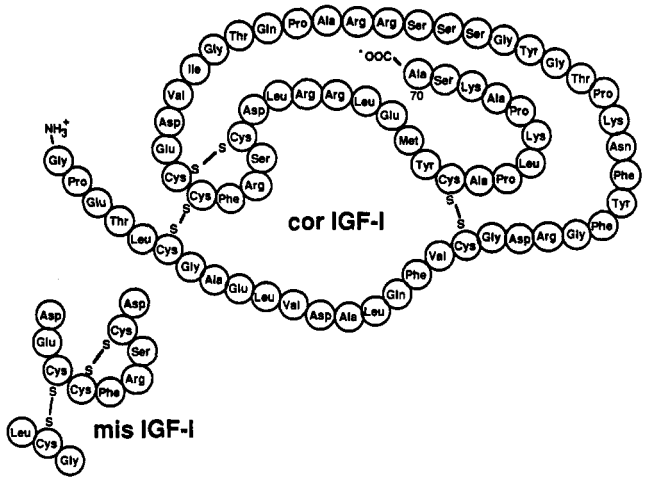

FIGURE 1: Two stable isomers of IGF-I. The primary amino acid sequence of the two disulfide isomers of human IGF-I is shown indicating the difference in disulfide bond pairing of the vicinal thiols. This is the only covalent change between the two isomers.

as the full-length mature IGF-I peptide by secretion into the *E. coli* periplasmic space. Mis-IGF-I and cor-IGF-I were made after refolding from inclusion bodies and preparatively collected after HPLC separation. Both isomers have been characterized previously (Canova-Davis et al., 1992; Hart et al., 1993). Bovine liver PDI was purchased from Pierce.

DsbA Preparation. DsbA was overexpressed from a pACYC-derived plasmid containing the *dsbA* gene downstream from an IPTG-inducible promoter, similar to Bardwell et al (1991). A four-way ligation was done where the (A) *Sau*3A–*Bss*HII and (B) *Bss*HII–*Hin*dIII fragments of p16-1 (Bardwell et al., 1991) encoding the *dsbA* gene were ligated with a (C) *Bsp*HI–*Xba*I fragment of pBR322tacII containing the *Tac*II promoter (deBoer et al., 1983) and (D) *Bsp*HI–*Hin*dIII-digested pACYC184. The *Xba*I and *Sau*3A sites were filled in with Klenow fragment prior to ligation. A W3110 lacIq derivative was grown in LB media with chloramphenicol (12.5 μg/mL) and induced with 1 mM IPTG at mid log of growth. Cell paste was stored at −20 °C. Twenty grams of paste (wet weight) was resuspended in 400 mL of 10 mM MOPS, pH 7.0, with the aid of a polytron tissue homogenizer. The resuspended cells were stirred on ice for 2 h. The soluble periplasmic proteins were separated from cells by centrifugation (5000*g*, 10 min, 4 °C). The supernatant was loaded on a 50-mL DEAE-Sepharose CL-6B column equilibrated in 10 mM MOPS, pH 7.0. The dsbA protein was eluted with a gradient of 0–0.1 M sodium chloride in the same MOPS buffer. Fractions were checked for dsbA by SDS–PAGE, pooled, and precipitated with 70% ammonium sulfate. After centrifugation (27000*g*, 20 min, 4 °C), the protein was resuspended in 10 mM HEPES·KOH, pH 7.5, and dialyzed versus 3 × 1L of the same buffer at 4 °C.

Reduction and Oxidation of DsbA. DsbA (190 μM) was incubated with either 10 mM DTT for reduction or 10 mM copper phenanthroline (Martin et al., 1993b) for oxidation. After 2 h at room temperature, the protein was separated from small molecules by chromatography on a 3-mL Sephadex G-15 column. Protein fractions were pooled, and the concentration was determined by a Bradford assay (Bradford, 1976). The modified protein was used immediately in a disulfide isomerase assay.

IEF Gel Electrophoresis. Novex (San Diego, CA) IEF gels were used according to the manufacturers' instructions. The gel was 5% polyacrylamide with ampholytes for separating in the pH 3–10 range. The anode buffer was 10 mM phosphoric acid, pH 2.4, and the cathode buffer was 17 mM arginine and 24 mM lysine, pH 10.2. The samples were loaded in cathode buffer with 30% glycerol. Gels were run for 1 h at 100 V, 1 h at 200 V, and then 30 min at 500 V followed by fixing in 5% (w/v) trichloroacetic acid, 10% (v/v) acetic acid, and 30% (v/v) ethanol.

Disulfide Isomerase Assay. Mis-IGF-I at a concentration of 15 μM was incubated with DsbA at 30 °C in 0.1 M Tris-HCl (pH 7.5)/0.1 M KCl. After 3 h, the samples were chilled on ice. The samples were stable on ice in the absence of added reducing agents. Chilling the samples was as effective as quenching with acid. Samples were passed through a 0.2-μm filter and placed in a chilled chamber for RP-HPLC analysis. The samples were analyzed by a C-18 (Vydac, 4.6 × 250 mm) column. The column was equilibrated in 29% acetonitrile/0.1% TFA (water/acetonitrile/TFA system). From 0 to 3 min, the acetonitrile concentration was raised linearly to 30%. From 3 to 5 min, the acetonitrile concentration was raised linearly to 32%. The column was then regenerated by raising the acetonitrile concentration to 50% and reequilibrated in 29% acetonitrile. Mis-IGF-I standard eluted at 4.7-min HPLC retention time, and cor-IGF-I standard eluted at 6.3-min retention time. DsbA eluted during regeneration of the column at 7.2-min retention time. The quantity of the two isomers was measured by integration of the peaks and comparison to fixed quantities of standards similarly analyzed. A plot of IGF-I concentration versus integrated area was linear within the concentrations used in these experiments. For the kinetic characterization of the disulfide isomerase activity, either 5 μM dsbA or 0.5 μM PDI was used. Analyses were performed every 10 min with the temperature controlled in the sample chamber at 37 °C. Each substrate concentration was repeated 3 times. Product accumulation was less than 10% for all initial velocity calculations.

Refolding from Completely Reduced IGF-I. IGF-I inclusion body protein was prepared from cell paste. Two grams of cell paste (wet weight) was resuspended in 25 mM Tris-HCl (pH 7.5) 5 mM EDTA. Lysozyme was added to 0.2 mg/mL and incubated for 15 min at room temperature. The lysed cells were placed on ice and sonicated with a Branson microtip probe sonicator for 10 × 1 min pulses with stirring between pulses. After centrifugation (12000*g*, 10 min, 4 °C), the pellet was resuspended in Tris-HCl/EDTA buffer with 1% lauroyl sarcosinate with the aid of a homogenizer. After 15 min at room temperature, the sample was centrifuged as described above. This extraction with sarkosyl/Tris-HCl/EDTA was repeated followed by two successive extractions with Tris-HCl/EDTA (no detergent). The pellet contained greater than 90% IGF-I as examined by SDS–PAGE (data not shown). Inclusion body IGF-I was solubilized and reduced with 2 M urea in 25 mM Tris-HCl, pH 9.0, 0.1 M KCl, 5 mM EDTA, and 20 mM DTT. After 4 h at room temperature, the sample was centrifuged at 15000*g*, 5 min, and loaded on a 3-mL Sephadex G-15 column equilibrated in 50 mM Tris-HCl (pH 9.0)/50 mM KCl. The IGF-I fractions were pooled, kept on ice, and later quantitated by RP-HPLC. There was no detectable fully oxidized protein at this point. To assess oxidative folding, the reduced IGF-I was incubated for 4 h at 37 °C in the presence of various reagents and chilled on ice. Samples were analyzed by RP-HPLC for quantitation of mis-IGF-I and cor-IGF-I concentrations.

RESULTS

Isolation and Characterization of DsbA. DsbA was purified from a strain overexpressing the protein. When frozen cell

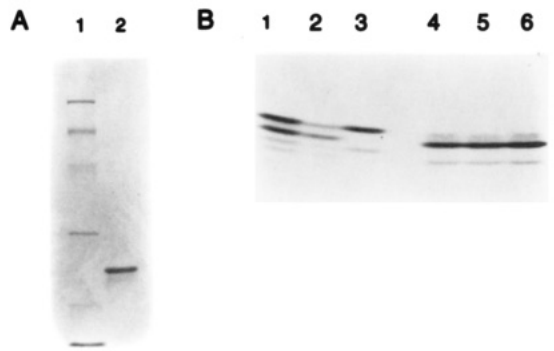

FIGURE 2: Electrophoresis of dsbA. (A) Reduced SDS–PAGE of dsbA purified after ammonium sulfate precipitation (see Experimental Procedures). Lane 1 is molecular mass standards (from the top of the gel: 97, 66, 45, 31, 21, and 14 kDa). Lane 2 is 1 μg dsbA. (B) Isoelectric focusing of dsbA after oxidation or reduction. Lane 1 is dsbA after ammonium sulfate precipitation. Lane 2 is dsbA after reduction and removal of DTT. Lane 3 is dsbA after oxidation and removal of copper phenanthroline. Lanes 4–6 are the same as lanes 1–3, respectively, except 10 mM DTT was included in the samples prior to electrophoresis. Both gels were stained with Coomassie blue.

paste is resuspended in low ionic strength buffer and stirred for 2 h, soluble dsbA and periplasmic proteins are separated from the cells by centrifugation. The resulting supernatant was then subjected to anionic exchange chromatography as described previously (Zapun et al., 1993). The purified protein was concentrated by ammonium sulfate precipitation. It migrates as a single band by SDS–PAGE (see Figure 2A, lane 2) but not by isoelectric focusing PAGE (Figure 2B, lane 1). A previous report had stated that dsbA was found solely in the oxidized form after purification by osmotic shock treatment of cells (Zapun et al., 1993). Another report stated that two forms of dsbA were isolated after purification by polymyxin B sulfate treatment of cells (Martin et al., 1993b). These forms are the oxidized and reduced versions of dsbA. We also note two predominant forms differing in their oxidation state (Figure 2B, lane 1). The amount of reduced and oxidized protein varied in different preparations even when the same cell paste was used. It seems that the time and conditions of handling the protein after anion-exchange chromatography, but before precipitation with ammonium sulfate, influence the relative amounts of the two forms. The minor variants migrating below the two main bands may be the result of carboxyl-terminal proteolysis. These bands are recognized by dsbA antiserum (data not shown). The last two residues of dsbA are both lysines, and the removal of these would increase the mobility with this gel system.

Previous reports on a disulfide isomerase activity of dsbA have differed depending on the substrate utilized (Zapun et al., 1993; Akiyama et al., 1992). We examined if dsbA had the ability to isomerize a stable but misfolded isomer of IGF-I (Figure 1) to the correctly folded isomer. The two isomers of IGF-I can be separated and quantitated by RP-HPLC (Canova-Davis et al., 1992; Hober et al., 1992; Miller et al., 1993; Hart et al., 1993). Addition of dsbA at one-tenth the molar concentration of mis-IGF-I yields a small amount of cor-IGF-I (data not shown). It appeared possible that not all of the dsbA was active and its activity might be dependent on its redox state. DsbA can be oxidized or reduced and the modifying agent removed. After oxidation, dsbA is very stable (Figure 2B, lane 3). After reduction, dsbA is less stable, and some of the protein is reoxidized after removal of the reducing agent (Figure 2B, lane 2). Purging solutions with argon does not completely prevent reoxidation. Quantitation of free sulfhydryl groups by Ellman's reagent yields an average of

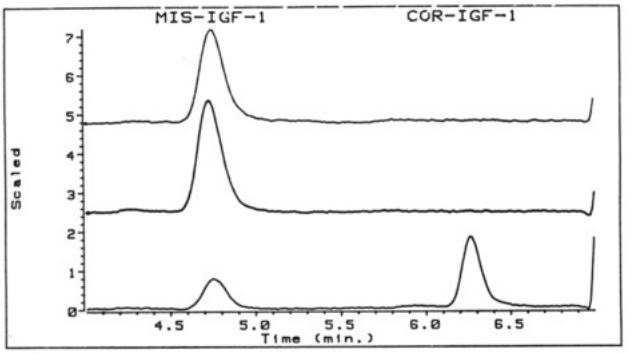

FIGURE 3: HPLC traces of IGF-I isomerization. The top profile is 15 μM mis-IGF-I injected alone. The middle profile is 15 μM mis-IGF-I after incubation with 15 μM oxidized dsbA. The bottom profile is mis-IGF-I after incubation with 15 μM reduced dsbA. All incubations were done at 30 °C, and all absorbances were measured at a wavelength of 280 nm. The time scale in minutes refers to the total HPLC retention time.

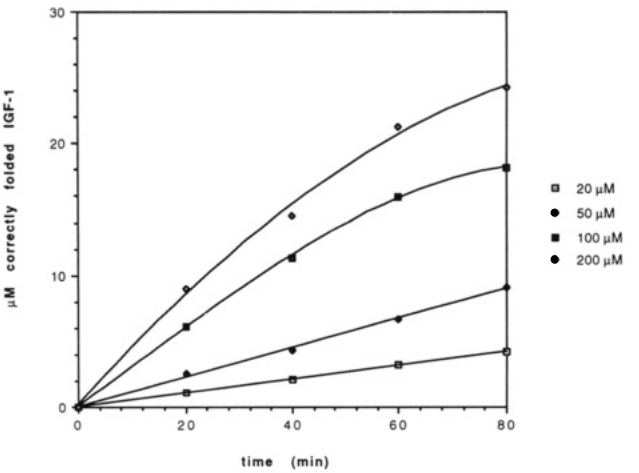

FIGURE 4: Time course of isomerization activity. Increasing levels of mis-IGF-I were incubated with 5 μM DTT-treated dsbA. Samples were incubated at 30 °C for the indicated times, and the concentration of cor-IGF-I was measured by HPLC analysis. The open squares refer to 20 μM mis-IGF-I, filled diamonds to 50 μM, filled squares to 100 μM, and open diamonds to 200 μM.

1.8 mol of SH/mol of protein after reduction and removal of the free reducing agent. When reduced dsbA is added, approximately 65% of the mis-IGF-I is converted to the cor-IGF-I isomer (Figure 3, bottom profile). This reaction is time-, temperature-, and pH-dependent (Figure 4 and data not shown). Most importantly, it is dependent on the presence of reduced dsbA. If the DTT solution alone is gel-filtered exactly as the reduced dsbA is, the corresponding fractions where dsbA normally elutes have no detectable isomerase activity. Concentrations of small molecular weight thiols equal to dsbA concentration do not isomerize mis-IGF-I at 37 °C and physiological pH (data not shown). Higher concentrations of thiols can induce isomerization as well as the formation of other IGF-I species (Miller et al., 1993). The sum of mis-IGF-I and cor-IGF-I is always greater than 90% of the starting mis-IGF-I when reduced dsbA is used. If the oxidized dsbA is incubated with mis-IGF-I, then no cor-IGF-I is produced (Figure 3, middle profile). The HPLC trace of this reaction is similar to without any dsbA added (Figure 3, top profile). The oxidation of dsbA is reversible. After copper-induced oxidation, dsbA can be reduced with excess DTT, and the protein regains its complete activity (data not shown).

Kinetic Characterization of Isomerase Activity. At this point, it was unclear if the reaction was stoichiometric or catalytic. When the concentration of dsbA is decreased to 5

Table 1: Initial Velocity Determinations for the Disulfide Isomerase Activity of DsbA at 5 μM Concentration, 37 °C, pH 7.5[a]

substrate concn (μM)	velocity (μM/min)	SD
20	0.33	0.11
40	0.46	0.15
50	0.53	0.03
80	0.60	0.02
100	0.71	0.06
200	1.00	0.17

[a] All measurements were done in triplicate as described under Experimental Procedures.

μM and the mis-IGF-I concentration increased, results indicate that the effect of dsbA on isomerization is catalytic. Greater than 5 μM cor-IGF-I is produced when the substrate-concentration is increased (Figure 4). Kinetic characterization of the conversion from mis-IGF-I to cor-IGF-I was then performed. Initial rates were measured before 10% of the substrate was converted to product and when product accumulation was linear with time. The enzyme shows saturation behavior in a velocity versus substrate concentration plot (data not shown). Nonlinear least-squares analysis of the velocity versus substrate concentration plot gives a K_m of 62 μM. A double-reciprocal plot of the data presented in Table 1 was linear and yielded a K_m of 43 μM, a v_{max} of 1.0 μM/min, and a k_{cat} of 0.2 min^{-1}. The uncatalyzed rate of isomerization is negligible (Figure 3, top profile) as the isomers of IGF-I are stable indefinitely with time in the absence of reducing agents (Miller et al., 1993). A thermodynamic equilibrium exists between the two disulfide isomers of mature IGF-I in the presence of disulfide reagents (Hober et al., 1992; Miller et al., 1993). This may result from refolding the protein without its natural carboxyl-terminal sequences. These sequences were deleted for production in *E. coli*. As a result of the equilibrium, dsbA can only convert a portion of the substrate to the product, approximately two-thirds. When reduced dsbA is incubated with cor-IGF-I only, approximately one-third is converted to mis-IGF-I (data not shown). This indicates that the isomerization is reversible. Similar ratios of cor-IGF-I to mis-IGF-I have been seen when IGF-I is refolded after treatment with small thiol reagents (Miller et al., 1993; Hart et al., 1993).

PDI can also isomerize mis-IGF-I to cor-IGF-I. In the presence of a redox buffer consisting of 100 μM reduced and 25 μM oxidized glutathione, PDI has a 17-fold higher turnover number (3.4 min^{-1}) but a similar K_m (36 μM, data not shown) as dsbA. In the absence of a redox buffer, PDI has no activity. The concentration of glutathione was chosen to minimize enzyme-independent isomerization that accompanies formation of other IGF-I species. Unlike PDI, the isomerase activity of dsbA is not stimulated in the presence of a redox buffer of glutathione. If PDI is reduced by excess DTT for 1 h and gel-filtered, the isomerase kinetics are similar to those for PDI with a redox buffer present (J. Joly, unpublished results). This activity is labile with time. The active-site cysteines may be reoxidized by dissolved oxygen in solution, thereby inactivating the isomerase activity.

Stimulation of Oxidative Folding. Previous work with dsbA has shown that it stimulated oxidative folding of reduced substrates (Akiyama et al., 1992; Zapun et al., 1993). We examined if dsbA could produce cor-IGF-I from a completely reduced substrate. Inclusion body IGF-I was solubilized and reduced with urea and DTT which were subsequently removed by gel filtration. The reduced IGF-I was then refolded at pH 7.0 in the presence of increasing concentrations of dsbA. In the absence of dsbA, only a small percentage of IGF-I refolded to cor-IGF-I (Figure 5). The completely reduced form of

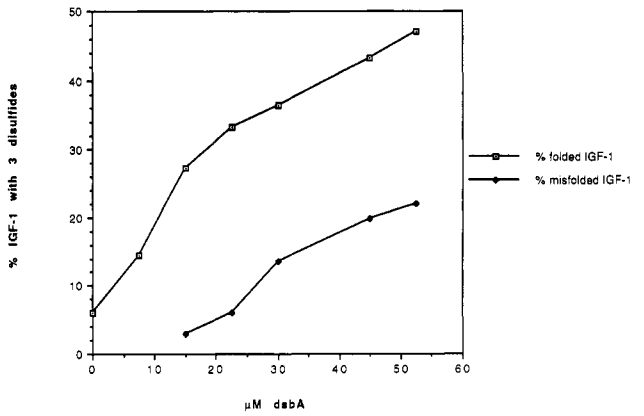

FIGURE 5: Oxidative folding of IGF-I. Reduced and solubilized IGF-I from inclusion bodies was incubated with increasing concentrations of dsbA for 4 h at 37 °C. The samples were analyzed by HPLC to quantitate the levels of mis-IGF-I (filled diamonds) and cor-IGF-I (open squares).

IGF-I is unstable at pH 7.0 in the absence of denaturants and precipitates from solution with time. The presence of dsbA relieves this problem. With an increasing concentration of dsbA, more cor-IGF-I is produced. Mis-IGF-I is also produced with greater dsbA concentration. The ratio of cor-IGF-I to mis-IGF-I is roughly consistent as evidenced by the two curves in Figure 5. The oxidized form of dsbA is a more potent stimulator of IGF-I refolding than the reduced form (data not shown). Catalysis of IGF-I refolding does not occur in this reaction as the concentration of dsbA necessary to produce completely disulfide-bonded protein is higher than the IGF-I concentration and roughly equal to the concentration of potential IGF-I disulfide bonds.

DISCUSSION

Previous work with dsbA demonstrated that the protein possessed the ability to isomerize scrambled RNase to enzymatically active RNase (Akiyama et al., 1992). Our work also demonstrates that dsbA has isomerase activity but additionally shows that dsbA is a true catalyst. It can perform multiple rounds of isomerization per molecule of enzyme. Not all misfolded substrates may work as Zapun et al. (1993) noted that dsbA had little or no effect on the rates of formation, rearrangement, or reduction of the three disulfides of BPTI. It is possible though that the dsbA used in that study may not have been in its active form for disulfide isomerase activity as the protein was completely oxidized after purification. While implicit in its ability to isomerize misfolded disulfide bonds, the fact that a reduced oxidoreductase enzyme is required for isomerization has not been previously demonstrated. The protocol for study of disulfide oxidoreductases with protein substrates involves adding a small amount of reducing agent. This may be DTT as in the case for the reduction of insulin (Holmgren, 1979; Bardwell et al., 1991) or a redox buffer consisting of reduced and oxidized glutathione (Lyles & Gilbert, 1991; Weissman & Kim, 1993) mimicking the folding environment of the ER (Hwang et al., 1992). We were able to eliminate the requirement for the small thiol reagents during the isomerization reaction, simplifying the number of molecules involved. Free thiol groups in reduced but native PDI can be modified with iodoacetic acid. This modified enzyme is then inactive (Hawkins & Freedman, 1991), which is consistent with the result that free sulfhydryl groups in the disulfide isomerase are necessary for isomerization. Our present work also then leads to the conclusion that a covalent intermediate exists between dsbA and the substrate.

The assay presented here is a simple and reliable indicator of disulfide isomerase activity. The population of substrate and product is uniform as there is only one isomer for each. Scrambled RNase contains many isomers, only some of which may be substrates for disulfide isomerases. RNase can also fold spontaneously although the rate is enhanced by either dsbA (Akiyama et al., 1992) or PDI (Lyles & Gilbert, 1991; Hawkins et al., 1991). The refolding of RNase does offer a continuous monitoring assay while IGF-I does not, but several time points followed by HPLC analysis allow effective monitoring of reaction rates with IGF-I. Additionally, substrates of a reduced or misfolded enzyme, such as cytidine cyclic monophosphate for scrambled RNase, present during refolding may induce or enhance the folding of certain domains of the enzyme.

The kinetics of disulfide isomerization by PDI (Hawkins et al., 1991; Weissman & Kim, 1993) and dsbA are rather slow *in vitro*. Turnover numbers are usually around 1 min^{-1} with any substrate tested. The Michaelis constants are usually in the low micromolar range which is impressive considering that these enzymes should be able to recognize a diverse set of substrates. The slow rates of isomerization may be due to the *in vitro* conditions employed but then again may not. This leads to a larger question of whether the isomerase activity is important *in vivo*. Clearly bacteria (Bardwell et al., 1991) and yeast (LaMantia & Lennarz, 1993) can survive without dsbA or the disulfide-forming activity of PDI although normal functions are impaired. The more important function of these enzymes may be disulfide formation and not isomerization. Isomerization could be a quality control mechanism or checkpoint after secretion. *In vivo* in *E. coli*, disulfide bonds form within seconds after translocation across the cytoplasmic membrane. Clearly the cell has efficient and capable means of forming these bonds. It is unclear how often the isomerization activity would be needed as most *E. coli* periplasmic and outer membrane proteins contain few disulfide bonds. In fact, examples of more than two disulfide bonds are rare in periplasmic and outer membrane proteins but do exist. A quick survey of the Dayhoff protein database revealed only 5 out of 58 periplasmic and outer membrane proteins in *E. coli* have the potential to form more than 2 disulfide bonds. Of these, none had more than four disulfide bonds per monomer. This is in contrast with many mammalian secretory proteins which contain a large number of disulfide bonds and show long transit times in the ER (Lodish & Kong, 1991). Both human serum albumin and tissue plasminogen activator have 17 disulfide bonds.

The redox buffer in the eukaryotic ER is glutathione (Hwang et al., 1992). It is unclear what, if any, is the redox buffer in the periplasm of *E. coli*. Glutathione can be detected in the media of bacterial cultures at micromolar levels (Owens & Hartman, 1986). Unlike the sealed environment of the ER, the outer membrane is readily permeable to small molecules. Another unknown factor is what regenerates or oxidizes dsbA *in vivo* after it has become reduced. All *in vitro* refolding experiments with reduced substrates have shown a stoichiometric effect rather than a catalytic one (Akiyama et al., 1992; Zapun et al., 1993). *In vivo*, dsbA must be reoxidized to reacquire disulfide bond formation activity. One theory is that dsbB, a cytoplasmic membrane protein with several cysteines in proposed periplasmic loops, performs this function (Bardwell et al., 1993). DsbB mutants are hypersensitive to DTT (Missiakas et al., 1993) and result in dsbA becoming completely reduced *in vivo* (Bardwell et al., 1993). What controls the redox state of dsbA and upstream redox events will be important for controlling the *in vivo* folding of secreted proteins.

ADDED IN PROOF

While this paper was under review, Wunderlich et al. (1993a) demonstrated catalytic refolding with dsbA at pH 4.0.

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