The Oxidase DsbA Folds a Protein with a Nonconsecutive Disulfide*5

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One of the last unsolved problems of molecular biology is how the sequential amino acid information leads to a functional protein. Correct disulfide formation within a protein is hereby essential. We present periplasmic ribonuclease I (RNase I) from Escherichia coli as a new endogenous substrate for the study of oxidative protein folding. One of its four disulfides is between nonconsecutive cysteines. In general view, the folding of proteins with nonconsecutive disulfides requires the protein disulfide isomerase DsbC. In contrast, our study with RNase I shows that DsbA is a sufficient catalyst for correct disulfide formation in vitro and in vivo. DsbA is therefore more specific than generally assumed. Further, we show that the redox potential of the periplasm depends on the presence of glutathione and the Dsb proteins to maintain it at ~165 mV. We determined the influence of this redox potential on the folding of RNase I. Under the more oxidizing conditions of dsb− strains, DsbC becomes necessary to correct non-native disulfides, but it cannot substitute for DsbA. Altogether, DsbA folds a protein with a nonconsecutive disulfide as long as no incorrect disulfides are formed.

Disulfide bond formation is an essential step in the folding of many proteins. Anfinsen et al. (1) have stated that “From chemical and physical studies of reformed enzyme, it may be concluded that the information for correct pairing of half-cystine residues in disulfide linkage, and for the assumption of the native secondary and tertiary structures, is contained in the amino acid sequence itself”. The reformed enzyme is bovine pancreatic ribonuclease A, a protein with four disulfides (three between nonconsecutive cysteines and one between consecutive). However, the rate of in vitro disulfide bond formation was much slower than the rates observed in living cells, and the yield of active enzyme was low (much of the protein containing the incorrect disulfide bonds). The explanation for the fast in vivo rates is resolved with the discovery that an enzyme is required to catalyze efficient disulfide bond formation (2–5), protein-disulfide isomerase (PDI). PDI is present in the lumen of the endoplasmic reticulum and is composed of four thioredoxin-like domains. This essential enzyme has the ability to catalyze both the oxidation of new disulfides and the isomerization of existing disulfides (6).

In Escherichia coli, the periplasmic protein DsbA is the catalyst of disulfide formation (4, 7). DsbA, however, is not a very specific oxidant and can introduce non-native disulfide bonds, which need to be corrected. To correct these non-native disulfides, the cell uses two protein disulfide isomerases, DsbC (8, 9) and DsbG (10, 11). The structural and enzymatic properties of both enzymes and their recycling by the inner membrane protein DsbD are reported in a recent review (7). Today, the native substrates of DsbG are not known.

Most of the substrates in the study of oxidative protein folding in E. coli are eukaryotic in origin (12–14). In the periplasm of E. coli, there are more than 300 proteins with at least two cysteines, suggesting that they are potential physiological substrates for DsbA (15). DsbA can properly fold the vast majority of these proteins. However, some of these proteins, like the peptidoglycan amidase MepA and the phytase AppA contain multiple disulfides, some of which are formed between cysteines in nonconsecutive positions (16, 17). Work on AppA and MepA suggests that the formation of these nonconsecutive disulfides is dependent on DsbC (18, 19).

We have determined the structure of another DsbC substrate (18), the periplasmic ribonuclease (RNase I) (20). We show that RNase I contains four disulfides, three consecutive and one non-consecutive and is therefore a physiologically relevant model protein to study oxidative protein folding in E. coli. Through studies of wild type and disulfide mutants of RNase I as substrates for the Dsb oxidoreductases, we show that a protein with a nonconsecutive disulfide may in vivo as well as in vitro fold to its proper structure with DsbA alone and that the dependence on DsbC is linked to the redox state of the environment.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification**—RNase I disulfide mutants are constructed. Wild type DsbA, DsbC, DsbG, RNase I, and the RNase I mutants are expressed and purified. The details can be found in the supplemental “Experimental Procedures” data.

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[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures.

The atomic coordinates and structure factors (code 2PQY) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[3] The abbreviations used are: RNase I, ribonuclease I; ruRNase I, reduced and unfolded RNase I; scRNase I, scrambled RNase I; GdnHCl, guanidine hydrochloride; Mes, 4-morpholineethanesulfonic acid.
Reduced and Unfolded RNase I (ruRNase I)—RNase I (~10 mg/ml) was reduced and unfolded in a buffer solution containing 50 mM Tris/HCl, pH 8.7, 50 mM NaCl, 6 mM GdnHCl, and 100 mM dithiothreitol. The mixture was incubated for 2 h at room temperature. To remove the excess of GdnHCl and dithiothreitol, the mixture was dialyzed against 50 mM Tris/HCl, pH 8.7, 0.6 mM GdnHCl, 50 mM NaCl, and 1 mM dithiothreitol at 15 °C. The dialysis buffer was argon-flushed for 30 min. All 8 cysteines were found to react with Ellman reagent indicating that all were reduced (21).

Scrambled RNase I (scRNase I)—To induce the formation of incorrect disulfides in ruRNase I (scrambled RNase I), the sample of ruRNase I was incubated with 50 μM CuCl2 and 2 mM H2O2 for 30 min at 25 °C. Excess of CuCl2 and H2O2 were removed by a final dialysis against 50 mM Tris, pH 8.7, 0.6 mM GdnHCl, and 50 mM NaCl.

In Vitro Folding—RNase I was refolded by mixing ruRNase I or scRNase I with Dsb protein (DsbA and/or DsbC and/or DsbG) in folding buffer solution to obtain a final concentration of 5 μM RNase I and 20 μM Dsb protein. The folding mixture was incubated for 3 min at 15 °C. All buffer solutions were argon-flushed for 15 min. Details about the folding buffer solution preparation are in the supplemental data.

RNase I Activity Assay—The RNA hydrolysis activity was measured in a methylene blue RNase I assay (22). The absorbance change was followed in function of time at 659 nm. The measured RNase activities are the initial velocities determined on the first 10% of the progress curves. A linear response was observed for RNase I concentrations between 9 and 36 nm. More details can be found in the supplemental “Experimental Procedures” data.

Dsb-refolded RNase I for Circular Dichroism (CD) Spectroscopy and Crystallization—After 3-min refolding at 15 °C, the reaction was stopped by injecting the sample on a Ni2+-Sepharose column equilibrated in 50 mM Hepes/NaOH, pH 8.0. As a reaction was stopped by injecting the sample on a Ni2+ column, the sample was dialyzed against 50 mM Tris/HCl, pH 8.7, 0.6 mM GdnHCl, 50 mM NaCl, and 1 mM dithiothreitol at 15 °C. The dialysis buffer was argon-flushed for 30 min. All 8 cysteines in ruRNase I (scrambled RNase I), the sample was dialyzed against 50 mM Tris/HCl, pH 8.7, 0.6 mM GdnHCl, 50 mM NaCl, and 1 mM dithiothreitol at 15 °C. The dialysis buffer was argon-flushed for 30 min. All 8 cysteines were found to react with Ellman reagent indicating that all were reduced (21).

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The crystals were first soaked for 1 min in a cryoprotectant solution containing 10 mM Tris, pH 7.5, 20% sucrose, and 2.5 mM EDTA. The equivalent of the pellet obtained from a 300-ml culture containing the mutants dsbA::kan, dsbC::cm, dsbG::kan, gshA::kan, CydD::kan, and the double mutant strains dsbA::kan/dsbC::cm, dsbC::cm/dsbG::kan were introduced by P1 transduction into MC1000 wild type E. coli. Cells were grown overnight at 37 °C in LB medium with their specific antibiotic(s). This pre-culture was diluted 100 times in a chemical defined M63 medium (25), except that the casaminoacids only contained isoleucine and leucine. The culture was grown at 37 °C until it reached an A600 of 1.0. After the determination of the exact volume and cell density, cells were harvested by centrifugation (7000 × g in JLA-8.1, 15 min) at 4 °C. The equivalent of the pellet obtained from a 300-ml culture with an A600 nm of 1 was resuspended in 1-ml buffer solution containing 10 mM Tris, pH 7.5, 20% sucrose, and 2.5 mM EDTA. The sample was left for 30 min on ice and subsequently diluted five times with H2O containing 1 mM gadolinium chloride to block cytoplasmic leakage (26). After another 15 min on ice, the supernatant of a 3500 × g × 30-min centrifuge run was 0.22 μM filtered and argon-flushed for 15 min.

Redox Potential of the Periplasm—The redox potential in the periplasm of wild type E. coli MC1000 and in the dsb− strains was measured at a sample temperature of 23 °C with a WTW Sentox ORP Ag/AgCl electrode. The electrode was calibrated with redox calibration solution (Schott Instruments) and corrected for $E^\circ_{Ag/AgCl} = 220$ mV according to $E_{measured_{Ag/AgCl}} - E^\circ_{Ag/AgCl} = E_{Ag/AgCl}$. The chemical redox potential ($E_{Ag/AgCl}$) at $[H^+] = 1 $ M was correlated to a biochemical redox potential at pH 7. Therefore, the $E_{Ag/AgCl}$ values were corrected to pH 7 with −59 mV/pH unit to obtain $E^\circ_{Ag/AgCl}$. Starting from a stock of 50 mM GSH and 50 mM GSSG in a buffer solution of 50 mM Hepes, pH 7.0, 4% sucrose, 0.5 mM EDTA, and 1 mM gadolinium chloride and based on the Nernst equation with $E^\circ_{GSH} = 240$ mV, a glutathione redox series was prepared with a calculated redox potential ($E^\circ_{GSH(cal)}$) ranging from −100 to −170 mV. After 15-min argon flushing, the redox potential of the glutathione series ($E^\circ_{GSH(cal)}$) was measured. The linear correlation between $E_{GSH(Ag/AgCl)}$ and $E_{GSH(cal)}$ was used to obtain the respective biochemical glutathione redox potentials of the periplasmic fractions ($E^\circ$).

Oxidative Protein Folding Revisited

The crystals were first soaked for 1 min in a cryoprotectant solution containing 0.1 M Mes, pH 6.1, 0.1 M ammonium acetate, 10% glycerol, 1 mM MgCl2 and 28% PEG3350 and subsequently flash-frozen in the cryostream. Data were integrated and scaled using the HKL suite of programs (23) (Table 1). The structure was refined using Crystallography and NMR System software 1.0 (24) starting from the refined co-ordinates of the original in vivo folded protein (Protein Data Bank code 2PQX). In the initial stages of refinement a slow cool protocol was alternated with the refinement of individual atomic B-factors. When the structure was near completion, the slow cool protocol was abandoned in favor of restrained positional refinement. Refinement statistics are given in Table 1.

Periplasmic Extract Preparation—The isogenic strains containing the mutants dsbA::kan, dsbC::cm, dsbG::kan, gshA::kan, CydD::kan, and the double mutant strains dsbA::kan/dsbC::cm, dsbC::cm/dsbG::kan were introduced by P1 transduction into MC1000 wild type E. coli.

3 J. Messens and R. Loris, unpublished data.
Oxidative Protein Folding Revisited

RESULTS AND DISCUSSION

All Disulfides of RNase I Are Essential for Optimal RNA Hydrolysis—We recently solved the crystal structure of E. coli RNase I at 1.4 Å resolution (Protein Data Bank code 2PQX). This structure shows an RNase T2-fold including three consecutive and one nonconsecutive disulfides (Fig. 1A). The N-terminal (1 SS), the nonconsecutive (2 SS), and the C-terminal disulfides (4 SS) of RNase I are structurally conserved (Fig. 1B).

All four disulfides must be correctly formed for RNase I to function properly. We mutated the disulfide bonds one by one by replacing the respective cysteines by alanines and measured RNA hydrolysis spectrophotometrically with methylene blue intercalating RNA as a substrate (22).

The Cys25-Cys39 disulfide is located within α-helix 1 between the β1 and β2 strands (Fig. 1A). After we mutated this disulfide, the RNA hydrolysis activity dropped 10-fold relative to wild type. Residues between Cys25 and Cys39 (Fig. 1A, orange) might be important for the correct positioning of the RNA substrate by base binding. A possible candidate residue is Phe24, which is also found on this position in the structure of Nicotiana alata Sps11-RNase (27).

The next disulfide is between the nonconsecutive cysteines Cys80 and Cys136 at the C-terminal end of the α-helix 6, which bears the conserved catalytic residues Glu129, Lys132, and His133. Removing this disulfide fully repressed the expression of the C80A/C136A double mutant. Here, the exposure of conserved hydrophobic residues located on the N-terminal site of α-helix 7 (Fig. 1A, green) and on α-helix 6 (Fig. 1A, blue) might result in the formation of aggregates or the misfolded protein might be proteolytically degraded.

Removing the consecutive Cys97-Cys124 disulfide located on the N-terminal site of α-helix 6 reduced the RNA hydrolysis activity to 10% of wild type. As such, the consecutive Cys97-Cys124 and the nonconsecutive Cys80-Cys136 disulfides, located on both sites of the α-helix 6 (Fig. 1A, blue), are essential for positioning the side chains of the residues of the active site and of the conserved hydrophobic pocket (Fig. 1A).

Less important for RNA hydrolysis is the conserved Cys197-Cys234 disulfide. Mutating this disulfide reduced the activity to 86% of wild type. The fact that RNase I is an endogenous enzyme that depends on the formation of a nonconsecutive disulfide for correct folding makes it a nice model to compare the ability of Dsb proteins to catalyze the formation of a nonconsecutive disulfide in vitro and in vivo.

In Vitro, DsbA Alone Folds a Protein with a Nonconsecutive Disulfide—Despite the presence of a nonconsecutive disulfide, DsbA on its own refolds reduced, unfolded RNase I. We have studied the specific role of DsbA, DsbC, and DsbG in the folding of reduced, unfolded RNase I (ruRNase I) and evaluated activity and structure (Fig. 2, A and B). The work of Haber and Anfinsen (28) on bovine pancreatic RNase A shows spontaneous folding and disulfide formation by molecular oxygen. Therefore, to discriminate between noncatalyzed, spontaneous folding and Dsb-catalyzed folding of recombinant RNase I, we performed experiments within 3 min at 15 °C, pH 7.2. Under these conditions, in glutathione redox buffer solution, the yield of noncatalyzed refolded RNase I was less than 10% in all the assays.

RNase I was correctly refolded by DsbA, as shown by using kinetics and CD spectroscopy measurements (Fig. 2, A and B). In addition, the crystal structure of DsbA-refolded RNase I (Table 1) turned out to be identical to the in vivo folded protein (root mean square deviation of 0.16 Å for 233 Cα atoms) (Fig. 1A) univocally proving that DsbA can correctly refold a protein with a nonconsecutive disulfide.

Remarkably, the refolding by DsbC results in about the same amount of functional RNase I as refolding by DsbA. DsbC can function as an oxidase in vitro; this result agrees with the observation of Zapun et al. (14).
We then prepared scRNase I in the presence of CuCl2 and H2O2, i.e., RNase I with incorrect disulfides (29). No free cysteines were detected with Ellman reagent (21), and scRNase I had completely lost its activity. DsbA was essentially unable to refold scRNase I, and only with the help of the isomerase activity of DsbC could RNase I regain its activity and structure (Fig. 2, A and B). DsbC seems to play an essential role in the handling of incorrect disulfides, which may form in a more oxidizing environment. On the other hand, DsbG, which has been shown to have significant isomerase activity (10), does not catalyze the refolding of scrambled or reduced-unfolded RNase I.

DsbA and DsbC Work in Concert—We studied the influence of the redox potential on the Dsb-catalyzed refolding of RNase I. The redox potential influenced the in vitro refolding of ruRNase I very little (Fig. 2C). Between −120 and −170 mV, the efficiency of refolding of ruRNase I by DsbA and DsbC alone was found within the same range. The highest yield of refolded ruRNase I was obtained under the condition where DsbA was combined with DsbC and this over the whole redox potential range tested (Fig. 3B). As such, DsbA and DsbC seem to work in concert to increase the efficiency of the refolding of ruRNase I.

In Vivo, DsbA Is Essential to Catalyze RNase I Folding—To check the Dsb-dependence for the folding of RNase I in vivo, single and double knock-out strains of the different dsb genes were constructed. To probe for structural integrity of RNase I, RNA hydrolysis activity in periplasmic fractions was used (Fig. 3A). Periplasmic fractions were prepared with osmotic shocks in the presence of 1 mM gadolinium chloride to block cytoplas-

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**TABLE 1**

| Structural statistics of DsbA-refolded RNase I | RNase I |
|---------------------------------------------|--------|
| Data collection                             |        |
| Beamline                                    | X12    |
| Wavelength (Å)                              | 0.95   |
| Space group                                 | P2,    |
| Unit cell                                   |        |
| a (Å)                                       | 39.6   |
| b (Å)                                       | 49.5   |
| c (Å)                                       | 53.3   |
| β (°)                                       | 77.7   |
| Resolution limits                           | 15.0-2.30 (2.38-2.30) |
| Number of measured reflections              | 31851 (3231) |
| Number of unique reflections                | 9192 (979) |
| Completeness                                | 100.0 (100.0) |
| Rmerge                                      | 0.072 (0.281) |
| Rfactor                                     | 11.9 (4.6) |

| Refinement                                   |        |
| R-factor                                     | 0.192 (0.231) |
| Rmerg-factor                                 | 0.240 (0.328) |
| r.m.s. bond lengths (Å)                      | 0.065   |
| r.m.s. angles (°)                            | 1.512   |
| Number of water molecules                    | 82      |
| Ligands                                      | Mes, Ca2+ |

| Ramachandran profile                         |        |
| Core                                         | 92.4%   |
| Other allowed                                | 7.6%    |
| Disallowed                                   | 0.0%    |

| Protein Data Bank code                       | 2PQY    |

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* r.m.s., root mean square.

Oxidative Protein Folding Revisited

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Excluded as a possible folding catalyst because a main folding catalysts involved. Oxidized DsbC can be recycled, indicating that DsbA and/or oxidized DsbC are the is unlikely to function as an isomerase because DsbC is not decrease of the RNase I activity. In the absence of DsbD, DsbC is still unclear (7). Also removing DsbD shows hardly any redox potential of the periplasm of E. coli. For each condition at least 10 replicates were measured. The ured redox potential was correlated to a glutathione biochemical redox potential at pH 7 (see “Experimental Procedures”).

In a dsbA strain, no RNase activity was detected, confirming the dependence of this protein on DsbA and suggesting that DsbC cannot substitute for DsbA. In a dsbC strain the activity drops 2-fold relative to wild type, indicating that DsbA can reasonably well catalyze the formation of the required nonconsecutive disulfide, even in a more oxidizing (vide infra) complex periplasmic environment.

dsb Strains Have a More Oxidizing Periplasm—The redox potential of the periplasm is not known. According to our knowledge, this is the first time that the redox potential of the periplasm of E. coli is determined. The redox potential is pH-dependent as soon as protons are involved. In the wild type dsb strains, the pH was found to be around 6.6 \( \pm 0.3 \) (n = 20), which is also the optimal pH for RNase I catalysis. The measured redox potential was correlated to a glutathione biochemical redox potential at pH 7 (see “Experimental Procedures”). For each condition at least 10 replicates were measured. The redox potential of the periplasm of E. coli was found to be \(-165 \pm 10\) mV (n = 15) (Fig. 3B). Removing DsbC shifts the redox balance to a more oxidizing condition. Unexpectedly, removing DsbA also increases the redox potential of the periplasm to a more oxidizing environment (from \(-165\) mV to \(-147\) mV) (Fig. 3B). For comparison, 30 mV is about the difference in redox potential observed between a differentiated and an apoptotic cell (31).

As in the dsbC/dsbG (\(-142\) mV) and dsbA/dsbD (\(-133\) mV) strains, the more Dsb proteins are removed, the more oxidizing the periplasm becomes. Altogether, our data where the redox potential is high and is in agreement with our in vitro results.

Glutathione and the Dsb Proteins Determine the Redox Balance of the Periplasm—We determined whether glutathione could play the role of a redox buffer in the periplasm, as it does in the cytoplasm. Removing one of the gene products required for the synthesis of glutathione (gshA) or the recently identified glutathione transporter (CydD) (32) increases the redox potential of the periplasm to \(-125\) mV. Our results suggest that glutathione does play the role of a redox balance in the periplasm. However, it has no effect on RNase I activity (Fig. 3). As such, the formation of correct disulfides in RNase I is clearly kinetically driven by DsbA and DsbC and not by its redox environment. The cell seems to have evolved to a DsbA-DsbC cooperating system balanced by the presence of GSH/GSSG and the Dsb proteins. Removing one of the redox components has consequences on the redox potential.

Oxidative Protein Folding Revisited—One of the established folding ideas is that there exists a correlation between the disulfide pattern and the DsbC-dependence. Proteins that contain nonconsecutive disulfides depend on DsbC for proper folding, whereas proteins with consecutive disulfides show no dependence on DsbC (12, 18, 19, 33). Our results on RNase I are surprising and in apparent contradiction with this view.

Hiniker and Bardwell (18) reported that dsbC strains show no RNase I activity and as such concluded DsbC-dependence of RNase I. However, in contrast to their results, we clearly observe RNase I activity in a dsbC strain, what makes that DsbA is reasonably good in folding a protein with a nonconsecutive disulfide in a more oxidizing environment. The discrepancy is likely to originate from a different technique used to measure RNase activity in vivo.

Berkmen et al. (19) obtained the same results as ours for phytase AppA, a nonconsecutive disulfide containing enzyme of E. coli. Also here, AppA refolds partially in a dsbC strain and shows almost base-line activity in a dsbA strain. However, the in vitro capability of DsbA to refold AppA was not studied, and the redox potential during the folding was not taken into account. They conclude that the nonconsecutive disulfide bond of AppA renders it dependent on DsbC. Our data show that there is another way to interpret these results. The observed
decrease of AppA activity in a dsbC− strain could be because of the increase of the redox potential causing the formation of incorrect disulfides.

For RNase I, the combination of the folding catalysts DsbA with DsbC results as well in vivo as in vitro in the highest folding efficiency even in a more oxidizing environment. On the other hand, DsbC on its own does not catalyze the folding of RNase I in vivo, whereas in vitro it was found to be as good as DsbA. This discrepancy emphasizes the difference in chemical environment between the complex environment of the periplasm and a buffer solution with glutathione. Mimicking an in vivo periplasm with the correct redox buffer solution at equilibrium in the presence of its Dsb proteins seems to be only part of the folding story. Living cells are not at equilibrium but are maintained at a steady state. In fact, a cell at true equilibrium is a dead cell. Therefore, the rates of the biochemical reactions coupled between DsbA and DsbB, and between DsbC and DsbD, and with all other compounds further downstream might be more relevant for proper folding than the equilibrium constants.

New in the oxidative folding interpretation of the in vivo results is the fact that until now nobody has ever taken the effect of redox potentials in dsb− strains into account. In a dsb− strain, the redox potential increases, and in this more oxidizing environment, incorrect disulfides might be introduced. Especially under this condition, the protein would benefit from the presence of DsbC to correct non-native disulfides. As such, for future interpretations of results obtained in dsb− strains, the redox potential change of the periplasm should be systematically taken into account.

By using dsb− strains from E. coli in combination with RNase I mutants, we showed in vitro and in vivo that although the presence of a nonconsecutive disulfide, RNase I folds to its native structure in the presence of DsbA alone, what makes DsbA more specific than previously assumed. Only in a more oxidizing environment when incorrect disulfide bonds are formed, the role of DsbC as an isomerase is essential. As such, the established oxidative folding idea that proteins with non-consecutive disulfides require DsbC has to be treated in a more subtle way or case-by-case.

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