Sulfur Denitrosylation by an Engineered Trx-like DsbG Enzyme Identifies Nucleophilic Cysteine Hydrogen Bonds as Key Functional Determinant

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Exposure of bacteria to NO results in the nitrosylation of cysteine thiols in proteins and low molecular weight thiols such as GSH. The cells possess enzymatic systems that catalyze the denitrosylation of these modified sulfurs. An important player in these systems is thioredoxin (Trx), a ubiquitous, cytoplasmic oxido-reductase that can denitrosylate proteins in vivo and S-nitrosoglutathione (GSNO) in vitro. However, a periplasmic or extracellular denitrosylase has not been identified, raising the question of how extracytoplasmic proteins are repaired after nitrosative damage. In this study, we tested whether DsbG and DsbC, two Trx family proteins that function in reducing pathways in the Escherichia coli periplasm, also possess denitrosylating activity. Both DsbG and DsbC are poorly reactive toward GSNO. Moreover, DsbG is unable to denitrosylate its specific substrate protein, YbiS. Remarkably, by borrowing the CGPC active site of E. coli Trx-1 in combination with a T200M point mutation, we transformed DsbG into an enzyme highly reactive toward GSNO and YbiS. The $pK_a$ of the nucleophilic cysteine, as well as the redox and thermodynamic properties of the engineered DsbG are dramatically changed and become similar to those of E. coli Trx-1. X-ray structural insights suggest that this results from a loss of two direct hydrogen bonds to the nucleophilic cysteine sulfur in the DsbG mutant. Our results highlight the plasticity of the Trx structural fold and reveal that the subtle change of the number of hydrogen bonds in the active site of Trx-like proteins is the key factor that thermodynamically controls reactivity toward nitrosylated compounds.

In eukaryotes, NO, a lipophilic gas generated by nitric-oxide synthases, plays an important role as a signaling molecule, in large part through the S-nitrosylation of cysteine thiols present in proteins (SNO proteins). For instance, the reversible S-nitrosylation of caspase 3 has been shown to regulate the activity of this pro-apoptotic protein (1). In addition to protein thiols, activated NO intermediates also react with low molecular weight thiols, such as GSH, resulting in the formation of S-nitrosoglutathione (GSNO). GSNO can then in turn cause the direct nitrosylation of proteins via transnitrosylation reactions (2).

Bacteria also encounter NO physiologically, when it is produced at low concentrations during anaerobic growth to serve as a terminal electron acceptor. Under these conditions, intracellular proteins can be nitrosylated, as recently reported in Escherichia coli (3). In this bacterium, the transcription factor OxyR, which controls an oxidative stress response, also serves as a regulator of protein S-nitrosylation and protects against endogenous nitrosative stress (3). Bacteria also face high levels of NO generated by the induction of nitric-oxide synthase in macrophages as part of the defense mechanisms of the host against invading microorganisms (4). These high NO levels cause a nitrosative stress and potentially lead to cell death. Detoxification of NO, as well as SNO, has also been shown to contribute to pathogenicity of bacteria (5).

Two enzymatic pathways play a major role in denitrosylation and the GSNO reductase and the thioredoxin (Trx) systems (1, 6). These systems are important both to regulate SNO-based signaling and to protect microorganisms from nitrosative stress. GSNO reductase is a NADH-dependent enzyme that is conserved from bacteria to human and that metabolizes GSNO to oxidized glutathione and NH$_3$ (6). GSNO reductase does not directly denitrosylate SNO proteins. However, because the GSNO pool is in equilibrium with protein thiols (2), reduction

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4 The abbreviations used are: SNO, S-nitrosothiol; GSNO, S-nitrosogluthion; Trx, thioredoxin; LMW, low molecular weight; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid.

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of GSNO by the GSNO reductase indirectly results in protein denitrosylation.

Trx is a ubiquitous oxidoreductase whose catalytic activity depends on a conserved CXXC motif that is maintained reduced by thioredoxin reductase at the expense of NADPH, although the best characterized function of Trx is the reduction of disulfide bonds that form in proteins during catalysis or as a result of oxidative stress (7), and Trx has recently been shown to also catalyze the direct denitrosylation of proteins in vivo (1). Trx is also able to reduce GSNO and other nitrosylated low molecular weight thiols in vitro, but the physiological relevance of this activity remains to be shown (8).

The fact that the GSNO and Trx systems exclusively function in the bacterial cytoplasm raises the question of how the proteins that are present in the periplasm are protected from reactive nitrogen species. To address this problem, we decided to investigate the role of DsbC and DsbG, two periplasmic oxidoreductases from the Trx family, in the protection of periplasmic proteins against nitrosative stress. DsbG and DsbC were selected because they both function in a reductive pathway that also involves DsbD, an inner membrane protein that transfers electrons from the thioredoxin system to the periplasm. Both DsbC and DsbG protect periplasmic proteins containing single cysteine residues from oxidation (9, 10), although DsbC mainly functions as a periplasmic disulfide bond isomerase (10–12).

Here, we show that DsbG and DsbC are poorly reactive toward low molecular weight nitrosothiols (GSNO and CysNO). Moreover, DsbG is also unable to catalyze the denitrosylation of its specific substrate protein YbiS. This prompted us to investigate the molecular factors that would allow DsbG to function in denitrosylation like E. coli Trx-1. Using site-directed mutagenesis, we transformed DsbG into a protein highly reactive toward GSNO and YbiS. We provide structural insights explaining how the mutation of only three amino acids alters the pK_a, redox, and thermodynamic properties to resemble those of Trx-1, thereby completely changing the function of DsbG.

Results

DsbG and DsbC Are Poor Denitrosylating Enzymes—As a first step, we asked whether DsbC and DsbG are able, like Trx-1, to catalyze the in vitro reduction of GSNO. If DsbC and DsbG are able to reduce GSNO, this should lead to the oxidation of their catalytic CXXC motifs, which can be monitored using AMS trapping experiments. AMS is a 490-Da reagent that covalently reacts with free thiol groups, leading to a mobility shift respective of the modified protein on SDS-PAGE gels (Fig. 1A). E. coli DsbG, DsbC, and Trx-1 were expressed and purified, using a His tag present at the C terminus and the purified proteins incubated with an excess of GSNO. As shown in Fig. 1A, both DsbG and DsbC remain reduced, even when the proteins are incubated with high concentrations of GSNO, in contrast to Trx-1, which becomes fully oxidized. In parallel, we tested the ability of DsbG and Trx-1 to reduce GSNO using a fluorescence assay, following the oxidation of the proteins by GSNO (Fig. 1B). This assay is based on the different fluorescence spectra of oxidized and reduced DsbG and Trx-1. Trx-1 rapidly reacts with GSNO, leading to the oxidation of Trx-1, in agreement with the data reported by Nikitovic and Holmgren (8). In contrast, DsbG reacts ~100 times slower with GSNO, confirming the results from the AMS trapping experiments. We also compared the reactivity of both enzymes to CysNO, an unstable low molecular weight thiol and obtained similar results (Fig. 1B). Thus, DsbG and DsbC are poorly reactive toward LMW nitrosothiols, in contrast to Trx-1.

Because Trx has been shown to catalyze the direct denitrosylation of proteins, we then sought to determine whether the periplasmic oxidoreductases are able to catalyze the denitrosylation of substrates proteins by determining the ability of DsbG to denitrosylate YbiS, one of its substrate proteins (9). YbiS was first S-nitrosylated by incubation with high concentrations of GSNO and then incubated with a 10-fold molar excess of reduced DsbG or Trx-1. The ability of DsbG or Trx-1 to denitrosylate YbiS was determined by following NO release using the Saville-Griess assay (Fig. 2). We found that DsbG is only poorly reactive toward YbiS-NO, in contrast to Trx-1, which is able to efficiently denitrosylate this nitrosylated protein.
Engineering DsbG into a Protein Reactive toward GSNO—To gain insights into the intrinsic properties of DsbG that contribute to its resistance against GSNO, we decided to engineer this oxidoreductase into a GSNO reducing enzyme. We first assessed the effect of the redox potential on the GSNO reducing activity. As the amount of NO released was measured using the Saville-Griess assay. The data are means ± S.E., n = 3.

Because the redox potential is directly linked to the Gibb's free energy $\Delta G$, which can be regarded as the thermodynamic driving force of the reaction, we determined the difference in stability between the oxidized and reduced forms of WT DsbG and DsbG CGPC by measuring their melting temperatures in a CD experiment (Fig. 4). For the WT, we measured a $\Delta T_m$ of 12 °C, the reduced form being more stable. For DsbG CGPC, the difference decreases to only 2 °C. Determination of the $pK_a$ of the nucleophilic cysteine shows a shift from a $pK_a$ of 3.5 for the wild type (14) to a value of 5 for DsbG CGPC (Table 1). Importantly, DsbG CGPC becomes also more reactive toward GSNO and CysNO than wild type DsbG (Fig. 5A), whereas introducing the CPYC active site of DsbG in Trx-1 makes Trx-1 less reactive (Fig. 5B). Importantly, DsbG CGPC also becomes reactive toward nitrosylated YbiS (Fig. 2). This experiment clearly shows the importance of the CGPC active site for the denitrosylation activity.

Combining the Trx Active Site CGPC Motif with a T200M Mutation Makes DsbG Highly Reactive toward LMW Nitrosothiols—Encouraged by these results, we decided to introduce an additional mutation in DsbG CGPC to further lower the redox potential. We chose to replace Thr200 by Met because this mutation has been shown by Hiniker et al. (13) to lower the redox potential of DsbG. We found that the resulting mutant has a strikingly lowered redox potential ($-280$ mV; Fig. 3 and Table 1), which is similar to that of Trx-1 (15). Remarkably, whereas for the WT, the reduced form is more stable than the oxidized form, the oxidized form of the double mutant becomes more stable ($T_m = 70$ °C) than the reduced form ($T_m = 59$ °C) (Fig. 4 and Table 1). The $pK_a$ of the nucleophilic cysteine is also further increased to 6.1. Thus, the triple mutant displays redox and thermodynamic properties similar to these of Trx-1 (15).

This double mutant also becomes highly reactive toward LMW nitrosothiols (Fig. 5). Based on pseudo first order kinetics in the presence of an excess of substrate, we estimated from $t_{1/2}$ that this double mutant reduces GSNO and CysNO with a second order rate constant in the range of $10^2$ M$^{-1}$ s$^{-1}$ (Fig. 5).

Hydrogen Bonds Influence Cysteine Reactivity—To gain more insight in the underlying mechanism by which these subtle mutations in DsbG cause such a drastic change in activity, we solved the crystal structures of DsbG CGPC and DsbG CGPC + T200M. Both proteins crystallized in similar conditions as the WT and in the same space group P21 (Table 2) (16, 17). The structures of DsbG CGPC and DsbG CGPC + T200M were solved to 1.7 and 1.96 Å, respectively. The overall structure of the mutants and the WT are the same with root mean square deviations between the Cα atoms of the wild type and the mutants of 0.25 Å (DsbG CGPC) and 0.39 Å (DsbG CGPC + T200M). The overall structure has also been reported to be unchanged for T200M (root mean square deviation 0.45 Å) and other DsbG mutants (13). As observed for WT DsbG (17), the loop between β3 and β4 (residues 43–48) was difficult to model, especially in DsbG CGPC, where a double conformation of the loop was modeled in.

The short distance between the Sγ of Cys109 and the backbone nitrogen of Cys112 in oxidized WT DsbG (3.1 Å) has been
reported to be one of the reasons why the reduced form is more favored than the oxidized form (17). Whereas DsbGCGPC is found in the reduced form in both chains in the asymmetric unit of the crystal structure (Protein Data Bank code 5G1L), both chains A and B of the DsbGCGPC-T200M structure (Protein Data Bank code 5G1K) show a mixture of the oxidized and reduced form of the protein (27% oxidized, 67% reduced in chain A, 38% oxidized, 62% reduced in chain B, as determined from the refined occupancies). However, the distance between the S\textsubscript{H}\textsuperscript{9253} of Cys109 and the backbone nitrogen of Cys112 (2.9 Å) is similar to the WT DsbG one (Fig. 6, A and B).

Change of the wild type DsbG CPYC motif to the Trx1 CGPC active site does not change the position of the \(\text{C}\alpha\) atoms in the active site motifs in both DsbGCGPC and DsbGCGPC-T200M. This has also been shown in other studies where the XX dipeptide is changed (14, 18). In DsbGCGPC-T200M where Thr\textsuperscript{200} is replaced by Met, however, there is a \(\sim\) 1 Å displacement of the \(\text{C}\alpha\) atoms at residues 198–201 comprising the \(\text{cis}\)-Pro loop. This displacement causes the backbone oxygen of Met\textsuperscript{200} to move further away from the disulfide bond of DsbGCGPC-T200M. As a consequence, two hydrogen bonds to the nucleophilic cysteine are lost (using a cutoff of 3.5 Å for a hydrogen bond) (Fig. 6C and Table 1): one between the backbone oxygen of Met\textsuperscript{200} and the sulfur of nucleophilic Cys\textsuperscript{109} and one caused by absence of the side chain of the Thr. In the oxidized and reduced form of wild type DsbG, the main chain O and O\textsubscript{\textsuperscript{H}}\textsuperscript{9253} of Thr\textsuperscript{200} are within H-bond distance with the S\textsubscript{H}\textsuperscript{9253} of Cys\textsuperscript{109} (Fig. 6), which has been shown to destabilize the oxidized form of the protein (14, 17). The lack of this interaction from Met\textsuperscript{200} in the DsbGCGPC-T200M mutant could lead to the observed stabilization of the oxidized form.

**Discussion**

\(S\)-Nitrosylation, resulting from the covalent attachment of an NO group to a cysteine or LMW thiol, has been regarded as a redox-based mechanism, allowing the post-translational regulation of all main classes of proteins (19). In addition, it also alters the intracellular thiol pool under nitrosative stress. Alterations in cellular NO and GSNO levels and in protein \(S\)-nitrosylation patterns have been shown to contribute to pathological conditions, e.g. in neurodegenerative diseases or bacterial pathogenesis (5, 20). Two major systems have been described to be involved in the denitrosylation of proteins and LMW nitrosothiols: the Trx system and the GSNO reductase system (1, 6). The latter cannot directly reduce nitrosylated proteins, but because of the equilibrium between GSNO and nitrosylated proteins, its reduction of GSNO indirectly leads to reduction of nitrosylated proteins (6). On the other hand, the Trx system directly denitrosylases by using its catalytic center with both cysteines in their reduced state. Trx establishes a disulfide bridge with the substrate SNO protein, allowing the release of NO, which will subsequently be protonated to HNO. An intramolecular disulfide bond is then formed between the Trx active site cysteines, releasing the substrate protein in the reduced state. Next, thioredoxin reductase restores the reduced state of the Trx catalytic cysteines through a NADPH-dependent reduction of the Trx intramolecular disulfide.

Here, we questioned whether the DsbG and DsbC oxidoreductases could be responsible for the reduction of periplasmic nitrosothiols in the *E. coli* periplasm. Upon the finding that neither could reduce the LMW nitrosothiols GSNO or Cys-
SNO (Fig. 1) and that DsbG was not even able to denitrosylate its specific substrate protein YbiS (Fig. 2), we decided to investigate the molecular factors that would allow DsbG to function in denitrosylation like Trx-1.

The CXXC active site motifs of Trx family proteins determine to a large extent their specific redox properties, and several studies have assessed the effect of changing the XX dipeptide within the CXXC catalytic signature motif of one thiol-disulfide oxidoreductase with that of another. Generally, this results in an oxidoreductase with redox properties similar to that of the oxidoreductase from where the dipeptide originated (18, 21–23). To this end, in two state-of-the-art papers, the Glockshuber group changed the XX dipeptide of the prototype reductase Trx (CGPC) and oxidase DsbA (CPHC) into the protein-disulfide isomerase type (CGHC), the glutaredoxin (Grx) type (CPYC), and the DsbA or Trx type, respectively (22, 23). The redox potential of Trx gradually increases from $-270$ mV for the WT enzyme, over $-221$ mV for the protein-disulfide isomerase type, to $-204$ and $-195$ mV for the DsbA and Grx types, respectively. Conversely, the DsbA redox potential decreased from $-122$ mV for the WT enzyme over $-146$ mV for the protein-disulfide isomerase type mutant, $-157$ mV for the Grx type, and $-214$ mV for the Trx type. Similarly, changing the dipeptide of the DsbG CPYC motif by that of Trx-1, CGPC, lowered the redox potential by 70 mV, making it more reducing ($-195$ mV in comparison to $-125$ mV for the WT enzyme (13)). In addition to affecting the redox potential, changes in the XX dipeptide of the CXXC active site motif also influence the $pK_a$ of the nucleophilic cysteine. For instance, the Trx CXXC motif conversion in the protein-disulfide isomerase/Grx/DsbA types resulted in a maximal $pK_a$ of 1.2 units, whereas the DsbA conversion to the protein-disulfide isomerase/Grx/Trx types led to a $pK_a$ of 2.93 units (22, 23). Here, we observe a $pK_a$ increase of 1.5 units between the WT DsbG and the CGPC DsbG (Fig. 3 and Table 1). Most importantly, the DsbGCGPC mutant shows increased denitrosylating activity (Fig. 5), toward both LMW nitrosothiols and nitrosylated YbiS. However, the XX active site dipeptide is not the only important feature determining the function of Trx family proteins. The loop containing the conserved cis-proline, which is in close proximity to the active site CXXC motif, also affects the function and the redox properties (14). A DsbG mutant where the residue located N-terminally from the cis-Pro loop was mutated, T200M, showed a more reducing redox potential of $-181$ mV and was shown to behave more like the disulfide isomerase DsbC (13). We prepared a DsbG mutant combining the CGPC Trx active site with this T200M mutation, DsbGCGPC-T200M. DsbGCGPC-T200M has a redox potential of $-280$ mV, even more reducing than WT Trx-1, $-271$ mV (15), and a nucleophilic Cys $pK_a$ of 6.1 (Table 1). A similar result was obtained for *E. coli* Trx-1, where combining the CPHC active site of DsbA with an I75T (the residue before the cis-Pro in *E. coli* Trx) mutation shifted the redox potential to $-180$ mV, 91 mV more oxidizing than the WT Trx.

**FIGURE 5.** *The active site determines the reactivity toward LMW nitrosothiols.* A, progress curves of the oxidation of DsbG and its CGPC mutant are shown. B, progress curves of Trx-1 and its CPYC mutant are shown. C, progress curves of the oxidation of WT and mutant DsbG by GSNO. D, progress curves of the oxidation of WT and mutant DsbG by CysNO. The reaction was followed as the decrease in fluorescence emission at 334 nm (excitation at 280 nm). Curves are fitted with a single exponential decay (dotted lines).
Remarkably, for DsbGCGPC-T200M, the oxidized form becomes 11 °C more stable than the reduced one (Fig. 4 and Table 1). The increase in $pK_a$ of the nucleophilic cysteine closer toward the more oxidizing form, thus stabilizing the reduced form. This effect was attributed to the formation of an extra hydrogen bond between the nucleophilic Cys and the T75 side chain, with respect to the WT enzyme (14).

In conclusion, mutations in the CXXC motif and the cis-Pro loop have been shown to change the thermodynamic properties of Trx family proteins (7). In general, the oxidized form of a reductase, such as Trx is more stable than the reduced form (7), whereas, conversely, the reduced form of an oxidase such as DsbA is more stable than its oxidized form (23). Mutations in the Trx CXXC motif to the protein-disulfide isomerase/Grx/DsbA type leads to a decrease in the stability of the oxidized form, whereas the conversion of the DsbA CXXC motif in the protein-disulfide isomerase/Grx/Trx type leads to an increase in the stability of the oxidized form. Similarly, here we see that whereas for the WT DsbG, the reduced form is more stable ($\Delta_{\text{red-ox}}G_m$ of 12 °C), for DsbGCGPC, this difference is only 2 °C. Remarkably, for DsbGCGPC-T200M, the oxidized form becomes 11 °C more stable than the reduced one (Fig. 4 and Table 1). Thus, the thermodynamic difference in stability between the oxidized and reduced form drives the reaction toward the reduction of nitrosylated substrates.

It is important to mention that the number of hydrogen bonds received by the sulfur cysteine determines the stability of the thiolate, a key factor that modulates the equilibrium between thiol (SH) and thiolate (S\(^{-}\)) (25). The crystal structure of DsbGCGPC-T200M indeed shows that introducing the T200M mutation leads to the loss of two hydrogen bonds to Cys\(^{109}\) (Fig. 6C and Table 1). This loss of hydrogen bonding can also be linked to the stability of the Cys\(^{109}\)–Cys\(^{112}\) disulfide bond. This disulfide bond has previously been shown to be unstable (17). Even upon oxidation using (1,10 phenanthroline) copper(II), exposure of WT DsbG crystals to synchrotron radiation leads to the reduction of the disulfide bond. Heras et al. (17) showed, however, that this is not merely due to radiation but is rather an intrinsic property of this disulfide bond. Thus, the fact that we observe the disulfide bond in the crystal structure of DsbGCGPC-T200M even though we did not chemically oxidize the proteins before crystallization and we used synchrotron radiation, indicates that the mutations indeed stabilize the oxidized form.

Heras et al. (17) argue that the short distance between the $S_N$ of Cys\(^{109}\) and the backbone nitrogen of Cys\(^{112}\) in oxidized WT DsbG (3.1 Å) could explain the destabilization of the oxidized form. However, whereas we actually clearly see a stabilization of the oxidized form for the mutants, especially for DsbGCGPC-T200M mutants, for which the oxidized form is 23 °C more stable than the WT oxidized form (in comparison with the respective reduced forms), this distance is the same as in the WT. Heras et al. (17) had also already hinted at the destabilizing effect of the Thr\(^{200}\)–Cys\(^{109}\) hydrogen bonds on the oxidized form. Indeed, as mentioned above, the crystal structure of DsbGCGPC-T200M shows the loss of two hydrogen bonds upon introducing the T200M mutation. Similarly, the I75T mutation in Trx-1 led to a more oxidizing form, thus stabilizing the reduced form. This effect was assigned to the formation of an extra hydrogen bond between the nucleophilic Cys and the T75 side chain, with respect to the WT enzyme (14).

Again, most importantly, the activity of the DsbGCGPC-T200M double mutant was drastically changed, changing a protein with hardly any denitrosylation activity into an enzyme with an estimated second order rate constant for the denitrosylation of GSNO and CysNO of $\sim 10^9 \text{M}^{-1} \text{s}^{-1}$ (Fig. 5). This rate is comparable with the published second order rate constant for GSNO reduction by E. coli Trx-1 ($760 \text{M}^{-1} \text{s}^{-1}$) (8).

In conclusion, we present the first evidence for the importance of the CXXC motif and of the cis-Pro residue in determining the activity of a Trx family oxidoreductase toward nitrosylated substrates. This property had never been investigated for any of the CXXC DsbA mutants. In addition, a recent paper describing the denitrosylating properties of the human Trx-related protein TRP14 does not study the role of the CXXC motif or the redox properties of this enzyme in relation to its function (26). In general, our study is a beautiful example of the plasticity of the Trx fold. We gave evolution a helping hand by showing how DsbG can be modified relatively easily from an enzyme that has been shown to reduce sulfenic acids on proteins (9) to an enzyme that is capable of reducing nitrosylated...
targets. Evolution of DsbG toward a denitrosylating enzyme could affect the pathogenicity of *E. coli*. In addition, our findings could contribute to the design of other mutant proteins with therapeutic potential, e.g. in neurodegenerative diseases.

**Experimental Procedures**

**Plasmids and Site-directed Mutagenesis**

The plasmids used for the expression of wild type *E. coli* DsbC, DsbG, YbiS, and Trx-1 have been described previously (9, 10, 21). The P110G1Y1LIP (CGPC) and T200M mutations were inserted in DsbG using the Stratagene QuikChange mutagenesis kit, using the CGPC-fw (3′-gcc gat ccc tgc gga cct tgt aaa cag ttc tgg-5′) and CGPC-rv (3′-cca gaa cta tca agg ttc gca gaa cgg atc ggc-5′) primers for the CGPC mutant and the T200M-fw (3′-gcc aat gtc atg ccg gct atc-5′) and T200M-rv (3′-gat agc cgg cat gac att tgc-5′) primers for the T200M mutant. The insertion of the desired mutations was confirmed by DNA sequencing. Construction of the Trx CPYC mutant was described previously (21).

**Protein Overexpression and Purification**

WT *E. coli* DsbG, DsbC, YbiS, and Trx-1 were overexpressed in BL21 cells and purified as described previously (9, 10, 21). Their corresponding mutants were expressed and purified using the same protocol.

**Protein Reduction and Oxidation**

DsbG, DsbC, Trx-1, YbiS, and their mutants were reduced by incubation in 10 mM DTT for 1 h at 4 °C. The reactions were then desalted on NAP-5 columns (GE Healthcare) and equilibrated in 50 mM phosphate buffer, pH 7.0, 50 mM NaCl and 1 mM EDTA.

**Synthesis of LMW SNOs**

*S*-Nitrosocysteine (CysNO) and GSNO were made freshly by reacting 50 mM cysteine or GSH, respectively, with 50 mM 150 mM EDTA. The relative concentrations of GSNO. The ability of DsbG and Trx-1 to denitrosylate *S*-nitrosothiols was investigated by monitoring the release of nitrite in the presence of these enzymes and Ybis-SNO using the Saville-Griess assay. The reaction is initiated by mixing a 10-fold molar excess of DsbG or Trx-1 with 25 μM of Ybis-SNO in 50 mM phosphate buffer, pH 8.0, 150 mM NaCl, and 1 mM EDTA, in the dark at room temperature. At different time points (5, 10, 15, 30, and 60 min), the amount of released NO was determined using the Saville-Griess assay as described above.

**Determination of the Protein Redox State by AMS Gel Shift**

1 μM of each freshly reduced oxidoreductase (Trx-1, DsbG, and DsbC) was incubated with 10 μM of GSNO or CysNO at room temperature in the dark. At different incubation times, the redox state of the proteins was determined by AMS gel shift, by alkylating the free thiol groups with AMS (Molecular Probes) as described (31, 32). AMS reacts with free thiol groups, resulting in a molecular mass increase of 490 Da. A 100-μl sample of each enzyme concentrated at 2 μM was precipitated by adding 10% TCA, incubated 15 min on ice, and centrifuged at 13,000 rpm for 5 min. The resulting pellet was then washed with acetone and resuspended in 30 μl of 50 mM Tris-HCl, pH 7.5, 20 mM AMS, 0.1% SDS, 10 mM EDTA. The sample was evaluated on 15% SDS-PAGE under non-reducing conditions.

**Kinetics of Oxidation of Trx-1, DsbG, and Their Mutants by LMW Nitrosothiols**

The ability of Trx-1, DsbG, and their mutants to be oxidized with GSNO or CysNO was investigated by following the decrease of fluorescence at the emission wavelength of 334 nm (excitation at 280 nm) in function of time by using a SLM-Aminco 8000C spectrofluorometer. The reaction was initiated after mixing 1 μM of each freshly reduced proteins with GSNO or CysNO (1 or 10 μM) in a total volume of 3 ml.

**Redox Potential Determination**

*Equilibrium against Glutathione*—The redox potentials of WT DsbG and DsbG_{CGPC} were determined by fluorimetry by measuring the equilibrium constant between WT or CGPC mutant DsbG and glutathione as described previously (33). Briefly, 1 μM of oxidized protein was incubated for 12 h at 293 K under a nitrogen atmosphere with 0.1 mM GSSG mixed with different concentrations of reduced GSH (0.2 μM to 1 mM) in 50 mM sodium phosphate, pH 7.0, 1 mM EDTA. The relative
amount of reduced protein at equilibrium was measured using the specific fluorescence at 330 nm (excitation at 280 nm).

Equilibrium against DTTred/DTTox—The redox potential of the DsbG double mutant CGPC-T200M and Trx-1 as control were determined by equilibrium against different DTTred/DTTox ratios as described previously (34). Briefly, 1 μM of oxidized protein was incubated for 4 h at 293 K under a nitrogen atmosphere with 10 mM DTTox, mixed with different concentrations of DTTred (2 μM to 200 μM) in 50 mM sodium phosphate, pH 7.0, 1 mM EDTA. After incubation, the redox state of each sample was investigated by AMS gel shift (see above). The ratio of the reduced and oxidized forms under each DTTox/DTTred conditions on Coomassie Brilliant Blue-stained SDS-PAGE (15%) was quantified with ImageJ software. The redox potential was calculated from the midpoint of the curves as described (35).

Determination of the pKa of the Nucleophilic Cysteine

The pH-dependent protonation of the nucleophilic cysteine thiolate was followed by the specific absorbance of the thiolate anion at 240 nm (36). As a reference, the pH-dependent absorbance for the oxidized form of the protein was monitored. All of the measurements were carried out at 298 K in buffer consisting of 10 mM K2HPO4, 10 mM boric acid, 10 mM sodium succinate, 1 mM EDTA, and 200 mM KCl, (pH 7.5) and an average initial protein concentration of 20 μM. The oxidized and reduced protein samples were prepared as described above. The pH of the protein solution was lowered stepwise to 2.2 by the addition of 0.2 or 0.1 M HCl. The absorbance at 240 and 280 nm was recorded on a Cary 50BIO UV-visible spectrophotometer and corrected for the volume increase. The pH dependence of the thiolate-specific absorbance signal (S = (A240/A280)reduced/(A240/A280)oxidized) was fitted according to the Henderson-Hasselbalch equation, $A_{exp} = A_{SH} + (A_{S-} - A_{SH})(1 + 10^{pK_a-pH})$, in which $A_{exp} = A_{240/A280}$ stands for the experimental determined value, $A_{SH}$ is the $A_{240/A280}$ value for the protonated form, and $A_{S-} = A_{240/A280}$ for the deprotonated form (25).

Unfolding and Thermal Stability

Oxidized and reduced wild type and mutant DsbG were prepared by incubation at room temperature with a 10 molar excess of diamide for 30 min or with 10 mM DTT for 30 min, respectively. The excess of diamide or DTT was removed by size exclusion chromatography on a Superdex 75 HR (10/30) column equilibrated in 50 mM boric acid, pH 8.0, 100 mM KF for the oxidized form and with the same buffer solution containing 1 mM DTT for the reduced form. For the thermal unfolding, samples were diluted to 0.2 mg/ml in the respective boric acid buffer solutions and slowly heated to 95 °C. The fraction of folded DsbG was recorded using CD on a J-715 spectropolarimeter (Jasco, Tokyo, Japan) at 220 nm for reduced wild type DsbG and the mutants. Oxidized wild type DsbG and the mutants were recorded at 232 nm. The change in ellipticity was plotted in function of temperature. The reversibility of the unfolding transitions was checked by cooling down to 4 °C and by rescan ning the far UV spectrum.

Crystallization, X-ray Diffraction, and Structure Solution

DsbGCGPC and DsbGCPC-T200M in 10 mM HEPES, pH 7, 100 mM NaCl, were crystallized at 298 K in 0.1 mM sodium citrate pH 3.75, 0.2 mM ammonium sulfate, 20% PEG 4000 by the hanging drop method as described for wild type DsbG and other DsbG mutants (13, 16, 17), at a protein concentration of 5 mg/ml. The crystals were dehydrated by increasing the PEG4000 concentration to 30% and cryoprotected by adding 10% glycerol before flash freezing in liquid nitrogen (16). Diffraction data were collected at the Proxima 1 Beamline at the SOLEIL synchrotron facility. The data were processed using the XDS package (37). The structures were solved by molecular replacement using the wild type DsbG structure, Protein Data Bank code 1V57, as a template, using the Phaser program (38) within the CCP4 suite (39). Further manual building and refinement were done using Coot, Refmac, and phenix.refine (40–42). The latter was used from the Phenix suite (42, 43). X-ray data collection parameters, processing, and refinement statistics are summarized in Table 2.

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