1. Introduction

The formation of correct disulfide bonds is critical in the folding process of many secretory and membrane proteins in bacteria, including toxins, adherence factors, and components of type III secretory systems [1,2]. Thiols:disulfide oxidoreductases belonging to the Dsb (disulfide bond) family of proteins catalyze this oxidative protein-folding step, which occurs in the periplasm in the case of Gram-negative bacteria [3]. In the best-characterized Escherichia coli K-12 strain, two distinct electron transfer pathways are involved in the formation of disulfide bonds in the periplasm [4]. In the oxidative (DsbA–DsbB) pathway, the strong dithiol oxidase DsbA randomly introduces disulfide bonds into newly synthesized polypeptides [5]. Upon oxidation of the substrate proteins, reduced DsbA is re-oxidized by an inner membrane protein DsbB [6,7]. DsbA can introduce incorrect disulfides, if disulfides need to be formed between non-consecutive cysteines. In the isomerization/reduction (DsbC/DsbG–DsbD) pathway, these non-native disulfides are corrected by the isomerase DsbC, and, to a lesser extent, by DsbG [4]. An inner membrane protein DsbD keeps both DsbC and DsbG in the active, reduced state [8]. DsbC and DsbG from E. coli share 26% sequence identity and they form a V-shaped dimer, with a CXXC active site motif in the thioredoxin-fold domain [9,10].

When proteins have an odd number of cysteines (or a single cysteine), the cysteines that are not involved in disulfides may be oxidized to form sulfenic acids. This oxidation could lead to inactivation of the protein function. E. coli DsbG rescues the catalytic cysteine residue of YbiS, the most active L,D-transpeptidase in the periplasm, from oxidation [11]. E. coli DsbG, whose negatively charged surface is better suited to interact with folded proteins, appears to be a key player in this reducing system, while E. coli DsbC, whose inner surface is lined with hydrophobic residues, seems to be designed to interact with unfolded proteins to correct non-native disulfides [11]. E. coli DsbC could also serve as a backup for E. coli DsbG [11].

Studies on Dsb proteins in other bacteria indicate that Dsb systems vary considerably among different genera and even strains of the same species [2,12]. Interestingly, pathogenic bacteria have developed distinct Dsb machineries that play a pivotal role in the biogenesis of virulence factors, thus contributing to their
pathogenicity [13–15]. In the *Helicobacter pylori* strain 26695, homologs of DsbG (HP0231; abbreviated as HpDsbG), DsbC (HP0377), DsbB (HP0595; DsbB), and DsbD (HP0265; CcdA) have been identified as putative Dsb proteins, whereas homologs of DsbA has not been identified [16,17], suggesting that *H. pylori* has a novel Dsb system. Among a total of eight *H. pylori* strain 26695 proteins (HP0096, HP0136, HP0231, HP0377, HP0390, HP0824, HP1458, and HP1563) that were identified to have the thioredoxin-like fold, four of them (HP0231, HP0377, HP0824, and HP1458) possessed the CXXC motif [17]. They were the two thioredoxins (HP0824 and HP1458), the putative DsbC (HP0377), and a hypothetical protein (HP0231) [17]. The amino acid sequence of HP0377 aligned with *E. coli* DsbC (with 12% identity), while that of HP0231 aligned with *E. coli* DsbG (with 6% identity) [17]. HpDsbG is one of the proteins secreted by *H. pylori* and was implicated in *H. pylori*-induced effects on the gastric epithelium [18]. It strongly reacts with the sera from *H. pylori*-positive patients with different gastric disorders and may represent a very immunogenic candidate for the development of a diagnostic assay to detect a variety of *H. pylori* strains [19]. In addition, it confers protective immunity in the mouse *Helicobacter* infection model [20].

To date, no structural data on Dsb proteins from *H. pylori* have been reported. To provide the structural framework to understand the role of HpDsbG in isomerization of disulfides and/or reduction of oxidized cysteines in the periplasm, we have determined the crystal structure of HpDsbG by single wavelength anomalous diffraction. To modify the catalytic cysteine residue (Cys176) of HP0518, the protein was incubated with 100-fold excess of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman’s reagent) (Sigma) for 2 h at room temperature. Excess DTNB was removed by HiPrep 26/10 desalting column using the same buffer as above. When DTNB reacts with a cysteine thiol, a mixed disulfide of 2-nitro-5-thiobenzoic acid (NTB) with the thiol group is formed [22]. Upon reduction of the mixed disulfide by a reductase such as DsbG, NTB− is released and absorbs light at 412 nm [11]. Fully reduced HpDsbG and HP0518-NTB were reacted at room temperature in a 1:1 molar ratio, with the initial concentration for each protein being 5.0 μM. Reduction assays were performed at room temperature by measuring the absorbance at 412 nm. Kinetic parameters were obtained by fitting the A412 curve after subtracting the small absorbance at 412 nm by the decreasing HP0518-NTB [23]. Protein concentrations were estimated using extinction coefficients ε280nm of 15,930 M−1 cm−1 and 54,780 M−1 cm−1 for HpDsbG and HP0518, respectively.

3. Results and discussion

3.1. Model quality and overall monomer structure

The model of HpDsbG was refined to Rwork/Rfree values of 22.0/24.5% for 20.0–2.10 Å data (Supplementary Table 1). In this model, only N- and C-termini are disordered; three residues (Met31, Lys264, and Ala265) and the C-terminal fusion tag (LEHHHHHH) are missing. The HpDsbG monomer consists of an N-terminal dimerization domain (residues 31–96) and a C-terminal catalytic thioredoxin-fold domain (residues 132–265), which are connected by a long linker α-helix (α2; residues 97–131) (Fig. 1A). The N-terminal dimerization domain is composed of one α-helix (α1) and a four-stranded antiparallel β-sheet (β1–β4). The characteristic CXXC motif of HpDsbG is present near the N-terminus of the first helix (α3) in its C-terminal domain and a cis-Pro loop is located adjacent to this motif (Fig. 1A). This arrangement is similar to other proteins of the thioredoxin-like fold [24]. However, the C-terminal domain of HpDsbG deviates from the typical thioredoxin fold (β1–α1–β2–α2–β3–α4–β3) [24] (i) by having an extra β-strand (β5) before the first β-strand (β1) of the thioredoxin fold and two inserted α-helices (α4.1 and α4.2) after the β1–α1–β2 motif of the thioredoxin fold, and (ii) by lacking a β-strand and an α-helix in the C-terminus, corresponding to β4 and α3 of the thioredoxin fold, respectively. These structural differences represent a significant variation among the thioredoxin-fold proteins [24]. The wide range of redox activities of thioredoxin-fold proteins is thought to be a consequence of modifications to the common scaffold [25].

3.2. Structural comparison of HpDsbG monomer and dimer with *E. coli* DsbG

Individual domains of HpDsbG show structural resemblance to the corresponding domains of *E. coli* DsbG [9]. The N-terminal dimerization domain of HpDsbG superimposes with that of *E. coli* DsbG (PDB ID: 1VS8, chain B in the reduced state) with a root mean square (r.m.s.) deviation of 2.9 Å for 54 Cx atoms and sequence identity of 12%. The C-terminal catalytic domain of HpDsbG superimposes with that of *E. coli* DsbG with an r.m.s. deviation of 2.9 Å for 114 Cx atoms and sequence identity of 17%. However, when we superimpose the whole HpDsbG monomer structure with *E. coli* DsbG, a much larger r.m.s. deviation of 5.6 Å is obtained with an overall sequence identity of 6%. When we align the whole sequences of HpDsbG and *E. coli* DsbG, the BLAST program aligns...
the sequences of the C-terminal catalytic domain only but fails to align the sequences of the N-terminal domain.

The following two reasons explain the large difference in the whole monomer structures despite the structural resemblance of individual domains. First, the conformation of a loop between $\beta_4$ and $\alpha_2$ in HpDsbG is very different from that of $E. coli$ DsbG. Second, the helical linker $\alpha_2$ of HpDsbG, which is nine residues longer than the counterpart of $E. coli$ DsbG, is bent by only $\sim 10^\circ$ at Ala116, whereas the linker helix of $E. coli$ DsbG is bent by $\sim 50^\circ$ at Pro75 (Fig. S1). As a consequence of these structural differences, the orientation of the C-terminal catalytic domain with respect to the N-terminal dimerization domain is considerably different between HpDsbG and $E. coli$ DsbG monomers (Fig. S1), resulting in considerably different dimer structures as discussed below. Interestingly, an imaginary line between the two cysteine sulfur atoms in the catalytic site of HpDsbG (red and green spheres in Fig. S1) is nearly orthogonal to that for $E. coli$ DsbG (purple and cyan spheres in Fig. S1), even though the two cysteine pairs occupy roughly similar positions, when the N-terminal domains are superimposed (Fig. S1).

Two monomers of HpDsbG, which are related to each other by crystallographic twofold symmetry in the crystal, associate through their N-terminal domains, displaying a V-shaped dimeric structure like the $E. coli$ DsbG dimer (Fig. 1B). The buried surface area at the inter-subunit interface is 960 Å² per monomer for HpDsbG. This is similar to the buried surface area of 1010 Å² per...
monomer for *E. coli* DsbG [9]. The dimer interface of HpDsbG is stabilized by hydrophobic interactions and hydrogen bonds involving α1, β3–β4 loop, β4, and β4–α2 loop. This pattern of dimerization is roughly similar, but not identical, to that in *E. coli* DsbG (Fig. 1B).

In HpDsbG and *E. coli* DsbG dimers, relative orientations of the two active sites in their C-terminal domains are considerably different due to significantly different overall monomer structures. In the HpDsbG dimer, the CXXC motifs face away from each other on the outside face of the V-shaped dimer, with the sulfur atoms of Cys159 in the dimer being separated from each other by 57 Å (Fig. 1C). When we view the dimer along the twofold axis, the line connecting the two active site cysteine sulfur atoms deviates ~20° from the longest dimension of the dimer (Fig. 1C). In contrast, the CXXC motifs of *E. coli* DsbG face each other toward the interior at two ends of the V-shaped dimer, with the equivalent sulfur–sulfur distance being 58 Å (Fig. 1D) [9]. When viewed down the dimer twofold axis, they lie along the longest dimension of the dimer (Fig. 1D). In *E. coli* DsbC, which is also a V-shaped dimer but with smaller dimensions than *E. coli* DsbG, the two CXXC motifs similarly lie along the longest dimension of the dimer, when viewed down the dimer twofold axis, with the equivalent sulfur–sulfur distance being 39 Å [10]. The observed structural differences between HpDsbG and *E. coli* DsbG dimers will likely affect substrate interactions and functions of HpDsbG, although we cannot rule out the possibility that the domain arrangement of HpDsbG changes during interactions with the substrate. In the case of *E. coli* DsbC, the dimer assumed a closed conformation on binding to a DsbDα monomer and the hinge movements observed in the DsbC linker helices resulted in the reduction of the distance between the active site sulfurs to 29 Å in the closed form [26].

Compared to the typical thioredoxin fold, the HpDsbG monomer has a pair of additional α-helices (α4.1 and α4.2) inserted after β6–α3–β7. An equivalent pair of extra helices is also present in *E. coli* DsbG [9]. This pair of helices is located similarly above the active site in both Dsb proteins (Fig. 2A). However, superposition of HpDsbG with *E. coli* DsbG shows that α4.1 helix of HpDsbG is significantly longer than that of *E. coli* DsbG by 2.5 turns, and α4.2 helix of HpDsbG and a following loop takes a considerably different path to α5 compared with that of *E. coli* DsbG (Fig. 2A). In addition, the C-terminal domain of HpDsbG lacks a β-strand and an α-helix, corresponding to β9 and α6 of *E. coli* DsbG, respectively, resulting in more solvent-exposed hydrophobic residues (Leu151 and Ile153 on β6; Phe259, Leu260, Tyr261, and His262 on β8). The location of the active sites outside the V-shaped cleft and the lack of one β-strand and an α-helix in HpDsbG at the C-terminus likely facilitate the access of the substrate protein into the active site.

### 3.3. Comparison of redox active sites of HpDsbG and *E. coli* DsbG

HpDsbG is in the reduced form in the crystal, with the active site sulfur atoms of Cys159 and Cys162 being 3.4 Å apart. Similarly, the equivalent distance is 3.4 Å in reduced *E. coli* DsbG, whereas it is 2.0 Å in the partially oxidized state [9]. In HpDsbG, the side chain...
of Cys159 interacts with the side chain of Cys162, the backbone amides of His161 and Cys162, and a water molecule as well as a positive charge from the dipole of an active site helix α3. Of the two cysteines of the CXXC motif in HpDsbG, only the N-terminal Cys159 is solvent exposed, which is expected to function by forming a mixed disulfide bond with substrate proteins. Two internal residues of the CXXC motif were reported to be the major determinant in modulating the redox properties by strongly affecting both the pKa value of the N-terminal thiolate cysteine and the relative stabilities of the oxidized and reduced forms of thioldisulfide oxidoreductases [27]. The central dipeptide in the CXXC motif is PY in many DsbG homologs including E. coli DsbG and Xy in DsbC homologs, whereas HpDsbG contains a PH dipeptide (Fig. S2). The PH dipeptide is much more common than PY among DsbAs from Gram-negative organisms [2].

Another interesting difference between HpDsbG and E. coli DsbG resides in the so-called cis-Pro loop between α5 and β8. The cis-Pro loop is a conserved feature among redox-active thio- redoxin-fold proteins and was shown to be important in substrate recognition [28]. Superposition of the C-terminal domains of HpDsbG and E. coli DsbG reveals that there exists a subtle difference in the conformation of their cis-Pro loops (Fig. 2B). The cis-Pro loop of HpDsbG is longer by three residues compared with E. coli DsbG. Interestingly, Val precedes cis-Pro in HpDsbG, whereas Thr precedes cis-Pro in E. coli DsbG (and also in E. coli DsbC). It was found that the minus 1 residue of cis-Pro is a general activity regulator of thio- redoxin-fold proteins [25]. It appears to control the activity of thioredoxin-family proteins by affecting both their redox properties and their ability to interact with partner proteins [25]. Mutations of the minus 1 residue of cis-Pro in E. coli DsbG gave rise to variants that gained E. coli DsbC-like isomerase activity [29]. These subtle differences between HpDsbG and E. coli DsbG redox active sites further hint a difference in their substrate specificities and functions.

3.4. HpDsbG functions as a reductase to protect HP0518 from oxidation

We asked whether HpDsbG has a reductase activity similar to E. coli DsbG [11]. E. coli YbiS, a putative L,D-transpeptidase in the periplasm, was found to be a substrate of E. coli DsbG and, to a lesser extent, E. coli DsbC [11]. To test the possibility, we speculated that a conserved hypothetical protein HP0518 from H. pylori [21] could be a substrate of HpDsbG, because it is secreted by H. pylori, like HpDsbG, and contains an L,D-transpeptidase domain with a single cysteine residue that may play a key catalytic role. There is no detectable overall sequence similarity between YbiS and HP0518.

When we performed the reductase assay using the DTNB-treated HP0518 as a potential substrate, HpDsbG showed a significant reductase activity, with a forward rate constant of 1.2 × 10^4 M^{-1} s^{-1} and a reverse rate constant of 9 M^{-1} s^{-1} (Fig. 3). This result supports the possibility that there is a specific interaction between HpDsbG and HP0518. To provide further evidence for the specific interaction between these proteins, we have isolated the reaction intermediate between HpDsbG C162A and HP0518 by size exclusion chromatography (Figs. S4 and S5) and confirmed by mass spectrometry that HpDsbG C162A and HP0518 form a stable 1:1 complex under high vacuum conditions (Fig. S6). These data support our hypothesis that HpDsbG may function as a reductase toward HP0518. HP0518 was shown to be involved in deglycosylation of flagellin, thereby regulating pathogen motility [21]. Motility of H. pylori is a prerequisite for successful colonization of human gastric tissues and the inactivation of HP0518 was shown to directly promote rapid cellular responses, including CagA phosphorylation and NF-kB activation [21]. Our work thus contributes to our understanding of the role of Dsb proteins in the pathogenicity of H. pylori.

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Appendix A. Supplementary data


References


