Disulfide bonds are required for the stability and function of a large number of proteins. Recently, the results from genome analysis have suggested an important role for disulfide bonds concerning the structural stabilization of intracellular proteins from hyperthermophilic Archaea and Bacteria, contrary to the conventional view that structural disulfide bonds are rare in proteins from Archaea. A specific protein, known as protein disulfide oxidoreductase (PDO) is recognized as a potential key player in intracellular disulfide-shuffling in hyperthermophiles. The structure of this protein shows a combination of two thioredoxin-related units with low sequence identity which together, in tandem-like manner, form a closed protein domain. Each of these units contains a distinct CXXC active site motif. Due to their estimated conformational energies, both sites are likely to have different redox properties. The observed structural and functional characteristics suggest a relation to eukaryotic protein disulfide isomerase. Functional studies have revealed that both the archaeal and bacterial forms of this protein show oxidative and reductive activity and are able to isomerize protein disulfides. The physiological substrates and reduction systems, however, are to date unknown. The variety of active site disulfides found in PDOs from hyperthermophiles is puzzling. Nevertheless, the catalytic function of any PDO is expected to be correlated with the redox properties of its active site disulfides CXXC and with the distinct nature of its redox environment. The residues around the two active sites form two grooves on the protein surface. In analogy to a similar groove in thioredoxin, both grooves are suggested to constitute the substrate binding sites of PDO. The direct neighbourhood of the grooves and the different redox properties of both sites may favour sequential reactions in protein disulfide shuffling, like reduction followed by oxidation. A model for peptide binding by PDO is proposed to be derived from the analysis of crystal packing contacts mimicking substrate binding interactions. It is assumed, that PDO enzymes in hyperthermophilic Archaea and Bacteria may be part of a complex system involved in the maintenance of protein disulfide bonds. The regulation of disulfide bond formation may be dependent on a distinct interplay of thermodynamic and kinetic effects, including functional asymmetry and substrate-mediated protection of the active sites, in analogy to the situation in protein disulfide isomerase. Numerous questions related to the function of PDO enzymes in hyperthermophiles remain unanswered to date, but can probably successfully be studied by a number of approaches, such as first-line genetic and in vivo studies.
Introduction

Covalent cross-linkages in proteins are mainly due to the existence of the disulfide bond, a covalent tertiary interaction which stabilizes a folded protein structure. Structural disulfide bonds usually link nonadjacent cysteines in a protein and result from oxidative, in most cases enzymatically catalyzed processes. The Cys–Cys linkage represents a stabilizing part of a folded protein and its formation may contribute to essential steps in the folding pathway and to the stability of the native state.

Proteins containing stable disulfide bonds are rare in the cytoplasmic compartments of most organisms, and this has been attributed to the reductive nature of the cytosol [1,2]. In bacteria, they are usually restricted to extracytoplasmic compartments with a more oxidative nature or secreted into the media, and in eukarya to the endoplasmatic reticulum or secreted into the external milieu.

In vitro protein folding studies have shown that the presence of oxygen or a strong oxidant (e.g. oxidized glutathione) is sufficient to promote formation of protein disulfide bonds in proteins [3]. However, this is not generally true for the situation in vivo. Genetic studies in bacteria and yeast revealed that the efficient formation of disulfides in proteins is dependent on the catalytic action of certain extracytoplasmic enzymatic systems. In the absence of such systems, protein disulfide bond formation is extremely slow.

A large number of enzymes which catalyze protein disulfide formation belong to a set of thiol-disulfide oxidoreductases found in all living organisms. Many of these exhibit the thioredoxin fold [4] and can be grouped into a subset, the thioredoxin superfamily. For the members of this family an active site containing a CXXC motif (i.e. cysteines separated by two amino acids XX) is a typical feature. These proteins act as oxidants in extracytoplasmic compartments; those with cytoplasmic location catalyze mainly reductive reactions. Oxidation and reduction of disulfide bonds is mediated by thiol–disulfide exchange reactions [6] between the active site cysteines of the enzyme and the cysteines in the target protein (Fig. 1).

Recently, enzymes are being described in still increasing numbers that are not members of the thioredoxin oxidoreductases found in all living organisms. From the very beginning of research on Archaea their proteins were, as a rule, suggested to be low in the content of cysteine residues and protein disulfide bonds. It was believed that cysteine side chains may not tolerate or may be prone to oxidative degradation by the harsh conditions and high temperatures under which these microorganisms grow. A recent exception to this rule, and to the restriction of stably disulfide bonded proteins to noncytoplasmic environments, is the discovery of large numbers of proteins with disulfide bonds in the cytoplasm of certain hyperthermophilic Archaea and Bacteria, in particular in the crenarchaea Pyrobaculum aerophilum and Aeropyrum pernix [7].

This finding implicates the importance of disulfide bonding in stabilizing thermostable proteins and points to largely unexplored biochemical and physio-
Table 1. Abundance of disulfide bonds in intracellular proteins from various archaeal and bacterial genomes (data taken from [7]). The abundance, f, is defined as the fraction of the total number of intracellular cysteines that are expected to form disulfide bonds.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth temperature (°C)</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrococcus abyssi</td>
<td>102</td>
<td>0.31</td>
</tr>
<tr>
<td>Methanococcus jannaschii</td>
<td>86</td>
<td>0.13</td>
</tr>
<tr>
<td>Thermotoga maritima</td>
<td>90</td>
<td>0.15</td>
</tr>
<tr>
<td>Methanobacterium thermoautotrophicum</td>
<td>90</td>
<td>0.17</td>
</tr>
<tr>
<td>Aquifex aeolicus</td>
<td>93</td>
<td>0.17</td>
</tr>
<tr>
<td>Aeropyrum pernix</td>
<td>100</td>
<td>0.40</td>
</tr>
<tr>
<td>Pyrococcus horikoshi</td>
<td>102</td>
<td>0.28</td>
</tr>
<tr>
<td>Archaeoglobus fulgidus</td>
<td>92</td>
<td>0.11</td>
</tr>
<tr>
<td>Pyrobaculum aerophilum</td>
<td>104</td>
<td>0.44</td>
</tr>
<tr>
<td>Methanococcus mazei</td>
<td>94</td>
<td>0.40</td>
</tr>
<tr>
<td>Sulfolobus acidocaldarius</td>
<td>90</td>
<td>0.15</td>
</tr>
<tr>
<td>Pyrococcus furiosus</td>
<td>92</td>
<td>0.11</td>
</tr>
<tr>
<td>Aquifex aeolicus</td>
<td>93</td>
<td>0.17</td>
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<td>Methanococcus jannaschii</td>
<td>86</td>
<td>0.13</td>
</tr>
<tr>
<td>Archaeoglobus fulgidus</td>
<td>92</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Within the thermophilic organisms there is even a cross-correlation between the abundance of disulfides and the maximum growth temperature. This observation suggests that intracellular disulfide bonds are very likely to be a result of selective pressure for thermostable proteins and presumably represent a strategy for adaption to high temperature. A role of disulfide bonds in the stabilization of thermophilic proteins has not been widely recognized, because it seemed to violate the general view of redox biochemistry in thermophilic microorganisms. However, an earlier statistical analysis of the optimization of electrostatic interactions as a stabilization factor in proteins [8] resulted in the following observation: a low level of spatial optimization of the electrostatic interactions is compensated by the statistical appearance of covalent cross-links in the protein structures and vice versa. In terms of protein stability, this means that the insufficiency of the electrostatic stabilization is compensated by the introduction of chemical cross-links, such as disulfide bonds. Another quite clear tendency is that smaller proteins (< 100 residues) are characterized by a lower electrostatic optimization value. This is in accordance with a higher disulfide-bridge density for small proteins. There exists complementarity in between those interactions in proteins which certainly has consequences for the understanding of the stability of proteins from thermophiles and hyperthermophiles [9].

The recently detected existence of disulfide bonds in proteins from hyperthermophiles suggests that these bonds may serve a role to stabilize proteins against thermal unfolding and denaturation. The underlying effect is probably an increase of the kinetic stability of a protein by a high activation energy barrier on the pathway from the folded to the unfolded structure, i.e. the thermostable structure rests in a kinetic trap. A number of structure investigations and stability studies on proteins from hyperthermophiles have provided experimental evidence to support a role of disulfide bonds. A decrease in the apparent melting temperature, $T_{m(app)}$, following the mutational or biochemical disruption of intramolecular disulfide bonds has been observed for A. pernix isocitrate dehydrogenase (Cys 87Ser, $\Delta T_{m(app)} = -9.6 \, ^\circ C$) [10] and P. aerophilum adenylosuccinate lyase (reduction by dithiothreitol, $\Delta T_{m(app)} = -18.5 \, ^\circ C$) [11]. In the structure of the soluble domain of the Rieske iron sulfur protein from Sulfolobus acidocaldarius, two disulfide bonds were detected [12]. One of them (C145-C172) is close to the iron–sulfur cluster and is probably involved in the stabilization of the cluster environment. In this way, even a certain influence on the redox potential of the cluster may be likely. The other disulfide bond (C46-C247) connects the cluster-binding domain with the main domain of the protein and has certainly a stabilizing function, too. The latter disulfide bond was not found in the mesophilic homologues from spinach chloroplasts and bovine mitochondria. Very recently, in line with the abundance of disulfide bonding in P. aerophilum, three disulfide bonds were found in the structure of a cysteine-rich protein of unknown function. Although one disulfide site showed some similarity to the sequence of a potential metal binding CXXC motif [13], the other two disulfides serve stabilizing roles within the protein fold [14]. Malate dehydrogenase from the moderately thermophilic bacterium Chloroflexus aurantiacus is a tetrameric enzyme. To explore the contribution of its dimer–dimer interface with regard to thermostability, an intersubunit disulfide was engineered at the interface in order to strengthen the dimer–dimer interactions [15]. The resulting mutant, containing two disulfide bridges in the tetramer, showed a 200-fold increase in half-life at 75 °C and an increase of 15 °C in the apparent melting temperature compared with the wild type. Remarkably, the mutant and the wild type had similar enzymatic activities at their
temperature optima. This experiment thus provided a clear example of stabilization by introduction of protein disulfides and a case of uncoupling of thermal stability and thermoactivity.

A considerable number of structural determinants have previously been implicated in the stabilization strategies of proteins from hyperthermophiles [9]. Inter- and intramolecular protein disulfide bonds can now without doubt be added to this list. Disulfide bonding provides an effective stabilization strategy, because the covalent sulfur–sulfur bond may be able to provide a stabilization equivalent, in terms of free energy $\Delta G_{\text{stab}}$, which is similar to a number of stabilizing non–covalent interactions acting in common.

Enzymatic catalysis of protein disulfide formation in Archaea: a hitherto insufficient structural basis

The in vivo situation of protein disulfide bond formation in prokaryotes and eukaryotes is known to be far from being perfect [2]. There is a certain probability that regularly non-native disulfides are introduced into proteins with more than two cysteines in their sequence. Thus there is a need for a safe-keeping system which works in parallel to disulfide oxidoreductases and can unscramble incorrect disulfide bonds in protein isomers. In *Escherichia coli* this function is provided by the periplasmic protein disulfide isomerase, DsbC, and a closely related enzyme, DsbG [16,17]. In eukaryotes, protein disulfide isomerase (PDI) promotes disulfide bond formation and isomerization [18].

The present knowledge concerning enzymes involved in disulfide shuffling in hyperthermophilic Archaea and Bacteria is still very limited. However, a recent comparative phylogenetic analysis [14] has identified a specific protein as a potential key enzyme in thermophilic intracellular disulfide maintenance by being most closely related with disulfide richness and thermophilicity. This protein was previously annotated ‘glutaredoxin-like’ based on its C-terminal similarity to glutaredoxin [19], however, it was later named protein disulfide oxidoreductase (PDO) by the authors of this review. In 1994, the same group purified a homologous protein from the hyperthermophilic archaeon *Sulfolobus solfataricus*. It showed the ability to reduce insulin disulfides and was named a thioredoxin; however, it had an unusual molecular weight of 25 kDa and four cysteine residues in its amino acid sequence [20]. Interestingly, as shown by bioinformatic evidence, these proteins seem to be exclusive to hyperthermophilic Archaea and Bacteria with a potential prevalence in a disulfide-rich subset of them. A complete family of PDO proteins was revealed with a strikingly precise correlation of the occurrence in those hyperthermophiles with high abundance of protein disulfide bonds [14]. Notably, proteins from this family were not observed in certain key organisms, such as *Methanococcus jannaschi*, *Methanococcus kandleri* and *Archaeoglobus fulgidus*. The PDO family is not only found on a single branch of the tree of organisms. Therefore, its precise correlation with richness in disulfides can be considered as strong evidence for a true relationship to this cellular property.

The first high-resolution structure of a member of the PDO family became available by the X-ray structure analysis of the PDO protein from *Pyrococcus furiosus* [21,22]. Recently, 8 years later, a functional and structural analysis of a homologous PDO from the hyperthermophilic bacterium *Aquifex aeolicus* was presented [23]. Both structures, which are in their overall appearance very similar, constitute the presently available structural information on hyperthermophilic PDO family members.

The crystal structure of *P. furiosus* PDO (PfPDO) revealed, very surprisingly, an uncommon combination of two thioredoxin-related structures which together, in tandem-like manner, formed a closed protein domain with a central eight-stranded $\beta$-sheet constituting the protein core and eight $\alpha$-helices distributed asymmetrically on both sides of the central sheet (Fig. 2). The topological arrangement of the secondary structure elements suggested that the PfPDO monomer can be divided into two structural units, which will be assigned, due to their location, ‘C-unit’ and ‘N-unit’ in...
Table 2. N- and C-terminal CXXC motifs in PDOs from selected hyperthermophilic Archaea and Bacteria.

<table>
<thead>
<tr>
<th>Organism</th>
<th>N-unit</th>
<th>C-unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archaea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrococcus furiosus</td>
<td>COYC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Pyrococcus horikoshi</td>
<td>COYC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Pyrococcus abyssi</td>
<td>COYC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Sulfolobus solfataricus</td>
<td>COYC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Sulfolobus tokodai</td>
<td>CHYC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Aeropyrum pernix</td>
<td>CETC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Thermoplasma acidophilum</td>
<td>CRYC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Thermoplasma volcanium</td>
<td>CRYC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Ferroplasma acidarmanus</td>
<td>CKYC</td>
<td>COYC</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermotoga maritima</td>
<td>COYC</td>
<td>CPYC</td>
</tr>
<tr>
<td>Aquifex aeolicus</td>
<td>CESC</td>
<td>CGYC</td>
</tr>
<tr>
<td>Thermoanaerobacter tengcongensis</td>
<td>CMPC</td>
<td>CAPC</td>
</tr>
</tbody>
</table>

the following text. These units should not be characterized as protein domains in the usual sense, because they show close packing and are structurally not separated. In fact, they are connected directly in between helix α₄ and α₅. The structural comparison with other PDO proteins has shown that each of the structural units in PfPDO is a thioredoxin fold motif, albeit with the insertion of an additional α-helix (α₁ or α₂) at the N-terminus (Fig. 2). Their sequence identity is rather low: the N-unit is only 18% identical to the C-unit. The C-unit has a conserved active site sequence of glutaredoxin family [19]. Each of the thioredoxin-like units contains one CXXC sequence motif (Table 2). Upon superposition of the two units (r.m.s.d. = 1.23 Å for 66 Cᵥ atoms), both active site disulfide bridges become spatially located at the same topological position. In addition, two conserved cis-prolines (cis-Pro80, cis-Pro194, see below), which were identified in the PfPDO monomer and are located close to one of the disulfides, respectively, become well aligned [21].

A structural comparison [21] revealed close similarities between both PfPDO units and the three-dimensional structures of E. coli thioredoxin [24], bacteriophage T4 glutaredoxin [25], the human PDI-a domain [26] and E. coli DsbA [26a]. As a result, however, the two PfPDO units resemble thioredoxin and PDI-a more closely than glutaredoxin and DsbA. In general, the sequence identities were low, with a range from 8 to 20% only. Glutaredoxin has shorter secondary structure elements and in DsbA a large α-helical domain insertion is found. Considerable structural differences between the PfPDO units and the four proteins mentioned above also occur at the N-terminal ends: with the exception of glutaredoxin, all the structures have additional residues forming secondary structure inserted at the N-terminus.

All these enzymes share a common biochemical reaction with the two active site cysteines in CXXC shuttling between the dithiol- and the disulfide form in order to catalyze the formation or reduction of disulfide bonds in their protein substrates [27,28]. In general, the N-terminal cysteine of the active site shows high reactivity and forms in a nucleophilic attack a mixed disulfide transition state with the substrate (see Fig. 1). This cysteine is solvent-exposed and has an unusually low $pK_a$ value ($pK_a = 3.5$ for DsbA [29] and $pK_a = 6.7$ for thioredoxin [30]). The C-terminal cysteine is usually buried and has a higher $pK_a$ value. It reacts exclusively with the transient mixed disulfide, finally resulting in a reduced substrate protein [5,27,31]. The low $pK_a$ value of the N-terminal cysteine is indicative for a highly reactive thiolate anion R$-$S$^-$ close to the N-terminal end of helix α₁. Its negative charge seems to be stabilized by the dipole moment of this α-helix, with an equivalent of a half positive unit charge [32]. However, it must be taken into account that the solution’s pH determines, in a crucial way, whether a thiolate anion state can be significantly populated in a thiol/thiolate equilibrium.

Oxidants or reductants? The influence of the redox potential and the subcellular environment

The physiological function of a disulfide oxidoreductase is expected to be correlated with the redox properties of its active site disulfide(s) CXXC. The redox potential of the active site disulfide in DsbA and thioredoxin is to a large extent determined by the amino acid residues XX between the two active site cysteines [33]. Among them, DsbA is the most oxidizing enzyme while thioredoxin has the strongest reduction power. Mutating the intervening amino acids of a reducing enzyme to those of a more oxidizing one will change the redox potential of the active site disulfide to become more oxidizing and vice versa [34–37]. The redox potential of thioredoxin, an important reduction catalyst in the cytoplasm, is around −270 mV, whereas that of DsbA, the main catalyst of oxidative disulfide bond formation in the periplasm, is around −120 mV [34,36,38,39]. In vitro, these enzymes can be forced, by changing their redox environment, to catalyze either disulfide bond oxidation or reduction, regardless of their active site redox potentials or their physiological
function [40]. The different redox properties of various members of the disulfide oxidoreductase family can be attributed to the stabilities of their reduced and oxidized states. Reducing enzymes, like thioredoxin, are usually more stable in their disulfide form compared with the dithiol form [41], and vice versa for an oxidizing enzyme like DsbA [38]. The conformational energy difference of their CXXC structures can amount to several kcal mol$^{-1}$ [39] depending, among other influences, on the chemical nature of the residues XX and on the conformational strain caused by them. To date there are no data available revealing the cysteine pK values or the redox potentials of the active sites of any archaeal or bacterial PDO. We are thus limited to conclusions which were derived from conformational stabilities and motional properties of the PDO N- and C-units.

PDO from *P. furiosus* has a typical CPYC sequence motif in the C-unit. This motif has been found at the N-terminus of all glutaredoxins. In addition, a CQYC motif was found in the N-unit of PfPDO (Fig. 3). The same motif also occurs in the enzymes from other *Pyrococcus* species; *Thermotoga maritima*, *S. solfataricus* and *Thermococcus kodakaraensis*. In spite of the existence of a glutaredoxin-like motif in the C-unit, there is no evidence whether PfPDO is a glutathione-dependent enzyme in vivo. There is also no indication for the existence of glutathione or related redox peptides in Archaea. Furthermore, no NADPH-dependent glutathione reductase was found in the cell extracts of *P. furiosus* and *S. solfataricus* [19].

The two active site disulfides found in PfPDO show remarkable differences in their geometrical parameters and, in fact, represent two extreme examples of disulfide conformations in disulfide oxidoreductases. The C-unit disulfide was found in a relaxed conformation with a torsional angle $\tau_3 = 80^\circ$ for the S–S bond and a $C_{\alpha}-C_{\alpha}$ distance of 5.5 Å, whereas the disulfide of the N-unit is in a most strained conformation with corresponding structural parameters of $47^\circ$ and 5.0 Å, respectively. The theoretical dihedral energy [42] calculated for the C-unit disulfide is $\sim 3$ kcal mol$^{-1}$, indicating a stable disulfide with low conformational strain. In contrast, the rather unfavourable dihedral angles of the N-unit disulfide lead to an extremely high dihedral energy of 13.8 kcal mol$^{-1}$, indicating strong conformational strain which may considerably destabilize this disulfide. Normally this value shows a variation from 0.5 to about 5 kcal mol$^{-1}$ for most protein disulfides [42] and the $C_{\alpha}-C_{\alpha}$ distance is found in a range of 5.2–5.4 Å in other active site disulfides. The refinement of crystallographic B-factors provided further evidence for a higher conformational flexibility of the N-unit.

**Fig. 3.** N- (A) and C-terminal (B) active site disulfides in PDO from *Pyrococcus furiosus* with the final $(2F_o - F_c)$ electron density map overlaid, contour level 1.2 $\sigma$. These two active sites exist in their oxidized states in the crystal structure and form two 14-membered disulfide rings; both disulfides are in the right-handed hook conformation [81], and the conformation of the disulfide ring in the C-unit is more relaxed than in the N-unit. Figure created using *PYMOL* [82].
disulfide of PfPDO. For 14 atoms forming the N-unit disulfide ring the average B-factor was 33.2 Å² vs. 17.8 Å² for the corresponding atoms of the C-unit disulfide ring. Even more surprising, the atoms in the N-unit as a whole have higher B-factors than those in the C-unit: the average B-factors for the Cα atoms in the N- and C-unit are 27.8 Å² and 22.8 Å², respectively. A similar distribution of B-factors was found in the N- and C-units of PDO from *A. aeolicus* (B. Ren, I. Haase, M. Fischer and R. Ladenstein, unpublished work). Thus, a similar behaviour was observed in two PDO structures, which have crystallized in different space groups. This leads us to the conclusion that the different conformational flexibilities in the N- and C-units of PDO do not result from crystal packing effects, but rather represent a true molecular property (Fig. 4). As seen from a functional viewpoint, it appears to be crucial for both PfPDO units to possess different flexibilities and conformational stabilities and to be involved in different functions during disulfide shuffling, too. The stability of the N-unit disulfide seems to be much lower than that of the C-unit. Consequently, the chemical nature of the N-unit disulfide may be more oxidative and that of the C-unit disulfide may be more reductive. The two thioredoxin domains a and a′ in PDI are known for their different conformational stabilities: the isolated a′ domain is much less stable than the isolated a domain [43]. However, it seems difficult to draw direct conclusions on the redox potentials, because they are in fact very similar in the human PDI domains [13]. The lower stability of the N-unit disulfide in PfPDO is partly a result of steric crowding as a consequence of the mutation of proline to glutamine adjacent to a tyrosine and the N-terminal cysteine in the CQYC site. This unfavourable conformational arrangement provides a steric hindrance to disulfide formation. In the C-unit active site CPYC, the proline residue has a stabilizing effect making the main chain more rigid by restriction of certain, otherwise accessible, conformations.

AqPDO from a hyperthermophilic bacterium, *A. aeolicus*, which was recently isolated and structurally characterized [23,44] showed 34% sequence identity with PfPDO and is also composed of two tandem thioredoxin folds with disulfide active sites on each unit. Its N-unit active site sequence, CESC, has not been observed in any other PDO, while the C-unit active site, CGYC, has already been found in DsbC, a protein disulfide isomerase of *E. coli*. Functional studies of AqPDO revealed high catalytic activity in reducing, oxidizing and isomerizing protein disulfide bonds. Site-directed mutagenesis suggested that its two active sites have similar functional properties, which is surprising in the view of expected differences in the redox potentials of those sites. A structural comparison of both disulfide active sites in AqPDO showed a remarkable degree of structural similarity with almost identical dihedral angle values of both disulfides. This finding is in strong contrast to the situation in PfPDO, with a strained N-unit disulfide and a relaxed C-unit disulfide (see above). In both AqPDO active sites the C-terminal cysteine residues are completely buried, whereas the N-terminal cysteine residues are equally exposed to the solvent. In PfPDO, however, both of the N-unit cysteine residues were found to be almost completely buried, which makes sense, because the N-unit disulfide is expected to carry the oxidative function of this enzyme.

Finally, it must be pointed out that not only the redox potential of the active sites, but also the nature of the redox environment has a strong influence on the catalytic properties of disulfide oxidoreductases. By manipulation *in vitro* they can be forced to perform either disulfide bond oxidation or reduction, regardless of the redox potential of their active site disulfides or their general physiological function [45,40]. In the case of thioredoxin, by knocking out thioredoxin reductase activity, this strong reductant can actually be turned into an enzyme catalyzing disulfide bond formation *in vivo* [46,47]. In a similar way, thioredoxin, which is exported into the periplasm, can act as an oxidant and partially counteract the disulfide bond formation defect in a *dsbA* null mutant strain [48]. However, this certainly does not mean that the redox potential of the active site disulfide is unimportant.
Searching for an archaeal protein disulfide isomerase

The observation of two sequential thioredoxin folds in the PDO proteins from *P. furiosus* and *A. aeolicus* [21,23] initiated a number of functional studies in view of the role that thioredoxin superfamily domains play in protein disulfide biochemistry, including reduction, oxidation and isomerization. Structure analysis of further members of this archaeal PDO family is likely to confirm the picture of two thioredoxin folds in tandem arrangement, with a CXXC motif on each unit. However, many questions still remain: Is the protein an oxidant, a reductant or an isomerase? What is the chemical nature of the substrates and the reducing systems? Do the active sites have distinct independent roles or do they function with synergy? Do their structural differences and stabilities lead to different redox and catalytic properties? Do any other archaeal PDO proteins exist with additional domains in between their thioredoxin motifs, CXXC-??-CXXC, as in eukaryotes and prokaryotes, with a certain substrate binding- or chaperone function? To date there is still only a limited number of clear answers to these questions. But there are some answers. At least, on structural grounds, it is tempting to see a relation of this archaeal protein family to the eukaryotic PDIs.

PDI is the major enzyme involved in protein disulfide bond formation in the endoplasmatic reticulum of eukaryotes. PDI catalyzes the formation and rearrangement, as well as breakage, of protein disulfide bonds depending on the redox conditions of the environment [18,49] and thus corrects non-native cysteine pairing such that the substrate protein can assume its proper native conformation. Recent biochemical studies of a cysteine mutant of PDI, with the second cysteine in both active sites mutated, has provided new insights into the net isomerization mechanism of protein disulfides [50,51]. The mutant PDI displayed only about 0.5% of wild-type activity in the scrambled RNase assay. The mutant PDI was found in a disulfide-bonded complex with the substrate protein and could not escape from it. This behaviour suggested that the function of the second cysteine is to release PDI from a complex with the substrate protein, finally leaving the substrate protein reduced and the PDI active site(s) oxidized. It was concluded that the majority of the net disulfide bond isomerization in a cell is in fact achieved in this way: reduction of the incorrect disulfide bonds and subsequent formation of the correct disulfide pairing. A collection of different functions, apart from its function as a redox catalyst, have been assigned to this molecule: activity as a chaperone [52], binding of calcium ions [51], dimer formation in solution [55], binding and hydrolysis of ATP [55]. The significance of some of these findings is still not well understood.

PDI from eukaryotes has a molecular weight of ~57 kDa and is a multidomain protein. The domains are arranged in the order a-b-b'-a. All four domains comprise the thioredoxin fold. Domains b and b' are inactive as redox catalysts; they do not contain CXXC motifs, however, they seem to be necessary for substrate binding. In domains a and a', the same active site sequence CGHC is found. Both CGHC motifs are involved in the formation of disulfide bonds in unfolded proteins [49,56]. Mutation of the Cys residues in the active sites of the domains a and a' resulted in a dramatic decrease of PDI activity [57]. The isolated a and a' domains are still able to catalyze disulfide formation, but do not show significant isomerase activity [58]. The recently determined crystal structure of yeast PDI [59] revealed that the four thioredoxin domains are arranged in the shape of a twisted ‘U’ with the active sites facing each other across the long sides of the ‘U’. The inside surface of the ‘U’ is covered by hydrophobic side chains, thereby facilitating interactions with misfolded proteins. Biochemical studies demonstrated that all PDI domains are required for full catalytic activity. A further structural study based on X-ray small angle scattering in solution arrived at similar conclusions, however, an annular model of the four PDI domains was proposed [60]. Besides the classical form of PDI an increasing number of proteins have been identified which contain two or more, directly adjacent or separated, thioredoxin-like domains in their primary structure [18].

More recently, functional studies have also suggested striking relations of the archaeal PDO family to eukaryotic PDI. These studies revealed that the archaeal protein not only has reductive and oxidative activity, but also isomerase activity, which was dependent on the presence of both thioredoxin units [61]. PpPDO was thus the first archaeal enzyme with a documented protein disulfide isomerase activity. Reductase activity was assayed by the turbidimetric method of Holmgren [62]. PpPDO reduces insulin disulfide in the presence of dithiothreitol at 30 °C. Both wild-type PpPDO and the mutant C35S were active in the insulin reductase assay, however, the activity of the mutants C146S and C35S/C146S were similar to the control. Thus, this result provided evidence that the C-unit active site CPYC is responsible for the reductase activity, in complete agreement with the suggestions above based on B-factor distribution and conformational energy of the disulfide motif obtained from crystallographic analysis.
The oxidative, disulfide-bond forming activity of PfPDO and several disulfide mutants was monitored by the so-called Ruddock test [63], using a synthetic decapeptide NRCSQGSCWNY with Cys residues at position 3 and 8, respectively. On oxidation of both thiols in this peptide, the fluorescent group (W) is brought closer to the charged group (R) and quenching of the signal from the fluorophore can be observed. In fact, fluorescence quenching was used as the basis for measuring the disulfide bond-forming activity of PfPDO. As expected, optimal conditions for this test were found at increased temperature, in this case 50–60 °C. Oxidative activity was determined in two ways: (a) by fluorescence quenching and (b) by calculation of the ratio between the peak areas of the oxidized and reduced peptide after separation by HPLC chromatography. However, both the oxidative and reductive activities were attributed by the investigations described above to the C-unit active site, CPYC. Apart from a residual oxidative activity, the role and function of the N-unit active site, CQYC, remained unclear. The isomerase activity of PfPDO was assayed by the method of Lambert and Freedman [64], based on the re-activation of scrambled RNase. The action of wild-type PfPDO in catalyzing interchange of intra-molecular disulfides in scrambled RNase resulted in restoration of the native disulfide pairing and return of RNase activity. Disulfide isomerase activity was obtained by incubation of scrambled RNase with PfPDO. At certain times aliquots were removed and RNase activity with RNA was measured. Reactivation of scrambled RNase was performed with the wild-type protein and the mutants C35S, C146S, C35S/C146S. Only wild-type PfPDO was able to refold and reactivate scrambled RNase, indicating that isomerase activity is dependent on the participation of both N- and C-unit active sites. However, it must be pointed out that the temperature used in this enzymatic test is very far from the usual ‘working temperature’ of PfPDO. To gain more realistic insights into protein disulfide reduction, oxidation and isomerization in archaean test systems have to be developed which are applicable to temperature ranges of, e.g. 80–100 °C. It is well known that enzymes from hyperthermophiles usually show low activity at room temperature. However, the scrambled RNase test needs quite some biochemical skills to be run properly.

PDO from *Pyrococcus horikoshii* (PhPDO) has similar reduction properties for insulin disulfides as PfPDO [65]. However, it showed no glutaredoxin activity, in line with the failure to detect glutathione (GSH and GSSG) in cell extracts of *P. horikoshii* and with the lack of genes coding for enzymes involved in GSH synthesis and -reduction, such as glutathione synthetase and glutathione reductase. These facts strongly indicate that PhPDO and related PDOs from other hyperthermophilic Archaea and Bacteria are not involved in a glutathione-dependent reduction system. Surprisingly, no isomerase activity of PhPDO was detected when scrambled RNase was used as substrate at 25 °C. However, a possible PDI-like activity, in vitro, at the optimum growth temperature of *P. horikoshii* was not excluded [65]. It was further tested in vitro whether thioredoxin reductase from *P. horikoshii* could reduce PhPDO. This flavoenzyme was apparently able to catalyze the NADPH-dependent reduction with an activity increase at higher temperature (60 °C) and an apparent *Km* value for PhPDO of 0.6 μM. In vitro, PhPDO and thioredoxin reductase from *P. horikoshii* formed a general disulfide reductase system [65], suggesting that thioredoxin reductase serves as a reduction catalyst of PhPDO in vivo. The high affinity of PhPDO to the thioredoxin in reductase system points to a critical role for the maintenance of a sufficiently low overall redox potential in the cytoplasm of *P. horikoshii*.

If PDO has isomerase function (which has been shown experimentally for PfPDO and AqPDO [23,61]), both units must have different functions, depending on redox potential, substrates and redox environment: Oxidation would involve transfer of an active site disulfide from PDO to a substrate protein. Isomerization requires the active site cysteines to be in the reduced state, to be able to attack non-native disulfides in substrate proteins and catalyze their rearrangement. Oxidation and isomerization would thus have opposite requirements in PDO. Does the protein primarily work as an oxidase or an isomerase? This question could be answered by in vivo mutation of the microorganism in the following way: CXXS–CXXS. Formation of a mixed disulfide with substrate proteins would be expected. In yeast, *Saccharomyces cerevisiae*, this kind of inactivation of PDI leads to a dramatic defect of *de novo* formation of disulfides in newly synthesized proteins [66], which suggests that yeast PDI plays an important role in catalyzing disulfide oxidation.

Formation of PDI dimers in solution has been well-studied, for example, for the protein purified from bovine liver [54]. For DsbC, the protein disulfide isomerase from *E. coli*, the dimeric arrangement of the molecule is fundamental for its role as an isomerase in the periplasm [67,68]. PfPDO forms a dimer in the crystalline state via two zinc binding sites and an ionic network [21]. Gel filtration and electrophoresis with native and SDS polyacrylamide gels suggested that PfPDO may exist in both monomeric and dimeric
forms in solution. Whether there is any functional significance of dimer formation is still unclear.

**The active site grooves and peptide binding**

The question on specificity of disulfide bond oxidoreductases is related to the existence of polypeptide binding sites on these proteins. It has been suggested that a relatively deep hydrophobic groove running alongside the active site of DsbA might participate in the binding of peptides [69]. NMR analysis of interactions between DsbA and model substrates suggested that DsbA binds peptides via hydrophobic interactions [70] and it was further concluded that only unfolded or partially folded proteins were able to serve as substrates of DsbA. Peptide binding was also reported for DsbC [71]. In the case of eukaryotic PDI, direct binding of peptides has been demonstrated [72,73].

The residues around the two active sites of PfPDO form two grooves on the protein surface, assigned as active site grooves N and C [21] (Fig. 5). Both grooves were found in close neighbourhood and span on top of the central β-sheet of PfPDO, a surface part of the molecule which is formed by six C-termini and two N-termini of the β-strands. A similarly located groove in thioredoxin has been shown to comprise the peptide binding site [74]. By extrapolation, it is suggested that these two grooves constitute the substrate binding sites of PfPDO. The shape of groove C is very similar to that of thioredoxin. It is formed exclusively by residues from the C-unit and is essentially hydrophobic. The residue cis-Pro194 abuts upon the active site disulfide with its pyrrolidine ring facing the Sγ atom of Cys149. As in other protein disulfide oxidoreductases, only the Sγ atom of Cys146 in the disulfide is exposed on the surface, while that of Cys149 is buried. The formation of groove N involves residues both from the C- and N-unit of the molecule. Groove N is narrower and deeper compared with groove C. Consequently the N-unit active site shows a decreased accessibility from the solvent, both Sγ atoms of Cys35 and Cys38 are almost completely buried. The N-unit active site of PfPDO constitutes thus the only completely buried active site disulfide in a disulfide oxidoreductase so far and gives rise to the question of how it can be accessed in its oxidized state. Comparison with the C-unit indicated that this unusual feature is created by the shifts of parts of the structure forming the active site groove. The loop involved in forming the active site C contains, like thioredoxin, DsbA and the PDI-a domain, three hydrophobic/polar residues (Thr143, Pro144, Thr145) which, together with Cys146, form a type VIII β-turn. As in the three proteins, the active site loop C adopts a more open conformation in the crystal structure as if the loop has moved away from cis-Pro80 and helix α7. As a consequence, groove N becomes narrower and the side chain of Cys35 points to the interior of the molecule with the Sγ atom of Cys35 buried (Fig. 6b). Access and substrate binding at the N-unit active site would therefore require substantial conformational adjustments of active site loop N and helix α3. Not surprisingly, these two regions possess the highest average B-factors in the whole PfPDO molecule besides both termini; 42.0 and 39.5 Å² for the Cα atoms in these two regions, respectively. A number of charged residues in the active site loop N are populated around the active site. They may possibly be involved in electrostatic interactions with the substrate and in this way induce
the necessary conformational adjustments to expose the S\(_s\) atom of Cys35 in order to become accessible for reaction with the substrate.

The two active site grooves are adjacent to each other on the protein surface. The distance between the active sites (Cys35S\(_s\)-Cys146S\(_s\)) is 22.1 Å, very close to the distance between the two active sites of yeast PDI (28 Å) [59]. Separately located in the grooves, with different accessibilities from solvent space, the two active sites of PfPDO function independently to some extent with different affinities to the substrates. Because the two disulfides are likely to have different redox potentials, it is suggested that the vicinity of the two grooves favours sequential disulfide shuffling reactions, such as formation of a mixed disulfide, reduction and re-oxidation (Fig. 1).

The hydrophobic surface around the active site of thioredoxin has earlier been suggested to be important for its interactions with a broad range of substrates [75]. The main residues which form the necessary hydrophobic surface patch in the thioredoxin fold include the highly conserved cis-proline, corresponding to cis-Pro80 and cis-Pro194 in both PfPDO units, and a glycine residue, corresponding to Gly97 and Gly210 in PfPDO, as well as the residues in close neighbourhood of the active sites. Peptides (such as those that are part of the transcription factor NF\(_{\kappa}\)B or the redox protein Ref1) can bind to the active site groove of the thioredoxin fold in two opposite orientations, either arranged parallel or antiparallel to the cis-proline and its preceding residue [74,76]. PfPDO probably shares similar features with thioredoxin concerning the binding of peptide substrates. In addition to the dimer interactions, described above, the PfPDO molecule forms a number of crystal packing contacts with symmetry related molecules in the crystal. While most of these contacts bury a surface area below 400 Å\(^2\) per molecule, one unusually large interface is observed, which shows a buried area of 755.9 Å\(^2\), i.e. 6.9% of the total surface area [77]. In contrast to the dimer interface observed in crystalline state, this interface comprises predominantly hydrophobic interactions with four possible hydrogen bonds, with a ratio of charged–polar–apolar residues of 4.8 : 34.4 : 60.8. Because the structures of both PfPDO units and thioredoxin are highly superimposable, potential peptide binding to the PfPDO molecule can be revealed and simulated by aligning the structures of the PfPDO C and N-units with those of the human thioredoxin complexes [74,76]. The two peptides NF\(_{\kappa}\)B and Ref1 can be aligned into the PfPDO active site groove C perfectly without collision (r.m.s.d. = 1.43 Å, for 65 C\(_\alpha\) atoms between the PfPDO C-unit and human thioredoxin) (Fig. 7). The positions of the aligned peptides superimpose well with the active site loop C from the symmetry-related molecule. This result is quite
surprising and suggests strongly that the observed crystal contacts together with the results from peptide alignment indeed mimick protein–substrate interactions. The alignment of the human thioredoxin-peptide complexes with the PfPDO C-unit has indicated easy access to the C-terminal active site. However, the alignment with the N-unit resulted in serious collisions between the peptide structures and the local structures forming the active site groove N, in line with the topology of this groove as discussed above.

Regulation of disulfide bond formation

In analogy to findings in Eukarya and Bacteria, it can be assumed that the redox environment or, more precisely, the nature of the redox partners, plays an important role in regulating the redox properties of an archaeal protein of the PDO family. In the cytoplasm of Bacteria, for example, where thioredoxin reductase reduces thioredoxin, this protein acts as a reductant [78]. In the periplasm where DsbB oxidizes the exported thioredoxin, this protein behaves as a protein oxidant. These observations are not surprising, because numerous studies have shown, that members of this family can perform electron transfer in either direction. One of the most striking examples of a so-called redox potential paradox is that of the protein DsbC, which is known as a reductant in the periplasm. However, its redox potential is only slightly different from that of the very strong oxidant, DsbA [2].

It is anticipated that PDO enzymes in hyperthermophiles may be only one facet of a complex system involved in the maintenance of protein disulfide bonds. Regulation of the formation of disulfide bonds may be prone to a distinct interplay of thermodynamic and kinetic effects. A ‘global redox potential’ which can be conceptually assumed as a synthesis of the redox potentials of the redox-active components of an entire cell, might favour a redox reaction in a thermodynamic sense. However, if a thermodynamically favoured reaction is kinetically impossible, by the presence of a high activation barrier, or by the absence of appropriate enzyme catalysts, the rate of such a reaction could be so slow that product formation appears completely insignificant. A striking example, which was discussed in [14], is given by the following: In the cell it is obviously possible for ribonucleotide reductase to reduce ribonucleotides to DNA synthesis, while at the same time the oxidative formation of structural protein disulfide bonds can proceed, provided both pathways are kinetically separated. Obviously, enzymatic recognition and catalysis make it possible for two such opposing redox processes to coexist.

The picture of disulfide metabolism in other organisms is very complex: in yeast there are five PDI homologues [79] and at least 17 different human proteins [80] are known, with various combinations of catalytic and noncatalytic thioredoxin domains as well as additional transmembrane and chaperone domains. All of them show the same type of disulfide sites in their domains. The complicated domain architecture in eukaryotes and yeast is poorly understood, as is the variability in the CXXC motifs in the PDO homologues of hyperthermophilic Archaea and Bacteria. A possibility is that the different domain combinations may dictate distinct redox functions for the thioredoxin units of the PDO homologues. To gain more insight into these unresolved problems, a strong focus on the measurement of redox potentials and in vivo functional studies of PDO enzymes will be needed.

Conclusion and future directions

Numerous problems related to protein disulfide oxidoreductases in hyperthermophiles remain unsolved to date, but can probably successfully be studied by genetic and in vivo approaches. Some of the questions that remain unanswered are: How would the phenotype of archaeal cells react upon a knock-out of a PDO gene?
What are the pathways and the regulation mechanisms for protein disulfide formation in Archaea? What is the global redox potential in archaeal cells?

The presence of disulfide bonds in proteins was suggested earlier to result from a simple process, spontaneous oxidation. The discovery of protein disulfide isomerases in Eukaryotes and Bacteria involved in protein disulfide formation and shuffling, however, has revealed pathways and cascades of unimagined variety and complexity. A similar complexity has to be expected for the Archaea. Concerning protein disulfide oxidation and reduction in Archaea, a number of very basic questions are still unanswered: What are the physiological substrates of archaeal PDOs? What is the redox system for archaeal PDOs? Could FAD thioredoxin reductase be the key player? What are the redox potentials of the various archaeal PDO active sites? Are these sites surrounded by peptide binding regions? Their identification should be possible by construction of the proper mutants.

In vivo, the redox state of PDI is determined by its interaction with Ero1p rather than by equilibrium with the bulk GSH/GSSG redox buffer in the endoplasmic reticulum [66]. How is the redox state of PDO determined? Is the interaction with the archaeal FAD thioredoxin reductase, which has been described for PhPDO, a crucial step? Can FAD thioredoxin reductase recognize both active sites in PDO? We have to establish better the interactions of PDOs with this enzyme, or might small, hitherto unknown redox compounds, be involved? By comparison with the redox buffer in the endoplasmic reticulum, which compounds make up the redox buffer in hyperthermophiles?

The PDI active sites have distinct roles: the a domain is mainly an isomerase while the a’ domain is an efficient oxidase. What are the roles of both PDO active sites? According to the stability analysis, described above, the N-unit site CQYC is more oxidizing and the C-unit site CPYC is more reducing. Surprisingly, the distribution of reducing and oxidizing functions in PDO seems to be reversed in comparison with PDI, however, a final conclusion has to await the determination of the redox potentials of both PDO sites. In extrapolation, the reducing site in PDO is suggested to represent the isomerase activity and the N-unit site the oxidase activity. Both sites can be characterized in analogy to PDI by functional asymmetry [33]. The functional asymmetry is likely to result from kinetic effects caused by interaction with the substrate protein.

In theory, the substrate protein, which may be a source of reductant, can influence the reaction rate in two ways: firstly, it may be a source of reductant for PDO; and second, it may be an unfolded protein which binds to PDO and alters the ability of FAD thioredoxin reductase to interact. Such an effect has recently been observed by the Weissman group by measuring the rate of oxidation in PDI in the presence of reduced and alkylated RNase A [33]. It was found that substrate binding specifically protected the N-terminal thioredoxin a domain of PDI from oxidation. Thus, the substrate-mediated protection of the N-terminal PDI active site magnified the intrinsic difference in oxidation rate of the PDI active sites and contributes to their functional asymmetry. Similar kinetic control mechanisms may be expected for archaeal PDOs. It is imaginable that substrate mediated protection of one of the active sites results from competition for PDO recognition between the protein substrate and the oxidizing or reducing moiety. Alternatively, substrate binding could induce conformational changes, slowing down the access to one of the thioredoxin units of PDO.

It is proposed that the documented asymmetry in the active sites would enable PDO to act in a dual way, as an oxidase and an isomerase. The N-unit active site CQYC is effective in oxidizing substrate cysteines; however, it is poor at catalyzing the formation of the correct native disulfides. In contrast, the C-unit active site CPYC is an inefficient oxidase, but contributes significantly to catalyzing the formation of native substrate disulfides, because it can, due to its reducing function, open incorrect disulfides and isomerize them subsequently. A possible difference/asymmetry in the oxidation rate of both active sites would ensure that the CQYC motif is in the oxidized state (for disulfide formation) while the CPYC motif is preferably reduced and can promote disulfide isomerization. In addition, a presumed regulation of the PDO redox state by the substrates would have important consequences for protein folding in archaeal cells. When there is a lower concentration of substrate proteins to fold, PDO can be kept in an oxidized state, ready to introduce disulfides in substrate proteins at maximum rate. Once it is engaged in disulfide oxidation (which causes reduction of its own disulfides), it may switch to the dual role of an oxidase and an isomerase. It is possible that the nature of the substrates would determine the extent of active site protection and thus be involved in tuning the roles of oxidation and isomerization depending on the requirements in the cell.

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