Extremophiles (2008) 12:29–38
DOI 10.1007/s00792-007-0076-z

REVIEW

Reconsideration of an early dogma, saying ‘‘there is no evidence for disulfide bonds in proteins from archaea’’

Rudolf Ladenstein · Bin Ren

Received: 12 December 2006 / Accepted: 27 February 2007 / Published online: 17 May 2007

Abstract Stability and function of a large number of proteins are crucially dependent on the presence of disulfide bonds. Recent genome analysis has pointed out an important role of disulfide bonds for the structural stabilization of intracellular proteins from hyperthermophilic archaea and bacteria. These findings contradict the conventional view that disulfide bonds are rare in those proteins. A specific protein, known as protein disulfide oxidoreductase (PDO) is recognized as a potential key enzyme in intracellular disulfide-shuffling in hyperthermophiles. The structure of this protein consists of two combined thioredoxin-related units which together, in tandem-like manner, form a closed protein domain. Each of these units contains a distinct CXXC active site motif. Both sites seem to have different redox properties. A relation to eukaryotic protein disulfide isomerase is suggested by the observed structural and functional characteristics of the protein. Enzymological 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 variety of active site disulfides found in PDO’s from hyperthermophiles is puzzling. 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.

Communicated by D.A. Cowan.

R. Ladenstein (✉) · B. Ren
Center of Structural Biochemistry, Karolinska Institutet
NOVUM, 14157 Huddinge, Sweden
e-mail: rla@csb.ki.se

Keywords Archaea · Hyperthermophiles · Protein disulfide isomerase · Protein disulfide oxidoreductase · Redox potential · Thermostability · Thioredoxin

Abbreviations

PDI	Protein disulfide isomerase
PDO	Protein disulfide oxido-reductase
DTT	Dithiothreitol
GSH	Reduced glutathione
GSSG	Oxidized glutathione

Introduction

The disulfide bond represents a covalent cross-linkage in a protein which is important for the integrity of a folded protein structure. Structural disulfide bonds usually link non-adjacent cysteines in the peptide chain and result from oxidative, in most cases enzymatically catalyzed processes. The Cys–Cys linkage represents a stabilizing part of a folded protein (Anfinsen and Haber 1961) and its formation may contribute to essential steps in the folding pathway and to the stability of the native state. (Fig. 1) Proteins containing stable disulfide bonds are rare in the cytoplasmic compartments of most organisms, due to the reductive nature of the cytosol (Derman and Beckwith 1991; Kadokura et al. 2003). In bacteria they are usually restricted to extra-cytoplasmic 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 (Anfinsen 1973). However, this is not generally true for the situation in vivo. Genetic

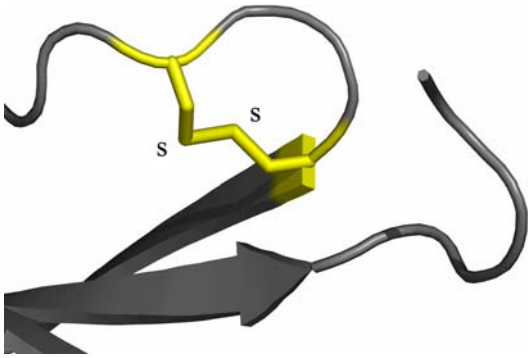

Fig. 1 Disulfide bond in a loop region of a protein molecule. Protein disulfides increase stiffness and mechanical strength, restrict protein motions and limit the number of conformations

studies in bacteria and yeast revealed that the efficient formation of disulfides in proteins is dependent on the catalytic action of certain extra-cytoplasmic 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 those exhibit the thioredoxin fold (Holmgren et al. 1975) 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 neighboured amino acids XX) is a typical feature. These proteins act as oxidants in extra-cytoplasmic compartments, those with cytoplasmic location catalyze mainly reductive reactions. Oxidation and reduction of disulfide bonds is mediated by thiol-disulfide exchange reactions (Bryk et al. 2002) between the active site cysteines of the enzyme and the cysteines in the target protein (Fig. 2).

The catalytic mechanisms of thiol-disulfide oxidoreductases have been the subject of numerous experimental investigations, however questions and problems remain regarding when and where they act in the cell, their specificities and the maintenance of the subcellular redox environment that critically determines their function.

Disulfide containing proteins in archaea: protein disulfides and thermal stability

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. Still in 1998 a knowledgeable referee has given the following comment: ''…there is no evidence, as far as I know, for proteins containing disulfide bonds in archaea…'' to one of our manuscripts which was sent in for publication. A recent exception to this rule, and to the restriction of stably disulfide bonded proteins to non-cytoplasmic environments, is the discovery of large numbers of proteins with disulfide bonds in the cytoplasm of certain hyperthermophilic archaea and bacteria, in

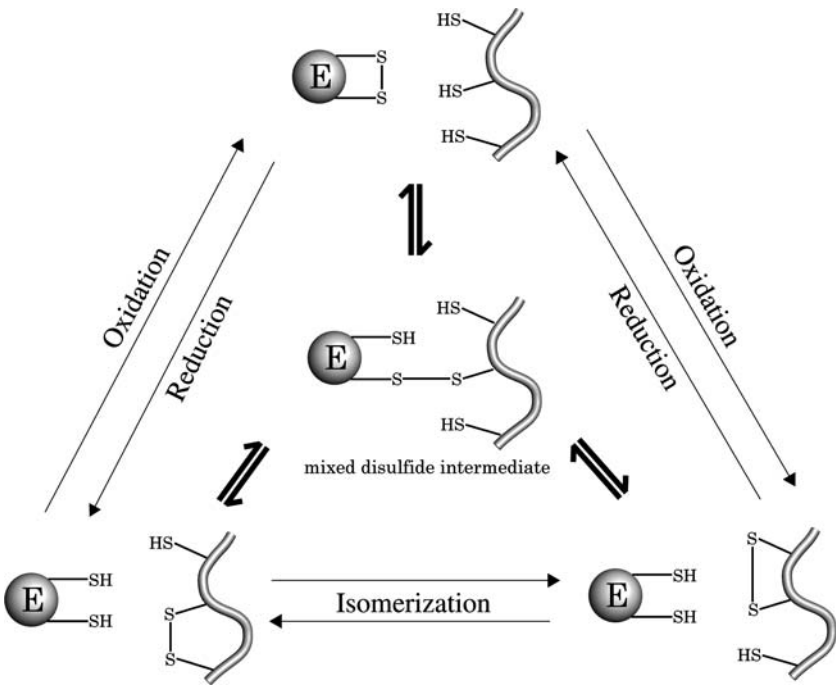

Fig. 2 Enzymatic disulfide bond reduction, oxidation and isomerization via a mixed disulfide intermediate between the enzyme (*E*) and a substrate protein

particular in the crenarchaea *Pyrobaculum aerophilum* and *Aeropyrum pernix* (Mallick et al. 2002).

This finding implicates the importance of disulfide bonding in stabilizing thermostable proteins and points to largely unexplored biochemical and physiological environments in the cells of these microbes. Specifically, the analysis of the protein coding regions of 25 fully sequenced genomes revealed several genomes with a strikingly high fraction of their total cysteine content expected to participate in the formation of intracellular disulfide bonds. (Fig. 3) Nine of the 25 analyzed genomes were predicted to contain more than 10% of their intracellular cysteines in disulfide bonds. This group of nine genomes includes seven archaea and two hyperthermophilic eubacteria (Table 1). Two *Pyrococcus* species have roughly 30% of their cysteines in disulfide bonds. Two other archaea, *P. aerophilum* and *A. pernix*, were predicted to have 40 and 44%, respectively, of their cysteine residues in protein disulfide bonds (Mallick et al. 2002).

Within the thermophilic organisms there is even a cross-correlation between the abundance of disulfides and the maximum growth temperature. (Fig. 4) This observation suggests that intracellular disulfide bonds very likely are 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.

The recently detected existence of disulfide bonds in proteins from hyperthermophiles suggests that these bonds

Table 1 Abundance of disulfide bonds in intracellular proteins from various archaeal and bacterial genomes (taken from Mallick et al. 2002, PNAS 99, 9680)

Organism	Growth T (°C)	f*
Pyrobaculum aerophilum	104	0.44
Aeropyrum pernix	100	0.40
Pyrococcus abyssi	102	0.31
P. horikoshii	102	0.28
Aquifex aeolicus	93	0.17
Methanobacterium thermoautotrophicum	90	0.15
Thermotoga maritima	90	0.13
Methanococcus jannaschii	86	0.13
Archaeoglobus fulgidus	92	0.11

*The abundance, f, is defined as the fraction of the total number of intracellular cysteines that are expected to form disulfide bonds

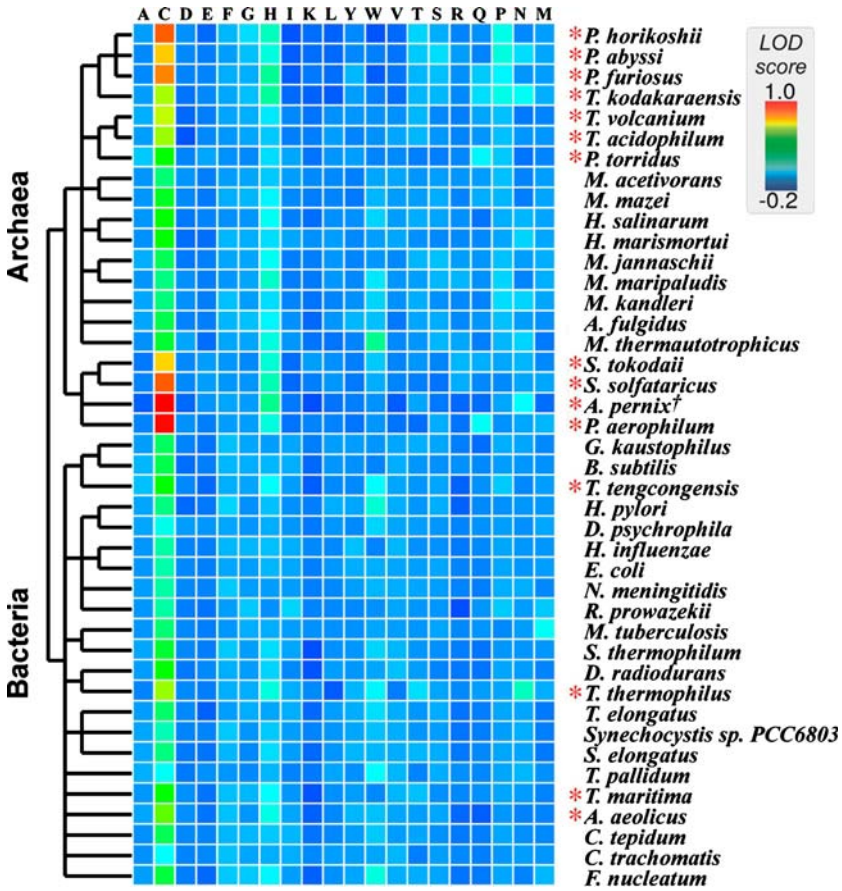

Fig. 3 Predicted protein disulfide abundance across thermophilic and mesophilic microorganisms. For each genome a *colored row* shows the tendency for cysteine residues in the proteins of that organism to occur close in 3D space to each of the 20 amino acids, given in one letter codes. The *color values* are log ratio of observed over expected occurrences of proximal amino acids. A *red asterix* close to an organism name indicates the presence of the PDO protein (With permission taken from Beeby et al. 2005)

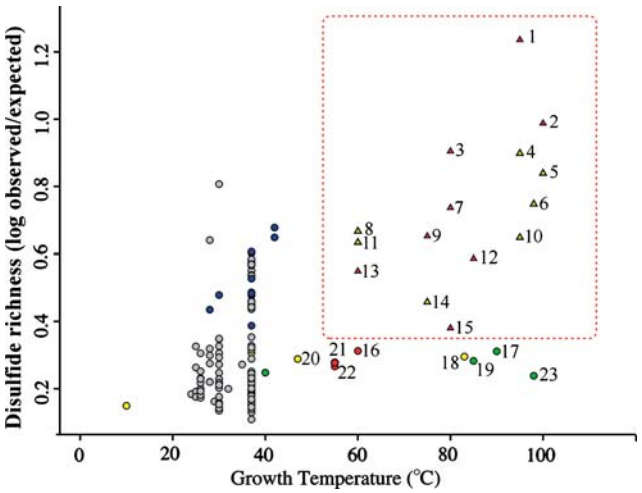

Fig. 4 Correspondence of growth temperature and richness in protein disulfides. Genomes containing the PDO protein are indicated by a *box* drawn in *red dotted lines*. *Numbers* indicate the following organisms: *1*, *A. pernix*; *2*, *P. aerophilum*; *3*, *S. solfataricus*; *4*, *Py. horikoshii*; *5*, *Py. furiosus*; *6*, *Py. abyssi*; *7*, *S. tokodaii*; *8*, *Thermoplasma volcanium*; *9*, *Thermus thermophilus* (both HB8 and HB27); *10*, *Thermococcus kodakaraensis*; *11*, *T. acidophilum*; *12*, *Aquifex aeolicus*; *13*, *Picrophilus torridus*; *14*, *Thermoanaerobacter tengcongensis*; *15*, *Thermotoga maritima*; *16*, *Symbiobacterium thermophilum*; *17*, *Methanothermobacter thermoautotrophicus*; *18*, *Archaeoglobus fulgidus*; *19*, *Methanococcus jannaschii*; *20*, *Synechococcus elongatus*; *21*, *Geobacillus kaustophilus*; *22*, *Thermosynechococcus elongatus*; and *23*, *Methanopyrus kandleri* (With permission taken from Beeby et al. 2005)

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, ΔT_m(app) = –9.6°C) (Karlström et al. 2005) and *P. aerophilum* adenylosuccinate lyase (reduction by DTT, ΔT_m(app) = –18.5°C) (Toth et al. 2000). In the structure of the soluble domain of the Rieske iron sulfur protein from *Sulfolobus acidocaldarius* two disulfide bonds were detected (Bönisch et al. 2002). 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 (Fomenko and Gladyshev 2003), the other two disulfides serve stabilizing roles within the protein fold (Beeby et al. 2005).

A considerable number of structural determinants have previously been implicated in the stabilization strategies of proteins from hyperthermophiles (Karshikoff and Ladenstein 2001). 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 ΔG_{stab}, which is similar to a number of stabilizing non-covalent interactions acting in common.

Enzymatic catalysis of protein disulfide formation in archaea

The in vivo situation of protein disulfide bond formation in prokaryotes and eukaryotes is known to be far from being perfect (Kadokura et al. 2003). 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 on a safe-keeping system which works in parallel to disulfide oxidoreductases and can unscramble incorrect disulfide bonds in protein isomers.

Concerning enzymes involved in disulfide shuffling in hyperthermophilic archaea and bacteria the present knowledge is still very limited. However, a recent comparative phylogenetic analysis (Beeby et al. 2005) 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 (Guagliardi et al. 1995), however, has later been renamed protein disulfide oxidoreductase (PDO) by the authors of this review (Ren et al. 1998). Interestingly, as shown by bioinformatic evidence, this protein seems 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 (Beeby et al. 2005). Notably, proteins from this family were not observed in certain key organisms,

such as *Methanococcus jannaschi, M. 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 *Py. furiosus* (Ren et al. 1998). Recently, eight years later, a functional and structural analysis of a homologous PDO from the hyperthermophilic bacterium *Aquifex aeolicus* was presented (Pedone et al. 2006a). 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 *Py. 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 β-sheet constituting the protein core and eight α-helices distributed asymmetrically on both sides of the central sheet (Fig. 5). 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 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 α_4 and α_5. The structural comparison with other PDOs has shown that each of the structural units in PfPDO is a thioredoxin fold motif, however, with the insertion of an additional α-helix (α_1 or α_5) at the N-terminus (Fig. 5). 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, which may explain why this protein has been initially classified as a member of the widely distributed glutaredoxin family (Guagliardi et al. 1995). Each of the thioredoxin-like units contains one CXXC sequence motif. Upon superposition of the two units (r.m.s.d = 1.23 Å for 66 C_α 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 (Ren et al. 1998).

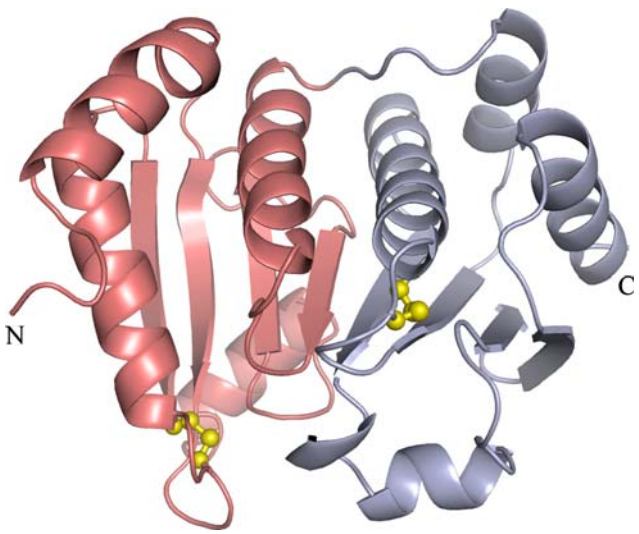

Fig. 5 Ribbon diagram of the PDO monomer from *Py. furiosus*, the two units are displayed in different colors

A structural comparison (Ren et al. 1998) revealed close similarities between both PfPDO units and the 3D structures of *Escherichia coli* thioredoxin (Katti et al. 1990), bacteriophage T4 glutaredoxin (Eklund et al. 1992), the human PDI-a domain (Kemmink et al. 1996) and *E. coli* DsbA (Kemmink et al. 1996; Martin et al. 1993). 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 8–20% only. Glutaredoxin has shorter secondary structure elements and in DsbA a large α-helical domain insertion is found.

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 (Holmgren 1995; Freedman 1995). 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. 2).

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 (Aslund et al. 1997; Huber-Wunderlich and Glockshuber 1998;). 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 (Aslund et al. 1997; Huber-Wunderlich and Glockshuber 1998;

Krause et al. 1991; Mössner et al. 1998). 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 its disulfide form compared to the the dithiol form (Holmgren 1972), and vice versa for an oxidizing enzyme like DsbA (Wunderlich et al. 1993). The conformational energy difference of their CXXC structures can amount to several kcal/mol (Zapun et al. 1993) 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.

Protein disulfide oxidoreductase from *Py. 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. 6). The same motif occurs also 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 *Py. furiosus* and *S. solfataricus* (Guagliardi et al. 1995).

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 $X_3 = 80°$ for the S–S bond and a C_α–C_α distance of 5.5 Å, whereas the disulfide of the N-unit is in a most strained conformation with corresponding structural parameters of 47° and 5.0 Å, respectively. The theoretical dihedral energy (Katz and Kossiakoff 1986) calculated for the C-unit disulfide is around 3 kcal/mol indicating a stable disulfide with low conformational strain. In contrast, the rather unfavorable dihedral angles of the N-unit disulfide lead to an extremely high dihedral energy of 13.8 kcal/mol, indicating strong conformational strain which may considerably destabilize this disulfide. The refinement of crystallographic B-factors provided further evidence for a higher conformational flexibility of the N-unit disulfide of PfPDO. For 14 atoms forming the N-unit disulfide ring the average B-factor was 33.2 versus 17.8 $Å^2$ 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 C_α atoms in the N- and C-unit are 27.8 and 22.8$Å^2$, respectively (Fig. 7). A similar distribution of B-factors was found in the N- and C-units of PDO from *A. aeolicus* (Ren et al. 2006). 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.

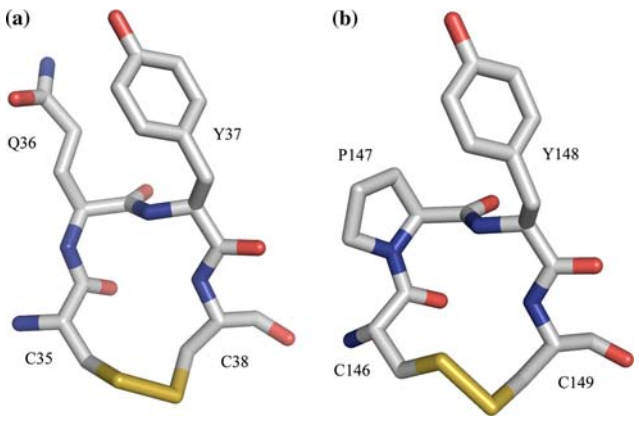

Fig. 6 N-terminal (**a**) and C-terminal (**b**) active site disulfides in PDO from *Py. furiosus*. These two active sites exist in their oxidized states in the crystal structure and form two 14-membered disulfide rings, both disulfides are in the right-handed hook conformation (Hutchinson and Thornton 1996), the conformation of the disulfide ring in the C-unit is more relaxed as in the N-unit

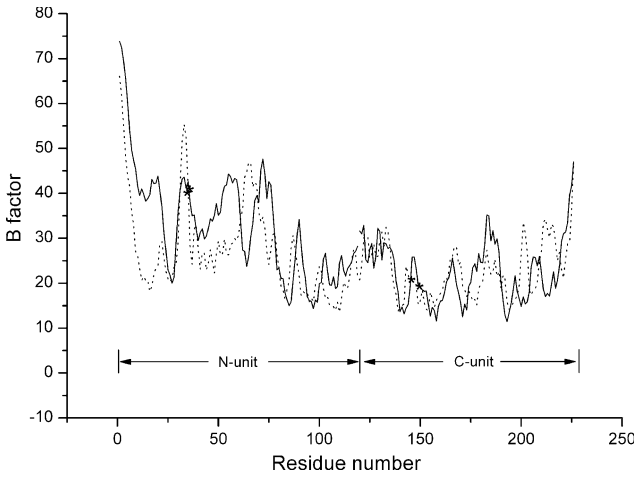

Fig. 7 Comparison of crystallographic temperature factors of the N- and C-units of PDO from *Pyrococcus furiosus* (*dotted line*) and *Aquifex aeolicus* (*solid line*) (Ren et al. 2006, unpublished), the positions corresponding to the C_α atoms of the N-terminal cysteines in both active sites are marked by *asterisks* (*)

AqPDO from a hyperthermophilic bacterium, *A. aeolicus*, which was recently isolated and structurally characterized (Pedone et al. 2006a; D'Ambrosio et al. 2004) 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 (PDI) 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).

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 (Bardwell et al. 1991; Pigiet and Schuster 1986; Derman et al. 1993; Prinz et al. 1997).

Searching for an archaeal protein disulfide isomerase

The observation of two sequential thioredoxin folds in the PDO proteins from *Py. furiosus* and *A. aeolicus* (Ren et al. 1998; Pedone et al. 2006a) 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 are likely to confirm the picture of two thioredoxin folds in tandem arrangement, with a CXXC motif on each unit. 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? 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 PDI.

Protein disulfide isomerase is the major enzyme involved in protein disulfide bond formation in the endoplasmatic reticulum of eukaryotes. PDI catalyzes the formation, rearrangement as well as breakage of protein disulfide bonds depending on the redox conditions of the environment (Bardwell and Beckwith 1993; Freedman et al. 1994) and thus corrects non-native cysteine pairing such that the substrate protein can assume its proper native conformation.

Protein disulfide isomerase from eukaryotes has a molecular weight of about 57 kDa and is a multi-domain 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 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 (Darby et al. 1994; Darby and Creighton 1995). Mutation of the Cys residues in the active sites of the domains a and a′ resulted in a dramatic decrease of PDI activity (Vuori et al. 1992). The isolated a and a′ domains are still able to catalyze disulfide formation, but do not show significant isomerase activity (Darby et al 1996). The recently determined crystal structure of yeast PDI (Tian et al. 2006) 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.

More recently, functional studies, too, have 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 (Pedone et al. 2004). PfPDO was thus the first archaeal enzyme with a documented PDI activity. Reductase activity was assayed by the turbidimetric method of (Holmgren 1979). The oxidative, disulfide-bond forming activity of PfPDO and several disulfide mutants was monitored by the so-called Ruddock test (Ruddock et al. 1996). The isomerase activity of PfPDO was assayed by the method of (Lambert and Freedman 1983), based on the re-activation of scrambled RNase. Quite recently ATP-dependent chaperone activity has been detected for the enzyme from *S. solfataricus* (Pedone et al. 2006b) suggesting a potential in vivo role of PDO.

Protein disulfide oxidoreductase from *Py. horikoshii* (PhPDO) has similar reduction properties for insulin disulfides as PfPDO (Kashima and Ishikawa 2003). It showed, however, no glutaredoxin activity, in line with the failure to detect glutathione (GSH and GSSG) in cell extracts of *Py. 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 PDO's 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. It was further tested in vitro whether thioredoxin reductase from *Py. 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 K_m value for PhPDO of 0.6 μM. In vitro, PhPDO and thioredoxin reductase from *Py. horikoshii* formed a general disulfide reductase system (Kashima and Ishikawa 2003), suggesting that thioredoxin reductase serves as a reduction catalyst of PhPDO in vivo. A similar conclusion was drawn in the work of (Pedone et al. 2006b) concerning PDO from *S. solfataricus*.

If PDO has isomerase function (which has been shown experimentally for PfPDO and AqPDO (Pedone et al. 2004, 2006a), 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.

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.

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 favor a redox reaction in a thermodynamic sense. However, if a thermodynamically favored 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.

The picture of disulfide metabolism in other organisms is very complex: in yeast there are five PDI homologs (Wang and Chang 1999) and, at least, 17 different human proteins (Ellgaard and Ruddock 2005) are known, with various combinations of catalytic and non-catalytic thioredoxin domains as well as additional transmembrane and chaperone domains. All of them show the same active site motifs. The complicated domain architecture in eukaryotes and yeast is poorly understood, as is the variability in the CXXC motifs in the PDO homologs of hyperthermophilic archaea and bacteria. A possibility is that the different combinations of CXXC motifs may dictate distinct redox functions for the thioredoxin units of the PDO homologs. 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.

Future directions

Numerous problems related to PDOs in hyperthermophiles remain unsolved to date, but can probably successfully be studied by genetic and in vivo approaches: 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 earlier suggested to result from a simple process—spontaneous oxidation. The discovery of PDIs 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 PDO's? The reduction system for archaeal PDO's is unknown? 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. 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? By comparison with the redox buffer in the ER, 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? Both sites can be characterized in analogy to PDI by functional asymmetry (Kulp et al. 2006). The functional asymmetry likely results from kinetic effects caused by interaction with the substrate protein. 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.

References

Anfinsen CB, Haber E (1961) studies on the reduction and re-formation of protein disulfide bonds. J Biol Chem 236:1361–1363

Anfinsen CB (1973) Principles that govern the folding of protein chains. Science 181:223–230

Aslund F, Berndt KD, Holmgren A (1997) Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein-protein redox equilibria. J Biol Chem 272:30780–86

Bardwell JCA, Beckwith J (1993) The bonds that tie: catalyzed disulfide bond formation. Cell 74:769–711

Bardwell JC, McGovern K, Beckwith J (1991) Identification of a protein required for disulfide bond formation in vivo. Cell 67:581–589

Beeby M, O'Connor BD, Ryttersgaard C, Boutz DR, Perry LJ, Yeates TO (2005) The genomics of disulfide bonding and protein stabilization in thermophiles. PloS Biol 3:1–10

Bönisch H, Schmidt CL, Schäfer G, Ladenstein R (2002) The structure of the soluble domain of an archaeal rieske iron–sulfur protein at 1.1 Å resolution. J Mol Biol 319:791–805

Bryk R, Lima CD, Erdjument-Bromage H, Tempst P, Nathan C (2002) Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein. Science 295:1073–1077

D'Ambrosio K, DeSimone G, Pedone E, Rossi M, Bartolucci S, Pedone C (2004) Crystallization and preliminary X-ray diffraction studies of a protein disulfide oxidoreductase from *Aquifex aeolicus*. Acta Cryst D 60:2076–2077

Darby NJ, Freedman RB, Creighton TE (1994) Dissecting the mechanism of protein disulfide isomerase: catalysis of disulfide bond formation in a model peptide. Biochemistry 33:7937–7947

Darby NJ, Creighton TE (1995) Functional properties of the individual thioredoxin-like domains of protein disulfide isomerase. Biochemistry 34:11725–11735

Darby NJ, Kemmink J, Creighton TE (1996) Identifying and characterizing a structural domain of protein disulfide isomerase. Biochemistry 35:10517–10528

Derman AI, Beckwith J (1991) *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. J Bacteriol 173:7719–7722

Derman AI, Prinz WA, Belin D, Beckwith J (1993) Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. Science 262:1744–1747

Eklund H, Ingelman M, Söderberg BO, Uhlin T, Nordlund P, Nikkola M, Sonnerstam U, Joelson T, Petratos K (1992) Structure of oxidized bacteriophage T4 glutaredoxin (thioredoxin): refinement of native and mutant proteins. J Mol Biol 228:596–618

Ellgaard L, Ruddock LW (2005) The human protein disulphide isomerase family: substrate interactions and functional properties. EMBO Rep 6:28–32

Fomenko DE, Gladyshev VN (2003) Identity and functions of CXXC-derived motifs. Biochemistry 42:1214–11225

Freedman RB (1995) The formation of protein disulfide bonds. Curr Opin Struct Biol 5:85–91

Freedman RB, Hirst TR, Tuite MF (1994) Protein disulfide isomerase: building bridges in protein folding. Trends Biochem Sci 19:331–336

Guagliardi A, de Pascale D, Cannio R, Nobile V, Bartolucci S, Rossi M (1995) The purification, cloning, and high level expression of a glutaredoxin-like protein from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Biol Chem 270, 5748–5755, with a published erratum in J Biol Chem (1997) 272, 20961

Holmgren A (1972) Tryptophan fluorescence study of conformational transitions of the oxidized and reduced form of thioredoxin. J Biol Chem 247:1992–1998

Holmgren A (1979) Thioredoxin catalyzes the reduction of insuline disulfides by dithiothreitol and dihydrolipoamide. J Biol Chem 254:9627–9632

Holmgren A (1995) Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. Structure 3:239–243

Holmgren A, Söderberg BO, Eklund H, Brändén CI (1975) Three-dimensional structure of *Escherichia coli* thioredoxin-S2 to 2.8 Å resolution. Proc Natl Acad Sci USA 72:2305–2309

Huber-Wunderlich M, Glockshuber R (1998) A single dipeptide sequence determines the redox properties of a whole enzyme family. Fold Des 3:161–171

Hutchinson EG, Thornton JM (1996) PROMOTIF-A program to identify and analyze structural motifs in proteins. Protein Sci 5:212–220

Kadokura H, Katzen F, Beckwith J (2003) Protein disulfide bond formation in prokaryotes. Annu Rev Biochem 72:111–135

Karlström M, Stokke R, Steen IH, Birkeland NK, Ladenstein R (2005) Isocitrate dehydrogenase from the hyperthermophile *Aeropyrum pernix*: X-ray structure analysis of a ternary enzyme-substrate complex and thermal stability. J Mol Biol 345:559–577

Karshikoff A, Ladenstein R (2001) Ion pairs and the thermotolerance of proteins from hyperthermophiles: a ''traffic rule'' for hot roads. TiBS 26:550–556

Kashima Y, Ishikawa K (2003) A hyperthermostable novel protein-disulfide oxidoreductase is reduced by thioredoxin reductase from the hyperthermophilic archaeon *Pyrococcus horikoshii*. Arch Biochem Biophys 418:179–185

Katti SK, LeMaster DM, Eklund H (1990) Crystal structure of thioredoxin from *Escherichia coli* at 1.68 Å resolution. J Mol Biol 212:167–184

Katz BA, Kossiakoff A (1986) The crystallographically determined structures of atypical strained disulfides engineered into subtilisin. J Biol Chem 261:15480–15485

Kemmink J, Darby N, Dijkstra K, Nilges M, Creighton TE (1996) Structure determination of the N-terminal thioredoxin-like domain of protein disulfide isomerase using multidimensional heteronuclear 13C/15N NMR spectroscopy. Biochemistry 35:7684–7691

Krause G, Lundström J, Barea JL, Delacues CP, Holmgren A (1991) Mimicking the active site of protein disulfide isomerase by substitution of proline 34 in *Escherichia coli* thioredoxin. J Biol Chem 266:9494–9500

Kulp MH, Frickel EM, Ellgaard L, Weissman JS (2006) Domain architecture of protein-disulfide isomerase facilitates its dual role as an oxidase and an isomerase in ero1p-mediated disulfide formation. J Biol Chem 281:876–884

Lambert N, Freedman RB (1983) Kinetics and specificity of homogeneous protein disulphide-isomerase in protein disulphide isomerization and in thiol–protein–disulphide oxidoreduction. Biochem J 213:235–243

Mallick P, Boutz DR, Eisenberg D, Yeates TO (2002) Genomic evidence that the intercellular proteins of archaeal microbes contain disulfide bonds. Proc Natl Acad Sci USA 99:9679–9684

Martin JL, Bardwell JCA, Kuriyan J (1993) Crystal structure of the DsbA protein required for disulfide bond formation in vivo. Nature 365:464–468

Mössner E, Huber-Wunderlich M, Glockshuber R (1998) Characterization of *E. coli* thioredoxin variants mimicking the active sites of other thiol-disulfide oxidoreductases. Protein Science 7:1233–1244

Pedone E, Ren B, Ladenstein R, Rossi M, Bartolucci S (2004) Functional properties of the protein disulfide oxidoreductase from the archaeon *Pyrococcus furiosus*: a member of a novel protein family related to protein disulfide-isomerase. Eur J Biochem 271:3437–3448

Pedone E, D'Ambrosio K, De Simone G, Rossi M, Pedone C, Bartolucci S (2006a) Insights on a new PDI-like family: structural and functional analysis of a protein disulfide oxidoreductase from the bacterium *Aquifex aeolicus*. J Mol Biol 356:155–164

Pedone E, Limauro D, D'Alterio R, Rossi M, Bartolucci S (2006b) Characterization of a multifunctional protein disulfide oxidoreductase from *Sulfolobus solfataricus*. FEBS J 273:5407–5420

Pigiet VP, Schuster BJ (1986) Thioredoxin-catalyzed refolding of disulfide-containing proteins. Proc Natl Acad Sci USA 83:7643–7647

Prinz WA, Aslund F, Holmgren A, Beckwith J (1997) The role of the thioredoxin and glutaredoxin pathways in reducing disulfide bonds in the *Escherichia coli* cytoplasm. J Biol Chem 272:15661–15667

Ren B, Tibbelin G, de Pascale D, Rossi M, Bartolucci S, Ladenstein R (1998) A protein disulfide oxidoreductase from the archaeon *Pyrococcus furiosus* contains two thioredoxin fold units. Nat Struct Biol 5:602–611

Ren B, Haase I, Fischer M, Ladenstein R (2006) unpublished work

Ruddock LW, Hirst TR, Freedman RB (1996) pH dependence of the dithiol-oxidizing activity of DsbA (a periplasmic protein thiol: disulphide oxidoreductase) and protein disulphide isomerase: studies with a novel simple peptide substrate. Biochem J 315:1000–1005

Tian G, Xiang S, Noiva R, Lennarz WJ, Schindelin H (2006) The crystal structure of yeast protein disulfide isomerase suggests cooperativity between its active sites. Cell 124:61–73

Toth EA, Worby C, Dixon JE, Goedken ER, Marqusee S, Yeates TO (2000) The crystal structure of adenylosuccinate lyase from *Pyrobaculum aerophilum* reveals an intracellular protein with three disulfide bonds. J Mol Biol 301:433–450

Vuori K, Myllylä R, Pihlajaniemi T, Kivirikko KI (1992) Expression and site-directed mutagenesis of human protein disulfide isomerase in *Escherichia coli*. This multifunctional polypeptide has two independently acting catalytic sites for the isomerase activity. J Biol Chem 267:7211–7214

Wang Q, Chang A (1999) Eps1, a novel PDI-related protein involved in ER quality control in yeast. EMBO J 18:5972–5982

Wunderlich M, Jaenicke R, Glockshuber R (1993) The redox properties of protein disulfide isomerase (DsbA) of *Escherichia coli* result from a tense conformation of its oxidized form. J Mol Biol 233:559–566

Zapun A, Bardwell JC, Creighton TE (1993) The reactive and destabilizing disulfide bond of DsbA, a protein required for disulfide bond formation in vivo. Biochemistry 32:5083–5092