Mini Review

Thioredoxin – Structural and Functional Complexity

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Abstract. Thioredoxins are small globular proteins that proved to be excellent model for investigating the relationship between the structure of protein and their physico-chemical and functional properties. The results from the experiments on thioredoxins offer the basic for the development of the new paradigms in the field of chemistry, biophysics and biology of proteins, with special attention to redox reaction in living cells, protein stability and design. It is a good example of broad class of sulphur-containing redox proteins.

Key words: Thioredoxin — Electrostatics — Redox reaction — Structure — Thermodynamic stability

Some background

Thioredoxins (Trx) are small proteins (molecular weight about 12 kDa) found in all living cells from archaebacteria to humans. They are involved in a wide variety of fundamental biological functions including dithiol hydrogen donation in ribonucleotide reduction, structural roles of coliphages such as f1 and M13, regulation of the activity of photosynthetic enzymes and some eukaryotic transcription regulation factors (Holmgren 1989; Jacquot et al. 1994; Holmgren 1995). The experimental evidence suggest the new roles of Trx as a signal for cancer cell growth and the protection of living cells from cytotoxicity caused by oxygen-free radicals (Fujji et al. 1991; Gasdaska et al. 1995).

All Trx possess a highly active site made up of two neighbouring cysteines in a conserved motif, Cys-Gly-Pro-Cys (CGPC), also referred to as the "Trx-motif". The oxidized form of the protein is reduced by NADP-linked system, present in all types of the cells, in which Trx obtains electrons from NADPH *via* the flavin enzyme Trx reductase (TrxR) (Fig. 1). The reduced Trx are the major cellular

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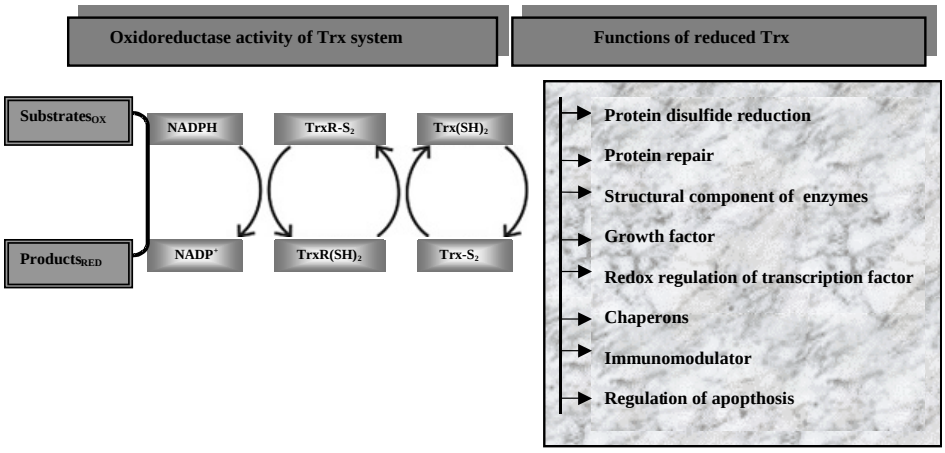

Figure 1. Oxidoreductase activity of Trx system and functions of reduced Trx (Trx-S_2).

protein disulfide reductases; therefore they also serve as an electron donor for specific enzymes of primary metabolism. However, the functions of Trx are by far not limited to this reaction (Fig. 1); and in fact, different Trx within one organism can serve different functions.

Primary structures of many Trx are known. These vary in length from 105 to 110 amino acids, and show 27–69 % sequence identity (Eklund et al. 1991). The Trx active site was found in Trx-like domains in several proteins, which represent a broad group of the multifunctional proteins with different roles in the living cells. These proteins can act in extra- or intra-molecular space and together form the Trx superfamily.

Structural studies of Trx

Three-dimensional structure of oxidized Trx (108-residues protein) from *Escherichia coli* has been determined to 2.8 Å and later redefined to 1.68 Å resolution by X-ray crystallography (Holmgren et al. 1975; Katti et al. 1990). This structure is reference structure for Trx. The protein molecule folds to form a core of β-pleated sheet flanked on either side by helices. The mixed β-sheet contains five strands and is twisted. The structure can be considered as being formed of two conformational domains, $\beta\alpha\beta\alpha\beta$ from residues 1 to 59 and $\beta\beta\alpha$ from residues 76 to 108, connecting with an 18-residues long segment that runs as distorted helices across the pleated sheet (Fig. 2). It is a highly structured molecule with 90 % of its residues involved in secondary structural elements. For other proteins, this number is usually less than 70 %. Thus, Trx is a very good protein for design and theoretical studies, because the secondary structural elements of the protein structure are relatively easily modelled.

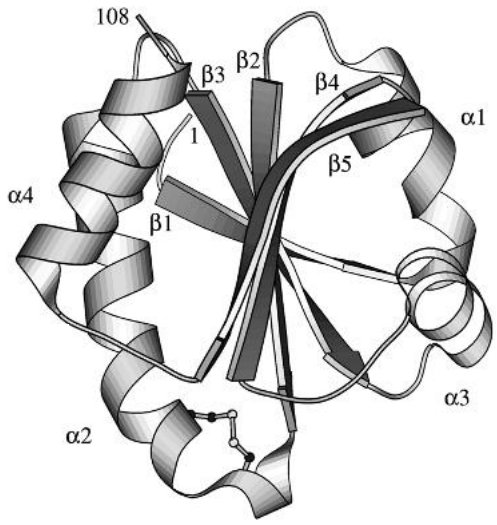

Figure 2. Schematic drawing of three-dimensional structure of *E. coli* Trx. It is a structure with a central five-stranded β-sheet with four flaking α-helices and a disulphide group in the active site (Holmgren et al. 1975; Katti et al. 1990).

First three-dimensional structure of Trx from photosynthetic organism was solved Trx 2 from *Anabaena* (Saarinen et al. 1995). The structural studies of the two chloroplast Trx *m* and Trx *f*, were published a few years later (Capitani et al. 2000). Crystal structures were determined for oxidized, recombinant Trx *f* (Trx *f-L*) and at the N terminus truncated form of it (Trx *f-S*), as well as for oxidized and reduced Trx *m* (at 2.1 and 2.3 Å resolution, respectively). Whereas Trx *m* crystallized as a monomer, both truncated Trx *f* and Trx *m* crystallized as non-covalent dimers. The structures of Trx *f* and Trx *m* exhibit the typical Trx fold consisting of a central twisted five-stranded β-sheet surrounded by four α-helices. Trx *f* contains an additional α-helix at the N terminus and an exposed third cysteine close to the active site. The overall three-dimensional structures of the two chloroplast Trx are quite similar. However, the two proteins have a significantly different surface topology and charge distribution around the active site (Capitani et al. 2000).

Human Trx was identified as the active component in many different biological processes and was given different names since initially it was unknown that the new molecules were identical with Trx (Tagaya et al. 1989; Clarke et al. 1991; Gasdaska et al. 1994). Determination of crystal structures of both reduced and oxidized wild type human Trx (at 1.7 and 2.1 Å nominal resolution, respectively) and of reduced mutant proteins offered many functional implications (Weichsel et al. 1996). Surprisingly, these Trx were in dimeric form (active form of the protein), covalently linked through a disulfide bond involving cystein residue from each monomer (third cysteine residue outside of the active site). Covalently linked dimer forms block of the Trx active site, which plays an important physiological role (Weichsel et al. 1996; Andersen et al. 1997). The dimerization of Trx from *E.*

coli was also observed under usual conditions for crystallization trials, i.e. pH values below its isoelectric point (pH 4.5) and high protein concentration. However, the above mentioned conditions are not physiologically relevant (Ladbury et al. 1993, 1994).

Trx arose also a lot of interest among NMR structural biologists. NMR structure has been solved for oxidized and reduced Trx from *E. coli* (Jeng et al. 1994), for reduced human Trx (Forman-Kay et al. 1991), for a thermostable Trx from *Bacillus acidocaldarius* (Nicastro et al. 2000), for cytosolic Trx *h* (Mittard et al. 1997) and for *m*-like Trx from green algae *Clamydomonas reinhardii* (Lancelin et al. 2000) and for Trx *m* from spinach (Neira et. al. 2001). There are no significant differences between corresponding structures from X-ray and NMR studies.

Proteins with Trx fold

Number of proteins was grown that showed sequence and structural similarity with Trx, which has one of the most common protein domain fold. In fact, some even contain a redox active Trx domain as a part of the structure such as the recently discovered sperm cell specific proteins hSptrx-1 (Jimenez et al. 2002). However, in most cases the redox active site is different, e.g., Cys-Gly-His-Cys in protein disulfide isomerase (PDI), and in calcium binding proteins 1 and 2 (CaBP1 and 2) (Lundstrom and Holmgren 1990; Lundstrom-Ljung et al. 1995). Many of these proteins can also be reduced by large TrxR, yet they lack other defining features of Trx – especially they do not provide reducing equivalent for ribonucleotide reductase. The casual use of the terms "Trx", "Trx domain", "Trx-motif", and particularly "Trx-like motif" for proteins that lack the classical core CGPC sequence (Matsuo et al. 2001; Anelli et al. 2002; Hosoda et al. 2003) and occasionally even Trx-typical redox activity (Matsuo et al. 2001; Anelli et al. 2002; Hosoda et al. 2003), is problematic.

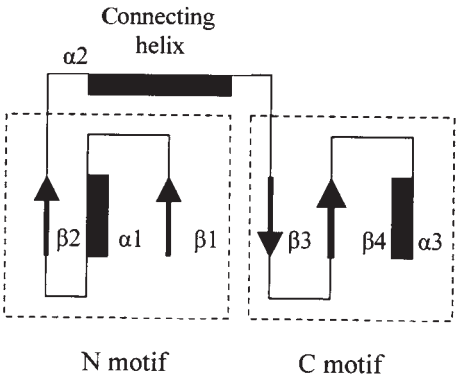

Figure 3. Architecture of Trx fold. β-sheet strands are drawn as arrows and α-helices as rectangles.

All Trx have similar three-dimensional structures despite the large variation in amino acid sequences (Eklund et al. 1991; Martin 1995). The Trx fold was found also in other 9 classes of proteins, including redox proteins: glutaredoxin, Dsb protein from *E. coli*, glutathione peroxidase, glutathione S-transferase, protein disulfide isomerase, cytochrome c oxidase (COX, proteins conserved among prokaryotes and eukaryotes), peroxiredoxins, evolutionary conserved Dim1 protein and iodothyronine selenodeiodinases. This fold is modified in above mentioned proteins by additional amino acid residues at the

N and/or C terminus of the Trx fold and some insertion in the specific regions of this fold (Fig. 3). The overall structural similarity between the Trx fold in all proteins is very interesting, despite it, proteins suggest functional differences and low sequence identity (Martin 1995; Kemmink et al. 1996).

Stabilities of Trx

Structural studies of oxidized and reduced form of *E. coli* and human Trx show that while the global structure is very similar, there exist some localized conformational differences, particularly in the active site region and regions close by (Jeng et al. 1994; Qin et al. 1994). When *E. coli* Trx is reduced, the temperature of thermal denaturation drops by about 12℃. Studies by guanidine hydrochloride and urea denaturation has shown that reduction of disulfide bond in the active site lowers the stability of native Trx by about 10–12.5 kJ/mol at neutral pH (Kelley et al. 1987; Lin and Kim 1991; Ladbury et al. 1994; Ghoshal et al. 1999). These changes of stabilities of *E. coli* Trx are too dramatic in relation to very small structural changes upon reduction of this protein. The behaviour of other Trx upon reduction or oxidation seems to be very similar. The mechanism is still not very well understood. One possible explanation lies in the significant changes in dynamic properties after reduction as demonstrated by differences in the hydrogen exchange behaviour and partial specific volume (Kaminsky and Richards 1992a,b). From experimental data, the lowered temperature of thermal denaturation of the protein indicates a decrease in stability upon reduction possibly due to fewer internal hydrogen donor bonds that normally increase the energy barriers for hydrogen exchange for the amide protons (Kaminsky and Richards 1992a). Because the structures of the oxidized and reduced Trx from *E. coli* are reported to be very similar, it is possible that alterations in the solvent layer in vicinity of the protein surface must play a very important role in producing changes in the apparent specific volumes and compressibility of Trx. These facts suggest for the significant role of the surrounding solvent in the stability of protein. In addition, the changes in flexibility around the active site upon the reduction should result in changes in compressibility (Kaminsky and Richards 1992b). The role of the solvatation energy in protein folding and binding belongs to the most interesting topics of nowadays proteins research. Mainly from this reason, Trx are very good candidates as model protein for such research.

Mutagenesis experiments in studies of Trx

New views on functions of Trx offer mutagenesis experiments together with determination of three-dimensional structures. For example, the Trx gene disruption experiments (Matsiu et al. 1996; Nonn et al. 2003) with mice resulted in embryonic lethality in homozygous animals, which indicated the importance of the Trx system. Recent site-directed mutagenesis experiments and structural studies have shown differences between wild type protein and various types of mutants in sta-

bility (Pedone et al. 1999, 2001; Rudresh et al. 2002), biochemical activity and pH dependence (Krimm et al. 1998), kinetics of reaction with TrxR (Lin 1999), and in structural consequence (Rudresh et al. 2002). Another site-directed mutagenesis experiments indicate that the local sequence within the active site region plays an important role in the modulation of redox potential (Chivers et al. 1996, 1997). The reduction potential of Trx is usually about −270 mV. Trx with mutation in close proximity of their active site show large differences in level of redox potential. *E. coli* Trx with mutated proline to histidine, with active site sequence Trp-Cys-Gly-His-Cys, similarly as in the domain of PDI, had a more oxidizing redox potential than the wild type Trx (−235 mV). Its catalytic properties were intermediate between Trx and PDI (Krause et al. 1991). The reduction potential of Trx is very sensitive to the changes of amino acid residues between cysteines in the active site; mutant with the active site changed to Cys-Trp-Gly-Cys has value of reduction potential −200 mV (Chivers et al. 1996).

Similar active sites as in Trx were also found in other proteins with Trx fold, i.e. protein disulfide isomerase (Cys-Gly-His-Cys) with reduction potential −180 mV, *E. coli* glutaredoxin 1 and 3 with identical active site sequence (Cys-Pro-Tyr-Cys) with reduction potential −233 mV and −198 mV, respectively (Aslund et al. 1997), and in the most oxidizing protein known, DsbA, with reduction potential −120 mV. The value of reduction potential is resulted from electrostatic interaction within protein matrix and other structural factors. An increase in reduction potential means that stability of the reduced form has increased relatively to the oxidized form. The substitution of the proline to another amino acid residue increases the conformation entropy of a polypeptide chain. This additional entropy contributes to the stabilisation of reduced state, because the oxidized form would be more strained. This is one reason why the mutants of Trx with substituted proline in the active site have higher reduction potential than wild type of Trx (Krause et al. 1991; Lin and Kim 1991; Chivers et al. 1996). These mutants of Trx with substituted proline in the active site indicate some structural, functional and thermodynamic differences (de Lamotte-Guery et al. 1997; Rudresh et al. 2002).

The ability of Cys-X-X-Cys (X, amino acid residue) motif to vary widely in their ability to assist electron flow makes the motifs vary as a molecular reostat, which can be set to a particular reduction potential to fit a particular need (Chivers et al. 1997).

Conclusion

Taken together, Trx has become an important system in understanding protein stability and folding. This small protein has 90 % of its residues involved in secondary structural elements. Also for this reason it becomes an excellent model for computer modelling and theoretical analysis by different theoretical methods (molecular dynamics, electrostatics). The Trx research offers also better understanding of fundamental aspects of electron transfer reactions in living cells.

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