Mini Review

Oxidative stress and thioredoxin system

M. Kohárýová and M. Kollárová

Department of Biochemistry, Faculty of Natural Sciences, Commenius University, Bratislava, Slovakia
E-mail: koharyova@fns.uniba.sk

Abstract. Oxidative stress plays an important role in the modulation of several important physiological functions. On the other side, oxidative stress is accountable for development of many unphysiological changes, which can be deleterious for cells. Consequently, at the present time there is increased interest about study mechanisms and changes evocated by oxidative stress. Despite the highly oxidizing environment (21% oxygen, at sea level), at normal conditions, the cell cytoplasm of all aerobic organisms is reduced and proteins contain free sulfhydryl groups. In the cytoplasm, two major systems were identificated responsible for maintaining a reduced state: thioredoxin and glutathione/glutaredoxin system. Thioredoxin in bacteria, thanks to the low redox potential is the major dithiol reductant in the cytosol, or an advanced equivalent to dithiothreitol of cells (Holmgren 1985). Thioredoxin system acts the dominant role in many physiological processes (see below) and it is also a cell antioxidant.

Key words: Oxidative stress — Thioredoxin system

Oxidative stress

At the end of 19th century and at the begining of 20th century, when McCord and Fridovich discovered the first detoxication enzyme – superoxide dismutase, intense research of free radicals and reactive metabolites arosed (McCord and Fridovich 1969). For clear explanation, it is good to remind that in literature are used exactly different terms – „free radicals" and „non-radical metabolites derived from oxygen and nitrogen". But for both groups are often used terms: reactive metabolites or reactive spieces. Reactive metabolites include: reactive metabolites derived from oxygen (ROS – reactive oxygen spieces) and reactive metabolites derived from nitrogen (RNS – reactive nitrogen spieces). The most important is the fact, that not all ROS and RNS are radicals (e.g. H_2O_2).

The creation of free radicals and reactive metabolites can be initiated by exogenous (e.g. ionizing, UV radiation, some medicaments, herbicide or insecticide contamination and cigarette smoke) and by endogenous factors (e.g. mitochondrial electron transport system, oxidative phosphorylation).

Correspondence to: Marta Kollárová, Department of Biochemistry, Faculty of Natural Sciences, Commenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia
E-mail: kollarm@fns.uniba.sk

Today it is well known that ROS and RNS play an important role in the modulation of several physiological functions. Mainly, there are cell signaling processes involved in inflammation, cell proliferation, angiogeneses, apoptosis and aging (Sies 1993a). Reactive metabolites are effective weapons of fagocytes against foreign elements and also their contribution is suggesting in reproduction (in fertilization, sperms need H_2O_2 and superoxide). In case that ROS and RNS are produced in abnormal excess, in wrong place and without sufficient antioxidating protection, it can lead to the break of equilibrium between pro-oxidants and antioxidant in favour of pro-oxidants. This effect is named oxidative stress. Increased oxidative stress has been proposed in many physiological changes, for example a pathomechanism in neurodegenerative diseases including Parkinson's disease, stroke and epilepsy (Schweizer et al. 2004; Varsik et al. 2005a,b, 2006; Kollar et al. 2006). Also it is believed to affect the development of diabetic-associated vasculopathy, endothelial dysfunction, and neuropathy within erectile tissue (Young et al. 2004). Oxidative stress can also influence forming of unregulated acute inflammatory response (Lee and Downey 2001) among acute lung injury and its most severe forms, the acute respiratory distress syndrome. These complications are frequent in critically ill patients and are responsible for significant morbidity and mortality (Lesur et al. 1999). Inflammation and oxidative stress also represent

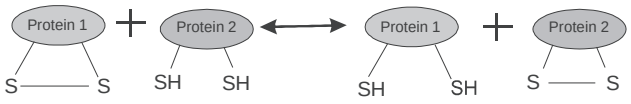

Figure 1. Modulation of redox regulation.

new features of the aertal and/or valvular calcification process and chronic micro-inflammation is also commonly observed in patients with chronic kidney disease (Kaysen 2001; Massy and Nguyen-Khoa 2002). Excessive ROS production has been implicated in the pathogenesis of atherosclerosis and hypertension (Alexander 1995; Araujo et al. 1998), in end-stage renal disease (Rigatto and Singal 1999) and also in diabetes mellitus (Baynes 1991).

The biological targets for highly ROS are DNA, RNA, proteins and lipids. In lipids, free radicals can attack directly polyunsaturated fatty acids in membranes and initiate lipid peroxidation. Primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can disrupt membrane-bound proteins. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products. Some of them, such as aldehydes, are very reactive and can damage molecules such as proteins (Humphries and Sweda 1998). Aldehydes, unlike reactive free radicals, are rather long lived and can therefore diffuse from the site of their origin and reach and attack targets, which are distant from the initial free-radical event, acting as „second toxic messengers" of the complex chain reactions initiated.

DNA is also main target. Active spieces attack both the base and the sugar moieties producing single- and double-strand breaks in the backbone, adducts of base and sugar groups, and cross-links to other molecules, lesions that block replication (Sies and Menck 1992; Sies 1993b). The spectrum of adducts in oxidized DNA *in vitro* and *in vivo* includes more than 20 known products, including damage to all four bases and thymine-tyrosine cross-links (Dizdaroglu 1992).

Proteins are ubiquitous in all cells and tissues, constituting more than 50% of the dry weight of cells, and are susceptible to oxidative (and also nitrosative) modifications. The oxidation of proteins includes: oxidation of sulfhydryl groups (the most frequent protein modification), oxidative adduction of amino acid residues close to metal-binding sites *via* metal-catalyzed oxidation, reaction with aldehydes, modification of prosthetic groups or metal clusters, protein-protein cross-linking and peptide fragmentation. The cellular redox state of proteins is a crucial mediator of multiple metabolic, signalling and transcriptional processes in cells and protein thiols in the form of cysteine residues are key players in redox sensing and regulation. The amino acid cystein is only one significant redox active component of proteins, which is critical for redox state. Cystein under normal atmospheric conditions will oxidize completely to form a disulphide bond (Dsb). The simplest scheme of modulation redox state is in Fig. 1.

Production and reduction of Dsbs is physiological feature. The redox state of protein thiols is dependent on the cellular location. Compartmentalization required for disulphide formation is important to protect cells from being exposed to nonspecific oxidation or reduction events. Dsbs are essential for the folding and stability of proteins that are secreted or localized through the secretory pathway. Similarly, many Dsbs are important in the quarternary structure of proteins, in the formation of homo- or hetero-multimers. In eukaryotic cells, Dsbs are formed in lumen of endoplasmatic reticulum by protein disulphide isomerases catalysis. In bacteria, Dsbs are formed in periplasm by catalysis during Dsb proteins (Raina and Missiakas 1997). The unwanted formation of Dsbs in the cytoplasm has been termed „disulphide stress" (Åslund and Beckwith 1999).

All these modifications are deleterious for the cell, since they lead to a loss of membrane function and also proteins, and block DNA replication or cause mutations (Cabiscol et al. 2000).

Aerobic organisms, despite the highly oxidizing environment where they live, maintain a reduced state inside the cell. Proteins in the extracellular environment or on the cell surface are rich in stabilizing disulphides, reflecting oxidizing condition. Inside of the cell is reduced and proteins contain many free sulfhydryl groups and disulphides are rare (Gilbert 1990). Also, at normal condition, it is bacterial cytoplasm maintained as a reducing environment. This state is under thiol-redox control. In *Escherichia coli*, the major thiol-disulphide redox buffer is the cysteine-containing tripeptide glutathione, which is presented at intracellular concentrations about ~5 mmol/l, 98–99.5% of it is in its reduced form. *In vitro*, these concentrations of reduced glutathione can inhibit Dsb formation in many proteins (Hwang et al. 1992). In the cytoplasms were indentificated further two major systems responsible for maintaining a reduced state inside cells: thioredoxin (Trx) and glutathione/glutaredoxin (GSH/Grx) systems (Holmgren 1985; Gilbert 1990; Williams 1992). In addition to protecting thiol groups from oxidation during oxidative stress, these systems provide a supply of electrons for enzymes, that undergo thiol-disulphide exchange as a part of their catalytic cycle. The Trx system, in which the major ubiquitous disulphide reductase responsible for the maintaining proteins in reduced state in cytoplasm is Trx, which is reduced by electrons from NADPH *via* Trx reductase (TrxR). The system together is called GSH/Grx system, in which other major factor generally responsible for low redox potential and high free SH level inside cells is GSH presented in millimolar concentrations and kept reduced by NADPH and GSH reductase (GSHR) (Gilbert 1990; Williams 1992). These reactions are catalyzed by Grxs (Fig. 2).

Trx system as the major cell reductant

In bacteria is thiol-Dsb balance generally maintained by Trx system and/or by GSH/GSHR system. Transcription of genes encoding Trx system in gram-negative bacteria *E. coli* is induced when cells are exposed to oxidizing agents like peroxide, further suggesting the importance of Trx system in the bacterial stress response (Ritz et al. 2000). Inactivation of *E. coli* TrxR increased Dsb formation, demonstrating that the cytoplasmic compartment is generally maintained in a reduced state (Derman et al. 1993). But *E. coli* has second system – GSH/GSHR, which also contributes to maintaining redox balance. In contrast to gram-negative bacteria, exemplified by *E. coli*, in which systems controlling the thiol state are well characterized, little is known about these systems in gram-positive bacteria. GSH has been detected in some bacteria of the family *Streptococcaceae* (Newton et al. 1996), although these bacteria seem to lack the enzymes to synthesize this compound. Thus, it appeared that Trx system may be the only system available to maintain intracellular thiol balance. Inactivation of the gene encoding Trx in gram-positive *Bacillus subtilis* or TrxR in gram-positive *Staphylococcus aureus* is reportedly lethal, further suggesting that a compensating GSH/GSHR system is under examined growth conditions absent or nonfunctional (Scharf et al. 1998; Uziel et al. 2004). Gram-positive bacteria *Streptomyces coelicolor* (and other streptomycetes) lacks GSH/Grx system. Instead, they contain a low-molecular-weight sugar-containing monothiol, called mycothiol, which plays a role analogous to GSH and protects actinomycetes against oxygen toxicity. But redox regulation of their proteins is almost exclusively under Trx control (Newton et al. 1996). Interesting observation was received by study of

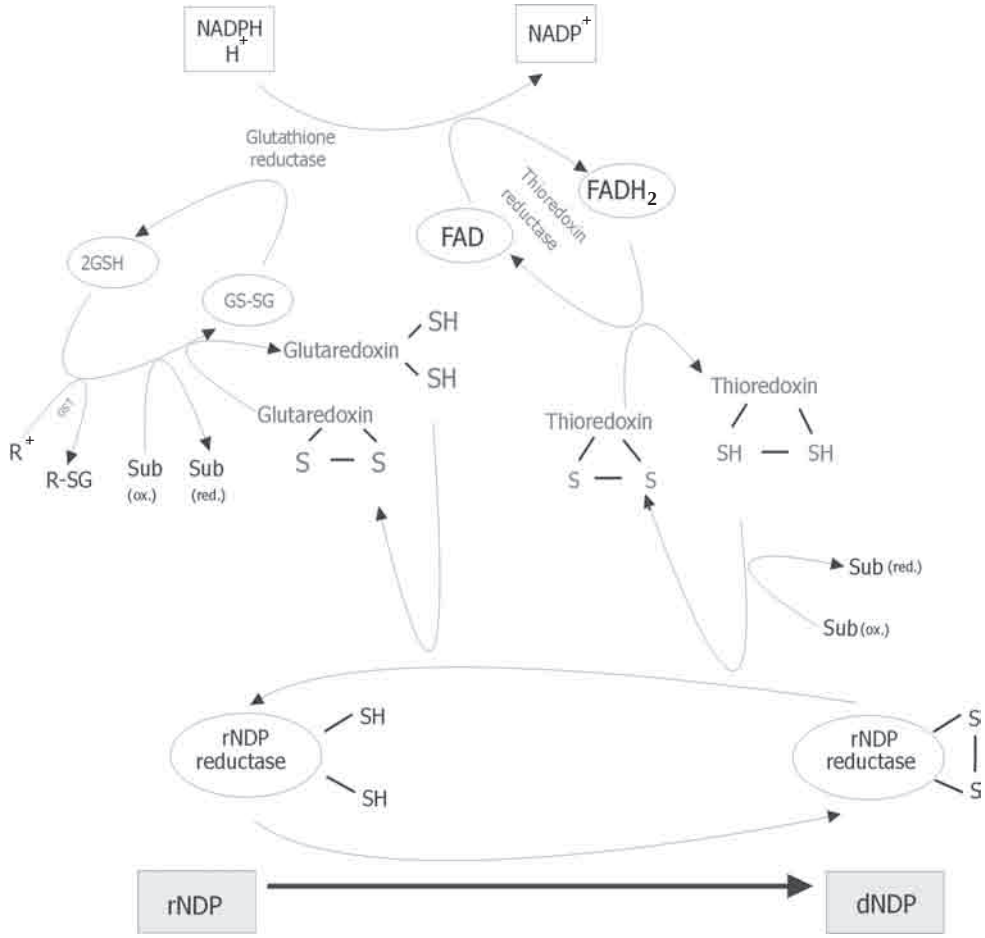

Figure 2. The scheme of thioredoxin (Trx) system and glutathione/glutaredoxin (GSH/Grx) system and role in transcription. Both systems are involved in the maintance of thiol status in mammalian cell. The Trx system (righ) with substrates for Trx, including oxidized proteins and some transcription factors and the GSH/Grx system (left). GSH can react with: 1. oxidized Grx, 2. electrophiles or oxidized macromolecules, 3. in a reaction with electrophiles catalysed by glutathione-S-transferase (GST). Sub, substrate; rNDP, ribonucleotide diphosphate; dNDP, deoxyribonucleoside diphosphate.

Lactococcus lactis trxB1 mutant. *L. lactis* is also gram-positive bacteria, lacking GSH biosynthesis pathway (Bolotin et al. 2001), it reportedly contains GSHR activity. This enzyme protects cell against oxygen toxicity only if GSH is supplied in the medium (Li et al. 2003). Inactivation of *trxB1* gene in *L. lactis* caused two additional effects previously not reported in other bacteria: i) induction of proteins involved in fatty acid or menaquinone biosynthesis, indicating that membrane synthesis is part of the cellular response to a redox imbalance, and ii) alteration of the isoforms of the glycolitic enzyme glyceraldehyde-3-phosphate dehydrogenase (Vido et al. 2005). In this case, for *L. lactis*, the TrxR is not essential.

Similarities of Trx system with GSH/Grx system

These systems were identificated together in many organisms, both of thiol systems play a key role in protecting cellular macromolecules from damage due to ROS and electrophilic spieces (Reed 1995). Common features of the Trx and GSH/Grx systems are in: i) an enzyme (TrxR, GSHR and also in enzymes: lipoamide dehydrogenase, trypanothione reductase and mercuric ion reductase (Williams 1992)), what is a member of homodimeric pyridine nucleotide-disulphide oxidoreductase family, ii) a small redox-active peptide (Trx and Grx, respectively), and iii) the ability to undergo to thiol-disulphide exchange.

Trx was first identificated as electron donor for ribonucleotide reductase, the essential enzyme that transcribes RNA to DNA, in *E. coli* (Laurent et al. 1964). The same role has Grx. The ribonucleotide reductase delivers electrons to the precursors of DNA synthesis – nucleotide diphosphates to deoxyribonucleotide diphosphates (Fig. 2).

Apart from this function, Trx (and also Grx) is an electron donor for 3′-phosphoadenosine-5′-phosphosulfate (PAPS) reductase in the sulfate assimilation pathway as well as for methioninesulfoxide (Met-SO) reductase in *E. coli* (Tsang and Schiff 1976; Ejiri et al. 1979). Met-SO reductase is involved in the repair of oxidized methionine. PAPS reductase is involved in the sulfate assimilation pathway and eventually leads to the cysteine synthesis. The cysteine biosynthesis needs to be always active. Without Trx the pathway backs up to PAPS, which acts as an intermediate enzyme for sulfate assimilation; in the absence of Trx or Grx, PAPS in mass quantities is toxic and may lead to the destruction of the cell (Russel et al. 1990).

In spite of a number of reports highlighting the roles of GSH/Grx system and Trx system in maintaining the redox homeostasis of the cell, until recently, there is no known functional interaction between these two systems. It was only well known, that Trx can reduce oxidated GSH (Kanzok et al. 2001).

Differences between Trx and GSH/Grx system

Differences lie in: i) the limited substrate specifity of GSHR, which only reduces GSH, and ii) the high intracellular levels of reduced GSH, which reduced electrophiles by both spontaneous and GSH transferase – catalysed mechanism.

In particular, mammalian TrxR shows a wider substrate specificity reducing not only Trx from different species but also nondisulphide substrates (Tab. 1). Grxs are able to catalyse reactions not only *via* a dithiol mechanism (as Trx do), but also *via* a monothiol mechanism (Bushweller et al. 1992), which is required for the reduction of protein GSH-mixed disulphides (deglutathionylation).

Dithiol mechanism in Trx system:

$$\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{Trx-(SH)}_2 + \text{NADP}^+$$
$$\text{Protein-S}_2 + \text{Trx-(SH)}_2 \rightarrow \text{protein-(SH)}_2 + \text{Trx-S}_2$$

Dithiol mechanism in GSH/Grx system:

$$\text{R-S}_2 + \text{Grx-(SH)}_2 \rightarrow \text{R-(SH)}_2 + \text{Grx-S}_2$$
$$\text{Grx-S}_2 + 2\ \text{GSH} \rightarrow \text{Grx-(SH)}_2 + \text{GSSG}$$

Table 1. Substrates for mammalian TrxR

Substrate	References
5, 5′- dithiobis (2-nitrobenzoic acid)	Williams (1992)
Vitamin K	Luthman and Holmgren (1982)
Alloxan	Holmgren and Lyckeborg (1980)
Sodium selenite	Kumar et al. (1992)
Sec	Björnstedt et al. (1997)
Selenodiglutathione	Björnstedt et al. (1992)
S-nitrosoglutathione	Nikitovic and Holmgren (1996)
Arachidonic acid hydroperoxides such as hydroperoxyeicosatetraenoic acid	Björnstedt et al. (1995)
Tumor-suppressor protein p53	Casso and Beach (1996)

Sec, selenocystein.

Monothiol mechanism in GSH/Grx system:

$$R\text{-}S\text{-}SG + Grx(SH)_2 \rightarrow R\text{-}SH + Grx\text{-}S\text{-}SG$$
$$Grx\text{-}S\text{-}SG + GSH \rightarrow Grx\text{-}(SH)_{2\,+}\, GSSG$$

R is protein, GSSG is oxidated GSH, R-S-SG is a mixed disulphide with GSH.

The study of *E. coli* redox pathways in cytoplasm revealed, that Grxs are generally less efficient reductants of Dsb than Trx (Holmgren 1989). This may be partially explained by the higher redox potentials of the Grxs (Trx = –270 mV, Grx 1 = –233 mV, Grx 3 = –198 mV) (Doig and Williams 1991). The low redox potential of Trx ensures that Trx-$(SH)_2$ is the major dithiol reductant in the cytosol, or an advanced equivalent to dithiothreitol of cells (Holmgren 1985).

Biochemical properties of Trxs

Trxs are small (M_r ~12,000) disulphide-containing redox proteins known to be present in all eukaryotic and prokaryotic organisms. Trx can be localized in the cytoplasm, in membranes, in mitochondrial eukaryotic cell fractions, as well as in the extracellular space. Trx is a member of the ubiquitous thiol-disulphide oxidoreductase family. The active sites of all Trxs have sequence: Cys-Gly-Pro-Cys, which is conserved throughout evolution. The oxidized form (Trx-S_2), where two cysteine residues are linked by an intramolecular Dsb, is reduced by flavoenzyme TrxR and NADPH (Holmgren 1985). The reduced form (Trx-$(SH)_2$) contains two thiol groups and can efficiently catalyze the reduction of many exposed disulphides. Therefore, Trx can interact with a broad range of proteins either in electron transport for substrate reduction or in regulation of activity by a seemingly simple redox mechanism based on reversible oxidation of two cysteine thiol groups to a disulphide, accompanied by the transfer of two electrons and two protons.

Oxidoreductase activity of Trx is illustrated through a multitude of functions, which can be grouped into two major categories. First, they act as electron carriers, providing reducing equivalents for the catalytic cycles of the biosynthetic and anti-oxidant enzymes, such as ribonucleotide reductases, methionine sulfoxide reductases and peroxiredoxins. Second, they protect the cytosolic proteins from aggregation and inactivation through intermolecular or intramolecular disulphides formation. Besides their antioxidative activity, Trxs have many other functions, some of them very specialized (subunit of T7 DNA polymerase, filamentous phage assembly), some others of high therapeutical significance, for example, their major regulatory effects on immune responses, including their ability to control the binding activity of immunologically active transcription factors (e.g. NFκB and AP-1) (Arnér and Holmgren 2000).

The first best characterized Trx with determinated three-dimensional structure obtained by X-ray crystallography comes from *E. coli*. It is an acidic protein (isoelectric point 4.5) containing 108 amino-acid residues of known sequence and it is devoid of metals and cofactors. The oxidation-reduction disulphide bridge is formed from Cys32 and Cys35 spaced by Gly33 and Pro34, thereby forming a 14-membered disulphide ring (Holmgren 1968). The molecule has about 75% of the residues in well-defined secondary structures. *E. coli* Trx is built up from a central core of three paralel and two antiparallel strands of pleated sheets surrounded by four helices (Holmgren et al. 1975). Interesting is, that all Trxs have 27 to 69% sequence identity to *E. coli* Trx, demonstrating that all Trxs have the same overall three-dimensional structure. This fact suggesting that all these proteins may possibly stem from a common ancestor (Martin 1995; Kemmink et al. 1996) and typical structure is often called the Trx fold (Euklund et al. 1991; Holmgren 1995). Trx fold consists from a central core of five stranded parallel β-sheets surrounded by four α helices with the archetypical active site sequence –Cys-X1-X2-Cys- (where X may represents any of 19 commonly occurring non-cysteine amino acids). While the GSH transferase and GSH peroxidase lacking the –Cys-X1-X2-Cys- active-site motif, share with the Grxs a specific interaction with GSH. Trx fold has been found in another nine protein classes: Trxs, Grxs, peroxiredoxins, Dsb proteins (Collet and Bardwell 2002), Dim1 proteins (Zhang et al. 1999), Sco1/2 proteins (Balatri et al. 2003), protein disulphide isomerase, GSH transferases, GSH peroxidases (Martin 1995; Ferrari and Söling 1999) and iodothyronine selenodeiodinases (Callebaut et al. 2003). This group is also named Trx-like-fold superfamily (Tab. 2). Among the recent additions to the Trx superfamily is a protein interacting with protein kinase C, coined protein kinase C-interacting cousin of Trx (Witte et al. 2000), a larger cytoplasmatic Trx-like protein of unknown function (Miranda-Vizuete et al. 1998) and a nuclear Trx-like protein with oxidoreductase activity, also of unknown function, named nucleoredoxin (Kurooka et al. 1997).

Table 2. The thiol-disulphide active sites of members of the thioredoxin (Trx) superfamily

Protein	Active site sequence
DsbA	Cys-Pro-His-Cys
DsbB	Cys-Val-Leu-Cys
DsbC	Cys-Gly-Tyr-Cys
DsbD	Cys-Val-Ala-Cys
DsbE	Cys-Pro-Thr-Cys
Trx	Cys-Gly-Pro-Cys
PDI	Cys-Gly-His-Cys

DsbA–E, disulphide bond protein A–E; PDI, protein disulphide isomerase.

Despite the high similarity in structure, Trxs have low sequence identity (vary in lengh from 105 to 110 amino acids) and have functional differences.

More information about structural and mutagenesis studies of Trxs are reviewed in previous article by Štefanková et al. 2005.

Catalytic mechanism of Trx

Two active site cysteine residues provide the sulfhydryl groups involved in the Trx-dependent reducing activity. This sequence is localized in a protrusion of the protein surface (Jeng et al. 1994) at the end of a β strand (β_2) and in the beginning of a long α helix. In all Trxs, the active-site α2 (residues 32 to 41) is divided into two parts by the presence of an invariant proline residue at position 41. The first part of this helix (residues 32 to 41) displays regular helical bonding and the central residues in this turn are Asn-Gly. The study of *E. coli* Trx revealed, that only the sulfur of Cys32 is exposed to solvent. The low acidic dissociation constant (pK_a) value of Cys32 (Cys35 showed a higher pK_a value) is suggested to arise from the partial positive charge from the α-helix dipole moment of helix α2 (Hol 1985). On this state is based the proposed mechanism of Trx-catalyzed protein disulphide reduction. The basic concepts of this mechanism are:

- Firstly, reduced Trx has a hydrophobic surface area, conserved in the Trx/Grx family (Eklund and Brändén 1984), which binds to a substrate protein, making a complex.
- Secondly, in the hydrophobic environment of the complex, the thiolate of Cys32, acting as a nucleophile, attacks the target protein to form a covalently linked mixed disulphide transition state.
- Finally, attack of the now deprotonated thiolate of Cys35 on the disulphide generates a dithiol in the target protein and a disulphide in Trx.

The mechanism involves a transient mixed disulphide intermediate and fast thiol-disulphide exchange in a hydrophobic environment. The reaction is reversible and Trx may either break or form disulphides depending on the redox potential of its substrate. Conformational changes in Trx and the target protein occur during binding and the subsequent electron-transfer steps (Holmgren 1995).

Another conserved residues near the active site of Trx

Most of the residues conserved in the 39 known Trx sequences are located around the active site (Saarinen et al. 1995). These highly conserved residues are: Asp26, Ala29, Trp31, Asp61, Pro76 (in the *cis* configuration), and Gly92 (using the *E. coli* numbering).

However, Trx is a substrate for two enzymes, TrxR and ribonucleotide reductase, the active center region of Trx must interact with the active centers of these two enzymes. Thus, in all probability, these enzymes will have active site clefts, which are suitably adapted to fit the active site protrusion in Trx. Asp26, apart from some of the residues in the protrusion, might participate in this enzyme interaction. Asp26 is the only negatively charged residue in the interior of the molecule. It is, however, accessible through a narrow pocket inside and above the protrusion and thus might interact with a positively charged residue in the reductases. Asp26 in the vicinity of the active-site is important for enzymatic activity (Saarinen et al. 1995). It is highly conserved in all Trxs and is the subject of numerous studies aimed at defining its role in Trx stability and function. A water molecule hydrogen bonds to the side-chain carboxylate of Asp26 and the main-chain carbonyl of the active site Cys36 (Katti et al. 1990; Saarinen et al. 1995). The conserved Asp residue serves as an acid/base in the oxidation/reduction reactions catalysed by Trxs, by protonating (during substrate oxidation) or deprotonating (during substrate reduction) the thiol of most buried substrate of cysteine residue *via* this bridging water molecule (Menchise et al. 2001).

Tryptophan lid over the redox disulphide, a ubiquitous feature of the Trx superfamily, is able to adopt different positions with respect to the redox disulphide, as noted in a mutant *E. coli* Trx (Nikkola et al. 1993), in chloroplast Trx (Capitani et al. 2000) and in the trypanosomatid protein called tryparedoxin. This tryptophan is a key residue in docking interactions of Trx and tryparedoxin with redox partners (Alphey et al. 2003).

Lysine residue, Lys36, is the only positively charged residue in the vicinity of the S-S bridge and thus might participate in the oxidation-reduction mechanism by stabilizing a negatively charged thiol ion intermediate.

Mammalian Trxs

In mammals have been identified two forms of Trxs and one special „truncated form" of Trx. Cytosolic/nuclear Trx1 and mitochondrial Trx2 are two Trxs isoforms encoded by two distinct genes (Arner and Holmgren 2000). Recently, based on protein sequence organization, a second group of Trxs has been distinguished composed of fusion proteins of Trx-like and additional domains (Sadek et al. 2003; Jimenez et al. 2004). Trx1 and Trx2 are parts of the so-called cytosolic and mitochondrial Trx systems including cytosolic and mitochondrial NADPH-dependent TrxRs (Miranda-Vizuete et al. 2000).

Human Trx1 was first purified from serum of leukemia patients as a secreted protein upregulating the IL-2 receptor and having co-cytokine activity. After transformation of human T-lymphocytes by T-lymphotropic virus 1, initially called adult T-cell leukemia derived factor, was secreted (Wakasugi et al. 1990). Only subsequently was shown that this factor and human Trx are the same protein.

Secretion of Trx under conditions of oxidative stress and inflammation has been observed from many normal or neoplastic cells (Rubartelli et al. 1995). Clinically, plasma Trx levels are raised in patients undergoing cardiopulmonary bypass operations (Nakamura et al. 1998) and in those with reumatoid arthritis (Yoshida et al. 1999) or HIV infection (Nakamura et al. 1996). Extracellular Trx has proinflammatory effects by potentiating cytokine release from fibroblasts (Yoshida et al. 1999) as well as monocytes (Schenk et al. 1996). Recently, Trx has been shown to act as a chemotactic protein causing migration of neutrophils, monocytes and T-cells with a potency similar to known chemokines including IL-8 (Bertini et al. 1999). Mutation of the active site residues to serines resulted in loss of chemotactic activity, suggesting that the redox activity is required. The concentration-dependent activity of Trx as a chemotactic factor is bell-shaped like other chemotactic agents and has a maximum in the range of the Trx plasma concentration (25 ng per milliliter).

For healthy individuals, the reference range of Trx in blood plasma is 15 to 40 mg per liter (Nakamura et al. 1998). Plasma Trx is not a standard clinical parameter, but a number of pathological conditions under which the serum level of Trx is affected have already been identified.

Mammalian mitochondrial Trx2 was first cloned and characterized in rat (Spyrou et al. 1997) and more recently in human (Chen et al. 2002; Damdimopoulos et al. 2002). Overexpression of mitochondrial Trx2 in human cells shows that Trx2 interacts with components of the mitochondrial respiratory chain and plays a role in the regulation of the mitochondrial membrane potential (Damdimopoulos et al. 2002) as well as in the protection against peroxide-induced apoptosis (Chen et al. 2002). Also, Trx2 was involved in the inhibition of apoptosis signal-regulating kinase 1-mediated apoptosis (Zhang et al. 2004). Finally, Trx2 inactivation in the chicken cell line and in the mouse revealed that mitochondrial Trx2 plays a crucial role in the regulation of the mitochondrial apoptosis signaling pathway, and is essential for normal development of mice, the embryonic lethality coinciding with maturation of mitochondria (Tanaka et al. 2002; Nonn et al. 2003). The absence of Trx2 in homozygous mouse causes massive apoptosis, exencephaly and early embryonic lethality despite of attendance of functional mitochondrial Grx2, MnSOD and also other functional antioxidant proteins present in other parts of the cell: GSH/phospholipid hydroperoxide GSH peroxidase (Knopp et al. 1999) and catalase (Radi et al. 1991). In the MnSOD knockout mouse, the neonates die by age 10 days of neurodegeneration and dilated cardiomyopathy (Li et al. 1995).

At the surface of monocytic cell lines was found a truncated form of Trx1, named Trx80, which is comprised of residues 1 to 80. Trx80 is most likely identical to the protein described as eosinophilic cytotoxicity enhancing factor, which enhances the capacity of eosinophils to kill larvae of *Schistosoma mansoni* (Lenzi et al. 1985; Silberstein et al. 1993). Recent results have shown that cloned Trx80 is a novel mitogenic cytokine for human peripheral blood mononuclear cells (Pekkari, Gurunath, Arnér and Holmgren, unpublished results).

TrxR

TrxR (EC 1.6.4.5) belongs to a family of GSHR-like homodimeric flavoenzymes, that catalyze the transfer of two electrons from NADPH, *via* FAD and an N-terminal disulphide active site to the substrate. The family includes also lipoamide reductase, GSHR and mercuric reductase, which vary only slightly in structure and mechanism depending on the source. In these three cases, catalysis have evolved in only one way. On the other hand, two distinct types of TrxRs have evolved (Tab. 3) (Luthman and Holmgren 1982;

Table 3. Characterization of an enzyme family: pyridine nucleotide-disulphide oxidoreductases

TrxR (2 distinct types)	Lipoamide dehydrogenase and GSHR
High M_r type Subunit M_r approximately 55 kDa (human and *Plasmodium falciparum*) Dimeric proteins with one FAD, one redox active disulphide and a third redox active group in each subunit. The third group is selenylsulphide in human TrxR and a disulphide in the *P falciparum* enzyme.	Subunit M_r approximately 55 kDa (all sources)
Low M_r type Subunit M_r approximately 35 kDa (*Escherichia coli* and other prokaryotes, yeast, mycoplasmas, *Giardia duodenalis*, *Arabidopsis thaliana* and *Methanococcus janaschii*) Dimeric proteins with one FAD and one redox active disulphide in each subunit	Dimeric proteins with one FAD and one redox active disulphide in each subunit

TrxR, thioredoxin reductase; GSHR, glutathione reductase.

Arscott et al. 1997). Both are dimeric, and catalysis is again brought about by FAD and a redox active disulphide. TrxR isolated from *Plasmodium falciparum, Caenohabditis elegans*, *Drosophila melanogaster* and other higher eukaryotes has a subunit M_r of 55,000, like that of GSHR and lipoamide reductase, while TrxR from lower spieces including lower plants and fungi, lacks a separate interface domain and has a subunit M_r of 35,000.

Catalytic mechanism of TrxR

The path of electrons in catalysis by GSHR or lipoamide dehydrogenase is clear: from the nicotinamide ring of NAD(P)H on the „re" side of the isoalloxazine ring to the disulphide on the „si" side of FAD and on to the disulphide substrate (Pai and Schulz 1983; Mattevi et al. 1991) („re" and „si" in nontechnical terms refer to specific sides of the isoalloxazine ring; the „re" side has the benzene ring on the left, when the ribytyl side chain is at the bottom and the „si" side is the opposite side). Interchange between the nascent dithiol and the substrate disulphide is catalyzed by a nearby histidine residue (Matthews and Williams 1976; Pai and Schulz 1983). Only one thiol can interact with the flavin (referred to as the flavin interacting thiol) and the remaining thiol can only participate in dithiol/disulphide interchange with the substrate (referred to as the interchange thiol) (Thorpe and Williams 1976, 1981). The milieu of the interchange thiol is more polar than that of the flavin interacting thiol (Pai and Schulz 1983).

The catalytic mechanism of TrxR is more complicated. The evolutionary distance between TrxR from prokaryotes and lower eukaryotes on one hand and the enzyme from higher eukaryotes on the other hand is further emphasized by the fact that catalysis occurs in a completely different manner (Sandalova et al. 2001).

Catalytic mechanism of low M_r TrxR

For catalytic mechanism of low M_r *E. coli* TrxR was suggested a model emulated the domain arrangement observed in GSHR (Waksman et al. 1994). A large rotation of the NADPH domain relative to the FAD domain would move the redox active dithiol to the protein surface where it could react with its protein substrate, and would position the nicotinamide of NADPH parallel to the flavin ring. Thus, interconversion between the observed and model conformations was proposed to occur at two steps in the catalytic cycle. A considerable body of mechanistic evidence supports the idea that two conformations are in equilibrium in solution (Wang et al. 1996). The initial crystal structure is referred to as the flavin oxidation (FO) conformation, because the flavin and the disulphide are juxtaposed for FO. Flavin reduction (FR) conformation is the alternate rotated structure in which the flavin and the pyridine nucleotide are juxtaposed to allow FR. Both conformations of the enzyme are essential for completion of the catalytic cycle. The flavin reduction conformation is required for FR by NADPH and for reduction of the large protein substrate Trx by the enzyme dithiol. The FO conformation is required for the transfer of electrons from the flavin to the enzyme disulphide (Lennon and Williams 1997).

Catalytic mechanism of high M_r TrxR

The high M_r TrxRs contain a C-terminal peripheral redox centre that communicates with the central redox-active catalytic site (Arscott et al. 1997). Whereas the peripheral central redox centre of *P. falciparum* TrxR is represented by Cys535-Cys540 and by Cys489-Cys490 in *D. melanogaster*, all known mammalian TrxRs possess a Cys-Sec sequence as the C-terminal redox pair (Gladyshev et al. 1996; Tamura and Stadtman 1996; Lee et al. 1999). Thus mammalian TrxR is a member of small and exclusive class of selenoenzymes (Stadtman 1996; Ganther 1999). *C. elegans* TrxR has been identified as the sole selenoprotein (Gladyshev et al. 1999). Selenocysteine (Sec) is rare amino acid, the so-called „21st" amino acid in proteins (Böck et al. 1991; Atkins and Gesteland 2000).

Conserved Sec residue in mammalian TrxR is a component of 16-residue C-terminal extension. This extension has two functions: i) it extends the electron transport chain from the catalytic disulphide to the enzyme surface, where it can react with Trx, and ii) it prevents the enzyme from acting as a GSHR by blocking the redox active disulphide. Many researches suggest, that mammalian TrxR evolved from the GSHR scaffold rather than from its prokaryotic counterpart (Sandalova et al. 2001). The catalytic mechanism of TrxR is based on high homology with GSHR, too.

In mammalian TrxR, the orientation of FAD and NADPH binding domain would allow transfer from NADPH to the disulphide of Trx without the necessity to invoke similar large conformational changes as in *E. coli* TrxR. The first step of catalysis, reduction by NADPH and formation of a charge-transfer intermediate, is common to GSHR and mammalian TrxR and does not depend on the presence of the Sec-Gly peptide of mammalian TrxR (Zhong and Holmgren 2000). The second part of the reaction is different. In oxidized wild-type enzyme, a selenylsulfide bond was found between Cys497 and Sec498, which can be reduced upon addition of NADPH (Arscott et al. 1997). It has therefore been proposed that in mammalian TrxR, the conserved Cys497-Sec498 motif acts as a second redox center and that electrons are transfered from the redox-active disulphide *via* the redox center at the C-terminal to the substrate, Trx (Zhong et al. 2000). This proposal is consistent with the observed three-dimensional structure of mammalian TrxR (Sandalova et al. 2001).

The variety of TrxR substrates, which includes proteins and low M_r molecules, give rise to the question at which redox centre of TrxR these different compounds are reduced. In this context, a main role plays not only the size of a substrate, but also its charge and polarity. The concept of the catalytic mechanism suggests that larger substrates react at the C-terminal redox active site whereas certain small compound may also take the shortcut *via* the flavin and/or the catalytic cysteines. Data obtained from TrxR with a proteolytically cleaved C-terminus support this idea as the artificial substrate 5,5'-dithiobis(2-nitrobenzoic acid) is still reduced by the modified enzyme whereas Trx is not (Zhong et al. 1998). Also small molecule Tris(2-carboxyethyl)phosphine also reacts exclusivelly at the C-terminal redox center. These aspects are of practical importance as inhibitors blocking the selenol or another function at the C-terminal redox centre could still allow reduction, when low M_r molecules is at the central dithiol (Williams et al. 2000).

Role of Sec in mammalian TrxR

Sec has been found in 25 human proteins (Kryukov et al. 2003). The codon that directs incorporation of Sec into a protein chain is the UGA codon, which normaly serves as a termination signal of protein synthesis. In the cases in which UGA directs the incorporation of Sec, a specific stem-loop structure is present in the mRNA. This structure, known as a 3´ untranslated region (UTR) mRNA secondary structures, termed Sec insertion sequence element, is responsible for the recording of the UGA codon as a sense codon for Sec by recruiting protein factors necessary to bind the specialized $tRNA^{Ser/Sec}$ needed to direct incorporation of Sec (Heider et al. 1992; Fagegaltier et al. 2000; Tujebajeva et al. 2000). The 3´ UTR of the TrxR gene has been shown to have two important regulatory functions: i) the Sec insertion sequence element directs a UGA codon to incorporate Sec rather than terminate protein synthesis, ii) AREs (ARE = AU rich elements, which form cluster in 3´ UTR TrxR) control Trx expression, by regulation mRNA levels by directing acceleration of the deadenylation process (Low and Berry 1996).

Non-specific incorporation of selenium into proteins occurs through substitution of selenomethionine for methionine and selenomethionine is a major constituent of selenized yeast used in chemopreventive studies (Uden et al. 1998).

The Cys-Sec center apperently involves a selenyl sulfide linkage that receives reducing equivalents from NADPH *via* the conserved Cys-Cys dithiol center (Nordberg et al. 1998). A number of experimental approaches confirm that the Sec center plays a key role in the novel, wide ranging functions of mammalian TrxR (Gromer et al. 1998a). A notable characteristic of the TrxR selenoprotein is its sensitivity to oxidizing conditions, leading to a change in conformation (Gorlatov and Stadtman 1998). A conformational change affecting interaction of TrxR with other molecules could be important with regard to triggering cell signaling in response to oxidative stress (Sun et al. 1999). Careful investigations to determine the role of Sec residue in TrxR during catalysis indicate that the selenium atom transfers electrons to oxidized Trx (Zhong et al. 2000).

Removal of C-terminal Sec in TrxR by carboxypeptidase treatment (Zhong et al. 1998), trypsin digestion (Gromer et al. 1998b) or alkylation of the Sec residue (Gorlatov and Stadtman 1998) all results in inactivation of the enzyme. Also substitution of cystein for Sec in other selenoenzymes (Axley et al. 1991) markedly diminishes their activity. Selenium is essential fot the activity of TrxR. Adding selenium at 1 μmol/l to the medium of cultured cells increases cellular TrxR activity by as much as 40-fold (Gallegos et al. 1997).

Controversial roles of Trx system in disease

The role of Trx system in antioxidant defense and the role of Trx in controlling physiological functions offer potential use in clinical therapy. But thanks to miscellanous biological properties, effect of Trx can be opposite in disease, depending on the type and stage of the condition. The anti-apoptotic properties of Trx are deleterious in cancer, because they can impair the effectiveness of chemotherapy strategies that trigger apoptosis. The growth-promoting effects of Trx are detrimental also in rheumatoid artritis (Powis et al. 2000; Gromer et al. 2004), but are beneficial in neurodegenerative disease in which promoting neurall-cell growth aids recovery (Masutani et al. 2004).

Conclusion

At the present time, the explosive increase in studies oriented to oxidative stress is recorded. It was indicated that some completely different diseases, e.g. heart attack, apopletic stroke, lungs cancer, after-cataract, diabetes and other have the same causality. In their genesis, the key role is played also by free radicals.

It is documented that Trx system protects also the cell against oxidative stress by scavenging ROS through a variety of direct or indirect mechanisms. Also Trx system plays a crucial role in the regulation of the intracellular redox state by reducing numerous protein substrates. The proteins that comprise this system, are unique in their characteristic and functions.

However, elevated levels of Trx system are also present in many forms of cancer with persistent oxidative stress and associated with many types of cancer, an understanding of how the expression of the Trx system is controlled under basal conditions and in response to oxidative stress,

may help in elucidating the link between the Trx system and cancer. Proteins of Trx system are attractive molecular targets for novel therapeutics not only in cancer, but also in many other diseases.

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