The mammalian thioredoxin reductases (TrxRs) are a family of selenium-containing pyridine nucleotide-disulphide oxidoreductases with mechanistic and sequence identity, including a conserved -Cys-Val-Asn-Val-Gly-Cys- redox catalytic site, to glutathione reductases. TrxRs catalyse the NADPH-dependent reduction of the redox protein thioredoxin (Trx), as well as of other endogenous and exogenous compounds. The broad substrate specificity of mammalian TrxRs is due to a second redox-active site, a C-terminal -Cys-SeCys- (where SeCys is selenocysteine), that is not found in glutathione reductase or Escherichia coli TrxR. There are currently two confirmed forms of mammalian TrxRs, TrxR1 and TrxR2, and it is possible that other forms will be identified. The availability of Se is a key factor determining TrxR activity both in cell culture and in vivo, and the mechanism(s) for the incorporation of Se into TrxRs, as well as the regulation of TrxR activity, have only recently begun to be investigated. The importance of Trx to many aspects of cell function make it likely that TrxRs also play a role in protection against oxidant injury, cell growth and transformation, and the recycling of ascorbate from its oxidized form. Since TrxRs are able to reduce a number of substrates other than Trx, it is likely that additional biological effects will be discovered for TrxR. Furthermore, inhibiting TrxR with drugs may lead to new treatments for human diseases such as cancer, AIDS and autoimmune diseases.

Key words: pyridine nucleotide-disulphide oxidoreductases, selenium, thiol redox system.

INTRODUCTION

The thioredoxin reductases (TrxRs) are enzymes belonging to the flavoprotein family of pyridine nucleotide-disulphide oxidoreductases that includes lipoamide dehydrogenase, glutathione reductase and mercuric ion reductase ([1] and references therein). Members of this family are homodimeric proteins in which each monomer includes an FAD prosthetic group, an NADPH binding site and an active site containing a redox-active disulphide. Electrons are transferred from NADPH via FAD to the active-site disulphide of TrxR, which then reduces the substrate [1]. The focus of this review is the mammalian TrxRs and the evidence to date of their structure, catalytic mechanism and biological function(s) in both normal and pathological processes.

TrxRs are named for their ability to reduce oxidized thioredoxins (Trxs), a group of small (10–12 kDa) ubiquitous redox-active peptides which have a conserved -Trp-Cys-Gly-Pro-Cys- redox catalytic site, to Trx and glutaredoxin respectively, and (iii) the ability to undergo thiol-disulphide exchange. Differences lie in (i) the limited substrate specificity of glutathione reductase, which only reduces glutathione, and (ii) the high intracellular levels of reduced glutathione, which removes electrophiles by both spontaneous and glutathione transferase-catalysed mechanisms.

STRUCTURE

The first mammalian TrxR to be cloned, TrxR1 from human placenta, was found to have only 31% sequence identity with...
prokaryotic TrxRs, but to have 44\% identity with eukaryotic and prokaryotic glutathione reductases [18]. The predicted domain structure of TrxR1 is given in Figure 2. The catalytic site of human TrxR, -Cys-Val-Asn-Val-Gly-Cys-, is also found in human glutathione reductase and is located in the FAD domain of the enzymes, whereas in the TrxR of *Escherichia coli* the catalytic site, -Cys-Ala-Thr-Cys-, is part of the NADPH domain [18,19]. The predicted molecular mass for the human TrxR1

Figure 1  Reactions and functions of TrxR in the cell

TrxR utilizes NADPH to catalyse the conversion of oxidized (ox.) Trx into reduced (red.) Trx, and to reduce the oxidized forms of ascorbate into reduced ascorbate. Reduced Trx provides reducing equivalents to (i) Trx peroxidase, which breaks down H$_2$O$_2$ to water, (ii) ribonucleotide reductase, which reduces ribonucleotides to deoxyribonucleotides for DNA synthesis, and (iii) transcription factors, which leads to their increased binding to DNA and altered gene transcription. In addition, Trx increases cell growth and inhibits apoptosis.

Scheme 1  Comparison of the TrxR/Trx and glutathione reductase/glutathione systems

Both systems are involved in the maintenance of thiol status in mammalian cells. (a) The TrxR/Trx system with substrates for Trx, including oxidized proteins and some transcription factors. (b) The GSHR/GSH system. GSH can react (1) with oxidized glutaredoxin, (2) with electrophiles or oxidized macromolecules, and (3) in a reaction with electrophiles catalysed by glutathione S-transferase. Abbreviations: Trx-(SH)$_2$ and Trx-S$_2$, reduced and oxidized Trx respectively; Sub$_{\text{ox.}}$ and Sub$_{\text{red.}}$, oxidized and reduced substrate respectively; GSHR, glutathione reductase; GST, glutathione S-transferase; Grx$_{\text{ox.}}$ and Grx$_{\text{red.}}$, oxidized and reduced glutaredoxin respectively; R$^-$, electrophilic compounds; R–SG, glutathione-conjugated compounds.

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The various domains and the TrxR2 cleavage site are indicated in the key. The catalytic-site sequences are indicated by triangles, and the position of the C-terminal penultimate SeCys residue is shown for the two human TrxRs.

As originally cloned, the predicted C-terminal amino acid sequence of human placental TrxR1 was -Gly-Cys [18]. Subsequent work showed that TrxR1 purified from A549 human lung cancer cells had a C-terminal -Gly-Cys-SeCys-Gly sequence (where SeCys is selenocysteine) and that human TrxR1 was, thus, a selenoprotein [22]. This difference can be explained by the predicted terminal UGA codon coding for SeCys instead of a stop codon (see below) and a second UGA, two codons 3′ of the first, being the actual stop codon. Human TrxR1 has an alternative translation start site 244 bp upstream of the predicted start site [23]. However, the use of this alternative start site has not been confirmed and no human TrxR protein of the predicted mass of 60 kDa has been demonstrated. The gene for human TrxR1 is located on chromosome 12q23-q24.1 [24]. It has a predicted molecular mass of 56.2 kDa and has been confirmed and no human TrxR protein of the predicted mass of 60 kDa has been demonstrated. The gene for human TrxR2 is located on chromosome 22q11.2 [26]. Based on preliminary work, it is highly likely that other members of the mammalian TrxR family will be identified [29].

**Mechanism**

The catalytic mechanism of *E. coli* TrxR has been extensively studied [19,30–32]. The spatial orientation of the NADPH and FAD domains of *E. coli* TrxR are such that the nicotinamide ring of NADPH bound to the enzyme does not make close contact with the isoalloxazine ring of FAD, as it does in other members of the pyridine nucleotide-disulphide oxidoreductase family [30]. However, if the NADPH domain of *E. coli* TrxR is rotated 66° while the FAD domain remains fixed, then the bound NADPH moves into close contact with the isoalloxazine ring; this allows electrons to pass to FAD and then to the active-site disulphide which, when reduced, moves to the surface of the enzyme, where it is accessible to oxidized Trx [30,33–36].

Mammalian TrxRs share a higher degree of sequence identity and mechanistic similarity with glutathione reductase than with...
E. coli TrxR [18,20,31]. In glutathione reductase the active-site Cys residues, which are in the FAD domain, and the bound NADPH are in close proximity to the isoalloxazine ring of FAD, allowing electrons to flow from NADPH to glutathione via the isoxaloxazine ring of FAD and the active-site disulphide without a major conformational change in the enzyme [37]. In the presence of excess NADPH, human TrxR [31], glutathione reductase and lipoamide dehydrogenase, but not E. coli TrxR, form a stable thiolate–flavin charge-transfer complex, indicative of the mechanistic similarity amongst these three enzymes [37,38]. However, titration of human TrxR with dithionite shows the presence of an additional redox-active site that is not present in glutathione reductase [31]. This finding is reminiscent of titration studies with mercuric ion reductase, an oxidoreductase with a second pair of redox-active Cys residues at the C-terminal end of the protein [31,39]. As discussed above, TrxR1 has a C-terminal SeCys residue that is required for catalytic activity, but is not part of the conserved active site. All other mammalian selenoproteins for which a function is known are redox enzymes with SeCys in the active centre [40].

Mammalian TrxRs are promiscuous enzymes capable of reducing Trxs of different species [41,42], proteins such as Nklysin [12] and p53 [16], a variety of physiological substrates [10,11,13–15], as well as several exogenous compounds [42–45]. It may be that it is the C-terminal catalytic SeCys that accounts for the broad substrate specificity of TrxR [46], allowing the enzyme to reduce bulky proteins as well as small molecules. One suggested catalytic mechanism for human TrxR is that the C-terminal end of the protein is flexible, allowing the -Cys-SeCys-Gly moiety to carry reducing equivalents from the conserved active-site Cys residues to the substrate [46] (Scheme 2).

SELENIUM AND TrxR ACTIVITY

Selenium (Se) is essential for the activity of TrxR, and adding Se at 1 μM to the medium of cultured cells increases cellular TrxR activity by as much as 40-fold [47–50]. Interestingly, cell lines of lymphoid origin, which have low levels of TrxR compared with epithelial cell lines, show little or no increase in TrxR activity when Se is added [47,51]. Studies utilizing 75Se have demonstrated that, as the 75Se concentration in the medium increased, the incorporation of 75Se into TrxR increased from a molar ratio of 0.01 Se unit per TrxR subunit at 27 nM Se to 0.98 Se unit per TrxR subunit (close to the predicted maximum value of 1.0) at 1 μM Se [47]. There was also an increase in both TrxR1 mRNA and TrxR1 protein levels as the concentration of Se in the medium increased, although these increases were less than the increase in TrxR activity [47]. When rat TrxR1 was transfected into COS-1 cells, TrxR1 activity increased as the Se concentration in the medium increased, without a concomitant elevation in TrxR1 protein levels [52]. These results indicate that most of the increase in TrxR activity seen following the addition of Se to the culture medium is due to an increase in the specific activity of the enzyme associated with increased incorporation of Se into TrxR. It should be noted that the Se concentration in human serum is between 1 and 5 μM [53] and that culture medium without added Se, containing 10% (v/v) fetal-bovine serum, has a Se concentration of < 0.01 μM [47]. Thus most cell culture conditions probably do not support physiological levels of TrxR activity, which could have consequences for our understanding of the biological role(s) of Trx and TrxR based on cell culture data alone.

Alterations in dietary Se intake affect TrxR activity in vivo. Rats fed a Se-deficient diet for several weeks showed decreased TrxR activity in the liver, kidney and lung, although brain TrxR activity was unchanged [54,55]. TrxR protein levels were also found to be decreased in these same rats. On the other hand, tissues from rats fed supranutritional levels of Se showed a transient increase in TrxR activity without an increase in TrxR protein above control levels [55]. These results are in agreement with results from cell culture studies, suggesting that the increased TrxR activity in vivo is due to increased Se incorporation into the enzyme without an increase in protein synthesis [55]. It is interesting that TrxR1 purified from human placenta has been found to contain Se at a molar ratio of between 0.6 and 0.9 Se unit per TrxR1 subunit [21,31,56]. These differences may reflect individual variation in Se nutritional status, with the lower levels present in individuals who have a lower Se intake. The in vivo consequences of alterations in TrxR Se content have not been thoroughly investigated and need to be addressed.

SELENOCYSTEINE INSERTION

SeCys incorporation into selenoproteins is encoded by a UGA codon which is more frequently the signal for the termination of protein synthesis [40,57]. The reading of UGA during translation as a SeCys codon occurs in the presence of specific 3′ untranslated region (UTR) mRNA secondary structures termed SECIS (selenoceysteine insertion sequence) elements. In TrxR SeCys is the penultimate C-terminal amino acid, and removal of the C-terminus by carboxypeptidase treatment [21], trypsin digestion [46] or alklylation of the SeCys residue [58] all result in inactivation of the enzyme. Expression in mammalian or insect cells of TrxR1 lacking the penultimate SeCys-Gly residues, or substitution of SeCys with Cys, decreases enzyme activity by up to two orders of magnitude with Trx or dithionitrobenzoic acid as substrate [59].

Figure 3 Structure of human TrxR1 and TrxR2 SECIS elements

Nucleotides conserved in all vertebrate SECIS elements are shown in red. Adapted from [59], with permission. ©1999 The American Society for Biochemistry and Molecular Biology.
Substitution of Cys for SeCys in other selenoenzymes [60,61] also markedly diminishes their activity.

Potential SECIS elements have been identified in the 3' UTRs of TrxR1 and TrxR2 [21,25]. Definitive evidence for a functional SECIS element has recently been obtained by subcloning of the TrxR1 SECIS element into an expression vector downstream of the coding region for another selenoenzyme, type 1 deiodinase, resulting in the production of active enzyme containing SeCys [59]. The SECIS sequence for TrxR1 is located between nucleotides 2184 and 2227 (Figure 3). Deletion analysis of the 3' UTR of rat TrxR1 has shown a putative SECIS element located between 1856 and 1915 bp that is necessary for expression of active enzyme in COS-1 cells [52].

REGULATION OF EXPRESSION

Sequences in the 3' UTRs of mRNA confer regulation of expression through a variety of mechanisms, including alterations in mRNA turnover, translation initiation, subcellular localization and, in the case of selenoenzymes as noted above, by dictating the choice between incorporation of SeCys or termination of protein synthesis [62]. One function of the 3' UTR SECIS element is to provide a hierarchy for the expression of selenoproteins under conditions of limited Se availability. Differences in the 3' UTRs of three selenoproteins, cytoplasmic glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase and type 1 deiodinase, result in 2-fold differences in protein expression in response to Se limitation [63]. The TrxR1 SECIS element is highly active under normal conditions, but is less responsive to Se supplementation than the SECIS element of type 1 deiodinase, suggesting that TrxR1 levels are better maintained when Se supply is low but that protein levels will not increase as dramatically under conditions of Se excess [59]. This is essentially what is seen when rats are fed supranutritional levels of Se [55] (see above).

The 3' UTR of TrxR1 also contains a cluster of six AU-rich elements (AREs), which function to regulate mRNA levels by directing acceleration of the deadenylation process [40,64]. These mRNA instability elements are typically found in cytokine, growth factor and proto-oncogene mRNAs that undergo rapid turnover [65]. Inactivation of AREs in growth factor and proto-oncogene mRNAs has been linked to promotion of cellular transformation and oncogenesis. For example, stabilization of c-Myc mRNA due to deletion of AREs promotes oncogenic transformation in vitro and is associated with a human T-cell leukaemia [66]. AREs in the gene encodingTrxR1 may serve to maintain stringent control of TrxR1 expression, thereby preventing the deleterious effects that may be associated with overexpression. It is interesting to note that the 3' UTR of TrxR2 does not contain AREs [25,27].

BIOLOGICAL FUNCTIONS OF TrxR

Not surprisingly, the involvement of TrxR in biological functions such as cell growth and protection from oxidative stress has, to date, centred around its role as a reductant for Trx. Further studies are needed to determine whether TrxR has biological functions that are not mediated by reduction of Trx.

Cell growth

As previously noted, Trx, a physiological substrate of TrxRs, has been shown to play an important role in regulating cell growth and inhibiting apoptosis [8,9]. Trx has to be in a reduced form in order to exert these effects, and mutant redox-inactive forms of Trx are unable to stimulate cell growth or inhibit apoptosis [2,3]. The only known mechanism for the reduction of Trx is through NADPH-dependent reduction by TrxR. It would be thought, therefore, that TrxRs could also play a role in regulating cell growth. However, TrxR activity in cultured cells can be increased several-fold by including Se in the growth medium without a marked effect on the growth rate of cells [47]. Transfection of MCF-7 breast cancer cells with the TrxR1 variant, Grim-12, results in a greater than 3-fold increase in TrxR activity, but a less than 50%, stimulation of cell growth [67]. It is possible that the lack of a correlation between increased TrxR activity and cell growth is due to the fact that most cell lines have been selected to grow in Se-deficient medium.

In contrast with the lack of effect of increased TrxR activity on cell growth, inhibiting TrxR activity to below normal levels is associated with inhibited cell growth. Several in vitro inhibitors of TrxR have been reported and, although many of these compounds only inhibit the reduced form of TrxR, it is likely that TrxR will be sensitive to these inhibitors in vivo, since TrxR is expected to exist predominantly in the reduced form due to the presence of cytosolic NADPH concentrations that are greater than the $K_m$ of TrxR for NADPH [31,54,68–75]. Two such inhibitors of TrxR are the anti-tumour quinones doxorubicin and diaziquone; treatment of cells with either of these compounds leads to secondary inhibition of ribonucleotide reductase and inhibition of cell growth [76].

p53 activity

p53 is a tumour-suppressor protein and transcription factor that is deleted in a number of human cancers [77]. As in mammalian cells, when wild-type (but not mutant) forms of the human tumour-suppressor p53 gene are expressed in the fission yeast Schizosaccharomyces pombe, strong growth inhibition occurs [78]. Using this as a model system to screen for genes whose function is required for normal activity of p53, a mutant yeast strain was found that was partially resistant to the effects of p53 expression with a recessive mutation in a novel gene, $trr1$, with strong identity with that encoding TrxR [16]. The levels and localization of the p53 protein were unchanged in the mutant yeast strain, suggesting that it was not p53 expression that was altered. Loss of $trr1$ function resulted in yeast with an increased sensitivity to the toxic effects of H$_2$O$_2$ and a 100% oxygen atmosphere. Studies in the budding yeast Saccharomyces cerevisiae have shown that deletion of the $trr1$ gene inhibits the ability of human p53 to stimulate reporter gene expression [79]. Whether TrxR exerts similar control over the function of p53 in mammalian cells is not known. However, it is known that the ability of p53 to bind to DNA is inhibited by oxidizing conditions [80], and p53 expression leads to alterations in the expression of a number of redox genes, including a decrease in TrxR expression [81].

Protection against oxidant stress

The continual formation of low levels of ROS is part of normal metabolism [82,83]; however, increased production of ROS, or a functional decrease in one or more of the protective systems present in the cell, can result in unrepaired macromolecular damage, i.e. oxidation of protein thiols, which may then lead to pathological processes, including apoptosis [84,85]. Trx has been shown to prevent apoptosis in cells treated with agents known to produce ROS [86]. The levels of TrxR1 mRNA and Trx mRNA are increased in the lungs of newborn baboons exposed to air or O$_2$ breathing [87], and increases in TrxR1 and Trx mRNA are
also observed in adult baboon lung explants in response to 95% O₂. It has been suggested that these increases in gene expression for TrxR1 and Trx play a protective role against O₂ breathing in the mammalian lung. There have also been reports that TrxR is highly expressed on the surface of human keratinocytes and melanocytes [74,88,89], where it has been suggested to provide the skin’s first line of defence against free radicals generated in response to UV light [90].

**Ascorbate recycling**

Humans lack the ability to synthesize ascorbic acid, an important antioxidant in the protection of cells from oxidative stress; therefore dietary intake and the recycling of ascorbate from its oxidized forms (dehydroascorbic acid and the ascorbyl free radical) are essential for maintenance of in vivo ascorbate levels. It has been demonstrated that maintenance of rats on a Se-deficient diet results in decreased liver ascorbate, glutathione peroxidase and TrxR levels, while liver glutathione levels are unchanged [14]. In another study, treatment of HL-60 cells with bithionine sulphoxamine or diethyl maleate resulted in decreases in cellular glutathione to approximately 10% of that in controls, but had no effect on the ability of these cells to reduce dehydroascorbic acid [91]. TrxR has also been shown to reduce the ascorbyl free radical to ascorbate with a Kᵣ of 2.8 μM, which is in the physiological range for this free radical in cells undergoing oxidant stress [15,92]. These studies suggest that, in addition to protecting the cell from oxidative stress by maintaining Trx in its reduced state, TrxR may play an additional role through the recycling of ascorbate.

**Human disease**

Although studies discussed in this review have demonstrated that TrxR levels and/or activity can be modulated, albeit modestly, by factors that are known to be associated with disease states (i.e. Se status), the evidence for a role for TrxR in human disease is, at this time, circumstantial. The best evidence that TrxR may play a role in human diseases comes from studies on cancer, AIDS, and autoimmune disease.

**Cancer**

It has been suggested, based on purification yields [93], that the level of TrxR in tumour cells is 10-fold or more greater than in normal tissues [22,41,42,94–96]. However, a study in which human primary colorectal tumours were compared directly with normal tissues [22,41,42,94–96]. However, a study in which human primary colorectal tumours were compared directly with normal tissue from the same patient showed that, while TrxR protein activity was higher in the tumours, the increase was only about 1-fold (100%−) [97]. TrxR has also been reported to be elevated in human primary melanoma and to show a correlation for TrxR1 and Trx play a protective role against O₂ response to UV light [90].

**Autoimmune disease**

Organic gold compounds are inhibitors of purified mammalian TrxR and are used to treat some autoimmune diseases [54,75]. Administration of a single dose of aurothioglucose to mice resulted in a marked and prolonged inhibition of TrxR activity in all tissues examined [104]. Whether inhibition of TrxR is responsible for the therapeutic effects of gold compounds has yet to be established.

**SUMMARY AND FUTURE DIRECTIONS**

Mammalian TrxRs are a class of Se-containing pyridine nucleotide-disulphide oxidoreductases that share sequence identity and mechanistic similarity with glutathione reductases. TrxRs also have a C-terminal -Cys-SeCys- active site located at a distance from the conserved -Cys-Val-Asn-Val-Gly-Cys- active site, and both sites are essential for enzyme activity. It is likely that the C-terminal -Cys-SeCys- active site is responsible for the relatively non-specific substrate specificity of mammalian TrxRs. There are currently two known forms of TrxR, TrxR1 and TrxR2, and it is likely that other forms will be identified. The 3’ UTR of the TrxR1 gene has been shown to have two important regulatory functions: (i) the SECIS element directs a UGA codon to incorporate SeCys rather than terminate protein synthesis, and (ii) AREs control TrxR1 expression. A current limitation to the study of TrxR is the difficulty of expressing recombinant active enzyme due to the complexity of SeCys synthesis and incorporation pathways in eukaryotic cells [40,105]. This problem will need to be overcome before structural modification of the enzyme can be accomplished. Structural NMR or X-ray crystallography studies are needed to establish how the C-terminal and conserved catalytic-site residues interact.

Because of the importance of the Trx system to many aspects of cell function, particularly with respect to cell growth and protection against oxidant damage and apoptosis, it would appear that TrxR should play a role in some of these functions. Although human tumours have been shown to have moderately increased levels of TrxR activity, studies using cultured cells have shown that increasing TrxR activity has no apparent effect on cell growth, although inhibiting TrxR is associated with decreased cell growth. However, extrapolations from work utilizing cultured cancer cells must be performed with caution because of the potential effects of Se deficiency in the culture medium on TrxR activity. In addition to the biological effects of TrxR that are mediated through Trx, it seems likely, given the ability of TrxR to reduce a broad spectrum of substrates, that other biological effects will be discovered for TrxR. One of these may be a role in the recycling of ascorbate from its oxidized forms.

While recent studies have provided intriguing insights into the complex role of Se in the activity of mammalian TrxRs and their unique properties, this field is only beginning to be investigated.
Elucidation of the mechanism(s) by which TrxR is involved in both normal and pathological cell functions, as well as its inhibition by drugs, may provide new insights into disease mechanisms and provide new treatments for cancer, autoimmune disease, and perhaps other human diseases. One challenge will be to discover agents that specifically inhibit TrxR and not other thiol- and Se-containing proteins.

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