Redox Potential of Human Thioredoxin 1 and Identification of a Second Dithiol/Disulfide Motif†

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Running Title: Redox States of Human Thioredoxin
Summary

Thioredoxin (Trx1) is a redox-active protein containing two active site cysteines (Cys32 and Cys35) that cycle between the dithiol and disulfide forms as Trx1 reduces target proteins. Examination of the redox characteristics of this active site dithiol/disulfide couple is complicated by the presence of three additional non-active site cysteines. Using the Redox Western blot technique and MALDI-TOF mass spectrometry, we determined the midpoint potential ($E_0$) of the Trx1 active site (-230 mV) and identified a second redox-active dithiol/disulfide (Cys62 and Cys69) in an alpha helix proximal to the active site, which formed under oxidizing conditions. This non-active site disulfide was not a substrate for reduction by thioredoxin reductase and delayed the reduction of the active site disulfide by thioredoxin reductase. Within actively growing THP1 cells, most of the active site of Trx1 was in the dithiol form while the non-active site was totally in the dithiol form. Addition of increasing concentrations of diamide to these cells resulted in oxidation of the active site at fairly low concentrations and oxidation of the non-active site at higher concentrations. Taken together, these results suggest that the Cys62-Cys69 disulfide could provide a means to transiently inhibit Trx1 activity under conditions of redox signaling or oxidative stress, allowing more time for the sensing and transmission of oxidative signals.
Introduction

Thioredoxin (Trx1) is a ubiquitous 12 kDa protein that functions as a reductant for ribonucleotide reductase, peroxiredoxins, and transcription factors (e.g. Fos, Jun, NF-kB, p53), controlling key aspects of cell proliferation and survival (1-4). The active site of Trx1, WCGPC, is conserved among species from cyanobacteria to humans (5). The active site cysteines are readily accessible on the surface of the protein and become oxidized to a disulfide upon reduction of a target protein. This disulfide is cycled back to the dithiol by Trx reductase (6).

Unlike Trx’s from lower species, mammalian Trx1 contains additional conserved cysteine residues (at positions 62, 69, and 73 of human Trx1; See Fig. 1). Whether these non-active site Cys residues have biologic function is unknown. Cys73 was present as an intermolecular disulfide bond (Trx1 homodimer) in X-ray crystal studies (7), suggesting a possible function for Cys73. However, a mutant Trx1 bearing a serine at this position still appeared as a homodimer in the crystal structure, suggesting that Cys73 was not essential for dimerization (7). More recently, S-glutathionylation of Trx1 at Cys73 has been found during oxidative stress (8). In addition, S-nitrosylation of Cys69 has recently been described (9).

The midpoint potential ($E_0$) for the active site dithiol of Trx is available for several lower species (10-14), but not for mammals. Equilibrium with NADPH in the presence of a catalytic amount of Trx reductase, where it is assumed that each mole of NADPH consumed translates into one mole of Trx reduced, indicated that E. coli Trx had a midpoint potential of $-270 \text{ mV}$ (10,11). An alternate approach using the equilibrium
between glutathione (GSH) redox buffers and E. coli Trx yielded a similar value for $E_0$ (15). In the latter approach, the relative amounts of reduced and oxidized Trx were determined by quantification of the number of thiols with Ellman’s reagent (dithionitrobenzoic acid; DTNB) because E. coli Trx contains only one redox-active pair of cysteines.

Oxidized and reduced forms of bovine Trx1 have been separated by carboxymethylation of thiols, native gel electrophoresis, and immunoblotting (16). The fully reduced (fully carboxymethylated) and fully oxidized forms of bovine Trx1 were identified, but intermediate bands on the immunoblot were only identified as “partially carboxymethylated” (16). In the present report, we have used mass spectrometry to positively identify the forms of human Trx1 that are resolved by this native gel electrophoresis/Western blot (Redox Western blot) method. The results show that the resolved bands include fully reduced Trx1, a band with the active site in the disulfide form, and a band in which the protein has 2 disulfides, one at the active site and the other involving Cys62 and Cys69. The midpoint potential ($E_0$) for the active site was found to be -230 mV. Using this value and Redox Western blot analysis of THP1 cells, the steady state redox ($E_h$) of Trx1 was -280 mV, a value that is comparable to the $E_h$ for GSH/GSSG in these cells (-259 mV). During oxidative stress induced by diamide, both the Trx1 active site and non-active site disulfides were observed. In vitro, the non-active site disulfide was found to inhibit the regeneration of the active form of Trx1 by thioredoxin reductase, supporting the interpretation that oxidation of this non-active site dithiol could provide a structural switch affecting Trx1 function during oxidative stress and redox signaling.
Experimental Procedures

Cell culture: THP1 human monocytes were obtained from American Tissue Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin. Cells were grown in a humidified atmosphere of 5% CO₂ at 37°C. Eₗₜ calculations for cells were done using the Nernst equation with E₀ values for pH 7.4 and assuming 5 µl cell volume per mg cell protein.

Glutathione redox buffers: Redox buffers were prepared in a 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. All solutions were de-oxygenated by bubbling with O₂-free nitrogen (O₂ < 0.5 ppm) for at least 2 h. The GSH/GSSG redox potential was calculated according to the Nernst equation (Eₗₜ = E₀ + 2.3*RT/nF*log([GSSG]/[GSH]²), where E₀ = -240 mV at pH 7.0 (17) and n = 2 for the 2-electron oxidation of 2GSH to GSSG). The following concentrations were used to achieve the desired potentials (Eₗₜ; [GSH], [GSSG]): -150 mV: 6.5 mM, 43.5 mM; -180 mV: 18 mM, 32 mM; -210 mV: 36 mM, 14 mM; -240 mV: 50 mM, 2.5 mM; -270 mV: 50 mM, 0.25 mM; -300 mV: 50 mM, 0.025 mM. After incubation with Trx1 at room temperature for 2 h, samples were taken for the measurement of GSH and GSSG, and for determination of the redox state of Trx1 (see details below). Preliminary studies showed that equilibration periods from 15 min to 4 h yielded similar results, indicating that equilibrium had been achieved. GSH and GSSG were quantified by HPLC as S-carboxymethyl, N-dansyl derivatives relative to γ-glutamylglutamate as an internal standard (18).
Redox Western blot analysis: Separation of the redox forms of Trx1 was based upon the procedures of Holmgren and Fagerstedt (19) and Fernando et al. (16). Trx1 was carboxymethylated in guanidine-Tris solution (6 M guanidine-HCl, 50 mM Tris, pH 8.3, 3 mM EDTA, 0.5% (v/v) Triton X100) containing 50 mM iodoacetic acid (IAA). Following incubation at 37°C for 30 min, excess IAA was removed by Sephadex chromatography (MicroSpin G-25 columns, Amersham-Pharmacia, Piscataway, NJ). Eluates were diluted in 5X sample buffer (0.1 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) and separated on a discontinuous native polyacrylamide gel (5% stacking gel, 15% resolving gel). Gels were electroblotted to PVDF membrane and probed for Trx1 using anti-Trx1 primary antibody (American Diagnostica, Greenwich, CT) and HRP-conjugated anti-goat IgG secondary antibody, followed by chemiluminescent detection (SuperSignal West Dura, Pierce, Rockford, IL) with X-Ray film. Band intensities were determined by densitometric analysis of exposed film with Un-Scan-It software (Silk Scientific Corporation).

In-gel digestion of Trx1: Coomassie-stained bands containing Trx1 were subjected to in-gel digestion with sequencing grade trypsin (Promega, Madison, WI) and the peptides were extracted as described (20). The extracted peptides were desalted using C18-ZipTip (Millipore, Bedford, MA) prior to their analysis by mass spectrometry.

ESI-MS: Flow injection ESI-MS analysis of the peptides was performed on a model API3000 triple quadrupole mass spectrometer (PE-Sciex, Foster City, CA) equipped with MicroIonSpray electrospray source and operated in the positive mode of operation. Low energy collision-induced dissociation spectra of the peptides (ESI MS/MS) were performed to confirm peptide identity.
MALDI-TOF MS: Positive-ion MALDI-TOF MS analysis was performed using model ReflexIII delayed-extraction MALDI-TOF mass spectrometer (Bruker Daltonics, Billarica, MA) equipped with a 337 nm nitrogen laser; alpha-cyano-4-hydroxycinnamic acid (MALDI-Quality, Hewlett-Packard) was used as the matrix.

Monobromobimane labeling and fluorescence detection of Trx1: For some experiments, samples were treated with 100 mM monobromobimane (mBBr, Molecular Probes, Eugene, OR) instead of IAA. Labeling was carried out in the guanidine-Tris solution for 30 min at 37°C, and excess mBBr was removed with G-25 MicroSpin columns. The mBBr-labeled samples were digested with trypsin in 50 mM Tris-acetate buffer, pH 8.5 (20 h at 35°C), and then 10% aqueous trifluoracetic acid (TFA; pH<2) was added. The resulting peptides were separated by microbore RP-HPLC on an Applied Biosystems model 140A solvent delivery system, using a C18 silica column (1x150 mm, Zorbax-SB300) with a linear gradient of acetonitrile in 0.1% TFA. The fluorescence of the effluent was monitored at an emission wavelength of 380 nm with an excitation wavelength of 290 nm. Eluted peaks were collected, and the identity of the peptide in each peak was established by Edman sequencing (Applied Biosystems, Foster City, CA).

Molecular Modeling: An energy minimization model for Trx1 with a disulfide bond between Cys62 and Cys69 was developed with the InsightII/Discover software package (Accelrys Inc., San Diego). The starting coordinates were from the reduced human crystal structure, PDB entry 1ERT. The model was first energy minimized using the cvff parameter file without forming any disulfide linkages. The Cys62-Cys69 disulfide was then formed and the model further minimized. Ribbon drawings of the resulting models were prepared using MOLSCRIPT (21), BOBSCRIPT (22) and RASTER3D (23).
Site-directed mutagenesis: Plasmid pcDNA3.1 encoding human Trx1 was a kind gift of Dr. Jiyang Cai of Emory University. Point mutations were introduced using the GeneEditor in vitro Site-Directed Mutagenesis System of Promega (Madison, WI). The C62,69S and C32,35S doubles mutants were generated by hybridization of the plasmid with oligonucleotides of the sequence 5’-
GTGGATGACTCTCAGGATGTTGCTTCAGAGTCTGAAGTCAAATGC-3’ and 5’-
TTCTCAGCCACGTGGTCTGGGCCTTCCAAAATGATCAAGCC-3’, respectively. Clones containing the desired mutations were selected by DNA sequencing.

Expression and purification of Trx1 mutants: For expression in E. coli, Trx1 mutants were subcloned into pET-15b (Novagen) via PCR amplification of the coding region of the pcDNA3.1/Trx1 mutants. A forward primer encoding an NcoI restriction site and a reverse primer encoding a 6X His tag followed by a stop codon and a BamHI restriction site were used. Both the pET-15b vector and the PCR products were digested with NcoI and BamHI and ligated overnight at 16°C. Competent DL21-Gold(DE3) cells (Stratagene) were transformed with pET/Trx1 plasmids. Expression of the mutant proteins was induced by 1 mM IPTG for 3 h, and His-tagged proteins were purified under native conditions using Ni-NTA agarose columns (Qiagen).

Reduction of oxidized thioredoxin by thioredoxin reductase and NADPH: Purified His-tagged Trx1(wt), Trx1(C32,35S), and Trx1(C62,69S) were oxidized with 1 mM H2O2 for 10 min at room temperature, then dialyzed overnight against two changes of Tris/EDTA buffer, pH 7.5 (reaction buffer). In a final volume of 0.5 ml Tris/EDTA buffer, Trx1 mutants were reduced in the presence of 0.3 mM NADPH and 14 µM bovine thioredoxin.
reductase (American Diagnostica). Oxidation of NADPH was monitored by the change in absorbance at 340 nm (24).

**Results**

Separation of reduced and oxidized Trx1: Purified Trx1 was reduced with DTT or oxidized with diamide, carboxymethylated with IAA, separated by native PAGE, and visualized by Coomassie staining (Fig. 2). DTT-treated Trx1 moved as a single band (labeled as Band 3 in Fig. 2). Diamide-treated Trx1 also moved as a single band (labeled Band 1 in Fig. 2), but migrated more slowly than DTT-treated Trx1. Fig. 3A shows the MALDI-TOF mass spectra of tryptic digests of reduced and oxidized Trx1. The predicted and observed masses of the relevant peptides are given in Table 1. All five cysteines were carboxymethylated in DTT-treated Trx1 (Band 3) (Table 1). The mass spectrum of diamide-treated Trx1 (Band 1) revealed the presence of two disulfides: one between Cys32 and Cys35, and one between Cys62 and Cys69 (Table 1). No masses were observed that corresponded to carboxymethylation at any of these four cysteines. Both unmodified and carboxymethylated Cys73 was present in diamide-treated Trx1. Thus, the redox state of the sulfur of Cys73 was unclear, perhaps indicating a mixture of redox states. The identities of the peptides representing the observed masses, as well as the positions of the carboxymethyl groups within these peptides, were verified by tandem mass spectrometry (Fig. 3B).

Redox states of Trx1 after equilibration with redox buffers: Purified Trx1 was equilibrated with 50 mM GSH/GSSG redox buffers over a range of potentials (-270 to -210 mV) likely to be found within cells (25,26), carboxymethylated, and separated on a...
native gel, as before (Fig. 4). The redox potentials of the redox buffers were verified by HPLC analysis of the GSH and GSSG content of the redox buffers at the beginning and the end of the equilibration period; these were found to be unchanged over the course of the equilibration (not shown). Experiments were also performed with GSH/GSSG redox buffers at 5 mM total concentration instead of 50 mM and identical results were obtained. Because IAA may shift the thiol-disulfide equilibrium of proteins (27), experiments were performed to compare results obtained with 10 mM and 50 mM IAA. The results were not distinguishable; thus, the conditions used here (50 mM IAA) did not allow significant changes in Trx1 redox state during processing. At –270 mV, the predominant band co-migrated with the DTT-treated Trx1. MALDI-TOF analysis showed that this band (Band 3) contained only fully carboxymethylated Trx1 (see Table 1). At –240 mV, Trx1 was distributed between Bands 2 and 3, and at –210 mV, Band 2 predominated. MALDI-TOF analysis showed that Band 2 contained both dithiol and disulfide forms of tryptic fragments T4 and T6, indicating that Band 2 is a mixture of two forms. One form contained a disulfide between Cys32 and Cys35 (active site) with Cys62 and Cys69 present as thiols, and the other form, which had a smaller signal, contained a disulfide between Cys62 and Cys69, with Cys32 and Cys35 present as thiols.

Calculation of the midpoint potential ($E_0$) of the active site (Cys32-Cys35) disulfide of human Trx1: Because immunoblotting yielded results comparable to those obtained with Coomassie staining of the different redox states of Trx1 (e.g., compare Fig. 2 and Fig. 4), redox state analyses were performed with the Redox Western blot technique. Trx1 was equilibrated with redox buffers from –300 to –150 mV, and Bands 1, 2, and 3 were quantified by densitometry. $E_0$ was calculated by fitting the data to the Nernst equation,
using the intensity of Band 3 for the reduced form of the active site and the total of Bands 1 and 2 as the corresponding disulfide (Fig. 5). The line represents the best fit to the Nernst equation, yielding a midpoint potential of –230 mV for n = 2.

To provide an independent confirmation of the E₀ of the active site, Trx1 was equilibrated at different redox potentials, labeled with the thiol-specific fluorescent probe mBBr, digested with trypsin, separated by C18 liquid chromatography, and detected by fluorescence (Fig. 6). Eluates corresponding to peaks of fluorescence were collected, and the identities of the peptides were established by Edman sequencing (Table 2). Because monobromobimane labels thiols, but not disulfides, the redox dependent change in fluorescence intensity of the peaks corresponding to peptides T4, T6, and T7 was reflective of the oxidation state of the thiols in each peptide. With this analysis, half of the fluorescence of the active site peptide (T4) was lost at a potential of –225 mV. These results are in good agreement with the value of –230 mV from the Redox Western blot approach, and show that the E₀ for the active site of human Trx1 is more positive than that for E. coli Trx, i.e., in the range of –230 mV rather than –270 mV (10,11).

Redox dependence of the non-active site disulfide: The mass spectrometry data showed that the oxidized form of Trx1 that moved as Band 1 on the Redox Western contained two disulfide bonds. Band 1 was detected at potentials as low as –270 mV, and the intensity of Band 1 increased at –240 mV and –210 mV. However, there was an overall loss of signal and the appearance of a fourth (minor) band at redox potentials more oxidizing than –210 mV; approximately half of the total signal (Bands 1, 2, and 3) was lost at –150 mV (Fig. 3). Thus, the E₀ for the non-active site is more positive than –210
mV. The nature of the changes responsible for the loss of signal in the Redox Western at potentials more positive than –210 mV are unclear.

**Redox state of endogenous Trx1 in cells:** To determine the redox state of intracellular Trx1, extracts from control and oxidant-exposed THP1 human monocytes were analyzed by Redox Western blot (Fig. 7), and compared to the redox state of Trx1 at known redox potentials (Fig. 4). About 95% of the Trx1 from proliferating cells was in the fully reduced state, with some Band 2, and no fully oxidized Trx1 (Fig. 7, lane 1). This corresponded to an $E_h$ value of $-280 \pm 5$ mV after correction for the effect of intracellular pH (7.4). By comparison, the redox state of glutathione in these cells was $-259 \pm 3$ mV (mean $\pm$ S.D. of three separate experiments). Upon exposure of the cells to diamide, there was a rapid, dose-dependent oxidation of the endogenous Trx1. The amount of the two-disulfide form of Trx1 (Band 1) went from undetectable to a maximum of 50% of the total Trx1 after 2 min exposure to 3 mM diamide, the highest concentration tested. At this concentration, there was a 75 mV oxidation of the active site relative to the untreated controls (i.e., from –265 to –190 mV). Even the lowest dose of diamide tested (0.1 mM) caused a 20 mV oxidation of the active site (Fig. 7).

**Modeling of the non-active site disulfide in the Trx1 structure:** The crystal structure of fully reduced human Trx1 is known, but not that of the Cys62-Cys69 oxidized structure. In the reduced structure, the sulfhydryls for Cys62 and Cys69 lie partially buried at opposite ends of a short $\alpha$-helix, about 10 Å apart. In this conformation, the Cys62-Cys69 disulfide bond cannot form. However, this helix is only loosely attached to the core of the protein through two loops, and this helix, in solution, could dynamically pull away from the protein core and sufficiently unravel to allow disulfide bond formation. We modeled
this possibility starting with the crystal coordinates for the fully reduced protein (PDB entry 1ERT), connecting Cys62 and Cys69, and energy minimizing. The resulting structure (Fig. 8) supports the hypothesis that local unfolding would allow the disulfide to form and substantially alter the structure in this region proximal to the active site.

**Reduction of Trx1 mutants by thioredoxin reductase:** Earlier studies showed that fully oxidized mammalian Trx1 was much more slowly reduced by thioredoxin reductase than was Trx1 that was oxidized only at the active site (24,28,29). These results imply that oxidation of the non-active site thiols could alter the rate of reduction by thioredoxin reductase and thereby provide a redox mechanism for control of Trx1 function. To determine whether the Cys62-Cys69 disulfide was responsible for the altered reactivity with thioredoxin reductase, a mutant of Trx1 bearing Cys → Ser mutations at positions 62 and 69 was generated and purified (C62,69S). Fig. 9 shows the reduction of wild type and mutant Trx1 by thioredoxin reductase and NADPH according to the reaction

\[
\text{NADPH} + \text{H}^+ + \text{oxidized Trx1} \rightarrow \text{NADP}^+ + \text{reduced Trx1}
\]

As reported previously (24,28,29), wild type Trx1 exhibited an early lag phase during which it was slowly reduced followed by a period of more rapid reduction. In contrast, C62,69S did not exhibit the lag period; the rate of reduction was greatest initially and slowed only as the amount of substrate (oxidized C62,69S) became limiting. The total amount of NADPH oxidized was consistent with the interpretation that the mutant form contained one less disulfide than the wild type Trx1.

An oxidized mutant of Trx1 in which the active site was mutated (C32,35S) was not a substrate for thioredoxin reductase (Fig. 9). However, upon addition of a small amount of wild type Trx1 (one-tenth of the amount of mutant Trx1 on a molar basis), one mole of...
NADPH was reduced per mole of the active site mutant. These results show that there was one disulfide in this mutant without active site thiols and that this non-active site disulfide was reduced by wild type Trx1. Thus, the non-active site disulfide is a substrate for reduction by the active site of Trx1, and the active site is, in turn, reduced by thioredoxin reductase.

**Discussion**

The three-dimensional structures of the thioredoxins are very similar among different species, even though there are regions of considerable sequence differences (5, 7). The structure of the sequence containing the active site cysteines is also highly conserved; Cys32 is located at the end of the α2-helix and Cys35 is in the linker region between the α2- and α3-helices (30). The $E_0$ of the active site dithiol of Trx1 is $-270$ mV in E. coli (10,11), $-240$ mV in yeast (12), and $-230$ mV in bacteriophage T4 (13). The present data show that the active site of human Trx1 has an $E_0$ of $-230$ mV, i.e., at the higher end of the range of known values. The redox state of endogenous E. coli Trx has been estimated to be 30% to 60% reduced (19, 31), although a more recent report has suggested that E. coli Trx1 is up to 90% reduced (32). In mammalian systems, bovine Trx1 was completely reduced in endothelial cells (16), and human Trx1 was 90% reduced in MCF-7 cells (33) and in HaCaT keratinocytes (34). Thus, our results show that human Trx1, like bovine Trx1, is maintained in a more reduced state within cells than is E. coli Trx1, and that the Redox Western blot yields an estimate of the redox state of cellular Trx1 that agrees well with previous estimates.
In addition to the active site cysteines, mammalian Trx1 contains three additional
cysteines that are not found in thioredoxins from other species or in mammalian
mitochondrial Trx2. Cys62 and Cys69 are within the α3-helix, and Cys73 is on a
hydrophobic patch on the surface of the protein (see Fig. 8). The data presented here
show that a disulfide bond can form between Cys62 and Cys69. In vitro analysis of
purified proteins demonstrated that oxidized forms of human (35), bovine (24), and rat
(28) Trx1 contained no detectable thiols as measured by reduction of DTNB (24,35),
supporting the interpretation that the non-active site thiols of mammalian Trx1 are subject
to oxidation. However, oxidation of Cys62 and Cys69 was not seen in the crystal
structure of oxidized human Trx1 (7). This could have resulted from the method of
sample preparation because the crystals of oxidized Trx1 were obtained by air oxidation
of the reduced form. The physical constraints within the crystallized reduced protein
could have prevented the formation of the second disulfide upon air oxidation (7).
Solution structures of oxidized and reduced human Trx1 are available (36), but do not
provide information on the non-active site disulfide because a mutant of Trx1 was used in
which all three non-active site cysteines were replaced with alanines. Thus, the present
study provides the novel and potentially important finding that non-active site cysteines
form a disulfide upon mildly oxidizing conditions.

A model for the Cys62-Cys69 linkage (Fig. 8) displays local unfolding of the intervening
helix. A likely mechanism for this disulfide formation would involve local unfolding
prior to bond formation. The helix is attached to the rest of the protein through two loops,
consistent with the possibility of helix dynamics. The temperature factors for the helix in
the X-ray structure, which is a measure of its disorder in the crystal, are about average for
the structure (14.7 Å² for the main-chain atoms vs. 14 Å² overall). However, movement of the helix is blocked in the dimer found in the crystal and so does not reflect its unrestricted dynamics. The loops connecting the helix are not restricted in the crystal and have higher temperature factors. Therefore, the possibility of disulfide bond formation between the Cys residues of the α3 helix is supported by the crystal structure.

The formation of a disulfide between Cys62 and Cys69 is predicted to have a profound effect by disrupting the helical structure of the α3 helix of Trx1 (see Fig. 8). In co-crystallization experiments, amino acids within this helix were shown to come in contact with Ref-1 and NF-κB peptides (37,38), two substrates for Trx1. Thioredoxin reductase most likely contacts Trx1 in this region as well; when Trx1 was in the homodimer form, stabilized by an intermolecular Cys73-Cys73’ disulfide bond in close proximity to the α3-helix, the active site was inaccessible to thioredoxin reductase (29). The biphasic reduction of the two-disulfide form of Trx1 (see Fig. 9) provides further support for the interpretation that the interaction between Trx1 and thioredoxin reductase is sensitive to presence of a non-active site disulfide in the α3-helix. A mutant of Trx1 (C62,69S) that was unable to form the Cys62-Cys69 disulfide was not inactivated as a substrate for thioredoxin reductase by extensive oxidation, highlighting the importance of these two residues. It should be noted that this mutant still contained Cys73 and was thus still capable of forming disulfide-bonded homodimers (29,35). Therefore, dimerization was probably not responsible for the early lag phase in the kinetics of reduction by thioredoxin reductase under the conditions used here.

The presence of the non-active site disulfide decreased the rate at which the active site of Trx1 was regenerated by thioredoxin reductase. Because the midpoint potential of the
non-active site dithiol is more negative than that of the active site, formation of this non-active site disulfide is more likely to occur under conditions in which the active site is also oxidized (and therefore inactive). Thus, the non-active site disulfide may provide a mechanism by which Trx1 can be temporarily inactivated and made resistant to regeneration by thioredoxin reductase. This would allow time for redox-dependent signaling processes to occur. Signaling processes that would benefit from the inactivation of thioredoxin include activation of the transcription factor Nrf2 via dissociation from Keap1 in the cytoplasm (39) and loss of inhibition of the pro-apoptotic kinase ASK1 (40).

In conclusion, $E_0$ for the active site of human Trx1 is $-230$ mV, among the highest of Trx proteins characterized to date. In addition to oxidation at the active site, human Trx1 also forms a second disulfide under relatively mild oxidizing conditions. Although the redox state of the active site is regulated by thioredoxin reductase, both Trx1 disulfides are at redox potentials within the range of the intracellular GSH/GSSG redox buffer. The location of the non-active site disulfide within the protein suggests a number of possible functions, such as substrate recognition (NF-κB), the regulation of other proteins through redox-sensitive binding to Trx1 (ASK1), and regulation of activity through interactions with thioredoxin reductase, as demonstrated in the current report. Thus, reversible oxidation of the conserved non-active site Cys residues of mammalian Trx1 may play an important regulatory role in the function of Trx1.
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References


*Structure* **3**, 289-297

*Structure* **4**, 613-620


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**Footnotes**

1 Abbreviations: Trx1, human thioredoxin-1; ASK1, apoptosis signal-regulating kinase  
1; Cys, cysteine; PAGE, polyacrylamide gel electrophoresis; mV, millivolts; NADPH,  
nicotinamide adenine dinucleotide phosphate; DTNB, dithionitrobenzoic acid; GSH,  
reduced glutathione; GSSG, oxidized glutathione; EDTA, ethylenediaminetetraacetic  
acid; IgG, immunoglobulin G; HRP, horseradish peroxidase; DTT, dithiothreitol; HPLC,  
high pressure liquid chromatography; ESI-MS, electrospray ionization triple quadrupole  
mass spectrometry; MALDI-TOF MS, matrix assisted laser desorption ionization time-  
of-flight mass spectrometry; RP-HPLC, reversed phase high pressure liquid  
chromatography; TFA, trifluoroacetic acid; CM, carboxymethyl; PTH,  
phenylthiohydantoin
Figure Legends

**Fig. 1: Amino acid sequence of human Trx1.** The peptides predicted to be formed upon digestion with trypsin are separated by a space and labeled T1 through T12. The five cysteines are shown in bold type.

**Fig. 2: Separation of oxidized and reduced forms of human Trx1.** [A] Purified recombinant human Trx1 (8 µg) was treated with either 4 mM dithiothreitol (lane 1) or 4 mM diamide (lane 2) for 10 min at room temperature. The reduced thiols were then carboxymethylated with 10 mM iodoacetic acid in the presence of 5 M guanidine. The proteins were separated by native PAGE and visualized by Coomassie Blue staining (see Materials and Methods). [B] Purified Trx1 was equilibrated in glutathione redox buffers of the indicated potentials for 2 h at room temperature, and then analyzed as in [A].

**Fig. 3A: Comparison of tryptic digests of oxidized and reduced Trx1.** Overlay of MALDI-TOF mass spectra of tryptic fragments for oxidized/carboxymethylated Trx1 (upper) and reduced/carboxymethylated Trx1 (lower). Identified tryptic fragments of Trx1 are labeled. Carboxymethyl is abbreviated “cm,” methionine sulfoxide is abbreviated “ox” and “a*” denotes autolytic fragments of trypsin.

**Fig. 3B: Product ion spectra of T4 tryptic fragment from the tryptic digest of reduced/carboxymethylated (upper) and oxidized carboxymethylated (lower) Trx1.** The reduced/carboxymethylated and oxidized/carboxymethylated proteins were digested with trypsin and the digests were then desalted and analyzed by nanospray ESI-MS/MS (positive mode) on a triple quadrupole. For the reduced species, the doubly charged ion at m/z 871.4, corresponding to the addition of two carboxymethyl groups (cm), was selected.
for fragmentation. The carboxymethyl group adds a mass of 58 Da for each group resulting in an increase in mass of 58 Da for the y2, y3, and y4 ions and an increase in mass of 116 Da for the y5 through y15 ions as compared to the non-carboxymethylated sequence. In addition, the b11 through b13 ions increase by 58 Da and the b14 and b15 ions increase by 116 Da due to the carboxymethyl groups. For the oxidized species, the doubly charged ion at m/z 812.3, corresponding to the disulfide, was selected for fragmentation.

**Fig. 4: Redox Western blot analysis of purified Trx1.** Trx1 (30 ng) was equilibrated in 50 mM glutathione redox buffers of the indicated potentials for 2 h at room temperature, then analyzed by Redox Western blot, as described in Materials and Methods.

**Fig. 5: Percent of Trx1 active site in the reduced form as a function of redox potential.** The experiment shown in Fig. 3 was repeated six times, and the relative amount of each band was determined by densitometry. The percent of Trx1 active site in the reduced form was calculated by the formula \( \% \text{ Reduced} = \frac{\text{band 3}}{\text{band 1 + band 2 + band 3}} \times 100 \). The line indicates the theoretical curve of a protein undergoing a 2-electron oxidation with a midpoint potential of –230 mV.

**Fig. 6: HPLC tracing of mBBr-labeled Trx1 peptides.** Trx1 (16 µg) was equilibrated in glutathione redox buffers, labeled with mBBr, digested with trypsin and separated by HPLC, as described in Materials and Methods. The peaks corresponding to the tryptic fragments containing one or two cysteines are labeled T4 (active site), T6, and T7.

**Fig. 7: Redox Western blot analysis of Trx1 in whole cell lysates.** THP1 cells were either untreated (lane 1) or treated with increasing concentrations of diamide for 2 min.
Cells were pelleted by centrifugation and proteins were extracted with guanidine lysis buffer containing 50 mM IAA (see Materials and Methods). For the last two lanes, THP1 proteins were extracted with guanidine lysis buffer in the absence of IAA, and the extracts were treated with either 5 mM DTT (labeled DTT) or 5 mM diamide (labeled diamide) for 10 min at room temperature. The reduced and oxidized extracts were then carboxymethylated with 50 mM IAA. All samples were analyzed by Redox Western blot, as described in the Materials and Methods. This experiment was repeated twice with essentially identical results.

**Fig. 8: Modeling of the Cys62-69 disulfide in human Trx1.** Energy minimization calculations were based on the crystal structure of the reduced form of human Trx1. The left panel shows Cys62 and Cys69 as thiols, and the right panel shows the predicted structure of Trx1 with a disulfide between Cys62 and Cys69. The residues of the α3 helix are shown in green, and the other helices are shown in blue. The beta sheets are shown in magenta. Each of the Cys side chains is shown in yellow (Cys73 is not visible in this orientation, but its position is indicated).

**Fig. 9: The non-active site disulfide of Trx1 inhibits reduction of the active site of Trx1 by thioredoxin reductase.** In a double beam spectrophotometer, 0.5 ml of reaction buffer (TE, pH 7.5) containing 300 µM NADPH and 14 µM bovine thioredoxin reductase was placed in both the sample and reference positions, and 15 nmol of Trx1(wt), 25 nmol Trx1(C62,69S), or 15 nmol Trx1(C32,35S) was added to the reference position. The oxidation of NADPH was monitored at 340 nm.
Table 1. Expected and observed monoisotopic masses (MH⁺) of tryptic fragments from Trx<sup>a</sup>.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Expected Mass (MH⁺)</th>
<th>MH⁺ observed in Band 1</th>
<th>MH⁺ observed in Band 2</th>
<th>MH⁺ observed in Band 3</th>
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<tbody>
<tr>
<td><strong>Cys-containing peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T4 disulfide</td>
<td>1622.8</td>
<td>1622.8</td>
<td>1622.8</td>
<td>ND</td>
</tr>
<tr>
<td>T4 (2x cm)</td>
<td>1740.8</td>
<td>ND</td>
<td>1740.8</td>
<td>1740.8</td>
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<tr>
<td>T6 disulfide</td>
<td>2717.2</td>
<td>2717.1</td>
<td>2717.2</td>
<td>ND</td>
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<tr>
<td>T6 (2x cm)</td>
<td>2835.2</td>
<td>ND</td>
<td>2835.2</td>
<td>2835.2</td>
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<tr>
<td>T7</td>
<td>1148.5</td>
<td>1148.5</td>
<td>1148.5</td>
<td>1148.5</td>
</tr>
<tr>
<td>T7 (cm)</td>
<td>1206.5</td>
<td>ND</td>
<td>1206.5</td>
<td>1206.5</td>
</tr>
<tr>
<td>T7 (cm+ox)</td>
<td>1222.5</td>
<td>ND</td>
<td>1222.5</td>
<td>1222.5</td>
</tr>
<tr>
<td><strong>Other identified peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>1336.6</td>
<td>1336.6</td>
<td>1336.6</td>
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<td>T5 (ox)</td>
<td>1479.8</td>
<td>1479.7</td>
<td>1479.7</td>
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<tr>
<td>T10</td>
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<td>908.4</td>
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<tr>
<td>T10-11</td>
<td>1165.6</td>
<td>ND</td>
<td>ND</td>
<td>1167.7</td>
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</tbody>
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<sup>a</sup> Trx1 was reduced with DTT, oxidized with diamide, or equilibrated with a redox buffer of –240 mV, carboxymethylated with IAA, and separated by native PAGE. Bands 1, 2, and 3 were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF. ND means that the expected mass was not detected. Figure 1 shows the amino acid sequences of the tryptic fragments. T4, T6, and T7 are the Cys-containing fragments. Carboxymethylation (cm) and Met oxidation (ox) are indicated.
Table 2. N-terminal sequence analysis of purified tryptic peptides of mBBr-labeled Trx1$^a$.

<table>
<thead>
<tr>
<th>Cycle #</th>
<th>PTH-amino acid (yield, pmol)$^b$</th>
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<tr>
<td></td>
<td>T4</td>
</tr>
<tr>
<td>1</td>
<td>L (5.0)</td>
</tr>
<tr>
<td>2</td>
<td>V (3.8)</td>
</tr>
<tr>
<td>3</td>
<td>V (3.8)</td>
</tr>
<tr>
<td>4</td>
<td>V (3.5)</td>
</tr>
<tr>
<td>5</td>
<td>D (4.2)</td>
</tr>
<tr>
<td>6</td>
<td>F (4.0)</td>
</tr>
<tr>
<td>7</td>
<td>S (1.7)</td>
</tr>
<tr>
<td>8</td>
<td>A (2.8)</td>
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<tr>
<td>9</td>
<td>T (1.5)</td>
</tr>
<tr>
<td>10</td>
<td>W (0.3)</td>
</tr>
<tr>
<td>11</td>
<td>CmB (nd)</td>
</tr>
<tr>
<td>12</td>
<td>G (0.7)</td>
</tr>
</tbody>
</table>

$^a$ Trx1 was derivatized with mBBr, digested with trypsin, and the resulting peptides were separated by HPLC. Eluted fractions corresponding to peaks of fluorescence were collected and subjected to N-terminal amino acid sequencing by Edman degradation.

$^b$ Raw PTH-amino acid yields uncorrected for background or carryover. Yield of the bimane derivative of Cys (CmB) could not be determined (nd) due to lack of a standard.

$^c$ Due to background contamination of T7, no PTH-amino acid could be positively assigned in the first cycle.
**Fig. 1:** Amino acid sequence of human Trx1. The peptides predicted to be formed upon digestion with trypsin are separated by a space and labeled T1 through T12. The five cysteines are shown in bold type.

```plaintext
<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<td>1</td>
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<td>QIESK</td>
<td>TAFQEA</td>
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<td>95</td>
<td>EK</td>
<td>LEATINELV</td>
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</tbody>
</table>
```
Fig. 2: Separation of oxidized and reduced forms of human Trx1. [A] Purified recombinant human Trx1 (8 µg) was treated with either 4 mM dithiothreitol (lane 1) or 4 mM diamide (lane 2) for 10 min at room temperature. The reduced thiols were then carboxymethylated with 10 mM iodoacetic acid in the presence of 5 M guanidine. The proteins were separated by native PAGE and visualized by Coomassie Blue staining (see Materials and Methods). [B] Purified Trx1 was equilibrated in glutathione redox buffers of the indicated potentials for 2 h at room temperature, and then analyzed as in [A].
**Fig. 3A: Comparison of tryptic digests of oxidized and reduced Trx1.** Overlay of MALDI-TOF mass spectra of tryptic fragments for oxidized/carboxymethylated Trx1 (upper) and reduced/carboxymethyl Trx1 (lower). Identified tryptic fragments of Trx1 are labeled. Carboxymethyl is abbreviated “cm,” methionine sulfoxide is abbreviated “ox” and “a*” denotes autolytic fragments of trypsin.
Fig. 3B: Product ion spectra of T4 tryptic fragment from the tryptic digest of reduced/carboxymethylated (upper) and oxidized/carboxymethylated Trx1 (lower).

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