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Oxidative Inactivation of Thioredoxin as a Cellular Growth Factor and Protection by a $Cys^{73} \rightarrow Ser$ Mutation

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ABSTRACT. Thioredoxin (Trx) is a widely distributed redox protein that regulates several intracellular redox-dependent processes and stimulates the proliferation of both normal and tumor cells. We have found that when stored in the absence of reducing agents, human recombinant Trx undergoes spontaneous oxidation, losing its ability to stimulate cell growth, but is still a substrate for NADPH-dependent reduction by human thioredoxin reductase. There is a slower spontaneous conversion of Trx to a homodimer that is not a substrate for reduction by thioredoxin reductase and that does not stimulate cell proliferation. Both conversions can be induced by chemical oxidants and are reversible by treatment with the thiol reducing agent dithiothreitol. SDS-PAGE suggests that Trx undergoes oxidation to monomeric form(s) preceding dimer formation. We have recently shown by X-ray crystallography that Trx forms a dimer that is stabilized by an intermolecular Cys^{73}-Cys^{73} disulfide bond. A $Cys^{73} \rightarrow$ Ser mutant Trx (C73S) was prepared to determine the role of Cys^{73} in oxidative stability and growth stimulation. C73S was as effective as Trx in stimulating cell growth and was a comparable substrate for thioredoxin reductase. C73S did not show spontaneous or oxidant-induced loss of activity and did not form a dimer. The results suggest that Trx can exist in monomeric forms, some of which are mediated by Cys^{73} that do not stimulate cell proliferation but can be reduced by thioredoxin reductase. Cys^{73} is also involved in formation of an enzymatically inactive homodimer, which occurs on long term storage or by chemical oxidation. Thus, although clearly involved in protein inactivation, Cys^{73} is not necessary for the growth stimulating activity of Trx. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1741–1747, 1996.

KEY WORDS. thioredoxin; growth factor; oxidation; homodimer

Trx[‖] is a redox protein found in both eukaryotes and prokaryotes [1]. The redox activity of Trx arises from a highly conserved Trp-Cys-Gly-Pro-Cys-Lys active site sequence where the 2 cysteine residues (Cys) undergo reversible oxidation to cystine. Reduction of Trx is catalyzed by thioredoxin reductase [2]. Trx was originally identified in *Escherichia coli* as a hydrogen donor for ribonucleotide reductase [3]. Trx has since been found to act as an intracellular dithiol-disulfide reductase and to modulate the activity of a number of intracellular proteins [4–6] including the DNA binding of transcription factors [7–10]. Trx-like sequences are found in other proteins including protein disulfide isomerase [11]. There is evidence that Trx may play a role in the growth and transformed phenotype of some cancers. Trx is over expressed by a number of human cancers compared with normal tissue [12–14]. We have recently shown that transfection of human cancer cells with a dominant-negative mutant human Trx inhibits anchorage-independent growth *in vitro* and tumor formation *in vivo* [15].

As well as having intracellular actions, Trx acts exogenously as a redox-active growth factor. Human Trx is identical to the leukemic cell autocrine growth factor adult T-cell leukemic factor [13], and stimulates the growth of both normal fibroblasts [16] and human hematologic and solid tumor cancer cells in culture [17, 18]. Trx appears to act by a helper mechanism that sensitizes the cells to growth factors secreted by the cells themselves [18]. Mutant human Trxs, where the Cys^{32} and Cys^{35} residues at the catalytic site¶ are converted to serines (Ser), either singly or together, are redox inactive and do not stimulate cell growth [16]. Trx is secreted from cells by a leaderless secre-

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‖ *Abbreviations:* Trx, thioredoxin; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle's medium; and NEN, *N*-ethylmaleimide.

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¶ Numbering of amino acid residues is from the N-terminal methionine, although this may be removed in some forms of Trx.

tory pathway [19] and could be acting as an autocrine factor for the growth of some cancer cells [18].

We have found that *E. coli* Trx, unlike human Trx, does not stimulate the growth of human solid cancer cells [18]. The structures of *E. coli* and human Trx are similar, and both are substrates for human thioredoxin reductase. However, the surface residues of the two forms vary considerably [20]. Human Trx has 3 additional cysteine residues, Cys^{62}, Cys^{69} and Cys^{73}, in addition to those in the active site, which do not normally form intramolecular disulfide bonds [20, 21]. Trx can also form a homodimer with a 1100 $Å^2$ interface domain and a disulfide bond between Cys^{73} from each monomer [20]. During our studies of cell growth stimulation by Trx we observed that storage of the Trx without a reducing agent for even a few days resulted in a loss of its growth-stimulating activity, although the Trx remained a substrate for reduction by thioredoxin reductase. We have, therefore, examined the role of spontaneous and induced oxidation of Trx and cysteine-deleted mutant Trxs, and their ability to stimulate cell proliferation.

MATERIALS AND METHODS

Preparation of Thioredoxins

Recombinant human Trx and $Cys^{32} \rightarrow Ser/Cys^{35} \rightarrow Ser$ mutant Trx (C32S/C35S) were prepared and purified as previously described [16]. $Cys^{73} \rightarrow Ser$ mutant human Trx (C73S) was prepared from single-stranded, sense strand human Trx cDNA ligated by polyethylene glycol precipitation into the pBluescript KS vector (Stratagene, La Jolla, CA) using R408 helper phage. The single-stranded cDNA was used for oligonucleotide-directed *in vitro* mutagenesis (Version 2.1 Kit, Amersham, Buckinghamshire, U.K.) using oligonucleotide 5′-TGTTGGCATGGATTT-GACTTC-3′. Point mutagenesis was confirmed by dideoxy sequencing of base-denatured double-stranded DNA using the Sequenase Version 2.0 kit (USB, Cleveland, OH). Novel *Nde*I and *Bam*HI restricted sites were introduced at the 5′ and 3′ ends of the mutant Trx cDNA by oligonucleotide-directed PCR. *Nde*I/*Bam*HI restricted fragments were extracted from agarose gels, ligated into a suitably restricted pET-3a expression vector [22], transformed into *E. coli* BL21 cells and confirmed by dideoxy sequencing. C73S Trx was expressed and purified as previously described [16]. All Trxs were stored at −20° as a 25 μM stock solution in 5 mM DTT. Before use, the DTT was removed by passing the Trx solution through a PD-10 desalting column (Pharmacia, Uppsala, Sweden). The Trx solution was kept at 4° and used within 2 hr (fresh) or stored in water or 0.1 M potassium phosphate-buffered 0.9% NaCl at 4° or −20° for specified times. Oxidized Trx for cell growth studies was prepared by adding a 5-fold molar excess of H_2O_2 to a 25 μM Trx stock solution without DTT and 1 hr later removing unreacted H_2O_2 using a PD-10 column.

Cell Growth Studies

MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD), maintained in DMEM containing 10% fetal bovine serum at 37° and 6% CO_2, and passaged at 75% confluence. The effect of Trx and modified Trxs on MCF-7 cell growth was determined as previously described [18]. Briefly, 10^5 cells were plated in a 35-mm culture dish in DMEM containing 10% fetal bovine serum and, after attachment for 24 hr, growth arrested using DMEM with 0.5% fetal bovine serum for 48 hr. The medium was then replaced with DMEM containing Trx or other additions for 2 days and cell number measured with a hemocytometer. All incubations were conducted in triplicate.

Thioredoxin Reductase Assay

Human placenta thioredoxin reductase, specific activity 33.3 μmol Trx reduced/min/mg protein, was prepared as previously described [23]. Reduction of Trx and C73S by thioredoxin reductase was measured by the oxidation of NADPH at 340 nM with insulin as the final electron acceptor as described by Luthman and Holmgren [2].

Electrophoresis

A 25 μM solution of fresh Trx, mutant C73S or C32S/C35S Trxs; Trxs that had been aged at room temperature for 48 hr, 7 days, 90 days; or Trxs treated for 1 hr with 1 mM diamide, 10 mM DTT, 3 mM 2-mercaptoethanol or 2:1 (v:v) H_2O_2, was mixed with an equal volume of loading buffer containing 3% SDS, 10% glycerol and 0.1% bromphenol blue in 0.05 M Tris-HCl, pH 6.8. Approximately 0.02 μg of the protein was loaded in each lane of a 24 × 45 cm 16.5% polyacrylamide resolving gel (pH 8.4) containing 0.3% SDS, a 10% spacer gel and a 6% stacking gel and separated by electrophoresis using an anode buffer of 0.2 M Tris-HCl, pH 8.9 and cathode buffer of 0.1 M Tris-HCl, 0.1% SDS, pH 8.2. The gel was run for 1 hr at 400 volts before loading the samples and then at 400 volts for 24 hr before fixing in 50% methanol, 7.5% acetic acid for 20 min, followed by 5% methanol, 7.5% acetic acid for 20 min, followed by 10% glutaraldehyde for 20 min. The gel was soaked in 2 L H_2O overnight to remove unbound SDS and then silver stained (ICN Silver Stain Kit, Irvine, CA). Similar observations were made when the gels were stained with Coomassie Blue.

RESULTS

Growth Stimulation

$Cys^{73} \rightarrow Ser$ mutant Trx (C73S) stimulated the proliferation of human MCF-7 breast cancer cells. The EC_{50} for growth stimulation by C73S was 350 nM and the maximum effect was seen at 1 μM, which is similar to values we have previously reported for stimulation of MCF-7 cell proliferation by recombinant human Trx [18]. Storage of Trx in the absence of a reducing agent such as DTT at 4° for 5 days resulted in a 78% loss, and for 90 days a 98% loss of cell growth stimulating activity (Fig. 1). In contrast, C37S

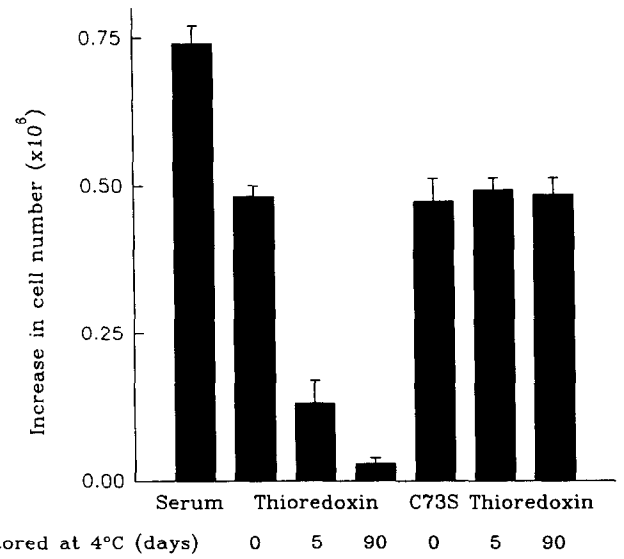

FIG. 1. Stimulation of MCF-7 breast cancer cell growth by fresh and aged Trx and C73S. MCF-7 cells were growth arrested and the stimulation of cell proliferation measured over 2 days using 1 μM Trx or C73S that was fresh or had been stored as a 25 μM stock solution without reducing agent for 5 days or 90 days at 4°. Also shown for reference is the effect of 10% fetal bovine serum. Each value is the mean of 3 determinations, and bars are SEM.

showed no loss of activity when stored under these conditions. Trx stored in the presence of bovine catalase at 1 unit/ml did not lose biological activity over a 5-day period (results not shown).

Reduction of Thioredoxins by Thioredoxin Reductase

C73S was a good substrate for reduction by human placental thioredoxin reductase with a K_m of 0.20 μM and a V_{max} of 6.3 nmol/min/μg. These values are similar to those we have previously found for fresh Trx, which were a K_m of 0.33 μM and a V_{max} of 5.9 nmol/min/μg [23].

The effect of storing Trx without DTT on its ability to act as a substrate for thioredoxin reductase was investigated (Table 1). When stored in H_2O either at −20° or at room temperature Trx showed a loss of activity with a half-life of 20–30 days. The loss of Trx activity was more rapid when stored in phosphate-buffered 0.9% NaCl, with a half life of 8 days. Phosphate buffer is known to contain small amounts of iron [24], which could catalyze an oxidative process increasing the loss of Trx activity. Alternatively, the lower pH of the solution in water could stabilize Trx or the increase in ionic strength of phosphate-buffered 0.9% NaCl could enhance the formation of the inactive homodimer of Trx. The aged Trx showed a slow, delayed reduction by thioredoxin reductase that was stimulated by catalytic amounts of fresh Trx (Fig. 2). It is important to note that the loss of activity of Trx as a substrate for thioredoxin reductase was much slower than the loss of activity as a stimulator of cell growth. C73S did not show a loss of activity as substrate for thioredoxin reductase upon storage for up to 30 days. The ability of Trx to act as a substrate for thioredoxin reductase was completely inhibited by treatment with 5 molar equivalents of H_2O_2, whereas C37S remained fully active after treatment with 100 molar equivalents of H_2O_2 (Fig. 3).

TABLE 1. Effect of storage of thioredoxins as substrates for thioredoxin reductase

	In H_2O		In PBS	
	−20° $t_{1/2}$ (days)	+21° $t_{1/2}$ (days)	−20° $t_{1/2}$ (days)	+21° $t_{1/2}$ (days)
Trx	30.5	20.1	8.2	7.8
C73S	stable*	stable*	stable*	stable*

25 μM stock solutions of Trx or C73S in H_2O or phosphate buffered 0.9% NaCl (PBS) free of DTT were stored frozen at −20° or at room temperature (+21°) for up to 60 days, and their ability to act as a substrate for reduction by human placental thioredoxin was measured. A first order decrease in activity was found for Trx, and the results are expressed as half-life ($t_{1/2}$).

* <10% loss of activity over 30 days.

Multiple Forms of Thioredoxin

Electrophoretic analysis of freshly prepared human Trx stored in DTT showed a mixture of 5 bands of apparent molecular weights ranging from 8.1 to 11 kDa (Figs. 4, 5, and 6, lane 1). Storage of Trx at room temperature without DTT resulted in a change in the banding pattern with disappearance of the 8.1-kDa band by 48 hr (Fig. 4, lane 2). Storage of Trx without DTT for 7 days resulted in the loss of additional bands and the appearance of a new band at 23 kDa due, apparently, to a Trx dimer (Fig. 4, lane 3). Storage of Trx without DTT for 90 days at 4° resulted in almost complete conversion to the Trx dimer (Fig. 4, lane 4). Treatment of 7-day aged Trx (Fig. 5, lane 2) with 2-mercaptoethanol resulted in the reappearance of the fresh Trx banding pattern, except for the 8.1-kDa band, which did not reappear (Fig. 5, lane 3). Loss of the smaller bands and dimer formation was seen when Trx was treated with di-

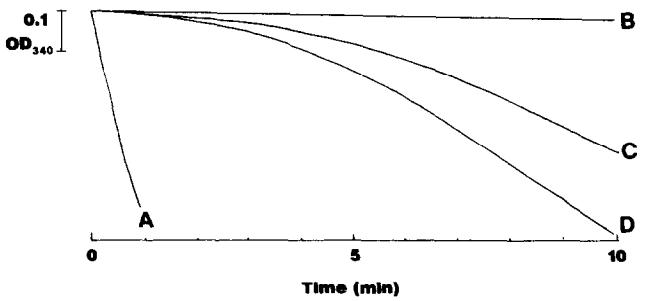

FIG. 2. Reduction of aged Trx by thioredoxin reductase. The incubation mixture contained 0.1 M HEPES buffer, pH 7.6, 5 mM EDTA, 17 μM insulin, 100 μM NADPH, 15 μg/ml human thioredoxin reductase. Traces show the oxidation of NADPH at 340 nm at room temperature with: A, 1 μM fresh Trx; B, 30 nM fresh Trx; C, 90-day aged 1 μM Trx; D, 1 μM 90-day aged Trx and 30 nM fresh Trx.

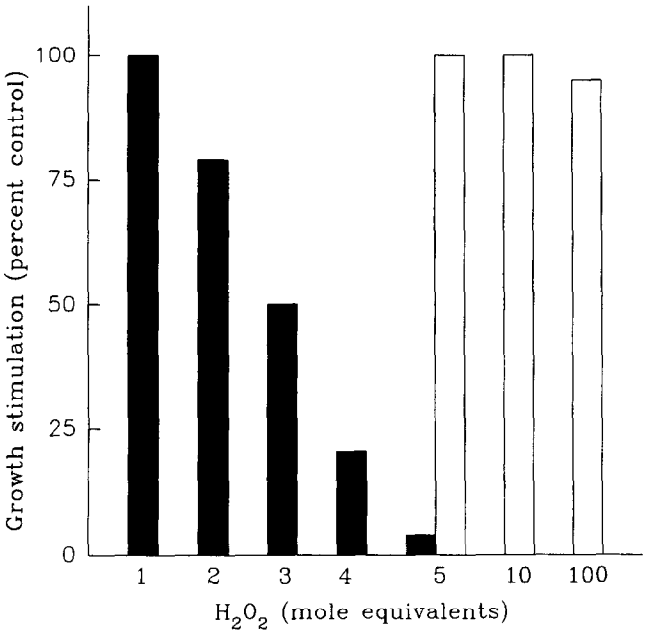

FIG. 3. The effect of H_2O_2 on the reduction of Trx (filled bars) and C73S (open bars) by thioredoxin reductase. Trx solutions were treated with varying amounts of H_2O_2 for 18 hrs at room temperature. Reductase activity was measured by adding treated samples to a solution of 0.1 M HEPES buffer, pH 7.6, 5 mM EDTA, 17 μM insulin, 100 μM NADPH, 15 μg/ml human thioredoxin reductase and measuring the rate of NADPH oxidation at 340 nm at room temperature. One hundred percent of thioredoxin reductase activity is defined as 0.1 absorbance unit/min/mM Trx or C73S Trx. H_2O_2 had no effect on the oxidation of NADPH.

amide, a protein thiol oxidizing agent [25] (Fig. 5, lane 5). The formation of Trx dimer following diamide treatment was also confirmed by gel permeation chromatography (results not shown). H_2O_2 treatment of Trx also caused dimerization but produced a different banding pattern to that produced by diamide (Fig. 5, lane 6). Treatment of Trx with NEM, a thiol alkylating agent [26], gave a single band with a slightly elevated apparent molecular weight, but no dimer formation (Fig. 5, lane 4). Treatment of 7 day aged Trx with NEM produced both the higher molecular weight band as in Fig. 5, lane 4, and the bands illustrated in Fig. 5, lane 2 (data not shown), suggesting that in the aged Trx not all the sulfhydryls are available for covalent modification. None of the changes caused by NEM were reversed with 2-mercaptoethanol treatment (data not shown). 2-Mercaptoethanol reversed Trx dimer formation caused by both diamide and H_2O_2 treatment (Fig. 5, lanes 7 and 8) but was less effective at reversing changes in the monomeric banding pattern of Trx produced by H_2O_2 (Fig. 5, lane 8).

Freshly prepared C73S Trx and C32S/C35S Trx showed fewer bands than wild type Trx (Fig. 6, lanes 2 and 3, compared with lane 1). Treatment of C32S/C35S Trx with diamide resulted in the formation of a 23-kDa dimer (Fig. 6, lane 6). Treatment of C73S Trx with diamide caused the bands to coalesce into a single band of around 10 kDa, but there was no 23-kDa dimer formed (Fig. 6, lane 4). The effects of diamide on C37S and C32S/C35S were reversed by treatment with DTT (Fig. 6, lanes 5 and 7).

DISCUSSION

The study shows that human recombinant Trx undergoes at least 2 levels of spontaneous and induced oxidative transformation. The first oxidation occurs spontaneously within a few days to a form(s) that can no longer stimulate cell growth but remains a substrate for thioredoxin reductase. The slower oxidation occurs over a period of weeks, or can be induced by the thiol oxidizing agent diamide, and leads to a disulfide bonded homodimer which not only fails to stimulate cell growth but is a poor substrate for thioredoxin reductase. The fact that similar changes in Trx can be induced by chemical oxidation, are protected against by catalase and are reversed by the thiol reducing agent DTT is consistent with the interpretation that the changes in Trx are due to oxidation. Cys^{73} appears to play a critical role in both levels of oxidant-induced inactivation since C73S does not lose the biological activity or its ability to act as a substrate for thioredoxin reductase upon aging.

We have shown by SDS-PAGE that fresh human recombinant Trx can exist in at least five different states, which probably reflect the fully reduces state of the protein as well as different intramolecular disulfide bonded states due to the five cysteine residues present in the protein. While the specific nature of these intramolecular disulfide bonds is not known, it is likely that some, at least, are due to non-

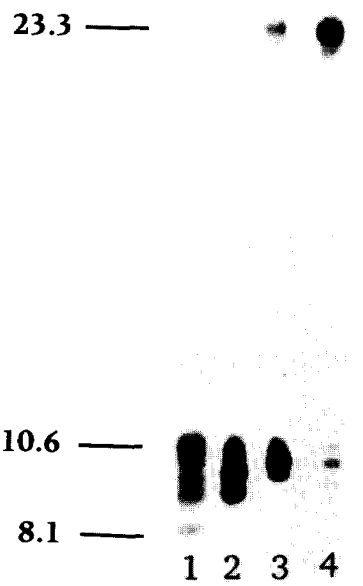

FIG. 4. Effect of storage on Trx studied by SDS-PAGE. Protein was stained with silver stain. Lane 1, fresh Trx; lane 2, Trx 48 hrs at room temperature without DTT; lane 3, Trx 7 days at room temperature without DTT; and lane 4, Trx stored 90 days at 4° without DTT. Position of molecular mass markers in kDa are shown on the left.

natural disulfide bonded structures which form during denaturation and the oxidizing conditions of extended electrophoresis [27]. The observation that C37S and C32S/C35S exhibit a simpler banding pattern than wild-type Trx upon SDS-PAGE also suggests that the banding pattern is due to disulfide bond formation. X-ray structural analysis indicates that in addition to a disulfide bond between Cys^{32} and Cys^{35}, the only other intramolecular disulfide bond that could form in the non-denatured Trx is between Cys^{73} and Cys^{32}, although even this would require a different conformation of the protein [20]. With the exception of a possible slight modification in Cys^{69}, there is no evidence that Cys^{32}, Cys^{35} or Cys^{62} are oxidized in Trx crystals formed in the presence of 5 mM DTT [20]. The fact that treatment of Trx with NEM produces only one band implies that prior to denaturation and electrophoresis fresh Trx exists as a single species. The number of free thiols in fresh Trx was determined to be 4.5 to 4.6/molecule by Ellman's reagent [28] (data not shown), indicating that all five cysteines are in the sulfhydryl form. Treatment of NEM-alkylated Trx with oxidizing or reducing agents produces no change in the banding pattern (data not shown), which is further evidence that all 5 sulfhydryls have been alkylated.

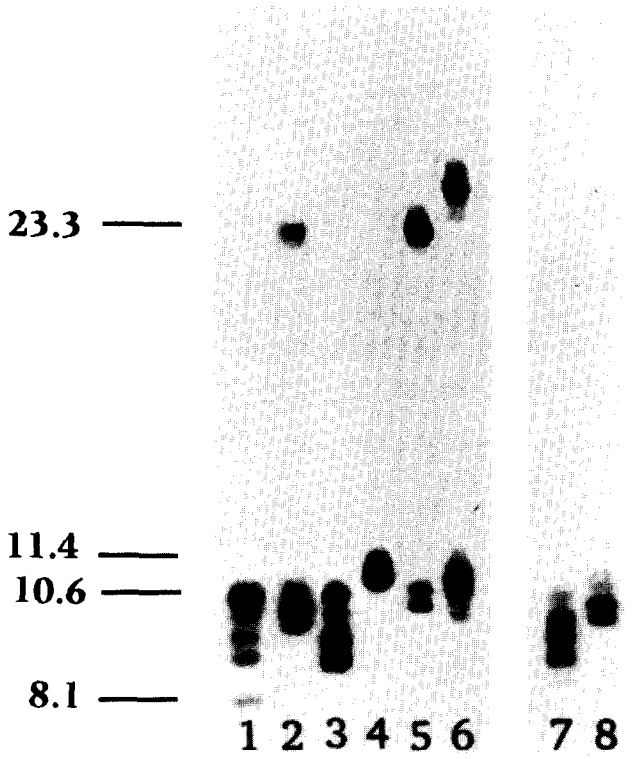

FIG. 5. Oxidation and alkylation of Trx studied by SDS-PAGE. Lane 1, fresh Trx; lane 2, Trx stored at room temperature without DTT for 7 days; lane 3, Trx as in lane 2 treated with 3 mM 2-mercaptoethanol; lane 4, fresh Trx treated with 1 mM N-ethylmaleimide; lane 5, fresh Trx treated with 1 mM diamide; lane 6, fresh Trx treated with 2:1 (v:v) H_2O_2; lane 7, Trx as in lane 5 treated with 3 mM 2-mercaptoethanol; and lane 8, Trx as in lane 6 treated with 10 mM 2-mercaptoethanol. Position of molecular mass markers in kDa are shown on the left.

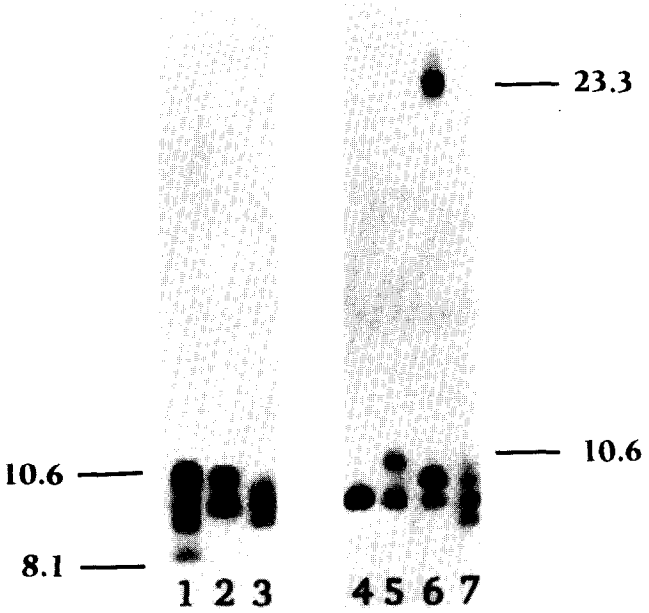

FIG. 6. Oxidation and reduction of mutant Trxs studied by SDS-PAGE. Lane 1, fresh Trx; lane 2, fresh C73S Trx; lane 3, fresh C32S/C35S Trx; lane 4, C73S Trx treated with 1 mM diamide; lane 5, C73S Trx as in lane 4 treated with 10 mM DTT; lane 6, C32S/C35S Trx treated with 1 mM diamide; and lane 7, C32S/C35S Trx as in lane 6 treated with 10 mM DTT. Position of molecular mass markers in kDa are shown on the left and right.

Oxidation of cysteines to sulfenic or sulfinic acids is unlikely to occur spontaneously [29]. It is noteworthy that H_2O_2 treatment of Trx gives rise to a different monomeric banding pattern than that of spontaneously oxidized Trx. The original monomeric banding pattern is also not regenerated by treatment with DTT. As has been previously suggested for NADH peroxidase [30], we speculate that H_2O_2 oxidizes the cysteines to sulfenic acids and to the irreversible sulfinic or sulfonic acid states.

During the same time interval that there was a loss of the growth stimulating activity of Trx, there was a shift of the electrophoretic banding pattern. There was a collapse of the banding pattern with loss of some of the Trx monomeric bands over 7 days, suggesting that Trx may be undergoing "native" intramolecular disulfide bond formation prior to electrophoresis, which prevents the formation of random disulfide-bond formation seen with denaturation and electrophoresis of fresh Trx. A similar phenomena has been observed with bovine pancreatic trypsin inhibitor [27, 31]. Alkylation of aged Trx with NEM gave more than one protein product, indicating that aged Trx exists in multiple forms and not all the sulfhydryls are available for reaction. Since C73S does not undergo a similar shift in banding pattern and does not undergo loss of growth stimulating activity, it can be assumed that Cys^{73} is involved in this intramolecular disulfide bond formation, perhaps with Cys^{32} (Fig. 7). Thus, spontaneous aging of Trx over a few days results in the inability of Trx to stimulate cell growth, although Trx is still a substrate for reduction by thioredoxin

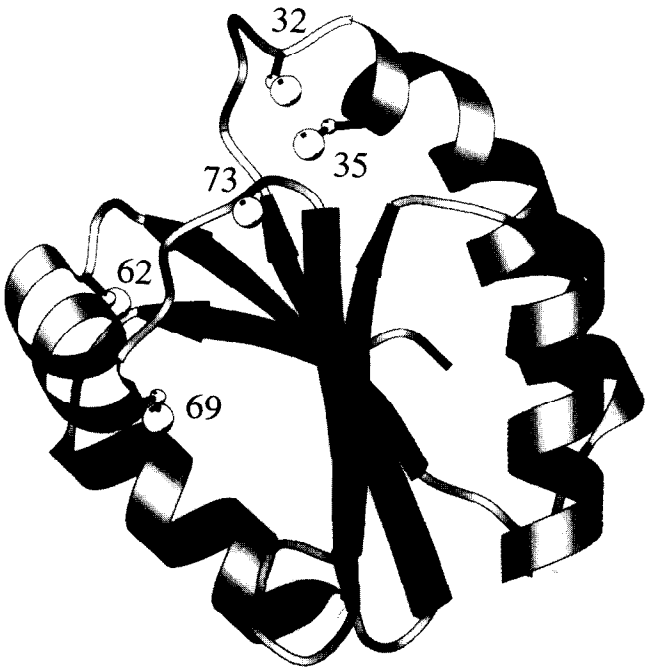

FIG. 7. Position of cysteines in human Trx. Ribbons and ball-and-stick representation showing the relative positions of Cys^{32}, Cys^{35}, Cys^{62}, Cys^{69} and Cys^{73}, based on the crystal coordinates for the wild type reduced protein [20]. None of the thiols are in a position for disulfide bond formation except for the redox active pair Cys^{32} and Cys^{35}. The intermolecular disulfide bond requiring the least distortion in the protein would be between Cys^{32} and Cys^{73}. The sulfhydryls for these residues are 9.1 Å apart in the model, but could possibly approach each other through local distortions in nearby residues. Both residues are in loops, making necessary distortions of energetically lower cost. The region near Cys^{32} has already been shown to adopt alternate conformations [20], in support of this possibility. This figure was made with MOLSCRIPT [35].

reductase. Analysis of the X-ray structure of Trx shows that Cys^{73} is by far the most accessible cysteine residue and possibly the most reactive [33].

If a solution of Trx is left long enough, or upon treatment with a strong oxidizing agent such as diamide or H_2O_2, there is formation of a 23-kDa Trx homodimer. Reduction of the Trx dimer by thioredoxin reductase is slow and delayed, and is stimulated by low concentrations of fresh Trx, suggesting there may be an autocatalytic process. A similar conclusion was reached by Ren *et al.* [34]. Formation of the Trx homodimer appears to involve the Cys^{73} residue since C73S, where Cys^{73} is replaced with serine, does not undergo oxidation-induced homodimer formation as do Trx and C32S/C35S. Ren *et al.* [34] have shown C73S does not undergo oxidative homodimer formation induced by selenodithioglutathione. We recently reported the X-ray crystal structure of Trx and identified a largely hydrophobic dimer forming interface that is stabilized by a Cys^{73}-Cys^{73} disulfide bond [20]. Our observation that Trx undergoes a faster loss of activity with thioredoxin reductase in PBS versus water indicates that iron-induced oxidation or an increase in ionic strength may stabilize and enhance dimer formation, which is consistent with the hydrophobic nature of the dimer interface observed in crystals of human Trx.

The importance of the monomeric oxidative form(s) of Trx is unknown. While the structural nature is yet to be identified, it does have different biological activity in our *in vitro* system. Trx is secreted by cells into the extracellular environment, which is predominantly oxidizing, and might be expected to undergo monomeric oxidation. Considering its ease of formation, it is reasonable to assume that monomeric oxidation will precede oxidative homodimer formation. Whether this might be sufficient to prevent Trx from acting as a growth factor is not known. The formation of the oxidized monomer inside the cell is less likely since it still can be slowly reduced by thioredoxin reductase and the interior of the cell is highly reducing.

The physiological significance of homodimer formation is also unknown. What might be Trx homodimer has been reported in diamide-treated Jurkat cells [35]. We have observed small amounts of the Trx homodimer by immunoblotting of untreated MCF-7 breast cancer and other cell lysates (Powis *et al.*, unpublished observations). It is intriguing to speculate that formation of an oxidized Trx monomer or homodimer in response to intracellular oxidants such as H_2O_2 might be a way mammalian cells detect oxidant formation. Trx is believed to exist in normal cells at concentrations from 1 to 10 μM [2, 12], though in selected tissues and specific cell compartments this value could be much higher. It is therefore not unreasonable to assume that Trx will undergo homodimer formation *in vivo*. As we observed with the enhanced inactivation of Trx in phosphate buffered saline, we expect dimer formation to precede faster *in vivo* than we observe *in vitro* in water. Whether dimer formation *in vivo* would prevent the faster oxidation to an intramolecular form is unknown. The slow autocatalytic reduction of the Trx homodimer to the monomer would be a way to restore the cell to normal operating conditions after the induction of oxidative stress.

In summary, we have found that human recombinant Trx undergoes relatively rapid (over a few days) spontaneous and oxidant-induced conversion to a form(s) that does not stimulate cell proliferation, but is still a substrate for reduction by thioredoxin reductase. There is much slower (over a period of weeks) spontaneous oxidation of Trx to a Cys^{73}-stabilized homodimer form that is not a substrate for thioredoxin reductase and that also does not stimulate cell proliferation. Both conversions can be reversed by treatment with the thiol reducing agent DTT, and both appear to involve the Cys^{73} residue. A $Cys^{73} \rightarrow$ Ser mutant Trx, which stimulates cell proliferation and is as effective a substrate for thioredoxin reductase as Trx, did not show age or oxidation-induced loss of these activities. Thus, with time Trx gradually loses its ability to stimulate cell proliferation and to be a substrate for thioredoxin reductase, unlike the $Cys^{73} \rightarrow$ Ser mutant Trx, which retains these activities with no loss. Thus, Cys^{73} is not critical for biological ac-

tivity but may play a critical role in the oxidative regulation of Trx activity.

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