Mutagenesis of Structural Half-Cystine Residues in Human Thioredoxin and Effects on the Regulation of Activity by Selenodiglutathione†

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ABSTRACT: A human thioredoxin cDNA was modified to optimize *Escherichia coli* expression and subcloned into the plasmid pACA, a vector for T7 RNA polymerase-directed expression. The substitution of structural (noncatalytic) half-cystines in human thioredoxin (hTrx) was made by site-directed mutagenesis. The recombinant wild-type (wt) hTrx and its mutants C61S, C72S, and C61S/C72S were expressed and purified to homogeneity. Characterization of the wt and mutant hTrx was done with respect to redox activity with thioredoxin reductase (TR), tryptophan fluorescence, and effects of incubation with GS–Se–SG, which is believed to be the major metabolite of inorganic selenium compounds in mammalian tissues. The K_m and k_{cat} of wild-type hTrx for human placenta thioredoxin reductase (HP-TR) at pH 7.0 were 2.0 μM and 2800 min^{-1}, respectively. The mutant proteins C61S, C72S, and C61S/C72S had K_m and k_{cat} values similar to those of the wt thioredoxin. Tryptophan fluorescence measurements showed that the wt and mutant proteins had similar stability to a denaturing agent. Incubation of fully reduced thioredoxin with 0.1 molar equivalent of GS–Se–SG resulted in continued oxidation of SH groups. After 3.5 h only 0.5 of initially 4.6 SH groups/thioredoxin remained. With the oxidized protein, a pronounced lag phase in thioredoxin reductase-dependent insulin disulfide reduction was present. Disulfide-linked dimers of the protein were present. The results clearly showed that noncatalytic cysteine residues in hTrx were oxidized accompanied by dimerization and inactivation. The activities of the mutant proteins C72S and C61S/C72S were unchanged after 3 h of incubation with GS–Se–SG. No dimer appeared of the C72S thioredoxin. Both wt and C61S hTrx were inhibitors of calf thymus thioredoxin reductase-dependent reduction of 5,5′-dithiobis(2-nitrobenzoic acid). Substitution of Cys 72 with Ser prevented this inhibition. The results show that Cys 72 is critical for the specific characteristics of hTrx related to potential regulation of activity via formation of intra- or intermolecular disulfides.

Thioredoxin is a small (M_r 12 000) multifunctional protein with the conserved active site structure -Trp-Cys-Gly-Pro-Cys-. The oxidized form (Trx–S_2)[1] contains a disulfide that can be reduced to a dithiol by NADPH and thioredoxin reductase. The reduced form (Trx–$(SH)_2$) is an effective protein disulfide reductase. Thus, together, thioredoxin and thioredoxin reductase form a powerful NADPH-dependent protein disulfide reductase system (Holmgren, 1985, 1989; Holmgren et al., 1986).

$$\text{Trx–S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{\text{TR}} \text{Trx–(SH)}_2 + \text{NADP}^+ \quad (1)$$

$$\text{Trx–(SH)}_2 + \text{protein–S}_2 \rightarrow \text{Trx–S}_2 + \text{protein–(SH)}_2 \quad (2)$$

$$\text{Net: NADPH} + \text{H}^+ + \text{protein–S}_2 \xrightarrow{\text{TR+Trx}} \text{NADP}^+ + \text{protein–(SH)}_2 \quad (3)$$

Thioredoxin has been isolated and sequenced from a variety of prokaryotic and eukaryotic species (Holmgren, 1985; Gleason & Holmgren, 1988; Eklund et al., 1991). *Escherichia coli* thioredoxin is the one that has been studied most extensively. Mammalian forms of thioredoxin show about 25% sequence identity when compared to the *E. coli* protein, but they also contain additional nonrelated regions in their primary structure. Thus, human thioredoxin with 104 residues contains three cysteine residues in addition to the two located in the active site. These structural or noncatalytic cysteine residues, Cys 61, 68, and 72, in the human thioredoxin, can undergo oxidation, a process which has been suggested to lead to inactivation and aggregation by unknown mechanisms (Holmgren, 1985). The solution structure of the reduced form of recombinant human thioredoxin has been determined by NMR spectroscopy (Forman-Kay et al., 1991). The overall three-dimensional structure, consisting of a five-stranded β-sheet surrounded by four α-helices with an active site protrusion, is similar to the crystal and NMR structures of oxidized and reduced *E. coli* Trx (Eklund et al., 1991).

Thioredoxin is involved in different fundamental biological phenomena; e.g., it acts as a hydrogen donor for ribonucleotide reductase, a key enzyme in the synthesis of precursors for DNA replication (Thelander & Reichard, 1979), or in regulating the activity of photosynthetic enzymes (Cseke & Buchanan, 1986). ADF (adult T-cell leukemia-derived factor), produced by both human T-lymphotropic virus type

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[1] Abbreviations: hTrx, recombinant human thioredoxin; Trx–S_2, oxidized thioredoxin; Trx–$(SH)_2$ reduced thioredoxin; TR, thioredoxin reductase; CT, calf thymus; HP, human placenta; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IPTG, isopropyl thiogalactoside; C61S, cysteine 61 replaced with serine; C72S, cysteine 72 replaced with serine; wt, wild type, GS–Se–SG; selenodiglutathione, IAA; iodoacetic acid.

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I- and Epstein-Barr virus-transformed lymphocytes, is a human thioredoxin (Tagaya et al., 1989). It has recently been suggested that human thioredoxin/ADF is involved in the induction of interleukin 2 receptor expression, acts as an autocrine growth factor, and synergizes with interleukin 1 and interleukin 2 (Rimsky et al., 1986; Tagaya et al., 1989; Wakasugi et al., 1990). ADF and human placenta thioredoxin are identical.[2] In general, expression of human thioredoxin is markedly elevated in cells undergoing cell division such as proliferating B-lymphocytes (Ericson et al., 1992).

Selenium is an essential trace element with a number of biological activities. Organic as well as inorganic selenium compounds have well-documented inhibitory effects on mammalian cell growth (Shamberger, 1985; Medina & Oborn, 1984; Milner, 1984). Inorganic selenium compounds are believed to undergo stepwise reduction by glutathione and to give rise to the central metabolite GS–Se–SG (Hsieh & Ganther, 1975). This compound is one of the most potent oxidants of reduced *E. coli* thioredoxin known and also a substrate for calf thymus thioredoxin reductase (Björnstedt et al., 1992). Independent of the presence of glutathione or glutathione reductase, calf thymus thioredoxin reductase and GS–Se–SG react, resulting in a continued nonstoichiometric oxidation of NADPH in the presence of oxygen (Björnstedt et al., 1992).

Oxidation of the structural cysteines in human thioredoxin by GS–Se–SG and the resulting inhibition of the activity is a potential mechanism for selenium toxicity and a possible physiological regulation of the protein disulfide reduction activity. In order to test this hypothesis, we have studied the protein disulfide reductase activity of fully reduced wild-type human thioredoxin and the activity after oxidation with a small amount of GS–Se–SG. To study the role and influence of the structural cysteine residues, we have used the mutant proteins C61S and C72S and the double mutant C61S/C72S.

MATERIALS AND METHODS

DTT, DTNB, and NADPH were from Sigma. Human insulin was from Nordisk Insulin A/S, Gentofte, Denmark. (α-^{35}S)dATP and restriction and DNA-modifying enzymes were from Boehringer Mannheim and Amersham Corp. The DNA sequencing kit was from United States Biochemical Corp. Oligonucleotides were synthesized by Scandinavian Gene Synthesis AB. The human placenta thioredoxin reductase was purified by the technique described for the rat liver enzyme (Luthman & Holmgren, 1982). Selenodiglutathione was synthesized and purified as described previously (Björnstedt et al., 1992).

Construction of hTrx Expression Vectors pUG11/Trx and pACA/Trx. A human thioredoxin cDNA (Tagaya et al., 1989) subcloned into pGEM4 (the generous gift of Dr. Kenichi Arai, DNAX, Palo Alto, CA) was digested with *Pst*I to release from the insert a 134-bp 5′-fragment containing 80 bp of untranslated sequence and 54 bp encoding the first 18 amino-terminal amino acid residues of hTrx. The above *Pst*I fragment was replaced by a synthetic, double-stranded 70-mer, designed with *Pst*I ends, an internal *Eco*RI site, and a prokaryotic ribosome-binding site derived from the rIIB gene of phage T4 (Reznikoff & Gold, 1986). The remaining downstream nucleotides encode the 18 amino-terminal amino acid residues and were optimized for *E. coli* codon usage (Reznikoff & Gold, 1986). From a selected subclone with a correctly oriented new *Pst*I insert, a 523-bp fragment was first isolated by *Eco*RI digestion and subsequently subcloned into the *Eco*RI site of the expression vector pUG11. This plasmid is composed of the *Bam*HI/*Eco*RI fragment of pGW7 (Schmidt & Chamberlin, 1984) ligated to the corresponding sites of pUG18, and it contains the $\lambda_{PL}/\lambda_{PR}$ promoters under the control of a plasmid-encoded temperature-sensitive λ_{cI857} repressor. The pUG11/Trx plasmid was transformed into *E. coli* strain JF521 [...(*lac–proAB*), *thi*, *supE*, *metE46*, *srl300::Tn10trxA2*(7004), *recA*, (F′*traD36*, *proAB*$^+$, *lacI*$_q$-*Z*δM15)] (Langsetmo et al., 1989), which offers an environment free from endogenous *E. coli* thioredoxin due to a deletion in the *trxA* gene.

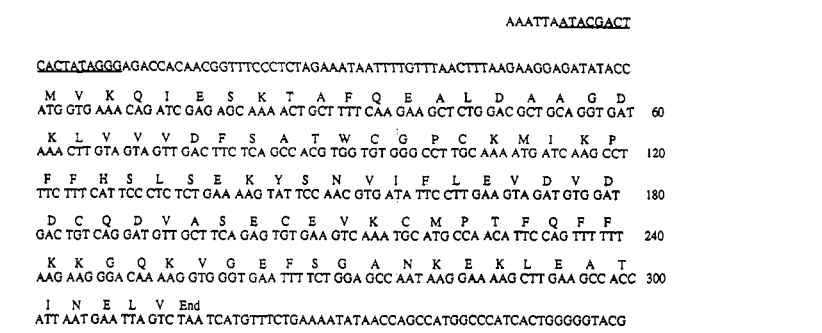

FIGURE 1: Nucleotide sequence of the promoter and coding region of the pACA/Trx expression vector. The amino acid sequence of thioredoxin is denoted by the one-letter code. The T7 RNA polymerase binding sequence is underlined. The N-terminal amino acid residue of thioredoxin is V, and the protein sequence numbering is based on this.

For achieving high efficiency of site-directed mutagenesis, we reconstructed hTrx cDNA into plasmid pACA, a selective expression vector for T7 RNA polymerase (the generous gift of Dr. Bengt-Harald Jonsson, Umeå University, Sweden) (Stanssens et al., 1989). Two designed oligonucleotides with the sequences of 5′ ATT CTA AGG AAA ACC ATG GTG AAA CAG 3′ and 5′ TTT AAA TAG CTC CAT GGC TGG TTA TA 3′ were used in PCR to amplify the hTrx cDNA fragment and generate two *Nco*I restriction sites at the start codon and downstream of the stop codon. The plasmid pACA was digested with *Nco*I and dephosphorylated with alkaline phosphatase. The target cDNA was inserted into pACA exactly after the start codon. The reconstructed pACA/Trx plasmid (Figure 1) was sequenced and transformed into *E. coli* strain BL21/DE3 [*hsdS*, *gal* (λ*cIts857*, *ind1*, *Sam7*, *nin5*, *lacUV5*-T7 gene 1)] (Rosenberg et al., 1987).

Mutagenesis Method. The three mutants of hTrx, C61S, C72S, and C61S/C72S, were made by *in vitro* oligonucleotide-directed mutagenesis. We employed the Muta-Gene system (Bio-Rad), which is based on the method of Kunkle (1985) where the mutant strand is synthesized using a template strand containing T to U substitutions. Mutations were verified by dideoxy sequencing (Sanger et al., 1977).

Protein Preparation. For expression, 10-L cultures of the *E. coli* strain BL21/DE3 harboring pACA/Trx were grown at 37 °C in Luria Broth medium supplemented with 1% glucose and 50 μg/mL ampicillin. At an OD_{550} = 0.5, IPTG was added to a final concentration of 0.5 mM to induce expression. After this induction, incubation was continued for another 6 h. Cells were harvested by centrifugation and stored frozen at −70 °C. The wt hTrx and all mutant proteins were purified by DEAE 52 and Sephadex G-50 chromatography (Luthman & Holmgren, 1982). In short, frozen cells were taken up in 5 times their wet weight of 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride and

[2] P. Lundman, K. Bhavani, J. Yodoi, and A. Holmgren, submitted for publication.

disrupted by a French pressure cell. Crude extracts were prepared by removing cell debris with high-speed centrifugation. Nucleic acids were precipitated by slow addition of 1/10 volume of freshly prepared 7% streptomycin sulfate solution. Protein was precipitated by adding ammonium sulfate to 85% saturation. After dialysis the crude extracts were preincubated with 2 mM DTT at room temperature for 30 min, and then applied to a DEAE 52 column (4.5 × 18 cm) equilibrated with 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 0.1 mM DTT. Elution of thioredoxin was achieved by applying a NaCl gradient from 0 to 0.3 M. Fractions exhibiting thioredoxin activity were pooled and concentrated by ultrafiltration with Diaflo cells and YM-3 membranes. The concentrated sample was preincubated with 2 mM DTT at room temperature for 30 min and applied to a Sephadex G-50 column (6.2 × 150 cm) equilibrated with 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 0.1 mM DTT. Fractions containing thioredoxin were pooled and concentrated *E. coli* Trx was removed by *E. coli* Trx antibody affinity chromatography. For the C61S and C72S proteins fast protein liquid chromatography on a MonoQ column, applying a 0–0.3 M NaCl gradient, was included as an additional purification step. The protein concentration was determined spectrophotometrically at 280 nm and calculated with $\epsilon_{280} = 8.05\ mM^{-1}\ cm^{-1}$.

Determination of K_m, k_{cat}, and k_{cat}/K_m. The assay mixture contained 100 mM potassium phosphate, pH 7.0, 0.2 mM EDTA, 0.16 mM insulin, 0.15 mM NADPH, and 0.5–10 μM wt or mutant Trx–$(SH)_2$. The reaction was started by addition of 5 nM CT-TR or HP-TR at 25 °C, andthe reaction rate was followed from the oxidation of NADPH at 340 nm. Eadie–Hofstee plots (Hofstee, 1952, 1959) were used for the calculation of kinetic constants.

Fluorescence Measurement. Protein fluorescence was measured with a thermostated Shimadzu RF-510 LC spectrofluorimeter at 15 °C. Excitation of fluorescence was at 280 nm, and emission spectra from 300 to 450 nm were recorded. The samples contained 1 μM oxidized or reduced Trx in 100 mM potassium phosphate buffer, pH 7.0. Guanidine hydrochloride was used as the denaturing agent.

Interaction of CT-TR and DTNB with Wt and Mutant Proteins of hTrx. The reaction was started after adding 5 μL of 360 nM CT-TR solution into 500 μL of the reaction mixture, which consisted of 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 0.24 mM NADPH, and 100 μM DTNB. After about 1.5 min, hTrx or mutant proteins were added to a final concentration of 4 μM. The reaction was monitored at 412 nm.

Enzyme Assay. The activity of the thioredoxin system was determined as the ability to reduce insulin disulfides according to the method described by Holmgren (1984). The reaction mixture consisted of 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 0.4 mM NADPH, and 0.16 mM insulin. The reactions were started by addition of human placenta thioredoxin reductase to 12 nM final concentration. The reduction of insulin disulfides was followed by 340 nm.

Reduction and Oxidation of Thioredoxin. The wild-type thioredoxin and the mutant proteins (30-75 μM in different experiments) were incubated at 37 °C for 15 min with a 20-fold molar excess of DTT which was removed by gel chromatography on a NAP-5 column from Pharmacia with ice-cold nitrogen equilibrated Tris–HCl buffer containing 1 mM EDTA. The reduced proteins were incubated with 0.1 molar equivalent of GS–Se–SG in air.

Table I: Recombinant Human Thioredoxin Mutant Construction

hTrx	mutagenesis primer (5′ → 3′)	codon change	change in protein
C61S	GTGGATGACTCTCAGGATGTT	TGT → TCT	Cys 61 → Ser
C72S	GAAGTCAAATCCATGCCAACA	TGC → TCC	Cys 72 → Ser
C61S/	GTGGATGACTCTCAGGATGTT/	TGT → TCT	Cys 61 → Ser
C72S	GAAGTCAAATCCATGCCAACA	TGC → TCC	Cys 72 → Ser

Determination of SH Groups. Solutions of human thioedoxin, 25 or 50 μL, were mixed with 0.50 mL of 6 M guanidine–HCl in 0.2 M Tris–HCl, pH 8.0, containing 1 mM DTNB. The absorbance at 412 nm was measured against a blank in order to calculate free SH groups using $\epsilon_{412} = 13.6\ mM^{-1}\ cm^{-1}$.

Separation of Different Molecular Forms of Human Thioredoxin after Oxidation. A mixture of human thioedoxin and GS–Se–SG was injected on an FPLC Superdex column. Immediately prior to injection, 20 μL of 50 mM IAA was added to the mixture (100 μL of 75 μM human thioredoxin) to block any remaining SH groups and thereby exclude the possibility of further dimerization during the elution. The column was eluted with 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 500 mM NaCl. Fractions of 1.0 mL were collected, and the flow rate was 1.0 mL min^{-1}.

RESULTS

Construction of an Expression Vector for hTrx. A modified cDNA fragment containing a complete hTrx gene was subcloned into the *Eco*RI site of the expression vector pUG11. The presence of recombinant human thioredoxin in lysates of *E. coli* JF521 harboring pUG11/Trx was confirmed by an insulin reduction assay and Western blot (data not shown). The expressed hTrx was free from endogenous *E. coli* Trx due to a deletion in the *trxA* gene of the host strain (data not shown). In order to achieve both high expression of Trx and high-efficiency site-directed mutagenesis, we reconstructed and subcloned the hTrx cDNA fragment into the plasmid pACA. The hTrx cDNA in the plasmid pACA was sequenced, and the deduced amino acid sequence was as expected identical to that published by Tagaya et al. (1989), as shown in Figure 1. The sequence of the first 22 N-terminal residues of the purified recombinant protein was further confirmed by amino acid sequencing. About $^2/_3$ of the hTrx molecules contained an amino-terminal valine residue, while approximately $^1/_3$ had an unprocessed *N*-formyl methionine.

Construction of Cys 61 and Cys 72 Mutants. For replacing the Cys 61 and Cys 72 codons in the cloned hTrx gene, 21-mer oligonucleotides (Table I) corresponding to the sense strand except for one central base mismatch were hybridized to the single-stranded uracil-containing templates. The mutants were verified by dideoxy sequencing of the expression plasmid. High efficiency of site-directed mutagenesis was achieved.

Expression of Mutant hTrx. Host strain *E. coli* BL21/DE3 contains a single copy of the gene for RNA polymerase of bacteriophage T7 in the chromosome under control of the inducible lac promoter. The pACA/Trx bears the T7 promoter (Figure 1) upstream of the hTrx gene. The expression of Trx was thus induced by IPTG. The mutant thioredoxins were obtained in good yield and were purified to homogeneity. The mutant thioredoxins behaved similarly to the wild-type protein during purification. The *E. coli* Trx from the host strain was removed efficiently by *E. coli* Trx antibody affinity chromatography (Nordström et al., 1981). In the wild-type or mutant protein solutions the activity of the *E. coli* Trx contaminant was not detectable using a sensitive insulin

Table II: Kinetic Parameters of Human Thioredoxin and Mutants for Calf Thymus or Human Placenta Thioredoxin Reductase[a]

hTrx	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
	CT-TR		
wt	3.0	3500	1160
C61S	2.3	3800	1650
C72S	4.1	4200	1020
C61S/C72S	2.5	3300	1320
	HP-TR		
wt	2.0	2800	1400
C61S	1.7	2800	1600
C72S	2.2	2900	1350
C61S/C72S	2.5	3200	1280

[a] The assay mixture contained 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 0.16 mM insulin, and 0.15 mM NADPH. Wt or mutant hTrx-$(SH)_2$ was added in a final concentration range from 0.5 to 10 μM. The reaction was initiated by adding CT-TR or HP-TR at a final concentration of 5 nM. The temperature was 25 °C.

reduction assay with *E. coli* TR, which is specific for the bacterial Trx (Holmgren, 1985).

Kinetic Parameters of Wt and Mutant hTrx for CT-TR and HP-TR. The redox reactions of reduced wt and mutant hTrx catalyzed by CT-TR or HP-TR were studied at pH 7.0 and 25 °C. The K_m, k_{cat}, and k_{cat}/K_m of wt hTrx for HP-TR were 2.0 μM, 2800 min^{-1}, and 1400 μM^{-1} min^{-1}, respectively. All C61S, C72S, and C61S/C72S hTrx had similar K_m and k_{cat} values, compared to wt hTrx (Table II). The results also showed that the Michaelis–Menten constants of hTrx for HP-TR and CT-TR exhibited no significant differences.

Stability to Denaturing Reagent. The fluorescence emission spectra from 300 to 450 nm were recorded for wt and mutant hTrx in both oxidized and reduced forms denatured by guanidine hydrochloride at pH 7.5. The results showed that C61S, C72S, and C61S/C72S hTrx had similar stabilities to that of the wt (Figure 2). The oxidized form of the proteins was more stable to denaturation than the reduced form. This difference in stability has previously been shown with the *E. coli* thioredoxin (Kelley et al., 1987).

Interaction of CT-TR and DTNB with Wt and Mutant Proteins of hTrx. It was known from previous work that rat liver or calf liver Trx was an inhibitor to CT-TR in a system with NADPH and a low concentration of DTNB, whereas *E. coli* Trx showed no detectable inhibition in that system (Luthman & Holmgren, 1982). The interactions of CT-TR in this DTNB system with wt and mutant hTrx proteins were investigated. The results (see Figure 3) showed that wt and C61S hTrx were inhibitors to this CT-TR, DTNB system, whereas C72S and C61S/C72S hTrx were not. The results strongly suggested that the SH group of the Cys 72 residue was somehow involved in the inhibition to CT-TR with DTNB, since substitution of Cys 72 with Ser made hTrx lose this property.

Oxidation of Reduced Thioredoxin. After incubation of fully reduced thioredoxin (30-75 μM) with 0.1 molar equivalent of GS–Se–SG there was a continued and fast oxidation of SH groups. After 30 min there was 1.9 SH groups per molecule, and after 24 h all SH groups were oxidized (Figure 4). This shows that GS–Se–SG and its cleavage products are efficient oxidants of both the structural and the active site cysteine residues of human thioredoxin. As a control fully reduced thioredoxin was kept in an open test tube and thus exposed to air for 24 h. During this time hardly any oxidation was observed (Figure 4).

NADPH Oxidation by GS–Se–SG and the Thioredoxin System. Incubations of human thioredoxin reductase with

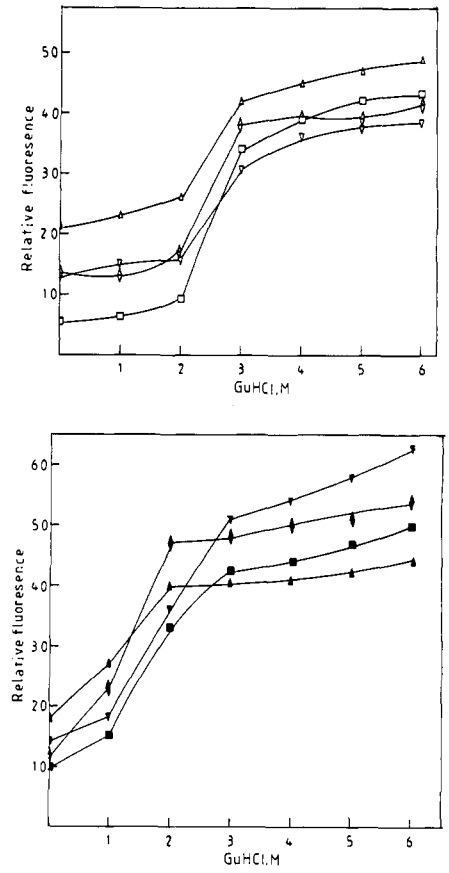

FIGURE 2: Denaturation of wt, C61S, C72S, and C61S/C72S hTrx by guanidine hydrochloride followed fluorimetrically. The oxidized form (A, top) and the reduced form (B, bottom) of wt (□, ■), C61S (△, ▲), C72S (▽, ▼), and C61S/C72S (◇, ◆) hTrx were exposed to guanidine hydrochloride, and the relative fluorescence emission values at 350 nm were measured after excitation at 280 nm.

GS–Se–SG resulted in a fast and nonstoichiometric oxidation of NADPH (Figure 5). This result has previously been shown with the calf thumus enzyme (Björnstedt et al., 1992). Addition of wt human thioredoxin resulted in an increased reaction rate (Figure 5). Addition of the double mutant protein (C61S/C72S) resulted in an even higher reaction rate (Figure 5).

Inhibition of the Activity of Thioredoxin by GS–Se–SG. The capacity to reduce insulin disulfides of fully reduced wt thioredoxin and mutant proteins C61S, C72S, and C61S/C72S is shown in Figure 6. After 3 h of incubation with 0.1 molar equivalent of GS–Se–SG the redox status of the proteins were 0.6 SH group per molecule of wt and C61S/C72S thioredoxin, 0.2 SH group per molecule of C61S thioredoxin, and 1.0 SH group per molecule of C72S thioredoxin. The insulin disulfide reducing capacity of wild-type thioredoxin was decreased and the reaction showed a marked lag phase (Figure 6B). The double mutant protein (C61S/C72S), despite the same degree of reduction (0.6 SH group per molecule) as the oxidized wt thioredoxin, showed the same reactivity as the fully reduced protein (Figure 6A,B). The oxidized C61S protein showed a slower reaction whereas the oxidized C72S thioredoxin had essentially the same reactivity, as the fully reduced proteins (Figure 6A,B). After 24 h of oxidation by GS–Se–SG (0 SH groups per molecule) the insulin disulfide reduction of wt thioredoxin showed a pronounced lag phase, and the change in A340 was only 0.03 after 5 min (Figure 6C).

Gel Filtration of Oxidized Wt Thioredoxin. After incubation of fully reduced wt thioredoxin with 0.1 molar equivalent

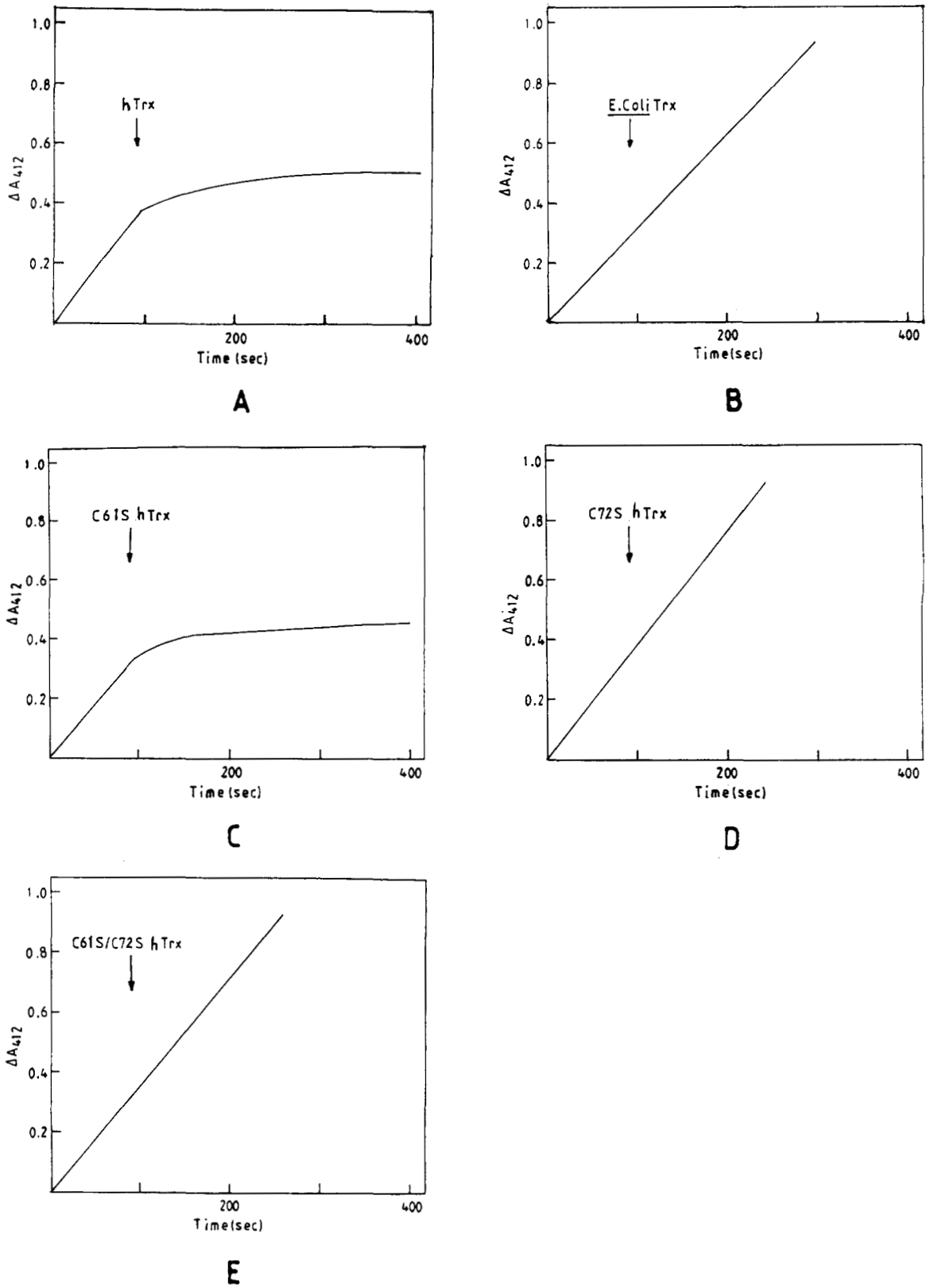

FIGURE 3: Interaction of CT-TR and DTNB with thioredoxins. The reaction mixture contained 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 0.24 mM NADPH, and 100 μM DTNB. The reaction was started by adding CT-TR to a final concentration of 3.6 nM. After 1.5 min, the following enzymes were added: (A) wt hTrx; (B) *E. coli* Trx; (C) C61S hTrx; (D) C72S hTrx; (E) C61S/C72S hTrx. The final concentration of the thioredoxins was 4 μM.

of GS–Se–SG most of the protein was in the form of a dimer whereas only a small peak was obtained in the 12-kDa area (corresponding to monomer thioredoxin) (Figure 7). After incubation of the reaction mixture with a 20-fold molar excess of DTT only one single peak appeared, in the 12-kDa area (Figure 7), and all higher molecular weight thioredoxin had been converted to the monomeric form. This suggests that the two molecules in the dimer were coupled by a disulfide bridge.

Analysis of the Properties of Thioredoxin by Gel Electrophoresis. After 3 h of incubation of the proteins (30-75 μM) with GS–Se–SG, dimers were formed from wt. C61S,

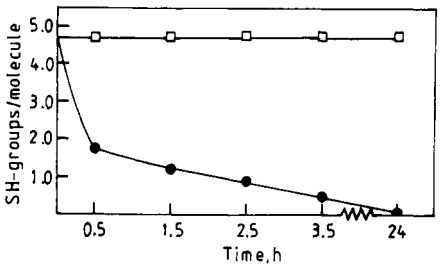

FIGURE 4: Oxidation of reduced wt human thioredoxin. Thioredoxin (30–75 μM) was reduced by a 20-fold molar excess of DTT, which was removed on a Sephadex G25 column (NAP-5). The number of SH groups per molecule was measured at various times after incubation in air (□) and after addition of 0.1 molar equivalent of GS–Se–SG (●).

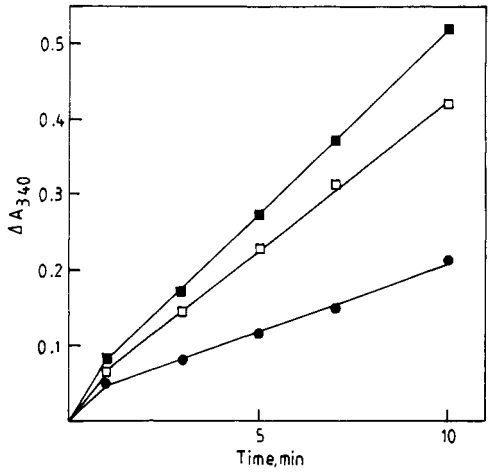

FIGURE 5: GS–Se–SG-dependent oxidation of NADPH catalyzed by the human thioredoxin system. The reaction was performed in 50 mM Tris–HCl, pH 7.5, and 1 mM EDTA containing 200 μM NADPH with 30 nM human thioredoxin reductase and 10 μM GS–Se–SG (●), plus 2 μM wt thioredoxin (□), or 2 μM C61S/C72S thioredoxin (■).

and C61S/C72S thioredoxin but not from C72S thioredoxin as observed from nonreducing gel electrophoresis (Figure 8, part II). After 24 h (0 SH groups per molecule in all four proteins) most of the wt, C61S, and C61S/C72S thioredoxins were in the form of a dimer, but despite complete oxidation no dimer was observed in the C72S thioredoxin preparation (Figure 8, part III).

DISCUSSION

Human thioredoxin as well as other mammalian thioredoxins contain additional or structural cysteine residues apart from the two in the conserved active site (Eklund et al., 1991). These structural cysteine residues have been implicated in causing inactivation and aggregation of mammalian thioredoxins upon air oxidation (Holmgren, 1985). In this study we have investigated the redox properties of human thioredoxin by site-directed mutagenesis of cysteine residues. The result clearly shows that the enzymatic activity of thioredoxin per se does not depend on the presence of the structural SH groups.

We have found that GS–Se–SG was an efficient oxidant of reduced human thioredoxin. Incubations of selenite or GS–Se–SG with calf thymus thioredoxin reductase results in continued oxygen-dependent nonstoichiometric oxidation of NADPH (Kumar et al., 1992; Björnstedt et al., 1992). The GS–Se–SG reaction has been proposed to start with cleavage of the molecule (Björnstedt et al., 1992). A proposed mechanism of the nonstoichiometric part is continuous reoxidation of HSe^- by oxygen (Kumar et al., 1992; Björnstedt et al., 1992). A small amount of GS–Se–SG added to human thioredoxin resulted in complete oxidation of SH groups. The mechanistic explanation is likely to be continued reoxidation of HSe^-, the suggested product of GS–Se–SG cleavage. The selenium anion HSe^- will thus serve as the electron-transfer catalyst to the final acceptor oxygen. The spontaneous oxidation of reduced thioredoxin by oxygen in air was only marginal after 24 h in the presence of 1 mM EDTA.

The protein disulfide reductase activity of wt human thioredoxin was markedly decreased after oxidation by GS–Se–SG. The reaction showed a pronounced lag phase consistent with reactivation of thioredoxin through reduction of structural cystine disulfide bridges. The mechanism of this reactivation is probably initiated by reduction of the active site of one molecule of thioredoxin. This molecule will then reduce a structural disulfide bridge of another molecule, leading to a reactivation cascade. However, a direct reduction of structural disulfides by thioredoxin reductase cannot be excluded. Although, there was the same amount of SH groups (0.6/molecule after 3 h) in the C61S/C72S protein compared to the wt, this protein showed the same reactivity as the fully reduced thioredoxin. This indicates that oxidation reactions involving cysteine 68 have a minor influence on enzymatic activity. After 3 h of incubation of C61S thioredoxin with GS–Se–SG, the reactivity was markedly decreased compared to the fully reduced protein. However, the insulin disulfide reducing capacity of oxidized C72S thioredoxin was unchanged, indicating the critical role of this residue.

We observed dimerization of thioredoxin upon exposure to GS–Se–SG. This dimer state was reversed by DTT, suggesting that the molecules were connected by S–S bridges. Again, the lack of dimerization in the C72S protein showed that this residue is crucial in inactivation/dimerization even though after complete oxidation by GS–Se–SG, there was probably an intramolecular disulfide bridge between C61 and C68 in this protein. The reduced form of C61S/C72S thioredoxin formed dimers after oxidation by GS–Se–SG. This was unexpected since the activity was essentially unchanged compared to the fully reduced protein. One explanation for this phenomenon could be that the structure of thioredoxin is not influenced by oxidation of the SH groups of Cys 68 and the formation of a disulfide bond involving Cys 68 in two molecules. Support for this result comes from the known three-dimensional structure of human thioredoxin. The Cys 68 residue is located at the end of the 3_{10} helix and far from the active site (Forman-Key et al., 1991; Eklund et al., 1991). Wollman et al. (1988) have suggested that an equilibrium between monomeric reduced human thioredoxin and dimeric oxidized human thioredoxin may exist physiologically. Exposure to selenium might alter this equilibrium toward the dimeric state. Ganther et al. (1969) described inactivation of reduced ribonuclease A by molar equivalents of selenite through cross-linking of the enzyme molecules by –S–Se–S– bridges. These are not likely to occur in our system since we used a low concentration of GS–Se–SG (1 GS–Se–SG/50 SH groups).

Calf thymus thioredoxin lacks Cys 68[3] and forms no dimers upon oxidation (as analyzed by gel electrophoresis). However, activity with thioredoxin reductase was very low initially, but gradual activation was seen during prolonged assays. In this study we did not examine the effect of a mutation of Cys 68 since calf thioredoxin is a natural mutant.

[3] C. Palmberg, H. Jörnvall, L. Hernberg, and A. Holmgren, submitted for publication.

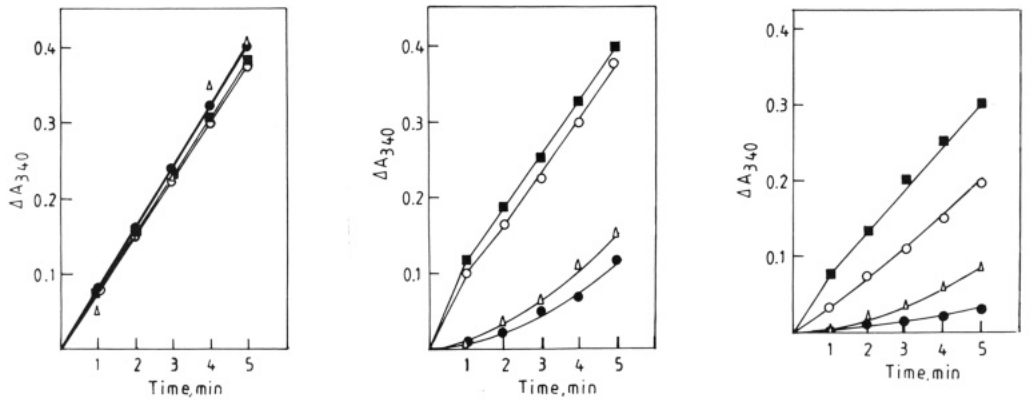

FIGURE 6: Insulin disulfide reduction by wt, C61S, C72S, and C61S/C72S human thioredoxin. A reaction mixture composed of 100 mM potassium phosphate, pH 7.0, 0.2 mM EDTA, 0.4 mM NADPH, and 0.16 mM insulin was used. To the cuvettes were added (●) wt, (△) C61S, (○) C72S, and (■) C61S/C72S human thioredoxin in a final concentration of 2 μM. The reactions were started by addition of human thioredoxin reductase to a final concentration of 12 nM. (A, left) shows the reaction with fully reduced wt and mutant thioredoxins. (B, middle) Wt and mutant thioredoxin were oxidized for 3 h with 0.1 molar equivalent of GS–Se–SG. (C, right) Wt and mutant proteins were oxidized for 24 h with GS–Se–SG.

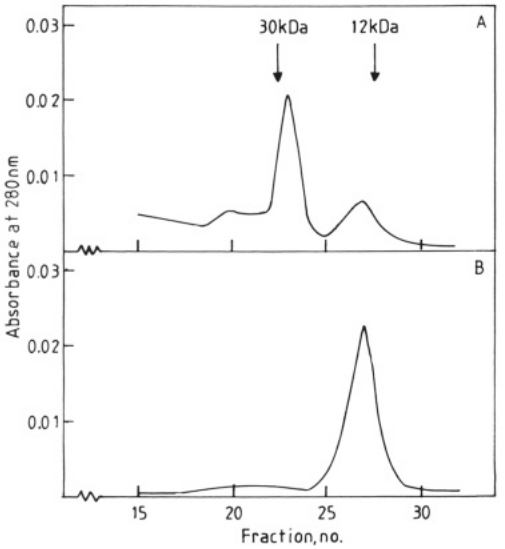

FIGURE 7: Gel filtration of dimer/monomer wt human thioredoxin. Reduced thioredoxin was incubated with 0.1 molar equivalent of GS–Se–SG for 24 h. The reaction mixture containing 60 μM thioredoxin and 6.0 μM GS–Se–SG in a final volume of 120 μL was injected on an FPLC Superdex column which was eluted with 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 500 mM NaCl. Fractions of 1.0 mL were collected, and the flow rate was 1.0 mL/min. (A) is for thioredoxin incubated with GS–Se–SG for 24 h. (B) is for thioredoxin reduced by a 20-fold molar excess of DTT after incubation with GS–Se–SG for 24 h.

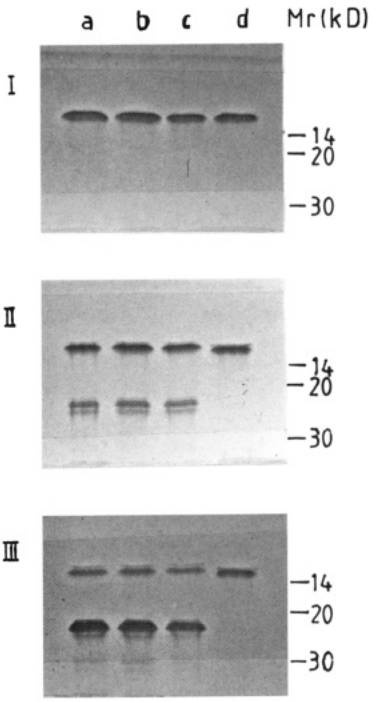

FIGURE 8: SDS–polyacrylamide gel electrophoresis of wt (a), C61S/C72S (b), C61S (c), and C72S (d) human thioredoxin: (I) reduced wt and mutant thioredoxin, (II, III) wt and mutant proteins (30–75 μM) oxidized with 0.1 molar equivalent of GS–Se–SG for 3 h (II) and for 24 h (III).

The results of oxidation of wt and mutant thioredoxins show that dimer formation and a lag phase of the reaction rate, in disulfide reduction of insulin with thioredoxin reductase, were both linked to the SH group of Cys 72. Furthermore, the inactivation of thioredoxin reductase upon addition of wt thioredoxin to a low concentration of DTNB was also dependent upon the SH group of Cys 72. From the published structure of reduced human thioredoxin, Cys 72 is located on the surface of the protein in a loop in proximity to the active site (Forman-Kay et al., 1991). Thus, formation of a disulfide involving the Cys 72 side chain either between or within thioredoxin molecules can be expected to severely disturb enzyme catalysis. A similar mechanism for the inactivation of thioredoxin reductase by DTNB and thioredoxin can be envisaged, where thioredoxin potentially becomes cross-linked to the reductase via the Cys 72 residue.

The functions of the thioredoxin system both as a hydrogen donor to ribonucleotide reductase and as a general protein disulfide reductase are effectively inhibited by selenium compounds (Kumar et al., 1992; Björnstedt et al., 1992). Two suggested mechanisms for these effects are NADPH depletion and competetive inhibition of thioredoxin reductase by inhibition of its reduction of thioredoxin. Oxidation of the structural cysteines by selenium compounds and the resulting inactivation is a third possible control mechanism of the human thioredoxin system. Thus, the combined effect by these inhibitory mechanisms on the human thioredoxin system could provide a possible explanation for the inhibitory effects of selenium compounds on mammalian cell growth and proliferation.

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