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Selenocysteine, identified as the penultimate C-terminal residue in human T-cell thioredoxin reductase, corresponds to TGA in the human placental gene

(selenium/thioredoxin reductase/TGA/selenocysteine)

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Contributed by Thressa C. Stadtman, February 27, 1996

ABSTRACT The possible relationship of selenium to immunological function which has been suggested for decades was investigated in studies on selenium metabolism in human T cells. One of the major $^{75}$Se-labeled selenoproteins detected was purified to homogeneity and shown to be a homodimer of 55-kDa subunits. Each subunit contained about 1 FAD and at least 0.74 Se. This protein proved to be thioredoxin reductase (TR) on the basis of its catalytic activities, cross-reactivity with anti-rat liver TR antibodies, and sequence identities of several tryptic peptides with the published deduced sequence of human placental TR. Physicochemical characteristics of T-cell TR were similar to those of a selenocysteine (Secys)-containing TR recently isolated from human lung adenocarcinoma cells. The sequence of a 12-residue $^{75}$Se-labeled tryptic peptide from T-cell TR was identical with a C-terminal-resolved sequence of human placental TR except that Secys was present in the position corresponding to TGA, previously thought to be the termination codon, and this was followed by Gly-499, the actual C-terminal amino acid. The presence of the unusual conserved Cys-Secys-Gly sequence at the C terminus of TR in addition to the redox active cysteines of the Cys-Val-Asn-Val-Gly-Cys motif in the FAD-binding region may account for the peroxidase activity and the relatively low substrate specificity of mammalian TRs. The finding that T-cell TR is a selenoenzyme that contains Se in a conserved C-terminal region provides another example of the role of selenium in a major antioxidant enzyme system (i.e., thioredoxin-thioredoxin reductase), in addition to the well-known glutathione peroxidase enzyme system.

Selenium is a trace element that is important for a number of physiological processes and for human health. Its essentiality is based on the fact that it is a specific component of Se-dependent enzymes and tRNAs. In known Se-dependent enzymes Se occurs as an active center amino acid residue, seleno-cysteine (Secys) (1, 2) or as a metal-coordinated labile cofactor (3, 4). In both cases Se is essential for the catalytic activities exhibited by the specific selenoenzymes. The mechanism of incorporation of Secys is established for prokaryotes (5), and many aspects of its incorporation in eukaryotic proteins are well understood (6).

The UGA codon directs incorporation of this amino acid residue both in prokaryotes and eukaryotes. In addition, conserved stem-loop structures immediately following UGA in prokaryotes (5) and in 3′ untranslated regions in eukaryotes (7) function as determinants of Secys incorporation instead of termination of translation.

Among eukaryotic proteins that contain Se as a Secys residue, the most studied are members of the glutathione peroxidase family (8). These enzymes that catalyze the glutathione- or thioredoxin-dependent reduction of hydroperoxides are important intra- and extracellular antioxidants in mammals and birds. A different metabolic role of selenium was established by the discovery of the selenium-dependent 5′ deiodinases (9). These enzymes function in the synthesis and maintenance of optimal levels of essential thyroid hormones required for growth and development. Selenoproteins of unknown function include selenoprotein P, the major plasma selenoprotein (10) that contains 10 Secys residues and selenoprotein W, a small Secys-containing protein present in muscle (11).

Recently a new selenoprotein purified from human lung adenocarcinoma cells was shown to be a homodimer of 57 kDa subunits containing FAD and Secys (12). This enzyme exhibited thioredoxin reductase (TR) activities similar to those of human placenta (13) and rat liver (14) TRs. Although the lung enzyme did not react with anti-rat liver TR antibodies, which suggests it might be a novel type of TR, it clearly is related to another form of the enzyme present in the lung cells that is immunoreactive (S.-Y. Liu and T.C.S., unpublished data).

In view of the fact that selenium has been indirectly implicated in immunological function in numerous nutritional studies and that HIV-infected individuals are reported to have decreased levels of plasma Se and selenium-containing glutathione peroxidase (15), we initiated studies on selenium metabolism in human T cells. Identification of one of the selenoproteins detected in mouse T cells as TR and demonstration that the location of Secys in this protein corresponds to a TGA codon in the cloned human placental gene (16) is reported in the present communication.

MATERIALS AND METHODS

Materials. The following materials were from the indicated sources: $^{75}$Se-selenious acid from the Research Reactor Facility (University of Missouri, Columbia); precast polyacrylamide gels and molecular weight standards from NOVEX (San Diego); isoelectrofocusing standards from Pharmacia; ECL immunoblotting detection system from Amersham; trypsin and 2',5'-ADP Sepharose from Sigma; C18 (218TP54) reversed-phase HPLC column from Vydatec; sulfopropyl (SP) HPLC column from TosoHaas (Montgomeryville, PA); and heat-inactivated fetal bovine serum and RPMI 1640 medium from Mediatech (Herndon, VA). Human thioredoxin, rat liver thioredoxin reductase, and rabbit polyclonal antibodies to rat liver thioredoxin reductase were kindly provided by Ho Zoon Chae (National Institutes of Health). Human lung adenocarcinoma.

Abbreviations: Secys, selenocysteine; TR, thioredoxin reductase; SP, sulfopropyl; DTNB, 5,5'-dithiobis(2-nitrobenzoate); MALDI, matrix-assisted laser desorption ionization.

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cinoma thioredoxin reductase labeled with $^{75}$Se was kindly provided by Song-Yuan Liu (National Institutes of Health). All other reagents were of the highest grade available.

**Cell Growth.** A human Jurkat T-cell line, JPX9 (29), was grown on RPMI 1640 medium in the presence of 10% fetal bovine serum and antibiotic-antimycotic solution in 5% CO$_2$. For preparation of $^{75}$Se-labeled cells, 12 $\mu$l of $^{75}$Se-selenious acid; 300 $\mu$Ci (1 Ci = 37 GBq), in 30% nitric acid was added to 40 ml of medium and cells were grown for 3 days under the same conditions. Cells were collected by centrifugation, washed twice with phosphate-buffered saline, and stored at $-80^\circ$C prior to use.

**TR Purification.** For purification of TR, unlabeled cells were mixed with $^{75}$Se-labeled cells. The cells were suspended in 2 volumes of cold 25 mM Tris-HCl (pH 7.8), 1.5 mM EDTA, and 2 mM DTT (buffer A) containing 10 $\mu$g of aprotinin per ml, 10 $\mu$g of leupeptin per ml, 2 mM MgCl$_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaF and were disrupted by sonication. The pellet, obtained after centrifugation of the disrupted cells, was washed with a minimal volume of buffer A and again collected by centrifugation. The supernatants were combined and applied to a DEAE-Sepharose anion-exchange column equilibrated with buffer A. The proteins were eluted with a linear 0–0.4 M gradient of sodium chloride in buffer A. The radioactivity of fractions containing $^{75}$Se was determined in a Beckman 5500 $\gamma$ counter and the peak $^{75}$Se-containing fractions were analyzed on SDS gels. Fractions containing a 55 kDa selenoprotein were combined, diluted four times with buffer A, and applied to a 2',5'-ADP Sepharose affinity column equilibrated with buffer A. The column was washed sequentially with buffer A, 0.2 M sodium chloride in buffer A, 0.3 M sodium chloride in buffer A, and 1 M sodium chloride in buffer A. The TR detected as a 75 kDa-containing protein was eluted with 1 M NaCl in buffer A. Ammonium sulfate (4 M, pH 8.0), was added to the enzyme solution to a final concentration of 0.5 M and the TR solution was applied to a phenyl-Sepharose column and eluted with a linear 0.5–0 M gradient of ammonium sulfate in 25 mM Tris-HCl (pH 7.8). The resulting $^{75}$Se-labeled TR that eluted at 0.2 M ammonium sulfate was concentrated to about 1 mg/ml and stored at $-20^\circ$C.

**Carboxymethylation and Tryptic Digestion of TR.** $^{75}$Se-labeled TR (100 $\mu$g, 3 $\times$ 10$^4$ cpm) in 160 $\mu$l of 100 mM Tris-HCl (pH 7.7) and 6 mM EDTA was denatured by mixing with 160 mg of dry guanidine–hydrochloride in a 1.5 ml microcentrifuge tube. The solution was stirred with argon for 20 min and after the addition of 15 $\mu$l of 100 mM DTT the reaction was incubated for 40 min at room temperature. For alklylation a 33 $\mu$l aliquot of 250 mM iodoacetate was added and the solution was incubated under argon at room temperature in the dark for 40 min. To quench the reaction, 15 $\mu$l of 1 M DTT was added. The solution was diluted to 650 $\mu$l with 4 M guanidine–hydrochloride and dialyzed with two changes against 2 liters of 20 mM Tris-HCl (pH 8.0). The resulting carboxymethylated TR was mixed in a 1.5 ml microcentrifuge tube with 2.5 $\mu$l of 0.66 mg/ml trypsin in 20 mM Tris-HCl (pH 8.0) and incubated at 37$^\circ$C for 20 h. After 8 h of incubation another 2.5 $\mu$l of the same trypsin solution was added. Five microliter aliquots of the digestion mixture were collected at 1 min, 8 h, and 20 h of incubation and analyzed by SDS/PAGE. Based on the Coomassie blue staining of the protein and the PhosphorImager detection of $^{75}$Se in the gel, most of the TR was digested 8 h and digestion was complete at 20 h. After 20 h of incubation a $^{75}$Se-containing peptide appeared as a diffuse 4-kDa species in SDS gels.

**Separation and Sequencing of Se-Containing Peptide.** The tryptic digestion mixture of carboxymethylated TR was acidified with HCl to pH 2.0, mixed with 600 mg of dry guanidine–hydrochloride and 5 $\mu$l of 0.5 M DTT, and loaded on a C$_{18}$ reversed-phase HPLC column, equilibrated in 0.05% trifluoroacetic acid. The 100-min gradient of acetonitrile from 0 to 50% in 0.05% trifluoroacetic acid was applied to elute peptides at 1 ml/min flow rate. A single $^{75}$Se-containing peptide (1.53 $\times$ 10$^5$ cpm) was eluted with 23% acetonitrile. A 35% portion of the isolated peptide was dried on a SpeedVac SC110 (Savant), dissolved in 6 M guanidine–hydrochloride and 2 mM DTT, and subjected to automatic sequencing on a Hewlett-Packard Protein Sequencer G100A. At each cycle, all eluates were collected and analyzed for the presence of $^{75}$Se by gamma counting. The remainder of the $^{75}$Se-containing peptide was loaded on a sulfopropyl (SP) HPLC column and eluted with a gradient from 0 to 0.5 M NaCl in 0.05% trifluoroacetic acid over a 30-min time period. A $^{75}$Se-containing peptide was eluted as a single peak, centered at 100 mM NaCl, that separated from the major impurities. This peptide was used directly for sequencing, and fractions corresponding to each cycle of the Edman degradation were collected and analyzed for the presence of $^{75}$Se.

**Other Methods.** Isoelectrofocusing (pH 3–7 range), SDS, and native PAGE analyses were performed according to standard procedures using 5%, 12%, or gradient 4–20% polyacrylamide gels. Gels were stained with Coomassie blue R-250. For detection of $^{75}$Se in gels, a PhosphorImager (Molecular Dynamics) was used. Thioredoxin- and 5,5'-dithiobis(2-nitrobenzoate) (DTNB)-dependent assays of catalytic activities were performed as described (12). The selenium content of TR was determined with a Perkin–Elmer model 4100 ZL atomic absorption spectrometer by using a palladium/magnesium nitrate modifier and temperature conditions as described (17). The molecular masses of purified TR and some of the tryptic peptides were determined by matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry with cinapinic acid as a matrix. Analytical HPLC gel-filtration for the determination of the molecular mass of the enzyme was performed as described (4). An immunoblotting procedure with an ECL detection system was used to study the cross-reactivity of human T-cell TR to polyclonal rabbit antibodies raised against rat liver TR. For this experiment, rat liver TR served as a control.

**RESULTS**

Analysis of extracts of $^{75}$Se-labeled human T cells by SDS/PAGE followed by PhosphorImager detection of radioactivity revealed the presence of several selenium-containing proteins. One of the most abundant selenium-containing proteins in the extracts, detected as a prominent $^{75}$Se-labeled 55-kDa protein band on the gels, was selected for preliminary characterization. About 40% enrichment of this protein was achieved by using DEAE Sephadex ion-exchange and phenyl Sepharose hydrophobic interaction chromatographic procedures. During these isolation steps it was observed that a flavin-containing species coeluted with the $^{75}$Se-labeled 55-kDa protein. This suggested that the T-cell protein might be a TR similar to the Secys-containing flavoprotein recently isolated from a human lung adenocarcinoma cell line (12). The lung enzyme, a homodimer of 57-kDa subunits, contained FAD and exhibited catalytic activities typical of mammalian TRs. To determine whether the T-cell selenoprotein is also a TR, the partially purified enzyme preparation was tested in immunoblot assays for interaction with anti-rat liver TR polyclonal antibodies. A strong signal exhibited by a 55-kDa protein band indicated the presence of TR.

For further purification of the 55-kDa selenoprotein, a 2',5'-ADP Sepharose affinity matrix that has been used routinely for efficient purification of mammalian TRs (18) was employed. As judged by SDS and native PAGE analyses (Fig. 1 A and D), an apparently homogeneous preparation of the enzyme was obtained by the modified isolation procedure described in Materials and Methods. The purified enzyme migrated as a 130-kDa species in gradient native PAGE gels.
The purified T-cell TR cross-reacted with the anti-rat liver TR antibodies in the immunoblot assay (Fig. 1C) and both of the protein bands detected on SDS gels appeared to be equally reactive. The TR isolated from human lung adenocarcinoma cells by a different procedure that involved absorption and elution from a heparin-agarose affinity matrix failed to react with the same antibody preparation (12). However, it now appears that a second form of the enzyme also is present in the lung cells, and this protein is recognized by the anti-rat liver TR antibodies in the immunoblot assay (S.-Y. Liu and T.C.S., unpublished).

The isolated T-cell enzyme exhibited catalytic activities typical of mammalian TRs. NADPH-dependent reduction of DTNB and human thioredoxin-dependent reduction of insulin with activities of 25 and 19 μmol NADPH oxidized/min/mg protein, respectively, were observed. These values are in the range of those reported for mammalian TRs from other sources (12–14, 19–21).

The electronic absorption spectrum of the T-cell TR revealed a characteristic flavin profile. The ratio of absorptivities at 280 nm and 450 nm was found to be 10 (ratio of absorptivities at maxima of 277 nm and 466 nm was 9), which is typical of mammalian TRs and indicative of an approximately full complement of FAD (two flavins per homodimer).

The amount of Se in T-cell TR determined by graphite furnace atomic absorption spectroscopy was 0.74 Se per 55-kDa subunit. The lower then stoichiometric amount of Se in T-cell TR presumably is due to the inaccuracies of determination of Se in proteins of similar molecular masses. Thus the T-cell TR probably contains 1 Se per enzyme subunit.

To determine the location of selenium in the human T-cell TR, the reduced 75Se-labeled enzyme was carboxymethylated and cleaved with trypsin followed by separation of peptides on a reversed-phase HPLC column (Fig. 2). The 75Se was eluted from the column in fractions of a single major radioactive peak. Further purification using a SP HPLC column separated the 75Se-labeled peptide from the major impurities (data not shown). The overall high (about 50%) recovery of 75Se in the resulting purified carboxymethylated peptide suggested the presence of a single stable selenium moiety, presumably carboxymethyl-selenocysteine.

The purified 75Se-labeled peptide isolated from the SP HPLC column was subjected to automated Edman degradation N-terminal sequencing analysis. Fractions corresponding to each cycle of the degradation were collected and analyzed for radioactivity. The resulting unique amino acid sequence matched exactly the deduced C-terminal 10-residue sequence
of placental TR derived from the cloned cDNA (16), except that the peptide contained two additional residues at the C terminus (Table 1). The $^{75}$Se in the peptide was eluted in cycle 11 where no amino acid was identified and this corresponded to the TGA codon in the gene. Cycle 12 contained a modest amount of glycine, and no additional amino acids were detected in cycles 13 and 14. From these data it is apparent that the TGA codon corresponds to Secys in the protein rather than serving as a termination codon as was concluded previously (16). Thus the three final triplets of the gene, TGA GGT TAA, actually encode Secys and a C-terminal glycine with TAA serving as the actual termination codon (Table 1).

The sequence of an additional major peptide was determined by Edman degradation analysis of the impure $^{75}$Se-labeled peptide fraction eluted from the reversed-phase HPLC column (Fig. 2). In addition to the sequence of the $^{75}$Se-labeled 12-residue C-terminal peptide (Ser-488–Gly-499), the sequence of another peptide which matched residues Phe-157–Arg-166 of the predicted gene sequence was clearly discernible. Identity of the latter peptide (expected mass 1159 Da) was confirmed by MALDI mass spectrometry. Sequences of three additional minor peptides that could be determined from the sequence analysis matched the sites of the deduced placental TR cDNA spanning Lys-299–Gly-306, Ile-316–Val-326, and Thr-313–Val-326. These data show that peptides comprising a total of 44 amino acids from T-cell TR share identity with deduced peptide sequences in placental TR.

### Table 1. Comparison of the deduced amino acid sequence of the C-terminal region of human placental TR with the Edman degradation sequence of a $^{75}$Se-labeled tryptic peptide from human T-cell TR

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Nucleotide sequence of C-terminal region of human placental TR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deduced amino acid sequence of C-terminal region of human placental TR</td>
<td>S - G - A - S - I - L - Q - A - G - C - $^\text{end}$ $^\text{489}$</td>
</tr>
<tr>
<td>Amino acid sequence of $^{75}$Se-labeled tryptic peptide from human T-cell TR</td>
<td>S - G - A - S - I - L - Q - A - G - C $^\ast$ - U$^\dagger$ - G $^\text{- end}$</td>
</tr>
<tr>
<td>Yields of amino acid residues during Edman degradation sequence analysis (pmol)</td>
<td>39$^\dagger$ - 119 - 130 - 25$^\dagger$ - 44$^\dagger$ - 98 - 83 - 93 - 75 - 52 - 6</td>
</tr>
</tbody>
</table>

$^\ast$Carboxymethylated Cys-497 was determined during Edman degradation based on the elution position of a carboxymethylated cysteine standard.

$^\dagger$U represents Secys. This residue is not detected during Edman degradation.

$^\ddagger$Yields for serine and isoleucine residues are usually lower than for other residues. The low yield of Gly-499 in cycle 12 is indicative of its poor binding to the sequencing column because this is the C-terminal residue in the peptide.
reduce thioredoxin or DTNB, whereas the alkylated enzymes exhibited greatly increased NADPH oxidase activities (23). In view of the known high reactivity of ionized selenol groups with alkylating agents, the Secys residue in the enzymes is the obvious target. Moreover, the slow rate of reaction of the alkylating agent with glutathione at pH 7.5 as compared with the almost 10^8 times faster rate of inactivation of the enzyme is consistent with the fact that a selenol is completely ionized at pH 7.5, whereas the cysteine thiol group of glutathione is only partially ionized. The ability of the alkylated enzyme to catalyze transfer of reducing equivalents from NADPH to oxygen suggests that a normal tightly coupled redox cycle involving the bound FAD moiety and Secys has been interrupted with the result that oxygen could then serve as direct electron acceptor for the reduced FAD.

In addition to disulfide-containing proteins such as thioredoxin and protein disulfide isomerase (24) that serve as substrates for mammalian TR, other unrelated substrates for the enzyme have been reported. These include DTNB, alloxan, menadione and oxidized lipoamide (14), Secys (25), and certain lipid hydroperoxides such as oxygenated forms of arachidonic acid (26). With the latter hydroperoxide substrates, a large rate enhancement of the reduction to the corresponding alcohols was observed when Secys (50-fold molar excess over enzyme) was included in the reaction mixtures. Similar large rate enhancements had been reported (27) for reduction of free and protein bound disulfides by selenols. The precise mechanism of the TR catalyzed peroxidase reaction, which presumably involves the Secys residues of the enzyme (two per protein dimer) together with the excess ionized free Secys formed enzymatically by reduction of selenocystine, is not established. In view of the fact that free Secys is present in biological systems in very low concentrations, the importance of TR as a peroxidase in vivo is unclear. However, based on these observations, one more type of antioxidant system with the potential of a protective role in the cell should be considered.

It is of interest that levels of TR in three different transformed cell lines (12, 19, 20) are approximately 10 times higher than the levels of enzyme reported in bovine liver (21), rat liver (14), human placenta (13), and human T cells. Whereas 3000- to 4000-fold enrichment was required to obtain homogeneous enzymes from the mammalian tissues, pure enzyme was obtained from transformed cells after only 150- to 500-fold enrichment (12, 19, 20). Depending on availability of NADPH, a ready supply of reducing equivalents can be maintained through the action of TR and this, together with the known high levels of glutathione in transformed cells, can be important factors in the rapid growth of tumors. Since selenium is a constituent of TR and other selenoproteins, the amount of this trace element in unsupplemented culture media may not be sufficient for the synthesis of the high levels of selenoenzymes found in transformed eukaryotic cells.

In view of the importance of the thioredoxin-TR system as a source of reducing equivalents for numerous essential cellular processes such as conversion of ribonucleotides to deoxyribonucleotides, reduction of protein disulfide groups and protein folding, reduction of methionine sulfoxide in proteins to methionine, reduction of thiol-specific peroxide reductase, redox regulation of DNA binding by transcription factors such as nuclear factor-κB and AP-1, and maintenance of the redox balance of cells, the recognition of Secys as a component of mammalian TR is especially significant (28). Previously the selenium-dependent glutathione peroxidases were the only Secys-containing enzymes known to have essential roles in antioxidant processes. The availability of the two NADPH-linked enzymes, TR and glutathione reductase, whose roles are to maintain levels of reduced thioredoxins and reduced glutathione in the cell, ensures the optimal function of numerous essential processes.

We thank Dr. Rodney L. Levine for helpful discussions and amino acid sequencing and MALDI analyses. We also thank Dr. Ho Zoon Chae for interesting discussions and gifts of proteins and antibodies as well as Dr. Song-Yuan Liu for providing us with a lung thioredoxin reductase preparation.


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**Table 2. Alignment of C-terminal regions of human glutathione reductase (GR), human TR, and C. elegans TR**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>*</th>
<th>***</th>
<th>*</th>
<th>####</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human TR</td>
<td>468 TIGHPVCAEVFTTLVTKRSAGSILQAGCUG</td>
<td>499</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. elegans TR</td>
<td>468 TIAIHPCSSEEFVKLHTRKSQDFPPTQGCG</td>
<td>499</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human GR</td>
<td>463 TVAHTPSSEELVLR</td>
<td>478</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Conserved residues in NADPH-dependent disulfide oxidoreductase enzyme family. H467 and E472 of GR and corresponding residues of other enzymes of this family are involved in interaction with active center cysteines. #, Conserved C-terminal regions of human and C. elegans thioredoxin reductases. U represents Secys residue. Numbering in the sequence of human TR starts from the methionine residue that was suggested to initiate translation (16).