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Original Research Communication

Efficiency of Selenocysteine Incorporation in Human Thioredoxin Reductase

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ABSTRACT

Thioredoxin reductase (TR) is a flavoenzyme, containing one selenocysteine (Sec) residue at the penultimate carboxyl-terminus, that catalyzes the NADPH-dependent reduction of oxidized thioredoxin. Sec is encoded by the UGA stop codon in the open reading frame of the mRNA, and the conserved stem–loop structure in 3′-untranslated regions functions as the determinant of Sec incorporation instead of termination of translation. The efficiency of Sec incorporation in Sec-containing enzymes in physiological or selenium (Se)-deficient condition remains unclear. To clarify this, we have developed monoclonal antibodies to human TR, and established a sandwich enzyme-linked immunosorbent assay to determine TR protein content. We observed that the specific activity of cytosolic TR in NCI-H441 cells increased with increasing concentrations of Se in a serum-free medium. The specific activity of TR purified from each cytosol was essentially equal to the calculated specific activity of each cytosolic TR. The Se content of TR increased with increasing concentration of Se in the medium, from 0.32 mol/mol of TR subunit (no SE) to 0.98 mol/mol of TR subunit (500 n*M* Se), and was directly correlated with the specific activity of TR. When calculated from the cytosolic TR specific activity of human peripheral mononuclear cell, the theoretical efficiency of Sec incorporation in physiological conditions is assumed to be 87%. Antiox. Redox Signal. 2, 643–651.

INTRODUCTION

SELENIUM (Se) is an essential trace element possessing remarkable biological effects that may be related to the unique functions of various selenocysteine (Sec)-containing enzymes (Stadtman, 1996; Ganther, 1999), including four types of glutathione peroxidase (GPx) (Takahashi *et al.*, 1990; Chu *et al.*, 1993; Ursini *et al.*, 1995), three types of iodothyronine deiodinase (Berry *et al.*, 1991; St. Germain and Galton, 1997), thioredoxin reductase (TR), and selenoprotein P (Saito *et al.*, 1999).

TR is a flavoenzyme that catalyzes the NADPH-dependent reduction of oxidized thioredoxin and many other substrates, and plays an important role in cell proliferation and thiol redox control (Holmgren and Bjornstedt, 1995; Ganther, 1999). TR is a dimeric enzyme with a redox-active disulfide and an FAD in each monomer, and it is a member of pyridine nucleotide-disulfide oxidoreductase family that includes glutathione reductase (GR) and lipoamide dehydrogenase (Holmgren and Bjornstedt, 1995; Arscott *et al.*, 1997). Although bacterial TR is highly specific for thioredoxin, mammalian TR has a variety of substrates in addition to thioredoxin (Tamura and Stadtman, 1996; Arscott *et al.*, 1997). TR contains one Sec residue, at the penultimate carboxyl terminus (Gladyshev *et al.*, 1996; Tamura and Stadtman, 1996), which is essential for the enzyme activity (Gorlatov and Stadtman, 1998; Zhong *et al.*, 1998). It has been shown that the removal of the carboxyl terminus by carboxypeptidase treatment (Zhong *et al.*, 1998) or alkylation of

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the Sec residue (Gorlatov and Stadtman, 1998) resulted in the inactivation of the enzyme, supporting that Sec has an essential role in TR activity.

Sec is encoded by the UGA stop codon in the open reading frame of the mRNA, and the conserved stem–loop structure in 3′-untranslated regions functions as the determinant of Sec incorporation instead of termination of translation (Berry *et al.*, 1994; Stadtman, 1996; Fujiwara *et al.*, 1999). The degree of efficiency of Sec incorporation in Sec-containing enzymes in physiological or Se-deficient conditions is unclear. Previously, we reported that there is a direct relationship between the recovery of GPx protein and activity in Se-deficient HL-60 cells transferred to a medium containing sodium selenite (Takahashi *et al.*, 1986). As cellular GPx contains one Sec residue at the 47th of 201 amino acid residues, the possibility of very rapid degradation of an incomplete protein cannot be eliminated. On the other hand, TR contains one Sec residue in the sequence Cys-Sec-Gly at the carboxyl terminus of each subunit, suggesting that a nearly full-sized TR protein could be synthesized even in Se-deficient conditions, although the resultant TR protein would be inactive. Various *in vivo* and *in vitro* studies on the Se-dependency of TR activity and protein (measured semiquantitatively by immunoblotting) have shown that TR protein does increase, but not to the same extent as TR enzyme activity (Berggren *et al.*, 1997, 1999; Gallegos *et al.*, 1997; Hill *et al.*, 1997).

To clarify this, we first developed monoclonal antibodies to human TR, and established a sandwich enzyme-linked immunosorbent assay (ELISA) to determine TR protein quantitatively. Second, we investigated the effect of Se on TR activity and protein content in human lung adenocarcinoma NCI-H441 cells. We report that the Se content of TR increased with increasing concentration of Se in the medium, and was directly correlated with the specific activity of TR. Amino acid sequence analysis of carboxy-terminal peptides from lysylendopeptidase-digests of TR protein indicates that the UGA codon acts as a stop codon under Se-deficient conditions. Finally, we consider the efficiency of Sec incorporation under physiological conditions.

MATERIALS AND METHODS

Materials

Frozen placentas were kindly provided by the Division of Obstetrics, Tonan Hospital (Sapporo, Japan). Recombinant human insulin and human transferrin were obtained from Wako (Osaka, Japan). Bovine serum albumin (BSA), DEAE-Sephacel, and 2′,5′-ADP-agarose were purchased from Sigma-Aldrich Co. (St. Louis, MO). Recombinant ADF/human thioredoxin was prepared as described previously (Mitsui *et al.*, 1992) and kindly provided by Ajinomoto, Co. Inc. (Kawasaki, Japan).

Cell culture

NCI-H441 cells, a human lung adenocarcinoma cell line (American Tissue Type Collection, Rockville, MD), were maintained in RPMI-1640 medium containing 5% fetal calf serum (FCS) at 37°C under an atmosphere of 95% air and 5% CO_2. For studies on the effects of Se, sodium selenite at various concentrations was added to a serum-free medium consisting of an RPMI-1640 containing 5 μg/ml human insulin, 5 μg/ml human transferrin, and 10 mg/ml BSA. After culture for 7 days, the cells were collected by centrifugation into an appropriate volume of 50 m*M* Tris-HCl, pH 7.4, containing 0.25 *M* sucrose, 0.1 m*M* EDTA, and 0.7 m*M* 2-mercaptoethanol. The cell suspension was sonicated with Ultra Sonic homogenizer VP-5s (Taitec, Tokyo, Japan), and centrifuged at 105,000 $\times$ *g* for 1 hr at 4°C to obtain a cytosolic fraction.

Purification of TR

TR was purified from human placenta as described previously (Oblong *et al.*, 1993), with some modification. Partially purified TR fractions obtained by acid precipitation and DEAE-Sephacel chromatography were applied to a 2′,5′-ADP-agarose column, washed with 100 μM $NADP^+$, and eluted with 500 μM $NADP^+$.

TR was also purified from NCI-H441 cells without acid precipitation, as described above. Each final preparation gave a single stained band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of monoclonal antibodies to TR

Hybridomas producing rat monoclonal antibodies were prepared as described previously (Kishiro *et al.*, 1995; Sado *et al.*, 1995). Briefly, the enlarged medial iliac lymph nodes from rats injected via hind footpads with an emulsion of purified placental TR and Freund's complete adjuvant were used for cell fusion, followed by hybridization, cloning, and the establishment of hybridomas. Two monoclonal antibodies, KF7 and KB12, were purified using 40% saturated ammonium sulfate precipitation and DEAE cellulose DE-52 (Whatman Int., Kent, England) column chromatography from culture media in which hybridomas were growing. KB12 was conjugated with horseradish peroxidase, as described (Nakane and Kawaoi, 1974).

Sandwich ELISA

Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated for 18 hr at 4°C with 50 μl of rat anti-human TR monoclonal antibody KF7 (3 μg/ml) in 0.05 *M* sodium bicarbonate buffer, pH 9.6 (Yamamoto *et al.*, 1995). The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and incubated for 1 hr at 37°C with 150 μl of Block Ace. After washing three times, 50 μl of TR standard or sample (diluted in PBS containing 0.05% Tween 20 and 0.1% BSA) was added to each well, and incubated for 1 hr at 37°C. After washing the wells three times, 50 μl of peroxidase-conjugated KB12 (0.5 μg/ml) was added and incubated for 1 hr at 37°C. Finally, the wells were washed three times. One hundred microliters of substrate solution containing 0.2 mg/ml 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (Wako, Osaka, Japan) and 0.02% hydrogen peroxide in 0.05 *M* citrate buffer, pH 5.0, was added to each well, and the enzyme–substrate reaction was allowed to proceed for 30 min. The absorbance was read at 405 nm in a Dual Wavelength Flying Spot Scanning Densitometer CS-9300 PC (Shimadzu Co., Kyoto, Japan).

Protein and Se assay

Protein concentrations of purified TR were determined by quantitative amino acid analysis. Se contents of purified TR were determined according to the fluorometric method (Bayfield and Romalis, 1985).

TR enzyme assay

Two assays were conducted to examine TR activity, as described previously (Luthman and Holmgren, 1982), with a slight modification. Method 1:5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) assay. The assay mixture contained 50 m*M* sodium phosphate, pH 7.0, 10 m*M* EDTA, 0.2 m*M* NADPH, and 5 m*M* DTNB. Activity was calculated as micromoles of NADPH oxidized per minute by $\Delta A_{412}/(13.6 \times 2)$, since 1 mol of NADPH yields 2 mol of thionitrobenzoate. Method 2: Spectrophotometric insulin reduction assay. The assay mixture contained 50 m*M* sodium phosphate, pH 7.0, 1 m*M* EDTA, 80 m*M* insulin, 0.2 m*M* NADPH, and 0.83 μ*M* human recombinant thioredoxin. The reaction rate was followed from the oxidation of NADPH at 340 nm, and activity was expressed as micromoles of NADPH oxidized per minute.

Other enzyme assays

GR activities were examined by following the NADPH oxidation in the presence of oxidized glutathione (Luthman and Holmgren, 1982). The assay mixture contained 50 m*M* sodium phosphate, pH 7.0, 1 m*M* EDTA, 0.1 m*M* NADPH, and 1 m*M* oxidized glutathione. Activity was calculated as micromoles of NADPH oxidized per minute. GPx activities were examined by following the oxidation of NADPH in the presence of GR (Sigma-Aldrich Co., St. Louis, MO), which catalyzes the reduction of oxidized glutathione formed by GPx and *t*-butyl hydroperoxide (Takahashi and Cohen, 1986). Activity was expressed as micromoles of NADPH oxidized per minute.

Isolation and amino acid sequence analysis of TR carboxy-terminal peptides

Three hundred micrograms of TR protein isolated from Se-deficient cells was *S*-carboxymethylated and digested with 10 μg of lysylendopeptidase for 20 hr at 37°C. The digest was applied on a Vydac 218TP52 high-performance liquid chromatography (HPLC) column, which was equilibrated with 0.05% trifluoroacetic acid (TFA). The peptides were eluted by a linear gradient from 0.05% TFA to 80% acetonitrile in 0.05% TFA. The Se contents of all peaks were analyzed, and the molecular masses of all peaks were determined using matrix-assisted laser desorption ionization (MALDI) mass spectrometry, Voyager RP-Jr (PerSeptive Biosystems, Houston, TX). Two peaks containing the candidates for TR carboxy-terminal peptides were further purified by a Vydac 218TP52 column with a linear gradient from 10 m*M* ammonium acetate to 80% acetonitrile in 10 m*M* ammonium acetate. Purified peptides were subjected to amino acid sequence analysis with an Applied Biosystems 477A gas-phase sequencer/120A PTH analyzer.

Preparation of human peripheral mononuclear cells

Human peripheral blood was collected from healthy volunteers in acid citrate-dextrose. Peripheral mononuclear cells were isolated by sedimentation using Dextran and Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden), as previously reported (Ohkuro *et al.*, 1994).

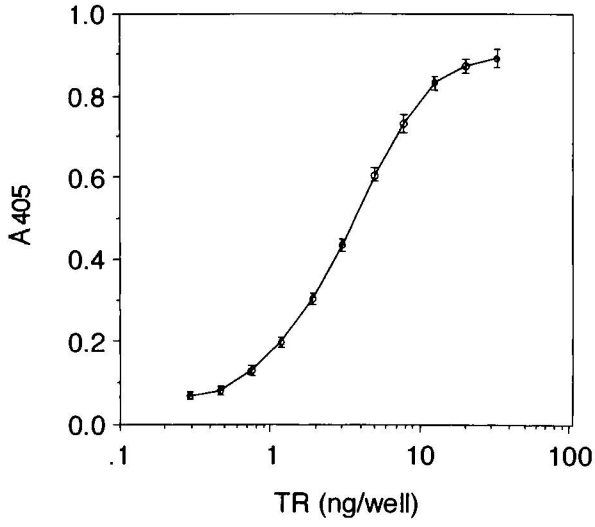

FIG. 1. Standard curve of ELISA for TR. The values represent the means ± SEM for triplicate.

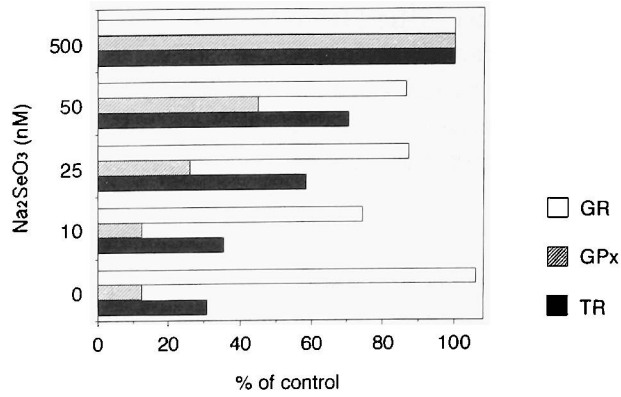

FIG. 2. Effect of Se concentration on GR, GPx and TR activity in NCI-H441 cells. The cells were grown for 7 days with various concentrations of sodium selenite in a serum-free medium. GR, GPx, and TR activity were determined, as described in Materials and Methods. Each point is expressed as percentage of control activity found in cells that cultured in a serum-free medium containing 500 n*M* sodium selenite. Data shown are representative of three independent experiments with similar results.

RESULTS

After immunization of TR purified from the human placenta, 15 hybridomas producing specific monoclonal antibodies against human TR were obtained. We selected two hybridomas, KF7 and KB12, and prepared monoclonal antibodies on a large scale. We then developed a sandwich ELISA for TR protein content. TR protein purified from the human placenta was serially diluted and introduced into the wells of microtiter plates coated with one anti-TR monoclonal antibody, KF7. Another monoclonal antibody, KB12, was conjugated with peroxidase, and used as a labeled second antibody. A typical standard curve is shown in Fig. 1. The results of this assay are highly reproducible, and 2–10 ng of TR could be determined from the slope.

Next, we investigated the effect of Se concentration on TR activity in NCI-H441 cells (Fig. 2). The cells were grown for 7 days with various concentrations of sodium selenite in a

serum-free medium. The enzyme activity of GR, an enzyme structurally related to TR (Holmgren and Bjornstedt, 1995), was not affected by the Se concentration in the medium. Decreases in the concentration of Se produced concentration-dependent decreases in the activities of Se-containing cytosolic enzyme, TR and GPx. Previously, we reported that there is a direct relationship between the recovery of GPx protein and activity in Se-deficient cells transferred to medium containing Se (Takahashi *et al.*, 1986). Therefore, a sandwich ELISA was used to determine TR protein.

The TR protein content decreased with a decrease in the Se concentration in the medium (Fig. 3). The calculated specific activity of TR also decreased with a decrease in the Se concentrations in the medium, *i.e.*, from 46.0 U/mg (500 nM Se) to 27.4 U/mg (no Se). This result suggests that 40% of TR protein may exist in an inactive form under Se-depleted conditions.

To confirm that the efficiency of Sec incorporation into TR protein declined in the absence of Se, we determined the Se content of TR proteins purified from the cells cultured at the various concentrations of Se. The determined specific activities of the purified TR were essentially equal to the specific activities

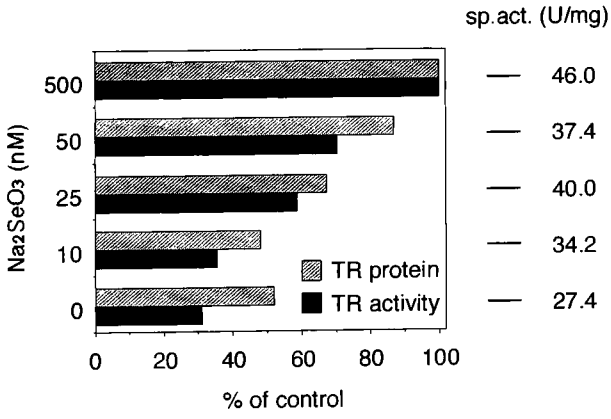

FIG. 3. Effect of Se concentration on TR protein and activity in NCI-H441 cells. The cells were grown for 7 days with various concentrations of sodium selenite in a serum-free medium. TR protein content was determined with a sandwich ELISA as described in Materials and Methods. Each point is expressed as percentage of control protein content or activity found in cells that cultured in a serum-free medium containing 500 n*M* sodium selenite. The calculated specific activities were shown on the right. Data shown are representative of three independent experiments with similar results.

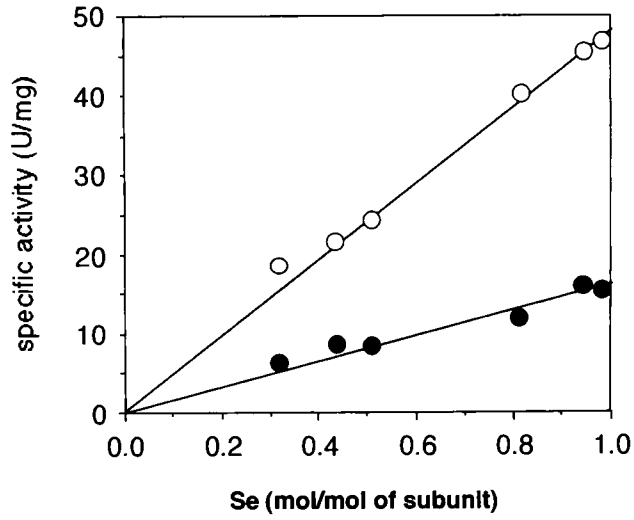

FIG. 4. Relationship between the Se contents and specific activities of TR purified from NCI-H441 cells cultured at the various concentrations of Se. After the cells were grown for 7 days with various concentrations of sodium selenite in a serum-free medium, TR was purified from the cytosol of the cells. Protein content, enzymatic activity, and Se content of purified TR preparations were determined as described in Materials and Methods. (○) DTNB assay; (●) insulin reduction assay.

calculated above. The Se content of TR increased with an increase in the Se concentration in the medium; *i.e.*, from 0.32 mol/mol of TR subunit (no Se) to 0.98 mol/mol of TR subunit (500 n*M* Se), and was directly correlated with the specific activity of TR (Fig. 4). In Fig. 4, not only DTNB-reducing activity of TR but also insulin-reducing activity of TR was determined. These results suggest that the efficiency of Sec incorporation into TR protein under Se-depleted and Se-replete conditions is 32% and 98%, respectively.

Because Sec-insertion machinery including selenocysteyl-tRNA is limited under Se-deficient conditions, premature termination at the UGA codon of TR mRNA during protein synthesis might occur. To confirm this possibility, we isolated two candidates for the carboxy-terminal fragment from lysylendopeptidase-digested TR by reverse-phase HPLC (Fig. 5). Peak (a) contained Se, and MALDI mass spectrometry revealed the presence of a compound with a mass of 1,386.88 Daltons. This peak was further purified and subjected to amino acid sequence analysis. The resulting sequence (Arg-Ser-Gly-Ala-Ser-Ile-Leu-Gln-Ala-Gly-Cys-Sec-Gly) matched exactly the deduced carboxy-ter-

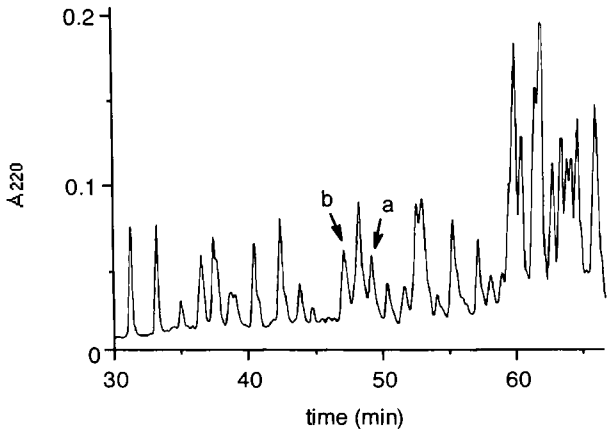

FIG. 5. Reverse-phase HPLC separation of a lysylendopeptidase digest of carboxymethylated TR. Peptides from carboxymethylated TR produced with lysylendopeptidase were separated by reverse-phase HPLC, as described in Materials and Methods. Peptides were followed at 220 nm. All fractions were subjected to analysis using mass spectrometry and Se analysis. The fractions marked "a" and "b" were subjected to amino acid sequence analysis.

minal 13-residue sequence of human placental TR derived from the cloned cDNA, although no amino acid was eluted in cycle 12, as described previously (Gladyshev *et al.*, 1996). Cycle 13 contained a modest amount of glycine. No additional amino acids were detected in cycle 14. The molecular mass calculated from amino acid composition was 1,384.37 Daltons, which corresponds closely to that observed by MALDI mass spectrometry. Because the TR protein used in this study was obtained from Se-deficient cells, a smaller, premature carboxy-terminal fragment (expected mass 1,119.24 Daltons) might also exist.

Peak (b) contained no Se, and contained a compound with a mass of 1,121.29 Daltons. This peak was further purified, and subjected to amino acid sequence analysis. The resulting sequence (Arg-Ser-Gly-Ala-Ser-Ile-Leu-Gln-Ala-Gly-Cys) matched exactly the deduced carboxy-terminal 11-residue sequence of placental TR, and no additional amino acids were detected in cycles 12 and 13. This indicates that the UGA codon partly acts as a stop codon under Se-deficient conditions, even in the presence of the conserved stem–loop structure that functions as the determinant of Sec incorporation instead of termination of translation.

The specific activity of TR purified from each cytosol was essentially equal to the calculated specific activity of each cytosolic TR. Furthermore, the specific activity of TR directly correlated with the Se content of TR (Fig. 4). Therefore, we attempted to determine the efficiency of Sec incorporation into protein under physiological conditions. When calculated from the specific activity (13.9 U/mg) of TR (as determined by method 2 from the cytosol of human peripheral mononuclear cells), the theoretical Se content of TR was 0.87 mol/mol of TR subunit. The Se content of placental TR calculated from the specific activity of purified enzyme was 0.88 mol/mol of TR subunit. Se analysis of purified placental enzyme (0.88 mol/mol of TR subunit) confirmed this value.

DISCUSSION

Mammalian TR is distinguished from a family of NADPH-dependent disulfide reductases containing FAD, which include GR and lipoamide reductase, by the presence of a carboxy-terminal peptide extension containing a Sec residue in the penultimate position (Holmgren and Bjornstedt, 1995). Several reports strongly suggest that Sec is an essential residue for TR activity and is lacking under Se-deficient conditions (Berggren *et al.*, 1997, 1999; Gallegos *et al.*, 1997; Hill *et al.*, 1997), but the precise efficiency of Sec incorporation into TR protein under physiological conditions has not been reported. This is mainly due to the absence of a quantitative immunological method for determining TR protein content.

Here, we report the development of rat monoclonal antibodies to human TR, and the establishment of sandwich ELISA for human TR protein. It has been reported that three mouse monoclonal antibodies to human TR recognize only one epitope (Soderberg *et al.*, 1998). Two antibodies prepared in this study can be used for a sandwich ELISA, suggesting that these two antibodies recognize the different epitopes. The rat monoclonal antibodies developed in this study may be useful for further studies on the distribution and localization of TR, including those performed in clinical situations.

Although several reports suggest the existence of premature inactive TR protein lacking Sec-Gly residues under Se-deficient conditions (Berggren *et al.*, 1997, 1999; Gallegos *et al.*, 1997; Hill *et al.*, 1997), there is no direct evidence to prove this. Using a sandwich ELISA, we show that TR protein content decreased to 50% with decreasing concentrations of Se in a serum-free medium. A combination of the decrease in TR mRNA levels and the decrease in the stability of TR mRNA under Se-deficient conditions reported previously (Fujiwara *et al.*, 1999; Gallegos *et al.*, 1997) may explain the decrease in TR protein. The degree of decrease in TR activity is not the same as that of TR protein. Therefore, the specific activity of TR decreased with decreasing concentrations of Se in the medium from 46.0 U/mg (at 500 n*M* Se) to 27.4 U/mg (without Se). This result suggests that 40% of TR protein may exist in an inactive form under Se-depleted conditions. In Se-deficient cells, Sec-insertion machinery including selenocysteyl-tRNA is limited, and premature termination at the UGA codon of TR mRNA during protein synthesis might occur. Previously, we reported that the specific activities of GPx from the cells cultured under Se-repleted conditions are the same as those cultured under Se-deficient conditions (Takahashi *et al.*, 1986). This can be explained by the inability to detect premature incomplete GPx protein by immunoassay, as GPx contains a Sec residue at the 47th of 201 amino acid residues. Because TR contains one Sec residue at the penultimate carboxyl-terminus, an immunologically detectable, nearly full-size TR protein could be synthesized, even under Se-deficient conditions, although the resultant TR protein would be inactive. To confirm this possibility, the Se contents of TR proteins purified from the cells cultured in the medium containing the various concentrations of Se were determined. The Se content in TR depends on the Se availability in the culture medium and correlates directly with the specific activity of TR.

TR preparations isolated form HeLa cells grown at a higher-than-optimum oxygen concentration have been reported to contain varying amounts of Se-deficient species that exhibited low specific activity (Gorlatov and Stadtman, 1998). Therefore, it was suggested that the oxidative decomposition of Sec in the protein had occurred during cell growth under high oxygen conditions. There are two possible mechanisms of Se-deficient TR protein production; one is by the premature termination at the UGA codon and the other is by the decomposition of Sec after the mature termination. Therefore, amino acids sequence analysis of TR carboxy-terminal fragments prepared from the Se-deficient cells was conducted. We observed two carboxy-terminal fragments—one derived from the mature TR and the other derived from the premature TR that lacks Sec-Gly at the carboxyl terminus. In this paper, we have clearly demonstrated the termination at the UGA codon of the mRNA during TR protein synthesis under Se-deficient conditions. Because TR is a dimeric enzyme, the formation of a dimer consisting of one full-length and one shortened subunit, which is 50% active, would occur.

When calculated from the cytosolic TR specific activities of human peripheral mononuclear cells, the theoretical efficiency of Sec incorporation under physiological conditions was 87%. Furthermore, purified placental TR contains 0.88 moles of Se per subunit. These results suggest the existence of premature inactive TR under physiological conditions. The ratio of premature inactive TR might, however, vary from tissue to tissue according to Se concentration; *i.e.*, with the availability of machinery for Sec incorporation. Se concentration in human serum or plasma is 1–2 μM, which is higher than that of the sodium selenite used in this study. We can not compare these values because we currently do not know which type of serum Se-containing compounds (selenoprotein or low-molecular-weight Se compounds) acts to transfer Se into the cells. The identification of the serum Se transporter remains to be done.

We have shown herein that the UGA codon partly acts as a stop codon under physiological conditions, even in the presence of the conserved stem–loop structure in 3′-untranslated regions of Sec-containing protein mRNA. However, further studies are still needed on the catalytic activity and function of truncated TR.

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ABBREVIATIONS

BSA, Bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GPx, glutathione peroxidase; GR, glutathione reductase; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel eletrophoresis; Se, selenium; Sec, selenocysteine; TFA, trifluoroacetic acid; TR, thioredoxin reductase.

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