# Purification of Human Thioredoxin Reductase: Properties and Characterization by Absorption and Circular Dichroism Spectroscopy†

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ABSTRACT: The flavoenzyme thioredoxin reductase (TR) and its natural substrate thioredoxin comprise a redox system generally found in all organisms. In order to better understand the biochemistry of this redox system, TR was purified (>4000-fold) from human placenta as a dimer of 60-kDa subunits. The molecular size of native TR was determined to be 160 kDa by gel filtration chromatography whereas migration on a sucrose gradient gave a molecular mass of 130 kDa. The p*I* of TR was determined to be 4.85. The temperature optima for DTNB and insulin reduction by TR were 52 and 40 °C, respectively. Preincubation of TR at 60 °C for up to 1 h showed no decrease in the enzymatic rates when assayed at 28 °C, while temperatures above 65 °C resulted in an irreversible loss of activity. Circular dichroism (CD) spectra of TR indicated that the secondary structural changes at 60 °C were only partly reversible at 28 °C. CD studies showed the flavoenzyme had a $T_M$ of 63 °C and above 45 °C began to exhibit changes in the secondary structure. Equilibrium denaturation of TR by temperature and guanidine hydrochloride suggested that FAD was not displaced during inactivation of TR and that the tertiary structure was primarily disrupted prior to denaturation of the secondary structure. The results of this study show that purified human TR is a relatively thermostable flavoenzyme whose tightly bound FAD group is not displaced by elevated temperatures up to 60 °C or by relatively low concentrations of guanidine hydrochloride.

The ability to maintain and regulate the intracellular thiol redox environment is critical to all cells. In mammalian cells, there are at least two thiol redox systems: the glutathione/glutathione reductase redox system and the thioredoxin/thioredoxin reductase (T/TR)[1] redox system. The T/TR redox system is important for protein folding through interactions with protein disulfide isomerase (Lundstrom & Holmgren, 1990), for reducing oxygen radicals (Tagaya *et al.*, 1989; Mitsui *et al.*, 1992), as an activating factor for certain receptors (Grippo *et al.*, 1983), and has been shown to be involved in the redox regulation of transcription factor activity (Abate *et al.*, 1991; Bannister *et al.*, 1991).

TR (EC 1.6.4.5) is a flavoenzyme which reduces the active-site cystine residue in T using NADPH as a cofactor. Two half-reactions comprise the actual sequence of catalytic events. The first involves the reduction of FAD by NADPH and electron transfer to the active-site cystine residue of TR. The second half of the reaction is the reduction of bound T. The reaction-site cysteines of bacterial and mammalian T are able to reduce proteins such as ribonucleotide reductase and insulin (Laurent *et al.*, 1964; Luthman & Holmgren, 1982, respectively). Kinetic analysis of *Escherichia coli* TR and T redox chemistry indicates that formation of the T/TR complex is rapid (Navarro *et al.*, 1991).

T and TR from *E. coli* have been extensively characterized [for a review, see Holmgren (1985)] and the crystal structures of both determined (Holmgren *et al.*, 1975; Kuriyan *et al.*, 1991). TR in both prokaryotes and eukaryotes is comprised of two subunits which form a homodimer. The structure of bacterial TR indicates that the symmetrically-shaped flavoenzyme can be divided into four domains. These consist of the NADPH and FAD binding pockets, a central core, and the dimer interface, which forms part of the active site (Kuriyan *et al.*, 1991). The mechanism of action of TR resembles that of human glutathione reductase and lipoamide dehydrogenase (Ghisla & Massey, 1988). Comparison of the crystal structure of *E. coli* TR with that of human glutathione reductase indicates that the two enzymes have specific similarities in overall tertiary structure. However, significant differences are seen between the active sites and dimer interfaces, suggesting that the similarities in catalytic activities were convergently evolved (Kuriyan *et al.*, 1991).

Mammalian forms of TR remain largely uncharacterized. Rat liver TR has previously been isolated as a 116-kDa homodimer of 58-kDa subunits (Luthman & Holmgren, 1982). Since the purification of mammalian TR includes a 65 °C incubation, the flavoenzyme presumably is able to retain an unfolded intermediate state, that upon cooling refolds to the enzymatically active species. This is the first report documenting the purification of a stable form of human TR. Attempts to isolate human forms of TR have been complicated, in part, by the inactivation of the flavoenzyme during purification, apparently due to a loss of the FAD prosthetic group.

We have purified TR from human placenta and used biochemical and biophysical techniques to characterize the structural changes in the flavoenzyme induced by elevated temperatures and guanidine hydrochloride (GuHCl). Absorption spectroscopy suggests that the FAD group is not displaced from the enzyme under these conditions. TR is reversibly inactivated at 60 °C while CD spectra analysis indicates that changes in the secondary structure at 60 °C are only partly reversible.

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[1] Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; GuHCl, guanidine hydrochloride; $K_M$, Michaelis–Menten constant, NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T, thioredoxin; TR, thioredoxin reductase; UV, ultraviolet.

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## MATERIALS AND METHODS

*Thioredoxin Reductase Assay.* Two separate assays were performed to monitor TR activity. The first assay involved the NADPH-dependent reduction of the disulfide bond in 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by TR (Holmgren, 1977). Briefly, 3 mL of 100 mM potassium phosphate buffer, 1 mM EDTA, and 0.2 mg/mL BSA was mixed with 30 μL of NADPH (20 mM) and 10 μL of DTNB (100 mM in dimethyl sulfoxide). Aliquots (0.5–5 μg) of samples containing TR were added to the assay mixture, and the change in optical density at 412 nm was monitored over 2 min. For thermal stability studies, assay volumes were decreased 10-fold. The second assay monitored the change in absorbance at 340 nm due to the oxidation of NADPH by TR in the presence of T and insulin (Luthman & Holmgren, 1982). Briefly, 250 μL of 100 mM potassium phosphate buffer, 1 mM EDTA, and 0.2 mg/mL BSA buffer was mixed with 3 μL of NADPH (20 μM), 30 μL of bovine insulin (80 μM), 5 μL of TR (25 nM), and 15 μL of T (2.5 mM) to a final volume of 300 μL. Initial enzyme rates were monitored over a 2–4-min period. Specific activity was calculated as previously described using the DTNB assay (Luthman & Holmgren, 1982). Kinetic constants were determined using "ENZFITTER" data analysis software (R. J. Leatherbarrow, Elsevier-BIOSOFT, 1987). *E. coli* T was purchased from Calbiochem and human recombinant T purified from an *E. coli* strain expressing a cDNA clone for human T (Gasdaka, Oblong, and Powis, unpublished results).

*Thioredoxin Reductase Purification.* A total of 150–200 g of frozen human liver (–80 °C) or 300–400 g of fresh placenta (University Medical Center, University of Arizona) was homogenized in 400 mL of 50 mM Tris-HCl/1 mM EDTA, pH 7.6 (solution A), in a Waring blender. The homogenate was centifuged at 30000*g* for 30 min and the supernatant adjusted to pH 5.0 by the dropwise addition of 1 M acetic acid with stirring. Precipitated protein was removed by centrifugation at 30000*g* for 30 min and the supernatant adjusted to pH 7.4 with 1 M $NH_4OH$. The sample was diluted with solution A to a final protein concentration of 3.0 mg/mL and applied to a DEAE-cellulose column (6 × 20 cm). Bound protein was eluted with a 400-mL linear 0–500 mM NaCl gradient in solution A, and fractions containing TR activity were dialyzed against solution A. The dialyzed sample was applied to a 2′,5′-ADP–agarose (Sigma Chemical Co., St. Louis, MO) column (1.5 × 14 cm) and washed with 3 column volumes of solution A and 1 column volume of 400 mM potassium phosphate buffer, pH 7.6. TR activity was eluted with 500 mM KCl in solution A. The sample was dialyzed as before, applied to a Blue Sepharose CL-6B (Pharmacia LKB) Piscataway, NJ) column (1.5 × 13 cm), and eluted with a 180-mL linear 0–300 mM NaCl gradient in solution A. The collected material was dialyzed as before and applied to a Mono Q HR 5/5 (Pharmacia LKB) column. Protein was eluted with a 60-mL linear 0–400 mM NaCl gradient in solution A, and 1-mL fractions were collected. Fractions containing TR were combined, diluted, and rechromatographed twice on Mono Q as before. Fifty-microliter aliquots of fractions collected from the final Mono Q column were analyzed by SDS–PAGE followed by silver-staining (Bio-Rad Silver-Stain Kit, Irvine, CA). Protein levels were determined either by the Bradford assay (Bio-Rad Protein Assay Kit, Irvine, CA) or by a modified Lowry method (Peterson, 1977). Molecular weight markers for SDS–PAGE were purchased from Sigma Chemical Co.

*Chromatofocusing.* Soluble homogenates from human liver and placental tissue were acid-precipitated and chromatographed on a DEAE resin as previously described. Combined fractions containing TR activity were dialyzed against 25 mM imidazole hydrochloride pH 7.35 (solution B), and 300–500 mg of total protein was applied to a PBE 94 (Pharmacia LKB) column (1 × 28 cm) preequilibrated in solution B. The resin was washed with 2 column volumes of solution B, and bound proteins were eluted with 300 mL of a 1:8 dilution of Polybuffer 74, pH 4.0 (Pharmacia LKB). Alternating fractions (4 mL) were prebuffered with 300 μL of 1 M Tris-HCl (pH 7.5) and assayed by DTNB reduction. The remaining fractions were used to determine the pH gradient.

*Gel Filtration.* A sample of purified TR was applied to a Sepharose 6B (Pharmacia LKB) column (1.5 × 17 cm) and eluted with solution A containing 150 mM NaCl. Fractions (1 mL) were collected, and the TR activity peak was determined using the DTNB reduction assay. Catalase (232 kDa, 11.3 S), aldolase (158 kDa, 7.7 S), and bovine serum albumin (BSA) (66 kDa, 4.3 S) (Pharmacia LKB) molecular mass standards were applied to the column separately and fractions collected as before. The eluting profile of the standards was determined by monitoring the absorbance at 280 nm.

*Sucrose Density Gradients.* Purified TR (10 μg) was mixed with 50 μg each of catalase, aldolase, and BSA molecular weight standards, layered onto a 5–25% linear sucrose gradient in solution A, and centrifuged at 238000*g* for 24 h at 4 °C. Fractions (500 μL) were collected and assayed for TR activity by DTNB reduction. The migration distances of the molecular weight standards were determined by the absorbance at 280 nm of each collected fraction and confirmed by the separation of respective subunits on 12% SDS–polyacrylamide gels, followed by Coomassie blue staining.

*CD Spectroscopy.* A stoppered 1-cm path-length quartz cuvette was used in a Aviv Model 60DS spectropolarimeter containing a water jacket attached to a circulating water bath. The actual temperature was read from the instrument's temperature probe. Scans were collected at 0.5-nm intervals with a bandwidth of 1.5 nm. The dynode voltage remained under 500 V. A buffer blank of 5 mM potassium phosphate (pH 7.0) or water was subtracted from each scan. A total of three scans were signal-averaged and smoothed to generate the final CD spectra. The temperature was increased at a rate of 1 °C/min and the sample allowed to equilibrate for a minimum of 10 min before being scanned. The $T_M$ was calculated as described in Kahn *et al.* (1992) by establishing a lower and an upper base line. The distance of each collected data point from the lower base line was divided by the distance to the upper base line to determine an equilibrium constant ($K_{eq}$). The derived $K_{eq}$ values were used to calculate van't Hoff enthalpy plots. The $T_M$ was calculated by interpolating from the point where ln $K$ equals zero. The estimated percentages of secondary structure were calculated from the CD spectra with the program PROSEC (PROtein SECondary structure estimator v2.1; Aviv Associates, Lakewood, NJ) based upon the method of Chang *et al.* (1978).

## RESULTS

*Purification of TR.* TR was purified to apparent homogeneity from human placenta by a modification of the method of Luthman and Holmgren (1987) (Table I). The final preparations of TR indicated the presence of a ~60-kDa polypeptide when analyzed on a silver-stained SDS–polyacrylamide gel (Figure 1, left panel). Two spectroscopic assays were utilized for detecting TR activity (see Materials and Methods). The first assay monitored the reduction of DTNB by TR and was used to detect enzymatic activity during all chromatographic procedures. The second assay monitored

Table I: Purification of Thioredoxin Reductase from Human Placenta[a]

| fraction | protein (mg) | total act. (units)[b] | sp act (units/mg) | purification (x-fold) |
|---|---|---|---|---|
| crude extract | 15500.0 | 3440 | 0.0082 | 1 |
| acid ppt | 5630.0 | 2044 | 0.0260 | 3 |
| DEAE column | 2550.0 | 500 | 0.0346 | 4 |
| 2′,5′-ADP column | 3.8 | 150 | 1.07 | 130 |
| Blue Sepharose column | 1.5 | 85 | 4.10 | 500 |
| Mono Q column[c] | 0.3 | 35 | 33.77 | 4120 |

[a] From a representative preparation of thioredoxin reductase. [b] NADPH (micromoles) oxidized per minute at 21 °C using DTNB assay. [c] Final Mono Q.

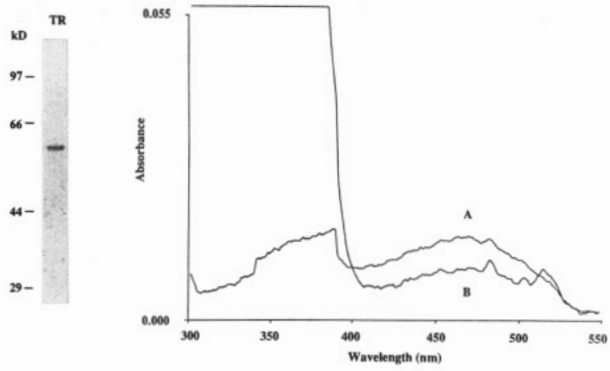


FIGURE 1: Spectral properties of purified human placental thioredoxin reductase. (Left panel) Aliquots (50 μL) of fractions (1 mL) collected from a final Mono Q ion-exchange column and containing purified TR activity were analyzed by SDS–PAGE followed by silver-staining (*TR*). A single polypeptide band corresponding to a molecular mass of 60 kDa was detected. Molecular mass markers are indicated at the left by size (kDa). (Right panel) The absorbance spectrum of purified TR (75 μg/mL) collected from a final Mono Q ion-exchange column was recorded from 300 to 550 nm (scan A). The same sample was then reduced with 500 μM NADPH and a new spectrum recorded from 300 to 550 nm (scan B).

Table II: $K_m$ Values of Human Placental Thioredoxin Reductase

| substrate | $K_m$ value (mM) |
|---|---|
| human thioredoxin[a] | 4.3 |
| *E. coli* thioredoxin[a] | 20 |
| DTNB[b] | 365 |
| NADPH[b] | 3.6 |

[a] Calculated by insulin reduction assay. [b] Calculated by DTNB reduction assay.

the reduction of insulin in the presence of T. A final specific activity of 33.8 units/mg was calculated for placental TR using the DTNB reduction assay. Purified TR was able to reduce both human recombinant T and *E. coli* T in the insulin reduction assay. The $K_M$ values for human TR are shown in Table II. Storage of purified placental TR at 4 °C over a 2-month period showed no detectable decrease in activity. The addition of 0.5 mM FAD did not increase the enzyme activity of partially purified placental TR.

TR purified from human liver samples was considerably less stable than placental TR. Crude human liver extract chromatographed on ion exchange resulted in an apparent decrease in the levels of TR activity. The addition of 0.3 mM FAD to the partially purified samples increased the enzymatic rate of DTNB reduction 3-fold (data not shown).

*Physical Properties of Placental TR.* Purified TR gave an absorbance maximum at 464 (Figure 1B, scan A), a wavelength in the region characteristic for the absorbance of the FAD group in flavoenzyme reductases (Kimura *et al.*, 1982; Vanoni *et al.*, 1992). When purified TR was reduced with 500 μM NADPH, there was a general decrease in the absorbance between 500 and 400 nm, indicating bleaching of the FAD moiety (Figure 1B, scan B).

In order to determine the p*I* of TR, a partially purified sample containing human placental TR was applied to a PBE 94 chromatofocusing resin as described under Materials and Methods. Bound protein was eluted with a linear pH gradient from 7.3 to 4.0. The p*I* of placental TR was calculated to be 4.85 ± 0.05 (Figure 2A). Chromatofocusing of a partially purified human liver sample containing TR gave a p*I* of 4.83 ± 0.06.

Two separate methodologies were used to determine the molecular mass of the flavoenzyme. The majority of TR activity fractionated by gel filtration chromatography eluted before and with aldolase (158 kDa) while a smaller amount eluted in the same volume as BSA (66 kDa) (Figure 2B). This suggests that TR was isolated as a dimer with a calculated molecular mass of 160 kDa but that a small fraction was present as a monomeric species. In contrast, when the molecular mass of purified TR was analyzed by sucrose density gradient centrifugation, the flavoenzyme migrated to a position slightly higher in the gradient than aldolase (158 kDa, 7.7 S), relative to the bottom of the gradient (Figure 2C). The calculated molecular mass was 130 kDa with a sedimentation coefficient of 6.8 S.

*Equilibrium Denaturation Monitored by Enzymatic Activity.* The thermal stability of TR was examined by monitoring the change in the initial activity rates as a function of temperature and GuHCl concentrations. The TR initial activity rate assayed by the DTNB and insulin reduction assays showed temperature optima of 52 and 40 °C, respectively. At 62 °C, there was a marked loss of activity in the DTNB reduction assay, and at temperatures above 62 °C, very little activity remained. Little, if any, activity was detected in the insulin reduction assay when the temperature was elevated to above 55 °C.

Since past protocols for purifying TR have utilized a 65 °C heat denaturation step (Luthman & Holmgren, 1982), the reversibility of the inactivation at 60 °C and above was examined. Purified TR was preincubated at 60, 65, and 80 °C for 2 min, cooled on ice, and assayed by DTNB reduction at 28 °C. Only preincubation at 60 °C resulted in sample that had 100% of the starting levels of activity. TR aliquots preincubated at 65 and 80 °C gave 20 and 10% of the starting initial activity rates, respectively. Purified TR preincubated at 60 °C for up to 60 min retained 100% of the starting levels of activity when assayed at 28 °C by both the DTNB and insulin reduction assays (data not shown). Furthermore, when recombinant T and insulin were preincubated at 60 °C prior to addition in the insulin reduction assay at 28 °C, 100% of the initial activity rate was maintained (data not shown).

*Equilibrium Denaturation Monitored by Activity and Absorbance Change.* The initial activity rates of DTNB reduction following increments of 5 °C were determined. The temperature of the DTNB assay buffer containing TR was raised by increments of 5 °C, allowed to equilibrate for 5 min, and mixed with DTNB and NADPH, and activity rates were determined as described before (Figure 3A, closed circles). The change in the absorbance spectrum of a purified TR sample was measured between 300 and 500 nm as a function of temperature. The temperature of the TR sample in the spectrophotometer was increased by 5 °C and allowed to equilibrate for 5 min and the $A_{464}/A_{450}$ ratio determined from each scan (Figure 3A, open circles), since the absorbance maximum of free FAD is at 450 nm. The $A_{464}/A_{450}$ ratio increased from 1.02 at 28 °C to 1.6 at 60 °C. Further increases

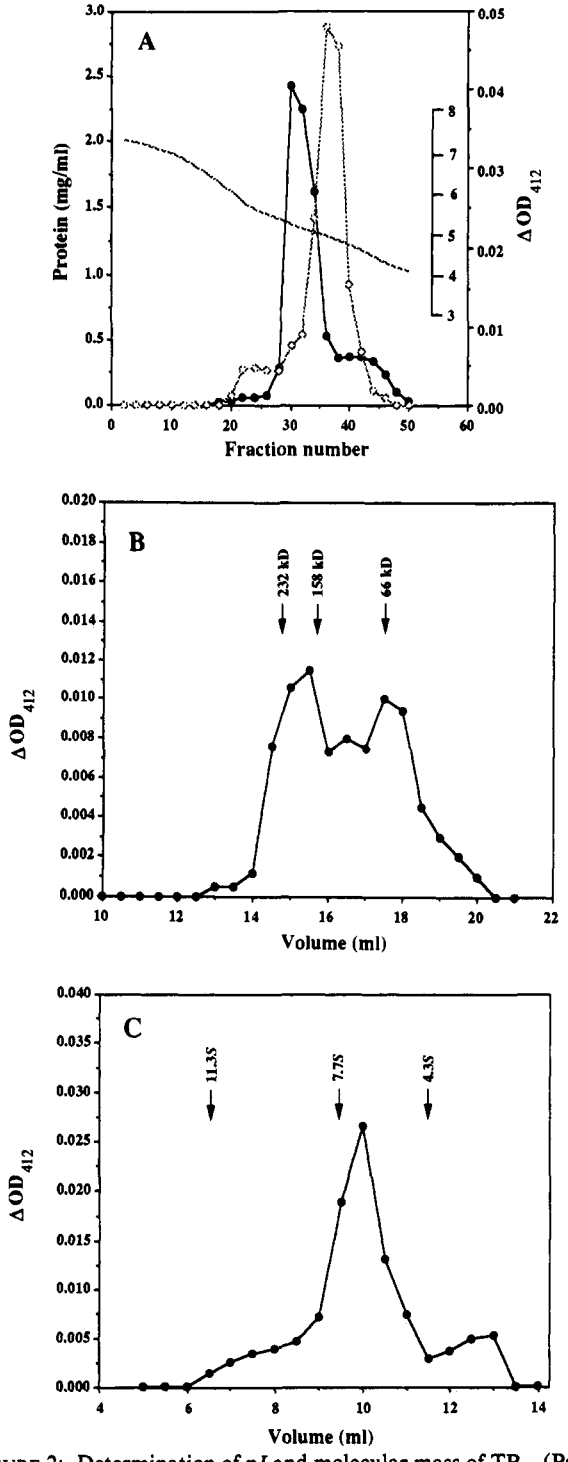


FIGURE 2: Determination of p*I* and molecular mass of TR. (Panel A) Partially purified placental TR collected from a DEAE ion-exchange column was applied to a PBE 94 chromatofocusing column, and bound proteins were eluted with a 7.3–4.0 linear pH gradient. The presence of TR activity was determined in alternating collected fractions by the DTNB reduction assay (open circles) as well as the relative protein concentrations (closed circles). The inset axis (unmarked solid line) denotes the pH gradient profile established by determining the pH in the remaining alternating collected fractions. (Panel B) Purified TR (10 $\mu$g) collected from a final MONO Q ion-exchange column was chromatographed on a Sepharose 6B gel filtration column and assayed by the DTNB reduction assay. The eluting volumes of molecular mass standards are indicated by vertical arrows. (Panel C) purified TR (10 $\mu$g) collected from a final Mono Q ion-exchange column was mixed with 50 $\mu$g each of molecular mass standards and separated on a 5–25% linear sucrose gradient. Aliquots (50 $\mu$L) of collected fractions (500 $\mu$L) were assayed by the DTNB reduction assay. The migration of molecular mass standards is indicated by vertical arrows. The molecular mass standards utilized in both panels B and C were catalase (232 kDa, 11.3 S), aldolase (158 kDa, 7.7 S), and bovine serum albumin (BSA) (66 kDa, 4.3 S).

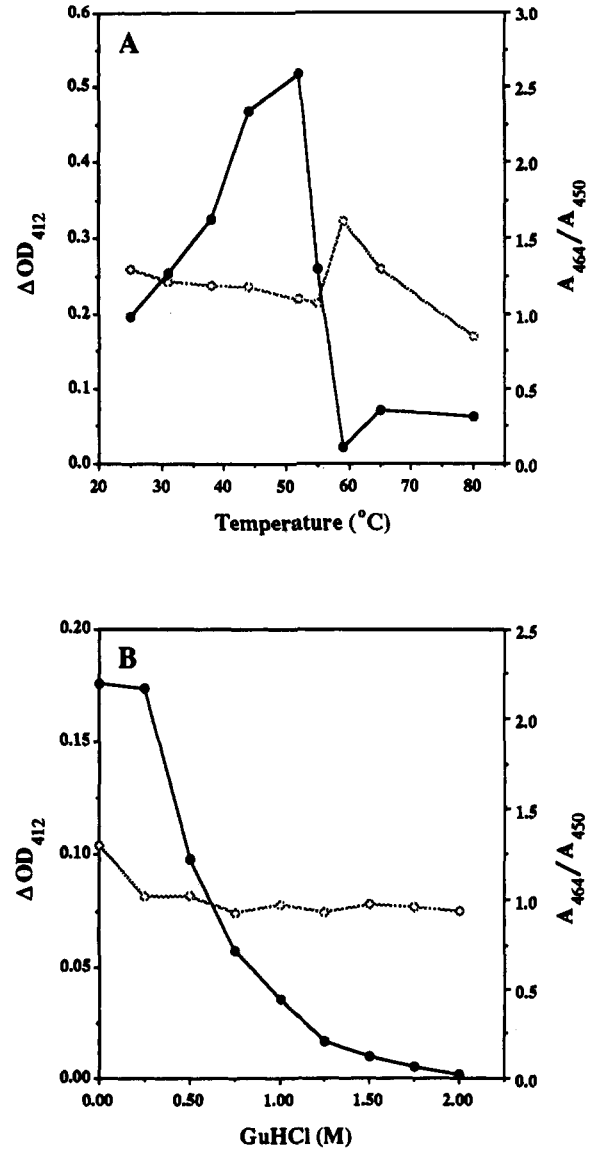


FIGURE 3: Equilibrium denaturation of TR as monitored by absorbance spectroscopy. (Panel A) Effect of temperature on the initial rates of TR activity and the $A_{464}/A_{450}$ ratio of purified TR. Initial activity rates were monitored as described for the DTNB reduction assay following equilibration after each temperature increment (closed circles). The absorbance spectrum of purified TR (75 $\mu$g/mL) collected from a final Mono Q ion-exchange column was recorded following equilibration after each temperature increment and the $A_{464}/A_{450}$ ratio calculated (open circles). (Panel B) Effect of GuHCl concentration on the initial rates of TR activity and the $A_{464}/A_{450}$ ratio of purified TR. Initial activity rates were monitored as described for the DTNB reduction assay following equilibration after each addition of GuHCl (closed circles). The absorbance spectrum of purified TR (75 $\mu$g/mL) collected from a final Mono Q ion-exchange column was recorded following equilibration after each addition of GuHCl and the $A_{464}/A_{450}$ ratio calculated (open circles).

in temperature above 60 °C resulted in a decrease of the $A_{464}/A_{450}$ ratio to values slightly below 1.0, suggesting that the structure of the FAD binding domain was affected by the elevation in temperature.

The initial activity rates of DTNB reduction at 28 °C were determined in the presence of increasing concentrations of GuHCl. Aliquots of 8 M GuHCl were added to DTNB assay buffer containing TR, allowed to equilibrate for 5 min, and mixed with DTNB and NADPH, and activity rates were determined as described before. A final concentration of 0.5 M GuHCl resulted in a 50% loss of TR activity, and 1.5 M GuHCl lowered the initial enzymatic rate to the background

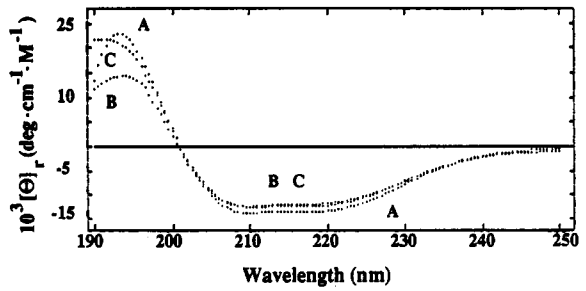


FIGURE 4: Far-UV circular dichroism spectra of placental TR. Purified TR (22 μg/mL) in 5 mM potassium phosphate (pH 7.0) collected from a final Mono Q ion-exchange column was used to record the far-UV CD spectra at 28 and 60 °C (scans A and B, respectively). The same sample of TR was then allowed to cool on ice and the CD spectrum recorded at 28 °C (scan C). The ellipticity is given as the molar ellipticity per residue.

Table III: Predicted Secondary Structure of Thioredoxin Reductase

| temp (°C) | α-helix (%) | β-sheet (%) | turn (%) | random coil (%) |
|---|---|---|---|---|
| 28 | 39.94 | 21.58 | 9.96 | 28.52 |
| 60 | 37.70 | 10.94 | 17.19 | 34.18 |
| 28[a] | 34.28 | 34.38 | 3.91 | 27.44 |

[a] Same sample from 60 °C scan after cooling on ice.

levels of the assay (Figure 3B, closed circles). When TR activity was assayed at 45 °C, final concentrations of 0.25 and 1 M GuHCl inhibited 50 and 100% of the activity, respectively (data not shown). The change in the absorbance spectra of TR in the presence of denaturant was determined. Purified TR was mixed with GuHCl at the indicated concentrations and allowed to equilibrate for 5 min, and the $A_{464}/A_{450}$ ratio was determined as before (Figure 3B, open circles). At 0.5 M GuHCl, the $A_{464}/A_{450}$ ratio changed from 1.3 to 0.96. Subsequent increases in the GuHCl concentration did not change this value, suggesting that the inhibition of TR activity was due in part to structural alterations in the FAD binding domain.

*Equilibrium Denaturation Monitored by Circular Dichroism.* Changes in the secondary structure of TR were examined by monitoring the change in CD as a function of temperature. CD spectra were recorded at 28 °C and then at 60 °C (Figure 4, scans A and B). Following the 60 °C scan, the cuvette containing the TR sample was cooled on ice, and spectra were recorded at 28 °C (Figure 4, scan C). The calculated changes in secondary structure are shown in Table III. The major changes in secondary structure which remain after the 60 °C incubation occur in the β-sheet and turn content. In contrast, the random-coil content returned to starting proportions, and the α-helical content did not change significantly during the temperature fluctuations.

The thermal denaturation profile of TR was plotted by recording CD spectra following increments in the sample temperature and determining the change in ellipticity at 233 nm (Figure 5A). The positive increases in the rotation values at 223 nm are the primary result of the decrease in the α-helical content of a polypeptide (Johnson, 1990). The transition temperature midpoint for unfolding during heat denaturation ($T_M$) of TR was determined to be 63 °C from the calculated van't Hoff enthalpy plot (Figure 5B). The changes in the secondary structure of TR caused by GuHCl were determined by monitoring the change in ellipticity at 223 nm as a function of increasing concentrations of GuHCl at 28 and 45 °C (Figure 5C).

The temperature-induced changes in the secondary structures of recombinant human T and insulin were investigated by CD since the temperature optimum of the insulin reduction assay was found to be lower than that for the DTNB reduction assay. While there was a rapid increase in the ellipticity at

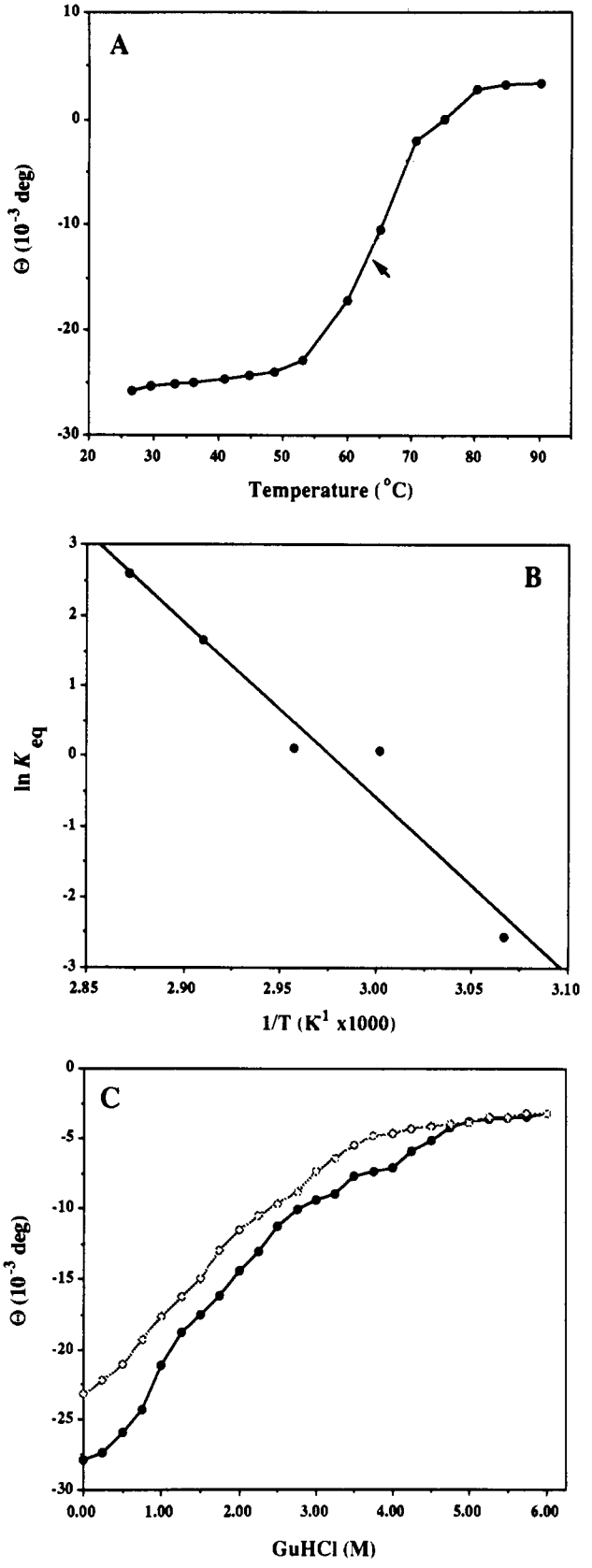


FIGURE 5: Equilibrium denaturation of placental TR as monitored by far-UV circular dichroism. (Panel A) Effect of temperature on the change in ellipticity at 223 nm of purified TR (22 μg/mL) in 5 mM potassium phosphate (pH 7.0) collected from a final Mono Q ion-exchange column. (Panel B) Calculated van't Hoff enthalpy plot of TR based on the change in ellipticity at 223 nm as a function of temperature. (Panel C) Effect of GuHCl concentration on the ellipticity at 223 nm of TR at 28 °C (closed circles) and 45 °C (open circles). The ellipticity is given as $10^{-3}$ mdeg.

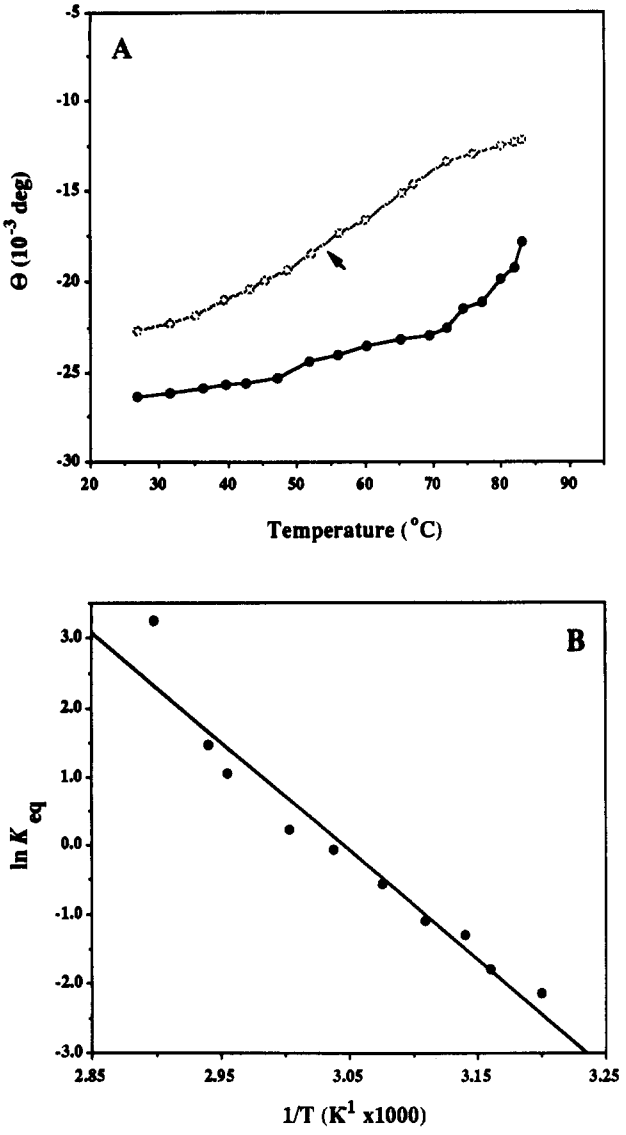


FIGURE 6: Equilibrium denaturation of human recombinant T and bovine insulin as monitored by far-UV circular dichroism. (Pane A) Effect of temperature on the ellipticity at 223 nm of human recombinant T (closed circles) and bovine insulin (open circles). The protein concentrations were 32 μg/mL (T) and 36 μg/mL (insulin) in water. (Panel B) Calculated van't Hoff enthalpy plot of insulin based on the change in ellipticity at 223 nm as a function of temperature. The ellipticity is given as $10^{-3}$ mdeg.

223 nm of human recombinant T above 75 °C, the lack of a plateau above 83 °C made it difficult to establish an upper base line and, hence, the $T_M$ (Figure 6A). The $T_M$ of insulin was determined to be 55 °C from the calculated van't Hoff enthalpy plot (Figure 6B).

## DISCUSSION

Human TR was isolated from both liver and placental tissues. Human liver TR was found to be less stable during purification, and all biochemical characterizations of TR were performed using the placental form. The specific activity of 33.8 units/mg for placental TR corresponds closely to a specific activity of 37.4 units/mg reported for rat liver TR (Mau & Powis, 1992). The p*I* of 4.85 for human placental TR was found to be nearly identical to the p*I* value of 4.9 determined for rat liver TR (Luthman & Holmgren, 1982). No apparent isoforms of human placental (or liver) TR, which might have eluted from the chromatofocusing resin at variant pH values, were detected by our studies. The 12 °C lower temperature optimum for the insulin reduction assay than the DTNB reduction assay (40 °C compared to 52 °C) may be due in part to the changes in the tertiary structure of TR, thereby potentially decreasing complex formation rather than directly decreasing the catalytic constant of TR. Furthermore, the CD data suggest that T or insulin does not undergo major changes in the respective secondary structures near the temperature optimum for the insulin reduction assay.

Human recombinant T was reduced by purified human TR ($K_M$ of 4.32 μM) with a 5-fold greater affinity than *E. coli* T ($K_M$ of 20 μM). It has been reported that *E. coli* TR has a decreased reactivity with human recombinant T ($K_M$ of 10 μM) compared to *E. coli* T ($K_M$ of 2 μM) (Jacquot et al., 1990). Our results suggest that mammalian TR is as selective for the eukaryotic T substrate as *E. coli* TR is for prokaryotic T. Human placental TR had a similar affinity for NADPH ($K_M$ of 3.57 μM) as *E. coli* TR ($K_M$ of 1.2 μM) (Williams, 1976).

The mass of human placental TR was estimated to be 160 kDa by gel filtration chromatography and 130 kDa with a sedimentation coefficient of 6.8 S by sucrose density gradient centrifugation. These results suggest that TR may be an asymmetrically shaped protein since the effective Stokes radius will cause asymmetrically shaped proteins to elute from gel filtration matrices with an aberrantly high molecular mass (Cantor & Schimmel, 1980; Stellwagen, 1990). Another explanation for the difference in the calculated molecular masses is that human TR may be glycosylated or an alternative covalent modification and thus, may migrate through the gel filtration matrix with an apparent higher molecular mass.

A proportion of the placental TR dimer complex dissociated into monomeric species during gel filtration chromatography but remained as a dimer during density gradient centrifugation. There is a precedent for such behavior with the soluble mitochondrial processing peptidase that can dissociate during gel filtration chromatography while remaining as a stable complex during density gradient centrifugation (Yang *et al.*, 1988). The active site of *E. coli* TR has been shown to be contained in the dimer interface, and the active form of human TR may be the dimer. If this is the case, the monomeric subunits must be able to reassociate to form an active dimer complex following gel filtration since there has been no report suggesting that a monomeric species of TR was catalytically active.

Proteins have been suggested to undergo transitions between three separate states during denaturation. These are the reversible transition between the native (N) and unfolded intermediates (U) and the irreversible transition between U and denatured protein (D) (Lepock *et al.*, 1992). The sigmoidal shape of the thermal denaturation curve of TR suggests that the transition from the initial N state to the final D state was biphasic and cooperative in nature. Comparing the calculated secondary structure from the CD spectra at 28 and 65 °C, the α-helical and random-coil content had not changed significantly whereas the β-sheet content had decreased 46% and the turn content had increased 96%. In contrast, the denaturation curve of TR by GuHCl was complex, suggesting that the transitions of TR from N to U and D occurred in multiple stages. While the change in TR secondary structure with 0.5 M GuHCl was minimal as determined by CD, the 50% decrease in activity suggests that the tertiary structure of TR was in some way disrupted. Further additions of GuHCl resulted in secondary structure alterations as evidenced by CD. These alterations presumably led to a molten globule intermediate since low concentrations of GuHCl have been suggested to induce such a state. This is supported by the concept that a principal way of increasing entropy in

proteins is to disrupt tertiary structure while maintaining secondary structure (Murphy *et al.*, 1992). The increase in kinetic energy at 45 °C resulted in TR becoming more sensitive to structural changes at lower concentrations of GuHCl.

One of the possible sites of equilibrium denaturation is the FAD binding domain, one of the four domains present in *E. coli* TR (Kuriyan *et al.*, 1991). However, on the basis of the $A_{464}/A_{450}$ ratios and initial activity rates, it is apparent that neither temperature nor GuHCl releases FAD from TR in the U state. Studies with human glutathione reductase, an enzyme with a homologous mechanism of action to TR, have shown that FAD is held tightly in place by 20 hydrogen bonds and during reduction FAD is the most immobile component of the domain (Schulz & Karplus, 1988). It appears that the FAD group in human placental TR is also tightly bound. Placental TR was able to maintain the U state at 60 °C and refold to an active form at lower temperatures, potentially to N. This may be explained in part by the presence of FAD. It is known that noncovalently bound ligands will help maintain the folded state of a protein under denaturing conditions when compared with proteins lacking a bound ligand (Jaenicke, 1987). However, the CD spectra of the secondary structure of TR at 28 °C following a 60 °C preincubation indicated that the changes in the secondary structure due to the increased temperature were not completely reversible. Furthermore, purified TR precipitated when the flavoenzyme was reduced with NADPH at 60 °C, suggesting that the reduced form of TR was less stable at the elevated temperature.

In summary, the results of this study show that purified human TR is a relatively thermostable flavoenzyme at temperatures up to 60 °C. Biochemical and biophysical characterization indicates that TR contains a tightly bound FAD group which was not displaced by elevated temperatures up to 60 °C or by low concentrations of GuHCl.

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