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Tissue Distribution and Subcellular Localization of Bovine Thioredoxin Determined by Radioimmunoassay†

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ABSTRACT: A double antibody radioimmunoassay for calf liver thioredoxin has been developed based on the use of rabbit antiserum and ^{125}I-labeled thioredoxin. The method permits determinations of as little as 2 ng (0.2 pmol) of thioredoxin in crude extracts, thus allowing measurements not possible with previous enzymatic determinations of thioredoxin. Since no competition is observed with thioredoxin from *Escherichia coli,* yeast, rat liver, Novikoff hepatoma, or man, the radioimmunoassay is specific for bovine thioredoxin; i.e., it is species specific. The immunoreactive thioredoxin in a week-old calf was determined in cell-free homogenates from different tissues, including platelets and erythrocytes. All tissues examined contain thioredoxin with the highest content in liver, kidney, thymus, erythrocytes, and brain. Immunoreactive thioredoxin in the different crude extracts represents from 0.03 to 0.07% by weight of the total protein; i.e., calf thymus contains 8×10^5 copies of thioredoxin per cell. Levels in bovine plasma were detectable but were below 0.05 mg per L. Calf liver and thymus were subjected to standard differential centrifugational techniques to separate subcellular fractions. Thioredoxin is present in homogenates of nuclei, mitochondria, microsomes, membranes, and in the 100 000*g* postmicrosomal supernatant. A homogenate of the plasma membrane fraction contains immunoreactive thioredoxin and the level increases several fold after treatment with 1% Triton X-100. This strongly suggests that a fraction of thioredoxin is associated with membrane structures or enclosed in vesicles. The ubiquitous presence of thioredoxin in tissues and subcellular fractions has important implications in view of the general thiol–disulfide oxidoreductase activity of the protein.

Thioredoxin is a well-characterized protein of 11 700 molecular weight (Laurent et al., 1964; Holmgren et al., 1975), containing a cystine residue, which is the electron acceptor in the transfer of electrons from NADPH catalyzed by the enzyme thioredoxin reductase (Thelander, 1968). The reduced form of thioredoxin, thioredoxin-$(SH)_2$, was until recently (Holmgren, 1976) the only known hydrogen transport system for the enzymatic formation of DNA precursors catalyzed by the essential enzyme (Fuchs et al., 1972), ribonucleotide reductase. The discovery of a second hydrogen transport pathway in *Escherichia coli* utilizing glutathione and a novel protein called glutaredoxin (Holmgren, 1976) raises questions about the function of thioredoxin in this reaction. Other functions for thioredoxin different from DNA precursor biosynthesis and as a general dithiol reductant have been described, e.g., the capacity to catalyze reduction of protein disulfide bonds in insulin (Moore et al., 1964; Engström et al., 1974; Holmgren, 1977), human choriogonadotropin (Holmgren & Morgan, 1976), or fibrinogen (Blombäck et al., 1974). Furthermore, a regulatory role has been suggested for thioredoxin in photosynthesis in spinach chloroplasts (Wolosiuk & Buchanan, 1977; Holmgren et al., 1977), and *E. coli* thioredoxin is known to be a subunit of T7 virus induced DNA polymerase (Mark & Richardson, 1976). Clearly more studies on the distribution and function of thioredoxins are required.

Thioredoxin was recently isolated in homogeneous form from calf liver (Engström et al., 1974), using an assay based on its capacity to promote disulfide reduction in insulin by NADPH and the homologous thioredoxin reductase (Holmgren, 1977). Since a thioredoxin has no obvious enzymatic activity by itself, measurements of thioredoxin have so far been performed by enzymatically coupled reactions using purified thioredoxin reductase or ribonucleotide reductase. These determinations have serious limitations since they cannot be applied to crude extracts without their prior purification. For

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the determination of the thioredoxin content in mammalian tissues and subcellular fractions, a highly sensitive assay is required. This paper describes the preparation of specific antibodies to calf liver thioredoxin and the development of a sensitive quantitative radioimmunoassay. The results of the application of this assay for the determination of the thioredoxin content in different calf organs and in subcellular fractions are described. The presence of comparatively large amounts of thioredoxin in cells with undetectable DNA synthesis strongly suggests that eucaryotic thioredoxin has major functions different from being the hydrogen donors for ribonucleotide reductase.

Experimental Procedure

Materials. NADPH, dithiothreitol, and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Sigma Chemical Co., St. Louis, Mo. Triton X-100 and Instagel were from Packard Instruments Inc., Stockholm, Sweden. Sodium [^{125}I]iodide for protein iodination was a carrier-free preparation obtained from the Radiochemical Centre, Amersham, England. Freunds adjuvant was from Difco Laboratories, Detroit, Mich. Bovine insulin (25 U/mg) was from Vitrum, Stockholm. Crystallized bovine serum albumin was bought from British Drug House Ltd., London, England. Sheep anti-rabbit γ-globulin (sheep 64/73) was obtained from Statens Bakteriologiska Laboratorium, Stockholm, Sweden. Thioredoxins from *Escherichia coli* (Holmgren & Reichard, 1967), yeast (Gonzales Porqué et al., 1970), phage T4 (Berglund & Sjöberg, 1970), and calf liver (Engström et al., 1974) were prepared by the previously described methods. Fully reduced and carboxymethylated calf liver thioredoxin was prepared by the method described (Engström et al., 1974). Thioredoxin reductase from calf liver was purified as previously reported (Holmgren, 1977). Rat liver Novikoff hepatoma thioredoxin (Herrmann & Moore, 1973) was a kind gift from Dr. E. C. Moore, Houston, Texas.

Preparation of Antiserum. Antiserum to calf liver thioredoxin was prepared by immunization of New Zealand rabbits. Initially, 1.2 mg of thioredoxin in 0.5 mL of 0.5 M potassium phosphate (pH 7.5) was emulsified with 0.5 mL of Freund's complete adjuvant and injected subcutaneously in the hind footpads. Four weeks later the same amount of antigen, emulsified in Freund's incomplete adjuvant, was distributed at the same sites. After 10 days blood was withdrawn, allowed to clot before separation of the antiserum, and this was stored at −20 °C. Preimmune serum was withdrawn before any immunization. All of four rabbits produced antibodies at the first bleeding. The γ-globulin fraction from the antiserum was prepared by repeated precipitation with 40% ammonium sulfate at +4 °C followed by extensive dialysis against 0.15 M NaCl–10 mM potassium phosphate, pH 7.0.

Iodination of Thioredoxin. Thioredoxin from calf liver was iodinated with ^{125}I to about 100 μCi/μg using the Chloramine-T method (Greenwood et al., 1963). Thioredoxin, 5 μg in 5 μL of 0.05 M potassium phosphate (pH 7.5) was mixed with 1 mCi of Na^{125}I (100 mCi/mL) and 50 μL of Chloramine-T (1.75 mg/mL) in 0.05 M potassium phosphate (pH 7.5) and allowed to react for 1 min in an ice bath. The reaction was stopped by addition of 10 μL of sodium metabisulfite (16 mg/mL) and 500 μL of KI (20 mg/mL). The labeled thioredoxin was separated from free iodine by chromatography on a column of Sephadex G-50 (1 × 20 cm) equilibrated with 0.15 M NaCl–10 mM potassium phosphate (pH 7.0) containing 1 mg/mL of bovine serum albumin. Fractions of 0.5 mL were collected, analyzed for radioactivity, and stored at −20 °C. Each preparation of [^{125}I]thioredoxin could be used for 1 to 2 months in the radioimmunoassay.

Radioimmunoassay Technique. Acid-washed glass tubes were used. All incubations and dilutions of samples were performed in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.0) containing 1 mg/mL of bovine serum albumin. Each tube in a series received 100 μL of a 1:1200 dilution of a sixfold concentrated γ-globulin fraction of an antiserum (138 A_{280}/mL); then 15 μL of [^{125}I]thioredoxin (~4 ng, 2 × 10^4 cpm) was added and 100 μL of a dilution series of unlabeled calf liver thioredoxin (2 mg per mL diluted from 1:400 to 1:102 400) or of extracts. Blanks received only buffer. The tubes were then incubated with shaking for 4 hr at 37 °C. After the incubation, 100 μL of a 1:4 dilution of sheep anti-rabbit serum was added; the incubations were continued for 18 h at +4 °C. The tubes were centrifuged for 15 min at 5000*g* in a Sorvall RCB2 centrifuge at +4 °C and 100 μL of the resulting supernatant fluid was carefully withdrawn for liquid scintillation counting. The radioactivity values reported are uncorrected. All assays included a standard curve of homogeneous thioredoxin (five points) and also control tubes which received no competing thioredoxin or antithioredoxin γ-globulin or sheep antirabbit serum.

Protein Determination. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard, or by reading the absorbance at 280 nm in a Zeiss PMQ 3 spectrophotometer (1-cm cuvettes; Laine, 1957). Samples containing Triton X-100 were precipitated with 10% trichloroacetic acid before analysis.

Animal Tissues. The liver and thymus from a 3-month old calf were obtained from a local slaughter house (Farmek, Uppsala, Sweden). The organs were removed from the animals and immediately stored on ice before preparation of subcellular fractions. The distribution of thioredoxin was measured in the various tissues of a 1-week old calf. The liver, kidney, spleen, thymus, lung, brain, heart, and skeletal muscle were removed and stored frozen at −20 °C until analyzed. The analysis of blood plasma, platelets, and red blood cells was performed on fresh blood collected in the presence of ACD solution (Blombäck et al., 1974). After centrifugation of the blood in a low-speed centrifuge, the red blood cells were collected and washed three times in 0.13 M NaCl–0.02 M trisodium citrate. The platelets were isolated from plasma by centrifugation at 2200*g* for 20 min at 4 °C. The pellet was resuspended and washed three times in 0.13 M NaCl–0.02 M trisodium citrate. The platelets were stored frozen at −20 °C with around 125 × 10^6 platelets per mm^3. The plasma from the isolation of platelets was also stored frozen at −20 °C before analysis.

Immunodiffusion. This was performed by the Ouchterlony (1949) double-diffusion technique. The wells of the agar plate contained 15 μL of antiserum and 15 μL of diluted thioredoxin in 0.15 M NaCl–0.01 M potassium phosphate, pH 7.0.

Inhibition of Thioredoxin Activity by Antiserum. The effect of antiserum on the thioredoxin activity was determined by following the thioredoxin dependent reduction of insulin disulfides by NADP-thioredoxin reductase (Engström et al., 1974; Holmgren, 1977). Thioredoxin (1.4 μg) was incubated with increasing volumes of the γ-globulin fraction at 37 °C for 30 min of an antiserum (10–50 μL) in a final volume of 200 μL containing 0.15 M Tris[1]-Cl (pH 7.5) and 2 mg/mL of bovine serum albumin. After 30 min at 4 °C the tubes were centrifuged for 5 min (5000*g*) and 20 μL from each fraction was taken for the standard assay with 20 munits of thioredoxin reductase (Holmgren, 1977). The reaction was terminated by

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

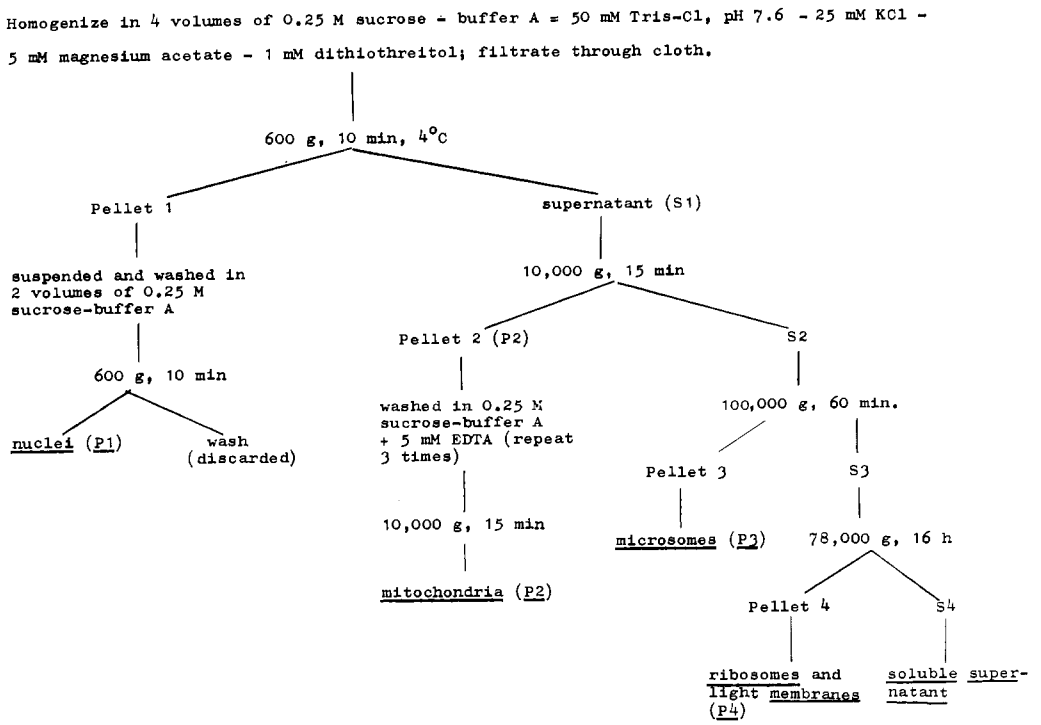

FIGURE 1: Methods for preparation of subcellular fractions from calf liver or calf thymus (Baril et al., 1971).

6 M guanidine hydrochloride–1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and the absorbance at 412 nm was determined (Holmgren, 1977). Controls of preimmune serum and a standard curve were run in parallel.

Preparation of the Plasma Membrane Fraction from Calf Liver. A particulate membrane fraction from calf liver was prepared as described by Takano et al. (1975). All operations were carried out at +4 °C. Fresh calf liver, 280 g, was trimmed of connective tissue, washed in cold 0.25 M sucrose, homogenized stepwise in 1 volume of 0.25 M sucrose with Omnix container, and further homogenized with Polytron PT-10 (Brinkman, West Germany) for 15 s at setting 5, 30 s at setting 7, and 15 s at setting 9. The membrane was purified by stepwise centrifugation at 600*g* for 10 min, at 1200*g* for 30 min, and the supernatant was finally centrifuged at 40 000*g* for 40 min (Beckman, SW-30). The pellet was suspended in approximately 80 mL of 0.05 M Tris-Cl (pH 7.4) and recentrifuged at 40 000*g*. The pellet was resuspended in about 3 volumes of 0.05 M Tris-Cl (pH 7.4) to give a protein content of 37 mg/mL and was kept frozen in small aliquots until thawed prior to use.

Preparation of Subcellular Fractions. The method described by de Duve et al. (1955) with some minor modifications was used (Baril et al., 1971). Calf thymus or liver was minced and homogenized in 4 volumes of 0.25 M sucrose–buffer A (50 mM Tris-Cl (pH 7.6 at 25 °C), 25 mM KCl, 5 mM magnesium acetate, and 1 mM dithiothreitol) using ten strokes with a loose-fitting Dounce homogenizer. After filtration through cheesecloth, subcellular fractions were isolated from the homogenate by centrifugation in a Spinco ultracentrifuge as outlined in Figure 1. The nuclear fraction and the mitochondrial, microsomal, and ribosomal fractions were isolated and each fraction was dissolved and split in two equal portions. One was homogenized with sonication, 2 × 0.5 min, with the addition of 1% Triton X-100 in 0.25 M sucrose–buffer A and the other without addition of Triton X-100. After centrifugation at 20 000*g* for 15 min the supernatant fractions were taken to assays for thioredoxin and protein.

Preparation of Crude Extracts from Calf Organs. Liver, kidney, spleen, thymus, lung, brain, heart, and skeletal muscle from a 1-week old calf which had been removed and stored frozen at −20 °C were used. From each organ a 2-g portion was homogenized in 6.0 mL of 0.05 M Tris-Cl (pH 7.5)–1 mM EDTA. Preparation of liver with the Polytron PT-10 (Brinkman) homogenizer gave at least a 100% better yield of thioredoxin than the Potter-Elvehjem homogenizer and the Polytron homogenizer was therefore used in the preparation of all other organs.

The homogenates were centrifuged for 45 min at 20 000*g* (Sorvall RCB2 centrifuge, SS-34 rotor) and the supernatant fractions were saved. The pellets after the first centrifugation were suspended in about 20 mL of 0.05 M Tris-Cl (pH 7.5)–1 mM EDTA, containing 1% Triton X-100. After continuous stirring for at least 3 h at cold room temperature, the remaining precipitate was removed by centrifugation for 45 min at 20 000*g* and the supernatants were withdrawn for analysis of thioredoxin and protein.

Some experiments with the acetone powder method of extraction were performed with established procedures (Morton, 1955).

Results

Preparation of Calf Liver Thioredoxin Antiserum. Immunization of rabbits with homogeneous calf liver thioredoxin resulted in production of antibodies. As shown in Figure 2, the antiserum gives a single line of precipitation when tested in a double-immunodiffusion system (Ouchterlony, 1949). No precipitation is obtained with either thioredoxin from *E. coli,* yeast, or bacteriophage T4 infected *E. coli,* demonstrating the specificity of the antibodies and structural differences of these thioredoxins.

The antiserum inhibits the enzymatic activity of calf thioredoxin in the reduction of disulfides of bovine insulin by NADPH catalyzed by thioredoxin and thioredoxin reductase (Figure 3). From these inhibition experiments, the titer of the antisera was calculated. By assuming that all antibody-thio-

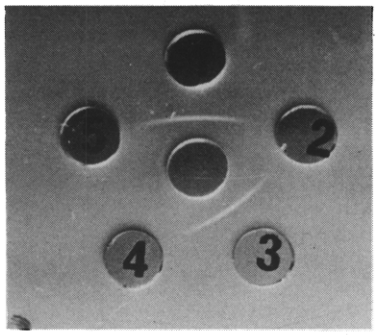

FIGURE 2: Double immunodiffusion of the anti-calf-liver thioredoxin γ-globulin fraction against various thioredoxins. The center well contained 10 μL of antiserum and the wells: (1) 1.5 μg of calf liver thioredoxin; (2) 2 μg of T4 thioredoxin; (3) 1.5 μg of calf liver thioredoxin; (4) 2 μg of yeast thioredoxin II; (5) 2 μg of *E. coli* thioredoxin. The photograph was taken after 8 h at room temperature.

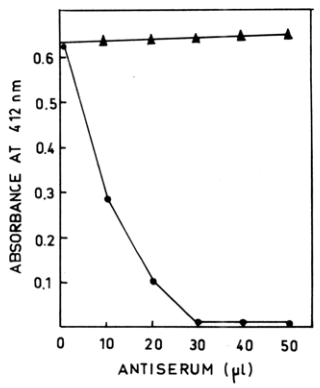

FIGURE 3: Inhibition of the enzymatic activity of thioredoxin by antiserum. Thioredoxin from calf liver, 1.4 μg, was incubated with the indicated amounts of antiserum in a final volume of 200 μL. After 30 min at 37 °C and 30 min at +4 °C, 20 μL from each fraction was assayed with NADP-thioredoxin reductase in the reduction of insulin disulfides. The activity is expressed as absorbance at 412 nm after addition of 5,5′-dithiobis(2-nitrobenzoic acid). (●—●) Antiserum used in radioimmunoassay; (▲—▲) preimmune serum.

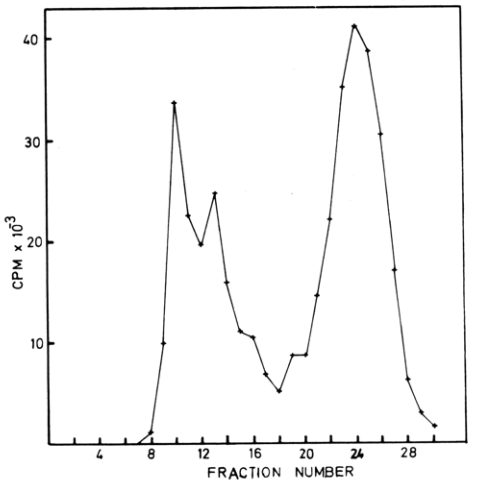

FIGURE 4: Separation of ^{125}I-labeled thioredoxin from excess reagents. The reaction mixture containing 5 μg of calf liver thioredoxin was applied to a column of Sephadex G-50 (1 × 20 cm) equilibrated with 0.15 M NaCl–10 mM potassium phosphate (pH 7.0)–0.1% bovine serum albumin. Fractions of 1 mL were collected and radioactivity was determined on 20-μL aliquots. The last peak is unbound [^{125}I]iodine.

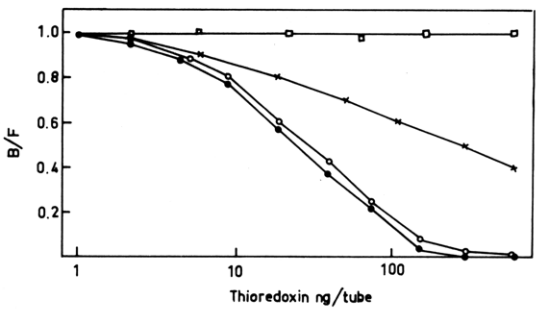

FIGURE 5: Competition for binding of [^{125}I]thioredoxin by thioredoxin of different oxidation states. (B) Bound; (F) free. The abscissa (log scale) shows the amount of unlabeled thioredoxin. (●—●) Fully oxidized standard calf liver thioredoxin; (○—○) calf liver thioredoxin after incubation with 2 mM dithiothreitol for 1 h. (X—X) reduced and carboxymethylated thioredoxin. (□—□) Thioredoxin from *E. coli*, yeast, or phage T4. For details, see Experimental Procedure.

redoxin complexes are inactive and have a 1:1 stoichiometry, the different antisera typically contain from 0.8 to 3.0 nmol of inhibiting antibodies per mL.

Development of a Radioimmunoassay. The preparation of [^{125}I]thioredoxin with the Chloramine-T method (Greenwood et al., 1963) results in the appearance of two main peaks of labeled thioredoxin when excess iodine is separated from the protein by chromatography on Sephadex G-50 as shown in Figure 4. The first peak eluting close to the void volume of the column is aggregated protein which is formed under oxidizing conditions. It contains mixed disulfides between structural sulfhydryl groups (Holmgren, 1977). The second peak is eluted in the position for a thioredoxin monomer (Holmgren, 1977) and was used in the experiments. However, material from the first peak binds to thioredoxin antibodies and may also be used as tracer in the radioimmunoassay with small differences in result. The specific activity of the [^{125}I]thioredoxin was around 100 μCi/μg.

All the radioactivity in an aliquot of the [^{125}I]thioredoxin was precipitated by the γ-globulin fraction of the antiserum and excess sheep anti-rabbit γ-globulin serum. This formed the basis for the development of a quantitative double antibody radioimmunoassay. We use about 4 ng of [^{125}I]thioredoxin and a small quantity of antithioredoxin giving around 50% binding. Competition for binding is obtained with unlabeled calf liver thioredoxin. A semilogarithmic plot of the concentration of cold thioredoxin vs. bound and free radioactivity in the supernatant is shown in Figure 5. The standard curve shows a linear portion in the range 2–300 ng of thioredoxin (0.2–25 pmol) per tube. This version of the radioimmunoassay which uses incubation for 4 h at 37 °C plus 16 h at +4 °C with a final volume of 320 μL was chosen for its speed. By diluting the system and prolonging the incubation times, the sensitivity could be increased. However, for the present purposes the sensitivity obtained is fully adequate.

The thioredoxin used to immunize the rabbits was a homogeneous but partly aggregated (approximately 50% dimers and higher molecular weight) fully oxidized material. The effectiveness of this material as a competitor after converting it into the fully reduced form by incubation with a final concentration of 2.0 mM dithiothreitol followed by repeated freezing and thawing cycles under aerobic conditions is shown in Figure 5. Similar competition curves are seen for the fully reduced thioredoxin, and the fully oxidized material. The radioimmunoassay is thus not distinguishing between oxidized or reduced thioredoxin. However, a fully reduced and carboxymethylated thioredoxin shows different properties (Figure 5) consistent with a partial immunological cross reaction of this modified protein.

TABLE I: Immunoreactive Thioredoxin in Liver from 1-Week-Old Calf Homogenized by Different Methods.[a]

homogenate prep	thioredoxin (mg/kg of tissue wet weight)	protein (g/kg of tissue wet weight)	sp act. (mg of thioredoxin/g of protein)
supernatant after Potter-Elvehjem	40.5	112.8	0.36
supernatant 1 after Polytron PT-10	99	115.9	0.85
supernatant 2 after Polytron PT-10[b]	21.4	57.4	0.37
sum of supernatant 1 and 2	120.4	173.3	0.69
soluble protein from acetone powder	38.4	44.8	0.86

[a] Thioredoxin was determined by radioimmunoassay. Protein was determined by the method of Lowry et al. (1951). For details of homogenate preparations, see Experimental Procedures. [b] Extraction of pellet after first supernatant with 1% Triton X-100.

TABLE II: Thioredoxin Content in Organs from a 1-Week-Old Calf.[a]

tissue	thioredoxin in Polytron crude extract	thioredoxin in Triton X-100 suspended pellet	total thioredoxin	thioredoxin/protein promille[b]
liver	99	21	120	0.7
kidney	44	12	56	0.7
thymus	38	7	45	0.6
spleen	27	8	35	0.5
lung	24	7	31	0.4
tongue	28	2	30	0.6
brain	19	6	25	0.5
heart	12	3	15	0.3
erythrocytes	7	30	37	
platelets	30	nd		nd
bovine plasma			0.05	0.001

[a] Thioredoxin was determined by radioimmunoassay. Extracts were prepared by Polytron PT-10. The values are given as mg of thioredoxin per kg of tissue wet weight. nd, not determined. [b] Promille, per thousand.

The specificity of the radioimmunoassay for calf liver thioredoxin was tested by using unlabeled thioredoxin from other species. No competition was observed with rat Novikoff hepatoma thioredoxin, rat liver thioredoxin, *E. coli* thioredoxin, or yeast thioredoxin up to 1.5 μg (Figure 5). A crude undiluted extract of human platelets previously shown to contain thioredoxin by enzymatic methods (Blombäck et al., 1974) gives no competition. The radioimmunoassay for bovine thioredoxin thus is highly specific, and the results show that thioredoxin from *E. coli*, yeast, rat, and man are immunologically different from the calf liver thioredoxin.

Thioredoxin Content in Liver Crude Extracts Prepared by Different Methods. The immunoreactive thioredoxin content in the supernatants of crude calf liver extracts prepared by the Potter-Elvehjem apparatus, Polytron, or acetone powder homogenization is shown in Table I. Polytron homogenization releases twice the amount of thioredoxin but essentially the same amount of protein. The most effective extraction of thioredoxin is obtained by Polytron treatment followed by suspension and reextraction of the first pellet by the detergent Triton X-100. Acetone powder extraction of a Polytron crude extract gave smaller amounts of thioredoxin and protein. Thus, treatments which effectively break intracellular structures release more thioredoxin. Control experiments where 1 or 2% final concentration of Triton X-100 was added to each tube in a standard radioimmunoassay showed slightly lower values (20%) of competition, thus ruling out artifacts as an explanation of the increase in thioredoxin content.

Distribution of Thioredoxin in Tissues from a 1-Week-Old Calf. The thioredoxin content in different organs from a 1-week-old calf was determined in crude extracts from liver, kidney, spleen, thymus, lung, brain, heart, and tongue. All the organs contain thioredoxin (Table II) and give parallel standard curves in the radioimmunoassay. Thus, no indication of any organ isothioredoxin is obtained from these results. The highest content of thioredoxin is present in the liver followed by kidney and thymus. The thioredoxin content of some calf organs is high, but when normalized in protein concentration the content is about the same in the different organs (see Table II).

Thioredoxin in Subcellular Fractions of Calf Thymus. The radioimmunoassay was used to study the distribution of thioredoxin in subcellular fractions from a calf thymus subjected to differential centrifugation (Table III). In all the determinations the standard and the extracts showed parallel competition curves, indicating the immunological identity of thioredoxin in all fractions. The thioredoxin content in the soluble supernatant fraction is highest. However, the homogenate of washed nuclei also contains a lot of thioredoxin. In fact the specific activity of thioredoxin is higher than in the soluble fraction, demonstrating the presence of thioredoxin in the nucleus. Also, the homogenates from the mitochondrial and the microsomal fractions contain thioredoxin. Inclusion of the Triton X-100 in the homogenization buffer increases the yield of thioredoxin significantly in the membrane-rich fractions, but also released more protein. The overall content of thioredoxin in the calf thymus is in good agreement with the previously calculated amount from preparative work employing enzymatic assay methods (Engström et al., 1974; Holmgren, 1977).

Thioredoxin in Subcellular Fractions from Calf Liver. Calf liver was subjected to the same preparation of subcellular fractions as outlined for thymus above and the various fractions were homogenized with or without Triton X-100 and analyzed for thioredoxin and protein. The results are shown in Table IV. Thioredoxin is present in soluble supernatant, nuclear fraction, mitochondria, microsomes, and finally also in the pellet containing ribosomes and light membranes. The presence of Triton X-100 in the buffer used to homogenize the various subcellular fractions increased the amount of thioredoxin threefold in the microsomal fraction and also had some effect on the extraction of the nuclear fraction. This fraction was about 20% of the total thioredoxin in the liver cells.

Thioredoxin in Calf Liver Membrane Fractions. The finding that more thioredoxin is liberated from the microsomal fraction when this was homogenized in buffer containing the detergent Triton X-100 suggested that some thioredoxin is membrane bound or enclosed in vesicles. To study this, plasma membranes containing binding sites for insulin and somatomedin were used (Takano et al., 1975). The washed and suspended membrane fraction which was homogenized contains thioredoxin as shown in Figure 6. Homogenization in the presence of Triton X-100 released three to six times more thioredoxin as seen in several experiments (Figure 6). The results confirm a membrane association of part of radioimmunoassayable thioredoxin in the plasma membrane and microsomal fractions from liver.

TABLE III: Thioredoxin Content of Calf Thymus Subcellular Fractions.[a]

fraction	total vol (mL)	protein (mg/mL)		thioredoxin (μg/mL)		sp act. (μg/mg of protein)	
		− Triton	+ Triton	− Triton	+ Triton	− Triton	+ Triton
first supernatant (S1)	58	12.2		3.7		0.30	
nuclear fraction (P1)	40	1.6	2.0	0.71	0.90	0.47	0.45
mitochondria (P2)	10	1.1	1.8	0.33	0.46	0.30	0.26
microsomes (P3)	10	1.6	1.8	0.40	0.50	0.25	0.28

[a] The data refer to a preparation of calf thymus (18.6 g) which was homogenized and fractionated according to the methods described in the Experimental Procedure and Figure 1. Thioredoxin was determined with radioimmunoassay using five different dilutions.

TABLE IV: Thioredoxin Content in Calf Liver Subcellular Fractions.[a]

fraction	total vol (mL)	protein (mg/mL)		thioredoxin (μg/mL)		sp act. (μg/mg)	
		− Triton	+ Triton	− Triton	+ Triton	− Triton	+ Triton
first supernatant (S1)	69	25.5		17.4		0.68	
nuclei (P1)	40	4.0	14.4	1.48	2.32	0.37	0.16
mitochondria (P2)	10	4.4	15.6	2.95	3.5	0.67	0.19
microsomes (P3)	10	3.7	19.5	1.65	5.10	0.44	0.26
ribosomes and light membranes (P4)	5	30.8	37.7	11.2	11.5	0.36	0.30
soluble supernatants (S4)	37	12.8		17.0		1.30	

[a] The data refer to a preparation of calf liver (20.2 g) which was homogenized and fractionated according to the methods described in Experimental Procedure and Figure 1. Thioredoxin was determined with radioimmunoassay.

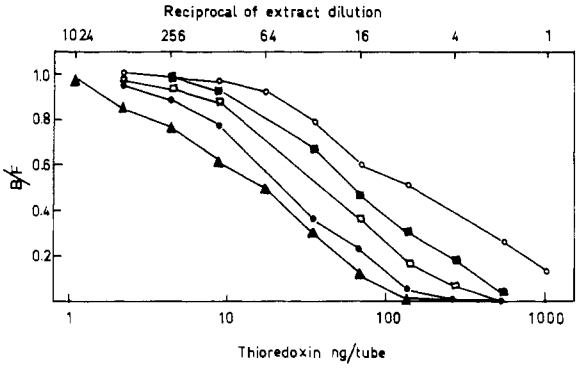

FIGURE 6: The radioimmunoassay determination of thioredoxin in the membrane fraction from calf liver. Radioimmunoassay was performed on 100 μL aliquots from standard dilutions and extracts from the membranes prepared as described in the Experimental Procedure. (●—●) Standard calf liver thioredoxin; (▲—▲) supernatant from preparation of liver after centrifugation at 12 000*g*; (○—○) supernatant after homogenization of membranes with Potter-Elvehjem homogenizer; (□—□) supernatant after homogenization of membranes with 1% Triton X-100; (■—■) supernatant after mixing of membranes with 1% Triton X-100 and centrifugation.

Discussion

The radioimmunoassay for bovine thioredoxin developed in this study permits highly sensitive and specific measurements of the content of this protein in cells. Previous methods for thioredoxin assay, which are based on the oxidoreduction function of thioredoxin coupled to the enzymes thioredoxin reductase or ribonucleotide reductase have serious limitations and require preliminary purification of an extract. Furthermore, since thioredoxin easily is inactivated by aggregation through oxidative thiol-disulfide interchange in the absence of dithiothreitol (Holmgren, 1977), these methods are subject to considerable technical difficulties. As shown here the radioimmunoassay measured both oxidized and reduced thioredoxin and is not sensitive to the presence of excess dithiothreitol, in contrast to the enzymatic methods.

The main conclusion from our results is that thioredoxin is present in all bovine tissues including erythrocytes. It is present in the subcellular fractions as separated in vitro. The highest content of thioredoxin is found in liver followed by kidney and thymus. In fact, a correlation of the thioredoxin content in these organs and the content of protein is observed. The originally discovered function of thioredoxin was to be hydrogen donor for the enzyme ribonucleotide reductase operative in DNA synthesis (Laurent et al., 1964). From the results shown here, other functions for thioredoxin in vivo have to be assumed to explain its distribution. The activity of ribonucleotide reductase is essentially undetectable in adult bovine liver or red blood cells both of which have large amounts of thioredoxin. The content of thioredoxin estimated in week old thymus (0.06%) corresponds to around 800 000 copies of thioredoxin per cell assuming a content of 4.6×10^9 cells in 1 g of thymus and a protein content of 0.15 g per g of cells (Bollum, 1975). Thioredoxin is thus an abundant protein of many parenchymatous organs. Generally, the levels are higher in rapidly growing tissues, as exemplified by higher levels in young animals.

Thioredoxin was until recently known as the only physiological hydrogen donor for procaryotic or eucaryotic ribonucleotide reductase (Herrmann & Moore, 1973). The identification of a mutant in *E. coli* lacking measurable thioredoxin (Holmgren et al., 1978) led to the discovery of a second hydrogen donor system consisting of NADPH, glutathione reductase, glutathione, and a new protein called glutaredoxin (Holmgren, 1976). Functions of thioredoxin as subunit of phage T7 DNA polymerase (Mark & Richardson, 1976), as regulator of the photosynthesis in chloroplasts (Wolosiuk & Buchanan, 1977; Holmgren et al., 1977), and as a potent protein disulfide reductase (Engström et al., 1974; Holmgren & Morgan, 1976; Holmgren, unpublished) have been described. This shows that thioredoxin may have a generalized function in many reactions. In support of this proposition, it was recently shown that thioredoxin has a general dithiol-

disulfide oxidoreductase activity and catalyzes the reduction of disulfide bonds in insulin with dithiothreitol and other reduced dithiols (Holmgren, unpublished results). Functions for thioredoxin in the synthesis of disulfide containing proteins may be consistent with its apparent presence in a ribosome and membrane-rich fraction.

The increase of immunoreactive thioredoxin in a membrane-rich fraction from bovine liver by homogenization with Triton X-100 is consistent with membrane localization of a thioredoxin fraction. Several experiments were carried out to exclude the possibility of artifacts as an explanation for the increase of thioredoxin by detergent extraction. The homogenization of the tissues was made in a buffer containing 1 mM dithiothreitol, a procedure which should exclude artifacts such as linkage of thioredoxin to other structures via S-S bonds. Experimental redistribution of proteins between different subcellular localizations may take place upon isolation of subcellular organelles in vitro. The presence of thioredoxin in all subcellular fractions that we observe is thus not unambiguous proof for its presence in vivo in all of these subcellular structures, although it is a strong suggestion. We are presently using immunohistochemical techniques to confirm the intracellular distribution of thioredoxin in mammalian cells. From a biochemical point, the presence of thioredoxin in homogenates of subcellular fractions and the soluble supernatant has important implications since thioredoxin catalyzes thiol-disulfide rearrangements between molecules (Holmgren, unpublished results).

Thioredoxin from *E. coli* has been extensively characterized since the three-dimensional structure of the molecule is known to 2.8-Å resolution by X-ray crystallography (Holmgren et al., 1975). Bovine thioredoxin is homologous to *E. coli* thioredoxin since the amino acid sequence around the oxidation-reduction two half-cystine residues (-Cys-Gly-Pro-Cys-) is conserved in the two structures (Holmgren, unpublished results). The ubiquitous occurrence of thioredoxin in both procaryotes and eucaryotes is consistent with some evolutionary stable function of the protein present in all living cells. The antibodies against thioredoxin and the radioimmunoassay described in this paper are versatile and valuable tools for further studies.

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