Characterization of Two Thioredoxins in Pig Heart Including a New Mitochondrial Protein

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Heart tissue contains two different thioredoxins. One is a specific mitochondrial protein and is best prepared from pre-isolated, intact heart mitochondria (mt-thioredoxin) whereas mitochondria-depleted tissue homogenates contain the major cellular thioredoxin of cytoplasmic origin (c-thioredoxin). Both heat-stable proteins are clearly differentiated chromatographically. They exhibit slightly different molecular weights (12300 *vs.* 12000) and isoelectric points (4.7 *vs.* 4.8) but differ remarkably in their cysteine content: mt-Thioredoxin has two cysteine residues like the bacterial proteins, and c-thioredoxin possesses six cysteines. Heart extracts were also shown to contain a NADPH-specific thioredoxin reductase of the known mammalian type. A specific function or target enzyme of mt-thioredoxin has not as yet been established.

Introduction

Thioredoxins from mammalian sources have been less thoroughly studied than those present in microorganisms and plants although the proteins isolated from rat and bovine tissues, tumor cells, and rabbit bone marrow have been known for long time [1–4]. Other members of the dicysteine protein family have attracted more interest because they occur in multiple species in green plants and in bacteria [5–9] whereas only one thioredoxin has so far been found in animal cells. Nevertheless an impressive number of biochemical effects has been linked to reduced (di-SH) thioredoxin in mammalian cells, including activation of protein synthesis, urea cycle enzymes, and glucocorticoid receptor binding, deiodination of thyroid hormones, regeneration of methionine from methionine sulfoxide, and inactivation of peptide hormones and growth factors [10–16]. In view of the diversity of specific thioredoxins available for thioredoxin-dependent reaction in other organisms it is indeed surprising that mammalian cells should rely on only one, unspecific thioredoxin system. The possible existence of more than one protein was not explored previously, although minor activity fractions are seen in some purification schemes [4, 17].

We have recently noted that mitochondria, like chloroplasts, possess their own thioredoxins [18] and that the organelle-specific proteins differ from other cellular thioredoxins in their enzyme-activating capacity. However, all known thioredoxins belong to a protein family with a high degree of sequence homologies [19, 20] and share basic properties such as molecular weight (12,000), heat stability, acidic isoelectric point, and a Cys-Gly-Pro-Cys tetrapeptide in the active site. The description of a novel thioredoxin and its physiologic significance is, therefore, only meaningful when all the (iso)proteins present in a tissue are characterized in parallel to that immediate comparisons can be made. Heart muscle is biochemically well known, and is a good source of mitochondria. We here report on the purification of two thioredoxins, a cytoplasmic and a mitochondrial protein from pig heart. Porcine thioredoxins have not been examined previously.

Materials and Methods

Materials and general methods

Chemicals and reagents were of the highest purity available. Tritiated cytidine diphosphate was

Abbreviations: CDP, cytidine 5′-diphosphate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); GSSG, oxidized glutathione; MDH, malate dehydrogenase; SDS, sodium dodecyl sulfate.

Enzymes: Isocitrate dehydrogenase (EC 1.1.1.42); NADP-malate dehydrogenase (EC 1.1.1.82); thioredoxin reductase (EC 1.6.4.5); catalase (EC 1.11.1.6); ribonucleoside diphosphate reductase (EC 1.17.4.1); fumarase (EC 4.2.1.2); carbamoyl phosphate synthase (EC 6.3.4.16).

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obtained from Amersham-Buchler, Braunschweig. Aquacide II for the concentration of aqueous solutions is a product of Calbiochem. Thioredoxin from *E. coli* was prepared as described [21]. Calf thymus glutaredoxin, and antisera against glutaredoxin and rat liver thioredoxin were kindly provided by Prof. A. Holmgren, Stockholm. Antibodies against *E. coli* thioredoxin were obtained from Dr. I. Häberlein, Kassel, and were coupled to CNBr-activated Sepharose by the standard procedure [22].

Protein concentrations were determined by the method of Lowry [23] or in the case of purified thioredoxin solutions were estimated using the equation $A_{280} - A_{310} = 1 \triangleq 1$ mg protein/ml. SDS polyacrylamide electrophoresis was carried out on 12% gels in a Tris/Tricine (pH 8.3) buffer system [24], and electrophoresis under native conditions on 10% gels. Isoelectric focussing was done on 5% polyacrylamide columns with Servalyt pH 4–7 carrier ampholyte, or on Servalyt Precote polyacrylamide sheets, pH 4–6, obtained from Serva, Heidelberg.

For amino acid analysis, reduced thioredoxins were carboxymethylated [25], desalted on a FPLC Sephadex G-25 column and hydrolyzed in 6 N HCl at 110 °C for 24 h. The mixture was derivatized with phenylisothiocyanate and the phenylthiocarbamyl amino acids were separated by reversed-phase HPLC [26] on a RP-Select B 5 µm column. *E. coli* thioredoxin served as reference protein.

Enzymes and thioredoxin assays

Ribonucleotide reductase from *E. coli* (substrate, [5-^{3}H]CDP) and NADP malate dehydrogenase from spinach were purified by the published procedures [27, 28] and were used for thioredoxin activity determination as described previously [8, 18]. Thioredoxin activities are expressed as rate increase over thioredoxin-free enzyme samples. Glutaredoxin was assayed by the NADPH/glutathione reductase (from yeast; Boehringer)/glutathione-coupled reduction of 2-hydroxyethyldisulfide [29].

Immunotitration of thioredoxin activity was done (in analogy to [30]) by incubating thioredoxin and antisera for 30 min at 37 °C and 30 min at 4 °C, followed by centrifugation and activity determination in the supernatant solution.

NADPH-dependent thioredoxin reductase activity was determined by reduction of 5,5′-dithiobis-(2-nitrobenzoate) (measured at 412 nm) without thioredoxin or by insulin reduction (measured at 340 nm) in the presence of thioredoxin [31].

Catalase, fumarase, glyceraldehyde 3-phosphate dehydrogenase and isocitrate dehydrogenase were determined by standard procedures. NADP isocitrate dehydrogenase and carbamoylphosphate synthase were partially purified from centrifuged pig heart mitochondrial lysates by gel filtration on Sephadex G-75 and Sephacryl S 400 columns to remove endogenous thioredoxins. The latter enzyme was assayed and characterized by its specifity as described [32].

Purification of mitochondria thioredoxin

Pig hearts were obtained from local slaughterhouses, chilled in ice, and processed immediately for the preparation of mitochondria. Following a standard procedure [33], two hearts (about 500 g) were cut into pieces, the tissue was ground and mixed with 3–4 volumes of icecold isolation medium (250 mM sucrose, 20 mM Tris-HCl buffer, pH 7.8, and 2 mM EDTA). The mixture was homogenized in a Waring Blender for 2 × 15 s, the pH was adjusted to pH 7.8 with 1 M Tris solution, the suspension centrifuged for 10 min at 1500 × *g*, and the precipitate was discarded. The supernatant was filtered through 4 layers of cheesecloth and centrifuged for 30 min at 11,000 × *g*. The supernatant was collected for the preparation of cytoplasmic thioredoxin (see below). The organelle pellet was resuspended in isolation medium, centrifuged again for 30 min at 11,000 × *g*, and the procedure was repeated three times. At this stage, washed mitochondria from 6 hearts were combined and kept at −20 °C (if desired) for routine preparation of mt-thioredoxin. Sucrose density gradient (23.4–53.9%) centrifugation of freshly prepared mitochondria in a TST 28.3 rotor for 17 h at 24,000 rpm [34] was carried out for analytical purposes.

Washed mitochondria were suspended in isolation medium and were sonicated for 3 × 30 s. After centrifugation (10 min at 39,000 × *g*) the yellow supernatant was heated to 80 °C and then rapidly chilled. The resulting pellet was removed by centrifugation and the clear supernatant solution, placed in dialysis tubing, was concentrated in Aquacide II. It was chromatographed on a column

(1.8 × 53 cm) of Sephadex G-75 equilibrated in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol. Fractions of 2.4 ml were collected at a flow rate of 9.5 ml/h. Active fractions were next applied to a column (1.0 × 3.5 cm) of DEAE cellulose (DE 52, from Whatman) equilibrated in the same Tris buffer. Elution was carried out with a linear gradient of 0–200 mM NaCl in buffer at a flow rate of 13 ml/h. Thioredoxin fractions had to be dialyzed against Tris buffer prior to activity determination with ribonucleotide reductase.

Purification of cytosolic thioredoxin

The red supernatant obtained in the first 11,000 × *g* centrifugation of a heart homogenate described above was heated to 80 °C and denatured proteins were removed by centrifugation for 30 min at 11,000 × *g*. Ammonium sulfate was added to 90% saturation and after stirring in the cold for 15 h the precipitate was collected. The pellet was redissolved in the minimum volume (≤ 300 ml) of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol, dialyzed against and applied to a column (2.8 × 10 cm) of DEAE cellulose equilibrated in the same buffer. The column was eluted with a 0–150 mM NaCl gradient in buffer at a flow rate of 50 ml/h. Thioredoxin activity could be determined using NADP-MDH.

Active fractions were combined, brought to 90% $(NH_4)_2SO_4$ saturation, and the precipitated protein collected after 4 h. The pellet was redissolved in a small volume (≤ 15 ml) of Tris buffer and chromatographed on a column (2.8 × 105 cm) of Sephadex G-75 at a flow rate of 33.5 ml/h. Fractions of 11.2 ml were collected. The active fractions were made 10 mM in dithiothreitol, incubated for 2 h, and then dialyzed against 20 mM Na-acetate buffer, pH 4.6, containing 1 mM dithiothreitol. A column (1 × 6 cm) of CM cellulose (CM 52, from Whatman) was equilibrated in the same buffer. Before application to the column, the thioredoxin sample was again brought to 10 mM dithiothreitol and then chromatographed using a 0–150 mM NaCl gradient in acetate buffer at a flow rate of 30 ml/h.

Thioredoxin reductase from pig heart

One heart was cut into pieces, ground and homogenized in a Waring Blender for 2 min in 2–3 volumes of 50 mM Tris-HCl buffer, pH 7.5. The pH of the suspension was readjusted to 7.5 and the mixture centrifuged for 15 min at 11,000 × *g*. The pellet was discarded, and the supernatant filtered through 4 layers of cheesecloth. The solution was applied to a column (2.8 × 8 cm) of DEAE cellulose which was equilibrated in the above buffer and eluted with a gradient of 0–350 mM Na-acetate in buffer at a flow rate of 18 ml/h. Fractions active in NADPH-dependent DTNB reduction assays were combined, concentrated by immersion in Aquacide II, and dialyzed against 10 mM K-phosphate, pH 7.6, containing 3 mM EDTA.

The sample was then chromatographed on a column (0.8 × 10 cm) of 2′5′-ADP-Sepharose (from Pharmacia) in the same phosphate buffer; unbound proteins were removed by washing with buffer, and the affinity-bound proteins were eluted with 1 mM NADPH solution in buffer at a flow rate of 7.5 ml/h. The latter fractions were finally applied to a column (1.2 × 100 cm) of Sephadex G-100 equilibrated in the above phosphate buffer, and chromatographed at a flow rate of 7.5 ml/h.

Results

Subcellular distribution of pig heart thioredoxins

Conventional purification schemes have led to the identification of one thioredoxin in mammalian cells [1–4]. With the existence of organelle-specific thioredoxins in mind [18] which will constitute a minor portion of the total thioredoxin content of a cell homogenate we considered separate analysis of subcellular fractions the only unambiguous way to differentiate individual, specific proteins. (In contrast, several thioredoxins are readily resolved in whole plant tissues [5–8]). Intact, respiration-active mitochondria were therefore prepared from pig heart by differential centrifugation as described in the Experimental section and were used as source for thioredoxin preparation. Likewise, the organelle-depleted, cellfree supernatants served as starting material. Washed mitochondria contained only 0.15% of the initial activity of a cytosolic marker enzyme, glyceraldehydephosphate dehydrogenase. They did contain substantial catalase activity because the separation of mitochondria and peroxisomes is difficult in muscle homogenates. However, when thioredoxin activities

were measured in a sucrose density gradient containing heavy and light mitochondria plus contaminating catalase (Fig. 1) the thioredoxin profile paralleled the mitochondrial fumarase and not the peroxisomal catalase activity, suggesting that peroxisomal proteins do not complicate the isolation of mt-thioredoxin.

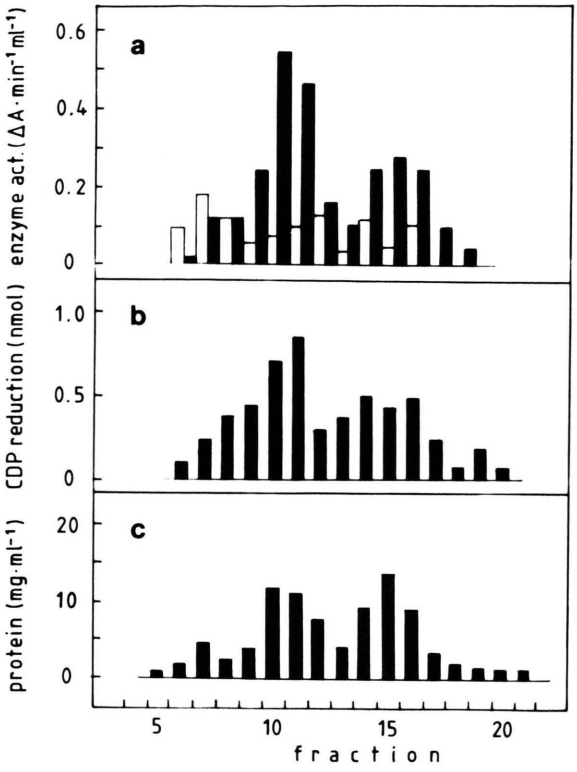

Fig. 1. Analysis of heart mitochondria fractionated in a sucrose density gradient. a: Fumarase (■) and catalase (□) activities, measured spectrophotometrically. b: Thioredoxin, measured as ribonucleotide (CDP) reduction. c: Mitochondrial protein.

Purification of mitochondrial thioredoxin

The identification of a heat-stable, $M_r = 12{,}000$ thioredoxin in mitochondrial lysates [18] required the unspecific ribonucleotide reductase of *E. coli* as indicator enzyme because a physiological target for the novel thioredoxin is unknown. Purification was achieved by gel filtration on Sephadex G-75 and ion exchange chromatography on DEAE cellulose or on FPLC Mono Q colums (Fig. 2). mt-Thioredoxin was enriched 50-fold over the activity of the total heat-stable protein (Table I); the true factor of purification must be considerably higher but cannot be calculated because ribonucleotide reductase measurements are inhibited in a crude mitochondrial lysate. The resulting protein was homogenous on native and denaturing electrophoresis gels (Fig. 5).

Isolation of cytoplasmic thioredoxin from pig heart

The protein-rich supernatant fraction remaining after removal of mitochondria and other organelles was subjected to heat (80 °C) denaturation and ammonium sulfate precipitation as in most thioredoxin purification protocols. In this case it was possible to use the more convenient spectrophotometric thioredoxin assay coupled to chloroplast NADP-malate dehydrogenase because the

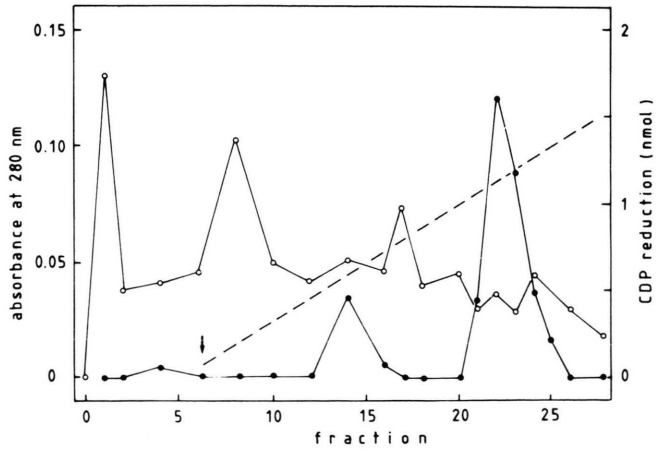

Fig. 2. Ion exchange chromatography of mt-thioredoxin from heart mitochondria on DEAE cellulose at pH 7.5. ○: Protein absorbance at 280 nm (left scale); ●: thioredoxin activity, measured as stimulation of ribonucleotide reductase-catalyzed CDP reduction (right scale). The arrow indicates onset of a 0–200 mM NaCl gradient (dashed line).

Table I. Purification of the two thioredoxins present in pig heart. Activities are expressed as stimulation of CDP reduction and of NADP-MDH-catalyzed oxaloacetate reduction, respectively. Activity determination prior to the heat denaturation steps is not possible due to inhibition of the test enzymes.

Purification step	Total protein [mg]	Total activity [nmol CDP · h^{-1}]	Specific activity [nmol CDP · h^{-1} · mg^{-1}]
Mitochondrial lysate[a]			
supernatant of heat denaturation	20.8	208	10
Sephadex G-75 chromatography	2.05	86	65
DEAE cellulose chromatography	0.22	85	500
Mitochondria-free homogenate[b]		[µmol NADP · min^{-1}]	[µmol NADPH · min^{-1} · mg^{-1}]
supernatant of heat denaturation	728	44.1	0.06
DEAE cellulose chromatography	143	33.1	0.23
Sephadex G-75 chromatography	2.8	21.3	7.6
CM-cellulose chromatography	0.78	10.2	13.0

[a] Washed mitochondria from 1.5 kg heart tissue were used.
[b] Obtained from 650 g tissue.

non-mitochondrial heart thioredoxin crossreacts with the plant enzyme. Further purification was achieved on DEAE cellulose and by gel filtration on Sephadex G-75 (Fig. 3). Chromatography of purified fractions on CM cellulose exhibited a second activity peak (Fig. 4). The size of the peak eluting at higher ionic strength increased in the absence of dithiothreitol in the buffer but it almost disappeared after preincubation of samples with 10 mM of the dithiol. Therefore, this material does not constitute an independent, additional thioredoxin but (partially) oxidized forms. When the two peaks were separated and rechromatographed in 1 mM dithiothreitol, the first one was again recovered as a double peak, and an obviously dimeric (M_r = 24,000) protein appeared on SDS-PAGE (Fig. 5, lane 11) whereas the fully reduced c-thioredoxin showed only one band (lane 4). The second peak lost activity altogether on rechromatography. This behaviour can be explained by the presence of more than two cysteine residues in the molecule *(vide infra)* and formation of intra- and intermolecular disulfide bonds.

Because only one thioredoxin, differing from mt-thioredoxin, is obtained in this purification scheme, that protein must constitute the major cellular, cytoplasmic (c-) thioredoxin. c-Thioredoxin from pig heart was enriched at least 200-fold. Its

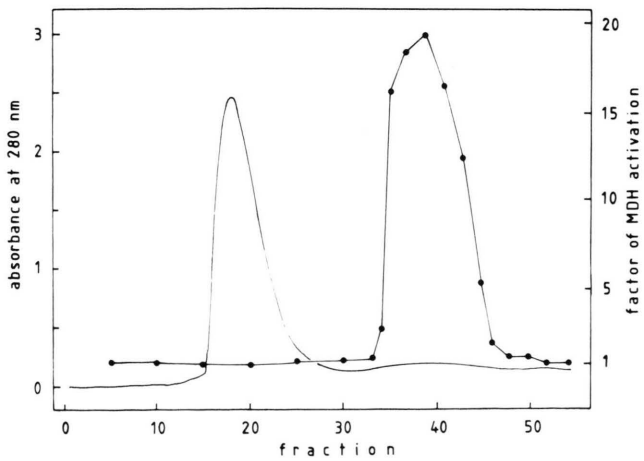

Fig. 3. Gel permeation chromatography of c-thioredoxin from pig heart on Sephadex G-75. Drawn line: Protein absorbance at 280 nm (left scale); ●: thioredoxin activity measured as NADP-MDH activation (right scale).

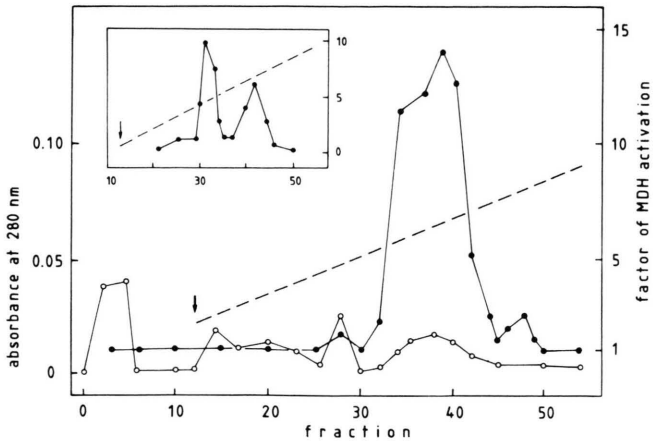

Fig. 4. Ion exchange chromatography of c-thioredoxin from pig heart on CM-cellulose at pH 4.6. ○: Protein absorbance at 280 nm (left scale); ●: thioredoxin activity measured as NADP-MDH stimulation (right scale). The arrow indicates onset of a 0–150 mM NaCl gradient (dashed line). Main panel: Chromatography in presence of 10 mM dithiothreitol; inset: activity distribution in dithiothreitol-free chromatography buffer.

purification is summarized in Table I and documented on the polyacrylamide electrophoresis gels shown in Fig. 5.

Chromatographic and activity differentiation of c- and mt-thioredoxin

Apart from the molecular differences of the two pig heart thioredoxins described below it was essential to verify that they can be safely distinguished by chromatography, because the organelle-free tissue extract will also contain proteins originating from broken organelles. Therefore, purified samples of both proteins were co-chromatographed (Fig. 6). The well-resolved pattern obtained confirms that different, specific thioredoxins were isolated in the preceding chromatography runs (Fig. 2 and 3).

An attempt was made to distinguish the heart thioredoxins immunologically; mt-thioredoxin could conceivably be related more closely to a bacterial *(E. coli)* and c-thioredoxin to another mammalian (rat liver) thioredoxin. However, no cross-reactivity of any sort was observed between the heart thioredoxins and antibodies against bacterial and rat liver thioredoxin in immunotitration with NADP-MDH, immunodiffusion tests, or during chromatography over immobilized antibodies.

The inactivity of heart mt-thioredoxin towards chloroplast MDH, and differential activities of mt- and c-thioredoxin with ribonucleotide reductase have been noted before [18]. In search of more

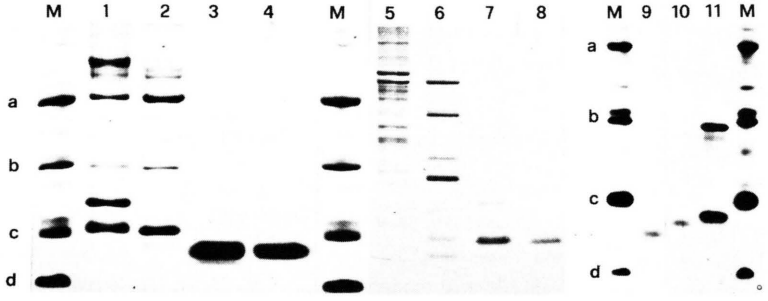

Fig. 5. Analysis of heart thioredoxins on SDS polyacrylamide gels. Marker proteins (M) from top to bottom; a, ovalbumin, $M_r = 45{,}000$; b, chymotrypsinogen, $M_r = 25{,}000$; c, cytochrome *c*, $M_r = 12{,}500$; d, aprotinin, $M_r = 6{,}500$. Left panel, lanes 1–4: Purification of c-thioredoxin. 1: Total-heat-stable proteins; 2: DEAE cellulose fraction; 3: Sephadex G-75 fraction; 4: CM cellulose fraction. Lanes 5–8: Purification of mt-thioredoxin. 5: mitochondrial lysate; 6: heat-stable fraction; 7: Sephadex G-75 fraction; 8: DEAE cellulose fraction. Right panel: Molecular weight of thioredoxins. Lane 9: *E. coli* thioredoxin; 10: heart mt-thioredoxin; 11: heart c-thioredoxin, not fully reduced.

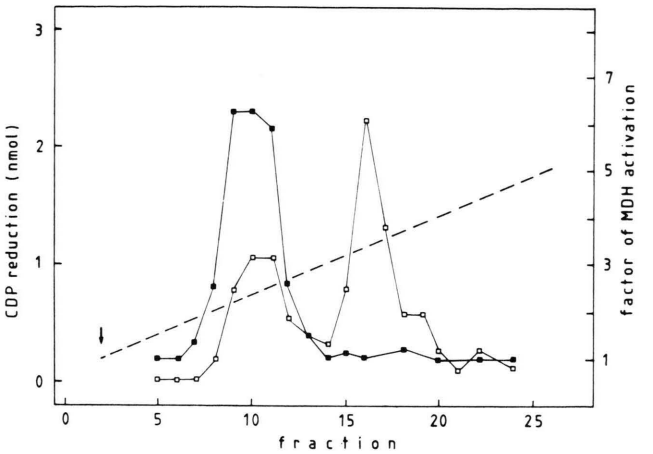

Fig. 6. Co-chromatography of purified samples of the two heart thioredoxins on DEAE cellulose. c-Thioredoxin elutes before mt-thioredoxin. ■: NADP-MDH activation (right scale); □: *E. coli* ribonucleotide reductase activity (left scale). Chromatographic conditions were as in Fig. 2.

physiological targets we have partially purified two enzymes from pig heart which had been linked to thioredoxin activation in other organisms, *viz.* NADP isocitrate dehydrogenase (in cyanobacteria, [35]) and carbamoylphosphate synthase (in rat liver, [11]). They were not stimulated by heart or by *E. coli* thioredoxins under a variety of conditions.

Mammalian cells contain another small dicysteine protein, glutaredoxin, which depends on reduced glutathione for activity but could be confused with thioredoxins under certain *in vitro* conditions. Using calf thymus glutaredoxin as positive control we have tested the heart thioredoxins for NADPH/glutathione reductase/glutathione-dependent transhydrogenase activity towards 2-hydroxyethyldisulfide [29]; very little, if any NADPH consumption was measured in these experiments. Furthermore an anti-glutaredoxin antibody did not precipitate the heart thioredoxins. These results rule out that we did purify a porcine glutaredoxin.

Molecular properties

Molecular weights of 12,000 and 12,300, respectively, were estimated for c-thioredoxin and mt-thioredoxin by SDS gel electrophoresis (Fig. 5). The two pig heart proteins also differ slightly but detectably in their isoelectric points, with mt-thioredoxin (IP 4.7) being more acidic than c-thioredoxin (IP 4.8) (*E. coli* thioredoxin: IP 4.5).

Table II. Amino acid composition of c-thioredoxin and mt-thioredoxin from pig heart.

Amino acid	c-thioredoxin	mt-thioredoxin
	[no. of residues]	
Ala	7	6
Arg	2	6
Asx	8	9
Cys[a]	6	2
Glx	16	16
Gly	9	9
His	1	1
Ile	3	2
Leu	12	13
Lys	6	8
Met	2	4
Phe	7	2
Pro	5	7
Ser	8	6
Thr	2	3
Trp[b]	not determined	
Tyr	4	6
Val	8	9
Total	106	109
calculated M_r[c]	12010	12360

[a] Determined as S-carboxymethyl cysteine.
[b] The absorption and fluorescence spectra indicate presence of at least 1 tryptophan residue.
[c] Without tryptophan.

The amino acid composition of both proteins is presented in Table II. The most striking difference is that mt-thioredoxin contains two cysteines whereas c-thioredoxin contains six cysteine residues. Thus, mt-thioredoxin resembles the microbial proteins in cysteine content and the cyto-

plasmic protein matches other mammalian thioredoxins which have 4–6 cysteines [2, 20, 36, 37]. With the exception of phenylalanine and arginine all other amino acids differ by only one or two residues. The small difference in molecular weight between the two proteins (Fig. 5) is confirmed in their amino acid analysis.

Thioredoxin reductase in pig heart

The reduced (di-SH) form of thioredoxins is required for most metabolic functions. It is generated by thioredoxin reductases on the expense of reduced nicotinamide coenzymes: these enzymes are flavoproteins related to glutathione reductase. Eukaryotic thioredoxin reductases are sometimes difficult to identify because they also catalyze unspecific reduction of disulfides in the absence of thioredoxin [31]. Using dithiobis(nitrobenzoate)- and insulin-coupled reduction assays we were able to demonstrate thioredoxin reductase activity in pig heart homogenates which had not been subjected to the heat denaturation step used in thioredoxin preparations. The enzyme was partially purified by the DEAE cellulose ion exchange, ADP-Sepharose affinity chromatography, and gel filtration steps described in the Experimental section. A molecular weight of about 70,000 was established which is in accord with data for thioredoxin reductases from various other sources [2, 38–40]. The specificities for NADPH and thioredoxin, but not glutathione, summarized in Table III also clearly qualify the pig heart protein as a typical eukaryotic thioredoxin reductase. However, as the heart muscle does not appear to be a particular rich source of enzyme, and the *in vitro* system did not exhibit selectivity for the homologous heart thioredoxin, no further characterization was attained at this stage. It has to remain open at present whether the mitochondria have the same, a different, or no thioredoxin reductase.

Discussion

Thioredoxins are widespread if not universally distributed in the living world. In mammals thioredoxin has been localized in many organs and subcellular fractions by radioimmunoassay and immunocytochemical techniques [30, 41, 42], in accord with the multitudinous metabolic functions. Nevertheless the distribution of thioredoxins appears to be uneven. High concentrations are found in liver, kidney, and thymus, preferentially in the cytoplasm and associated with cellular membranes, whereas bovine heart contained much less thioredoxin, and mitochondrial antibody labeling was scarce. The existence of thioredoxin in pig heart and heart mitochondria described in our present studies is not *per se* unusual: the demonstration of a specific mitochondrial protein, however, greatly expands our picture of the occurrence

Table III. Specificity of partially purified pig heart thioredoxin reductase.

Substrates (concentration)[a]		Enzyme activity [nmol NADPH oxidation · min^{-1}]
Dithiobis(nitrobenzoate) reduction		
NADPH		0
NADPH, DTNB		1.3
NADH (125 μM), DTNB		0.05
NADPH, GSSG (125–250 μM)		0.02
NADPH, DNTB, heart thioredoxin (4–20 μg)		1.3
Insulin reduction assay		
NADPH, insulin		0
same, + heart thioredoxin	(20 μg)	0.21
same	(60 μg)	0.61
same, + *E. coli* thioredoxin	(7 μg)	0.97
same,	(13 μg)	2.3

[a] Fixed concentrations were: NADPH, 125 μM (= saturating); DTNB, 25 μM; insulin, 60 μM. Other conditions: enzyme protein, 120 μg; 1 mM EDTA, pH 7.0, 25 °C.

and relation of the thioredoxin protein family in general.

Both new porcine proteins described here are fully characterized as thioredoxins by molecular weight, isoelectric point, and activity with ribonucleotide reductase. Their most significant structural difference is the cysteine content. The number of cysteines in mt-thioredoxin (2) and c-thioredoxin (6) is in agreement with a different, endosymbiotic origin of the mitochondrial protein. As the mammalian mitochondrial genome does not code for thioredoxin one expects (at least) two, differently regulated nuclear thioredoxin genes but such a genetic analysis has not yet been reported. Other mammalian thioredoxins of presumed cytoplasmic origin also possess more than two cysteines (rat, calf: 4; rabbit, human: 5) [2, 20, 36, 37]. Amino acid sequence determination of the new thioredoxins, for example by advanced mass spectrometric techniques [47] will allow homology comparisons in the future. If mt- and c-thioredoxin sequences in the same organism were quite dissimilar, this could explain the observed ambiguities in immunological studies [30, 42].

mt-Thioredoxin represents as much as 0.5% of the total soluble proteins in pig heart mitochondria. Its specific function is unknown and is hard to predict. Several dithiol-requiring reactions such as oxidative phosphorylation [43] and mitochondrial glycine metabolism [44] could be affected and have to be tested for thioredoxin involvement. As described above, two candidates, isocitrate dehydrogenase and carbamoylphosphate synthase could be excluded but the urea cycle is not an important pathway in heart mitochondria anyway. Rat cytochrome oxidase is also unaffected by mitochondrial or bacterial thioredoxin (D. Steverding, personal communication). Thioredoxin activates the CF_0-CF_1-ATPase γ-subunit in chloroplasts [45] but this effect is unlikely to have a counterpart in mitochondria because the corresponding mitochondrial ATPase subunit does not have a reactive disulfide bond. Although mitochondria possess their own deoxyribonucleotide pools for DNA synthesis [46] the existence of thioredoxin-requiring ribonucleotide reductase in the organelles is also unknown.

Pig heart cytosolic thioredoxin plus NADPH-thioredoxin reductase represent another typical mammalian thioredoxin system and therefore need not be discussed in detail. Because of the presence of six cysteine residues the protein can apparently exist in several forms which differ in the number of intra- and/or intermolecular disulfide bonds. Those with 0 or 1 cysteine bridges are active in enzyme stimulation and are separable on CM cellulose (Fig. 1) whereas activity appears to be lost in completely oxidized, probably denatured forms with 2–3 disulfide bonds. A similar behaviour, which may represent an extra control mechanism regulating cellular thioredoxin activities [36] has been described for calf thymus thioredoxin.

Acknowledgements

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