Free Radical Biology & Medicine, Vol. 24, No. 2, pp. 370–376, 1998

0891-5849/98 $19.00 + .00

ELSEVIER

PII S0891-5849(97)00216-5

Brief Communication

PURIFICATION OF MITOCHONDRIAL THIOREDOXIN REDUCTASE AND ITS INVOLVEMENT IN THE REDOX REGULATION OF MEMBRANE PERMEABILITY

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(*Received* 27 *March* 1997; *Accepted* 3 *June* 1997)

Abstract—The isolation to purity of a rat liver mitochondrial thioredoxin reductase is reported. The mitochondrial enzyme shows a chromatographic behavior different from that of the cytosolic enzyme. The purified enzyme, after sodium dodecylsulfate-polyacrylamide gel electrophoresis, yields a single band with a molecular weight of approximately 54 kDa. The apparent K_m for *E. coli* thioredoxin is about 13 μM, while the apparent K_m for 5,5′-dithiobis (2-nitrobenzoic acid) is 530 μM, values comparable to those reported for the cytosolic enzyme. Mitochondrial thioredoxin reductase, in addition to its natural substrate thioredoxin, is also able to reduce chemically unrelated compounds such as 5,5′-dithiobis (2-nitrobenzoic acid), selenite, and alloxan; the enzyme is inhibited by classical inhibitors of the cytosolic enzyme such as 1-chloro-2,4-dinitrobenzene and 13-*cis*-retinoic acid. A strong inhibitory action is also elicited by Mn^{2+} and Zn^{2+} ions. Thiol status appears critically involved in the control of membrane permeability and, therefore, a thiol/disulfide transition involving reduced pyridine nucleotides, matrix soluble thiols, and inner membrane thiols appears to play a fundamental role. The potential role of thioredoxin/thioredoxin reductase system in the control and redox regulation of the mitochondrial membrane permeability, is discussed.

Keywords—Permeability transition, Rat liver mitochondria, Redox control, Thiol groups, Thioredoxin, Thioredoxin reductase

INTRODUCTION

Mitochondria from animal sources contain several NAD(P)H-linked oxidoreductases distributed in the outer membrane, inner membrane, and in the matrix.[1] The reductases of the latter compartment are lipoyl dehydrogenase, glutathione reductase, and DT-diaphorase.[1] Recently, a mitochondrial matrix DTNB reductase, independent of the presence of glutathione and inhibited by arsenite,[2–5] has been recognized and shown to be a thioredoxin reductase.[3] The latter enzyme is widely distributed in bacteria, plants, and animals and, in the presence of NADPH, acts as a reducing equivalents donor to the ubiquitous small protein thioredoxin.[6] Mammalian thioredoxin reductase shows a dimeric structure of 58 kDa subunits, while the *E. coli* enzyme is a dimeric protein composed of 34 kDa subunits.[7,11]

Thioredoxin has been localized by radioimmunoassay in the cytosol and in most subcellular organelles of calf liver and thymus, including mitochondria.[8] A mitochondrial thioredoxin from pig heart has also been purified and characterized.[9,10] Therefore, the mitochondrion is endowed with the complete NADPH/thioredoxin reductase/thioredoxin system that, in addition to other functions as a donor of reducing equivalents, can also act as a general protein disulfide reductase.[6,11]

In the present article, the isolation to purity of a rat liver mitochondrial thioredoxin reductase is reported. The role of this enzyme is discussed with reference to a possible redox regulation of the permeability of the inner mitochondrial membrane.

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MATERIALS AND METHODS

Thioredoxin reductase was purified from rat liver mitochondria by a modification of the methods of Luthman and Holmgren[12] and Williams et al.[13] Rat liver mitochondria were isolated with differential centrifugation essentially as described by Myers and Slater[14] using a medium containing 220 mM mannitol, 70 mM sucrose, 5 mM Hepes (pH 7.0), 0.1 mM EDTA, and 5 mg/ml of bovine serum albumin. Protein of the mitochondrial preparation was measured with the biuret test.[15] Preparations of mitochondria (stored frozen at −20°C) were pooled to obtain about 5–6 g of proteins that were frozen and thawed three times and then subjected to sonic irradiation (three times for 20 s). Disrupted mitochondria were then centrifuged at 105,000 × *g* for 60 min and the clear supernatant fraction (mitochondrial matrix) was collected and submitted to ammonium sulfate fractionation. According to Williams et al.[13] the ammonium sulfate precipitation prior to heat treatment (see below) gives a better recovery of the activity. Ammonium sulfate precipitation was carried out in three steps of 30, 50, and 85% saturation, respectively; the pellets obtained were rapidly dissolved in 10 mM Tris/HCl (pH 7.5) with 1 mM EDTA and dialyzed overnight against the same buffer. Afterwards, fractions were heated up to 60°C and rapidly cooled at 4°C in an ice bath; the precipitate was removed by centrifugation at 105,000 × *g* for 3 h and the resulting supernatant was assayed for thioredoxin reductase (method 1, see below) and glutathione reductase.[16] The fraction obtained with 50% ammonium sulfate was enriched in thioredoxin reductase, while glutathione reductase was prevailing in the 85% ammonium sulfate fraction. This preliminary separation appears necessary in order to achieve a good separation in the DEAE cellulose chromatography. The latter was performed in a 2.5 × 15 cm column equilibrated in 10 mM Tris/HCl (pH 7.5), 1 mM EDTA and eluted with 300 ml of a NaCl gradient (0 to 0.3 M with a volume of 150 ml for each reservoir). Fractions of 4.5 ml were collected every 3 min and aliquots of each fraction (0.1 ml) were utilized for testing glutathione reductase activity[16] and thioredoxin reductase activity with method 1 (see below). At variance with the cytosolic form, thioredoxin and glutathione reductase were coeluted at 0.13–0.15 M NaCl. Proteins of the fractions obtained between 0.13 and 0.15 M NaCl were pooled and precipitated with 85% ammonium sulfate, resuspended in 50 mM Tris/HCl (pH 7.5), 0.1 mM EDTA and dialyzed overnight against the same buffer. The enzyme preparation was then chromatographed on 2′,5′-ADP Sepharose, applied to a 0.8 × 10 cm column equilibrated with 50 mM Tris, 0.1 mM EDTA (pH 7.5) and eluted with the indicated steps of phosphate and NaCl (Fig. 1). Thioredoxin reductase active fractions were eluted at 0.8 M NaCl, at variance with the cytosolic enzyme that was eluted at 0.2 M phosphate. After 2′,5′-ADP Sepharose chromatography the enzyme was separated from glutathione reductase. The peak fraction was concentrated in an Amicon pressure dialysis system using a YM/10 membrane with the addition of 0.2% octylglucoside to avoid loss of enzymatic activity. At this step of the preparation the enzyme was electrophoretically pure giving a single band on SDS-PAGE stained with Coomassie brilliant blue or silver. SDS-PAGE was performed according to Laemmli.[17] Proteins of the fractions collected from the columns and of the enzyme preparations were determined according to Lowry et al.[18]

Thioredoxin reductase activity was measured with three different assays essentially according to Luthman and Holmgren.[12] Method 1 was utilized for the measurement of thioredoxin reductase activity in the mitochondrial matrix and in the eluates obtained from the columns. With this method the enzyme activity was followed as the reduction of DTNB in the presence of NADPH. The assay mixture (1 ml) contained 0.2 M Na^+-K^+-phosphate buffer (pH 7.6), 1 mM EDTA, 0.25 mM NADPH, and 1 mM DTNB. The increase of absorbance at 412 nm was followed over 5 min at 25°C. The other two methods (2 and 3) are based on a coupled system where *E. coli* thioredoxin (from Sigma Chemical Co., St. Louis, MO), reduced by NADPH in the presence of thioredoxin reductase, in turn, reduces insulin. In method 2 the oxidation of NADPH is monitored at 340 nm and at 30°C. Thioredoxin reductase (about 10–12 μg protein) was incubated in a final volume of 0.5 ml in the following medium[19]: 50 mM phosphate buffer (pH 7.0), 20 mM EDTA, 0.25 mM NADPH, 16 μM thioredoxin, and 80 μM insulin. In method 3, thioredoxin reductase (about 1 μg protein) was incubated in a final volume of 120 μl containing 0.12 M Hepes/Tris buffer (pH 7.0), 3.4 mM EDTA, 0.85 mM NADPH, 30 μM thioredoxin, and 0.35 mM insulin. Samples were incubated at 37°C for the indicated times (Fig. 2, inset). Reactions were quenched by the addition of 0.5 ml of 6 M guanidine hydrochloride in Tris/HCl 0.2 M (pH 8) containing 10 mM EDTA and 1 mM DTNB. Reduced insulin formation was followed by the increase of absorbance at 412 nm.

RESULTS AND DISCUSSION

Table 1 gives a summary of the purification of thioredoxin reductase from rat liver mitochondria to apparent homogeneity. The enzyme exhibits a behavior completely different from that of the cytosolic enzyme

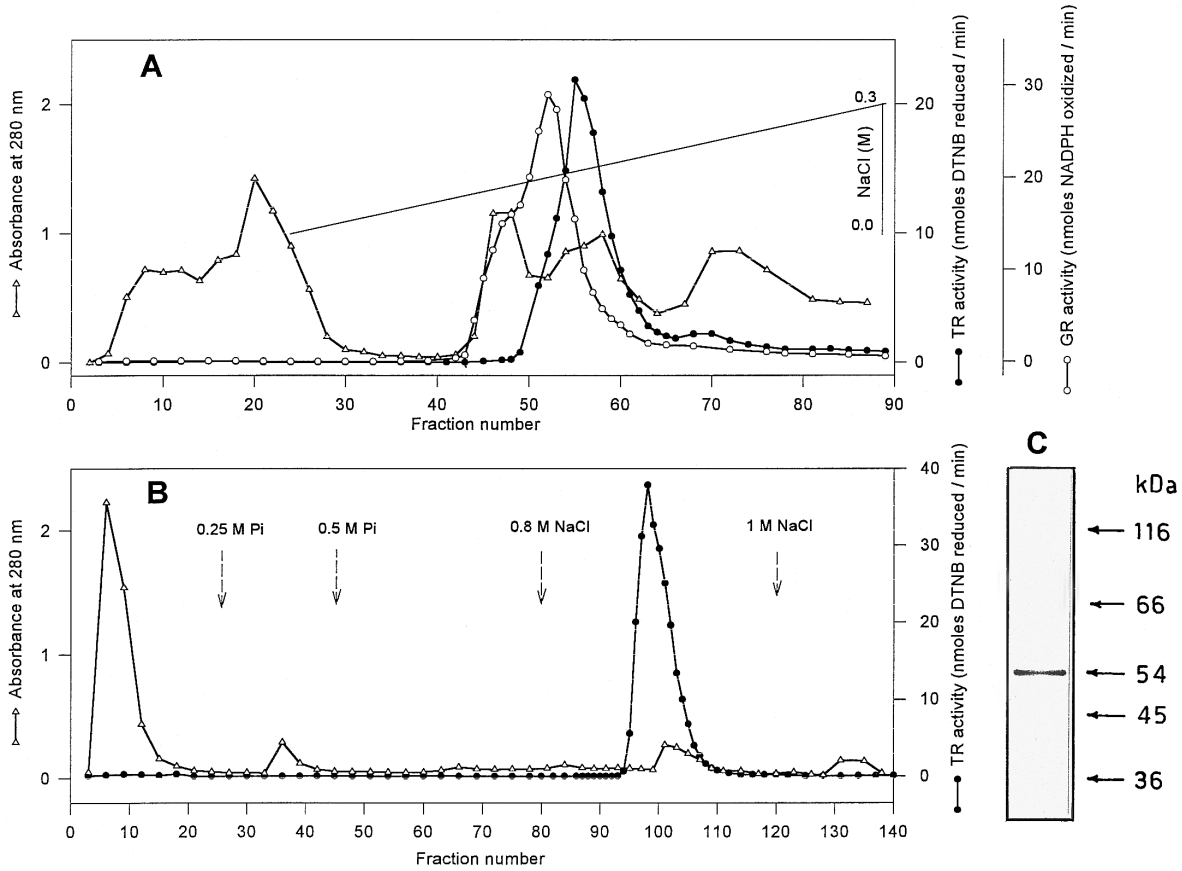

Fig. 1. Chromatographic purification of mitochondrial thioredoxin reductase. (A) DEAE-cellulose chromatography of ammonium sulfate precipitate. Column was eluted with a linear gradient of NaCl. Aliquots of 100 μl of each fraction were utilized for testing glutathione reductase activity (○) and thioredoxin reductase activity (●), the latter with DTNB reduction at 412 nm (method 1, see Materials and Methods). Absorbance at 280 nm (△). (B) 2′,5′-ADP Sepharose chromatography of thioredoxin reductase fractions obtained from DEAE cellulose column. Thioredoxin reductase active fractions (●) were eluted with 0.8 M NaCl. Absorbance at 280 nm (△). (C) SDS-PAGE of the mitochondrial thioredoxin reductase. The purified enzyme obtained from the 2′,5′-ADP Sepharose column was analyzed by SDS-PAGE followed by Coomassie brilliant blue staining. A single polypeptide band showing a molecular weight of about 54 kDa was detected. The details for the purification are reported under Materials and Methods.

both on DEAE-cellulose and 2′,5′-ADP Sepharose chromatography (Fig. 1A and B). Mitochondrial thioredoxin reductase and glutathione reductase are partially overlapping when eluted from the DEAE-cellulose column, while the cytosolic enzyme is completely separated from glutathione reductase, which is eluted at lower salt concentrations.[12] The purified enzyme was subjected to SDS-PAGE (Fig. 1C) under reducing conditions and shows a single band stained with Coomassie brilliant blue. A similar result was obtained after staining with silver (not reported). The obtained band shows a molecular weight of approximately 54 kDa that is a little lower than that reported for the mammalian cytosolic enzyme (57 kDa).[12] The mitochondrial enzyme, in addition to its ability to directly reduce DTNB, is also able to cause the reduction of insulin measured with two different assays as reported in Fig. 2, therefore confirming its specificity as a thioredoxin reductase. The apparent K_m for *E. coli* thioredoxin (calculated utilizing method 2) is about 13 μM, while the apparent K_m for DTNB in the presence of saturating concentration of NADPH is 530 μM; these values are close to those reported for the mammalian cytosolic thioredoxin reductase.[12,32]

Figure 3A shows that thioredoxin reductase, in addition to its natural substrate thioredoxin and DTNB, is also able to deliver reducing equivalents to different chemically unrelated compounds such selenite and alloxan; at variance with the cytosolic form, menadione appears almost inactive as a substrate for the mitochondrial thioredoxin reductase (not reported). In Fig. 3B the inhibitory effects of 1-chloro-2,4-dinitrobenzene (CDNB) and 13-*cis* retinoic acid are reported; both inhibitors appear to

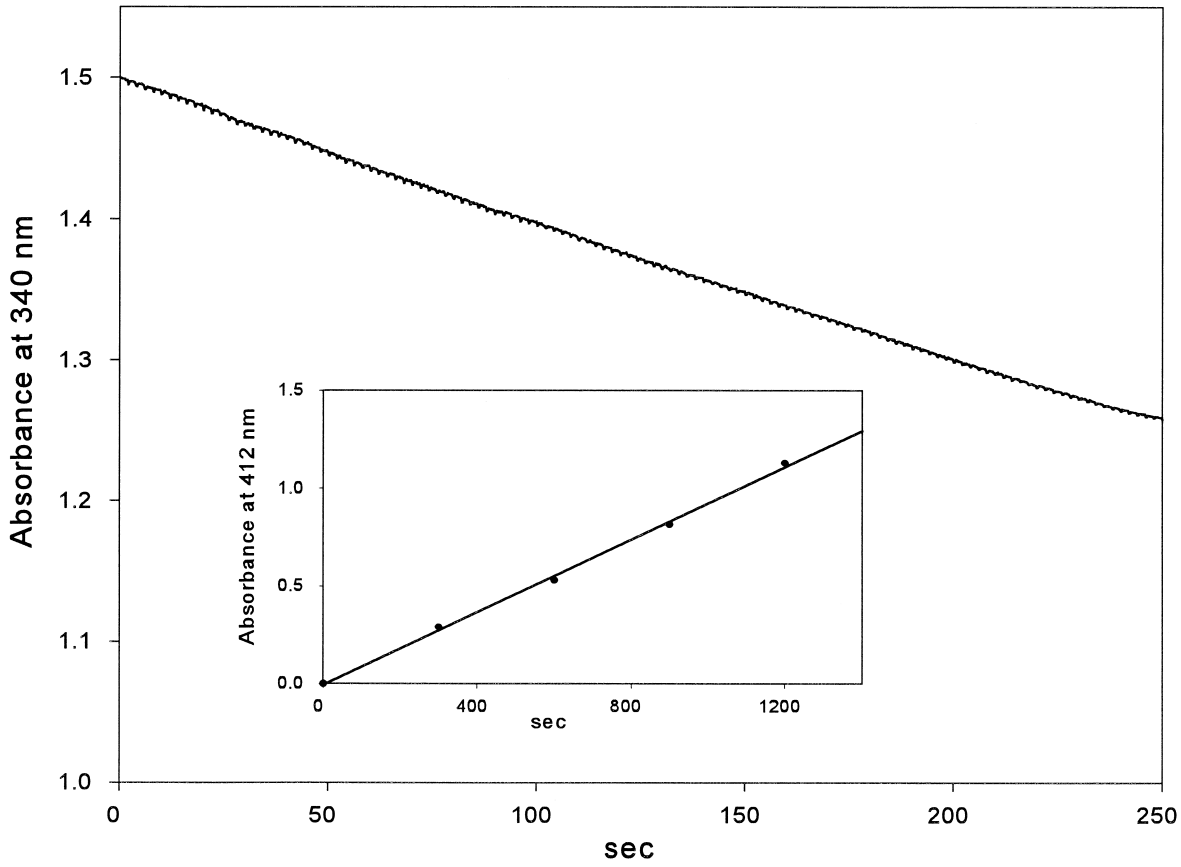

Fig. 2. Assay of mitochondrial thioredoxin reductase. The activity of the enzyme was followed as NADPH oxidation at 340 nm (method 2) and DTNB reduction at 412 nm (method 3; inset). Details are reported under Materials and Methods.

act by blocking a sulfhydryl group at the active site of the enzyme[7,33]; nevertheless, CDNB appears active at lower concentration as compared to 13-*cis* retinoic acid. A strong inhibitory effect on mitochondrial thioredoxin reductase is elicited by Mn^{2+} and Zn^{2+} ions (Fig. 3B, inset).

The permeability of the mitochondrial inner membrane can be increased by Ca^{2+} ions and several other factors including phosphate, thiol reagents, and oxidative stress-inducing agents.[20] This condition, also called ''permeability transition,'' is considered to depend on the opening of an ''unselective pore.''[20–23] Membrane thiol status is critically involved in the control of membrane permeability because a shift towards a more oxidized condition determines a general increase of the membrane permeability, while the opposite occurs in the presence of reducing agents. Therefore, the thiol/disulfide transition appears to play a fundamental role in controlling the permeability of the mitochondrial inner membrane[24–27] and involves reduced pyridine nucleotides, matrix soluble thiols, and inner membrane thiols.

Table 1. Purification of Thioredoxin Reductase from Rat Liver Mitochondria

	Protein (mg)	Total Activity (units)[a]	Specific Activity (units mg^{-1})
Mitochondria	5400	19170	3.55
Mitochondrial matrix	1920	11328	5.90
50% Ammonium sulfate	963	8185	8.5
Heated fraction	235	3995	17
DEAE cellulose column	30	2700	91.5
2′5′ ADP sepharose column	1	880	880

[a] Nanomoles of NADPH oxided per minute using the DTNB assay (method 1).

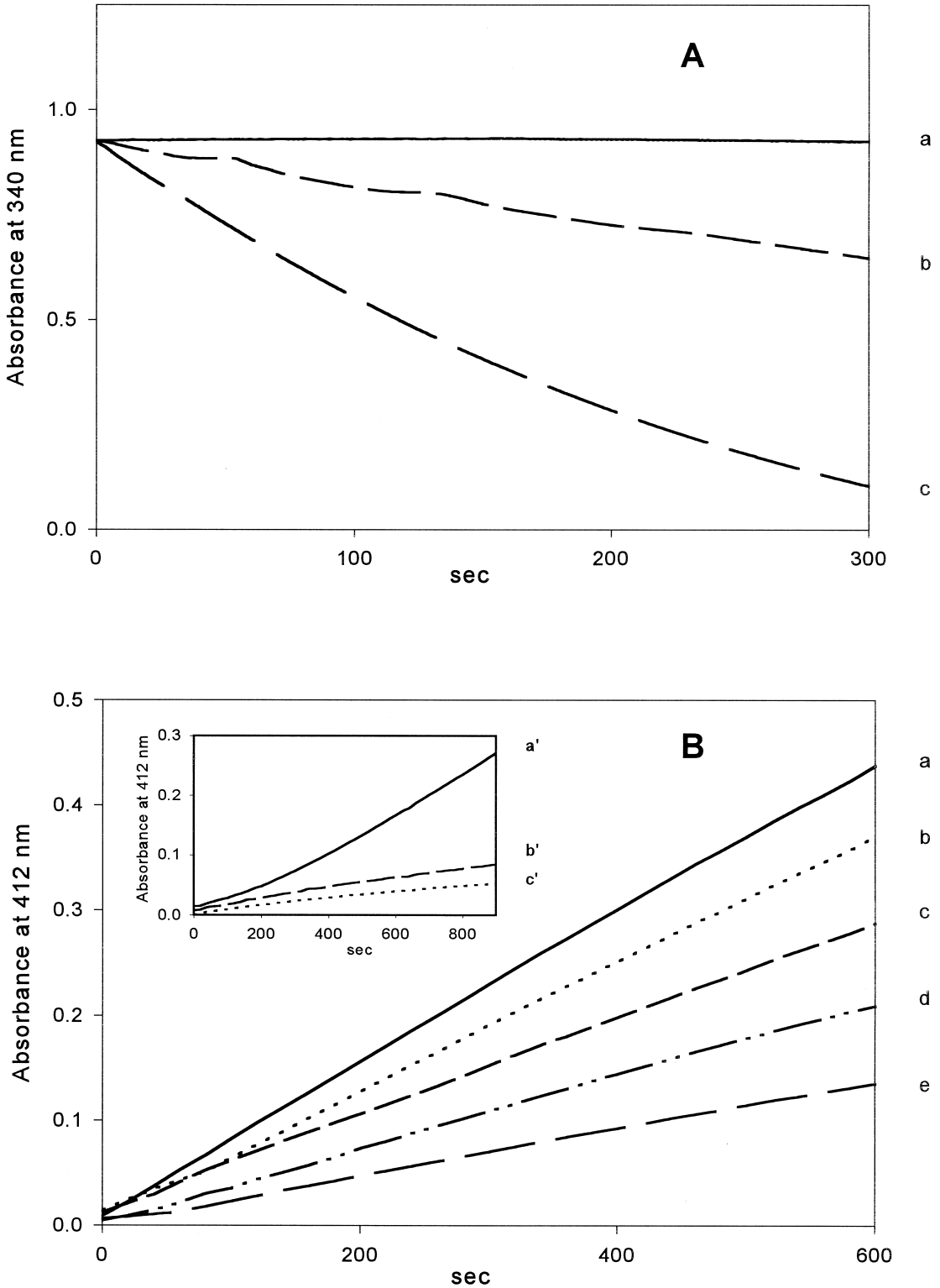

Fig. 3. Effect of different substrates (A) and inhibitors (B) on rat liver mitochondria thioredoxin reductase. The indicated substrates and inhibitors were incubated in 0.2 M Na^+-K^+ phosphate buffer (pH 7.6) containing 1 mM EDTA, 0.2 mM NADPH and 3–4 μg protein of thioredoxin reductase in a final volume of 0.7 ml and at 30°C. In (A), activity was followed by the decrease of absorbance at 340 nm. Substrates (1 mM) are: selenite (b) and alloxan (c); control, no addition (a). In (B) reaction was started by the addition of 1 mM DTNB and the activity was followed as the increase in absorbance at 412 nm (method 1). Inhibitors were: 25 μM CDNB (d), 50 μM CDNB (e), 100 μM 13-*cis* retinoic acid (b), 500 μM 13-*cis* retinoic acid (c); control, no addition (a). The inset reports the inhibitory effect of 50 μM $MnCl_2$ (b′) and 25 μM Zn acetate (c′) on mitochondrial thioredoxin reductase. Medium was as above but without EDTA; control, no addition (a′).

Thiol oxidizing agents such as diamide, *tert*-butyl hydroperoxide, or phenylarsine oxide are also able to enhance the binding of mitochondrial cyclophilin to inner membrane together with the stimulation of the ''pore'' opening.[28] Cyclophilin possesses a peptidyl-prolyl *cis-trans* isomerase activity, is inhibited by cyclosporin A and is able to interact with membrane protein components to induce ''pore'' opening.[23] On the contrary, conditions known to increase the mitochondrial NADH/NAD^+ ratio decrease the cyclophilin binding.[29]

Recently, Costantini et al.[30] reported that the mitochondrial membrane permeability transition is controlled at two sites; the first site is in apparent redox equilibrium with the pyridine nucleotides, while the second site, being a redox sensitive dithiol, is in equilibrium with the glutathione pool. Glutathione is considered the major soluble thiol of the matrix (5 mM), and appears to act mostly as a detoxifying agent towards H_2O_2. All the above reported indications suggest that also the thioredoxin/thioredoxin reductase system might have a role in the control and regulation of the mitochondrial membrane permeability because of its potential ability in reducing protein disulfides. In fact, thioredoxin, in association with thioredoxin reductase, constitute a redox system utilizing the reducing equivalents supplied by NADPH. In turn, the oxidation of the respiratory substrates allows a continuos rereduction of the pyridine nucleotides.

The present work describes the purification to homogeneity of a rat liver mitochondrial thioredoxin reductase that might be involved in the redox regulation of the mitochondrial membrane permeability. The chromatographic behavior of the mitochondrial enzyme is different from that of the cytosolic enzyme, and also the molecular weight of the monomer is slightly different from that of the cytosolic enzyme. Mitochondrial thioredoxin reductase, similarly to the cytosolic mammalian enzyme,[7] shows a rather broad substrate specificity because, in addition to thioredoxin and DTNB, also selenite and alloxan are good substrates, while menadione, at least in our conditions, is scarcely active. It is of interest that all these agents are able to induce swelling and Ca^{2+} release in mitochondria[20]; they might also act by oxidizing pyridine nucleotides and diverting electrons from the thioredoxin/thioredoxin reductase system. In addition, quinonoid compounds can also be easily reduced by the mitochondrial reducing systems[31] and thioredoxin can, therefore, play a role in this reductase activity. Mitochondrial thioredoxin reductase activity is inhibited by CDNB and 13-*cis* retinoic acid, the former reported to be efficient inhibitor of thioredoxin reductase obtained from the soluble fraction of human placenta[7,32] and calf thymus[7] and the latter of human melanoma tissue.[33] In addition, Mn^{2+} and Zn^{2+} ions also strongly inhibited the activity; this is in line with previous observations[3] indicating that in lysate of mitochondria a DTNB reductase activity is inhibited by these ions. Interestingly, according to Lenartowicz and Wudarczyk[3] the detection of thioredoxin reductase activity requires the presence of cation chelators during the preparation of mitochondria and the enzyme assay. This implies that mitochondrial physiological chelators such as ADP, ATP, and citrate might prevent the permeability transition by enhancing thioredoxin reductase activity and maintaining an intact balance of mitochondrial sulfhydryl groups.[3]

Acknowledgements — This work was supported by grants from National Research Council of Italy (CNR) and Progetto Finalizzato ''Invecchiamento.'' The authors wish to thank Professor L. Galzigna for the critical reading of the manuscript.

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ABBREVIATIONS

CDNB—1-chloro-2,4-dinitrobenzene
DTNB—5,5′-dithiobis (2-nitrobenzoic acid)
PAGE—polyacrylamide gel electrophoresis
SDS—sodium dodecyl sulfate