Distribution of protein disulphide isomerase in rat liver mitochondria

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Here we report the localization of protein disulphide isomerase (PDI) in the mitochondrial compartments, comparing it with that of thioredoxin reductase. The latter enzyme is present mostly in the matrix, whereas PDI is located at the level of the outer membrane. We characterize the different submitochondrial fractions with specific marker enzymes. PDI, whether isolated from whole mitochondria or from purified outer membranes, exhibits the same electrophoretic mobility, indicating identical molecular masses. Moreover, immunoblot analysis with monoclonal anti-PDI antibody shows immunoreactivity only with the microsomal PDI, indicating the specificity of the mitochondrial isoform. The significance of these findings is discussed with reference to the potential role of PDI and thioredoxin reductase in regulating the mitochondrial functions dependent on the thiol–disulphide transition.

Key words: mitochondrial outer membrane, redox regulation, thiol-disulphide oxidoreductases, thiol groups, thioredoxin reductase.

INTRODUCTION

Glutathione is largely present in mitochondria of various sources together with glutathione reductase and glutathione peroxidase [1,2]. Thioredoxin [3–5] and the related enzymes thioredoxin reductase (EC 1.6.4.5) [6–10] and peroxiredoxin [11,12] have also been localized in mitochondria. Protein disulphide isomerase (PDI; EC 5.3.4.1), a microsomal enzyme involved in the folding of proteins, belongs to the thioredoxin family [13–15] and can be reduced by both thioredoxin reductase and thioredoxin. A PDI was recently isolated from rat liver mitochondria [16]. However, no information was available on its distribution in the submitochondrial compartments. Here we report that mitochondrial PDI is located almost exclusively at the level of the outer membrane, whereas thioredoxin reductase is present essentially in the matrix. The potential functional significance of these findings is discussed.

MATERIALS AND METHODS

Preparation, purification on Percoll gradient and subfractionation of mitochondria

Rat liver mitochondria were isolated by differential centrifugation with the use of conventional procedures. The resulting pellet, representing the crude mitochondrial fraction, was resuspended in 220 mM mannitol/1 mM EDTA/25 mM Hepes (pH 7.0) and used for purification on a Percoll gradient. The procedure of Hovius et al. [17] was followed, with minor modifications. Mitochondria (5 ml; 60 mg/ml) were loaded in centrifuge tubes containing 45 ml of 30 % (v/v) Percoll in 225 mM mannitol/1 mM EGTA/25 mM Hepes (pH 7.4) and spun down at 95000 ***g*** for 30 min. Mitochondria were collected from the lower fraction (relative density 1.070–1.100 g/ml) and washed twice by centrifugation at 8000 ***g*** for 10 min.

Swelling and shrinking of mitochondria were performed as described by Sandri et al. [18], with minor modifications. The membrane fractions were separated by differential centrifugation essentially as described by Bandlow [19]. The first centrifugation was run at 6000 ***g*** for 10 min. Pellet I, formed of unbroken mitochondria, was discarded. The resulting supernatant was again centrifuged at 15000 ***g*** for 15 min and the resulting pellet (pellet II) essentially consisted of mitoplasts. The supernatant obtained was centrifuged at 65000 ***g*** for 30 min and yielded a pellet (pellet III) mostly containing inner membranes and the fraction corresponding to the contact sites between inner and outer membrane. The resulting supernatant was diluted with an equal quantity of medium containing 20 mM Tris, pH 7.5, and 0.25 M mannitol, then centrifuged at 105000 ***g*** for 60 min. The resulting pellet (pellet IV) contained outer membranes; the supernatant constituted the soluble fraction. The latter, essentially containing the enzymes of the intermembrane space and those of the matrix released from ruptured mitochondria and mitoplasts, was concentrated by an ultrafiltration system consisting of a YM/10 membrane (Amicon) under argon pressure. Pellets II–IV were further purified through a discontinuous sucrose gradient as described by Maisterrena et al. [20]. The protein contents of the homogenate, whole mitochondria and mitochondrial subfractions were measured by the biuret method [21].

Purification of PDI

Mitochondrial and microsomal PDI were purified as previously described [16]. The purified enzyme was essentially free of thioredoxin as tested by SDS/PAGE. PDI was also directly purified from the outer mitochondrial membrane as follows. Pooled fractions of outer mitochondrial membranes were frozen (−70 °C) and thawed three times in 0.1 M sodium phosphate buffer (pH 7.5)/5 mM EDTA/0.1 % (v/v) Triton X-100 containing a protease inhibitor cocktail ('Complete' Roche; 1 tablet in 50 ml of medium) and further homogenized in an Ultra-Turrax (Janke & Kunkel, Staufeu, Germany), three times for 10 s each. The resulting homogenate was centrifuged at 18000 ***g*** for 30 min; the pellet was discarded and the supernatant was dialysed against 25 mM citrate buffer, pH 5.3. Afterwards, the procedure described in [16] was followed. The protein contents of purified PDI and thioredoxin reductase were quantified by the method of Lowry et al. [22]. Bovine liver PDI, used as microsomal

Abbreviation used: PDI, protein disulphide isomerase.

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reference isoform, was obtained from Sigma (St Louis, MO, U.S.A.).

Enzyme assays

PDI activity was determined at 37 °C with a turbidimetric assay depending on insulin precipitation and measured spectrophotometrically at 650 nm [23]. The enzyme preparation was preincubated for 20 min at 37 °C in 1 ml of 0.2 M sodium/potassium phosphate buffer, pH 7, containing 2 mM EDTA and 1 mM dithiothreitol; the reaction was initiated by the addition of 0.130 mM insulin. Enzyme activity was expressed as the change in A_{650} after 15 min of incubation. The results were corrected for the non-enzymic reduction of insulin due to dithiothreitol and measured in samples lacking the enzyme preparation. Thioredoxin reductase activity was measured by the method of Luthman and Holmgren [24] at 25 °C in 0.2 M Tris/HCl (pH 8.1)/50 mM EDTA/0.25 mM NADPH/1 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The absorbance was followed spectrophotometrically at 412 nm.

To assess the purity of the obtained fractions, the following marker enzymes were tested: monoamine oxidase [25], NADPH:cytochrome *c* reductase (EC 1.6.99.1) [26], succinate dehydrogenase (EC 1.3.99.1) [27] and glutathione S-transferase (EC 2.5.1.18) [28]. All these assays were performed at 30 °C.

SDS/PAGE and Western blotting

SDS/PAGE was performed by the method of Laemmli [29]. Membrane fractions to be tested for PDI immunoreactivity were separated by SDS/PAGE under reducing conditions, transferred electrophoretically to a nitrocellulose membrane [30] and incubated with a monoclonal antibody (Stress Gen Biotechnologies, Victoria, BC, Canada). The enzyme was detected with an enhanced chemiluminescence detection system (ECL®; Amersham, Little Chalfont, Bucks., U.K.), using the horseradish-peroxidase-catalysed luminol chemiluminescence detected by a light-sensitive autoradiography film.

RESULTS AND DISCUSSION

The integrity of the mitochondrial membranes involves both the permeability functions and the activities of the associated enzymes and depends not only on the glutathione redox systems but also on the thioredoxin redox system [1–10]. We have previously observed that, after the induction of mitochondrial pyridine nucleotide oxidation under specific conditions, a decrease in thiol groups, not involving glutathione, is apparent [31], indicating the existence of a redox link in which pyridine nucleotides, matrix thiols and membrane thiols are involved. We also observed that mitochondria show a marked disulphide reductase activity in the presence of low-molecular-mass disulphides [32]. All these observations prompted us to investigate the presence of the enzyme thioredoxin reductase in mitochondria [6]; afterwards, in exploring the protein targets of thioredoxin reductase, we also found the enzyme PDI in rat liver mitochondria [16]. Both enzymes have been purified and characterized [6,16]. However, their relative distributions in the mitochondrial subcompartments seemed to be of major importance for the purpose of defining their functions in the mitochondrial physiology.

To find out more precisely the submitochondrial localization of PDI and thioredoxin reductase, their activities were measured in various subfractions characterized by specific marker enzymes (Table 1). Before subfractionating mitochondria, the Percoll purification procedure was adopted so as to limit, as far as possible, the contamination from the endoplasmic reticulum. After a mitochondrial swelling/shrinkage/sonication procedure, the suspension was subjected to differential centrifugation to obtain a crude separation of the various subfractions. Table 1 shows the relative specific activities of marker enzymes in the mitochondrial subfractions. As is apparent, the fraction corresponding to the mitoplasts (pellet II) is particularly enriched with succinate dehydrogenase, whereas in the 'contact sites' fraction (pellet III) there is a decrease in succinate dehydrogenase activity and a corresponding increase in monoamine oxidase (EC 1.4.3.4) and glutathione S-transferase activities. The increase in activity of the latter two enzymes is clearly evident in the outer-membrane fraction, whereas succinate dehydrogenase activity is strongly decreased. When the outer-membrane fraction was further purified through a discontinuous sucrose gradient, there was a marked increase in monoamine oxidase activity. All of the above-mentioned enzyme activities are almost absent from the soluble fraction. NADPH:cytochrome *c* reductase is a typical microsomal marker; in gradient-purified mitochondrial membranes its activity was less than one-tenth that of microsomes, indicating that the microsomal contamination was very low in our mitochondrial preparations. Moreover, this should be considered to be an upper limit of microsomal contamination because an NADPH:cytochrome *c* reductase is also present in the mitochondrial outer membrane [33].

Table 1 Relative specific activities of typical marker enzymes in mitochondria and purified submitochondrial fractions from rat liver

The figures compare the specific activities of NADPH:cytochrome *c* reductase in mitochondria, submitochondrial fractions and microsomes. The preparation of mitochondria, submitochondrial and microsomal fractions and the assays of marker enzymes were as described in the Materials and methods section. Monoamine oxidase (MAO), succinate dehydrogenase (SDH) and glutathione S-transferase (GST) activities were measured in the presence of 50, 40 and 35 μg of protein respectively. NADPH:cytochrome *c* reductase activity was measured in the presence of 50 μg of mitochondrial or submitochondrial fraction protein and 10 μg of microsomal protein. For MAO, SDH and GST the specific activities (nmol/min per mg of protein) are reported relative to the whole mitochondrial fraction; NADPH:cytochrome *c* reductase activities are reported as nmol/min per mg of protein. Results are means ± S.D. ($n = 8$).

Fraction	Relative specific activity			NADPH:cytochrome *c* reductase activity (nmol/min per mg)
	MAO	SDH	GST	
Mitochondria	1	1	1	5.23 ± 1.21
Pellet II	0.74 ± 0.16	2.31 ± 0.40	1.33 ± 0.22	6.02 ± 0.8
Pellet III	1.41 ± 0.23	1.82 ± 0.20	1.49 ± 0.21	12.51 ± 1.1
Pellet IV	6.52 ± 1.15	0.43 ± 0.08	2.93 ± 0.34	18.45 ± 0.42
Pellet IV (gradient purified)	15.04 ± 1.52	0.27 ± 0.25	3.01 ± 0.1	14.23 ± 0.65
Soluble fractions	0.43 ± 0.02	0.05 ± 0.02	0.89 ± 0.07	–
Microsomes	–	–	–	150 ± 6.9

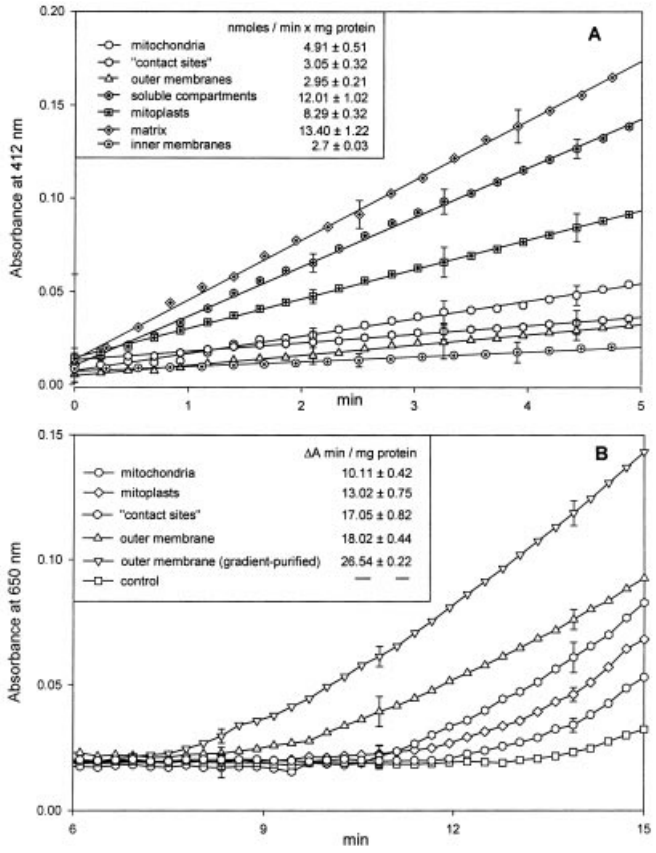

Figure 1 Estimation of the activities of thioredoxin reductase (A) and PDI (B) in mitochondria and submitochondrial fractions

Thioredoxin reductase (200 μg of protein) was tested with the 5,5′-dithiobis-(2-nitrobenzoic acid) reduction method; PDI (350 μg of protein) was estimated with the turbidimetric assay as described in the Materials and methods section.

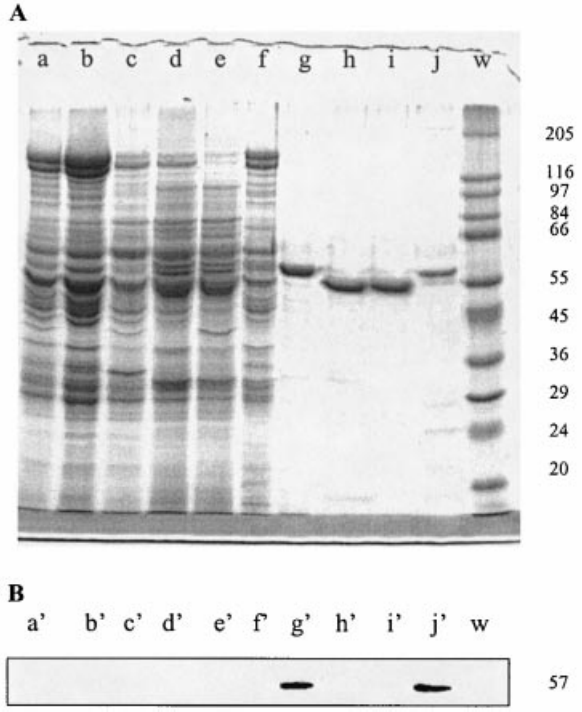

Figure 2 SDS/PAGE of proteins of mitochondria and submitochondrial fractions (A) and immunoblot analysis of the same fractions (B)

(**A**) Proteins were separated by SDS/PAGE [10% (w/v) gel] and stained with Coomassie Brilliant Blue. Lane a, whole mitochondria; lane b, mitoplasts (pellet II); lane c, 'contact sites' (pellet III); lane d, outer membrane (pellet IV); lane e, outer membrane after gradient purification; lane f, soluble fraction; lane g, PDI from bovine liver; lane h, PDI from the outer membrane; lane i, PDI from whole mitochondria; lane j, PDI from rat liver microsomes; lane w, molecular mass markers (molecular masses are indicated at the right, in kDa). The amounts of protein loaded were 10 μg for mitochondria and submitochondrial fractions, and approx. 1 μg for the isolated enzymes. (**B**) Proteins of the various submitochondrial fractions, separated by SDS/PAGE, were subjected to Western blotting with a monoclonal anti-PDI antibody. Immunocomplexes were detected by the use of a horseradish-peroxidase-conjugated secondary antibody and the enhanced chemiluminescence technique. Lanes are labelled to correspond to those in (**A**).

The various preparations obtained after subfractionation of mitochondria were used to estimate the activities of the mitochondrial isoforms of thioredoxin reductase and PDI. Thioredoxin reductase exhibits a marked activity in the soluble compartments (matrix and intermembrane space), whereas purified membrane fractions (contact sites, inner and outer membranes) are poorly active (Figure 1A). The mitoplast fraction contains a high activity because it includes the matrix fraction, which is rich in thioredoxin reductase. Considering the activity of PDI (Figure 1B), a completely different picture emerges because the highest activity was found in the fraction corresponding to the outer membranes. This activity showed a further increase when outer membranes were purified through sucrose gradient. Some activity was also found in the contact sites fraction because these sites are formed by apposition of the inner and outer membranes. The preparations obtained after differential and gradient centrifugation were subjected to SDS/PAGE and, as is apparent in Figure 2(A), the bands corresponding to PDI isolated from whole mitochondria (lane i) and PDI isolated from the outer membrane (lane h) exhibit the same electrophoretic mobility, indicating an identical molecular mass of approx. 54 kDa, as reported previously [16]. Moreover, in Figure 2(B) the immunoblot analysis of the various fractions with a monoclonal anti-PDI antibody shows that the antibody reacts only with proteins of the lanes corresponding to PDIs from bovine liver (g′) and from rat liver microsomes (j′), again indicating the specificity of the mitochondrial isoform.

In the present paper we report evidence that thioredoxin reductase is localized mostly in the matrix space, whereas PDI is localized in the outer membrane. Both enzymes are probably involved in the redox regulation of mitochondrial functions and might co-operate in the maintenance of thiol homoeostasis. Taking into account their redox potentials, thioredoxin reductase seems to exert essentially a reducing action, whereas mitochondrial PDI could act as the oxidizing counterpart. These considerations raise some analogies with the *Escherichia coli* Dsb enzymes, which are a family of thiol:disulphide oxidoreductases comprising several different proteins endowed with oxidizing (DsbA and DsbB) or reducing (DsbD and DsbE) actions towards the thiol–disulphide couple [34].

The major site of localization of PDI in the cell is the lumen of the endoplasmic reticulum, where it assists in the folding of newly synthesized proteins [13–15]. However, it was shown to be present at the surface of plasma membrane of different cell types such as platelets [35], B lymphocytes [36], pancreatic cells [37], hepatocytes [38] and thyroid cells [39], where it is involved in several functions essentially linked to transport processes. The presence of a PDI in the mitochondrial outer membrane could

contribute to some extent to the assembly and functioning of the enzyme systems endowed with different functions, including pore-forming proteins, receptors devoted to protein import and the systems involved in the mitochondrial-dependent control of cell death such as pro-apoptotic members of the Bcl-2 family.

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