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Distribution of phospholipid hydroperoxide glutathione peroxidase (PHGPx) in rat testis mitochondria

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Abstract

The distribution of phospholipid hydroperoxide glutathione peroxidase (PHGPx) in isolated rat testis mitochondria was investigated, using a reverse sucrose density gradient centrifugation procedure for the separation of the inner and outer membranes and the contact sites between the two membranes. The results indicate that PHGPx is largely localized in the contact sites fraction. This finding might therefore suggest that the enzyme has more than just an antioxidant function.

Key words: Mitochondrion; Phospholipid hydroperoxide; Glutathione peroxidase; Selenium; Glutathione; (Rat testis)

1. Introduction

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) belongs to the super-family of selenium-dependent peroxidases: while the 'classical' glutathione peroxidase (GPX) and its variants are tetrameric and use small hydrophilic peroxidic substrates, PHGPx is monomeric and higly specific for hydroperoxy derivatives of lipids inserted in natural or artificial membranes [1–3].

In the complex mechanism of the lipid peroxidation, the propagation of the peroxidative chain is efficiently inhibited by the sinergistic role of both PHGPx and vitamin E, being the hydroperoxides, generated through the scavenging of the peroxyl radicals by the vitamin, reduced by the enzyme [4].

The distribution of the PHGPx in various mammalian tissues has been already studied and described [3]: interestingly, the authors found that PHGPx activity in rat testis is at least two orders of magnitude higher than in other tissues, and that it is gonadotropin-dependent. Moreover, the enzymatic and immunologic localization of the enzyme, mainly in mitochondria and in the nuclear envelope of maturating spermatogenic cells [3], points to an involvement of PHGPx far beyond its antioxidant capacity [5].

On the basis of these findings, data concerning the occurrence of PHGPx in isolated outer and inner membranes and contact sites of rat testes mitochondria are presented. The results obtained indicate that PHGPx is a mitochondrial membrane-bound enzyme with peculiar localization in the contact sites between the two membranes.

2. Materials and methods

Rat testis mitochondria were prepared from 2-month-old male albino rats essentially according to Sulimovici et al. [6], with minor modifications, aimed to keep the mitochondria intact: 0.5% BSA, 2 mM Hepes (pH 7.4), 1 mM EGTA (pH 7.4) and 0.1 mM PMSF were routinely added to the homogenization medium and 0.5% BSA and 0.1 mM PMSF to the washing medium.

The further fractionation of swollen, shrunken and sonicated mitochondria was performed according to Sandri et al. [7]. Mitochondrial subfractions obtained after a reverse sucrose density gradient centrifugation [7] were indicated as IB (outer membrane), IIB (contact sites) and P (inner membrane).

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Abbreviations: BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

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The contact sites (about 1.5 mg protein/ml) and the inner membrane (about 6 mg protein/ml) fractions were further processed by 7-fold and 40-fold dilutions, respectively, in 5 mM Tris-HCl buffer (pH 7.4), followed by a sonication (10 and 20 s, Branson Sonifier equipped with a micro tip, operating at 2.8 A) and a centrifugation at $200\,000 \times g$.

The activities of the following marker enzymes were measured at 30°C according to the methods used in the references given: rotenone-insensitive NADH–cytochrome-*c* reductase (EC 1.6.99.3) [8]; succinate–cytochrome-*c* reductase (EC 1.3.99.1) [8]; malate dehydrogenase (EC 1.1.1.37) [8]; creatine kinase (EC 2.7.3.2) [9]. PHGPx was assayed according to Maiorino et al. [1].

Protein content was measured by means of the bicinchoninic acid method [10].

All the operations were performed at 4°C, using chemicals of the highest available purity grade.

3. Results and discussion

Table 1 shows data concerning the specific activity of PHGPx in mitochondria from testis, brain and liver. The activity found in rat testes mitochondria is close to that reported by Roveri et al. [3] and is two orders of magnitude higher of that one reported by the same authors for rat liver mitochondria and that determined by us in brain cortex mitochondria.

Fig. 1 shows the submitochondrial distribution and the specific activities, relative to the whole mitochondria, of the marker enzymes and of PHGPx. The histograms clearly indicate the following situation: the rotenone-insensitive NADH–cytochrome-*c* reductase (marker of the outer membrane) is enriched in the IB fraction; the succinate–cytochrome-*c* reductase (marker of the inner membrane) is enriched in the P fraction; the creatine kinase, as well as hexokinase (data not shown), which are markers of the junctions between the two membranes [11], show a pronounced enrichment in the IIB fraction (contact sites), which, as expected, contains also part of both rotenone-insensitive NADH-cytochrome-*c* reductase and succinate–cytochrome-*c* reductase activities, originating from outer and inner membrane respectively.

Table 1
Specific activity of PHGPx in mitochondria isolated from different tissues

Mitochondria	nmol/min per mg protein
Rat testis	108.20 ± 11.95 *
Rat brain cortex	1.27 ± 0.66 *
Rat liver (ref. 3)	2.30 ± 0.20

PHGPx activity was measured as described by Maiorino et al. [1].
* Means ± S.D. of at least five experiments.

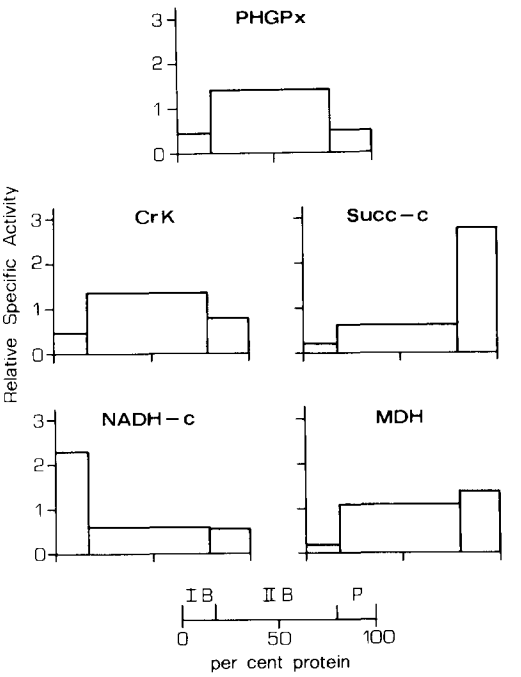

Fig. 1. Distribution pattern of marker enzymes and PHGPx after subfractionation of rat testes mitochondria according to Sandri et al. [7]. Abscissa: percentage of proteins recovered in the single fraction: first band (IB, outer membrane), second band (IIB, contact sites) and sediment (P, inner membrane). Ordinate: specific activity relative to that of mitochondria. Crk, creatine kinase; Succ.-c, succinate–cytochrome-*c* reductase; NADH-c, rotenone-insensitive NADH–cytochrome-*c* reductase; MDH, malate dehydrogenase; PHGPx, phospholipid hydroperoxide glutathione peroxidase.

It is important to note that, at this stage of separation, the soluble malate dehydrogenase activity, marker of the matrix content, is still present mainly in the vesicles constituted by the contacts (IIB), and the inner membrane (P).

The distribution pattern of the PHGPx is practically identical to that of creatine kinase; however, this fact alone does not yet allow us to attribute the PHGPx activity to the contact sites. The question is still open as to whether the enzyme, as well as the creatine kinase for instance, is soluble in the matrix content entrapped in the contacts and in the inner membrane vesicles, or whether it is bound to membranes.

To solve this question, both contact sites (IIB) and inner membrane (P) fractions were subjected to a further processing as described in Materials and methods.

Fig. 2 shows the histograms which refer to the matrix content (soluble) and the membrane moiety of the contact sites (m) obtained from the diluted, sonicated and spun original IIB fraction. The distribution of succinate–cytochrome-*c* reductase and creatine kinase activities clearly indicates that both the enzymes were recovered in the membranes. As expected, the bulk of malate dehydrogenase became soluble. The

PHGPx pattern, identical to those of succinate–cytochrome-*c* reductase and creatine kinase, thus indicates a contact sites-bound localization for the enzyme. From data shown in Figs. 1 and 2, the amount of PHGPx present in this membrane domain can be evaluated in terms of about 75% of the total activity measured in intact mitochondria.

Fig. 3 shows the results obtained by a similar procedure carried out with the inner membrane (P) fraction. Again, the matrix content, monitored by malate dehydrogenase, was set free in the supernatant (soluble), whereas the same pattern of both succinate-cytochrome-*c* reductase and PHGPx indicates that the latter remains bound to the matrix-free inner membrane (m). The percentage of the PHGPx bound to this membrane accounts for about 15–20% of the total mitochondrial PHGPx, taking into account the slight contamination of outer membrane and contacts still present in the P fraction.

In conclusion, submitochondrial localization of PHGPx in rat testis appears to be rather peculiar. Moreover, this localization hardly fits with just an antioxidant function, which would be expected to be more diffusely localized. On the other hand, the presence of this enzyme in the contact sites between outer and inner membrane might be reminiscent of a function in some way related to molecule trafficking between intra- and extra-mitochondrial space.

The recent observation in the gene of PHGPx of regulatory elements related to hormonal signalling (Ursini, F., personal communication) fits this still undefined function, apparently linked to metabolic control. In this light, the enzyme could be a key regulatory element of either the peroxide tone [12] in a specific mitochondrial site, or of the redox status of thiols in specific proteins. In other words, the submitochondrial localization of PHGPx supports the already proposed function of the enzyme as a very specific modulator of the redox status [5].

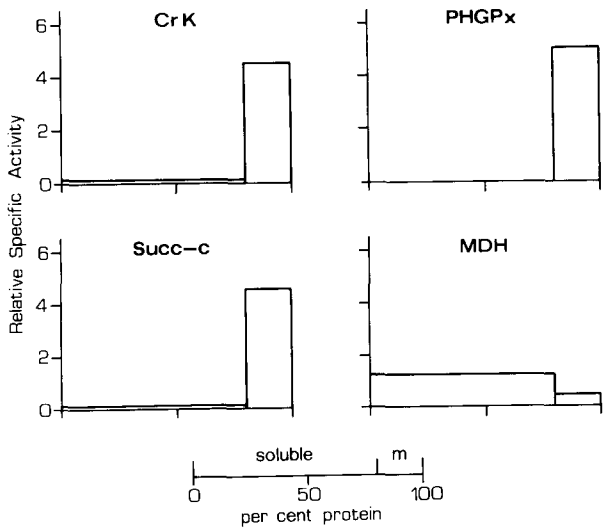

Fig. 2. Distribution pattern of marker enzymes and PHGPx after fractionation of the IIB fraction obtained by reverse density gradient centrifugation of rat testes mitochondria (see Fig. 1). Experimental conditions as indicated in Materials and methods. Abscissa: percentage of proteins recovered in the single fraction: supernatant (soluble) and sediment (m). Ordinate: specific activity relative to the original IIB fraction. Marker enzymes as in Fig. 1.

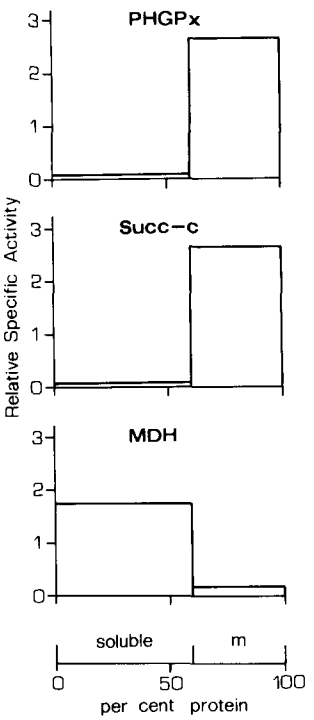

Fig. 3. Distribution pattern of marker enzymes and PHGPx after fractionation of the P fraction obtained by reverse density gradient centrifugation of rat testes mitochondria (see Fig. 1). Experimental conditions as indicated in Materials and methods. Abscissa: percentage of proteins recovered in the single fraction: supernatant (soluble) and sediment (m). Ordinate: specific activity relative to the original P fraction. Marker enzymes as in Fig. 1.

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