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Distribution of glutathione peroxidases and glutathione reductase in rat brain mitochondria

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The distribution of glutathione reductase (GR), glutathione peroxidase (GPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) in isolated rat brain mitochondria was investigated, using a fractionation procedure for the separation of inner and outer membranes, contact sites between the two membranes and a soluble fraction mainly originating from the mitochondrial matrix. The data indicate that GR and GPx are concentrated in the soluble fraction, with a minor portion of the two enzymes being associated with the contact sites. PHGPx is localized largely in the inner membrane. The possible functional significance of these findings is discussed.

Glutathione reductase; Glutathione peroxidase; Phospholipid hydroperoxide glutathione peroxidase; Mitochondria; Contact site; Rat brain

1. INTRODUCTION

The role of both mitochondrial glutathione peroxidase (GPx, E.C. 1.11.1.9) and glutathione reductase (GR, E.C. 1.6.4.2) in the removal of H_2O_2 of mitochondrial origin is well-recognized [1]. Apart from the superoxide radical and H_2O_2 generated by the respiratory chain and superoxide dismutase [2–6], H_2O_2 is also produced by the two forms of monoamine oxidase (E.C. 1.4.3.4) localized in the outer mitochondrial membrane [7–9]. We have recently investigated this type of H_2O_2 production in rat brain mitochondria [10], chiefly from the point of view of changes in glutathione levels connected with H_2O_2 accumulation, and with the availability of the reducing power necessary for the GR and GPx activities. Our evidence indicated that the localization of the two enzymes is not only the matrix, as has been reported [11–13], but may be both the matrix and the intermembrane space, as demonstrated by other authors [14,15]. We found that both intra- and extramitochondrial reduced glutathione (GSH) and oxidized glutathione (GSSG) plus NADPH can react with mitochondrial GPx and GR respectively, suggesting that at least a fraction of the two enzymes may be located at the contact sites between the two membranes. In addition, brain tissue contains the enzyme phospholipid hydroperoxide glutathione peroxidase (PHGPx, E.C. 1.11.1.9), which has been reported to be heterogeneously distributed in different membranes [16–18].

Since the distribution of these enzymes has not yet been described in brain mitochondria, we are here presenting data concerning the occurrence of GR, GPx and PHGPx in isolated outer and inner membranes and contact sites, comparing their location with those of well-established marker enzymes for these fractions. The results obtained are consistent with the suggested location of a fraction of GR and GPx in the contact sites between the inner and outer membranes of these mitochondria. Moreover, the data indicate that a major part of the PHGPx in brain mitochondria is associated with the inner membrane.

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2. MATERIALS AND METHODS

Non-synaptosomal rat brain mitochondria were prepared from two-months-old male albino rats by the procedure of Rehnerona et al. [19].

The fractionation of swollen, shrunken and sonicated mitochondria was performed according to Sandri et al. [20]. The fractions, indicated as soluble (S), outer membrane (IB), contact sites (IIB) and inner membrane (P) were obtained after a reverse sucrose density gradient centrifugation [20].

The contact-site fraction (IIB) was further processed by 5-fold dilution in 5 mM Tris-HCl buffer (pH 7.4), followed by a brief sonication (5 s at 0°C, Branson Sonifier equipped with a micro tip, operating at 2.8 A).

The inner membrane fraction (P) was further processed by 30-fold dilution in 5 mM Tris-HCl buffer (pH 7.4), followed by sonication for 5 s under the same conditions as described above.

The activities of the following enzymes were determined according to the methods quoted in parentheses: rotenone-insensitive NADH-cytochrome *c* reductase (E.C. 1.6.99.3) [21]; succinate cytochrome *c* reductase (E.C. 1.3.99.1) [21]; malate dehydrogenase (E.C. 1.1.1.37) [21]; creatine kinase (E.C. 2.7.3.2) [22]; glutathione reductase [23]; glutathione peroxidase (with H_2O_2 as substrate) [24]; phospholipid hydroperoxide glutathione peroxidase (with phosphatidylcholine hydroperoxides as substrate) [16].

Protein content of the mitochondria and the various subfractions

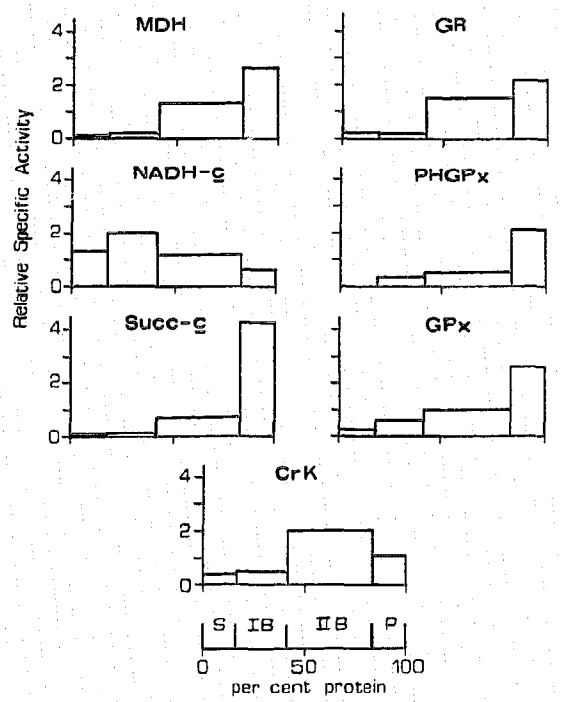

Fig. 1. Distribution pattern of marker enzymes after subfractionation of rat brain mitochondria according to Sandri et al. [20]. Abscissa: percentage of protein recovered in the single fraction: soluble (S), first band (IB), second band (IIB) and sediment (P). Ordinate: specific activity relative to that of mitochondria. CrK, creatine kinase; Succ.-*c*, succinate-cytochrome *c* reductase; NADH-*c*, rotenone-insensitive NADH-cytochrome *c* reductase; MDH, malate dehydrogenase; GPx, glutathione peroxidase; PHGPx, phospholipids hydroperoxide glutathione peroxidase; GR, glutathione reductase.

was measured according to the bicinchoninic acid method [25].

All chemicals were of the highest available purity grade.

3. RESULTS AND DISCUSSION

Fig. 1 shows the submitochondrial distribution and the specific activities, relative to whole mitochondria, of the marker enzymes and of GR, GPx and PHGPx.

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, which together with hexokinase and glutathione transferase, is a marker for the contact sites [26], shows a pronounced enrichment in the IIB fraction. This fraction also contains some rotenone-insensitive NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities, originating from the outer and inner membranes. Finally, the bulk of the activity of malate dehydrogenase (marker of the matrix content) is, at this stage of separation, still included in the vesicles of both the contact sites (IIB) and the inner membrane (P).

The GR, GPX and PHGPx activities show a complex pattern of distribution. The distribution of GR is rather similar to that of malate dehydrogenase, whereas those

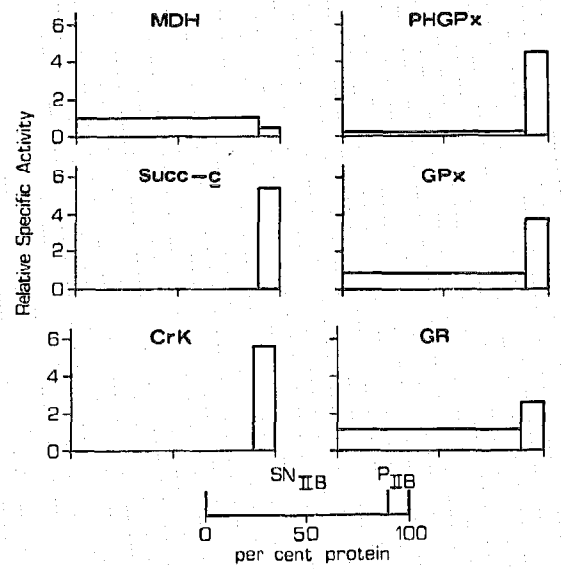

Fig. 2. Distribution pattern of marker enzymes after further fractionation of the IIB fraction obtained by reverse density gradient centrifugation of rat brain mitochondria after swelling, shrinkage and sonication (see Fig. 1). Experimental conditions were as indicated in section 2. Abscissa: percentage of protein recovered in the single fraction: soluble (SN_{IIB}) and sediment (P_{IIB}). Ordinate: specific activity relative to the intact IIB fraction. Marker enzymes as in Fig. 1.

of GPx and PHGPx resemble that of succinate-cytochrome *c* reductase. These data, however, leave the question open as to whether these enzymes are present in the matrix that is entrapped in the contact-site and inner membrane vesicles, or whether they are real membrane components.

In order to answer this question, we subjected the contact-site fraction (IIB) to a further disruption by sonication and subsequent centrifugation. The results are shown in Fig. 2, where the histograms refer to the supernatant (SN_{IIB}) (matrix content) and the sediment (P_{IIB}) (contact sites). Succinate-cytochrome *c* reductase, creatine kinase and malate dehydrogenase showed the expected pattern: practically all the activity of the first two enzymes remained in the membranes, whereas the bulk of malate dehydrogenase became soluble. On the other hand, the GR activity was not completely released by this treatment, about 20% remaining bound to the membrane fraction. More, up to 37%, of the GPx activity, and even more, about 75%, of the PHGPx, were also recovered in the contact sites.

A similar procedure was used for inner membrane (P) vesicles: Fig. 3 shows that in this case, only the PHGPx behaved as a membrane-bound enzyme, similar to the succinate-cytochrome *c* reductase, whereas GR and GPx showed a distribution similar to that of malate dehydrogenase.

From the data in Figs. 1–3 it can be concluded that the bulk of GR and GPx is located in the soluble fraction—mainly in the matrix—of rat brain mitochondria and that a minor portion, estimated at 10–15% of both enzymes, is concentrated in the contact sites between the

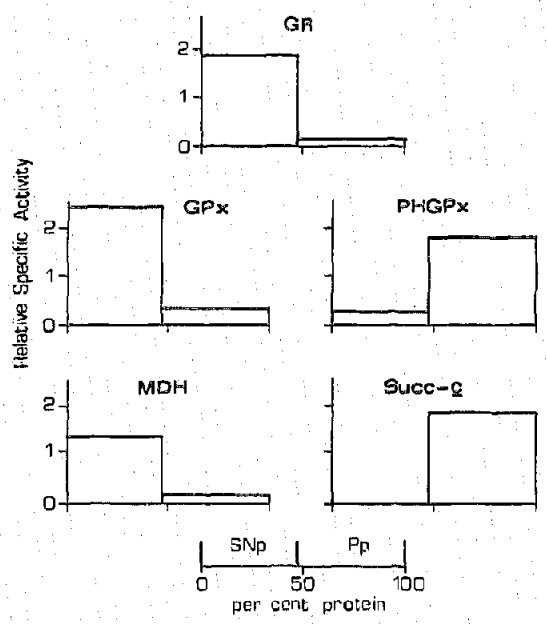

Fig. 3. Distribution pattern of marker enzymes after further fractionation of the P fraction obtained by reverse density gradient centrifugation of rat brain mitochondria after swelling, shrinking and sonication (see Fig. 1). Experimental conditions were as described in section 2. Abscissa: percentage of protein recovered in the single fraction: soluble (SN_P) and sediment (P_P). Ordinate: specific activity relative to the intact P fraction. Marker enzymes as in Fig. 1.

outer and inner membranes. This result is in accordance with our earlier finding [10] that brain mitochondria can utilize both endogenous and externally added substrates for these enzymes. The present evidence for a differential distribution of GPx and PHGPx in relation to the mitochondrial inner membrane is of special interest, suggesting a strategic role of the two enzymes in eliminating, on one hand, H_2O_2 generated in the inner and outer mitochondrial compartments by superoxide dismutase and by monoamine oxidase; and, on the other hand, phospholipid hydroperoxides formed in the inner membrane which is an important potential site of lipid peroxidation (27–29].

The data reported here further underline the functional importance of the contact sites between the inner and outer mitochondrial membranes, including their role in the transport of proteins [30–32] and lipids [33,34], as well as various enzyme activities [26], ion conductance [35] and, in general, the metabolic interplay between mitochondria and the rest of the cell.

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