Rat liver mitochondrial ADP-ribose pyrophosphatase in the matrix space with low K_m for free ADP-ribose

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A study involving markers of subcellular and submitochondrial fractions, gradient centrifugation, latency measurements and extraction with digitonin, demonstrates the association of a specific ADP-ribose pyrophosphatase with rat liver mitochondria

and its localization in the matrix space. The enzyme hydrolyses ADP-ribose to AMP, with a K_m of 2–3 μM . The results support the occurrence of a specific turnover pathway for free ADP-ribose and its relevance in mitochondria.

INTRODUCTION

Free ADP-ribose is formed in the turnover of NAD^+ [1–7], protein-bound poly(ADP-ribose) [8–11] or mono(ADP-ribose) [12] and the novel Ca^{2+} regulator cyclic ADP-ribose [13,14]. It may be a widespread (low-concentration) metabolite. It has been measured at 0.5 μM concentration in human red cells [15]. A specific ADP-ribose pyrophosphatase (EC 3.6.1.13) from rat liver hydrolyses ADP-ribose to AMP and D-ribose 5-phosphate, with a K_m of 0.5–1 μM [16,17]. Free ADP-ribose may have a specific role or may be just a catabolic intermediate disposed of through a specific pathway. It forms covalent adducts [18] and may cause non-enzymic protein modification, including specific ADP-ribosylation of mitochondrial proteins [2,4]. However, there is no information on free ADP-ribose levels in mitochondria; its formation in submitochondrial compartments by NAD^+ breakdown is under discussion [3,4,7,19], and ADP-ribosyltransferase activities have been also reported in mitochondria [3,20]. ADP-ribosylation has been related to mitochondrial Ca^{2+} -release elicited by compounds that induce oxidation and breakdown of intramitochondrial nicotinamide nucleotides [19,21,22].

We now study further the relevance of intramitochondrial free ADP-ribose by showing the association of rat liver ADP-ribose pyrophosphatase with mitochondria and its localization in the matrix space.

MATERIALS AND METHODS

Rats were treated with Triton WR-1339 to diminish the buoyant density of hepatic lysosomes, and crude mitochondria pellets were prepared as described [23]. Pelleted mitochondria were suspended and fractionated by gradient centrifugation: for analytical or preparative purposes the conditions were respectively as in [23] or [24]. Fractions from analytical gradients (Figure 2) were diluted 6-fold in 50 mM Tris (pH 7.5; all buffers were adjusted at the indicated pH, at room temperature, with conc. HCl)/1 mM EDTA/6 mg/ml Triton X-100, and samples were prepared and frozen in series of convenient size for the later assay of enzyme activities. In preparative gradients, mitochondria were localized by assay of glutamate dehydrogenase and the relevant fractions were pooled, diluted 3-fold in 5 mM Tris (pH 7.5)/1 mM EDTA/0.25 M sucrose, and pelleted (25000 g , 15 min). The purified mitochondrial pellets were used for

submitochondrial fractionation with digitonin, latency measurements or purification of ADP-ribose pyrophosphatase.

For digitonin treatment, mitochondria were suspended at 10 mg of protein/ml, mixed with 1 vol. of digitonin solution, shaken gently for 15 min on ice, diluted 4-fold and centrifuged for 20 min at 12500 g . All the steps were performed in 5 mM Tris (pH 7.5)/1 mM EDTA/0.25 M sucrose. Enzymes (see below) were assayed in the supernatants to assess the solubilization of submitochondrial compartments. Compared with results obtained by others [7,25,26], in our experiments only a minor proportion of monoamine oxidase (outer-membrane marker) was solubilized by digitonin concentrations that extracted almost quantitatively the matrix marker glutamate dehydrogenase (see Figure 3). This discrepancy, which does not affect the validity of conclusions regarding ADP-ribose pyrophosphatase location in the matrix space, may be related to the treatment of rats with Triton WR-1339 before killing. This detergent is captured by liver lysosomes, facilitating their separation from mitochondria upon gradient centrifugation [27]. Perhaps Triton WR-1339 also affects the behaviour and/or composition of the outer membrane of mitochondria.

ADP-ribose pyrophosphatase was partially purified from supernatants obtained either from mitochondria (dissolved in 20 mM Tris, pH 7.5, containing 5 mg/ml Triton X-100, and centrifuged for 15 min at 27000 g) or, in some experiments, from total-liver isotonic homogenates (prepared in 5 mM Tris, pH 7.5, 0.5 mM EDTA, 1 mM phenylmethanesulphonyl fluoride, 0.25 M sucrose, centrifuged at 27000 g for 20 min, and the supernatant was collected and re-centrifuged at 100000 g for 60 min). The purification protocol involved gel filtration and DEAE-cellulose chromatography, with columns equilibrated in 20 mM Tris, pH 7.5, essentially as described by Miró et al. [16].

Enzyme assays: in all cases they were carried out at 37 °C; one unit represents 1 μmol of substrate/min. Unless otherwise indicated, initial rates of hydrolysis of ADP-ribose or ADP-glucose (each at 450 μM ; from Sigma) were measured by coupling to alkaline phosphatase in 50 mM Tris, pH 7.5, 1 mM EDTA, 6 mM MgCl_2 , followed by assay of P_i [16].

Saturation kinetics of ADP-ribose pyrophosphatase was studied by computer analysis of rate-versus-[substrate] data derived from complete reaction progress curves recorded by monitoring the increase in A_{265} caused by the hydrolysis of ADP-ribose coupled to alkaline phosphatase and adenosine deaminase [17]. The irreversibility of the process was thus ensured. The reactions

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were initiated by addition of ADP-ribose to a mixture containing 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 3 μg of alkaline phosphatase (Boehringer cat. no. 108146), 1.2 μg of adenosine deaminase (Boehringer cat. no. 102121) and 7 μg of partially purified ADP-ribose pyrophosphatase (final volume 1.2 ml). At the end of the first progress curve, a second one was recorded by repeating the addition of ADP-ribose. Successive progress curves recorded in the same assay mixture were superimposable and yielded identical saturation parameters, which indicated that neither enzyme inactivation nor product inhibition interfered in the results [17].

Subcellular and submitochondrial marker assays were performed as in the cited references: glutamate dehydrogenase (mitochondria and mitochondrial matrix), acid phosphatase (lysosomes), catalase (peroxisomes) and glucose-6-phosphatase (endoplasmic reticulum) as in [23]; monoamine oxidase (mitochondrial outer membrane) as in [25]; adenylate kinase (mitochondrial intermembrane space) as in [28], except that Tris buffer was used; cytochrome *c* oxidase (mitochondrial inner membrane) with ascorbate-reduced 10 μM cytochrome *c* as in [29]; phosphodiesterase I (plasma membrane and endoplasmic reticulum) by measuring the increase in A_{405} in reaction mixtures with 50 mM Tris, pH 8, 5 mM $MgCl_2$ and 1 mM dTMP *p*-nitrophenyl ester, after terminating the reaction with NaOH.

RESULTS AND DISCUSSION

In the particulate fraction of rat liver, ADP-ribose is hydrolysed by nucleotide pyrophosphatase or phosphodiesterase I (EC 3.6.1.9/EC 3.1.4.1), a plasma-membrane and endoplasmic-reticulum hydrolase [30] that attacks ADP-ribose and ADP-glucose at similar rates, as well as other pyrophosphate- or phosphodiester-containing substrates [31]. The presence of specific ADP-ribose pyrophosphatase in crude mitochondria was first shown by comparing ADP-ribose and ADP-glucose hydrolase activity profiles after gel filtration (Figure 1) or gradient centrifugation (Figure 2). Detergent extracts of crude mitochondria were analysed by gel filtration, which showed two major ADP-ribose hydrolase peaks: one attacked also ADP-glucose and may correspond to phosphodiesterase I [31], but the other did not, and corresponds to specific ADP-ribose pyrophosphatase [16] (Figure 1). On the other hand, after centrifugation of crude mitochondria suspensions in sucrose gradients, ADP-ribose and ADP-glucose hydrolase showed simi-

Figure 1 Identification of ADP-ribose pyrophosphatase (arrow) by gel filtration

A 350 μl sample of a crude mitochondrial suspension was diluted 14-fold in 50 mM Tris (pH 7.5)/1 mM EDTA/5 mg/ml Triton X-100, centrifuged, and the supernatant was applied to a Sephadex G-100 column (1.4 cm $\times$ 127 cm) in 50 mM Tris (pH 7.5)/1 mM EDTA.

Figure 2 Gradient-centrifugation analysis of crude mitochondria

(a) A 5 ml sample of a crude mitochondrial preparation was fractionated by density-gradient centrifugation [23], and the fractions (each approx. 1 ml) were solubilized with Triton X-100 (see the Materials and methods section) for enzyme assays. Activities were measured in the presence of 5 mg/ml Triton X-100. I–IV, detergent-treated fraction pools used for analysis of ADP-ribose pyrophosphatase distribution (Table 1). (b) Activity profiles near the bottom of the gradient, with fraction-11 activities normalized to 100%. Subcellular markers are as mentioned in the Materials and methods section.

lar sedimentation profiles, except in mitochondria-rich fractions, where the former was more active (Figure 2a); in the denser side of the mitochondrial peak, where ADP-glucose hydrolase activity was low, the profile of ADP-ribose hydrolase was identical with that of the mitochondrial marker glutamate dehydrogenase (Figure 2b). The gradient profile of ADP-glucose hydrolase was like that of phosphodiesterase I (Figure 2a).

In addition to the evidence based on comparisons between ADP-ribose and ADP-glucose hydrolase activities, association with mitochondria of specific ADP-ribose pyrophosphatase was also shown by using data obtained after separation from phosphodiesterase I or ADP-glucose hydrolase by gel filtration. The distribution of ADP-ribose pyrophosphatase among four gradient-fraction pools resembled mitochondria or peroxisomes (Table 1), but association with peroxisomes can be discarded, as

Table 1 Distribution of ADP-ribose pyrophosphatase and subcellular enzyme markers in gradient-fraction pools

Fraction pools I–IV are those indicated in Figure 2(a). Data for subcellular markers are a computation from Figure 2(a) profiles. ADP-ribose pyrophosphatase was assayed after gel filtration (Figure 1) of detergent-treated fraction pools.

Enzyme	Activity in fraction pool (% of total activity in gradient)			
	I	II	III	IV
ADP-ribose pyrophosphatase	0.6	80	5	14
Glutamate dehydrogenase	< 0.1	99	1	< 0.1
Catalase	11	67	7	15
Phosphodiesterase I	0.3	28	51	21
Acid phosphatase	0.4	18	53	29
Glucose-6-phosphatase	0.1	55	42	3

Figure 3 Submitochondrial location of ADP-ribose pyrophosphatase

Assays were performed in supernatants obtained after treatment of density-gradient-purified mitochondria with digitonin (see the Materials and methods section). ADP-ribose pyrophosphatase was quantified by gel-filtration chromatography (Figure 1). Results are percentages of controls solubilized by 5 mg/ml Triton X-100 instead of digitonin. Blanks without detergent were processed and supernatant activities subtracted from those obtained with digitonin or Triton X-100. Submitochondrial markers are as mentioned in the Materials and methods section.

the gradient bottom contained catalase but not ADP-ribose hydrolase (Figure 2b, fractions 1–6).

Submitochondrial fractionation with digitonin showed parallel solubilization of matrix glutamate dehydrogenase and ADP-ribose pyrophosphatase (Figure 3). The location of the latter in the matrix space was confirmed by latency data: 75% of total ADP-ribose hydrolase activity was latent, i.e. measurable in the presence of Triton X-100 but not its absence, compared with 85% of glutamate dehydrogenase and none of the adenylate kinase (intermembrane-space marker).

Mitochondrial ADP-ribose pyrophosphatase was partially (6-fold) purified from detergent extracts of gradient-purified mitochondria (see under the Materials and methods section), with a 33% yield and a final specific activity about 0.1 unit/mg. The partially purified enzyme showed: M_r 35000 by gel filtration (Figure 4); requirement for Mg^{2+} partly fulfilled by Mn^{2+} (results not shown); 2–3 μM K_m for ADP-ribose (Figure 5); formation of AMP as a product (h.p.l.c. results not shown); absence of activity on ADP-glucose or diadenosine 5',5'''-pyrophosphate in the presence of Mg^{2+} (results not shown). Very similar properties are displayed by the ADP-ribose pyrophosphatase present in 100000 g supernatants prepared from rat liver in hypotonic medium [16]; and gel-filtration results indicative of M_r 38000; A. Miró and J. C. Cameselle, unpublished work), except perhaps

Figure 4 Apparent M_r of mitochondrial ADP-ribose pyrophosphatase

ADP-ribose pyrophosphatase ($\square$) partially purified from mitochondria by gel filtration and DEAE-cellulose chromatography was analysed in a Sephadex G-75 column (1 cm $\times$ 130 cm) equilibrated and eluted with 20 mM Tris (pH 7.5)/0.1 M KCl, and calibrated with BSA (M_r 66000; $\circ$), ovalbumin (45000; $\triangle$) and cytochrome *c* (12500; $\blacklozenge$) as protein standards.

Figure 5 Saturation kinetics of mitochondrial ADP-ribose pyrophosphatase

Rate values at different substrate concentrations were obtained from two successive reaction progress curves, each initiated by the addition of 8 μM ADP-ribose (final concn.), as described in the Materials and methods section and in [17]. The curves were identical, and the Figure shows the combined results from both. Non-linear regression of the hyperbolic plot [17] yielded saturation parameters ($\pm$ S.E.) (K_m 2.5 $\pm$ 0.2 μM ; V 97 $\pm$ 3 nmol/min per mg of protein) identical with those obtained by linear regression of the Lineweaver-Burk plot (inset; K_m 2.4 $\pm$ 0.2 μM ; V 94 $\pm$ 4 nmol/min per mg of protein).

for a somewhat lower K_m value, 0.5–1 μM [16]. Therefore, the 100 000 g supernatant enzyme may come in part from damaged mitochondria. However, it is likely that ADP-ribose pyrophosphatase, besides being located in the mitochondrial matrix, as shown in the present work, is also present in another subcellular compartment (e.g. cytosol). This conclusion is based upon the following data. (a) The mitochondrial enzyme, partially purified by gel filtration of Triton X-100 extracts, accounted for about 20 m-units/g of liver (estimated after correction for losses of mitochondria). (b) A slightly higher activity of specific ADP-ribose pyrophosphatase (30–40 m-units/g of liver) was recovered, also after partial purification, from 100 000 g supernatants from rat liver homogenates prepared in either hypotonic medium [16] or isotonic buffered sucrose (the present work; results not shown). (c) In the latter case, glutamate dehydrogenase activity in liver supernatants was less than 0.5% of total-homogenate activity measured in the presence of Triton X-100, indicating that mitochondrial-matrix contamination in isotonic 100 000 g supernatants was very low and cannot account for ADP-ribose pyrophosphatase activity in those supernatants.

When rat liver ADP-ribose pyrophosphatase was partially purified from 100 000 g supernatants of homogenates prepared in hypotonic medium, besides the specific enzyme, another one of lesser specificity (different from phosphodiesterase I) was found after a DEAE-cellulose chromatography step [16]. In mitochondria, after the same chromatographic steps, we observed only one ADP-ribose pyrophosphatase, similar to the specific enzyme described by Miró et al. [16].

The formation of free ADP-ribose from NAD^+ has been suggested as one step to the (non-enzymic) ADP-ribosylation of mitochondrial proteins [2,4,19,21]. A recent paper has questioned the role of intramitochondrial free ADP-ribose, as it is reported that NAD^+ glycohydrolase activity is apparently absent from the matrix space [7]. In our view, the presence of a low- K_m specific ADP-ribose pyrophosphatase in the mitochondrial matrix favours the possibility that free ADP-ribose in fact occurs within mitochondria, possibly at low concentration and perhaps produced by a pathway different from NAD^+ glycohydrolase, for instance as a turnover product of protein-bound (oligo)ADP-ribose.

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