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Location of dinucleoside triphosphatase in the matrix space of rat liver mitochondria

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The submitochondrial location of dinucleoside triphosphatase (EC 3.6.1.29), previously shown to be in part associated with mitochondria, has been studied in rat liver. The precipitability and latency of activity in organelle suspensions, and the profile of solubilization by digitonin, were like those of the matrix space marker glutamate dehydrogenase, and differed from those of other submitochondrial fractions. This, and the synthesis of diadenosine polyphosphates by mitochondrial aminoacyl-tRNA synthetases, suggest the occurrence of a pathway for the intramitochondrial turnover of diadenosine 5',5'''-P^1,P^3-triphosphate (Ap_3A).

Dinucleoside triphosphatase; Diadenosine triphosphate; Dinucleoside polyphosphate; Mitochondria (rat liver); Digitonin; Submitochondrial fractionation

1. INTRODUCTION

Ap_3A is synthesized by aminoacyl-tRNA synthetases [1–3] and has potential significance in the response to different kinds of stress [4–6], blood platelet function [7,8], vasomotor activity [9] and hepatic function [10] control. Two enzymes hydrolyze Np_3N compounds in rat liver. PDE hydrolyzes phosphodiester- and pyrophosphate-containing compounds, including Np_nN [11]. It is a membrane-bound enzyme located, at least in part, in the outside of the plasma membrane [12–15]. Np_3Nase [16–18] hydrolyzes specifically Np_3N to the cognate nucleoside 5'-mono- and diphosphates, e.g., Ap_3A to ADP and AMP. It is partitioned between the cytosolic and mitochondrial fractions [13,19]. In sucrose gradients, part of rat liver Np_3Nase co-sediments with mitochondria with a pattern different to other subcellular fractions [19]. Mitochondrial Np_3Nase is solubilized by freeze-thawing, which suggests that it is not an integral membrane protein [13,19].

The cytosolic and mitochondrial Np_3Nases are so far undistinguishable from each other except for their location [13,16]. This picture can be explained by separate, cytosolic and intramitochondrial Np_3Nases or by one cytosolic enzyme binding to the outer mitochondrial membrane as, e.g., hexokinase or glycerol kinase [20,21]. The submitochondrial location of Np_3Nase is relevant as, depending on it, the enzyme bound to mitochondria could act on the same pool of Ap_3A as the cytosolic Np_3Nase. Here we show the location of Np_3Nase in the mitochondrial matrix, what may be put together with evidence that mitochondrial aminoacyl-tRNA synthetases may synthesize Ap_nA [22,23].

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Abbreviations: Ap_nA, diadenosine 5',5'''-P^1,P^n-*n*-phosphate; buffer A, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mg/ml bovine serum albumin; buffer TS, 5 mM Tris-HCl, pH 7.5, 0.25 M sucrose; Np_nN, dinucleoside 5',5'''-P^1,P^n-*n*-phosphate; Np_3Nase, dinucleoside triphosphatase (EC 3.6.1.29); PDE, phosphodiesterase I/nucleotide pyrophosphatase (EC 3.1.4.1/EC 3.6.1.9)

2. MATERIALS AND METHODS

2.1. *Materials*

Female Wistar rats weighing 200–250 g were used. Digitonin was from Calbiochem; Triton X-100, Triton WR-1339, Ap_3A, Ap_2A, cytochrome *c* (type III) and hexokinase from Sigma; other enzymes and bovine serum albumin (fraction V) from Boehringer; sucrose, Tris, $MgCl_2$ and EDTA from Merck.

2.2. *Preparation of mitochondria*

In order to better separate mitochondria from lysosomes (see below), a s.c. injection of 0.75 g Triton WR-1339 per kg was given to rats 4 days before killing by decapitation. The livers were minced, washed in saline and in buffer TS, homogenized in 3 ml of buffer TS per gram of tissue, and filtered through cotton gauze. A crude mitochondrial fraction was prepared by differential centrifugation as follows: the homogenate was centrifuged for 10 min at 150 × *g*; the pellet was resuspended in buffer TS up to the initial volume and centrifuged again; both supernatants were separately centrifuged for 20 min at 40000 × *g*; the 40000 × *g* pellets were resuspended together (final volume 0.55 ml/g liver) in buffer A with 0.8 M sucrose. Crude mitochondria (5 ml samples) were centrifuged for 2 h at 60000 × *g*, in 50 ml, 1–2 M sucrose gradients prepared in buffer A. Fig. 1 shows the profiles of one mitochondrial (glutamate dehydrogenase; EC 1.4.1.3) and one lysosomal marker (acid phosphatase; EC 3.1.3.2).

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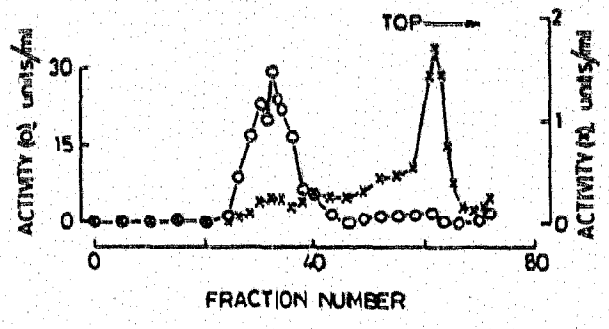

Fig. 1. Purification of mitochondria by sucrose gradient centrifugation of crude mitochondrial pellets obtained from rats treated with Triton WR-1339. (○) Mitochondria (glutamate dehydrogenase); (×) lysosomes (acid phosphatase).

Lysosomal density was lighter than normal due to the previous treatment with Triton WR-1339, that facilitates the purification of mitochondria [24]. Mitochondrial fractions were pooled, diluted with 2 vols of buffer TS with 0.5 mM EDTA, centrifuged for 15 min at 25000 × *g*, and the pellet was gently resuspended in the same buffer to a concentration of 10 mg protein/ml. All the steps were carried out at 4°C or on an ice-water bath.

2.3. *Extraction of mitochondrial enzymes*

Mitochondria diluted to 5 mg protein/ml in buffer TS, 0.5 mM EDTA, and detergent as indicated, were shaken gently for 15 min on ice, and centrifuged for 20 min at 12500 × *g*. Activities were assayed in the supernatants.

2.4. *Enzyme assays*

Except when indicated, glutamate dehydrogenase and acid phosphatase were assayed as in [19], monoamine oxidase as in [25], and adenylate kinase as in [20] except that, in the latter case, Tris buffer was used. Cytochrome *c* oxidase was assayed with 10 μM cytochrome *c* (reduced with ascorbate [26]), in 0.2 M Tris-HCl, pH 7.5, 1 mg/ml Triton X-100, recording the decrease in A_{550}. Total $Ap_3A(Ap_2A)$-hydrolase activity was assayed [17] measuring P_i formed in the presence of alkaline phosphatase, 50 mM Tris pH 7.5, 5 mM $MgCl_2$, and 225 μM $Ap_3A(Ap_2A)$. When the assay of Ap_3A-hydrolase was intended to reflect the activity of the specific Np_3Nase, mitochondrial extracts (1.3 ml) were chromatographed in a Sephadex G-100 column (0.9 × 49 cm) equilibrated and eluted with 20 mM Tris-HCl, pH 7.5, at 6 ml/h. Np_3Nase eluted as a peak with V_e about 18 ml (e.g. Fig. 3) whose Ap_3A-hydrolase activity was quantified.

3. RESULTS AND DISCUSSION

To find out the submitochondrial location of Np_3Nase, we studied, in a preparation of rat liver mitochondria, the latency, the precipitation due to association to sedimentable particles, and the solubilization by digitonin of the Ap_3A-hydrolase activity and of enzyme markers of submitochondrial fractions.

Latency of an enzyme is defined as its inaccessibility to exogenously added substrate and, in particulate preparations, it is shown by enzymes entrapped within compartments delimited by membranes not freely permeable to substrate(s). Latency of mitochondrial enzymes may be lost as a consequence of damage to mitochondrial membranes, which may also give rise to leakage of enzymes from the intermembrane or matrix spaces. We have estimated enzyme latency in mitochondrial suspensions by measuring, in isotonic sucrose reaction mixtures, the increase in activity promoted by Triton X-100. In the same experiments in which latency was measured, the precipitability of enzymes was also assessed. The results are summarized in Fig. 2. Glutamate dehydrogenase was 95% bound to precipitable particles and 90% latent, reflecting its location in the mitochondrial matrix as well as the action of the inner membrane as a barrier to enzyme and substrates. Adenylate kinase, a marker of the intermembrane space, was only 70% precipitable, indicating some damage to the outer membrane, and not latent at all, indicating the permeability of the outer membrane to kinase substrates. Ap_3A-hydrolase activity behaved like glutamate dehydrogenase.

Two experiments confirmed that the major part of the Ap_3A-hydrolase activity in mitochondrial preparations was due to the specific Np_3Nase rather than to the unspecific PDE (see the introductory paragraph). On the one hand, the hydrolytic activity on Ap_2A, measured in the preparation of Fig. 2 (not shown), was about 25% of the Ap_3A-hydrolase activity. Since Ap_2A and Ap_3A are hydrolyzed at the same rate by rat liver PDE [11], it can be concluded that only 25% of the total Ap_3A-hydrolase activity of Fig. 2 may correspond to that unspecific enzyme. On the other hand, Np_3Nase and PDE can be solubilized from the particulate fraction of liver with Triton X-100 and resolved by gel-filtration chromatography [13]. Such a procedure, applied to mitochondrial preparations, showed that about 90% of the solubilized Ap_3A-hydrolase activity eluted devoid of Ap_2A-hydrolase and corresponds in fact to Np_3Nase (Fig. 3).

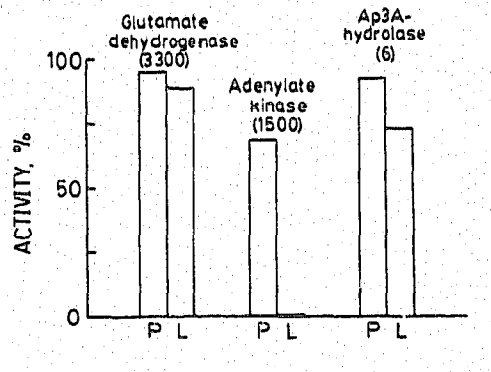

Fig. 2. Enzyme latency and precipitability in mitochondria. Total activities (100%), measured in 5 mg/ml Triton X-100 and 0.25 M sucrose, are shown in parentheses (mU/ml). Latency (L) was obtained by subtracting from 100% the activities measured omitting Triton X-100 (i.e., the non-latent activities). To measure precipitability (P), mitochondria were diluted 1:6 in buffer TS with 0.5 mM EDTA, like for the enzyme assays, and non-precipitable activities were assayed in 12500 × *g* supernatants and subtracted from 100% values.

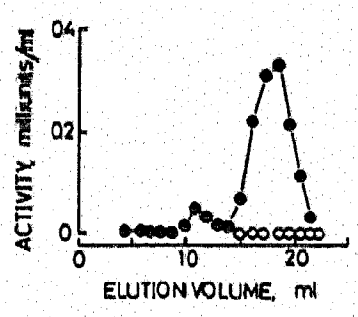

Fig. 3. Np_3Nase identification and quantification by gel filtration. Mitochondrial extracts (1.3 ml) prepared with 5 mg/ml Triton X-100 were analyzed in a Sephadex G-100 column (see section 2). The Ap_3A-hydrolase peak that did not split Ap_2A is Np_3Nase [13]. (●) Ap_3A-hydrolase; (○) Ap_2A-hydrolase; fraction volume, 1 ml.

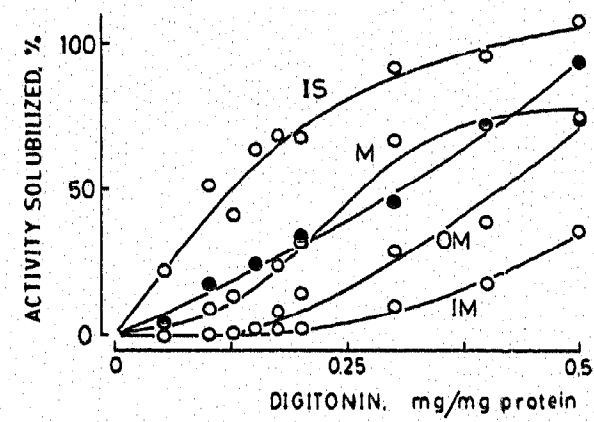

Fig. 4. Solubilization of mitochondrial enzymes by extraction with digitonin. (●) Np_3Nase (see Fig. 3); (○) submitochondrial fractions (marker) as follows: IS, intermembrane space (adenylate kinase); M, matrix (glutamate dehydrogenase); OM, outer membrane (monoamine oxidase); IM, inner membrane (cytochrome *c* oxidase). Percentages are relative to values found with 5 mg/ml Triton X-100 substituting for digitonin. Blank extract (no Triton or digitonin used) activities were subtracted from detergent-extracted activities.

Another proof that Np_3Nase is located in the matrix of mitochondria was provided by the sequential solubilization of submitochondrial compartments with digitonin (Fig. 4). The solubilization profile of the specific Np_3Nase (assayed after chromatography of digitonin supernatants as described in Fig. 3), within the range 0–0.5 mg digitonin/mg protein, was like that of the matrix marker glutamate dehydrogenase and clearly different to those of the markers of other submitochondrial fractions: adenylate kinase (intermembrane space), monoamine oxidase (outer membrane) and cytochrome *c* oxidase (inner membrane) (Fig. 4).

Ap_3A and other adenylated dinucleoside polyphosphates are formed by aminoacyl-tRNA synthetases of several origins and specificities [1–3]. Mitochondrial synthetases from yeast [22] and *Euglena gracilis* [23] synthesize Ap_4A and may be also Ap_3A. The matrix location of Np_3Nase reinforces the idea of an intramitochondrial pool of Ap_3A or other dinucleoside triphosphate. The possible role of diadenosine polyphosphates in relation with mitochondrial functions was discussed in an earlier paper [19].

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