The characterization of a new enzyme from rat liver mitochondria, oligophosphoglyceroyl-ATP 3'-phosphodiesterase

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By using an assay based on the precipitation of intact ^{14}C -labelled substrate, an activity has been located in the mitochondrial fraction of rat liver which selectively hydrolyses the 3' ester link in the fairly recently discovered oligomeric tetraphosphate derivative of ATP and glyceric acid for which the structure 3-phospho[glyceroyl- γ -triphospho-5'-adenosine-3'-3-phospho] $_n$ -glyceroyl- γ -triphospho-5'-adenosine has been proposed [Hutchinson, Morris & Mowbray (1986) *Biochem. J.* **234**, 623–627]. This enzyme activity (M_r 85000) has been purified approx. 30-fold from washed mitochondria by $(\text{NH}_4)_2\text{SO}_4$ precipitation and f.p.l.c. The apparent K_m for substrate (adenosine equivalents) is around 35 μM . The recovery of total activity is about 20%, and this, allied to the relatively low V_{max} found in contrast with the rapid turnover of oligomer seen in post-ischaemic tissues, suggests that some activating factors have been lost in purification. Percoll-gradient studies confirm that the activity is mitochondrial and not lysosomal or endoplasmic-reticular. The activity is latent in intact mitochondria; it is not, however, associated with intact inner-membrane vesicles but released during their preparation, implying an intermembrane-space location. The product of the enzyme is proposed to be the monomeric unit 3-phosphoglyceroyl- γ -triphospho-5'-adenosine, from which digestion with snake-venom phosphodiesterase releases ADP.

INTRODUCTION

Systematic changes in the amounts of adenine nucleotides in steady-state perfused rat hearts which could not be explained by interconversion with known derivatives or polymers led us to propose the existence of some unknown derivative [1–3]. Subsequent labelling studies located an oligomeric species which rapidly exchanges units with ATP in rat heart [4], kidney [5] and liver (B. Patel, C. D. Gove, W. L. Hutchinson & J. Mowbray, unpublished work). Selective digestion and chromatography along with ^{31}P n.m.r. and m.s. studies led us to propose a structure and the name of oligophosphoglyceroyl-ATP (oPG-ATP) for this compound [7]. Here we describe how, using radiolabelled substrate and the property that the oligomer can be precipitated in cold aq. 50% (v/v) ethanol, we have partially purified and characterized from rat liver an activity which specifically cleaves the oligomer to a single product and have shown that this enzyme appears to be confined to the intermembrane space of liver mitochondria. A preliminary account of this work has been presented [8].

EXPERIMENTAL

Materials

$(\text{NH}_4)_2\text{SO}_4$ (AR grade) was from Fisons, Loughborough, Leics., U.K.; benzamide hydrochloride and phenylmethanesulphonyl fluoride were bought from BDH, Poole, Dorset, U.K.; Percoll, 2,6-dichlorophenol-indophenol, Sephacryl S-300 and snake-venom phosphodiesterase VIII-S were from Sigma Chemical Co., Poole, Dorset, U.K.; digitonin was from Calbiochem, San Diego, CA, U.S.A. All other materials were as described previously [6].

Methods

Preparation of [^{14}C]oPG-ATP. Radioactive labelling of heart nucleotides by Langendorff perfusion with 8-[^{14}C]adenosine and

the recovery of the trichloroacetic acid/methanol-insoluble material was carried out as described previously [4]. The ^{14}C -labelled substrate was purified from the insoluble dried precipitates from four or five hearts (~0.5 g) by resuspension, differential phenol extraction and anion-exchange chromatography as described in [7]. Finally the substrate (sp. radioactivity approx. 80000 d.p.m./ μmol of adenosine equivalents) was precipitated over 14 days at -20°C from column buffer made 50% (v/v) with cold ethanol. The product, collected by centrifugation and washed twice with 10 ml of ethanol, was dried under a stream of dry N_2 gas and stored at -20°C .

Precipitation assay. Up to 40 nmol (adenosine equivalents; ϵ_{259} $15.4 \pm 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) of oPG-ATP were taken up in 10 mM- MgCl_2 /1 mM-adenosine/25 mM-Hepes buffer, pH 7.4, and incubated for up to 30 min at 37°C with enzyme (total volume 150–300 μl). The reactions were terminated by the addition of 1 vol. of cold ethanol and the mixtures stored at -20°C for 2–3 h. The mixtures were then syringe-filtered (Millipore HA; 0.45 μm pore size, 12.5 mm diam.), washed with 200 μl of ethanol and the radioactivity trapped on the filter and in the wash and filtrate (breakdown products) were both assayed on each sample using 4 ml of Ecoscint A scintillation cocktail (National Diagnostics, Manville, NJ, U.S.A.) in a Packard Tri-Carb 900CA liquid-scintillation counter (Canberra Packard, Pangbourne, Reading, Berks., U.K.) and compared with a non-enzymic blank (no significant breakdown). The amounts of substrate, enzyme sample and incubation time giving linear breakdown of substrate with time, where this was required, were established by preliminary examination of the time course of substrate solubilization (see the Results section). All of the solubilized radioactivity within experimental error was present in a single product which could be purified by either two-dimensional t.l.c. or h.p.l.c. (see below); its production could thus be assayed quantitatively as solubilized radioactivity.

Cell fractionation. Livers from Sprague-Dawley rats weighing 180–200 g were chopped at 4°C in 210 mM-mannitol/

Abbreviations used: oPG-ATP, oligophosphoglyceroyl-ATP; PMSF, phenylmethanesulphonyl fluoride; PEI, polyethyleneimine.

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70 M-sucrose/1 mM-EDTA/10 mM-Tris/HCl buffer, pH 7.4, containing 2 mM-benzamidine and 0.01 % (w/v) phenylmethanesulphonyl fluoride (PMSF) to inhibit proteinase action, rinsed in the same buffer and homogenized with six strokes of a Teflon/glass homogenizer, the pestle rotating at 500–1000 rev./min, with 2 ml of buffer/g of wet tissue. The homogenate was centrifuged in a Sorvall RC-5B centrifuge using the SS-34 head at 1600 rev./min (310 $g_{av.}$) for 10 min and the resulting supernatant at 2300 rev./min (640 $g_{av.}$) for 10 min to remove the nuclei and tissue debris. The 2300 rev./min supernatant was centrifuged at 9000 rev./min (9680 $g_{av.}$) for 15 min and the resulting mitochondrial pellet washed twice with the homogenization buffer; the mitochondria could be fractionated into membranes and soluble extract by resuspending in 20 mM- KH_2PO_4 buffer, pH 7.4, containing 0.01 % PMSF and subjected to sonic disintegration in an MSE Soniprep 150 using the 3 mm exponential microprobe at 23 kHz and an amplitude of 10 μ m for 4 $\times$ 15 s with 15 s intervals. The mixture was then centrifuged at 35000 rev./min (90000 $g_{av.}$) for 45 min using the 70.1 Ti head in a Beckman L-8 centrifuge. The post-mitochondrial supernatant was centrifuged in the Sorvall centrifuge for 10 min at 17000 rev./min (34500 $g_{av.}$) and the pellet discarded before the supernatant was centrifuged at 45000 rev./min (140000 $g_{av.}$) for 45 min in the 70.1 Ti head of a Beckman L-8 centrifuge to give microsomal and liver cytosol fractions.

Chromatography. F.p.l.c. was performed by using a Pharmacia FPLC System (Pharmacia, Milton Keynes, Bucks., U.K.) using an H10/10 Mono Q column equilibrated with 20 mM-Bistris propane, pH 6.6, and developed with an NaCl gradient (0–1 M). Gel filtration was carried out at 4 °C using Sephacryl S-300 (total volume 275 ml) equilibrated and run (25 ml/h) with 50 mM- KH_2PO_4 /0.1 M-NaCl buffer, pH 7.4, calibrated with Blue Dextran (M_r 2×10^6), rabbit muscle aldolase (M_r 150000) and BSA (fraction V; M_r 68000). H.p.l.c. analysis of nucleotides was performed using a Gilson 303 system (supplied by Anachem, Luton, Beds., U.K.) and developed with a linear gradient (1–400 mM) of KH_2PO_4 pH 3.0. T.l.c. on polyethyleneimine (PEI)-cellulose used the two-dimensional system previously described [7].

Mitochondrial analysis. Percoll-gradient examination of the mitochondrial fraction was performed exactly as detailed by Blume [9], using 1 ml fractions. The fractions were assayed for acid phosphatase [10], succinate dehydrogenase [11], glucose-6-phosphatase [12] and protein content [13]. The actual densities were monitored by measuring the fraction refractive index using a Bellingham and Stanley refractometer calibrated with suitable standard solutions.

Subfractionation of mitochondria. Mitochondria prepared from two rat livers were separated into mitoplasts (intact inner-membrane vesicles) and outer-membranes-plus-intermembrane-space enzymes essentially as described previously [14]. After protein determination, the mitochondrial suspension was adjusted to 2 mg of protein/ml with cold 0.25 M-mannitol, and 40 μ l of a 10 mg/ml solution of digitonin in ethanol was added per ml of suspension. The mixture was placed on ice with occasional shaking for 15 min, at the end of which time it was diluted with an equal volume of cold 0.25 M-mannitol and centrifuged in a Sorvall RC-5B/SS-34 head at 9000 rev./min (9680 $g_{av.}$) for 10 min. The resulting mitoplasts were washed once by resuspension in 20 ml of 0.25 M-mannitol and finally resuspended in the same solution.

RESULTS AND DISCUSSION

There were two problems to be overcome in looking for an activity in the cell which would specifically cleave oPG-ATP. The

first, the extreme lability of the substrate in neutral solution, was overcome by the somewhat surprising finding that concentrations of Mg^{2+} or Mn^{2+} above 2 mM remarkably stabilized the compound [15], allowing its incubation at 37 °C for a number of hours with no detectable breakdown. All assays were routinely carried out in 10 mM- $MgCl_2$ thereafter.

The second was that some strategy was required to allow partial purification of any enzyme away from the phosphatases and nucleotidases present in cell homogenates which could be expected to modify any initial product of oligomer lysis. This was tackled by making use of oPG-ATP's property of being precipitable with cold aq. 50 % (v/v) ethanol. Radioactive substrate was purified after prior perfusion of rat hearts in the presence of 8- $[^{14}C]$ adenosine (see the Materials and methods section); lytic activity in a fraction was defined as the subsequent inability of ethanol to precipitate substrate and allow it to be trapped on a Millipore filter and assayed for radioactivity. For quantitative estimation of activity, conditions were adjusted to ensure that 30 % of substrate was broken down (see the Materials and methods section). It is not clear how large the oligomers need to be to confer this ethanol-precipitability. Thus precipitation of partially digested substrate could lead to serious underestimation of activity. In the event, the enzyme found appears to produce a single product from the oligomer (see below), which is in contrast with the results of non-enzymic breakdown (e.g. in the absence of Mg^{2+}), which produces, in aq. 50 % ethanol solution, compounds which are both less polar and much more polar than ATP as judged by anion-exchange h.p.l.c. One might therefore have expected to detect some dimers or trimers of PG-ATP units if random hydrolysis of the 3'-OH bonds in the oligomer had taken place; that only PG-ATP monomers have been found may imply that this enzyme is an exo-phosphodiesterase.

Cellular location and partial purification of a lytic activity

Rat liver homogenized in proteinase-inhibitor-containing mannitol/sucrose/EDTA/Tris/HCl buffer, pH 7.4, was fractionated by differential centrifugation (see the Materials and methods section) and each fraction tested for breakdown activity. Only the crude 'mitochondrial' fraction had appreciable activity, and then only after it had been treated to sonic disintegration (results not shown). When this sonicated fraction was separated by high-speed centrifugation into extract and membranes (see the Materials and methods section), the activity was found to be in the soluble extract. Treatment of this soluble extract with 60 %-(w/v)-satd. $(NH_4)_2SO_4$ precipitated about 80 % of the recoverable activity, along with around 50 % of the extract protein; the $(NH_4)_2SO_4$ precipitate was redissolved in 1 mM-EDTA/2 mM-benzamidine/20 mM-Bistris propane buffer, pH 6.6, containing 0.01 % (w/v) PMSF, desalted by dialysis against the same buffer and concentrated to about 22 mg of protein/ml using an Amicon 8050 ultrafiltration cell with a 30 kDa cut-off membrane. A portion (2 ml) of the resulting protein solution was applied to an f.p.l.c. anion-exchange column and eluted with an NaCl gradient; an example of such an experiment is shown in Fig. 1. All of the activity was eluted between 0.13 M- and 0.15 M-NaCl. The specific activity of the recovered activity increased ~15-fold; the recovery of activity was, however, disappointingly low at 15–20 % and was not improved by re-addition of the other protein-containing fractions to the active fractions. The active fractions were combined, desalted and concentrated as above to ~10 mg/ml and stored at 4 °C in assay buffer.

Fig. 2 shows a Lineweaver–Burk plot of the rate of solubilization of $[^{14}C]$ oPG-ATP in aq. 50 % cold ethanol over a range of substrate concentrations. In the inset to Fig. 2 is shown an example of a preliminary time-course assay to establish the

Fig. 1. F.p.l.c. chromatographic separation of the lytic activity

The 60% -satd.- (NH₄)₂SO₄ precipitate from the soluble extract of the crude mitochondrial fraction was dissolved in column buffer, desalted by dialysis and concentrated by ultrafiltration (see the text for additional details). A 44 mg portion of this protein in 2 ml was applied to a Pharmacia H10/10 Mono Q column which had been equilibrated with 1 mM-EDTA/2 mM-benzamidine/20 mM-Bistris propane, pH 6.6, containing 0.01% (w/v) PMSF and developed at 4 ml/min in this buffer with a 0–1 M-NaCl gradient. The fractions were assayed for protein and oPG-ATP breakdown activity (▨).

Fig. 2. Lineweaver-Burk plot of data from the breakdown assay

Between 7 and 40 nmol (adenosine equivalents) of [¹⁴C]oPG-ATP in 10 mM-MgCl₂/1 mM-adenosine/25 mM-Hepes buffer, pH 7.4, were incubated at 37 °C for 2 min with 0.4 mg of enzyme protein. The reaction was stopped by the addition of an equal volume of cold ethanol and the samples left at -20 °C for 2 h before they were filtered and assayed for radioactivity (see the Materials and methods section). The inset shows the time course of solubilization of 7 nmol equivalents of substrate under the same conditions.

Fig. 3. Distribution of lytic activity, protein and some marker enzymes after centrifugation of the crude mitochondrial fraction on a Percoll density gradient

Fractions (1 ml each) were collected from the top of the gradients, the density monitored and the protein content measured. The activities of acid phosphatase (lysosomes), succinate dehydrogenase (mitochondria), glucose-6-phosphate (endoplasmic reticulum) and lytic activity were assayed in each fraction. For further details, see the Materials and methods section.

linear region of substrate solubilization. The apparent K_m for adenosine equivalents of oPG-ATP can be estimated at about $35 \mu\text{M}$ and the V_{max} at 6 nmol/min per mg of protein. Comparable values were obtained with dialysed 60 % -satd. $(\text{NH}_4)_2\text{SO}_4$ precipitates from isolated mitochondria. Although the K_m compares not unreasonably with estimates of purine equivalents in oPG-ATP ($> 1 \text{ mM}$ in heart [4] and kidney [5]; at least 5 mM in liver (B. Patel, C. D. Gove, W. L. Hutchinson & J. Mowbray, unpublished work)), the relatively low V_{max} does not accord with the attainment of specific-radioactivity equilibrium with (soluble) ATP within 10 min in heart and kidney [4,5] and the less complete equilibration, but comparable rate of exchange, seen in perfused livers (B. Patel, C. D. Gove, W. L. Hutchinson & J. Mowbray, unpublished work). This, coupled with relatively poor recoveries of total activity noted above, could imply that some activating or regulatory function has been lost during purification. In earlier studies with a similar activity from rat heart [15], evidence for AMP- or adenosine-dependent activation was found. However, in the heart preparation (B. Patel, unpublished work), as in this one, no dependence on purine nucleotides or nucleosides can be shown with the mitochondrial activity.

In order to make an approximate estimate of the molecular size of this enzyme, it was subjected to gel-filtration chromatography at 4°C in 0.1 M-NaCl/50 mM- KH_2PO_4 buffer, pH 7.4, on Sephacryl S-300 calibrated with Blue Dextran ($M_r 2 \times 10^6$), rabbit muscle aldolase ($M_r 150\,000$) and BSA fraction V ($M_r 68\,000$); it ran with an M_r corresponding to 85 000 (results not shown).

Subcellular localization of the lytic activity

Percoll-gradient centrifugation studies (see the Materials and methods section) were undertaken to try to determine exactly in which cellular fraction, isolated as the crude mitochondrial pellet, this latent enzyme activity lay. Fig. 3 shows the findings from such a separation. The breakdown activity was distributed most closely in register with succinate dehydrogenase, notably in what appears to be a light mitochondrial fraction which separates clearly (unlike the heavy mitochondria) from the marker activities for lysosomes and endoplasmic reticulum.

The earlier observation that the activity is latent in intact vesicle preparations might suggest that it is located in the mitochondrial matrix. However, this does not exclude the possibility that access to the oligomeric substrate may be restricted by the outer membrane. In an effort to resolve this point, liver mitochondria were subfractionated into intact-mitoplasts and outer-membranes-plus-intermembrane-space enzymes by controlled digitonin treatment (see the Materials and methods section). The result was a clear separation of the enzyme from the intact mitoplasts (lysed before assay). The finding that very little activity was found associated with the mitoplasts makes it less likely that the enzyme could be associated with the outside of the inner membrane and released from there by digitonin. In addition, the observation reported above that the enzyme is found in the soluble extract when the crude mitochondrial fraction was separated into membranes and extract in the absence of detergent, would suggest that it is free in the intermembrane space.

Identification of the breakdown product

The aq.-ethanol-soluble radioactive product of [^{14}C]oPG-ATP breakdown co-migrated as a single spot with authentic ATP on PEI-cellulose two-dimensional t.l.c. (see [7]). However, this product eluted from the plate was not identified as ATP by either the luciferase-based assay [16] or the less specific hexokinase/glucose-6-phosphate dehydrogenase assay [17]. The estimated

Fig. 4. H.p.l.c. of the product of enzymic breakdown of oPG-ATP

(a) The aq.-50 %-ethanol-soluble product of enzymic digestion of oPG-ATP was purified by t.l.c. (see the text), extracted in 10 mM- MgCl_2 /25 mM-Hepes buffer, pH 7.4, and applied with 5 nmol of authentic adenosine, AMP, ADP and ATP to a 15 cm $\times$ 4 mm column packed with APS-Hypersil 5 and developed with a linear gradient of 1–400 mM- KH_2PO_4 pH 3.0. See the Materials and methods section for additional details. (b) Partial digestion for 10 min at 25°C in assay buffer of the breakdown product with phosphodiesterase 1 (type VIII-S from *Crotalus durissimus terrificus* venom) that had previously been dialysed against 10 mM-Tris/HCl buffer, pH 7.4. The mixture was Millipore-filtered and examined by h.p.l.c. as in (a). The products eluted before 5 min are present in incubations devoid of substrate.

phosphate/purine ratio (see [4]) was 3.8:1 (ATP gives 2.7:1), implying that the product is a whole unit of the oligomer (theoretical ratio 4:1). The finding that it migrates on t.l.c. in the borate buffer system (unlike oPG-ATP) strongly suggests (see [18]) that both the 2'- and 3'-OH of the ribose are free. Hence a reasonable proposal for the identity of the product would be that it is the phosphoglyceroyl-ATP monomer p3Gri1pp5'A (PG-ATP) (where p is phosphate, Gri is glyceric acid and A is adenosine).

Despite its polar behaviour on t.l.c. and its high phosphate content, this lytic product behaves in an anomalously much-less-polar fashion on anion-exchange h.p.l.c. in phosphate buffer, where it migrates between AMP and ADP (Fig. 4a). More than 90 % of the applied radioactivity co-chromatographed with this product. On the assumption that the product is PG-ATP monomer, it might be expected that phosphodiesterase 1, an exonuclease which requires a free 3'-OH group and liberates nucleoside 5'-phosphates, would hydrolyse this product to yield AMP or ADP, depending on the specificity of the enzyme. Fig. 4(b) shows that partial digestion with snake-venom phosphodiesterase yields a product which can be shown to be co-eluted with ADP; within experimental error the amounts of ADP match the disappearance of absorbance from the PG-ATP peak.

The identity of the ADP was confirmed by converting it into ATP using phosphoenolpyruvate and pyruvate kinase and identifying the ATP using luciferase [16]. The digestion also yields a product which co-migrates on t.l.c. [4] with authentic phosphoglycerate.

Thus we propose that the reaction catalysed by this liver mitochondrial intermembrane space enzyme is:

We gratefully acknowledge project-grant support from the Medical Research Council, the British Heart Foundation and The Wellcome Trust.

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Received 3 August 1990/19 September 1990; accepted 3 October 1990