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STUDIES ON THE NATURE OF ADENOSINE DIPHOSPHATASE ACTIVITY FROM RAT LIVER MITOCHONDRIA

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Adenosine diphosphatase (ADPase) activity was studied in rat liver with [β-^{32}P]ADP as a substrate. Mitochondria and outer mitochondrial membrane fractions were isolated and assayed for ADPase and various marker enzymes. ADPase activity was strikingly reduced when the outer membranes were removed from the mitochondria whether by digitonin treatment or osmotic shock. Addition of the inter-membrane space subfraction to the purified outer membranes resulted in enhanced ADPase activity. Addition of the inter-mitochondrial membrane enzyme adenylate kinase to outer membranes also produced a large stimulation of activity. The ADPase activity could also be reconstituted in vitro with adenylate kinase and either mitoplast ATPase or ouabain-sensitive ($Na^+ + K^+ + Mg^{2+}$)-ATPase. Chloroform-released ATPase, however, was not capable of producing an ADPase activity when combined with adenylate kinase. Gel permeation chromatography of Triton-solubilised outer mitochondrial membranes was unable to resolve ADPase activity from contaminating ATPase. These results suggest that the majority of ADPase activity in rat liver mitochondria consists of the coupled activity of adenylate kinase and ATPase.

Introduction

It is well established that ADP is important in platelet aggregation [1]. It has also been shown that the breakdown products of ADP, AMP and adenosine, will inhibit this ADP-induced aggregation. In line with this model an ADPase activity has been shown to be associated with plasma [2], vascular cells [2–5], heart[6], lung [7], smooth muscle [8] and cultured arterial cells [9]. ADPase activity has also been demonstrated in the rat liver [10]. Analytical subcellular fractionation of this tissue showed that ADPase had the same unimodal distribution profile as succinate dehydrogenase, a mitochondrial marker, and could be resolved from markers for the plasma membrane, the lysosomes and the endoplasmic reticulum. Further mitochondrial subfractionation with digitonin located the ADPase activity to the outer membrane fraction. Purification of the enzyme was hampered by large losses of activity after subfractionation by digitonin. The present study describes an explanation for this large loss of activity and illustrates the true nature of ADPase activity in rat liver mitochondria.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Materials and Methods

Preparation of rat liver mitochondria and outer mitochondrial membranes

Sprague-Dawley rats (180–220 g) were killed by cervical dislocation, the liver excised and placed in ice-cold mannitol medium (220 mM D-mannitol,

70 mM sucrose, 2 mM Hepes, 1 mM EDTA, 0.5 mg/ml bovine serum albumin adjusted to pH 7.4 with KOH). 10 g of minced rat liver were homogenised in 20 ml of mannitol medium in a glass Potter-Elvehjem homogeniser with a motor driven pestle (1000 rev/min for five passes) (Jencons Ltd., Leighton Buzzard, U.K.). The homogenate was filtered through 50 μm nylon gauze (J. Stanier and Co., Manchester, U.K.) and diluted to 40 ml with mannitol medium and centrifuged for 10 min at $700 \times g$. The resulting nuclear pellet was washed once and the supernatants centrifuged for 15 min at $7000 \times g$. The mitochondrial pellet obtained was washed twice and outer mitochondrial membranes were prepared by one of the following methods.

(a) Digitonin treatment. Freshly prepared mitochondria were treated with digitonin according to the method of Greenawalt [11]. Mitochondria were added to digitonin in mannitol medium at a concentration of 0.12 mg digitonin/mg of mitochondrial protein and stirred for 15 min at 4°C. The mixture was then diluted 1 in 4 with fresh medium and centrifuged at $9000 \times g$ for 10 min. After washing once, the combined supernatants were centrifuged at $144\,000 \times g$ for 60 min to pellet the outer mitochondrial membranes.

(b) Osmotic shock. Mitochondria were swollen in 20 mM NaH_2PO_4 containing 0.2 mg/ml bovine serum albumin, pH 7.2 according to the method of Parsons et al. [12]. The mitochondria were then centrifuged at $35\,000 \times g$ for 20 min. The pellet was resuspended in 20 mM phosphate and centrifuged at $1900 \times g$ for 15 min to pellet the mitoplasts. The resulting supernatant was then centrifuged again at $35\,000 \times g$ for 20 min to pellet the outer mitochondrial membranes. The pellet was resuspended in 4 ml of 20 mM phosphate and 1 ml aliquots loaded onto each of three discontinuous sucrose density gradients. Each gradient consisted of 1.2 ml of density 1.25 $g \cdot cm^{-3}$, 1.2 ml of density 1.17$g \cdot cm^{-3}$ and 1.2 ml of density 1.11 $g \cdot cm^{-3}$ sucrose. After centrifugation for 1 h at $115\,000 \times g$, the bands at each interface were collected and combined. Purified outer mitochondrial membranes formed a layer at the interface between the 1.11 $g \cdot cm^{-3}$ and 1.17 $g \cdot cm^{-3}$ sucrose bands.

Purification of mitochondrial ATPase was performed by a modification [13] of the chloroform extraction technique of Beechey et al. [14].

Enzyme assays

Radioassay for adenosine diphosphatase (EC 3.6.1.-) was performed [15] with [β-^{32}P]ADP as substrate. Assay of Mg^{2+}-dependent adenosine triphosphatase (EC 3.6.1.3) was performed with [γ-^{32}P]ATP as a substrate [16]. ATPase and ADPase activities were expressed as milliunits (mU) per mg protein, where 1 mU is equal to the hydrolysis of 1 nmol of ATP or ADP per min. Assays for the inner membrane marker succinate dehydrogenase (EC. 1.11.1.6) [17] and the outer mitochondrial membrane marker monoamine oxidase (EC 1.4.3.4) [18] were performed according to published methods.

Chromatography of Triton-solubilised outer mitochondrial membrane fractions on Sepharose 6B

Outer mitochondrial membranes were prepared by the digitonin procedure as described above. The membrane pellet (1 g) was stirred with 5 ml of 0.05 M Tris-HCl buffer, pH 8.0 containing 0.1% Triton X-100 for 30 min at 4°C. The extract was then centrifuged at $106\,000 \times g$ for 60 min and the supernatant applied to a 2.2×68 cm column of Sepharose 6B (Pharmacia, Uppsala, Sweden). The column was eluted with 0.05 M Tris-HCl (pH 8.0) containing 0.05% Triton X-100 at a flow rate of 20 ml/h. The protein content of the fractions was determined by a modification of the method of Lowry [19].

Materials

[β-^{32}P]ADP and [γ-^{32}P]ATP were purchased from Amersham International PLC. Nucleoside phosphate and ouabain sensitive ATPase were purchased from Sigma London Ltd. Digitonin was obtained from Calbiochem. Adenylate kinase was purchased from Boehringer. All other reagents were from BDH Ltd., Poole, Dorset and were of Analar grade.

Results

The recoveries of ADPase, ATPase, succinate dehydrogenase and monoamine oxidase activities following isolation and disruption of rat liver

TABLE I

ADPase, ATPase, SUCCINATE DEHYDROGENASE AND MONOAMINE OXIDASE ACTIVITIES OF SUBCELLULAR FRACTIONS (DIGITONIN TREATMENT)

The upper half of the table shows the recovery of ADPase, ATPase, succinate dehydrogenase and monoamine oxidase activities for a typical preparation of mitochondria by differential centrifugation of 10 g of rat liver homogenate. The lower half shows the recoveries of the same enzymes following disruption of the mitochondria by digitonin treatment. The percentage distributions of the recovered activities are shown alongside the relevant fractions. Total recoveries are shown for each enzyme for the two stages in the fractionation.

	Total enzyme activity (units)			
	Adenosine diphosphatase	Adenosine triphosphatase	Succinate dehydrogenase	Monoamine oxidase
Homogenate	30.4	208	45.2	2.6
Nuclear	8.67 (31%)	66.8 (35%)	12.0 (34%)	0.664 (26%)
Mitochondrial	12.6 (45%)	64.4 (33%)	21.8 (62%)	1.34 (53%)
Post-mitochondrial supernatant	6.62 (24%)	64.3 (32%)	1.46 (4%)	0.563 (22%)
Recovery	(92%)	(95%)	(78%)	(99%)
Mitochondria	12.6	64.4	21.8	1.34
Mitoplasts	0.64 (21%)	102 (73%)	21.7 (91%)	0.122 (11%)
Outer membrane	2.36 (79%)	35.5 (25%)	1.89 (8%)	0.719 (67%)
Soluble (inter-membrane space)	< 0.01	2.79 (2%)	0.32 (1%)	0.237 (22%)
Recovery	(24%)	(217%)	(110%)	(80%)

TABLE II

ADPase, ATPase, SUCCINATE DEHYDROGENASE AND MONOAMINE OXIDASE ACTIVITIES OF SUBCELLULAR FRACTIONS (HYPOTONIC TREATMENT)

The upper half of the table shows a typical experiment in which the ADPase, ATPase, succinate dehydrogenase and monoamine oxidase activities were assayed following disruption by osmotic shock of mitochondria prepared from 10 g of rat liver homogenate. The lower half of the table shows the activities following purification of the outer membrane fraction on a discontinuous sucrose density gradient. Fraction A corresponds to the interface between the sample layer and the layer of density 1.11 $g \cdot cm^{-3}$ and contains lipid droplets and aggregated protein. Fraction B corresponds to the interface between density 1.11 and 1.17 $g \cdot cm^{-3}$ and should contain mainly outer membrane. Fraction C corresponds to the interface between density 1.17 and 1.24 $g \cdot cm^{-3}$ and should contain inner membrane ghosts. The remaining fraction, the pellet, should contain unbroken inner membrane ghosts [12]. The percentage distributions of the recovered activities are shown alongside the relevant fractions. The total recoveries are also shown for each enzyme at each stage of the fractionation.

	Total enzyme activity (units)			
	Adenosine diphosphatase	Adenosine triphosphatase	Succinate dehydrogenase	Monoamine oxidase
Mitochondria	8.46	48.3	20.3	0.731
Mitoplasts	< 0.01	21.8 (76%)	8.44 (76%)	0.286 (44%)
Outer membrane	0.523 (100%)	7.09 (24%)	2.68 (24%)	0.322 (50%)
Soluble (inter-membrane space)	< 0.01	< 0.01	< 0.01	0.042 (6%)
Recovery	(6.1%)	(60%)	(56%)	(89%)
Outer membrane	0.523	7.09	2.68	0.322
A	0.012 (2%)	0.086 (1%)	< 0.01	0.014 (5%)
B	0.127 (26%)	0.841 (11%)	0.020 (1%)	0.186 (66%)
C	0.298 (60%)	2.26 (30%)	0.197 (13%)	0.061 (20%)
Pellet	0.059 (12%)	4.27 (57%)	1.28 (86%)	0.043 (44%)
Recovery	(95%)	(105%)	(56%)	(95%)

mitochondria with digitonin are shown in Table I. Succinate dehydrogenase, an inner membrane enzyme, was found with good recovery in the mitochondrial and subsequent mitoplast fractions. Mg^{2+}-dependent ATPase activity was also found mainly in the mitoplast fraction with about a 220% recovery. The outer mitochondrial membrane marker monoamine oxidase was found with good recovery in the mitochondrial and outer membrane fractions. Adenosine diphosphatase, although located mainly in the mitochondrial fraction with good recovery, showed a 75% loss of activity after digitonin treatment to prepare outer mitochondrial membranes. The recovered activity was located principally in the outer mitochondrial membrane fraction. This loss of activity could have been due to the action of digitonin so a second method of preparing outer membranes was attempted.

Table II shows the recoveries of total activity for the same enzymes as in Table I after mitochondria were disrupted by osmotic shock to prepare outer mitochondrial membranes. Succinate dehydrogenase was located primarily to the mitoplast fraction and the pellet obtained after discontinuous sucrose density gradient centrifugation. Total recovery of activity after osmotic shock was however lower than that obtained after digitonin treatment. Adenosine triphosphatase activity, although recovered in the same fractions as succinate dehydrogenase, again showed a lower recovery of total activity than that obtained after digitonin fractionation. No increase in total mitochondrial ATPase activity was seen after osmotic shock, suggesting that digitonin was having an effect on the latency or degree of coupling of ATPase. Monoamine oxidase was found with good recovery of activity in the fractions attributed to the outer mitochondrial membrane. Adenosine diphosphatase activity was associated with the outer mitochondrial membrane. However, the total recovery of activity was very low after removal of the outer mitochondrial membrane by osmotic shock. This suggested that the large losses of ADPase activity were not attributable to the method of preparing the membranes but could be due to the physical separation of the mitochondrial components. Further experiments were performed to examine this possibility with digitonin prepared outer membranes.

Table III shows the enhancement of ADPase activity of outer mitochondrial membranes by addition of inter-membrane space fraction. Addition of increasing concentrations of the inter-membrane fraction resulted in enhanced activity not attributable to the ADPase activity of the in-

TABLE III

STIMULATION OF ADPase ACTIVITY IN OUTER MITOCHONDRIAL MEMBRANES BY ADDITION OF INTER-MEMBRANE SPACE FRACTION

Outer-mitochondrial (OM) membranes (50 μg protein), prepared by digitonin treatment, were incubated with increasing concentrations of inter-mitochondrial (IM) fraction and assayed for ADPase. Inter-membrane fraction was also boiled, added to outer mitochondrial membranes and assayed for ADPase activity. n.a., not applicable.

Inter-membrane protein (μg/ml)	ADPase activity (mU/ml incubation medium)			
	OM + IM	OM + IM (boiled)	IM	IM (boiled)
1	6.20	n.a.	n.a.	n.a.
10	6.45	5.82	< 0.1	< 0.1
20	7.34	6.74	< 0.1	< 0.1
40	9.99	6.39	0.36	< 0.1
80	13.46	6.55	0.91	< 0.1
160	22.12	6.81	3.46	< 0.1

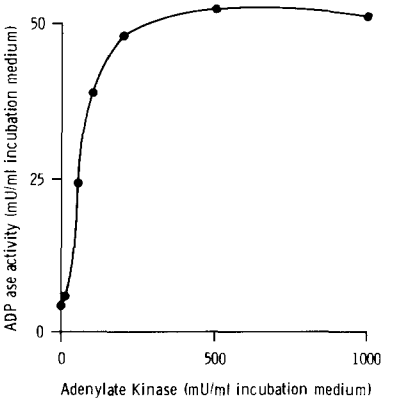

Fig. 1. Stimulation of ADPase activity in outer mitochondrial membranes by addition of adenylate kinase. Outer mitochondrial membranes (50 μg protein), prepared by digitonin treatment were incubated with increasing concentrations of adenylate kinase and assayed for ADPase as described.

ter-membrane fraction alone. Boiling of the inter-membrane fraction before addition of outer mitochondrial membranes resulted in the loss of all enhancement of activity. Boiling of outer mitochondrial membrane fraction also resulted in total loss of ADPase activity. These results suggested that removal of a protein component present in the inter membrane fraction could be responsible for the loss of ADPase activity during fractionation of mitochondria. One enzyme present at high concentrations in the inter-membrane fraction is adenylate kinase [20]. Fig. 1 shows the effect of adding purified adenylated kinase to an outer mitochondrial membrane fraction. Increasing concentrations of adenylate kinase added to the membrane fraction produced a large stimulation of ADPase activity. No ADPase activity was attributable to the adenylate kinase preparation.

One possible explanation for this stimulation of ADPase activity in outer mitochondrial membranes would be the liberation by ATPase of [γ-^{32}P] from the [γ-^{32}P]ATP produced by adenylate kinase. As shown in Tables I and II, outer mitochondrial membranes are contaminated by Mg^{2+}-ATPase activity. Fig. 2 shows the reconstitution of ADPase activity in vitro with adenylate kinase and ouabain-sensitive ($Na^+ + K^+ + Mg^{2+}$)-ATPase. The addition of increasing concentrations of adenylate kinase to ATPase resulted in a similar enhancement of ADPase activity. The stimulation of activity was saturable and a preparation of outer mitochondrial membrane incubated in parallel followed a similar stimulation pattern as that for 100 mU ATPase. The ATPase preparation, however, did contain a significant amount of ADPase activity in the absence of adenylate kinase. This could be due to contamination of the ATPase preparation with adenylate kinase. Assay of the preparation to determine the levels of contamination are difficult to interpret as most adenylate kinase assays involve the use of an ATP trapping system which would obviously be interfered with by the ATPase. Another possibility is that ATPase may exhibit an ADPase activity [21].

Fig. 3 shows an experiment where equivalent amounts of ATPase activity from mitoplast and chloroform-released ATPase preparations were incubated with increasing concentrations of adenyl-

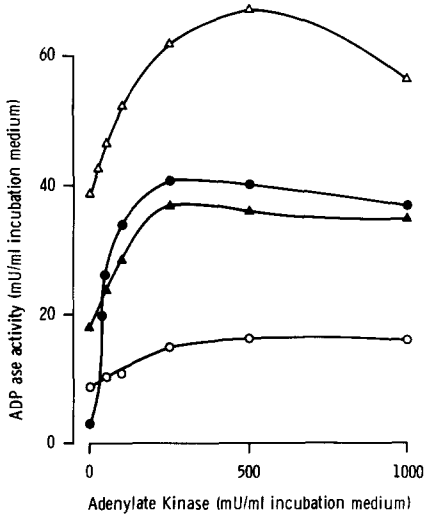

Fig. 2. Reconstitution of ADPase activity with ATPase and adenylate kinase. Ouabain sensitive ATPase was incubated with adenylate kinase and assayed for ADPase activity. The figure also shows the stimulation of ADPase activity in outer mitochondrial membrane fraction by addition of adenylate kinase. ○———○, 50 mU ATPase/ml; ▲———▲, 100 mU ATPase/ml; △———△, 200 mU ATPase/ml; ●———●, outer membrane (50 μg protein/ml).

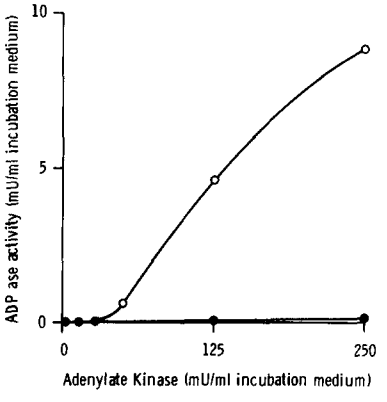

Fig. 3. Reconstitution of ADPase with adenylate kinase and mitoplast ATPase, or chloroform-released ATPase. Chloroform-released mitochondrial ATPase and mitoplast ATPase, prepared by subfractionation of mitochondria, were incubated with increasing concentrations of adenylate kinase and assayed for ADPase. The concentration of the two preparations in terms of their ATPase activity was identical in each experiment (16 mU/ml incubation medium). The ADPase activity of mitoplast ATPase (○———○) and chloroform-released ATPase (●———●) are shown plotted against adenylate kinase concentration.

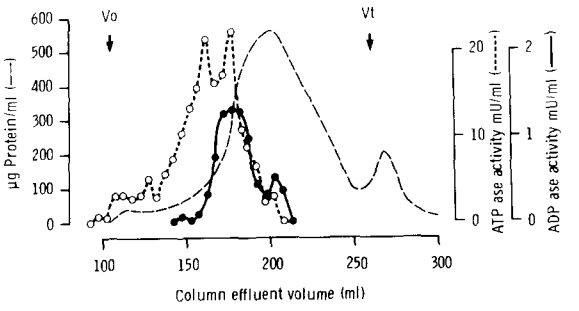

Fig. 4. Chromatography of Triton-solubilised outer mitochondrial membrane on Sepharose 6B. Outer mitochondrial membranes (2.2 g) prepared by digitonin treatment were stirred slowly in 11 ml of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1% Triton X-100 for 30 min. Following centrifugation at 106000 × *g* for 60 min, the Triton-solubilised extract (10.5 ml) was applied to a column of Sepharose 6B (2.2 × 68 cm) and eluted with two column volumes of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05% Triton X-100 at a flow rate of 20 ml/h. The total column volume was 258 ml with $V_0 = 103$ ml. The diagram shows the protein profile, ADPase (●) and ATPase (○) activities.

ate kinase. No ADPase activity could be detected in the mitoplast or chloroform ATPase extract in the absence of adenylate kinase. The results show that chloroform-released ATPase, although capable of ATP hydrolysis, is not able to produce apparent ADPase activity even with high levels of exogenous adenylate kinase.

These experiments, therefore, suggest that loss of the majority of ADPase activity on subfractionation of mitochondria is due to separation of adenylate kinase from Mg^{2+}-ATPase, the components of an artefactual ADPase activity. To investigate the nature of the residual ADPase in outer mitochondrial membranes, the preparation was partially solubilised in Triton X-100. About 10% of the total ADPase and ATPase activities could be released from the outer mitochondrial membrane preparations. The solubilised fraction was then chromatographed on Sepharose 6B as shown in Fig. 4. Adenosine diphosphatase and ATPase activities were well resolved from the majority of the protein components. The ATPase activity was resolved into two major peaks with the ADPase activity cochromatographing with the second peak of activity. Assay of adenylate kinase was not possible due to dilute concentrations of protein present and the problem of interference in the assay by ATPase.

Discussion

Earlier workers in this laboratory [10] demonstrated that rat liver contained an ADPase activity localised to the outer mitochondrial membrane. The possibility that the activity could be the result of the combined action of ATPase and adenylate kinase was considered, but was rejected largely on the basis that solubilised ATPase and adenylate kinase in combination, did not exhibit ADPase activity. However, work presented here demonstrates that it is likely that the majority of rat liver mitochondrial ADPase activity in vitro could be due to the concerted action of these two enzymes.

Subfractionation of rat liver mitochondria to obtain an outer membrane fraction results in a large loss of ADPase activity. The loss of activity is independent of the method of fractionation. This would suggest that an event such as removal of a cofactor or required ion had occurred, i.e. an inactivation due to a physical separation of components, not a chemical modification. Monoamine oxidase was found with good recovery in both the outer mitochondrial membrane preparations.

Reconstitution studies demonstrated that addition of the inter-membrane fraction could enhance the ADPase activity of outer mitochondrial membranes. Furthermore, addition of purified adenylate kinase resulted in a massive stimulation of ADPase activity in outer membranes. Addition of a ouabain-sensitive ($Na^+ + K^+ + Mg^{2+}$)-ATPase to adenylate kinase produced an ADPase reaction in vitro.

The major evidence proposed by Smith et al. [10] against a coupled ADPase was the lack of reconstitution of activity with adenylate kinase and chloroform-released ATPase, a finding confirmed in the present study. Lowe and Beechey [22] have reported differences in the properties of chloroform-released ATPase compared with other preparations of soluble mitochondrial ATPase. It has also been suggested that soluble ATPase preparations of low ATP hydrolysing activity (e.g., chloroform ATPase) more closely resemble the in vivo ATP synthetase complex [23]. It is also possible that a membrane-protein interaction is required for the coupled reaction to occur. Certainly the ($Na^+ + K^+ + Mg^{2+}$)-ATPase, which also exhibits ADPase activity in the presence of adenylate

kinase, is a membrane protein.

It is therefore apparent that the majority of the ADPase activity of rat liver mitochondria consists of a coupled reaction involving adenylate kinase and ATPase. Subfractionation destroys this activity by separating the intermembrane adenylate kinase from the inner membrane Mg^{2+}-ATPase. Whether this reaction would proceed in vivo in coupled mitochondria is unlikely. The K_m for the adenine nucleotide transporter is about 0.001–0.01 mM depending on the energy state of the mitochondria [24] and the K_m reported for adenylate kinase in bovine liver is about 1 mM [25]. Therefore ADP would be preferentially transported into the mitochondria and rephosphorylated. In addition, in coupled mitochondria the F_1-ATPase is an ATP synthetase not hydrolyase.

The nature of the residual ADPase in the outer membrane of rat liver mitochondria after subfractionation is not known. Treatment with digitonin results in outer mitochondrial membranes containing both ATPase and adenylate kinase [10]. Further purification of Triton-solubilised outer mitochondrial membranes failed to provide ADPase activity free from ATPase. It is not known where the rat liver adenylate kinase, that contaminates the outer mitochondrial fraction, chromatographs on the Sepharose column. However, human liver adenylate kinase has been purified and shown to be of molecular weight 26000 [26]. It is therefore likely that the enzyme would elute at the total column volume. Therefore at least three possibilities could be considered as to the nature of the ADPase activity after chromatography on Sepharose 6B: (1) An enzyme exists in the outer membrane capable of hydrolysing ATP or ADP. (2) The activity in the outer membrane consists of a complex of ATPase and adenylate kinase. (3) The ATPase and ADPase activities of the outer membrane fraction were not separable under the conditions of chromatography. It is therefore possible that the outer membranes of rat liver mitochondria do contain an ADPase activity but the majority of the activity is due to adenylate kinase and ATPase.

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