LOCALIZATION OF OLIGOMYCIN-SENSITIVE ADP-ATP EXCHANGE ACTIVITY IN RAT LIVER MITOCHONDRIA

Carl A. Schnaitman[1] and Peter L. Pedersen

Department of Physiological Chemistry, The Johns Hopkins School of Medicine, Baltimore, Maryland

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Intact rat liver mitochondria catalyze an ADP-ATP exchange which is partially inhibited by oligomycin and dinitrophenol (Wadkins and Lehninger, 1963; Guillory and Slater, 1965). It has been postulated that the dinitrophenol- and oligomycin-sensitive ADP-ATP exchange reaction is catalyzed by the enzyme(s) participating in ATP formation during oxidative phosphorylation (Wadkins and Lehninger, 1958; 1963). However, the presence of large amounts of adenylate kinase and nucleoside diphosphokinase, which catalyze oligomycin-insensitive ADP-ATP exchanges, has rendered further investigation of the oligomycin-sensitive exchange difficult.

In this communication it is shown that when rat liver mitochondria are fractionated with digitonin the oligomycin-sensitive ADP-ATP exchange activity is recovered with the inner membrane-matrix fraction, while all of the nucleoside diphosphokinase and adenylate kinase activity is released with the outer membrane. The exchange activity found in the inner membrane-matrix fraction is localized in the inner membrane and is completely inhibited by oligomycin, dinitrophenol, and atractyloside when assayed in the absence of added Mg^{++}. Thus, the oligomycin-sensitive ADP-ATP exchange activity observed in intact mitochondria does not appear to be catalyzed by nucleoside diphosphokinase or adenylate kinase.

Materials and Methods

Rat liver mitochondria were isolated as described by Schnaitman and Greenawalt (1968). They were suspended in isolation medium containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES buffer, pH 7.4, and 0.5 mg/ml crystalline bovine serum albumin to a

[1] Postdoctoral Fellow of the American Cancer Society. Present address: Department of Microbiology, The University of Virginia Medical School, Charlottesville, Va.

concentration of 100 mg mitochondrial protein/ml. Equal aliquots of the mitochondrial suspension and a solution containing 12 mg digitonin/ml in the same isolation medium were mixed and stirred at 0° for 15 min. The suspension was diluted with 3 vols. of isolation medium and centrifuged twice at 9,000g for 10 min. The pooled supernatant fractions contained essentially all of the outer membrane, and the pellet contained essentially all of the inner membrane and matrix (Schnaitman and Greenawalt, 1968; Schnaitman, Erwin and Greenawalt, 1967). The inner membrane-matrix fraction was separated into inner membrane and matrix by treating with 0.1 mg Lubrol WX (obtained from I.C.I. Organics, Inc., Providence, Rhode Island) per mg protein followed by sedimentation of the inner membrane at 144,000g for 1 hr (Schnaitman and Greenawalt, 1968).

The ADP-ATP exchange reaction was assayed exactly as described by Wadkins and Lehninger (1963). Samples (0.1-0.8 mg protein) were equilibrated for 8 min at 30° in a medium containing, in 0.45 ml, 11 mM imidazole buffer, pH 6.9, 54.5 mM sucrose, 5.3 mM ADP, and where indicated, 0.56 mM 2,4-dinitrophenol, 111 μM atractyloside, 1.0 μg oligomycin, and 5.3 mM $MgCl_2$. The ATP (10 mM) and 0.1 μc ^{14}C-ADP were then added to start the reaction (total vol. 0.60 ml). In all assays initial rates were measured; the reaction was not allowed to approach isotopic equilibrium except as noted in Table II. ATPase activity was assessed in the same assay by measuring the inorganic phosphate formed.

Nucleoside diphosphokinase activity was estimated by two different procedures. The reaction ATP + CDP → ADP + CTP was measured spectrophotometrically using the coupled lactic dehydrogenase-pyruvate kinase assay described by Glaze and Wadkins (1967). The enzyme was activated by the addition of 0.1% Lubrol to the assay; the observed rates were corrected for ATPase activity by omitting CDP from the assay. The reaction ADP + dCTP → ATP + dCDP was measured spectrophotometrically by a modification of the assay described by Goffeau, Pedersen, and Lehninger (1967). The assay contained, in 1 ml, 0.5 mM NADP, 10 mM glucose, 7.5 I.U. hexokinase, 0.4 I.U. glucose-6-phosphate dehydrogenase, 0.45 mM KCN, 5 mM $MgCl_2$, 40 μM ADP, 0.1 mM AMP, 3 mM dCTP, 70 mM glycylglycine, pH 8.0, and 0.3 mg Lubrol/mg mitochondrial protein. The rate observed with the complete assay system was corrected for the slow reaction observed in the absence of enzyme (due to the direct reaction of dCTP with hexokinase and glucose) and for that observed in the absence of dCTP (due to adenylate kinase activity). The validity of these corrections was confirmed by the addition of purified nucleoside diphosphokinase from beef liver to the assays.

Adenylate kinase was also estimated by two different procedures. The reaction ADP → AMP + ATP was measured by following the formation of ^{14}C-AMP from ^{14}C-ADP.

The separation of the nucleotides by paper electrophoresis and the assay procedure were exactly as described by Bygrave and Lehninger (1966). The same reaction was measured spectrophotometrically in an assay containing, in 1 ml, 0.75 mM NADP, 15 mM glucose, 10 I.U. hexokinase, 0.4 I.U. glucose-6-phosphate dehydrogenase, 0.45 mM KCN, 3 mM ADP, 5 mM $MgCl_2$, 70 mM glycylglycine buffer, pH 8.0, and 0.3 mg Lubrol/mg mitochondrial protein. Monoamine oxidase and malic dehydrogenase were assayed as previously described (Schnaitman, Erwin and Greenawalt, 1967).

Results and Discussion

Table 1 shows the distribution of nucleoside diphosphokinase and adenylate kinase in relation to oligomycin-sensitive and total ADP-ATP exchange activity, as well as the enzymatic markers for outer membrane (monoamine oxidase) and matrix (malic dehydrogenase).

Table 1. Localization of Enzymes in Mitochondrial Fractions

	Inner Memb. + Matrix	Outer Memb.[1]	Recovery[2]
^{14}C-ADP-ATP Exchange[3] Activity:			
Total	40.7%	37.6%	78.3%
Oligomycin-sensitive	65.0%	0	65.0%
Malic Dehydrogenase	88.1%	15.4%	103.5%
Monoamine Oxidase	2.6%	97.0%	99.6%
Nucleoside Diphosphokinase:			
ADP + dCTP → ATP + dCDP	2.4%	112.0%	114.4%
ATP + CDP → ADP + CTP	0	115.0%	115.0%
Adenylate Kinase:			
ADP → ATP + AMP	0	90.2%	90.2%
^{14}C-ADP → ^{14}C-ATP + ^{14}C-AMP	0.3%	73.0%	73.3%

[1]This fraction also contains soluble proteins localized between the inner and outer mitochondrial membranes (Schnaitman and Greenawalt, 1968).

[2]Based on whole, unfractionated mitochondria.

[3]Assayed in the absence of added Mg^{++}. All assay details are given in the methods.

The inner membrane-matrix fraction, which contains all of the recovered oligomycin-sensitive ADP-ATP exchange activity, contains virtually no detectable nucleoside diphosphokinase or adenylate kinase activity. The validity of this observation is supported by the fact that the assays which employed the glucose-hexokinase ATP-trapping system are not affected

Table II. ADP-ATP Exchange Activity of Mitochondrial Fractions

Assay Conditions	ADP-ATP Exchange Rate	ATPase
	(nmoles product formed per min per mg)	
Whole Mitochondria	65.5	41.5
+ Oligo	21.2	10.5
+ DNP	10.9	332
+ Atract	15.9	28.6
+ Mg^{++}	isotopic eq.[1]	49.7
+ Oligo + Mg^{++}	isotopic eq.[1]	22.8
+EDTA	10.9	12.8
Inner Memb. + Matrix	34.0	35.7
+ Oligo	4.1	6.4
+ DNP	0	263
+ Atract	0	15.5
+ Mg^{++}	34.0	71.5
+ Oligo + Mg^{++}	35.2	9.6
+ EDTA	12.8	56.0
Outer Membrane	86.0	78.0
+ Mg^{++}	1750.0	191.0
+ Oligo + Mg^{++}	2170.0	78.0

by the levels of ATPase activity found in the inner membrane-matrix preparation. Hence it is possible to detect very low levels of nucleoside diphosphokinase or adenylate kinase in this fraction. Since the non-ionic detergent Lubrol was included in these assays it is unlikely that any enzymatic activity is "masked" by the inner membrane. Neither digitonin nor Lubrol inhibit purified nucleoside diphosphokinase or adenylate kinase at concentrations much higher than employed in this study.

[1]This activity is greater than 660 nanomoles/min/mg protein.

Table II shows a comparison of the properties of the ADP-ATP exchange activity associated with these mitochondrial fractions and with intact mitochondria. The ADP-ATP exchange activity associated with the inner membrane-matrix fraction is completely inhibited by oligomycin, dinitrophenol, and atractyloside in the absence of added Mg^{++}. In the presence of Mg^{++} the exchange becomes insensitive to oligomycin. The inner membrane-matrix preparation shows respiratory control in the absence of added Mg^{++}, but not in its presence (Schnaitman and Greenawalt, 1968). These properties are consistent with the finding of Bygrave and Lehninger (1966) that oligomycin-sensitivity of the ADP-ATP exchange occurs only in mitochondria with respiratory control. The inhibition of exchange activity by EDTA indicates that this activity may require some Mg^{++}. This requirement may be met by endogenous Mg^{++}, since almost all of the endogenous Mg^{++} found in intact mitochondria is retained in the inner membrane-matrix fraction after digitonin treatment (unpublished data). About 70% of this Mg^{++} is released from the inner membrane by Lubrol. This may explain the lower exchange rate observed with the isolated inner membrane in the absence of added Mg^{++}. Thus the presence of an intact outer membrane may be necessary to protect mitochondria from deleterious effects of a high external Mg^{++} concentration.

Additional experiments to be described in detail elsewhere showed that the ADP-ATP exchange of mitochondria remained with the inner membrane when the inner membrane-matrix fraction was separated into inner membrane and matrix with the non-ionic detergent Lubrol.

These results demonstrate that the oligomycin-sensitive ADP-ATP exchange activity observed in intact rat liver mitochondria is clearly distinct from the oligomycin-insensitive ADP-ATP exchange activity catalyzed by nucleoside diphosphokinase and adenylate kinase observed in intact mitochondria in the presence of added Mg^{++}. They do not support the suggestion of Glaze and Wadkins (1967) that oligomycin and dinitrophenol-sensitive ADP-ATP exchange activity is catalyzed by a bound form of nucleoside diphosphokinase. These data do, however, support the suggestion of Bygrave and Lehninger (1966) that the oligomycin-sensitive ADP-ATP exchange activity observed in intact mitochondria is catalyzed by an enzyme participating in oxidative phosphorylation.

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