SOLUBILIZATION OF ATRACTYLOSIDE-SENSITIVE ADP(ATP) BINDING ACTIVITY OF RAT LIVER MITOCHONDRIA

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Summary - Treatment of rat liver mitochondria with the nonionic surfactant Brij 56 yields a non-sedimentable supernatant fraction containing over 60 percent of the original atractyloside-sensitive ADP(ATP) binding activity and a sedimentable (membrane) fraction showing atractyloside-insensitive ADP binding. The supernatant fraction exhibited no atractyloside-sensitive binding of AMP, GDP, or CDP. Atractyloside-sensitive binding of ADP was not inhibited by oligomycin, antimycin A, 2,4-dinitrophenol, or azide. The atractyloside-sensitivity of ADP binding was lost on heating. The properties of the non-sedimentable ADP(ATP) binding factor agree closely with those of the ADP-ATP translocase of the inner mitochondrial membrane.

The inner mitochondrial membrane contains a carrier or translocase, specifically inhibited by atractyloside, that facilitates an equimolar exchange of external for internal ADP or ATP (reviews: 1, 2). Most of the available information on the properties of the ADP-ATP translocase has been deduced from kinetic and ligand-binding studies carried out on intact mitochondria (1, 2). Although atractyloside-sensitive ADP binding and/or translocation have been found to take place in submitochondrial membrane vesicles (3, 4), preparation of a soluble, non-sedimentable form of the ADP-ATP translocase has not been reported.

In this paper we describe the preparation and some properties of a surfactant-solubilized fraction from rat liver mitochondria that exhibits many of the properties of the ADP-ATP translocase of the inner membrane.

MATERIALS AND METHODS

The Brij 56 solutions were prepared by dispersing 2.0 g of Brij 56 in 100 ml H_2O, heating to 42°C to yield a clear solution, and cooling to room temperature; some turbidity appeared below 29°C but the solutions remained homogenous. The final pH was 6.5.

Mitochondria were isolated from the livers of male Sprague-Dawley albino rats, washed 3 times with 0.25 M sucrose, and suspended in cold 0.25 M sucrose - 10 mM Tris chloride pH 7.4 (hereafter referred to as sucrose-Tris) at 25 mg mitochondrial protein per ml. Brij extracts containing non-sedimentable atractyloside-sensitive ADP binding activity were prepared by adding 0.25 ml of 2 percent Brij solution to 2 ml of the mitochondrial suspension at 0°C (0.1 mg Brij per mg protein). The mixture was stirred

30 min at 0°C and then centrifuged at 144,000 xg for 60 min. The supernatant fraction consisted of two layers, the upper somewhat turbid and the lower clear and yellow in color; the pellet packed firmly. The lower supernatant layer, which contained about 80 percent of the solubilized protein and all of the atractyloside-sensitive ADP binding activity, was used for most of these studies.

To determine the amount of nucleotide bound by solubilized mitochondrial fractions, a gel exclusion method was employed in which [^{14}C]-labeled nucleotide was allowed to partition between Sephadex G-25 and the excluded phase containing the mitochondrial components. [^{14}C]-labeled nucleotide in 0.60 ml of the sucrose-Tris was equilibrated with 100 mg Sephadex G-25 for at least 4 hours at room temperature and cooled to 0°C. The solution being tested (0.25 ml) was added to the equilibrated system (final nucleotide concentration of 10-15 μM) and the system stirred for 30 min at 0°C. In control-blank experiments, 0.25 ml of sucrose-Tris replaced the solution to be tested. The nucleotide concentration in the excluded phase was determined by scintillation counting. Paired tubes, one containing 12 μM atractyloside and the other no inhibitor, were used to determine atractyloside-sensitive and atractyloside-insensitive nucleotide binding.

Due to the combined action of adenylate kinase and F_1 ATPase, present in the unfractionated Brij-treated mitochondrial suspension, the endogenous ADP or ATP was completely converted into AMP prior to assay. The supernatant fraction contained no ATPase activity, which is localized entirely in the pellet (membrane) fraction.

The rate of dialysis toward equilibrium across a cellophane membrane was useful as a semiquantitative assay of nucleotide binding. Such assays agreed with results obtained by the gel exclusion method.

Brij 56 was a gift from Imperial Chemical Industries of America, Inc. Atractyloside was a gift from Professor R. Santi, University of Padua, Italy.

RESULTS

Effect of Brij 56 concentration on extraction of atractyloside-sensitive ADP binding activity. Figure 1 shows the fraction of the total mitochondrial protein extracted and the distribution of atractyloside-sensitive ADP binding activity between the supernatant and pellet fractions at various concentrations of Brij 56. About 80 percent of the original ADP binding activity of the mitochondrial pellet was removed (144,000 xg; 60 min) at 0.1 percent Brij and essentially 100 percent at 0.2 percent. At 0.22 percent Brij, the supernatant fraction contained about 70 percent of the original atractyloside-sensitive ADP binding activity and about 74 percent of the total mitochondrial

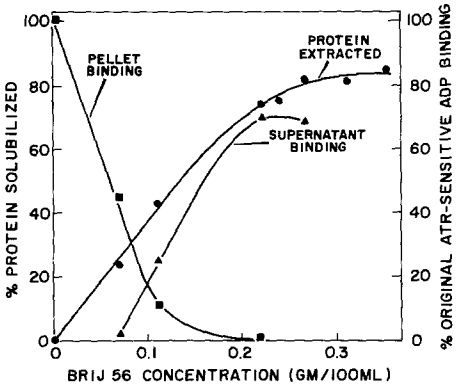

Figure 1. Effect of Brij 56 concentration on ADP binding by mitochondrial fractions. For assay, the clear supernatant fraction was added directly (0.25 ml). The drained pellet was resuspended in sucrose-Tris, then assayed. [^{14}C]-ADP was added at 8.8 μM and atractyloside (ATR) at 12 μM.

protein. The Brij 56 concentration used in all subsequent experiments was 0.22 percent in the ratio of 0.1 mg Brij per mg mitochondrial protein.

Fractionation of ADP binding activity. Table 1 shows the atractyloside-sensitive and atractyloside-insensitive ADP binding activity of the supernatant and pellet fractions of the Brij-treated mitochondria. The large amount of atractyloside-insensitive ADP binding evoked by the Brij treatment is expected, since several ADP binding enzymes (F_1 ATPase, amino acyl-CoA synthetases, carbamyl phosphate synthetase, etc.) are located in the internal matrix of intact mitochondria and are thus not able to bind extramitochondrial ADP in the presence of atractyloside. Disruption of the membrane by Brij makes these enzymes accessible to ADP in the presence of atractyloside.

About 75 percent of the ADP binding activity in the supernatant fraction is atractyloside-sensitive; in some experiments it exceeded 90 percent. In contrast, none of the ADP binding activity in the pellet fraction was atractyloside-sensitive.

Yield of non-sedimentable atractyloside-sensitive ADP binding activity. If it is assumed that rat liver mitochondria contain about 270 pmoles of total atractyloside-sensitive ADP binding sites per mg protein (2,5), then each batch of 50 mg of mitochondrial protein contains about 13,500 pmoles of atractyloside-sensitive ADP binding activity prior to extraction. As shown in Table 1, the average atractyloside-sensitive ADP binding activity recovered in the clear supernatant fraction of the Brij extracts (average volume, 1.7 ml; protein content, 18 mg per ml) was 272 pmoles per mg protein

TABLE 1

BINDING OF [^{14}C]-ADP TO FRACTIONS OBTAINED WITH BRIJ 56

Fraction	Specific ADP Binding (pmole per mg protein) Total	ATR-sensitive	ATR-insensitive	Percent ATR sensitivity	Percent total ATR-sensitive activity recovered
Supernatant					
Exp. 1	420	314	106	75	71
Exp. 2	344	340	4	99	77
Exp. 3	222	171	51	77	39
Exp. 4	454	316	138	70	72
Average of 8 experiments	381	272	109	71	62
Pellet					
Average of 2 experiments	423	6	417	1.4	0.6

[^{14}C]-ADP was added at 12 μM and atractyloside, where added, was 12 μM. The supernatant assays contained 4.5 mg protein per tube and the pellet assays 3.25 mg per tube.

TABLE 2

SPEICIFICITY OF NUCLEOTIDE BINDING

Nucleotide	Conc (μM)	Specific Nucleotide Binding (pmole per mg protein) Total	ATR-sensitive	ATR-insensitive
ADP	13	262	236	26
ATP	13.5	235	218	17
AMP	9	293	2	291
CDP	16.1	15	1	14
GDP	11.6	106	0	106

The [^{14}C]-labeled nucleotides were added in the concentrations shown; atractyloside was added at 12 μM.

or about 8300 pmoles per batch, some 62 percent of the original activity.

Nucleotide specificity. Data in Table 2 show that the supernatant fraction from Brij 56 extracts of rat liver mitochondria binds ATP, ADP and AMP. Binding of ATP and ADP was almost entirely inhibited by atractyloside, whereas the binding of AMP was insensitive. GDP was bound to a lesser extent

but was totally insensitive to atractyloside, as was the small amount of CDP binding.

Effect of other inhibitors. Atractyloside-sensitive ADP binding in the clear supernatant fraction was not appreciably inhibited by 15 μM oligomycin, 15 μM antimycin A, 45 μM 2,4-dinitrophenol, or 100 μM azide (Table 3), in agreement with the properties of the ADP-ATP translocase of intact rat liver mitochondria (6).

TABLE 3

THE EFFECT OF INHIBITORS ON ATR-SENSITIVE ADP BINDING

Inhibitor	Concentration (μM)	ATR-Sensitive ADP Binding (pmole per mg protein)
None	—	316
Azide	100	332
Antimycin A	15	313
Oligomycin	15	274
2,4-dinitrophenol	45	231

The inhibitors were added to the extract 15 min prior to assay. [^{14}C]-ADP was added at 17.5 μM and atractyloside (ATR) at 12 μM.

Selective effect of heat. The atractyloside-sensitive ADP binding activity was totally destroyed by heating the clear supernatant fraction from the Brij extract for 1 min in a water bath at 100°C (Table 4). This treatment did not, however, destroy the atractyloside-insensitive activity. In fact, heating usually caused an increase in atractyloside-insensitive ADP binding.

DISCUSSION

The properties of the solubilized atractyloside-sensitive ADP binding activity described in this paper closely resemble those of the ADP-ATP translocase of the inner mitochondrial membrane (5). The similarities may be listed. (1) The extracted factor is macromolecular, since it does not pass cellophane membranes and is excluded by Sephadex G-25. (2) It is probably a protein, since it, or its atractyloside sensitivity, is heat-labile. (3) It is sensitive to low concentrations of atractyloside. (4) It shows atractyloside-sensitive binding of ADP and ATP, but not of AMP, GDP or CDP. (5) It is insensitive to oligomycin, antimycin A, 2,4-dinitrophenol, and

TABLE 4

THERMAL INACTIVATION OF ATRACTYLOSIDE-SENSITIVE ADP BINDING

	Specific ADP Binding (pmole per mg protein)			
	Total	ATR-sensitive	ATR-insensitive	Percent ATR sensitivity
Before heating	339	224	95	72
After heating	170	2	168	1

The supernatant fraction (19 mg protein per ml) was placed in a boiling water bath for 1 min, cooled, and resuspended with a glass-teflon homogenizer. [^{14}C]-ADP was added at 13 μM and atractyloside (ATR) at 12 μM.

azide. The similarity in these cardinal properties strongly indicates that the Brij-solubilized atractyloside-sensitive ADP binding activity represents a portion of or perhaps the entire ADP-ATP antiport system of the inner mitochondrial membrane. This represents, to our knowledge, the first report in which atractyloside-sensitive ADP binding activity has been obtained in non-sedimentable form from mitochondria.

Brij 56 (ethoxylated cetyl alcohol) was the only solubilizing agent tested that successfully extracted atractyloside-sensitive ADP binding activity in non-sedimentable form from rat liver mitochondria. A series of ethoxylated octylphenols (Tritons) and other ethoxylated straight chain alcohols, cholate, digitonin, sodium dodecyl sulfate, chaotropic agents, sonication, and osmotic shock, either totally destroyed the activity or left it in the pellet fraction. In instances where both the pellet and the supernatant fractions were inactive, the factor may have been extracted, but in inactive form.

The solubilized atractyloside-sensitive ADP binding factor described here very likely occurs as a micelle in which Brij 56 molecules surround at least that portion of the carrier normally surrounded by lipid molecules in the intact membrane, leaving the nucleotide binding sites exposed and accessible. Such micelles may of course contain several proteins, or even portions of membrane in non-sedimentable form.

Since heating the solubilized ADP binding factor caused loss of atractyloside sensitivity with some increase in atractyloside-insensitive ADP binding, it appears possible that atractyloside binds to sites other than the

nucleotide binding site on the carrier molecule.

Further studies of the properties and purification of the Brij-extracted atractyloside-sensitive ADP(ATP) binding protein are under way.

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