Adenine Nucleotide Transport in Submitochondrial Particles and Reconstituted Vesicles Derived from Bovine Heart Mitochondria*

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SUMMARY

Uptake of ATP by submitochondrial particles from bovine heart was stimulated 15-fold by internal ADP. The transport was inhibited by atracyloside and bongkrekic acid. Vesicles prepared with soybean phospholipids and a protein fraction from mitochondria also catalyzed the ADP-dependent and inhibitor-sensitive uptake of ATP. This reconstituted system provides a suitable assay for the purification of the mitochondrial adenine nucleotide carrier.

The inner mitochondrial membrane catalyzes the generation of ATP from ADP and Pi on the matrix side of the membrane. To accomplish the delivery of ADP into the mitochondria and of ATP into the cytoplasm the inner mitochondrial membrane contains a translocation system that exchanges ADP for ATP (cf. Refs. 1 and 2). The inhibition of the process by atracyloside (3) and bongkrekic acid (4) has greatly facilitated the study of this transport system, but attempts to purify a biologically active transport protein have been unsuccessful, partly because no suitable assay system was available. In this communication, we show that an ADP-dependent and inhibitor-sensitive transport system can be observed in submitochondrial particles as well as in reconstituted vesicles.

MATERIALS AND METHODS

Mitochondria from bovine heart (5), ETPH (6), and partially purified soybean phospholipids (7) were prepared as described in the references with modifications described in the text. The cholate-insoluble protein fraction was prepared by incubating ETPH particles (at 30 mg per ml) for 30 min at 0° in the presence of 2% sodium cholate, 1 mM dithiothreitol, 20 μM EDTA, 125 mM sucrose, and 10 mM Tricine-KOH (pH 8). After centrifugation at 150,000 X g for 60 min, the pellet was suspended at about 40 mg of protein per ml in 1 mM dithiothreitol-10 mM Tricine-KOH (pH 8) and frozen at -80°. [γ-32P]ATP was prepared by photophosphorylation of ADP with ATP, by spinach chloroplasts (8). ADP (9) and proteins (10) were measured as described. Reconstitution was performed by two methods.

Cholate Dialysis Procedure—Soybean phospholipids (180 μmoles of organic phosphorus) were sonicated at room temperature until clear in the presence of 2 mM EDTA, 1 mM dithiothreitol, 154 mM KCl, 2% sodium cholate, and 20 mM Tris-Cl (pH 7.5) in a total volume of 5 ml. ADP (5 mM) and atracyloside (200 μM) were present in some preparations. The mixture was cooled to 0°, the cholate-insoluble protein fraction (2 mg) was added, and sonication was continued an additional 10 min at 0°. The mixture was then dialyzed at 4° for 18 hours against the sonication medium minus cholate. The vesicles were sedimented at 150,000 X g for 60 min and suspended in 104 mM KCl at about 1.5 mg of protein per ml.

Instant Vesicles—The sonication medium for the preparation of instant vesicles was the same as that described for the cholate dialysis procedure but with cholate omitted. The phospholipids without the protein were sonicated at room temperature until clear; the mixture was cooled to 0°, protein was added, and sonication was continued at 0° for 10 min. The vesicles were isolated as described above.

RESULTS AND DISCUSSION

Submitochondrial particles were prepared from mitochondria by the described procedure (5) except for the addition of 5 mM ADP instead of ATP and of 200 μM atracyloside where indicated. The particles, washed once in ice-cold 0.25 M sucrose, were stable for at least 72 hours at 0°.

Fig. 1 shows the dependency of the extent of ATP uptake on the concentration of ADP present during sonication. It can be seen also that the ADP content of the isolated particles increased in proportion to added ADP. Since the extent of ADP uptake by the process during sonication is approximately proportional to the amount of ADP present in the particles, there is apparently a 1:1 exchange of ATP for ADP as has been shown for mitochondria (1). There appears to be a small amount of endogenous ADP in the particles which does not exchange with external ATP and which might be tightly bound to noncarrier sites in the membrane. Furthermore, the extent of ATP uptake in ETPH prepared without added ADP compares favorably with the number of atracyloside removable ADP binding sites (0.3 nmole per mg of protein) in bovine heart mitochondria (11). The extent of AP uptake was found to be linear with protein concentration up to 5 mg per ml. At room temperature the adenine nucleotide exchange was very rapid, being essentially complete within 15 s. At 0° the rate of ATP uptake was much slower and time-dependent (Fig. 2). The ADP-dependent uptake of ATP was of the same order of magnitude as in mitochondria (1, 2) and strongly inhibited by 100 μM bongkrekic acid or by 200 μM internal atracyloside. However, atracyloside added externally had little effect on ATP uptake in ETPH. The amount of atracyloside used was in great excess. Observed Kᵢ values were about 3 μM at 1 mM ATP.

Adenine nucleotide transport was also demonstrated in phospholipid vesicles reconstituted with a hydrophobic protein fraction prepared from submitochondrial particles as described under “Materials and Methods” (Fig. 3). The rate of ATP uptake was stimulated severalfold by the inclusion of ADP during reconstitution. The values in the absence of ADP were usually between 0.25 to 0.45 nmole per mg of protein. Similarly in the absence of phospholipid vesicles ATP binding usually did not exceed 0.3 to 0.5 nmole per mg of protein and in fact it was slightly inhibited in the presence of ADP. Atractyloside (200 μM) inhibited this binding less than 50%.

Bongkrekic acid inhibited ATP uptake in ETPH and in reconstituted vesicles to the level observed without internal ADP. Most interesting was the effect of atracyloside. Inhibition was
FIG. 1. The effect of ADP concentration in the sonication medium on the extent of ATP uptake and ADP content of ETPH. The assay medium for ATP uptake contained 2 mM EDTA, 8 μg per ml of rutamycin, 250 mM sucrose, 1 mM ATP, 10 mM Tris-HCl, pH 6.5, about 10^6 cpm of [γ-32P]ATP per amole of ATP, and about 0.2 mg of protein in 1.0 ml total volume. The reaction was started by addition of protein and performed at room temperature. An aliquot was filtered at 1 min through a 0.45-μm Millipore filter, washed with 10 ml of 250 mM sucrose, dried, and counted in a Nuclear Chicago gas flow counter. 32P was determined to be about 1% of the radioactivity on the filters. Close to 99% of the 32P was released as P_i after hydrolysis in 2 N HCl for 7 min at 100°C.

FIG. 2. Rate of ATP uptake in ETPH at 0°C. Preparation of ETPH was described in the text. The reaction conditions were described in Fig. 1 except the reaction temperature was 0°C. Values indicate ATP uptake of particles prepared in the presence of 5 mM ADP minus the uptake rate of particles prepared in the absence of ADP. Atractyloside (200 μM) and bongkrekic acid (100 μM, 10-min preincubation at room temperature) were present where indicated.

FIG. 3. Rate of ATP uptake by reconstituted vesicles. The preparations are described in the text. The assay medium for reconstituted vesicles contained 2 mM EDTA, 8 μg per ml of rutamycin, 154 mM KCl, 100 μM ATP, 10 mM Tris-HCl, pH 6.5, about 10^6 cpm of [γ-32P]ATP per amole of ATP, and about 0.2 mg of protein in 1.0 ml total volume. The reaction was started by addition of protein. The reaction temperature was 21°C ± 2°C, aliquots were filtered through 0.20-μm Millipore filters and washed with 10 ml of 154 mM KCl. Values indicate ATP uptake of particles prepared in the presence of 5 mM ADP minus the rate of particles prepared in the absence of ADP. Concentrations of inhibitors are 200 μM atractyloside and 100 μM bongkrekic acid (10-min preincubation at room temperature).

There have been earlier indications for the presence of an ADP carrier in digitonin fragments (14) and lubrol particles (15) but this is the first report of inhibitor-sensitive adenine nucleotide transport in sonicated submitochondrial particles as well as in reconstituted vesicles. These reconstituted vesicles may serve as an assay system for the purification of the adenine nucleotide carrier.

REFERENCES
Adenine Nucleotide Transport in Submitochondrial Particles and Reconstituted Vesicles Derived from Bovine Heart Mitochondria
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