Fatty Acyl Coenzyme A-sensitive Adenine Nucleotide Transport in a Reconstituted Liposome System*

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The adenine nucleotide translocase was purified from bovine heart mitochondria and incorporated into membranes of phospholipid liposomes. The rate of transport of the adenine nucleotides was competitively inhibited by oleoyl coenzyme A with an approximate \( K_i \) of 1.0 \( \mu \)M. Significant inhibition was limited to those fatty acyl coenzyme A esters which are carnitine dependent for their oxidation in isolated mitochondria. Octanoyl coenzyme A was almost completely inactive as was palmitic acid and palmitoyl carnitine. By comparing the inhibitory characteristics of carboxyatractylate and bongkrekic acid with those of oleoyl-CoA, it was determined that the fatty acyl-CoA esters could produce inhibition whether the carrier was inserted into the liposome in either the conventional (65%) or reverse (30%) orientation. The results demonstrate that the interaction of long chain fatty acyl-CoA esters with the ADP/ATP carrier in a purified reconstituted system mimics their effects with isolated mitochondria and inverted submitochondrial particles. In general, these findings are consistent with the role of acyl-CoA esters acting as natural ligands and biological effectors of the translocator.

Long chain fatty acyl coenzyme A esters are potent reversible inhibitors of adenine nucleotide translocation in mitochondria (1), submitochondrial particles with inverted sidedness (2), and mitoplasts prepared by lubrol extraction (3). Because of their strikingly similar effects to those of the classical inhibitors carboxyatractylate and bongkrekic acid, long chain fatty acyl-CoA esters have been proposed to function as natural ligands for the ADP/ATP carrier (4). However, not all investigators agree with this concept and some regard the acyl-CoA esters to be only nonspecific inhibitors of the translocase (5, 6). In view of the increasing amount of evidence which indicates that the adenine nucleotide translocase is the rate-limiting step for oxidative phosphorylation (6–8), it is very important to document the biochemical specificity of the acyl-CoA effect if the ligands are to be seriously considered as biological effectors of the carrier.

Analytical techniques for the isolation and purification of the ADP/ATP carrier from bovine heart mitochondria and its subsequent reconstitution in a liposome system have recently been developed (9–11). The use of these procedures offers a unique opportunity to establish rigorous criteria with which to test the specificity and sensitivity of the adenine nucleotide translocase to the fatty acyl-CoA esters. The present communication provides results which demonstrate that the reconstituted carrier is sensitive to the acyl-CoA in a specific manner. Furthermore, the interaction of the ligand with the receptor corresponds appropriately with the asymmetric orientation of the carrier in the liposome membrane.

MATERIALS AND METHODS

\(^{14}C\)ADP (99.9% radiochemically pure) was purchased from New England Nuclear; hydroxyapatite and anion exchange resin AG 1-X8, 100–200 mesh, formate form, were purchased from Bio-Rad; carboxyatractylate was purchased from Boehringer Mannheim; bongkrekic acid was a gift from Professor W. Berends, University of Delft, The Netherlands; fatty acyl-CoA and carnitine esters were purchased from P-L Biochemicals, Inc.; phosphatidylcholine (egg, >99% pure), phosphatidylethanolamine (egg, >99% pure), and cardiolipin (beef heart) were purchased from Avanti Polar Lipids, Inc., Birmingham, AL.

Heavy beef heart mitochondria were prepared according to the procedure of Green et al. (12) and protein was determined by the method of Lowry et al. (13). The ADP/ATP carrier was purified according to published procedures (9–11) with slight modification. A mitochondrial pellet containing 50–60 mg of protein was dissolved in 2.5% Triton X-100, 100 mM Na2SO4, 10 mM Tricine-KOH, pH 7.4, and 1.0 mM EDTA at a concentration of 7 mg/ml. The suspension was incubated at 0 °C for 20 min and then centrifuged at 20,000 × g for 10 min. A 1-ml aliquot of the supernatant was placed on a hydroxyapatite column (6 cm × 0.7 cm) that had been equilibrated with an elution buffer containing 0.5% Triton X-100, 100 mM Na2SO4, 10 mM Tricine-KOH, pH 7.4, and 0.1 mM EDTA, and the carrier protein which was eluted in the pass through fraction within 15 min was monitored by protein determinations.

The purified ADP/ATP carrier protein was incorporated into liposomes composed of phosphatidylethanolamine/phosphatidylincholine/cardiolipin in a ratio of 70:22:8 (9–11). A chloroform solution of the phospholipids was dried under nitrogen at room temperature and then Vortex suspended in a medium containing 20 mM ATP, 100 mM NaCl, 0.5 mM MgCl2, and 10 mM Tricine-KOH, pH 7.5, at a concentration of 33 mg/ml. The phospholipid suspension was sonicated at 40–50 watts using a Branson Sonifier with a microtip for 15 min in an ice bath and centrifuged at low speed for 10 min. Approximately 200–300 μg of carrier protein in 0.2–0.4 ml of Triton X-100 solution was added to the sonicated liposome suspension and incubated at 0 °C for 20 min. The mixture was then subjected to a second sonication of 15–20 s in an ice bath. External ATP was removed by passage of the reconstituted carrier through an anion exchange column (25 cm × 0.7 cm) (AG 1-X8, 100–200 mesh, formate form) that had been equilibrated with 130 mM glycerol solution (14). The fractions containing the proteoliposomes which were eluted with the glycerol buffer were pooled and used for the transport studies.

Adenine nucleotide transport was measured by a standard radioactive forward exchange assay (1). In a typical assay, 0.2–0.5 ml of the reconstituted carrier was added to a 1.0 ml incubation media containing 250 mM sucrose, 0.5 mM EDTA, and 20 mM 4-morpholino-propenesulfonic acid, pH 7.0, Vortex dispersed, and incubated for 4 min at room temperature. The reaction mixture was then incubated for an additional 2 min with or without the inhibitors and the forward exchange initiated by addition of 3.3 μCi of \(^{14}C\)ADP at a final concentration of 1.0–2.0 μM. The reaction was terminated by the addition of 1.0 ml of 100 mM Na2SO4, 100 mM Tricine-KOH, pH 7.4, and 1.0 mM EDTA, and the carrier protein which was eluted in the pass through fraction was monitored by protein determinations.
concentration of 10 μM. After incubation for 15 min at room temperature, the exchange was terminated by addition of 10 μM carboxyatractylate and 10 μM bongkrekic acid and the external ["C"]ADP removed by passage of the reaction mixture through an anion exchange column (6 cm x 0.7 cm) (AG 1-X8, 100-200 mesh, formate form) which was equilibrated with 136 mM glycerol. The proteoliposomes were eluted with 136 mM glycerol and 1.5-ml aliquots were collected until the liposomes were quantitatively recovered. The radioactivity of a 0.2-ml aliquot dissolved in 10 ml of Bray's solution (15) was determined using a Tri-Carb liquid scintillation spectrometer.

Sodium dodecyl sulfate gel electrophoresis was performed on 10% acrylamide and 0.27% bisacrylamide gels using the discontinuous buffer system of Neville (16) as previously described (17).

RESULTS

In order to prevent denaturation of the carrier protein following its extraction from the inner mitochondrial membrane, it is characteristically preloaded with the tight binding ligand, carboxyatractylate, which confers almost complete protection during the purification (18). This procedure is not applicable when the objective is to obtain a protein which carries out active transport. Conditions have, therefore, been modified and with the use of a very small hydroxyapatite column, a more rapid purification has been accomplished permitting a considerable amount of the unliganded carrier to be actively reconstituted into the liposome system (11). The carrier protein purified by hydroxyapatite chromatography as described under “Materials and Methods” is a dimer of 60,000 daltons (18). Fig. 1 is a representative example of the purified preparation used in these experiments which has been subjected to sodium dodecyl sulfate gel electrophoresis. A distinct protein band with the mobility equivalent to a molecular weight of 30,000 is the subunit of the carrier (18). Using the standard more lengthy purification procedure, an almost homogeneous protein (70–80% pure) can be obtained by chromatography on hydroxyapatite (17, 18).

The results in Table I demonstrate rates of adenine nucleotide transport in the reconstituted liposome system which are comparable to values reported (9–11) and are almost completely inhibited by the combined addition of carboxyatractylate and bongkrekic acid. The critical observation in this series of experiments is the one showing that adenine nucleotide translocation is also extremely sensitive to oleoyl-CoA.

The concentration curve for oleoyl-CoA inhibition of adenine nucleotide transport in the liposome system shown in Fig. 2 is almost identical with that obtained with either mitochondria or submitochondrial particles (2). Furthermore, the inhibition is competitive with ADP as it is in isolated mitochondria (2, 3). The K for ADP of 14 μM obtained in these experiments is similar to the value recently reported for the reconstituted carrier system (11,19). A K, for oleoyl-CoA was calculated to be 1.0 μM. The value is similar to that reported for rat liver mitochondria (3) but lower than the figure given for palmitoyl-CoA inhibition of a reconstituted carrier protein prepared from cholate-extracted bovine heart mitochondria (20).

Fatty acyl-CoA inhibition of adenine nucleotide translocation in isolated mitochondria is limited to those thioesters which are carnitine dependent for their subsequent oxidation (2, 3). In fact, inhibition is released upon addition of carnitine to the incubation medium which permits the transacylation of the acyl-CoA to the acylcarnitine ester by the carnitine palmitoyltransferase enzyme (2, 3). In the experiments shown in Table II, all of the acyl-CoA esters tested except octanoyl-CoA produced a strong inhibition of adenine nucleotide transport, again illustrating the marked similarity of the responses in isolated mitochondria and the reconstituted liposome sys-

![Fig. 1. Sodium dodecyl sulfate gel electrophoresis of the peak protein fraction obtained by hydroxyapatite chromatography. Experimental and analytical techniques are described under “Materials and Methods.” A, standard molecular weights. B, protein bands from peak fraction; →30,000 daltons represents the ADP/ATP carrier subunit.](image)

![Fig. 2. Effect of increasing concentrations of oleoyl-CoA on the rate of transport in the reconstituted ADP/ATP carrier. Inset, double reciprocal plot of oleoyl-CoA inhibition of the concentration-dependent rate of ADP uptake. The assay for transport was carried out as described under “Materials and Methods.” 1/V represents 1/ADP bound (nanomoles/min/mg of protein); ○—○, control; ●—●, oleoyl-CoA, 4 μM; Δ—Δ, oleoyl-CoA, 9 μM.](image)
The transport assays were carried out as described under “Materials and Methods.” The amount of protein in the reconstituted carrier was 0.2 μg and the concentration of the added ligands was 10 μM.

| Additions      | Bound [3]C]ADP | nmol/min/mg protein | %   |
|----------------|--------------|---------------------|-----|
| None           | 660          | 100                 |     |
| Octanoyl-CoA   | 526          | 75                  |     |
| Lauryl-CoA     | 204          | 29                  |     |
| Myristoyl-CoA  | 200          | 29                  |     |
| Palmitoyl-CoA  | 182          | 26                  |     |
| Oleoyl-CoA     | 90           | 13                  |     |
| Palmitic acid  | 700          | 100                 |     |
| Palmitoyl carnitine | 730    | 100                 |     |

**DISCUSSION**

The present results provide evidence that the interaction between the ADP/ATP carrier and long chain fatty acyl-CoA esters in a purified reconstituted system is identical with that observed in isolated mitochondria and submitochondrial particles. An important finding brought out in previous studies (2, 23, 24) was that, unlike atracylate and bongkrekic acid which bind and inhibit the carrier asymmetrically, long chain fatty acyl-CoA esters recognize receptor sites on both the cytosolic and matrix side of the inner mitochondrial membrane. Moreover, on the cytosolic side of the membrane, the acyl-CoA esters resemble atracylate in their kinetic effects, while on the matrix side, they resemble bongkrekic acid (4). Whereas the present results might seem predictable, it was, nevertheless, reassuring to find that they confirm the fact that in a reconstituted system as in the natural environment, the acyl-CoA esters were still able to recognize either conformation of the receptor as dictated by the orientation of the carrier in the membrane.

**TABLE III**

| Additions       | Bound [3]C]ADP | Orientation of the carrier |
|-----------------|----------------|---------------------------|
|                 | nmol/min/mg protein | % | % |
| None            | 700            | 100 |     |
| Carboxyatractylate | 400      | 57 |     |
| 5 μM            | 293            | 42  |     |
| 10 μM           | 263            | 38  | Conventional |
| 20 μM           |                |     |     |
| Bongkrekic acid | 48             | 7   |     |
| 5 μM            | 20             | 3   |     |
| 10 μM           | 12             | 2   | Reverse |
| 20 μM           |                |     |     |
| Oleoyl-CoA      | 167            | 24  |     |
| 5 μM            | 56             | 8   |     |
| 10 μM           | 23             | 3   |     |
| Carboxyatractylate | 3        | 1   | Undetermined |
| 10 μM           |                |     | <5 |
| Bongkrekic acid | 12             | 2   |     |
| 10 μM           |                |     |     |
| Oleoyl-CoA      | 12             | 2   |     |
| 10 μM           |                |     |     |

The role of the ADP/ATP carrier in energy-linked respiration is an important factor in assessing the potential significance of the present study. Whereas there has been disagreement in the past, more recent evidence (6-8) favors the probability that the adenine nucleotide translocase is the rate-limiting step in oxidative phosphorylation. This condition implies that the carrier, like other rate-limiting enzymes, must be carefully regulated. It also seems reasonable to consider the possibility that the receptors on the carrier fortuitously recognize atracylate and bongkrekic acid, and that the fatty acyl-CoA esters may represent the natural ligands and biological effectors of the translocator. The present study supports
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this concept and future efforts will be directed to providing evidence for the existence of physiological or pathophysiological conditions under which the ADP/ATP carrier is regulated and, in turn, affects energy-linked respiration.

Acknowledgment—We appreciate the helpful discussions of H. A. Lardy, Enzyme Institute and Department of Biochemistry, University of Wisconsin, Madison, WI.

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