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THE RECOGNITION OF TWO SPECIFIC BINDING SITES OF THE ADENINE NUCLEOTIDE TRANSLOCASE BY PALMITOYL CoA IN BOVINE HEART MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

Gebretateos Woldegiorgis and Earl Shrago

Departments of Medicine and Nutritional Sciences
University of Wisconsin
Madison, Wisconsin 53706

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SUMMARY: Palmitoyl CoA which is an effective inhibitor of adenine nucleotide transport is able to remove bound [^{14}C] ADP and [^{3}H]atractylate from the translocator on the outer side of the inner mitochondrial membrane. Bongkrekic acid, when added to the incubation medium prior to palmitoyl CoA, can prevent the removal of bound [^{14}C]ADP from the membrane by palmitoyl CoA, however, bongkrekic acid is ineffective if palmitoyl CoA is added first. Upon incubation with inverted submitochondrial particles, both palmitoyl CoA and bongkrekic acid prevent the uptake and transport of [^{14}C]ADP by the particles. Moreover, when the submitochondrial particles are preincubated with [^{14}C]ADP, palmitoyl CoA, like bongkrekic acid, is unable to remove the bound nucleotide from the inner face of the carrier. Thus, palmitoyl CoA which has a high affinity for the translocator on both sides of the inner mitochondrial membrane, nevertheless, interacts differently with the carrier on each side of the membrane. This suggests that the translocase contains binding sites in two specific states both of which accommodate palmitoyl CoA.

The binding properties and inhibitory characteristics of atractylate and bongkrekic acid for the adenine nucleotide translocator are strikingly different. Atractylate competes with ADP and ATP for the receptor side and is able to remove the bound nucleotides from the carrier (1), whereas, bongkrekic acid, which does not inhibit competitively, binds the adenine nucleotides tightly to the carrier (2). Intact mitochondria are impermeable to atractylate which binds exclusively to the carrier on the outer or C side of the inner membrane, while bongkrekic acid which can penetrate the inner mitochondrial membrane binds to the inner or M side (3). However, in sonicated submitochondrial particles with inverted sidedness, atractylate is still unable to bind to the now exposed M side of the inner membrane (4). These differences in binding characteristics of atractylate and bongkrekic acid can be attributed either to the different chemical structures of the two inhibitors or they may indicate an asymmetry of the translocator with respect to its orientation to the membrane

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side. Like atractylate and bongkrekic acid, long chain fatty acyl CoA esters are potent inhibitors of the adenine nucleotide translocase (5,6). However, in contrast to the other two ligands, long chain acyl CoA esters bind to the translocator from either side of the inner mitochondrial membrane with equally high affinity (7-9). This rather unique characteristic of the acyl CoA esters make them ideal probes to delineate the orientation of the carrier with respect to the membrane sidedness. The present report which is an investigation of the binding properties of palmitoyl CoA to the carrier provides good evidence that the adenine nucleotide translocase contains binding sites which exist in two specifically different states.

MATERIALS AND METHODS

Heavy beef heart mitochondria were prepared according to Green et al. (10) and where indicated were partially depleted of endogenous nucleotides (11). Submitochondrial particles were prepared from frozen thawed mitochondria according to the method of Hanson and Smith (12) as modified by Klingenberg (8). [^{3}H]atractylate was prepared by the decarboxylation of carboxyatractylate according to the procedure of Brandolin et al. (13). [^{14}C]ADP uptake by mitochondria was carried out as previously described (14) and with submitochondrial particles according to the method of Shertzer and Racker (4). The incubation media for binding studies contained in a total volume of 1.0 ml: 250 mM sucrose, 20 mM MOPS[1] and 1.0 mM EDTA, pH 7.2, or pH 6.8 for studies involving bongkrekic acid. The binding of [^{14}C]ADP and [^{3}H]atractylate to the mitochondria was determined following their preincubation by assaying the radiolabeled compounds in the mitochondria after separating the suspension by centrifugation at 9,000 x g for 5 minutes in a Beckman Microfuge and processing the pellet for counting according to published procedures (15,16). Submitochondrial particles were separated from the reaction mixture by rapid filtration on a 0.45 mμ Millipore filter, washed twice with the incubation media, dried and radioactivity on the filter determined in a Tricarb liquid scintillation spectrometer. Protein was measured by the Lowry procedure (17).

RESULTS

The similar inhibition pattern of adenine nucleotide translocation in isolated beef heart mitochondria by atractylate and palmitoyl CoA is shown in panel A of Figure 1. Neither of the ligands can penetrate the inner mitochondrial membrane and the binding which occurs on the C side of the inner membrane initiates marked inhibition of the translocase which is competitive in type (3,14). Based on appropriate use of inhibitors, it was suggested by Weidemann et al. (15) that the total binding of nucleotides could be divided

[1] morpholinopropane sulfonic acid

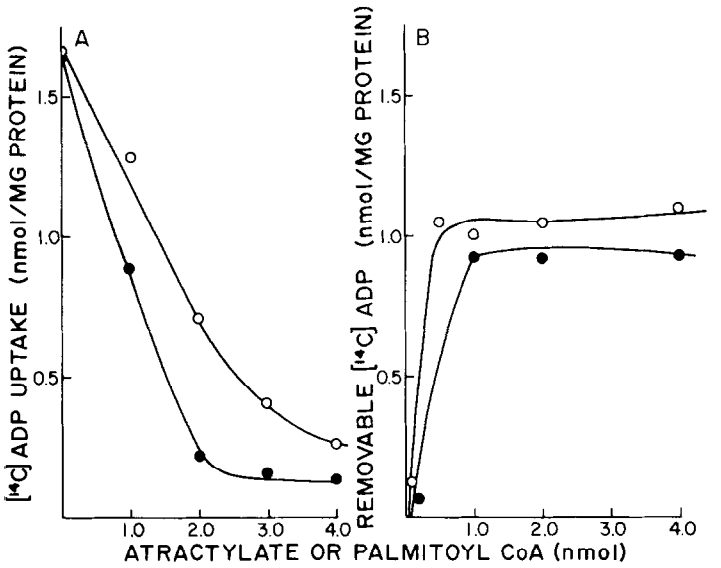
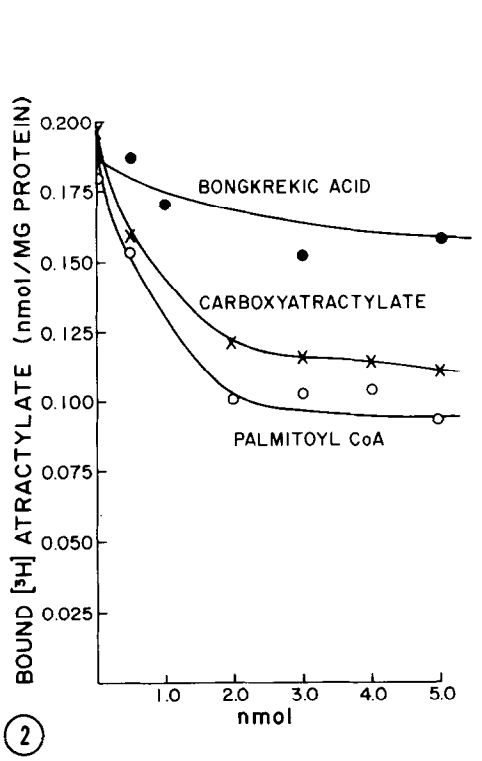


Figure 1 - Effect of Atractylate and Palmitoyl CoA on ADP Uptake By and Removal From Bovine Heart Mitochondria. Panel A: The uptake of ADP was determined using intact mitochondria incubated with 0.03 µCi carrier free [^{14}C]ADP (14). Panel B: Removal of bound ADP was measured with mitochondria (2.1 mg protein/ml) partially depleted of endogenous nucleotides (11) and then preincubated with 30 µM [^{14}C] ADP, pH 7.0. After 2 min at 0°, atractylate or palmitoyl CoA was added and the incubation continued for another 2 min. Subsequent treatment of the reaction mixture for determination of radioactivity was carried out according to Weidemann et al. (15) as described under Materials and Methods. Atractylate (●—●—●) and palmitoyl CoA (0—0—0) added in nmol/ml.

into binding to the carrier sites, uptake into the matrix space and nonspecific binding. Atractylate added after preincubation of the mitochondria with [^{14}C]ADP is able to remove the nucleotide bound specifically to the carrier site (15), and, as shown in panel B, palmitoyl CoA acts in an identical manner to atractylate under the same experimental conditions.

The ability of palmitoyl CoA and atractylate to compete with and remove the adenine nucleotides from receptor sites on the carrier implies that the two ligands are also competitive with each other. This is shown to be the case in Figure 2 where palmitoyl CoA can remove approximately 50% of the bound atractylate. By contrast bongkrekic acid which, under conditions where atractylate occupies the receptor sites on the C side of the inner membrane may or may not interact at the M side, does not have this capacity to the same extent (see Figure 2).



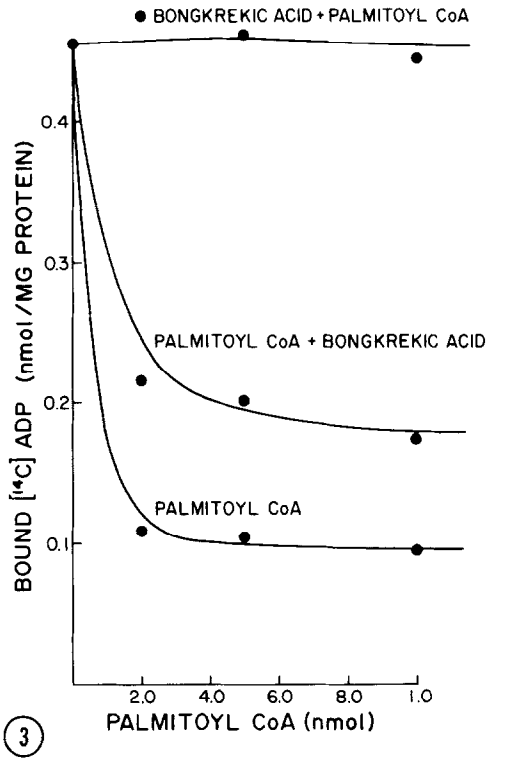


Figure 2 - Removal of Bound Atractylate by Palmitoyl CoA, Carboxyatractylate And Bongkrekic Acid From Bovine Heart Mitochondria. Mitochondria (1 mg protein/ml) depleted of endogenous nucleotides (11) were incubated with [^{3}H]atractylate (2 nmol/mg protein) for 2 min. at 0°. The ligand was then added (with bongkrekic acid 10 μM ADP was included) and the reaction continued an additional 30 min. Following centrifugation, the washed pellet was dissolved in 0.2 ml 2% lubrol and the radioactivity counted.

Figure 3 - Competition Between Palmitoyl CoA And Bongkrekic Acid For Removal of Bound ADP From Bovine Heart Mitochondria. Mitochondria (1 mg protein/ml) depleted of endogenous nucleotides were incubated with 10 μM [^{14}C]ADP for 2 min. at 0°. The ligands were then added in the sequence shown. When palmitoyl CoA was added alone or first in the sequence, the reaction was continued for 2 min. and, following addition of bongkrekic acid (10 μM) the reaction was continued for an additional 2 min. period. When bongkrekic acid was added first, the reaction was incubated for 10 min. prior to addition of palmitoyl CoA and then continued for another 2 min. Subsequent treatment of the reaction mixture for determination of radioactivity was carried out according to Weidemann et al. (15) as described under Materials and Methods.

Under appropriate incubation conditions of pH and protein concentration, bongkrekic acid can penetrate the inner mitochondrial membrane where it characteristically binds to the carrier on the M side (3,18). In contrast to atractylate, which removes bound nucleotides, bongkrekic acid inhibits by binding the nucleotide in a tight configuration to the carrier. As shown in Figure 3,

if bongkrekic acid is added prior to palmitoyl CoA, the acyl CoA ester is unable to remove bound [^{14}C]ADP from the carrier. Presumably bongkrekic acid after binding on the M side immobilizes the carrier preventing access of palmitoyl CoA on the C side to the bound nucleotide. However, if palmitoyl CoA is added prior to bongkrekic acid, it is able to remove bound [^{14}C]ADP almost as easily as if bongkrekic acid were not included in the incubation. These studies with intact mitochondria are similar to those previously reported by Klingenberg et al. (19).

A critical characteristic of the binding of the three ligands is the contrast between atractylate and bongkrekic acid which bind asymmetrically to the to the C and M sides of the translocator respectively, and long chain fatty acyl CoA esters which have a high affinity for either side (7-9). This particular property of the carrier was defined with the use of sonicated submitochondrial particles with the inner membrane turned inside out. Typical results with inverted submitochondrial particles are shown in Table 1, experiment 1. In this case atractylate is unable to inhibit the binding and subsequent transport of ADP into the submitochondrial particle. As originally shown by Shertzer and Racker (4) atractylate is only effective when added to the mitochondria prior to sonication. It is then internalized facing the C side of the submitochondrial membrane. By contrast, palmitoyl CoA, which acts in a similar manner to atractylate when incubated with intact mitochondria, inhibits more like bongkrekic acid when added to inverted submitochondrial particles. The critical observations are shown with particles preincubated with [^{14}C]ADP in experiment 2. When [^{14}C]ADP is preincubated with the submitochondrial particles, bongkrekic acid does not remove the bound nucleotide though the bound [^{14}C]ADP is able to exchange freely with added cold ADP. More important, palmitoyl CoA which was shown to remove bound [^{14}C]ADP from the C side is unable to remove the nucleotide from the M side of the membrane. Thus, the experiments clearly demonstrate that palmitoyl CoA which binds to the translocator on either side of the inner mitochondrial membrane initiates effects typical of atractylate on the C side and bongkrekic acid on the M side of the inner membrane.

TABLE 1

Effect of Inhibitory Ligands of the Adenine Nucleotide Translocase on the Uptake and Removal of [^{14}C]ADP from Submitochondrial Particles.

ADDITIONS	BOUND [^{14}C]ADP nmol	% OF CONTROL
EXPT. 1		
None	1.74	100
Atractylate	1.76	100
Bongkrekic Acid	0.12	7
Palmitoyl CoA	0.33	19
EXPT. 2		
None	1.70	100
ADP	0.40	23
Bongkrekic Acid	1.64	96
Palmitoyl CoA	1.65	97

In experiment 1, submitochondrial particles (0.94 mg protein/ml) were preincubated with or without the ligands (5 μM) for 2 min and then 10 μM [^{14}C]ADP was added and the incubation continued for an additional 4 min at room temperature. The reaction mixture was then filtered on a 0.45 mμ Millipore filter, the residue on the filter washed twice with the incubation medium, dried and the radioactivity of the filter counted. In experiment 2 the submitochondrial particles were preincubated with 10 μM [^{14}C]ADP for 2 min and then incubated with 40 μM ADP or 5 μM bongkrekic acid or palmitoyl CoA for an additional 4 min. Subsequent treatment of the reaction mixture was as described in experiment 1.

DISCUSSION

The characteristic inhibition of the adenine nucleotide translocase by atractylate and bongkrekic acid implies an asymmetry of the translocator with respect to its orientation to the membrane side (19). By contrast studies (7) comparing these ligands with long chain fatty acyl CoA esters showed that palmitoyl CoA was equally efficient in the inhibition of adenine nucleotide translocation with mitochondria and inverted submitochondrial particles. The present investigation, however, more carefully delineates the specific effects of the ligands with respect to bound adenine nucleotides and provides a clearer picture. The results substantiate the conclusion for the asymmetry of the carrier (19). Moreover, they provide additional information with which to define a mechanism for adenine nucleotide translocation through the use of

a ligand, palmitoyl CoA, which has an affinity for both the C and M side of the inner mitochondrial membrane. From the present experimental results it is clear that the asymmetry with respect to binding of the ligands must be intrinsic to the carrier. The difference in the interaction of palmitoyl CoA with the carrier on the C and M side indicates that there must be two distinct receptors or the translocator must exist in at least two different conformational states. In either case both are recognized by the acyl CoA ester. This interpretation is consistent with the isolation of separate carboxyatractylate and bongkrekic acid protein-complexes which appear to be subunits of the carrier (20,21).

It seems very unusual that, unlike atractylate and bongkrekic acid, long chain fatty acyl CoA esters are able to coincidentally recognize both receptors or conformations of the translocator. In addition, it may be more than fortuitous that long chain acyl CoA esters, which themselves cannot penetrate the inner mitochondrial membrane, can be physiologically translocated across by the carnitine-acyl carnitine transport system (22). We interpret this evidence to support the hypothesis that long chain fatty acyl CoA esters, which have access to both sides of the inner mitochondrial membrane, are natural ligands for and effectors of the adenine nucleotide translocase.

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