The Relation of Phospholipid and Membrane-Bound ATPase in Mitochondrial Electron Transport Particles

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Introduction

The effects of specific modifications of membrane phospholipids on membrane structure have been discussed in the preceding paper.[1] Four compositional states were examined, and the disappearance of the projecting 90 Å headpiece-stalk complexes in three of these states prompted us to examine the ATPase activity in the various states. This paper represents a study of the oligomycin sensitive, membrane bound ATPase in relation to phospholipid composition, and the correlation of these studies with ultrastructural findings of the preceding paper.[1] It also attempts to briefly review and compare our findings with those of other investigators.

Materials and Methods

Materials

Lysolecithin, lysophosphatidyl ethanolamine, and ATP were purchased from Sigma Chemical Co. All other chemicals and materials including beef heart mitochondria and electron transport particles (ETP) were obtained as described in the preceding communication.[1]

Methods

Mg^{2+}-stimulated ATPase was measured according to the procedure described by Ernster *et al.*[1] Assays were performed at 30° C for 10 min. Oligomycin was added to a final concentration of 5 $\mu g/ml$ as indicated. Inorganic phosphate was determined according to Lindberg and Ernster.[3]

All other methods used were performed as noted in the preceding paper.[1]

Results

Oligomycin Sensitive ATPase Activity in the Phospholipid Compositional States

Partial lyso state. The major forms of phospholipids in the membranes after phospholipase A treatment, with or without BSA extraction as described in the preceding paper,[1] are outlined in Table I.

The partial lyso state is characterized by a low recovery of oligomycin sensitive ATPase activity (30·1%), although most of the oligomycin insensitive activity (92·7%)

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TABLE I. Outline of phospholipid composition of membranes after treatment

State	Treatment*	Major phospholipid composition*
1. Partial lyso	Low level phospholipase A	Lyso PE, lyso PC, cardiolipin
2. Partial lipid depleted	Same as 1 but extracted with BSA	Cardiolipin
3. Lyso	High level phospholipase A	Lyso PE, lyso PC, lyso cardiolipin
4. Lipid depleted	Same as 3 but extracted with BSA	None

* For details concerning treatments and phospholipid composition see the preceding communication.

is retained (Table II). It thus appears that conversion of phosphatidyl ethanolamine and phosphatidyl choline to lysophosphatides results in the conversion of the ATPase to a predominantly oligomycin insensitive form.

Partial lipid depleted state. The transformation from the partial lyso to the partial lipid depleted state results in a measurable increase of oligomycin sensitive ATPase activity (Table II). Activity insensitive to oligomycin decreases slightly to 79·0%, while the

TABLE II. ATPase activity of the phospholipid compositional states

Phospholipid compositional state of ETP	ATPase specific activity*	% Oligomycin inhibition	Net oligomycin sensitive activity	% Recovery of oligomycin sensitive activity
Normal	4·38			
+ oligomycin	0·227	94·8	4·15	100
Partial lyso state	4·06			
+ oligomycin	2·81	30·8	1·25	30·1
Partial lipid depleted state	3·45			
+ oligomycin	1·65	52·2	1·81	43·6
Lyso state	4·09			
+ oligomycin	3·33	19·6	0·76	18·3
Lipid depleted state	0·287			
+ oligomycin	0·162	43·6	0·125	3·0

* μmoles P_i/10 min/mg protein.

recovery of oligomycin sensitive activity increases to 43·6%. The fact that nearly 50% of the lysocompounds are removed in the compositional transformation, indicates partial removal of lysocompounds restores oligomycin sensitive activity.

Lyso state. The total ATPase activity in the lyso state is 93·4% of untreated ETP

(Table II). However, only 19·6% of it is sensitive to oligomycin. ATPase in the lyso state membranes is, therefore, even more in the oligomycin insensitive form than ATPase in the partial lyso state.

Lipid depleted state. When the lysocompounds are removed in the lyso to lipid depleted transformation, there is a great decrease in ATPase activity. The total activity recovered is only 6·6% of control ETP and only 3% of oligomycin sensitive activity is recovered. Table III summarizes this data along with the findings in the previous paper concerning the loss of visible projecting headpiece-stalk complexes in certain phospholipid compositional states.

TABLE III. Correlation of phospholipid compositional states, ATPase activity and visible projecting headpiece-stalk complexes as observed in negative staining

Phospholipid compositional state*	ATPase activity†	Projecting headpiece-stalk complexes*
Control ETP (PE, PC, DPG)	Active ATPase 95% inhibited by oligomycin	Visible
Partial lyso state (lyso PE, lyso PC, DPG)	Active ATPase 31% inhibited by oligomycin	Visible
Partial lipid depleted state (lyso PE-depleted, lyso PC-depleted, DPG)	Active ATPase 52% inhibited by oligomycin	Less than 50% visible
Lyso state (lyso PE, lyso PC, lyso DPG)	Active ATPase 20% inhibited by oligomycin	Not visible
Lipid depleted state (lyso PE-depleted, lyso PC-depleted, lyso DPG-depleted)	Inactive ATPase	Not visible

* See preceding paper for the quantitative values of the phospholipid composition and the electron micrographs showing the presence or absence of projecting headpiece-stalk complexes.
† See Table II for actual specific activities.

ATPase Activity and the Collapse of the Headpiece-Stalk Complexes

It is well documented that the ATPase of ETP membrane is localized in the 90 Å projecting headpiece-stalk complexes. The disappearance of these projecting structures in conversion to partial lipid depleted and lyso states however does not result in significant loss in ATPase activity (Tables II and III). No ATPase activity is found in the supernatant after the treatments and essentially all the ATPase activity remains in the membrane fractions. These results indicate that the 90 Å headpiece-stalk complexes are not destroyed but have lost their original morphology.

Disappearance of the projecting 90 Å headpiece-stalk complexes may be due to severe conformational changes in the membrane as a result of compositional changes in

the phospholipids. Such changes could result in the collapse of the headpiece-stalk complexes onto the membrane, so that they are no longer visible in negatively stained specimens. In such a situation the complexes may be buried in the membrane, coated on the surfaces of the membrane, or partially embedded in the membrane and partially on the surfaces. The finding that the membranes lacking visible headpiece-stalk complexes have different staining characteristics, and might also be thicker than untreated ETP, can be interpreted in terms of the collapse of the ATPase particles onto the membrane, but also might be due solely to the changes in phospholipid composition.[1,9]

Protein recovery data also substantiates the retention of the collapsed ATPase by the membrane. The washing with BSA in the partial lyso to partial lipid depleted state transformation results in only small losses in protein. In high level treatments with phospholipase A (lyso state) not more than 12% of the total protein is released into the supernatant, and NADH dehydrogenase, which is almost completely released from the membrane in this treatment,[10] accounts for about 8% of the total membrane protein. Thus the remaining 4% soluble protein cannot account for the headpiece-stalk protein which has been reported by Stasny and Crane to make up 20–25% of the total membrane protein.[11]

Inactive Form of ATPase in Lipid Depleted Membranes and Its Reactivation by Phospholipid

The conversion of the lyso state to the lipid depleted state by means of BSA washing followed by centrifugation, results in the extreme loss of total ATPase activity. Is the ATPase converted to an inactive form, or is the ATPase actually detached from the membrane during the conversion? Analysis of the supernatant after BSA washing of the lyso state showed only negligible ATPase activity in the supernatant. It has been reported, however, that ATPase detached from ETP membranes may be cold labile.[5] If the ATPase was actually detached from the membrane proper, the inactivated protein may be sticking to the membranes or may be in the BSA supernatant. We there-

TABLE IV. Protein and enzyme recovery of ATPase in the lipid depleted ETP at 0° and 25° C

Fraction	ATPase specific activity*	Total protein (mg)	Total activity	% Activity recovered
Untreated ETP	4·15	50	207·5	100
0° C				
Lipid depleted state pellet	0·290	45·2	13·1	6·31
Lipid depleted state supernatant	0·154	4·8	0·74	0·33
25° C				
Lipid depleted state pellet	0·266	44·7	11·9	5·73
Lipid depleted state supernatant	0·162	5·3	0·86	0·41

* μmoles P_i/10 min/mg protein.

TABLE V. The effect of exogenous phospholipid and lysophosphatides on ATPase activity in the lipid depleted state

Compositional state of ETP membrane	ATPase specific activity	% oligomycin inhibition	Net oligomycin sensitive activity	% Recovery of oligomycin sensitive activity
Untreated ETP	3·14			
+ oligomycin	0·333	89·4	2·81	100
Lipid depleted state	0·420			
+ oligomycin	0·210	50·0	0·21	7·47
Lipid depleted state + phospholipid*	1·65			
+ oligomycin	0·724	66·1	0·926	32·9
Lipid depleted state + lysocompounds†	0·802			
+ oligomycin	0·546	31·9	0·256	9·11

* Incubated with phospholipid micelles equivalent to 20 μg P/mg ETP protein, 5 min, 30° C.
† Incubated with 0·5 μmole of a 50:50 mixture of lysophosphatidyl choline and lysophosphatidyl ethanolamine per mg ETP protein, 5 min, 30° C.
‡ μmoles P_i/10 min/mg protein.

fore carried out the lyso to lipid depleted transformation at 25° C as well as at 0° C and did an enzyme recovery study. As can be seen in Table IV, both the membrane fractions and supernatants were similarly depleted of ATPase activity at 25° C and 0° C. Thus the loss of ATPase activity during BSA washings cannot be attributed to cold inactivation. Addition of total mitochondrial phospholipid micelles to the lipid depleted ETP reactivates oligomycin sensitive ATPase activity (Table V). The reconstitution results in a fourfold increase in both total and oligomycin sensitive activity. This strongly suggests that at least a part of the ATPase is still attached in an inactive state to the lipid depleted membranes.

The Relationship Between Lysophosphatides and Oligomycin Insensitive ATPase

By combining data from Table I of the preceding page and Table II of this paper, the amount of membrane phospholipid converted to lysophosphatides, and the amount of ATPase converted to the oligomycin insensitive form, can be related. The results

TABLE VI. The relation between lysophosphatide content and oligomycin sensitivity

Compositional state	% Phosphorus as lysophosphatide in membrane*	% Recovery of oligomycin sensitive ATPase†
Partial lyso state	70	30·1
Partial lipid depleted state	55	43·6
Lyso state	>95	18·3

* Calculated from data presented in Table I of the preceding paper.
† Data taken from Table II.

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presented in Table VI show a progressive decrease in the amount of oligomycin sensitive ATPase as the percentage of lysophosphatides in the membrane increases. Lysophosphatides and free fatty acids had no effect, however, on either total ATPase activity or oligomycin sensitive activity (Table VII), when added to untreated ETP at levels formed during high level phospholipase A digestion.

TABLE VII. The effect of lysophosphatides and free fatty acids on ATPase activity in untreated ETP

Fraction	ATPase specific activity‡	% Oligomycin inhibition
ETP	4·10	
+ oligomycin	0·325	92·1
ETP + lysophosphatides*	4·11	
+ oligomycin	0·352	91·4
ETP + free fatty acid†	4·21	
+ oligomycin	0·331	92·1

* Incubated with 50:50 mixture of lysophosphatidyl choline and lysophosphatidyl ethanolamine, 0·5 μmole/mg ETP protein, 30° C, 90 min.

† 0·35 μmoles potassium oleate/mg ETP protein, 90 min, 30° C.

‡ μmoles P_i/10 min/mg protein.

Conversion of Inactive ATPase (Lipid Depleted State) to the Active Oligomycin Sensitive and Insensitive Forms

If complete phosphatides are needed for oligomycin sensitive ATPase, then addition of phospholipid to the lipid depleted state should convert inactive ATPase to the active oligomycin sensitive form. As shown in Table V, there is a more than fourfold increase in oligomycin sensitive activity which amounts to a 25·4% increase in total recovery. By analogy, the addition of lysophosphatides to the lipid depleted membranes should convert ATPase from the inactive form to the active oligomycin insensitive form. Table V shows that although the total ATPase activity doubles, the increase in recovery of oligomycin sensitive ATPase is only 1·6%.

Discussion

Phospholipid compositional states and ATPase activity. It is suggested that the membrane bound ATPase can exist in three different forms depending on the phospholipid compositional state of ETP membranes. In the untreated state, where all three phospholipids are present, the ATPase is active and fully sensitive to oligomycin. Hydrolysis of phosphatidyl choline and ethanolamine to lysophosphatides (partial lyso state) results in the conversion of most of the ATPase to an active oligomycin insensitive form. Conversion to the active-oligomycin insensitive form is increased when cardiolipin is cleaved as well (lyso state).

When the lyso state is converted to the lipid depleted state, most of the lysophos-

phatides are removed, and ATPase is in an inactive form. Transformation from partial lyso to partial lipid depleted results in a decrease of 15% in the percentage of lysophosphatides in the total membrane phospholipid. There is a corresponding increase in the percentage of recovered oligomycin sensitive ATPase activity of 13·5%. Addition of phospholipids to the lipid-depleted membranes results in a fourfold increase in ATPase activity and of the recovery of oligomycin sensitive ATPase. In contrast, added lysophosphatides increase the ATPase activity about twofold but this is mainly oligomycin insensitive.

To summarize, the membrane bound ATPase is inactive when the membrane is depleted of phospholipid. There are two forms of membrane bound active ATPase. In a lysophosphatide rich phospholipid environment, the active ATPase is oligomycin insensitive. In a phosphatide rich phospholipid environment, the active ATPase is oligomycin sensitive.

Correlations between the phospholipid compositional state, oligomycin sensitive ATPase activity and ultrastructure. Racker and his colleagues[5–8] in isolating ATPase from inner mitochondrial membranes, found that the factor conferring oligomycin sensitivity is retained in the ATPase stripped membranes. Addition of the membrane fraction CFo to the isolated (F_1) results in restoration of oligomycin sensitive activity. Workers in Green's laboratory (Kopaczyk et al.[4]), maintain that the oligomycin sensitivity factor is localized in the stalk. They claimed to have isolated headpiece-stalk complexes which have oligomycin sensitive ATPase activity and suggest that the CFo of Racker and his group contains stalks. The ATPase activity of the headpiece-stalk complex or P_2 preparation of Green and his group is very low.[4] Addition of phospholipid results in a more than tenfold increase of ATPase activity which is oligomycin sensitive. In the absence of added phospholipid the headpiece-stalk complexes aggregate and are not recognizable as such. When phospholipid is added 90 Å particles are clearly seen attached by stalks to phospholipid micelles. From this the Wisconsin investigators conclude that both ATPase activity and oligomycin sensitivity depends on the headpiece-stalk being in the proper configuration. The headpieces must be projecting by stalks from some base, be it the membrane proper or phospholipid micelles.

Our results, however, indicate that there is no such ultrastructural requirement for oligomycin sensitive ATPase activity. For one thing, the oligomycin sensitivity is low in the partial lyso state, even though the projecting headpiece-stalk complexes appear intact. This perhaps could be explained by subtle conformation changes in the headpiece-stalk complex, or its attachment to the membrane, which are not easily seen with the electron microscope. If this is so, then the visible loss of many of the projecting headpiece-stalk complexes in the partial lyso to partial lipid depleted transformation should correspondingly result in the loss of all or most of oligomycin sensitive activity. However, this is not the case. On the contrary, there is an increase in oligomycin sensitivity. Oligomycin sensitive ATPase is also regained by addition of phospholipid to the lipid depleted membranes, without the concurrent reappearance of projecting headpiece-stalk complexes.

All of this suggests that oligomycin sensitivity can be present without the ultrastructural necessity of projecting headpiece-stalk complexes. It further suggests that a particular phospholipid compositional state, namely unhydrolyzed phospholipid, is needed for such sensitivity.

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The Oligomycin Sensitivity Factor and Phospholipid Composition

A protein factor conferring oligomycin sensitivity on ATPase activity has been isolated independently by both MacLennan and Tzagoloff[12] and by Bulos and Racker.[13] Both laboratories found that binding of ATPase to the membrane and oligomycin sensitivity are separate factors. In the absence of the factor conferring oligomycin sensitivity, however, the membrane bound ATPase was oligomycin insensitive. Our findings substantiate the possible transitions between oligomycin sensitive and oligomycin insensitive forms of membrane bound ATPase, but emphasize the active role that phospholipid plays in such processes. The findings of Bulos and Racker[14] also suggests that there may be very specific interactions between phospholipids and the oligomycin conferring factor which are important in the functioning of the ATPase. Both Kopaczyk *et al.*[4] and Kagawa and Racker[7] have previously indicated that phospholipid is necessary for high ATPase activity.

We therefore conclude that oligomycin sensitive ATPase activity is dependent on both a specific oligomycin conferring protein and a specific phospholipid environment, namely unhydrolyzed phospholipid. With regard to the work of Bulos and Racker,[14] it is very likely that the phospholipid acts by means of interactions with the oligomycin conferring protein factor. Since Kopaczyk *et al.*[4] have isolated an oligomycin sensitive ATPase which resemble the projecting headpiece-stalk complexes in the presence of phospholipid, we propose that the oligomycin sensitivity factor may be located at the base of the stalk, perhaps at the point of attachment of the stalk to the membrane. Conversion of the membrane phospholipids to lysophosphatides could then result in conformational changes in the area where the factor is located and result in its inactivation. Addition of phospholipid to the lipid depleted membranes restores the proper phospholipid compositional state, and the oligomycin sensitivity factor becomes active again.

Summary

1. Membrane bound ATPase activity can exist in three forms depending on the phospholipid composition of the membrane.

2. When the membrane phospholipids are mainly in the intact unhydrolyzed form, ATPase activity is in an active oligomycin sensitive form.

3. ATPase activity is converted to an active oligomycin insensitive form when the phospholipids are hydrolyzed to their respective lyso derivatives.

4. Removal of phospholipid from the membrane results in an inactive ATPase.

5. Addition of intact, unhydrolyzed phospholipid to the lipid depleted membranes converts the inactive ATPase to an oligomycin sensitive activity. In contrast, lysophosphatide addition results in an oligomycin insensitive ATPase activity.

6. It is suggested that the oligomycin sensitivity conferring protein[12,13] may be located at the point of attachment of the headpiece-stalk complex to the membrane surface where it can interact with membrane phospholipid.

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