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Reconstitution and photolabeling of the purified ($Na^+ + Mg^{2+}$)-ATPase from the plasma membrane of *Acholeplasma laidlawii* B with phospholipids containing a photosensitive fatty acyl group

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The purified membrane ($Na^+ + Mg^{2+}$)-ATPase of *Acholeplasma laidlawii* B was reconstituted into vesicles composed of phospholipids containing a photoactivatable aryl nitrene-generating fatty acyl group. The reconstitution with phospholipid resulted in an enhancement of ATPase activity and a reduction in the sensitivity of the enzyme to radiation inactivation. The incorporation of the enzyme into the lipid vesicles results in a broadening of the gel-to-liquid-crystalline phase transition of the photolabeled phospholipid and the appearance of two partially resolved endotherms in the calorimetric traces. The temperatures and the total enthalpy of these overlapping transitions are higher than in the absence of incorporated enzyme. After photolysis of the lipid-reconstituted ATPase and separation of the polypeptide subunits by sodium dodecyl sulfate (SDS) gel electrophoresis, a significant labeling of the α-subunit of the enzyme was demonstrated. These results indicate that at least the α-subunit of this ATPase must penetrate into or traverse the phospholipid bilayer.

Introduction

The ($Na^+ + Mg^{2+}$)-ATPase of *Acholeplasma laidlawii* B is an intrinsic membrane-bound enzyme which is dependent upon associated membrane lipids for the full expression of its activity [1–3]. This enzyme has been purified from a detergent-solubilized extract of *A. laidlawii* B membrane [4] and was found to be an assembly of five polypeptide subunits of apparent molecular weights ranging from 16000 to 68000 [4,5]. A characterization of the purified ATPase suggested that the enzyme was isolated intact with respect to the determinants of its kinetic and lipid-dependent properties (see Refs. 4 and 6) and suggested that this ATPase is unlike any other that has been described in the literature. We describe here the use of a phospholipid containing a photosensitive fatty acyl group to identify the subunit or subunits of this ATPase which penetrate into or traverse the lipid bilayer.

Materials and Methods

The methods for culturing the organism *Acholeplasma laidlawii* B, membrane isolation, purification of the ($Na^+ + Mg^{2+}$)-ATPase and assay of its ATPase activity have been described in detail in previous publications [4,6,7]. The photosensitive phospholipid used was obtained by the acylation of the cadmium chloride complex of L-α-glycerophosphorylcholine (Sigma) with the mixed anhydride made from a 4:1 (mol/mol) mixture of unlabeled palmitic acid or 1-[^{14}C]palmitic acid and 12-[*N*-(4-azido-2-nitrophenyl)]aminododecanoic acid, and was purified as described by Lewis and McElhaney [8]. 12-[*N*-(4-Azido-2-nitrophenyl)]aminododecanoic acid was

synthesized by the chemical reactions used by Bisson and Montecucco [9]. The ATPase was reconstituted into lipid vesicles by the procedure used by George and McElhaney [7] and the thermotropic properties of the lipid vesicles were studied in a Microcal MC-2 high-sensitivity differential scanning calorimeter. The photolyses were performed under conditions identical to those described by Chong and Hodges [10] and phospholipid was estimated by the procedure described by Raheja et al. [11]. All other materials and methods were the same as previously used in this laboratory [4,6–8].

Results

The specific activity of the purified ATPase was 159 units at 37°C under the standard assay conditions (see Table I). When reconstituted with the photosensitive phospholipid (arylazidophosphatidylcholine (arylazidoPC)), the specific activity increased to 189 units. The latter value was considerably lower than the 279 specific activity units obtained for the ATPase sample reconstituted with dimyristoylphosphatidylcholine (DMPC). This result was entirely unexpected, since our preliminary experiments showed that a reconstitution of ATPase activity comparable to that obtained with DMPC was possible with a similar phospholipid made from 12-[*N*-(4-azido-2-nitrophenyl)]aminododecanoic acid and a fatty acid mixture obtained from egg yolk phosphatidylcholine. The latter was not considered suitable for photolabeling experiments because the acyl chains contain double bonds which may act as scavengers of the nitrene free radical. Given the above and the fact that 80% of the acyl chains of the photosensitive phospholipid sample were palmitic acid, we suspected that the relatively low specific activity of the ATPase reconstituted with the arylazidoPC (see Table I) may be related to the phase-state properties of this phospholipid. We therefore investigated the thermotropic properties of the arylazidoPC vesicles, with and without the incorporated ATPase, by differential scanning calorimetry (DSC).

TABLE I

SPECIFIC ACTIVITIES OF THE PURIFIED AND PHOSPHOLIPID-RECONSTITUTED ATPase BEFORE AND AFTER PHOTOLYSIS FOR 1 h AT 4°C

Specific activities are expressed in units of μmol P_i/h per mg protein.

Sample	Specific activity of ATPase		Residual activity (% control)
	before photolysis	after photolysis	
Purified ATPase	159	26	17
ArylazidoPC-reconstituted ATPase	189	56	30
DMPC-reconstituted ATPase	279	119	43

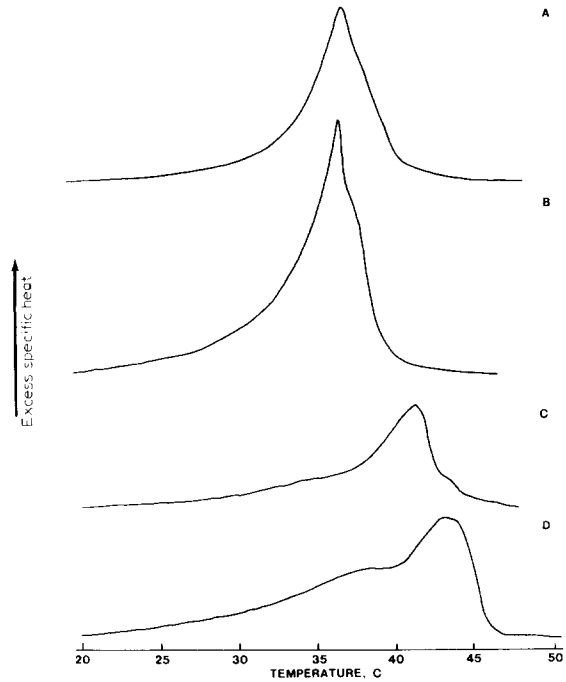

Fig. 1. High-sensitivity DSC heating endotherms of: (A) A multilamellar aqueous dispersion of the photosensitive arylazidoPC; (B) unilamellar vesicles composed of the photosensitive arylazidoPC; (C) proteoliposomes composed of the photosensitive arylazidoPC and the purified *A. laidlawii* ATPase (first heating scan); (D) the same sample as C after three cycles of heating and cooling. The proteoliposomes contained 7.8 μmol phospholipid per mg ATPase protein.

The thermotropic phase behavior of an aqueous, large multilamellar dispersion of the arylazidoPC alone (see Fig. 1A) is characterized by a broad phase transition between 24 and 45°C with

a maximum rate of heat absorption at 36.4°C and an enthalpy of 5.7 kcal · mol^{-1} (see Table II). There was also evidence of a small shoulder on the endotherm at 37.4°C and this may be indicative of some lateral phase separation and/or nonideality in the gel-state miscibility of the components of the phospholipid mixture used. The thermotropic phase behavior of the unilamellar vesicles composed of the arylazidoPC alone (see Fig. 1B) was in general similar to that of the multilamellar dispersion. However, the transition enthalpy was lower (3.8 kcal · mol^{-1}), the transition occurred over a wider temperature range (19–43°C) and the shoulder near 37°C was more pronounced. Both the unilamellar and multilamellar vesicles were stable as judged by the reproducibility of their thermotropic phase properties on repeated heating scans. Fig. 1C and D show the thermotropic properties of the proteoliposomes composed of the ATPase and the photosensitive phospholipid. These vesicles were characterized by a still broader transition (19–47°C) and the maxima in the heat-absorption endotherms occurred at temperatures higher than in the absence of the ATPase. In addition, we also found that these vesicles exhibited stable and reproducible thermotropic properties only after repeated heating scans to temperatures near 50°C. Once the behavior of the vesicles had stabilized, the heating endotherm showed evidence of two partially resolved peaks with maxima occurring at 38 and 43°C. The total enthalpy values of the transition(s) were also initially unstable to repeated scanning and were higher than that found in the absence of the ATPase, ranging from 5.2 kcal · mol^{-1} (first scan) to values which stabilized near 6.9 kcal · mol^{-1} (see Table II). It is also clear from the endotherms shown in Fig. 1 that at the ATPase assay temperature (37°C) most of the lipids in the proteoliposomes were in the gel state. This observation probably accounts for the relatively low specific activity of the ATPase reconstituted with the photosensitive arylazidoPC, since previous studies from this laboratory have shown that the catalytic activity of this ATPase is impaired by gel-state lipids [3].

Table I also lists the specific activities of the purified ATPase and the lipid-reconstituted ATPase samples after exposure to ultraviolet irradiation for 1 h at 4°C. All the samples tested were subject to radiation inactivation. However, the reconstitution of the purified ATPase with phospholipid appeared to offer some protection against photolytic inactivation, and DMPC appeared to be more efficient in this regard than the photosensitive phospholipid used in these studies. After photolysis of the sample reconstituted with the radioactively labeled arylazidoPC, the sample was dissagregated with SDS and the polypeptide subunits separated by gel electrophoresis. The distribution of radioactivity among the subunits is shown in Fig. 2 along with that obtained from an unphotolyzed sample which served as a control. It

TABLE II

CHARACTERIZATION OF THE THERMOTROPIC PROPERTIES OF THE LIPID VESICLES AND PROTEOLIPOSOMES MADE FROM THE ARYLAZIDO-PC

Sample	Temperature (°C) range [a]	T_m (°C) [b]	Enthalpy (kcal · mol^{-1})
ArylazidoPC (multilamellar vesicles)	23–42	36.4	5.7
ArylazidoPC (unilamellar vesicles)	18.5–43	36.3	3.8
ArylazidoPC/ATPase (first scan) [c]	17.4–49	41.3	5.2
ArylazidoPC/ATPase (fourth scan) [c]	18.8–46.7	42.7	6.9

[a] The temperature range refers to that bracketed by the beginning and completion of the phase transition.

[b] T_m refers to the temperature of maximal heat absorption.

[c] The ATPase was reconstituted with phospholipid in the ratio of 7.8 μmol phospholipid per mg ATPase protein.

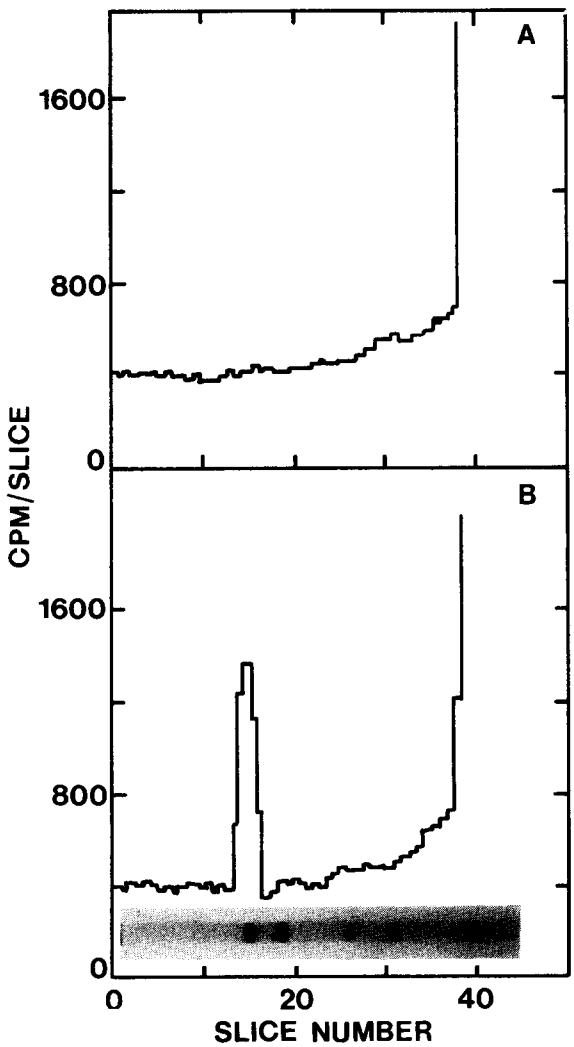

Fig. 2. Distribution of radioactivity on an SDS gel of a sample of *A. laidlawaii* ATPase reconstituted with ^{14}C-labeled arylazidoPC, before (A) and after (B) photolysis. The SDS gel shown is an analytical gel showing the position of the subunits on the labeled gel.

is clear that once photolyzed, some of the [^{14}C]palmitic acid-labeled PC migrated with the α-subunit, and that the β-, γ- and δ-subunits were not significantly labeled under the experimental conditions. The ϵ-subunit migrated too cose to the dye-front and unbound labeled lipids to enable any judgement as to whether it was labeled or not. To guard against the possibility that the observed labeling might have occurred via a water-soluble intermediate (see Ref. 12), the photolysis of the labeled lipid-reconstituted sample was carried out in the presence of dithiothreitol, which served as an aqueous-phase, free-radical scavenger. After disaggregation of the protein and subsequent gel electrophoresis, we found that the labeling pattern of the photolyzed and unphotolyzed samples was not significantly different from that illustrated in Fig. 2. Given this, we believe that the labeling pattern illustrated in Fig. 2 can be attributed to a direct interaction of the α-subunit with the phospholipid photosensitive fatty acyl group in the proteoliposomes.

Discussion

We have demonstrated that the *A. laidlawii* B ATPase can be successfully reconstituted into lipid vesicles containing arylazidoPC and that this probe will photolabel at least one of the subunits of this enzyme. This type of photoactivable, nitrene-generating lipid probe has also been successfully used to label a variety of other membrane proteins [9,13–17], even though there have been problems associated with the use of this type of reagent to cross-link membrane proteins to membrane lipids (see Ref. 18 and references cited therein). In this study, we used the phospholipid mixture obtained by the acylation with the mixed anhydride described above instead of the species used by other workers (see Ref. 14 for a model of its structure), primarily because its synthesis was relatively simple and inexpensive. The mixture was expected to contain 64% dipalmitoylphosphatidylcholine (DPPC), 4% di(12-[*N*-(4-azido-2-nitrophenyl)]amino)dodecanoylphosphatidylcholine and 32% of the two positional isomers of the mixed-chain species. With this mixture, we were apparently unble to reconstitute the ATPase activity as well as with DMPC. However, from the calorimetric data, it seems unlikely that this was the result of poor reconstitution, since at the ATPase assay temperature (37°C) most of the lipids in the proteoliposomes were in the gel state, and this ATPase requires liquid-crystalline lipid for maximal activity [3].

The DSC data also provided some evidence for direct interaction of the ATPase with the lipids, as evidenced by the change in the thermotropic properties of the unilamellar vesicles composed of this

lipid and the increase in the transition enthalpy. We have observed similar effects when this ATPase is reconstituted with other lipids and the physical basis of these phenomena will be discussed elsewhere. In addition, the thermotropic properties of the lipid vesicles hinted at the possibility of some heterogeneity in the population and/or behavior of the lipid vesicles, especially when the ATPase was incorporated into them. This may be indicative of some nonideality in the gel-state miscibility of DPPC and the phosphatidylcholine species containing the arylazido-substituted fatty acyl chain. Furthermore, it is not clear from these studies whether the effect of the ATPase involved the accentuation of an intrinsic property of this arylazidoPC or a reflection of inhomogeneity brought about by preferential interaction of the ATPase with one or more of the individual molecular species present in the photosensitive lipid mixture used. These issues have not been rigorously addressed in the literature and, in principle, can set limits to the usefulness of this type of photoactivable lipid probe. Indeed, miscibility and/or phase-separation problems should be expected whenever attempts are made to incorporate a relatively polar nitrene precursor group of the type used here into the hydrophobic domain of a lipid bilayer.

Inspite of the above, we have demonstrated that the α-subunit of this ATPase can be photoaffinity labeled by the lipid mixture used. This suggests that at least the α-subunit of the enzyme is interacting with the hydrophobic domain of the membrane lipids and further supports the conclusion that the enzyme was purified intact with respect to the determinants of its lipid-dependent properties. It is also clear that this ATPase behaves differently from the F_1 particle of the F_1F_0 type ATPase, since the F_1 particle is not labeled in experiments using a similar type of lipid probe [13]. This further supports our previous conclusion that the *A. laidlawii* $(Na^+ + Mg^{2+})$-ATPase is not of the F_1F_0 family of ATPases. In studies using an aqueous-phase protein cross-linker [4], we have previously shown that the α-subunit can be cross-linked to other subunits, an observation which indicates that there must be some exposure of this subunit to the aqueous phase. This has been supported by studies using group-specific agents and nucleotide analogues (Ref. 7; Lewis, R.N.A.H., George, R. and McElhaney, R.N., unpublished observations). The results described here also suggest that the α-subunit does form a significant part of the intramembrane domain of this enzyme. While the lack of any significant labeling of the other subunits cannot exclude the possibility of a penetration of the hydrophobic core of the lipid bilayer by these subunits, these results may indicate a geometric arrangement of the subunits in which the α-subunit is arranged so as to shield the other subunits from a direct interaction with the hydrocarbon chains of the membrane lipids, if indeed they do penetrate the membrane.

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