COMPLETE CONTROL OF THE LIPID ENVIRONMENT OF MEMBRANE-BOUND PROTEINS: APPLICATION TO A CALCIUM TRANSPORT SYSTEM

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1. Introduction

Most biological membranes are complex heterogeneous systems with respect to both their lipid and their protein composition and are intractable to detailed biochemical and biophysical studies of lipid–protein interactions. Definitive studies of a membrane-bound protein are simplified by the availability of homogeneous complexes of the protein with phospholipid, in which the composition of the phospholipid can be completely controlled. So far, control of the lipid composition has been limited to those proteins which can be completely stripped of endogenous lipid without irreversible loss of protein integrity [1–4]. Many membrane-bound proteins will not survive this treatment without irreversible denaturation. Thus, complete delipidation of the calcium transport protein ($(Mg^{2+} + Ca^{2+})$-ATPase) from the sarcoplasmic reticulum of rabbit muscle abolishes the calcium-dependent hydrolysis of ATP and this activity cannot be restored by the addition of exogenous phospholipids [5–7]. In an attempt to define the role of lipids in this transport system, we have devised a new method for controlling the lipid composition of membrane-bound proteins. This method involves the direct displacement of endogenous lipids by defined exogenous lipids in a dispersing agent, so that the protein is at no time without a lipid environment. We have used this method to prepare lipid complexes of the calcium transport protein consisting almost entirely of dioleoyl lecithin (DOL), dimyristoyl lecithin (DML), or dipalmitoyl lecithin (DPL). We have also been able to apply this new technique successfully to rhodopsin. This suggests that the technique may be widely applicable to membrane-bound proteins.

As an example of the procedure we discuss here the large-scale preparation of the calcium transport protein substituted with DOL. A preliminary report of some of this work has already appeared [8].

2. Materials and methods

The calcium transport protein with associated lipids (ATPase) was purified from sarcoplasmic reticulum vesicles as described previously [8] except that, instead of deoxycholate, 0.5 mg of cholate were added per mg of protein. The enzyme is more than 95% pure (by weight) and of the 90 lipid molecules associated with each molecule of ATPase in native sarcoplasmic reticulum vesicles, about 25 remain associated with the purified protein. Purified ATPase (150 mg) was incubated for 1 hr at 0°C in a total volume of 3 ml of 250 mM sucrose, 50 mM potassium phosphate buffer pH 8 and 1 M KCl containing 225 mg of [^{14}C] cholate (~ 25 000 dpm/mg) and 225 mg of (N-methyl-[^{3}H]-dioleoyl lecithin [9] (~ 35 000 dpm/mg). Samples (1 ml) were then carefully layered on a two step discontinuous gradient comprising 1 ml of 20% (w/v) sucrose and 0.1 ml of 50% (w/v) sucrose in 50 mM potassium phosphate buffer pH 8 and 1 M KCl. The samples were then subjected to an average centrifugal field of 160 000 *g* for 20 hr at 5°C and

the pellet of ATPase in which the lipids have been substituted with DOL was collected at the interface of the 20% and 50% sucrose layers. The yield of protein at this stage was between 60 and 75 mg. The above substitution procedure was then repeated by incubating the ATPase after a single substitution with DOL in a total volume of 1.5 ml containing 113 mg each of cholate and DOL. Samples (0.5 ml) were layered on 1.5 ml of 20% (w/v) sucrose and the system centrifuged as before. The ATPase after the double substitution procedure was finally dialyzed for 2 days at 0–10°C against 1000 volumes of 250 mM sucrose, 50 mM potassium phosphate buffer pH 8 and 1 M KCl containing XAD-2 to sequester any residual cholate. The final yield of ATPase varied between 45 and 55 mg.

Fatty acid analysis by GLC and the assay of protein and ATPase activity have all been described previously [8].

3. Results and discussion

The effectiveness of this lipid substitution technique depends on two factors.

Firstly, in the presence of cholate, complete equilibration of lipid occurs between DOL and the endogenous lipids of the ATPase. This is clearly shown in table 1, where the fatty acid composition of the substituted ATPases observed by GLC analysis is very close to that expected for complete lipid equilibration. Although GLC analysis gives no information concerning the substitution by DOL of minor lipids (< 15%) such as cholesterol and sphingomyelin, rigorous extraction of the lipids [10] bound to the substituted ATPases followed by thin layer chromatography showed that there was no selective retention of these or any other lipid class.

Secondly, the complexes of the ATPase protein with associated lipid and cholate, sediment in a high centrifugal field, whereas excess lipid and cholate do not sediment under these conditions and remain in the supernatant. The amount of lipid associated with the ATPase rises slightly during the first DOL substitution step but then remains constant at approximately 30–35 molecules of lipid/molecule of ATPase (table 1). During the sedimentation of the substituted ATPase through the detergent-free, discontinuous sucrose gradient, the bound cholate diffuses away from the complex and the DOL-substituted ATPase sediments in a particulate form. As a result, only 5–7% of the original cholate (20–30 moles of cholate/mole of ATPase, table 1) remains associated with the substituted ATPase. Prolonged dialysis against XAD-2 is however necessary to reduce the level of detergent to an average of less than 1 molecule/molecule of ATPase. It should be noted that the preparation of substituted ATPase by the small

Table 1
DOL substitution of ATPase

	Purified ATPase	ATPase; single DOL substitution	ATPase; double DOL substition
ATPase activity (i.u./mg):			
– 1 mM EGTA	8.8	8.1	6.3
+ 1 mM EGTA	0.14	0.10	0.19
Lipid composition (moles/mole ATPase):			
Total lipids*	23	32	33
Endogenous lipids	23	3	< 1
DOL	–	29	33
% DOL substitution expected:	–	91.5	98.9
% DOL substitution observed:	–	91.0	98.0
Bound cholate (moles/mole ATPase)	–	27	< 1

* Estimated by quantitative GLC analysis of the transesterified fatty acids [8] or by calculation from [^{3}H] DOL and the % DOL substitution. The mol. wt. of ATPase was 115 000 [8] and the assumed mol. wt. of the lipids was approx. 775.

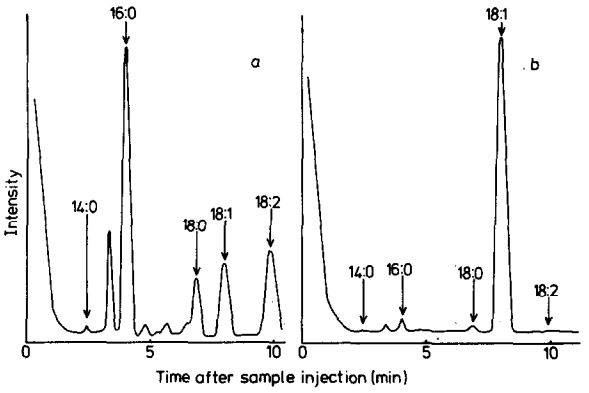

Fig. 1. GLC analysis (DEGS, 195°) of the transesterified fatty acids derived from the lipids associated with (a) ATPase and (b) doubly DOL-substituted ATPase. The illustrated portion of the fatty acid spectrum represents more than 75% of the total fatty acids. The quantity of each fatty acid is proportional to the area under the appropriate peak.

scale procedure [8] results in the retention of less lipid and cholate.

The ATPase doubly-substituted with DOL, prepared by the large-scale technique, retains an average of less than 1 molecule of endogenous lipid/molecule of ATPase. This is clearly illustrated by the GLC traces of the major fatty acids from both the purified and the doubly-substituted ATPase (fig. 1). After substitution, the fatty acid chains of DOL (18:1) dominate the fatty acid spectrum of the substituted ATPase and, in fact, the 16:0 and 18:0 peaks that are observed for the doubly-substituted ATPase are derived predominantly from the low levels of these fatty acid chains present as impurities in the purified DOL [8]. Negligible amounts of the minor fatty acids present in purified ATPase (18:3 and longer fatty acid chains) remain after the double substitution procedure.

The calcium transport protein remains active after almost complete substitution of its associated lipid with DOL (table 1). We have also found that activity is retained after substitution with DML, or DPL, provided that the enzyme is assayed above the transition temperature of the substituted lipid. The protein does not therefore exhibit an absolute lipid specificity. Preliminary experiments have however delineated the qualitative lipid specificity of the enzyme with respect to lipid headgroup, fatty acid chain length and the number and position of unsaturated double bonds; so far, the DOL substituted ATPase has the highest enzyme activity that we have obtained.

We expect this lipid substitution procedure to be applicable to many membrane-bound proteins providing that the integrity of the protein will withstand the presence of detergent. We have used deoxycholate, cholate, dodecyltrimethylammonium bromide and Triton X-100 as detergent and we have found that for the calcium transport protein, cholate causes the least protein denaturation when used with buffers of high ionic strength containing sucrose.

The large-scale preparation of lipid–protein complexes containing the lipid of choice should allow a precise evaluation of the role of lipid in maintaining and determining the structural and functional integrity of the membrane-bound protein.

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