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Reconstitution of a Calcium Pump Using Defined Membrane Components

[$(Mg^{2+} + Ca^{2+})$ATPase/sarcoplasmic reticulum/active transport/membrane protein purification/lipid substitution]

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ABSTRACT **A $(Mg^{2+} + Ca^{2+})$ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been purified from sarcoplasmic reticulum using a single step centrifugation procedure. The preparation is >95% pure by weight and contains only 25–30% of the lipid associated with the enzyme in native sarcoplasmic reticulum. The purified enzyme is unable to accumulate Ca^{2+}. Using a sedimentation-substitution technique, >98% of the lipid associated with the purified enzyme can be replaced by dioleoyl lecithin without grossly affecting the ATPase activity of the enzyme. The Ca^{2+} pump can be restored to this dioleoyl lecithin-substituted enzyme by addition of excess sarcoplasmic reticulum lipids in the presence of cholate. Removal of the cholate by dialysis generates a system which accumulates Ca^{2+} at a rate and to a level comparable to native sarcoplasmic reticulum. Significant reconstitution of the Ca^{2+} pump can also be achieved using excess dioleoyl lecithin, but since the full expression of the capacity to accumulate Ca^{2+} requires the presence of oxalate, these vesicles would appear to be more leaky than those reconstituted with an excess of sarcoplasmic reticulum lipids. Of about 90 lipid molecules which are associated with one molecule of ATPase in native sarcoplasmic reticulum, an average of less than one lipid molecule remains in these reconstituted systems. We have therefore achieved a fully functional Ca^{2+} pump containing essentially a single protein and exogenous lipid.**

Physical and biochemical studies of membrane structure are complicated by the multiplicity of components in native membranes. One solution is to reconstitute specific membrane functions using the minimum number of defined lipid and protein components. This would allow both the determination of the tolerated range of lipid composition and the insertion of specifically labeled components into the reconstituted structure to serve as physical probes. We selected the active Ca^{2+} transport system of sarcoplasmic reticulum (SR) for reconstitution because the major protein in the membrane is the $(Mg^{2+} + Ca^{2+})$ATPase (ATP phosphohydrolase, EC 3.6.1.3); this enzyme has been purified to homogeneity by MacLennan (1) and has been shown to be the sole protein responsible for Ca^{2+} transport (2).

There are at least two approaches to the reconstitution of a defined functional structure. The protein may be freed of lipid during the purification procedure and the pure protein reactivated with exogenous lipid; examples of this approach include the galactosyl transferase system described by Rothfield (3) and the isolation of rhodopsin by Hong and Hubbell (4). Alternatively, the endogenous lipid can be directly replaced by defined lipid with retention of activity throughout the substitution. This conservative technique leads to the same final state and can provide a technically easier route.

Early work on the SR membrane by Martonosi *et al.* (18) demonstrated that after phospholipase digestion of up to 60% of the total SR phospholipid, the ATPase activity and Ca^{2+} uptake could be restored by addition of sonicated dispersions of phospholipids. Complete delipidation of SR ATPase results in the inactivation of the enzyme (ref. 5 and P. Hardwicke and N. M. Green, personal communication). Where reactivation has been possible, it has resulted in a Ca^{++}-independent ATPase (5). The technique of substitution described here is the first successful attempt to replace essentially all the lipids of the ATPase whilst retaining the normal Ca^{2+}-dependent activity.

Defined reconstitution of the Ca^{2+} pump has been effected in two stages. Firstly, purification and dioleoyl lecithin (DOL) substitution of the ATPase involve a *very rapid* removal of detergent from the lipid–protein complex, by centrifuging the complex into a detergent-free sucrose gradient. The resulting complex is unable to accumulate Ca^{2+}. Secondly, reconstitution of the Ca^{2+} pump involves the *slow* removal of detergent using the dialysis technique described by Racker (2). The dialysis technique was used by Racker to reconstitute a membrane transport system using ATPase and soybean lipids.

By a combination of these two basic techniques we have been able to restore the Ca^{2+} pump to systems which contain an average of less than one molecule of endogenous SR lipid for every molecule of ATPase.

METHODS

SR membranes were prepared from white muscle from rabbit leg (6) in the presence of 5 μM phenylmethylsulphonyl fluoride (to inhibit proteolysis) and 1 mM dithiothreitol (DTT). The ATPase accounts for 70–80% of the total protein on polyacrylamide gels.

ATPase activity was estimated using a coupled enzyme assay in a medium containing 100 mM triethanolamine hydrochloride-KOH (pH 7.2), 5 mM $MgSO_4$, 2 mM ATP, 0.1 mM $CaCl_2$, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume of 3 ml of 37°. ATPase (<0.3 IU) was added to start the reaction which was monitored continuously at 340 nm. Controls were carried out in the presence of 1 mM

Abbreviations: DOC, deoxycholate; DOL, dioleoyl lecithin; DTT, dithiothreitol; SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid; EDTA, ethylenediaminetetraacetate; GLC, gas-liquid chromatography.

ethylene glycol bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA).

Uptake of $^{45}Ca^{2+}$ was measured in the presence and absence of 5 mM oxalate in a medium similar to that described above and containing $^{45}CaCl_2$ (3 to 6 × 10^6 cpm/μmol) in a total volume of 1 ml at 23°. The SR or reconstituted vesicles were added to start the reaction which was terminated by passage through a 0.45-μm Millipore filter. The filter was washed with 10 ml of a solution containing 100 mM-triethanolamine hydrochloride-KOH buffer (pH 7.2) and 0.2 mM $CaCl_2$, and no significant loss of protein through the filter could be detected. The filter was vigorously shaken with 15 ml of Triton X-100:toluene:water [4:8:1 (v/v/v)] containing 0.4% 2-(4′-tert-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole, and counted. Controls were carried out in the presence of ethylenediaminetetraacetate (EDTA).

Polyacrylamide gel electrophoresis was carried out in the manner described by Weber and Osborn (7). The molecular weight of the ATPase was 115,000. Protein was estimated by the method of Goa (8) in the presence of 0.1% deoxycholate (DOC).

Fatty acid analysis of DOL (9) showed that the lecithin contained 98.7% 18:1, 0.8% 16:0, and 0.5% 18:0. [^{3}H]-Methyldioleoyl lecithin was prepared by the method of Stoffel *et al.* (10). Membrane lipids (typically 0.1–4 mg) were extracted by chloroform/methanol (2:1) and transesterified (11), and the fatty-acid methyl esters were analyzed by gas-liquid chromatography (GLC) (DEGS, 195°). The areas of the peaks were compared with those given by known weights of transesterified defined phospholipids and extracted SR lipids. Certain lipids such as cholesterol and sphingomyelin are not detected. However, rigorous extraction of the lipids (12) of substituted ATPases followed by thin-layer chromatography gave no indication of selective retention of these or any other lipid class.

RESULTS

Purification of ATPase. The SR proteins sediment in a detergent layer as a function of their molecular weight (see Fig. 1). When the lipid–protein complexes reach the boundary between the DOC and the sucrose gradient, they break through, reform particulate material, and sediment to an equilibrium position in the sucrose gradient. Polyacrylamide gels clearly revealed the pattern of events. Low molecular weight proteins such as "calsequestrin" (13) remained in the detergent layer [Fig. 1 (*b*), fraction 16]. The main protein peak was heterogeneous, the lower part containing the majority of the high-molecular-weight contaminants and polymeric forms of ATPase [Fig. 1(*b*), fraction 9]. The upper part of this particulate protein band, however, was essentially pure ATPase (>95%, specific activity 10–14 IU/mg).

Using this sedimentation procedure, less than 0.3% of the added DOC remains bound to the purified enzyme, corresponding to < 1 molecule of DOC per ATPase molecule. Furthermore, only 25–30% of the original SR lipid is retained in the purified ATPase when compared on a protein basis with the intact SR membrane (Table 1). There is no evidence for the selective removal of any particular phospholipid class during the stripping process. This preparation contrasts with the ATPase purified by MacLennan (1), which retains the same proportion of endogenous SR lipid as in the SR membrane. However, neither preparation can accumulate Ca^{2+}

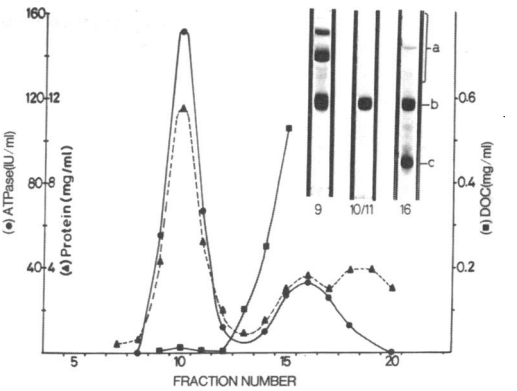

FIG. 1. Purification of ATPase. SR vesicles (13 mg of protein per ml) in 250 mM sucrose, 50 mM potassium phosphate (pH 8), 1 M KCl, and 2.5 mM DTT were treated with 10% [^{14}C]DOC (7300 cpm/mg) at a ratio of 0.4 mg/mg of protein. The resulting mixture was centrifuged at 190,000 × *g* for 20 min at 5° and the clear supernatant was collected; 3-ml aliquots were layered on a 16 ml [20%–60% (w/v)] sucrose gradient in 50 mM potassium phosphate (pH 8), 1 M KCl, and 2.5 mM DTT. This system was centrifuged at 130,000 × *g* for 24 hr at 5° and purified ATPase obtained from fractions 10 and 11 (see *inset*). *Inset:* polyacrylamide gels of the indicated fractions. (a) High-molecular-weight contaminants and polymeric forms of ATPase. (b) Pure ATPase. (c) "Calsequestrin."

(ref. 1; Fig. 2.). The activity of the ATPase is stable for at least 4 weeks at 4°.

Substitution of DOL for endogenous SR Lipid. DOL can be substituted for the residual SR lipid in the purified ATPase by dissolving the enzyme in a mixture containing DOL and either DOC or cholate and then centrifuging the protein into a detergent-free sucrose gradient (see Table 1 for details). There are three points of note. Firstly, the purified ATPase–lipid complex contains the same proportion by weight of lipid irrespective of the amount of added DOL (Table 1). Secondly, the proportion of DOL in the substituted ATPase, as estimated by GLC (Table 2) or by using [^{3}H]DOL, is that expected for complete equilibration of the lipid pools. Thirdly, there is little indication of preferential substitution of DOL for any lipid of specific fatty-acid composition, although at very high levels of DOL substitution there may be some preferential displacement of lipids with polyunsaturated fatty-acid chains.

Very high levels of DOL substitution can be achieved with a very large excess of DOL or, more conveniently, by repeated substitution of a partially substituted DOL–ATPase complex. Active ATPase preparations can be prepared in which more than 99% of the endogenous SR lipids have been replaced by DOL (see Table 1 for details). This corresponds to a residual level of < 1 SR lipid molecule per molecule of ATPase. Preparation of active singly substituted (DOL–ATPase) and the twice substituted (DOL^2–ATPase) ATPase is critically dependent on the experimental conditions (see Table 1); the substituted enzymes are stable for at least 4 weeks at 4°.

Reconstitution of a Ca^{2+} Pump from Purified ATPase and ATPase–DOL Complexes. Reconstitution of the Ca^{2+} pump utilizes the dialysis technique described by Racker (2). ATPase, DOL–ATPase, or DOL^2–ATPase was suspended

TABLE 1. *DOL-substitution of ATPase and reconstitution of the calcium pump*

	SR	Purified ATPase	DOL–ATPase	DOL²–ATPase
% Lipids	100	28	28	24
% DOL substitution:				
Expected	—	—	85	98.8
Observed	—	—	85	98.0
Endogenous lipids:				
%	100	28	4	0.5
mol/mol ATPase*	90	25	4	<1
ATPase activity (IU/mg):				
− 1 mM EGTA	2.6	8.3	5.9	3.8
+ 1 mM EGTA	0.37	0.41	0.13	0.08
Calcium accumulated (nmol of Ca^{2+}/mg of protein)				
Reconstitution with:				
SR lipids:				
− oxalate	(350)†	130	340	355
+ oxalate	(3800)†	4400	—	4900
DOL:				
− oxalate	(350)†	40	40	45
+ oxalate	(3800)†	1800	2800	3150

Preparation of DOL–ATPase: 4 mg of ATPase, 4 mg of sonicated DOL, and 4 mg of DOC in 250 mM sucrose, 50 mM potassium phosphate (pH 8), 1 M KCl, and 2.5 mM DTT were incubated in a total of 0.3 ml for 2 hr at 2°. The mixture was then layered on a 2-step discontinuous sucrose gradient in 50 mM potassium phosphate (pH 8), 1 M KCl, and 2.5 mM DTT and centrifuged at 160,000 × *g* for 17 hr at 5°. DOL–ATPase was removed from the interface of the 15 and 50% sucrose layers.

Preparation of DOL²–ATPase: The procedure was similar to that described above, except that 4 mg of DOL–ATPase was incubated with 8 mg of sonicated DOL and 6 mg of DOC.

Reconstitution was carried out as described in the *text* using a 20-fold excess (by weight) of lipid to ATPase protein.

* Intact SR contains 62% protein and 38% lipid by weight.

† Values for intact SR.

in 30% (w/v) sucrose, 50 mM potassium phosphate (pH 8), 1 M KCl, and 2.5 mM DTT to a concentration of 2.5 mg protein per ml, and 1 mg of this resuspended enzyme was added to 400 mM potassium phosphate (pH 7.5) containing 1–40 mg of sonicated SR lipid or DOL and 0.64 mg of [^{14}C]-cholate (5–10,000 cpm/mg)/mg of lipid to a total volume of 1 ml. Sonication of the lipid and cholate mixtures to optical clarity was performed in a closed glass vial under nitrogen in a sonicating bath. The mixture was immediately dialyzed against 500 ml of 400 mM potassium phosphate (pH 7.5) for 16 hr at 5° and a further 4 hr in the presence of Amberlite XAD-2 to reduce the cholate level to < 0.03 mg/mg of lipid.

TABLE 2. *The equilibration of exogenous DOL with the endogenous lipid pool*

	% of total fatty-acid content				
	SR	DOL-substituted SR			
Fatty acid		1:1*		5:1	20:1
16:0	26	15	14	8	4
18:0	12	7	6	3	2
18:1	14	61	57	85	93
18:2	19	8	11	2	0.5
20:4	16	7	8	—	—
22:5	6	3	3	—	—
	% DOL substitution				
Expected	—	50	50	83	95
Observed by GLC	—	53	50	82	92

Conditions for DOL-substitution were similar to those described in Table 1, except that the incubation contained 1 mg of SR protein and either 1 mg of DOL (1:1), 5 mg of DOL (5:1), or 20 mg of DOL (20:1). Cholate was present at 0.64 mg/mg of (lipid + protein).

* Values for two separate experiments are given.

Although a Ca^{2+} pump can be reconstituted in the absence of any added lipid (Fig. 2.), the steady state level of Ca^{2+} accumulated is low both in the absence (30 nmol of Ca^{2+}/mg of protein) and the presence (200 nmol of Ca^{2+}/mg of protein) of oxalate. This probably reflects the low level of lipid present in the purified enzyme (Table 2).

Reconstitution of the Ca^{2+} Pump Using SR Lipid. Reconstitution using the purified ATPase in the presence of an increasing excess of SR lipid has a marked effect on the level of Ca^{2+} accumulated in the absence of oxalate (Fig. 2.). With a 20-fold excess by weight of SR lipid to protein, a final preparation is obtained which accumulates Ca^{2+} to a steady state level of 130 nmol of Ca^{2+}/mg of protein. Higher levels of SR lipid do not increase the level of Ca^{2+} accumulated.

A Ca^{2+} pump with high levels of exogenous, defined, lipids was obtained by a similar reconstitution of the DOL-substituted ATPases. Fig. 3 summarises the results obtained with a 20-fold excess of SR lipids in the absence of oxalate. The similarity between the Ca^{2+} uptake curves for native SR and vesicles reconstituted from DOL²–ATPase is striking (Fig. 3.). To our knowledge this is the first report of a membrane-bound transport protein functioning in a system with very high levels of defined exogenous lipids (>99%). The level of Ca^{2+} accumulated appears to increase with the degree of substitution of the purified ATPase with DOL (Fig. 3.);

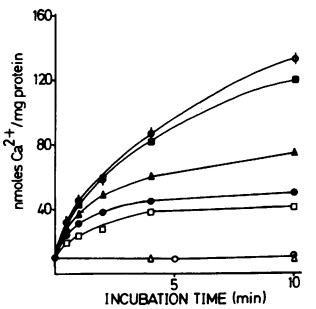

FIG. 2. The effect of excess lipid on the reconstitution of the Ca^{2+} pump. Details of the reconstitution procedure may be found in the text. O = control, 1 mM EDTA; Δ = purified ATPase; □ = 1 mg ATPase reconstituted in the presence of 0.5 mg of cholate; 1 mg of ATPase reconstituted in the presence of SR lipids = 1 mg (●), 5 mg (▲), 10 mg (■), 20 or 40 mg (ϕ).

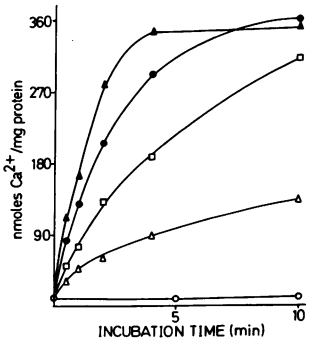

FIG. 3. Reconstitution of the Ca^{2+} pump using 1 mg of ATPase substituted to differing extents with DOL, and 20 mg of SR lipid. O = Control, 1 mM EDTA; Δ = ATPase; □ = DOL–ATPase; ● = DOL^2–ATPase; ▲ = intact SR for comparison.

this distinction is not so apparent in the presence of oxalate (Table 1).

Reconstitution Using DOL. The Ca^{2+} pump can also be reconstituted with an excess of DOL. With either 1 or 20 mg of DOL per mg of purified ATPase, a final steady state level of Ca^{2+} accumulation of 40 nmol/mg of protein is achieved (Table 1). This value is similar to that of the Ca^{2+} pump reconstituted from purified enzyme in the absence of excess lipid (30 nmol/mg of protein). However, the level of Ca^{2+} accumulated by the DOL-reconstituted vesicles in the presence of oxalate (1800 nmol/mg of protein) is considerably higher than that accumulated by the Ca^{2+} pump reconstituted from purified ATPase alone (200 nmol/mg of protein). Since the presence of oxalate renders the process of Ca^{2+} uptake much less sensitive to leak of Ca^{2+} from the vesicles, we conclude that the vesicles reconstituted in the presence of excess DOL are probably more leaky than those reconstituted with excess SR lipids under these experimental conditions.

An excess of DOL can also be used to restore Ca^{2+} accumulation to both DOL–ATPase and DOL^2–ATPase. Although the level of Ca^{2+} accumulated is low (Table 1), these values are substantially increased by the presence of oxalate. It is of importance that the DOL^2–ATPase can be reconstituted with an excess of DOL, since this strongly suggests that a significant degree of reconstitution can be effected using essentially one protein and one phospholipid class.

DISCUSSION

The techniques described in this paper allow complete control of the lipid composition of purified ATPase and the reconstituted membrane. The reproducibility of the sedimentation-substitution technique is essential for a systematic study of the functional parameters of the reconstituted system.

The experiments strongly suggest that a single protein species acts as both ATPase and ionophore in conferring Ca^{2+} uptake activity. They further suggest that the lipid composition is probably an important structural feature for optimal function, since we have shown that under our reconstitution conditions DOL is much less effective than SR lipid in restoring Ca^{2+} uptake in the absence of oxalate; this we attribute to the inability of DOL to prevent Ca^{2+} leakage from the vesicle. The cholesterol present in the SR lipid extract, although at a low level, might possibly be important in controlling leakage. However, it is also possible that the difference in uptake reflects variations in the optimal conditions for reconstitution using different phospholipids rather than any intrinsic difference in their functional competence. If the phospholipids play a direct catalytic role in the reaction pathway of the ATPase, then the above experiments would suggest that DOL can perform this function without grossly affecting the integrity of the enzyme.

The high levels of Ca^{2+} accumulated by the most active reconstituted systems strongly suggest that their structures approximate that of intact SR membranes. The parameters which define the functional status of the Ca^{2+} pump are the ATPase activity, the extent to which it is coupled stoichiometrically with Ca^{2+} uptake, the rate of nonspecific efflux from the vesicles, and the internal volume per ATPase pump unit. Since a number of permutations of these parameters could yield similar uptake curves to those observed experimentally, this precludes direct quantitative comparison between native and reconstituted vesicles. The separation of functionally reconstituted vesicles from any inactive material would also be an essential step before these quantitative comparisons can be made.

The ability to reconstitute an active membrane transport system with defined lipids will simplify the study of these systems by spectroscopic techniques. Evidence from magnetic resonance studies suggests that although some of the lipids in SR membranes undergo rapid lateral diffusion (14, 15), there may also be a segregation of the lipids with restricted inter-diffusion between different lipid regions (6). We have suggested that the ATPase molecules may be surrounded by a bilayer shell of lipid which exchanges relatively slowly with lipid in regions of unperturbed bilayer (16). This dynamic model of the structure is similar to that proposed by Jost *et al.* (17) involving immobilized boundary lipid in purified cytochrome oxidase particles. There is also interesting recent evidence for restricted lateral motion in the vicinity of the cytochrome P_{450}-cytochromeP_{450}-reductase hydroxylating enzyme system from the work of Stier and Sackman (19). The use of ^{13}C-labeled lipids in the reconstitution procedure should allow these models to be tested.

Finally, we note that the techniques of lipid substitution and reconstitution described here may have wider application to membrane proteins which withstand dispersal in DOC and other detergents.

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