SINGLE-PHASE BUTANOL EXTRACTION: A NEW TOOL FOR PROTEOLIPID ISOLATION

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SUMMARY

An alternative method to the chloroform/methanol extraction of proteolipids is presented. The proteolipid fraction from sarcoplasmic reticulum membranes is isolated by a single-phase n-butanol extraction and subsequently precipitated by diethyl ether. The two step procedure described is successfully applied in the purification of the dicyclohexylcarbodi-imide binding protein from submitochondrial particles.

INTRODUCTION

The isolation of highly hydrophobic membrane proteins often has been hampered by the absence of suitable nonaqueous techniques. While classical extraction methods utilizing organic solvents have been widely employed (1-6), a biphasic system was always present. This report describes a new technique for proteolipid extraction using n-butanol as organic solvent in a single phase system. n-Butanol is known as a pronounced solubilizing agent for membrane components, disorganizing lipid-protein interactions (7-9). The miscibility of n-butanol with small amounts of aqueous solutions, without forming a two-phase system, favors an easy separation of the solubilized fraction. By means of the technique detailed here the proteolipid from sarcoplasmic reticulum membrane and the mitochondrial membrane DCCD-binding protein have been isolated in a rapid and facil two step procedure.

METHODS AND MATERIALS

Sarcoplasmic reticulum membranes were prepared from rabbit skeletal muscle (10) in the presence of 5μM phenylmethylsulfonyl fluoride. The final membrane fraction was suspended in 100mM KCl, 5mM imidazole pH 7.4 and stored at -70°C. Before isolation of the proteolipid fraction, sarcoplasmic reticulum membranes (15-20 mg/ml) were sedimented by centrifugation at 24,000 g for 60 minutes. The membrane pellet was suspended in 5mM $CaCl_2$ or water, conserving the initial protein concentration.

Isolation of rat liver mitochondria was carried out according to Johnson and Lardy (11). Submitochondrial particles were prepared with a Branson sonifier in a medium containing 2mM EDTA pH 8.5 and were subsequently twice washed (105,000 g, 30 minutes) with a buffer containing 250mM sucrose, 10mM Tris-HCl pH 7.5. The isolated membranes were stored at -70°C. Submitochondrial particles were incubated with DCCD (2μg per mg protein) for 18 hours at 0°C (6,12). ATP'ase activity is defined and assayed as in reference 6 in the absence of an ATP regenerating system. The labeled membranes were washed twice with sucrose-Tris buffer and collected by

Abbreviations: DCCD - N,N'-Dicyclohexylcarbodi-imide
SDS - Sodium dodecylsulfate

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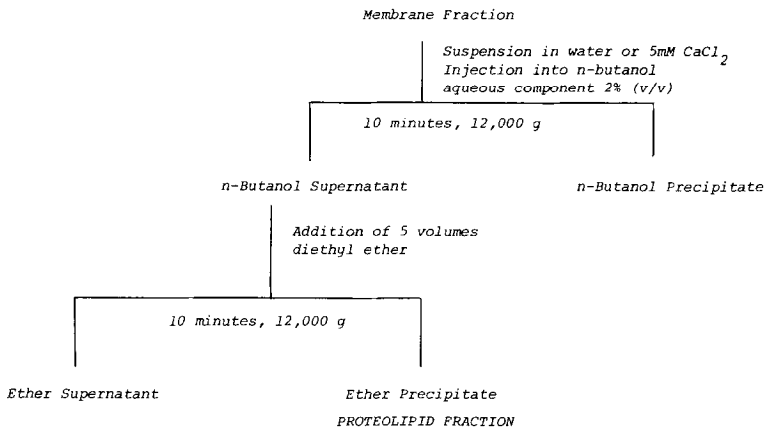

Fig. 1. Schematic representation of the proteolipid isolation procedure.

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centrifugation at 105,000 g for 30 minutes. The final pelleted membranes were suspended in water (6-8mg/ml). Protein was determined in the presence of 0.1% SDS according to Lowry et. al (13). Samples containing organic solvents were dried under nitrogen before assaying for protein. Total phosphate was measured by the method of Chen et. al (14).

SDS gel electrophoresis was carried out in accordance with Weber and Osborn (15). The electrophoresis was performed in 0.1% SDS, 50mM sodium phosphate buffer pH 7.2 using 10% polyacrylamide gels. Gels were stained with coomassie brillant blue and destained with 7% acetic acid. For molecular weight determinations bovine pancreatic insulin (MW 5,700) and egg white lysozyme (MW 14,300) served as standards in the co-electrophoretic analysis. When ^{14}C-DCCD-labeled samples were electrophoresed, identical samples were applied to separate gels. One gel was stained; the duplicate gel was immediately cut into 2mm slices. The individual gel slices were extracted overnight with 10mM Triton X-100. Scintillation fluid was added and the slices were counted for radioactive content.

All chemicals and reagents were of the highest purity commercially available. ^{14}C-DCCD (45mCi/mM) was a generous gift of Dr. H.R. Kaback, Roche Institute of Molecular Biology, Nutley.

RESULTS

Isolation procedure:

The isolation procedure entails injection of the membrane suspension into n-butanol followed by addition of diethyl ether to the butanol fraction (fig. 1). The widely applicable procedure is described as follows:

Step I: Using a tuberculin syringe the membrane suspension is injected into n-butanol at room temperature. Final concentration of the aqueous component is 2%. The immediately formed precipitate is separated by repeated centrifugation, usually three times at 12,000 g for 10 minutes.

Step II: The n-butanol supernatant is combined with 5 volumes of diethyl ether, precooled to -20°C. The finely dispersed precipitate (proteolipid fraction) sediments when centrifuged at 12,000 g for 10 minutes (4°C). The sedimentation of the sarco-

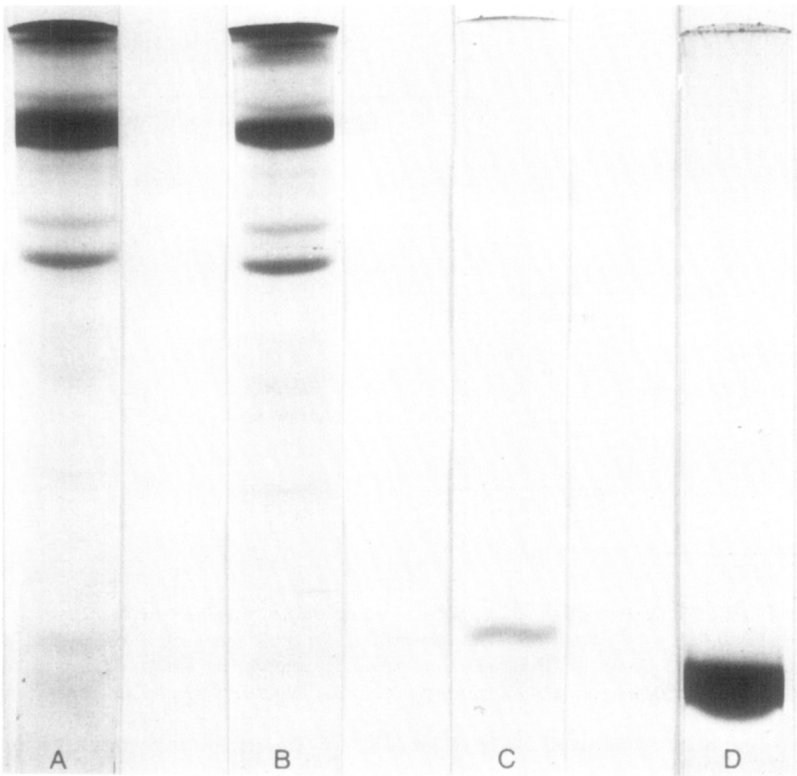

Fig. 2. Protein patterns of fractions obtained during the sarcoplasmic reticulum proteolipid isolation. SDS gel electrophoresis, staining and destaining were performed as described in METHODS. The amount of protein applied is given in brackets. A. Sarcoplasmic reticulum membranes (60µg); B. Butanol precipitate (40µg); C. Ether precipitate (proteolipid) (5µg); D. Insulin (12µg).

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plasmic reticulum proteolipid fraction is performed immediately after the addition of diethyl ether, whereas the n-butanol/diethyl ether mixture containing the mitochondrial proteolipid is stored at -20°C overnight.

Sarcoplasmic proteolipid fraction:

The proteolipid purification was routinely followed by SDS gel electrophoresis (fig. 2). All major proteins of the sarcoplasmic reticulum, excepting the proteolipid, could be identified in the gel pattern of the butanol precipitate (fig. 2B). The proteolipid was present in gels made of the ether precipitate (fig. 2C). In the gel system employed, an apparent molecular weight of 9,000 daltons was determined. A faint band attributable to the lipid components was present in all gels although to a greatly reduced extent in gels of the butanol precipitate. In preliminary experiments it was noted that the presence of low concentrations (5mM) of Ca^{++} increased the amount of proteolipid solubilized into butanol. When Ca^{++} was absent during the ad-

TABLE I

Isolation of proteolipid fractions from sarcoplasmic reticulum membranes and carbodi-imide - treated submitochondrial particles: Recoveries of total protein and phosphate.

Fraction		Protein		Phosphate	
		mg	%	µmoles	%
Sarcoplasmic reticulum membranes		78.02	100.0	58.01	100.0
Butanol precipitation:	Precipitate	75.01	96.2	2.93	5.0
	Supernatant	1.03	1.3	53.47	92.1
Ether precipitation:	Precipitate	0.12	0.15	0.44	0.7
	Supernatant	2.40	3.0	54.38	93.5
DCCD-treated submitochondrial particles		6.03	100.0	4.65	100.0
Butanol precipitation:	Precipitate	5.77	95.6	0.50	10.7
	Supernatant	0.20	3.4	3.90	83.8
Ether precipitation:	Precipitate	0.12	1.9	0.44	9.4
	Supernatant	0.32	5.4	3.65	78.4

dition of sarcoplasmic reticulum membranes to n-butanol, small amounts of proteolipid were detected in the butanol precipitate.

The recoveries of both protein and total phosphate during the isolation of the proteolipid fraction from sarcoplsamic reticulum and the DCCD-labeled submitochondrial particles are listed in table I. As demonstrated by gel electrophoresis, n-butanol effectively precipitated almost all protein, approximately 96% being present in the butanol precipitate. Addition of ether to the separated butanol supernatant of sarcoplasmic reticulum membranes served to precipitate the proteolipid fraction leaving 94% of the total phosphate in the ether supernatant. When submitochondrial particles were identically treated 78% of the total phosphate remained in the ether supernatant. The protein content of the supernatant in step II is most probably overestimated due to the simultaneous presence of concentrated lipids known to interfere with the Lowry determination (16). Additionally, the absence of protein in this fraction was confirmed by gel electrophoresis where protein bands could not be detected (see also fig. 3C).

Mitochondrial proteolipid fraction:

The versatility of the proteolipid isolation procedure was demonstrated by the successful purification of the DCCD-binding protein from mitochondrial membranes previously characterized as a proteolipid by Catell et. al. (5). Under the described con-

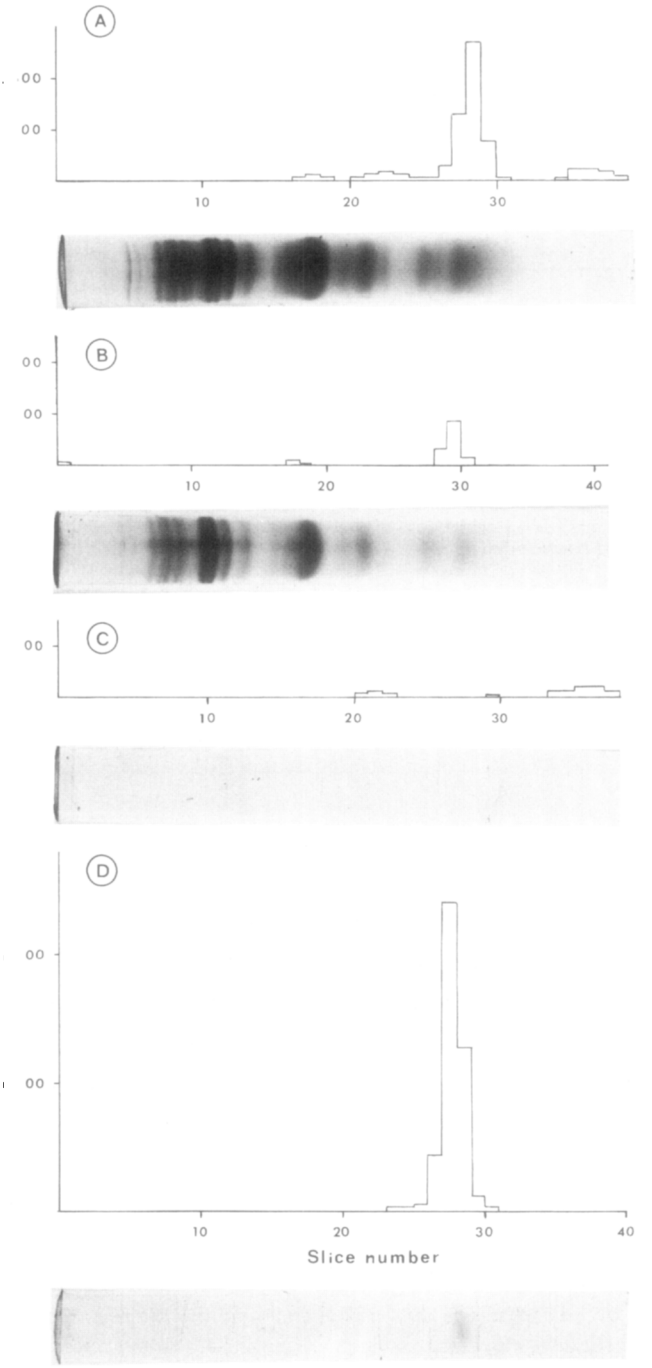

Fig. 3. Isolation of the ^{14}C-DCCD-binding protein from submitochondrial particles: Distribution of protein and radioactivity in polyacrylamide gels. A. ^{14}C-DCCD-treated submitochondrial particles (40μg); B. Butanol precipitate (32μg); C. Ether supernatant (equivalent to 4μg); D. Ether precipitate (proteo lipid) (5μg).

ditions nonradioactive DCCD as well as ^{14}C-DCCD (both 2μg/mg membrane protein) inhibited the ATP'ase activity, 97% and 98% respectively, compared to a membrane suspension without inhibitor. These results are in agreement with the data reported by Steckhoven et. al. (6) and Cattell and co-workers (5). Figure 3 presents SDS gel analysis of submitochondrial particles, butanol precipitate, ether precipitate (proteolipid), and ether supernatant fractions, as well as the patterns of corresponding ^{14}C-DCCD labeling. As previously reported (5,6) DCCD labeled highly selectively one protein band in the submitochondrial particles (fig. 3A). Extraction of the DCCD-binding proteolipid was not totally quantitative, DCCD-labeled protein being found in the butanol precipitate (fig. 3B). Protein banding was absent when concentrated samples of the ether supernatant (fig. 3C) were subjected to SDS gel electrophoresis. Moreover, no radioactivity was found on the gels at the position of the proteolipid's relative mobility, confirming complete precipitation of the protein-bound radioactivity by ether. In the ether precipitate (fig. 3D) all radioactivity was present in the corresponding protein band. An apparent molecular weight for the DCCD-binding protein of 11,000 daltons was determined (molecular weights reported in ref. 6, 10,000 daltons; ref. 5, 13,000-14,000 daltons).

DISCUSSION:

In various membrane systems DCCD is reported to restore impared respiratory-linked functions (17-19), presumably by decreasing proton permeability. The identification of a DCCD-binding protein as a proteolipid (5) and the possible involvement of the sarcoplasmic reticulum proteolipid in transmembrane ion translocation (4) emphasize the significance of this protein fraction. To further study the functional and structural aspects of this class of membrane proteins an alternative isolation method was developed.

The proteolipid isolation procedure described here presents the advantages of simplicity combined with rapidity and purity of the isolated fractions. Other procedures reported for proteolipid extraction from either sarcoplasmic reticulum membranes (2-4) or submitochondrial particles (5,6) involve more laborious techniques and longer preparation times. n-Butanol, a weakly protic nonaqueous solvent was used to effect rapid precipitation of all globular proteins, extracting the proteolipids and lipids into the organic phase. The use of a single-phase extraction additionally avoids the problems of the classical two-phase systems in that denaturated interfacial protein is absent.

Conformation and resulting functional properties of highly hydrophobic complexes such as the proteolipids described here may be critically dependent upon associations with a nonaqueous environment. Since the present extraction procedure avoids extensive exposure of the proteolipid to an aqueous phase, the functional conformation may be retained to a greater extent than by other treatments. Concentrated butanol supernatant (step I) can be used to form injected liposomes (20) composed of endogenous lipid and proteolipid. Indeed, preliminary studies by the present authors utilizing this technique indicate that

the proteolipid from sarcoplasmic reticulum does possess ionophoric activity. Further investigations on the butanol extracted proteolipid may help to elucidate the conflicting data reported for the ionophoric activity of the sarcoplasmic reticulum proteolipid (2,4,21)

Finally, the general applicability of the proteolipid isolation technique has been further demonstrated by the successful isolation of a proteolipid fraction from chloroplast membranes (N. Nelson, personal communication).

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