FORMATION OF DECANE-SOLUBLE PROTEOLIPIDS: INFLUENCE OF MONOVALENT AND DIVALENT CATIONS

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1. Introduction

In a previous communication [1] we attempted to establish in model systems the principles which govern the formation of decane-soluble proteolipids. We found that cytochrome *c* and a mixture of acidic and neutral phospholipids could not be extracted into decane unless the protein carboxylates were neutralized by protonation. Thus nearly quantitative extraction of cytochrome *c* and a mixture of phosphatidylcholine and phosphatidylserine, phosphatidylinositol or cardiolipin could be achieved by lowering the aqueous phase pH to 2.4. It was theorized that the acidic phospholipids formed ion-pairs with the protein cationic residues and when the protein carboxylates were neutralized, the overall complex became hydrophobic and could then partition into the apolar solvent. A similar pH dependent extraction of beef heart cytochrome oxidase and bovine erythrocyte acetylcholinesterase was noted.

One of the limitations in this method of proteolipid formation was the possible denaturation of the proteins due to the required low pH. In order to overcome this problem, we attempted to extract the proteolipid in the presence of cations which, at neutral pH, might be able to neutralize the protein carboxyl moieties. In the present communication we show that cytochrome *c*-containing proteolipids can be formed readily in the presence of $CaCl_2$, $MgCl_2$, LiCl, KCl or NaCl. The proteolipid thus extracted forms stable bilayers whose electrical properties are similar to those of the protonated proteolipid but significantly different from those formed with the component lipids alone.

2. Methods and materials

The procedure used to prepare the decane-soluble proteolipids, the assay of protein and phospholipid content in both aqueous and hydrocarbon phases, as well as the method of thin-proteolipid film formation were essentially as previously described [1].

3. Results

3.1. *Formation of decane-soluble proteolipids at neutral pH*

No partition of cytochrome *c* or lipids into n-decane could be observed at neutral pH unless monovalent or divalent cations were present in the aqueous phase during the extraction step. As presented in table 1, the extent of extraction of cytochrome *c*-containing proteolipid is dependent on the nature and concentration of the cations. Complete extraction (up to 96% of both lipid and protein) is achieved with 2.0 mM $CaCl_2$, 10 mM $MgCl_2$, 100 mM KCl or LiCl and 200 mM of NaCl; for comparison it is also shown that 4 mM of H^+ is required for maximal extraction. It is worth pointing out that the concentrations of cations required for proteolipid extraction are in the physiological range. It should be noted that while the decane phase is optically clear in the case of Ca^{2+}, Mg^{2+}, Li^+ or H^+, it is turbid when the extraction is performed in the presence of Na^+ or K^+. A possible explanation for this difference in optical appearance is a higher concomitant extraction of water into the decane. The water content of the

Table 1
Effect of monovalent and divalent cations on proteolipid extraction into n-decane

Cation	Conc. (mM)	Protein extracted into n-decane (%)	Phospholipid extracted into n-decane (%)	Relative effectiveness
	25	0	70	
	50	74	75.7	
Na^+	100	81	66.1	1
	150	82	58.4	
	200	93	95.5	
	50	92	80.4	
Li^+	100	93	95.0	2
	200	92	98.0	
	25	63	83.8	
K^+	50	89.5	97.0	2
	100	93.4	97.8	
	0.5	0	48.2	
	1.0	56.4	61.3	
Mg^{2+}	2.0	75.4	74.0	20
	5.0	93.38	98.5	
	10.0	96.4	98.5	
	0.1	0	25.7	
Ca^{2+}	0.5	0	38.7	100
	1.0	64.1	67.6	
	2.0	95.3	96.7	
$H^{+(*)}$	4.0	94.0	92.7	50

Phosphatidylserine (1 mg/ml) and phosphatidylcholine (2 mg/ml) were dispersed in a final volume of 4.0 ml of water by shaking in a Vortex mixer for 10 min. Cytochrome *c* (0.125 mg/ml) was then added and shaking was continued for a further 10 min period. n-Decane (1.0 or 2.0 ml) was then carefully layered on top of the aqueous dispersion and 0.5 ml of an aqueous buffer or salt solution was rapidly introduced and the two-phase system was immediately shaken for a 15 min period. Phase separation was allowed to occur; the tubes were centrifuged for 10 min at 600 *g* in a refrigerated MSE centrifuge. The uppermost decane phase was carefully removed; the lower decane phase and a portion of the upper aqueous layer were discarded. The remaining aqueous phase was used to determine protein and phosphorous as previously reported [1].

* Data taken from the published results [1].

Table 2
Comparison of the electrical properties of thin proteolipid films prepared in the presence of H^+ or Ca^{2+} ‡.

Electrical property	Thin proteolipid films H^+ in extraction*	Ca^{2+} in extraction+
Membrane resistance (ohm · cm^2 × 10^8)	0.5 –1.0	0.5 –1.0
Membrane capacity** ($\mu F/cm^2$)	0.29 ± 0.05	0.31 ± 0.03

Bathing solution: 1 mM NaCl and 1 mM TEA-Cl, pH 7.0.
*pH of aqueous phase = 2.4.
+2 mM $CaCl_2$ in aqueous phase.
‡The membrane forming solution consisted of: the material extracted into 1.0 ml of n-decane after shaking this solvent with an aqueous phase containing 8 mg egg lecithin, 4 mg of phosphatidylserine and 0.5 mg of cytochrome *c* in a final volume of 4.0 ml adjusted at pH 2.4 with formate buffer or in the presence of 2 mM $CaCl_2$.
**In the determination of the membrane capacity, care was exercised to maintain the film area constant. The published values [1] for membrane capacity are high because of an underestimation of the membrane area. Dividing the reported values [1] by a correction factor of 1.5, one arrives at the correct values.

decane containing the different proteolipids is, at present, under study.

3.2. *Formation of thin proteolipid films*

The proteolipid solution in decane is used to prepare thin films as previously described [1]. A comparison of the electrical properties of thin proteolipid films prepared from an extract carried out in the presence of H^+ or Ca^{2+} is illustrated in table 2. No significant differences in either membrane resistance or capacity are measured, indicating that a similar structure is responsible for the observed responses and it is therefore independent of the way by which charge neutralization and ion pair extraction is achieved.

4. Discussion

In 1951 Folch and Lees [2] reported the isolation from brain tissue of a proteinaceous material insoluble

in aqueous solvents and soluble in mixtures of chloroform: methanol. They coined the name "*proteolipid*" to stress the fact that even though they were lipid–protein complexes they exhibited some of the properties of lipid, such as solubility, in contrast to the "*lipoproteins*" which behave as water soluble proteins. Since then, proteolipids have been identified in all the tissues where they have been looked for and its localization is referred to the membranes of organelles such as mitochondria and chloroplasts (cf. [3]).

The attempts to design model systems to understand the structure, properties and function of this membrane fraction have been scarce but significant. Das and Crane [4], Reich and Wainio [5] and Leslie et al. [6, 7] have studied the cytochrome *c* proteolipid; Lesslauer et al. [8] that of the basic protein of myelin; DeRobertis et al. [9–11] the proteolipids associated to the acetylcholine and norepinephine receptors; Beechey et al. [12, 13] that associated to the mitochondrial ATPase system and Strominger et al. [14] that of the isoprenoid phosphokinase system associated to the cell wall synthesis in *Staphylococcus aureus.*

At the realm of these findings lies the fundamental problem of the nature of lipid–protein interactions. Our model studies are directed towards the establishment of the elementary physical principles underlying the formation of the proteolipid and its topography in the membrane.

The present results indicate that proteolipids soluble in decane may be formed if the conditions leading to overall charge neutralization of the complex are met, the net extraction being independent of the nature of the ionic species used to achieve it. In addition, they indicate that ion-pairs may occur in the apolar regions of membranes. This suggestion is also supported by the evidence of Packter and Donbrow [15] that the association constant, and therefore the free energy, of ion pair formation in water increases dramatically when both anion (sulfate) and cation (trimethylammonium) possess a long hydrocarbon chain of 12 carbon atoms ($-\Delta G$ association = 8.4 Kcal/mole) as compared to those with chains of 6 carbon atoms ($-\Delta G$ association = < 1 Kcal/mole).

Important implications from this model system with regards to membrane dynamics and plasticity can be derived. The fact that physiological ions allow the extraction of proteolipids into n-decane, suggests that they may induce neutralization of the residual charges of a lipid–protein complex in a membrane and hence allow its penetration into the apolar core of the bilayer. It is well known that an alteration of the ratio of univalent/divalent cations in favor of the divalent results in a phase transition from an "oil in water" state to a "water in oil" state [16]. Taking into consideration that the recombination rates of ion-pairs in liquids of low dielectric constant are in the nanosecond time range [17], such transitions (lipoprotein → proteolipid) in location of the lipid–protein complex could be extremely fast. This would be in line with the reported rotational relaxation rates of membranous lipid–protein complexes, such as rhodopsin in the retinal rods [18, 19] and cytochrome oxidase in the mitochondria [20]. It is possible to envisage a mechanism based on the results hitherto reported by which hydrocarbon-soluble complexes between aqueous solutes and membrane elements could be formed. This would allow water soluble solutes to diffuse across the liquid hydrocarbon chains of the bilayer phospholipids [21], a process which is normally energetically unfavorable [22]. This mechanism is conceptually similar to that underlying the solubilization of ions in hydrocarbons mediated by the cyclic macrolide and peptide antibiotics (neutral lipid soluble molecules) [22] in which the substitution of the hydration shell of the ion by the polar groups of the peptide antibiotic renders the overall complex lipid soluble and allows its diffusion across biological and artificial membranes [23]. The detailed aspects of this proposal will be published elsewhere.

Finally, the fact that thin proteolipid films are formed with the decane-soluble proteolipid extracted at neutral pH in the presence of physiological concentrations of cations offers a starting point towards the reconstitution of complex membrane functions.

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