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Probing the extended lipid anchorage with cytochrome *c* and liposomes containing diacylphosphatidylglycerol lipids

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ABSTRACT

Experiments investigating the adsorption and desorption of cytochrome *c* onto and from liposomes containing 50 mol% 1,2-diacylphosphatidylglycerol lipids [10:0, 12:0, 14:0, 16:0, 18:1(Δ9 cis)] with 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) in pH 7.4 buffered solutions of low to moderate ionic strength are reported. Fluorescence experiments show that cytochrome *c* has a similar adsorption affinity for the five labeled 50 mol% PG liposome systems investigated. Fluorescence recovery experiments reveal the extent of cytochrome *c* desorption upon the addition of > 10× excess of unlabeled 100% 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG) liposomes is dependent on the lipid's acyl chain length. The extent of desorption is also shown to be independent of temperature, albeit over a narrow range. The differences in the extent of cytochrome *c* desorption from liposomes containing PG lipids with different acyl chain lengths is attributed to the varying contribution of the binding motif involving the extended lipid anchorage in response to lipid packing stress.

1. Introduction

Cytochrome *c* (cyt *c*) is a highly conserved, highly water soluble, heme-centered protein with a molar mass of 12,400 Da and a +8 charge at physiological pH [1]. Cyt *c* is one of the essential molecules in the electron transport chain contained in the inner mitochondria membrane by transferring electrons from cyt *c* reductase to cyt *c* oxidase where the electrons are used to convert oxygen to water and pump protons out of the mitochondrial matrix [2]. In the cytosol, cyt *c* is incorporated in the apoptosome complex facilitating the activation of caspases leading to cell death [3]. For this work, cyt *c* is the prototypical example of a peripheral membrane protein having been the focus of numerous studies examining its interactions with membranes and model membrane structures [4].

With its large positive charge, cyt *c* had been thought to simply bind reversibly to deprotonated acidic lipid domains though electrostatic interactions. However, it has been reported that the protein-lipid interaction can withstand gel permeation chromatography, ultracentrifugation, and centrifugal concentration [5–7]. Also, cyt *c* has been shown to undergo conformational changes upon adsorption onto membrane surfaces and a preferred orientation [8–14]. Consequently a second mode of protein-lipid binding, involving an irreversible hydrophobic interaction between cyt *c* and phospholipids, is also thought to be important [5,6,15,16].

To explain the hydrophobic interaction of cyt *c* with membrane bound lipids, Kinnunen and co-workers have proposed that acidic phospholipids, such as phosphatidylglycerol or cardiolipin, have the ability to adopt an extended conformation and anchor cyt *c* to the membrane [17–21]. As shown in Fig. 1, acidic phospholipids in the outer layer of a membrane in an extended conformation undergo a gauche to antiperiplanar conformational change to allow the non-polar tails of a lipid to extend in opposite directions. This conformation change removes an acyl chain from the membrane's outer layer reducing phospholipid packing stress. The formation of the extended conformation is favored by frayed phospholipids that have a propensity to adopt an inverted hexagonal phase (H_{II}) and occurs when a membrane-bound peripheral protein presents a hydrophobic grove or channel to accept the extended acyl chain.

In a more recent and comprehensive study, Kalanxhi and Wallace have studied the dynamics of the cyt *c* binding process and evaluated the effects of ionic strength, pH, liposome size and composition, and the inclusion of nucleotides and various metabolites on the extended lipid anchorage [22]. They proposed a mechanism involving a fast and reversible electrostatic interaction between cyt *c* and a lipid membrane followed conformational changes in both the protein and lipid leading to insertion of an acyl chain into cyt *c* occurring on a much longer time scale.

In our own work with three *N*-acylated phosphatidylethanolamines

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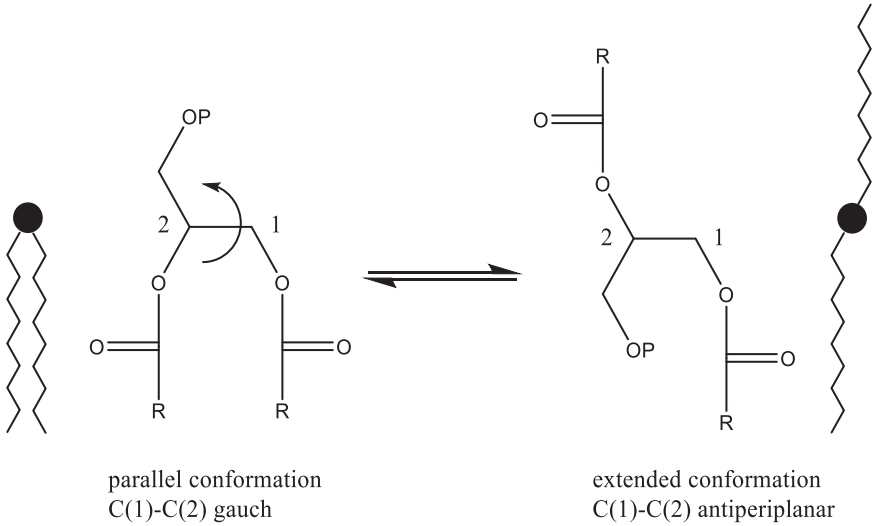

Fig. 1. A schematic representation of the conformation change experienced by normal diacyl phospholipids leading to the extended lipid conformation.

with *N*-acyl chains of differing lengths, we have shown that alterations at the head group interface that lead to an expansion of the lipids within a layer and a reduction in packing stress lead to a decrease in the fraction of cyt *c* bound through the extended lipid anchorage [23].

The extended lipid anchorage is potentially a universal mechanism for peripheral membrane protein-lipid interactions as well as vesicle fusion, but the phospholipid structural scope and the physiological relevance of this mode of interaction remains to be broadly tested. Interestingly, recent studies have shown that the loss of the Fe—S(Met) bond in cyt *c*, alters the Fe redox potential making oxidation under aerobic conditions possible [24]. It is not known how this structural switch is triggered under biological conditions, but a membrane-induced interaction seems an intuitive possibility.

In this work, we describe the interactions of cyt *c* with a series of liposomes containing 1,2-diacylphosphatidylglycerol lipids with different acyl chain lengths under conditions of low to moderate ionic strength. We find that the extent of association of cyt *c* to the 50 mol% PG containing liposomes is similar for all the diacylphosphatidylglycerol lipids investigated but the relative contributions of the electrostatic and hydrophobic binding modes vary with the phospholipid acyl chain length as indicated by the fraction of irreversibly bound cyt *c*.

2. Materials and methods

1,2-Dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) [DOPG], 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) [DPPG], 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) [DMPG], 1,2-dilauroyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) [DLPG], 1,2-didecanoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) [PG 10/10] and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine [DOPC] were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. The β-pyrene-C10-PG fluorescent label (1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol ammonium salt) was purchased from Invitrogen (Carlsbad, CA) and used as received. Beef heart cytochrome *c* was purchased from Acros Organics USA (Morris Plains, NJ) and used as received. HEPES (99.5%) was purchased from Sigma Aldrich (St Louis, MO) and all buffers were 20 mM and adjusted to pH 7.4 with concentrated sulfuric acid and contain 0.1 mM EDTA.

3. Liposome preparation

Stock solutions (2 mg/mL) of each lipid were prepared in $CHCl_3$ or $CHCl_3$:MeOH:H_2O (65:35:8) as recommended by Avanti Polar Lipids. Labeled liposomes are 50 mol% diacylphosphatidylglycerol with the remainder DOPC and the β-pyrene-C10-PG label at a level of 0.1 mol%. The CH_3Cl or CH_3Cl:MeOH:H_20 was removed following placement for 1 h in a room temperature vacuum jar. The lipids were hydrated in 0.01 M HEPES buffer for 30 min at 30 °C but were removed two times for 30 s of agitation in an ultrasonic bath. Liposomes were formed by 21 passes through a track-etched polycarbonate membrane (0.1 μm dia.) in a mini-extruder (Avanti Polar Lipids, Alabaster, AL) at room temperature except liposomes containing dipalmitoyl lipids that were extruded at 51 °C. The lipid suspensions were diluted as needed using the HEPES buffer and used within 24 h after preparation.

Representative liposome samples were sized using a Viscotek DLS instrument (Malvern Instruments, Malvern, Worcestershire, UK) and found to have a prominent scattering peak indicating an average radius in the range of 103 to 140 nm.

4. Fluorescence experiments

All fluorescence experiments were conducted in a manner similar to those of Kinnunen and co-workers using a FluorMax-3 spectrofluorimeter (HORIBA Jobin Yvon, Edison, NJ) equipped with a Neslab RTE 7 refrigerated bath (Thermo Scientific, Newington, NH) [18,25,26]. The β-pyrene-C10-PG label was excited (λ_{ex}) at 344 nm, and the emission was monitored (λ_{em}) at 398 nm. The excitation slit width was 1 nm and the emission slit width was 16 nm. All data reported is the average of two or more replicate experiments.

4.1. Cyt c adsorption experiments

A 3.00 mL aliquot of a 50 mol% PG labeled vesicle suspension was transferred with a stirring bar to a cuvette at 23 °C and allowed to thermally equilibrate for 10 min. An initial measurement of the fluorescence intensity was recorded. Eight aliquots of cyt *c* were sequentially added to the sample the fluorescence intensity was recorded after 5 min of gentle stirring. The observed decrease in the fluorescence intensity is a result of the quenching of the lipophilic pyrene label by the heme in cyt *c*.

4.2. Cyt c desorption experiments

A 3.00 mL aliquot of the 50 mol% PG labeled vesicle suspension was transferred with a stirring bar to a cuvette and allowed to thermally equilibrate for 10 min. After the initial measurement of the fluorescence intensity was recorded (F_0), a 6.0 μL aliquot of a 2.0 mg/mL solution of