Quantification of Protein-Lipid Selectivity using FRET: Application to the M13 Major Coat Protein

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ABSTRACT Quantification of lipid selectivity by membrane proteins has been previously addressed mainly from electron spin resonance studies. We present here a new methodology for quantification of protein-lipid selectivity based on fluorescence resonance energy transfer. A mutant of M13 major coat protein was labeled with 7-diethylamino-3-(4’-iodoacetyl)amino)phenyl-4-methylcoumarin to be used as the donor in energy transfer studies. Phospholipids labeled with N-(7-nitro-2-1,3-ben佐xadiazol-4-yl) were selected as the acceptors. The dependence of protein-lipid selectivity on both hydrophobic mismatch and headgroup family was determined. M13 major coat protein exhibited larger selectivity toward phospholipids which allow for a better hydrophobic matching. Increased selectivity was also observed for anionic phospholipids and the relative association constants agreed with the ones already presented in the literature and obtained through electron spin resonance studies. This result led us to conclude that fluorescence resonance energy transfer is a promising methodology in protein-lipid selectivity studies.

INTRODUCTION

For integral membrane proteins, the interaction with lipids is dependent, among other factors, on the hydrophobic segments and interface properties of both lipids and protein. Minimal bilayer perturbation is achieved when the hydrophobic length of the protein matches that of the surrounding lipid (Mouritsen and Bloom, 1984). Deviations from perfect hydrophobic matching at the protein-lipid interface create a tension arising from the exposure of hydrophobic residues or acyl-chains to the hydrophilic medium. It is considered that the protein-lipid system adapts to hydrophobic mismatch conditions through several alternative and nonexclusive strategies such as ordering or disordering of perturbed phospholipids (change in bilayer thickness), lipid phase transition to nonlamellar phases, protein oligomerization/aggregation (minimization of interface area), helices tilting or side-chain rotation of a helical terminal residue (reduction in effective hydrophobic length), change in protein orientation, and decrease in the bilayer partitioning of the protein (for reviews see Killian, 1998; Dumas et al., 1999).

In the case of proteins incorporated in lipid systems containing lipids with different electrostatic properties or hydrophobic lengths, selectivity to one lipid component at the protein-lipid interface or preferential phase partitioning (depending on the lipid miscibility) may occur (Dumas et al., 1997, Lehtonen and Kinnunen, 1997; Fahsel et al., 2002).

In this study, we focused on the process of lipid selectivity at the protein-lipid interface. The problem of protein-lipid selectivity quantification has been addressed mainly from electron spin resonance (ESR) studies (see Marsh and Horváth, 1998, for a review) or other techniques which focus only on the protein-lipid interface, like tryptophan fluorescence quenching by brominated phospholipids (Everett et al., 1986; Williamson et al., 2002; O’Keeffe et al., 2000). The results obtained from ESR studies agree well with an annular model for protein-lipid selectivity, in which only the first shell of lipids around the integral protein, and in direct contact with it, is significantly disturbed by the protein incorporation in the bilayer (Lee, 2003; Marsh and Horváth, 1998).

ESR results report the fraction of motionally restricted lipids, whereas fluorescence collisional quenching depends on molecular contact. On the other hand, fluorescence resonance energy transfer (FRET) only depends on donor-acceptor distances and is an alternative technique to quantify lipid selectivity. Gutierrez-Merino derived approximate analytical expressions for the average rate of FRET ($k_{\text{FRET}}$) in membranes undergoing phase separation or protein aggregation (Gutierrez-Merino, 1981a,b) and extended this formalism to the study of protein-lipid selectivity (Gutierrez-Merino et al., 1987). His model has proved to be useful to the study of the lipid annulus around the oligomeric acetylcholine receptor (Bonini et al., 2002; Antollini et al., 1996). However, there are some limitations to the model, namely, the simplification that underlies the formalism, which consists of considering resonance energy transfer (RET) only to neighboring acceptor molecules. On the other hand, with the experimental observable being the average RET efficiency given by...
where $k_D$ is the donor intrinsic decay rate coefficient, the relation with $(k_T)$ is not straightforward. It is proposed that if the setting of experimental conditions is such that $(E)$ is low (namely, $(k_T)$ is much smaller than $k_D$), then $(E) \approx (k_T)/k_D$ (Gutierrez-Merino, 1981a). However, accurate low RET efficiencies are difficult to measure experimentally.

In the present work a new FRET formalism for an annular model of protein-lipid selectivity is proposed, and used in the quantification of M13 major coat protein selectivity toward different phospholipids. M13 major coat protein is the main protein component of the filamentous bacteriophage M13 with $\sim 2800$ copies. It contains a single hydrophobic transmembrane segment of $\sim 20$ amino-acid residues, apart from an amphipathic N-terminal arm and a heavily basic C-terminus with a high density of lysines (for reviews see Stopar et al., 2003; Hemminga et al., 1993).

The present study is separated in two sections. The first section focuses on the effect of hydrophobic length, and the selectivity of M13 toward 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) ((18:1) 2-PE-NBD) was determined in unsaturated phosphatidyldicholine bilayers of different acyl chain lengths (14:1, 18:1, and 22:1). Whereas for 18:1 chains the chain length matches the hydrophobic length of the protein, there is significant hydrophobic mismatch for the other lipids used. The second part deals with specificity of M13 major coat protein to different phospholipid headgroups, some zwitterionic and other negatively charged. The results are compared to the results from the other methodologies for quantification of protein-lipid selectivity. Conclusions on the validity of the annular model for the M13 coat protein interaction with lipids are obtained.

### MATERIALS AND METHODS

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC; (18:1)-PC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DMPC; (14:1)-PC), 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) ((18:1)-PE-NBD), 1-Oleoyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanooyl-sn-Glycero-Phosphocholine (18:1-(12:0-NBD)-PC), 1-Oleoyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-Glycero-Phosphoethanolamine (18:1-(12:0-NBD)-PE), 1-Oleoyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-Glycero-Phosphoserine (18:1-(12:0-NBD)-PS) (Sodium salt), 1-Oleoyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-Glycero-Phosphate (18:1-(12:0-NBD)-PA) (Monosodium salt), and 1-Oleoyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-Glycero-3-[3-phospho-rac-(1-glycerol)] (18:1-(12:0-NBD)-PG) (Sodium salt), were obtained from Avanti Polar Lipids (Birmingham, AL). 7-diethylamino-3-(4’iodoacetyl)amino)phenyl-4-methylcoumarin (DCIA) was from Molecular Probes (Eugene, OR). Fine chemicals were obtained from Merck (Darmstadt, Germany). All materials were used without further purification.

### Coat protein isolation and labeling

The T36C mutant of the M13 major coat protein was grown, purified from the phage and labeled with DCIA as described previously (Spruijt et al., 1996). For the removal of free label, DNA and other coat proteins, the mixture was applied to a Superdex 75 prep-grade HR 16/50 column (Pharmacia, Amersham Biosciences, Piscataway, NJ) and eluted with 50 mM sodium cholate, 150 mM NaCl, and 10 mM Tris-HCl pH 8. Fractions with an $A_{280}/A_{260}$ absorption ratio $>1.5$ were collected and concentrated by Amicon filtration (Amicon, Millipore, Bedford, MA).

### Coat protein reconstitution in lipid vesicles

The labeled protein mutant was reconstituted in DOPC ((18:1)-PC), DMoPC ((14:1)-PC), and DeoPC ((22:1)-PC) vesicles using the cholate-dialysis method (Spruijt et al., 1989). The phospholipid vesicles were produced as follows: the chloroform from solutions containing the desired NBD labeled and unlabeled phospholipid amount was evaporated under a stream of dry $N_2$, and last traces were removed by further evaporation under vacuum. The lipids were then solubilized in 50 mM sodium cholate buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA) at pH 8 by brief sonication (Branson 250 cell disruptor, Branson Ultrasonics, Danbury, CT) until a clear opalescent solution was obtained, and then mixed with the wild-type and labeled protein. Samples had a phospholipid concentration between 0.5 and 1 mM (phospholipid concentration was determined through the analysis of inorganic phosphate according to McClare, 1971) and the lipid to protein ratio ($L/P$) was always kept at 700. Dialysis was carried out at room temperature and in the dark, with a 100-fold excess buffer containing 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA at pH 8. The buffer was replaced five times every 12 h.

### Fluorescence spectroscopy

Absorption spectroscopy was carried out with a Jasco V-560 spectrophotometer (Tokyo, Japan). The absorption of the samples was kept $<$0.1 at the wavelength used for excitation.

Steady-state fluorescence measurements were obtained with an SLM-Aminco 8100 Series 2 spectrophotometer (Rochester, NY; with double excitation and emission monochromators, MC400) in a right-angle geometry. The light source was a 450-W Xe arc lamp and for reference a Rhodamine B quantum counter solution was used. $5 \times 5$ mm quartz cuvettes were used. All measurements were performed at room temperature.

The quantum yield of DCIA-labeled protein was determined using quinine bisulfate dissolved in 1 N $H_2SO_4$ ($\phi = 0.55$; Eaton, 1988) as a reference.

Fluorescence decay measurements of DCIA were carried out with a time-correlated single-photon timing system, which is described elsewhere (Loura et al., 2000). Measurements were performed at room temperature. Excitation and emission wavelengths were 340 and 450 nm, respectively. The timescales used were between 3 and 12 ps/ch, depending on the amount of NBD-labeled phospholipid present in the sample. Data analysis was carried out using a nonlinear, least-squares iterative convolution method based on the Marquardt algorithm (Marquardt, 1963). The goodness of the fit was judged from the experimental $\chi^2$ value, weighted residuals, and autocorrelation plot.

In all cases, the probe fluorescence decay was complex and described by a sum of exponentials,

$$I(t) = \sum_i a_i \exp(-t/\tau_i),$$

where $a_i$ are the normalized amplitudes and $\tau_i$ are the fluorescence lifetimes.
THEORETICAL BACKGROUND

Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) can be used to characterize the lateral distribution of labeled coat protein mutants in the bilayer. In the case of energy heterotransfer, the degree of fluorescence emission quenching of the donor caused by the presence of acceptors is used to calculate the experimental energy transfer efficiency \( E \):

\[
E = 1 - \frac{\tau_{DA}}{\tau_D}.
\]  (3)

Here \( \tau_{DA} \) is the donor lifetime-weighted quantum yield in the presence of acceptor and \( \tau_D \) is the donor lifetime-weighted quantum yield in the absence of acceptor. In turn, lifetime-weighted quantum yields are defined by Lakowicz (1999) as

\[
\tau = \sum_i \alpha_i \tau_i.
\]  (4)

The Förster radius is given by

\[
R_0 = 0.2108 (J \kappa^2 n^{-4} \phi_D)^{1/6},
\]  (5)

where \( J \) is the spectral overlap integral, \( \kappa^2 \) is the orientation factor, \( n \) is the refractive index of the medium, and \( \phi_D \) is the donor quantum yield. \( J \) is calculated as

\[
J = \int f(\lambda) \varepsilon(\lambda) \lambda^4 d\lambda,
\]  (6)

where \( f(\lambda) \) is the normalized emission spectrum of the donor and \( \varepsilon(\lambda) \) is the absorption spectrum of the acceptor. If the \( \lambda \)-units in Eq. 6 are nm, the calculated \( R_0 \) in Eq. 5 has \( \AA \) units (Berberan-Santos and Prieto, 1987).

Annular model for M13 coat protein selectivity toward phospholipids

To analyze the FRET results, a model for transmembrane protein selectivity toward phospholipids was derived. The model assumes two populations of energy transfer acceptors, one located in the annular shell around the protein and the other outside it. The donor fluorescence decay curve will have energy transfer contributions from both populations,

\[
i_{DA}(t) = i_D(t) \rho_{\text{annular}}(t) \rho_{\text{random}}(t).
\]  (7)

Here \( i_D \) and \( i_{DA} \) are the donor fluorescence decay in the absence and presence of acceptors respectively, and \( \rho_{\text{annular}} \) and \( \rho_{\text{random}} \) are the FRET contributions arising from energy transfer to annular labeled lipids and to randomly distributed labeled lipids outside the annular shell, respectively.

The acceptors in the annular shell (Fig. 1) are at a constant distance \( d \) to the coumarin fluorophore located in the center of the transmembrane domain, and therefore we can assume that the energy transfer to each of these acceptors is described by the rate constant

\[
k_i = \frac{1}{\tau_D} \left( \frac{R_0}{d} \right)^6,
\]  (8)

where \( \tau_D \) is the donor lifetime (in the absence of acceptor). The NBD fluorophores in the acceptor probes used in this study (phospholipids labeled with NBD in the headgroup or in the acyl-chain) are assumed to be located in the bilayer surface. For the chain-labeled lipids, this is justified because the NBD group “loops up” to the surface when attached to the end of the phospholipids acyl-chain (Chattopadhyay, 1990). The donor fluorophore is labeled in the M13 major coat protein 36th residue, located near the center of the bilayer (Spruijt et al., 1996). Therefore, to calculate \( d \) it is necessary to estimate the average distance \( l \) between the donor plane (center of bilayer) and the acceptors planes (both leaflets), as well as the lateral separation between both probes inside the transmembrane protein-annular shell lipids complex. For DOPC bilayers the position of the NBD fluorophore \( l \) in the derivatized phospholipids has been calculated through the parallax method (Abrams and London, 1993), and it was 18.9 and 19.8 \( \AA \) from the bilayer center for the phospholipids labeled at the headgroup and at the acyl-chain, respectively. These values agree with other studies which employed different techniques to obtain the fluorophore position (Wolf et al., 1992; Mázeres et al., 1996). The reason for a position of NBD closer to the surface of the membrane while labeled at the acyl-chain is probably the increase in flexibility that the C12 chain allows. The

FIGURE 1 Molecular model for the FRET analysis ((A) side view; (B) top view). Protein-lipid organization presents a hexagonal geometry. Donor fluorophore from the mutant protein is located in the center of the bilayer, whereas the acceptors are distributed in the bilayer surface. Two different environments are available for the labeled lipids (acceptors), the annular shell surrounding the protein and the bulk lipid. Energy transfer to acceptors in direct contact with the protein has a rate coefficient dependent on the distance between donor and annular acceptor (Eq. 8). Energy transfer toward acceptors in the bulk lipid is given by Eq. 11 (see text for details).
lateral separation between the probes was assumed to be 8 Å. The estimated \( d \) was then 20.5 and 21.4 Å for NBD derivatized in the headgroup and acyl-chain, respectively. For the DMoPC and DEuPC bilayers the values used for \( l \) were 15.4 and 22.4 Å, respectively, as for each additional carbon in the phospholipid chain in the liquid crystalline phase the bilayer thickness increases 1.75 Å (Lewis and Engelman, 1983).

Considering a hexagonal type geometry for the protein-lipid arrangement (Fig. 1b), each protein will be surrounded by 12 annular lipids. In bilayers composed by both labeled and unlabeled phospholipids, these 12 sites will be available for both of them. The probability (\( \mu \)) of one of these sites to be occupied by labeled phospholipid is given by

\[
\mu = K_S \frac{n_{\text{NBD}}}{n_{\text{NBD}} + n_{\text{lipid}}}.
\]

Here, \( n_{\text{NBD}} \) is the concentration of labeled lipid, and \( n_{\text{lipid}} \) is the concentration of unlabeled lipid. \( K_S \) is the relative association constant, which reports the relative affinity of the labeled and unlabeled phospholipid. Using a binomial distribution we can calculate the probability of each occupation number (0–12 sites occupied simultaneously by labeled lipid), and finally the FRET contribution arising from energy transfer to annular lipids,

\[
\rho_{\text{annular}}(t) = \sum_{n=0}^{n=12} e^{-nk_2} \binom{12}{n} \mu^n (1 - \mu)^{12-n}.
\]

The FRET contribution from energy transfer to acceptors randomly distributed outside the annular region in two different planes at the same distance to the donor plane (from the center of the bilayer to both leaflets) is given by Davenport et al. (1985) as

\[
\rho_{\text{random}} = \exp\left\{ -4n_2 \pi l^2 \int_0^{R_c} \frac{1 - \exp(-tb^3 \alpha^6)}{\alpha^4} d\alpha \right\},
\]

where \( b = (R_0^2/l)^{2/3} \), \( n_2 \) is the acceptor density in each leaflet, \( l \) is the distance between the plane of the donors and the planes of acceptors, and \( R_c \) is the distance between the protein axis and the second lipid layer (exclusion distance for bulk-located acceptors). In the present system, \( l \) is the unlabeled lipid bilayer thickness, and the exclusion distance is 16 Å assuming a radii of 5 Å and 4.5 Å for the protein and the phospholipid, respectively; see Fig. 1b). The value \( n_2 \) must be corrected for the presence of labeled lipid in the annular region, which therefore is not part of the randomly distributed acceptors pool.

**RESULTS**

**M13 coat protein selectivity toward phospholipids with different hydrophobic thickness**

The DCIA-labeled protein quantum yield was determined (\( \phi = 0.41 \)). Using Eqs. 5 and 6, and assuming \( \kappa^2 = 2/3 \) (the isotropic dynamic limit) and \( n = 1.4 \) (Davenport et al., 1985), \( R_0 = 39.3 \) Å is obtained for the DCIA-NBD FRET pair (Fig. 2). The value \( \kappa^2 = 2/3 \) was used, because for fluorophores in the center of a liquid crystalline bilayer, the rotational freedom should be sufficiently high to randomize orientations (for a detailed discussion see Loura et al., 1996).

FRET selectivity studies were performed in bilayers of one lipid component (DOPC, DMoPC, or DEuPC) using T36C M13 major coat protein mutant labeled with DCIA as the donor and (18:1)2-PE-NBD (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine derivatized with NBD at the headgroup) as the acceptor.

The donor fluorescence intensities ratio (\( \tau_{DA}/\tau_D \)), which is related to the energy transfer efficiency, decreases upon increasing the acceptor (Eq. 3). The results are presented in Fig. 3. The results of fitting the derived formalism to the data are also shown in this figure, and the corresponding \( K_S \) values are summarized in Table 1.

**M13 coat protein selectivity toward phospholipids with different headgroups**

Energy transfer studies were also performed to determine the selectivity properties of M13 coat protein toward phospholipids with different headgroups. Again, the donor was T36C coat protein mutant labeled with coumarin (DCIA), but various probes were used as acceptors, all studies being made in DOPC vesicles. The probes used as acceptors were
phospholipids of identical acyl-chains (18:1 and 12:0) and different headgroups (PC, PE, PS, PG, and PA) classes, derivatized with NBD at the 12:0 chain. The results are shown in Fig. 4, together with the model fits. Table 1 summarizes the recovered $K_S$ values.

**DISCUSSION**

**M13 coat protein selectivity toward phospholipids with different hydrophobic thickness**

The M13 coat protein is known to form large irreversible aggregates under specific conditions. These aggregates are not found in vivo, and therefore are regarded as an artifact (Hemminga et al., 1993). Due to their β-sheet conformation, they are detected by CD spectroscopy, and because of the hydrophobic mismatch between the protein and DEuPC (longer) or DMoPC (shorter) lipids, it was possible that while incorporated in pure bilayers of these components, the M13 coat protein could be submitted to irreversible aggregation. This hypothesis has been ruled out for both lipids in recent studies (Meijer et al., 2001; Fernandes et al., 2003). Nevertheless, M13 major coat protein was shown recently by us to aggregate reversibly while in these con-

**TABLE 1** Labeled phospholipids' relative association constants toward M13 major coat protein

<table>
<thead>
<tr>
<th>Labeled phospholipid</th>
<th>Bilayer composition</th>
<th>$K_S$</th>
<th>$K_S/K_S(\text{PC})^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(18:1)$_2\text{-PE-NBD}$</td>
<td>DOPC (18:1)$_2$PC</td>
<td>1.4</td>
<td>—</td>
</tr>
<tr>
<td>$(18:1)$_2\text{-PE-NBD}$</td>
<td>DEuPC (22:1)$_2$PC</td>
<td>2.1</td>
<td>—</td>
</tr>
<tr>
<td>$(18:1)$_2\text{-PE-NBD}$</td>
<td>DMoPC (14:1)$_2$PC</td>
<td>2.9</td>
<td>—</td>
</tr>
<tr>
<td>(18:1)-(12:0-NBD)$_2$-PE</td>
<td>DOPC</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(18:1)-(12:0-NBD)$_2$-PC</td>
<td>DOPC</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(18:1)-(12:0-NBD)$_2$-PG</td>
<td>DOPC</td>
<td>2.3</td>
<td>1.1</td>
</tr>
<tr>
<td>(18:1)-(12:0-NBD)$_2$-PS</td>
<td>DOPC</td>
<td>2.7</td>
<td>1.3</td>
</tr>
<tr>
<td>(18:1)-(12:0-NBD)$_2$-PA</td>
<td>DOPC</td>
<td>3.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*K_S(\text{PC}) is the relative association constant of (18:1)-(12:0-NBD)-PC.*
ditions from a study of BODIPY labeled protein steady-state fluorescence emission (Fernandes et al., 2003). However, in that study much higher concentrations of protein were used when compared with the present one, and assuming the aggregation constants obtained in that work, only up to 5% at the very most of protein could be aggregated at the protein concentration used throughout this study. Therefore we can consider that our results report the phospholipid selectivity properties of the monomeric M13 major coat protein.

The fitting of the annular model for protein-lipid interactions to the FRET data (Figs. 3 and 4), converged always to \( K_S \) values above 1 (Table 1), as the energy transfer...
efficiencies \((1 - \tau_{DA}/\tau_D)\) are above the expected value for random distribution of the labeled phospholipids. As our annular model assumes a random distribution outside the protein-lipid interface (which should be true for one-component bilayers in the liquid crystalline phase; Loura et al., 1996), this result is rationalized as an increase in local concentration of probe in the lipid annular shell around the protein. For \((18:1)_{2}\)-PE-NBD probe in DOPC (\((18:1)_{2}\)-PC) bilayers (Fig. 3 A), the value of \(K_S\) was 1.4, pointing to almost complete randomization of the probe distribution in the bilayer, and therefore identical selectivity to the DOPC lipid. This was expected, because the probe acyl-chains are identical to the unlabeled lipid and allow a perfect hydrophobic matching of the protein.

The results from Fig. 3, A–C, all report energy transfer efficiencies to the \((18:1)_{2}\)-PE-NBD probe, but in different bilayers of one lipid component. In DOPC bilayers the value of \(K_S\) was 1.4 as discussed above, but in DMoPC ((14:1)\(_2\)-PC) and DEuPC ((22:1)\(_2\)-PC) bilayers the relative association constant values were 2.9 and 2.1, respectively, confirming a greater selectivity toward the hydrophobic matching unlabeled phospholipid (DOPC).

**M13 coat protein selectivity toward phospholipids with different headgroups**

Results from analysis of the data on M13 coat protein selectivity toward phospholipids headgroups are presented in Fig. 4. Clearly the anionic-labeled phospholipids exhibit larger selectivity to the lipid annular region around the protein, especially the 18:1-(12:0-NBD)-PA and 18:1-(12:0-NBD)-PS probes (\(K_S = 3.0\) and 2.7, respectively). The 18:1-(12:0-NBD)-PG probe presents an intermediate selectivity (\(K_S = 2.3\)), whereas 18:1-(12:0-NBD)-PC and 18:1-(12:0-NBD)-PE have identical relative association constants (\(K_S = 2.0\)). The selectivity for anionic phospholipids must be a consequence of electrostatic interaction of these with the highly basic C-terminal domain of the protein, which contains four lysines.

Overall the selectivity of annular lipid-M13 coat protein is not large, which is common for intrinsic membrane proteins (Lee, 2003). Additionally, it has been shown that selectivity of some proteins toward anionic lipids is significantly decreased in the presence of increasing ionic strength (Marsh and Horváth, 1998). In our case the ionic strength was kept high, because it is necessary to keep the protein in the monomeric state (Spruijt and Hemminga, 1991), and this further explains our results.

Phospholipid selectivity ESR studies have already been performed with aggregated forms of M13 major coat protein (Peelen et al., 1992; Wolfs et al., 1989, Datema et al., 1987), resulting in similar selectivity patterns of M13 coat protein to phospholipid headgroups. Peelen et al. (1992), using a identical buffer type, ionic strength, and pH to that used in the present study, obtained the following relative association constants ratios \((K_S(PX)/K_S(PC))\) of M13 coat protein incorporated in 1,2-dimiristoyl-sn-glycero-3-phosphocholine (DMPC): \(K_S(PA)/K_S(PC) = 1.6, K_S(PS)/K_S(PC) = 1.2, K_S(PG)/K_S(PC) = 1.1, and K_S(PE)/K_S(PC) = 1.0\). Overall the selectivity pattern is the same, and the relative association constants ratios are almost identical. The M13 coat protein was aggregated in that study, and according to the authors the number of first shell sites was five, that is, for each protein only a maximum of five lipids could be motionally restricted due to contact with the protein surface. For a monomeric helix, however, a value of 12 should be expected (Marsh and Horváth, 1998), and that was the number used in our model for the data analysis. Therefore it is particularly interesting that the ratios of the relative association constants remain almost identical. Apparently protein aggregation lowers the selectivity degree of each protein for phospholipids only through a decrease of available area for protein-lipid contacts but the relative association ratio with phospholipids of different headgroups remains the same. Even though the protein presents higher selectivity for the NBD-labeled phospholipids than for DOPC (\(K_S(PC) = 2.0\)) (possibly due to electrostatic interactions with the NBD at the bilayer interface), the result presented above clearly shows that the presence of NBD at the bilayer interface does not change significantly the relative association ratios of the phospholipids.

Sanders et al. (1992) were not able to determine the selectivity of the M13 coat protein monomeric species toward phospholipids using ESR, because the monomer was not able to produce a sufficiently long-living boundary shell of lipids that could be detected by ESR spectroscopy. The fact that it was possible to clearly quantify relative association constants using FRET in the present study presents this technique as an alternative to ESR in protein-lipid studies.

One important difference between the ESR and FRET techniques is that the latter is not restricted to the lipids adjacent to a given protein molecule. Not only labeled lipids in the first shell of lipids will be potential acceptors to a donor-labeled integral protein, but also the acceptors in the other lipid shells surrounding the protein will contribute to the final result. For that reason, this study also seems to confirm the hypothesis of selectivity to anionic phospholipids by the protein to largely confine itself to an annular shell of lipids in direct contact with the protein, in the case of the M13 major coat protein.

In case that the annular region would extend beyond this first shell, our FRET analysis methodology (based in transfer to a single annular shell and also to the bulk) would recover substantially larger values for the relative association constants. Moreover, as commented above, our recovered \(K_S/P_S(18:1)\) match those obtained from ESR measurements, which only detects immobilization of annular lipids upon incorporation of protein. The existence of a single annular
lipid layer for this protein might be related to the fact that it has a sole transmembrane segment.

The FRET methodology has three interesting features. First, by choosing donor-acceptor pairs with different Förster radii it is possible to specifically study mainly the first-shell of lipids or also the outside shells, as was the case in the present study. The joint analysis of results coming from these different donor-acceptor pairs could allow for an even more detailed description of the protein-lipid arrangement in more complex systems. In our study, the relatively large $R_0$ value for the used donor-acceptor pair meant that the experimental quenching curves shown in both Figs. 3 and 4 look similar at first sight. Nevertheless it is impressive that the analysis methodology is able to retrieve significant $K_S$ values. Of course, this methodology could still be improved by the use of a donor-acceptor pair with a smaller $R_0$ value, closer to the distances under measurement. Second, the more economic character of fluorescence studies, which requires much smaller amounts of material than ESR, should be stressed. And third, although this model leads to a somewhat complex decay law (Eqs. 7, 10, and 11), it is actually not necessary to analyze the decay curves with this law to recover the relevant parameters, unlike in other FRET studies (e.g., of lipid phase separation; see Loura et al., 2001). The theoretical curves are conveniently simulated and integrated in a worksheet to calculate the theoretical FRET efficiencies. These can be matched to experimental values by varying the $K_S$ value (the sole unknown parameter). The experimental FRET efficiencies could also be obtained from steady-state data. In our case, we obtained them from integration of donor decay curves because these are less prone to artifacts (e.g., light scattering, inner filter effects, measurement of absolute intensities), which in any case, could in principle be corrected for in a steady-state experiment.

CONCLUSIONS

In the present study FRET has been applied with success in the characterization of the M13 major coat protein selectivity toward phospholipids. As expected, the protein has no significant selectivity for the lipid probe containing two oleoyl acyl-chains while in DOPC bilayers, but exhibits larger selectivity for the same probe while in bilayers with different hydrophobic thickness due to hydrophobic mismatch stress. The protein also presents larger selectivity for anionic lipids, particularly for phosphatidic acid and phosphatidylserine phospholipids. FRET was shown here to be a promising methodology in protein-lipid selectivity studies.


REFERENCES


