A small protein in model membranes: a time-resolved fluorescence and ESR study on the interaction of M13 coat protein with lipid bilayers

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Abstract. Model membranes with unsaturated lipid chains containing various amounts of M13 coat protein in the α-helical form were studied using time-resolved fluorescence and ESR spectroscopy. The lipid-to-protein (L/P) ratios used were > 12 to avoid protein-protein contacts and irreversible aggregation leading to β-polymeric coat protein. In the ESR spectra of the 12-SASL probe in dioleoyl phosphatidylcholine (DOPC) bilayers no second protein induced component is observed upon incorporation of M13 coat protein. However, strong effects are detected on the ESR lineshapes upon changing the protein concentration. The ESR lineshapes are simulated by assuming a fixed ratio between the parallel (D||) and perpendicular (D⊥) diffusion coefficients of 4, and an order parameter equal to zero. It is found that increasing the protein concentration from L/P 3 to L/P 15 results in a decrease of the rotational diffusion coefficient D⊥ from 3.4 x 10⁷ to 1.9 x 10⁷ s⁻¹.

In the time-resolved fluorescence experiments with DPH-propionic acid as a probe, it is observed that increasing the M13 coat protein concentration causes an increase of the fluorescent lifetimes, indicating an increase in bilayer order. Analysis of the time-resolved fluorescence anisotropy decay allows one to quantitatively determine the order parameters ⟨P₂⟩ and ⟨P₄⟩, and the rotational diffusion coefficient D⊥ of the fluorescent probe. The order parameters ⟨P₂⟩ and ⟨P₄⟩ increase from 0.34 to 0.55 and from 0.59 to 0.77, respectively, upon adding M13 coat protein to DOPC bilayers with an L/P ratio of 35. The rotational diffusion coefficient D⊥ of the DPH-propionic acid probe decreases on incorporating M13 coat protein, in accordance with the ESR results. It is concluded that M13 coat protein in the α-monomeric state is not able to produce a long living lipid boundary shell and consequently an immobilization of the lipids. An overall effect on the lipids is induced, resulting in a reduction in the dynamics and an increase in average lipid order. The hydrophobic region of M13 coat protein is proposed to perfectly match the lipid bilayer, resulting in a relatively small distortion of the bilayer structure of the lipid system.

Key words: M13 coat protein – Lipid protein interaction – ESR spectroscopy – Time-resolved fluorescence spectroscopy – Order parameter – Diffusion coefficient

Introduction

The infection of Escherichia coli with the bacteriophage M13 starts with the entry of the phage via a sex pilus and the subsequent release of the viral DNA into the cytoplasm and storage of the major coat protein as an integral membrane protein in the cytoplasmic membrane of the host (Pratt et al. 1969). Newly synthesized coat protein is inserted into the membrane as a preprotein and is converted to mature protein with a molecular weight of 5240 Da, by cleavage with a leader peptidase. This protein and probably also the stored parental protein are used for the membrane-bound assembly of new bacteriophage particles (Model et al. 1982). The secondary structure of the coat protein in the bacteriophage, as determined by neutron scattering data and Raman experiments, is about 100% α-helical (Day 1969). It has been shown that in model membranes, M13 coat protein can adopt two different forms: an α-oligomeric and a β-polymeric form. The presence of either one of these forms critically depends on the headgroup type and the degree of saturation of the acyl chains of the lipids used (Nozaki et al. 1978; Fodor et al. 1981; Spruijt et al. 1989; Spruijt and Hemminga 1991).

The β-polymeric form is an aggregated β-sheet protein complex (Datema et al. 1987; Wolfs et al. 1989; Spruijt and Hemminga 1991; Van Gorkom et al. 1990), whereas the protein in the α-oligomeric form has a high amount of α-helix structure and forms only small aggregates, in dynamic equilibrium with protein monomers (Spruijt et al.
accompanying change in conformation, are avoided contacts that may result in protein aggregation and the and Hemminga 1991). In this L/P region, protein-protein lipids will surround the protein (Wolfs et al. 1989; Spruijt and Hemminga 1991). It has been argued by Spruijt and Hemminga (1991) that this form is the biologically active state of the protein. This form has been studied recently using $^2$H-NMR and $^{31}$P-NMR spectroscopy (Sanders et al. 1991). In the present paper, we study the effects of M13 coat protein in the $\alpha$-oligomeric form on bilayers consisting of DOPC with time-resolved fluorescence and ESR spectroscopy. These techniques are suitable for investigating changes in order and dynamics of the lipid chains upon incorporation of the M13 coat protein. The results obtained from both techniques can be compared directly, since they are both sensitive to motions on the nanosecond time scale. The lipid-to-protein (L/P) molar ratios taken are > 12, because for an ideal single trans-membrane $\alpha$-helix it is expected that 12 lipids will surround the protein (Wolfs et al. 1989; Spruijt and Hemminga 1991). In this L/P region, protein-protein contacts that may result in protein aggregation and the accompanying change in conformation, are avoided (Spruijt et al. 1989; Spruijt and Hemminga 1991).

Materials and methods

**Chemicals:** DOPC was obtained from Sigma (St. Louis, USA). The fluorescent probe 3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-propionic acid, Fig. 1A) was obtained from Molecular Probes, Inc. and spin labelled fatty acid (12-SASL, Fig. 1B) from SIGMA. All chemicals were used without further purification.

**Sample preparation:** Bacteriophage M13 was grown and purified as described elsewhere (Spruijt et al. 1989). After removing the chloroform with nitrogen gas, the desired amounts of lipids were lyophilized for at least 12 hours. If desired, DPH-propionic acid and 12-SASL were added to the chloroform solution at a molar ratio of 1:500 and 1:100, respectively. The lyophilized samples were solubilized in buffer (50 mM cholate, 10 mM Tris, 0.2 mM EDTA, pH 8.0). To the buffer solution the desired amount of protein in the same buffer was added. The coat protein was purified as described previously (Spruijt et al. 1989). Cholate was removed by extensive dialysis at 25°C against a 100 fold excess of buffer (10 mM Tris, 0.2 mM EDTA, pH 8.0) for a total of 48 hours with buffer changes every 12 hours. The samples used for optical experiments were diluted to an OD$_{280}$ of 0.1. The corresponding reference samples without protein were prepared and diluted in the same way as the protein containing samples. The ESR samples were lyophilized, re-solubilized and concentrated using an Amicon Stirring cell. The L/P ratio of the samples was determined with a phosphate and protein determination. The conformation of M13 coat protein was checked with circular dichroism spectroscopy. An average of at least five scans was recorded at room temperature on a Jobin-Yvon Auto-Dichograph Mark V in the wavelength range 200–240 nm, using a sample cell with a 1-mm path length. The aggregation state was checked with HPLC as described by Spruijt et al. (1989).

**ESR studies:** ESR spectra were recorded on a Bruker ER 200 spectrometer with nitrogen gas flow temperature regulation ($\pm 1^\circ$C). ESR settings were: 5 mW microwave power, 0.1 mT modulation amplitude, 200 ms time constant, 200 s scan time, 10 mT scan width and 339 mT centre field. Up to 12 spectra were accumulated to improve the signal to noise ratio. The ESR simulations were performed with a computer program developed by Schneider and Freed (1989). The parameters used for the simulation were as follows: components for the hyperfine coupling tensor (in mT) 0.62, 0.56, 3.25; components for the $g$-factor tensor 2.0088, 2.0061, 2.0027. The $g$-factor tensor components were similar to those used by other workers for the same type of radical (Meirovitch et al. 1984; Moser et al. 1989).

**Time-resolved fluorescence studies:** The experimental set-up for the polarized fluorescence decay measurements has been described previously (Visser et al. 1985). The excitation wavelength was 340 nm and the emission wavelength (437 nm) was selected by a filter (Balzer 437). The fluorescence decay was detected by time-correlated single photon counting (Phillips et al. 1985). The analysis was performed using a global analysis program, which uses a non-linear fitting least square procedure (Beechem and Graton 1989). The quality of the fit was determined by the weighted residuals and the chi-square.

After correction for the background fluorescence, the fluorescence decay ($I_f(t)$) of DPH-propionic acid in DOPC bilayers with various amounts of M13 coat protein was analyzed as a sum of exponentials:

$$I_f(t) = \sum_{i=1}^{2} a_i e^{-t/t_1},$$

in which $t_i$ is the fluorescence lifetime and $a_i$ a pre-exponential factor. Typically, two exponential components were sufficient to describe the fluorescence decay.

After correction for background fluorescence, the time-resolved fluorescence anisotropy decay of DPH-propionic acid in DOPC bilayers with various amounts of M13 coat protein was analyzed as a sum of exponentials, which were expressed in terms of four variables, $<P_2>$, $<P_3>$, $D_1$ and $r(0)$ (Ameloot et al. 1984). The parameters $<P_5>$ and $<P_4>$ are the orientational order parameters expressed in Legendre polynomials of the sec-

![Fig. 1. Chemical formulas of DPH-propionic acid (A) and 12-SASL (B)](image-url)
ond and fourth rank. In view of the approximate cylindrical shape of the DPH-propionic acid probe, its rotational diffusion in the membrane is assumed to be axially symmetric with a parallel \((D_{II})\) and perpendicular \((D_{I})\) diffusion coefficient. Because \(D_{I}\) is much smaller than \(D_{II}\), for the analysis of the fluorescence experiments only \(D_{I}\) needs to be taken into account (Ameloot et al. 1984). The initial anisotropy, \(r(0)\), depends on the angle between the absorption and emission moments. This parameter was fixed to a value of 0.39 as has been obtained by other authors (Ameloot et al. 1984; Pottel et al. 1986).

**Results**

**Protein studies:** M13 coat protein is able to adopt two conformations, an \(\alpha\)-helix or a \(\beta\)-sheet, depending on its lipid environment and history of preparation (Spruijt et al. 1989). Since the purpose of this study was to investigate the protein in an \(\alpha\)-helix structure, care was taken to avoid protein-protein contacts and protein aggregation that may result in the formation of protein in a \(\beta\)-sheet conformation, by using \(L/P\) ratios > 12. Therefore, the conformation and aggregation state of the protein in the lipid bilayers was carefully checked. The circular dichroism spectra of M13 coat protein show that the \(\alpha\)-helix content of the protein is approximately 90%, whereas HPLC demonstrated the absence of large aggregates in the reconstituted samples. After the experiments the conformational and aggregation state of M13 coat protein was checked again. No changes could be detected, showing that the \(\alpha\)-helical state of the protein is conserved at all \(L/P\) ratios used.

**ESR:** ESR spectra of 12-SASL in DOPC bilayers at 10°C, which have a liquid crystalline transition temperature of \(-16°C\) (Dijck et al. 1976), with various amounts of M13 coat protein are given in Fig. 2. At low \(L/P\) ratios no distinct bound component can be observed, as has been reported previously for the \(\beta\)-polymeric form of the M13 coat protein in similar bilayers (Datema et al. 1987; Wolf's et al. 1989), but strong effects on the lineshape are observed (Fig. 2). Similar, but somewhat less pronounced effects are observed for 14-SASL (results not shown). To describe the spectral changes found upon incorporation of M13 coat protein in terms of changes in order parameters and rotational diffusion coefficients, ESR spectra with and without protein were simulated using the program of Schneider and Freed (solid lines in Fig. 2).

The motional behaviour of the probe is found to be well described by an anisotropic Brownian diffusion. The choice of other motional models (free or jump diffusion) gave a less good fit. Axial diffusion is assumed with a fixed ratio between the parallel \((D_{II})\) and perpendicular \((D_{I})\) diffusion coefficients \((D_{II}/D_{I} = 4)\), as found in simulations performed by Schneider and Freed (1989). All simulations were carried out with an order parameter equals to zero. The introduction of a non-zero order parameter was also tried, but did not result in an improved fitting of the experimental ESR spectra. The simulations resulted in a consistent set of data, in which the spectra at the various \(L/P\) ratios were considered as single component spectra characterized by different values of \(D_{I}\). The resulting values of \(D_{I}\) at various \(L/P\) ratios are displayed in Fig. 3.

An important factor in the application of the present simulation model was the observation that the same ESR spectra were found for lower \(L/P\) ratios at higher temper-
The fluorescent probe DPH-propionic acid also senses the order parameters ($\phi$) and ($P_4$) to increase.

Table 1. Fluorescence lifetimes ($\tau_i$), the relative contribution ($e_i$) and the values for $\langle P_2 \rangle$, $\langle P_4 \rangle$, and $D_\perp$ of DPH-propionic acid in DOPC membranes with various amounts of M13 coat protein at 5 and 20°C. The error in the values is estimated to be less than 5% for all parameters (10% for $D_\perp$).

<table>
<thead>
<tr>
<th>L/P</th>
<th>T (°C)</th>
<th>$\alpha_1$</th>
<th>$\tau_1$ (ns)</th>
<th>$\alpha_2$</th>
<th>$\tau_2$ (ns)</th>
<th>$\langle P_2 \rangle$</th>
<th>$\langle P_4 \rangle$</th>
<th>$D_\perp$ ($\times 10^9$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\infty$</td>
<td>5</td>
<td>0.47</td>
<td>2.94</td>
<td>0.53</td>
<td>6.00</td>
<td>0.34</td>
<td>0.59</td>
<td>0.10</td>
</tr>
<tr>
<td>55</td>
<td>5</td>
<td>0.43</td>
<td>3.10</td>
<td>0.57</td>
<td>6.21</td>
<td>0.39</td>
<td>0.62</td>
<td>0.09</td>
</tr>
<tr>
<td>35</td>
<td>5</td>
<td>0.45</td>
<td>3.28</td>
<td>0.55</td>
<td>6.42</td>
<td>0.55</td>
<td>0.77</td>
<td>0.08</td>
</tr>
<tr>
<td>$\infty$</td>
<td>20</td>
<td>0.38</td>
<td>1.97</td>
<td>0.62</td>
<td>5.40</td>
<td>0.32</td>
<td>0.57</td>
<td>0.18</td>
</tr>
<tr>
<td>55</td>
<td>20</td>
<td>0.37</td>
<td>2.29</td>
<td>0.63</td>
<td>5.78</td>
<td>0.36</td>
<td>0.59</td>
<td>0.14</td>
</tr>
<tr>
<td>35</td>
<td>20</td>
<td>0.40</td>
<td>2.73</td>
<td>0.60</td>
<td>6.08</td>
<td>0.50</td>
<td>0.68</td>
<td>0.14</td>
</tr>
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</table>

This also happens when the L/P ratio is lowered by an increasing protein concentration. An increase of the temperature from 5 to 20°C leads to an increase of $D_\perp$ by nearly a factor of two; $D_\parallel$ decreases only slightly upon increasing the protein concentration in the bilayers.

**Discussion**

During the infection process of *Escherichia coli* by the M13 bacteriophage, high amounts of old and newly synthesized M13 coat protein are stored in the cytoplasmic membrane of the host. Therefore, it is interesting to know how M13 coat protein affects the membrane properties. In this paper, model membranes with unsaturated lipid chains with various amounts of the M13 coat protein in the presumed biologically active $\alpha$-monomeric form are studied with ESR and time-resolved fluorescence spectroscopy to probe the effect of the protein on the lipid bilayers. Care was taken to avoid protein-protein contacts (formation of $\alpha$-oligomers) and the concomitant formation of $\beta$-polymers. It is known that $\beta$-polymers strongly affect the lipid membrane by giving rise to non-bilayer lipid structures around the aggregates (Sanders et al. 1992). Therefore, L/P ratios > 12 were used and the reconstitution approach was taken as described by Spruijt et al. (1989) that conserves the $\alpha$-helical monomeric state (Spruijt and Hemminga 1991). In the present study, the absence of protein aggregation is clearly demonstrated with HPLC and M13 coat protein is predominantly in an $\alpha$-helical conformation, as shown by circular dichroism spectroscopy.

**Diffusion coefficient:** The changes in the ESR spectra of the 12-SASL spin probe upon introduction of M13 coat protein can be analyzed by a computer simulation in terms of a decrease in the rotational diffusion coefficient $D_\perp$, $D_\parallel$ is assumed to be related to $D_\parallel$ by the equation $D_\parallel = 4D_\perp$ (see Results). The order parameter is fixed at zero. This is a good approximation since the order parameter found for the 12-SASL probe in our simulations is less than 0.1 (results not shown), in agreement with other studies (Wassall et al. 1990). Because of this low value, it is not possible to determine changes in order parameter induced by the protein with a sufficient accuracy. In addition, a variable order parameter would increase the number of parameters in the simulations, thereby increasing unnecessarily the complexity of the calculations. The rotational diffusion coefficient $D_\perp$ shows a non-linear dependence on the protein concentration (Fig. 3): a strong decrease is observed, especially at low L/P ratios. This effect may be expected, since on going to very low L/P ratios totally immobilized lipid would eventually result by squeezing the lipids between the aggregated $\alpha$-oligomeric protein molecules (Peelen et al. 1992).

The fluorescent probe DPH-propionic acid also senses a decrease of the diffusion coefficient $D_\perp$ upon incorporating M13 coat protein. However, the effect of M13 coat protein on $D_\perp$ in this case is less when compared to the changes observed by ESR. Also the rate of the motion of the DPH-propionic acid probe is 2.5 times faster than the
rate of the 12-SASL probe. Although this small difference is difficult to explain in a quantitative way, it will be related to differences in size and molecular properties of the probes (see Fig. 1). These molecular differences of the probes also affect the order parameters as will be discussed below.

**Order parameters:** The molecular structure of the 12-SASL probe gives rise to a high flexibility, which arises from a trans-gauche isomerization of the acyl chain to which the nitroxide moiety is attached. This gives rise to an order parameter that is about zero. However, the DPH-propionic acid probe is a rigid molecule, as a result of the presence of the conjugated bonds (Fig. 1). Therefore, it senses the "average" order over different depths in the membrane. In Table 1 it can be seen that increasing the protein concentration causes an increase in both order parameters, \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \), of the DPH-propionic acid probe. This indicates that the molecular order of the DOPC bilayers as sensed by the probe has increased upon incorporation of the M13 coat protein. The increase in order correlates with the observed increase of both lifetimes on adding protein to the bilayers (Table 1). It has been shown previously that lifetimes of DPH analogues show a remarkable correlation with order (Straume and Litman 1987). The increase of the lifetimes is found to result from a reduced water penetrability into the lipid bilayer by a reduced lipid flexibility (Best et al. 1987).

The value of \( \langle P_4 \rangle \) is larger than the value of \( \langle P_2 \rangle \) and shows the same variation with protein concentration as \( \langle P_2 \rangle \). The increase of \( \langle P_4 \rangle \) with increasing protein concentrations can be related to a decrease of the width of the angular distribution of the lipid probe in the membrane. If \( \langle P_2 \rangle \) is plotted versus \( \langle P_4 \rangle \) at various temperatures and at various protein concentrations, an almost linear relation is observed (Fig. 4). This suggests that changing the temperature is comparable to adding protein to lipid bilayers. This is in agreement with the finding from the ESR experiments, that increasing the protein concentration results in a change in diffusion coefficient, which can be compensated by increasing the temperature. From Fig. 4 it can also be seen that, in agreement with the findings of Best et al. (1987), the relation between \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) can not be considered as being described by the cone model. The relation between \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) is best described by a \( P_4 \)-distribution model, indicating that the interaction energy of the DPH-propionic acid probe is proportional to \( P_2 (\cos \beta) \), where \( \beta \) is the angle between the probe molecule and the normal to the bilayers. This is in agreement with results obtained by Pottel et al. (1986) for DPH molecules in the phospholipids DMPC and DPPC.

In Fig. 5 the angular distribution function, calculated using information theory, is given for a sample with and without protein (Ameloot et al. 1984), constructed from the experimental values of \( \langle P_2 \rangle \) and \( \langle P_4 \rangle \) using information theory applied to DPH-propionic acid in DOPC bilayers with and without M13 coat protein.

Phospholipid model membranes consisting of DOPC specifically deuterated in both chains at the 11-position (1,2-[11,11-2H_2]-DOPC) containing various amounts of M13 coat protein in the \( \alpha \)-helical form have been studied with \(^2\)H-NMR spectroscopy (Sanders et al. 1992). These experiments indicate that on the \(^2\)H-NMR time scale the order in the bilayer is decreased in the presence of the coat protein. This shows that \(^2\)H-NMR spectroscopy is much more sensitive in detecting small differences in the order parameter than is ESR spectroscopy on the 12-SASL probe. Whereas, the order of the lipid bilayers, as seen by the fluorescent DPH-propionic acid probe, increases upon incorporation of the M13 coat protein, the \(^2\)H-NMR experiments show the opposite. This difference may be due to the presence of bulky amino acid side chains of M13 coat protein in the hydrophobic membrane region. These groups will provide protrusions with holes in between, that will be sensed by the deuterium nuclei on the flexible acyl chains. This may result in an
increased disorder when more M13 coat protein is introduced in the phospholipid system. However, the relatively long, rigid fluorescent probe will only experience the "average" protein surface and sense an increased order. Previously, protrusions and holes have been proposed to play a role in the packing of M13 coat protein incorporated in membranes (Dunker and Jones 1978). Bulky side chains may be provided by the aromatic amino acids of M13 coat protein (tyrosine 21 and 24, tryptophan 25, and phenylalanine 42).

**General conclusions:** As observed from the ESR experiments in this paper, M13 coat protein in the α-oligomeric state at high L/P ratios (L/P > 12), is not able to induce a long living lipid boundary shell and consequently an immobilization of the lipids. This is in contrast with ESR studies performed by Peelen et al. (1992) on M13 coat protein in the α-oligomeric state at low L/P ratios (L/P < 12), where an immobilized component was observed of which the intensity depended on the M13 coat protein concentration (Peelen et al. 1992). For this system, it was estimated that about four lipids bind per protein monomer (Peelen et al. 1992). This low number of boundary lipids indicates that protein-protein contacts take place at these low L/P ratios (Wolfs et al. 1989) and that lipid sites are shared by two or more protein monomers. Protein-protein contacts result in the formation of α-oligomers. This effect gives rise to an immobilized ESR spectral component, as has also been observed in studies with the β-polymeric M13 coat protein (Datema et al. 1987; Wolfs et al. 1989).

A long range perturbation of α-oligomeric M13 coat protein at low L/P ratios has been observed by Peelen et al. (1992): A fluid component, which was characteristic of a lower temperature than at which the corresponding spectra of the lipid protein systems was recorded, had to be used in the spectral simulations. This indicates that also in the case where protein-protein contacts take place, M13 coat protein induces an overall lipid effect, similar to that found for the monomeric form.

M13 coat protein in the α-helical conformation has a rigid trans-membrane α-helix exposing a hydrophobic surface to interact with the lipid chains. In general, it has been assumed that immobilization of lipids by proteins originates from the rigidity of the protein backbone of the membrane bound domain of the protein (Jost et al. 1973; Marsh 1981; Devaux 1983). Based on our experiments, it is clear that not only the rigidity of the trans-membrane part of the protein is important, but also the size of the hydrophobic surface of the protein region: a single M13 coat protein molecule is not able to cause a strong immobilization, in contrast to α-oligomers consisting of aggregated protein molecules with an α-helix conformation.

When small trans-membrane α-helical M13 coat protein monomers are incorporated in lipid systems, an gated protein molecules with an e-helix conformation. The coat protein molecule is not able to cause a strong immobilization, in contrast to e-oligomers consisting of aggregated hydrophobic molecules, such as cholesterol, in lipid bilayers (Marsh and Smith 1973; Schreier-Muccillo et al. 1973; Hemminga 1975). This conclusion is in agreement with the observation that the hydrophobic region of M13 coat protein perfectly matches the lipid bilayer, resulting in a relatively small distortion of the bilayer structure of the lipid system (Sanders et al. 1992).

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