The Use of Fluorescent Probes for Studying the Interaction of Proteins with Black Lipid Membranes

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The number of 1-anilino-8-naphthalene sulphonate (ANS) molecules bound to black phosphatidylcholine (PC) and phosphatidylinositol (PI)-membranes was calculated. The fluorescence change of membrane-bound ANS was measured after the addition of positively charged proteins to the same side as ANS. Cytochrome c caused a fluorescence decrease, lysozyme and protamine an increase. These effects were completely reversible in the case of cytochrome c and lysozyme and only partly reversible in the case of protamine by increasing the ionic strength. The fluorescence polarization of membrane bound ANS was not significantly changed by protein addition. The results are discussed with respect to the binding of proteins to black lipid membranes and the use of ANS as a probe compared with other fluorescent probes.

Introduction

The lipid bilayer is accepted as a very important structural feature of most biological membranes. Therefore optical black lipid membranes [1] (BLM) are widely used as models for biological membranes. The importance of proteins for the function of biomembranes prompted much effort to incorporate proteins into black lipid membranes. A direct method to quantitate incorporation is necessary for such studies. Electrical measurements are most sensitive but not applicable in many cases. Spectroscopic methods on the other hand will be applicable in most cases. Proteins carrying groups with a high molar extinction coefficient can directly be monitored by absorption spectroscopy [2]. Another approach is to incorporate or adsorb fluorescent labels into the lipid phase which change their fluorescent properties when proteins interact with the membrane. In the present study we have used the fluorescence method because it is more sensitive than absorption measurements at comparable experimental sophistication and examined how useful it is for studying the interaction between proteins and black lipid membranes. ANS has been chosen as fluorescent probe because its properties are well known [3–5]. As model compounds cytochrome c, lysozyme and protamine were used because they induce only small changes of ANS fluorescence in the aqueous phase. Experiments with another fluorescent probe Dansyl-phosphatidyl-ethanolamine (Dansyl PE) [6–8] have been started. The results [9] fit well into the picture obtained with ANS.

Materials and Methods

Oxidized cytochrome c (Horse heart, Type III) was obtained from Sigma, protamine sulfate (Clupein) pract. from Fluka or Merck, egg white lysozyme cryst. from Boehringer and all used without further purification. 1-anilino-8-naphthalene sulphonate was obtained from Eastman or Serva, Heidelberg, and twice recrystallized from water as the Mg-salt in the dark. Dierucoylphosphatidylcholine (di-22:1-PC, synthesized by K. Janko in our laboratory) and Phosphatidylinositol (bovine brain, Folch Fr. 1, lyoph) from Koch-Light were used for membrane formation. All other chemicals were reagent grade from Merck and Fluka. Water was bidistilled, either unbuffered (pH between 5.3 to 6.3) or buffered with phosphate at pH 6.3. Buffer was usually added after formation of the membranes. Ionic strength was varied between 1 mM and 100 mM with KCl. Fluorescence emission spectra of solutions were recorded with a FICA 55 fluorimeter, fluorescence polarization of solutions with a Doignon-Magot PF-1 apparatus.

Fluorescence spectra and fluorescence polarization of black lipid membranes were measured in an instrument of our own design, which has been described [10, 11] previously. An OSRAM XBO 150 W/1 xenon-high pressure lamp or a 250 W halogen-

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wolfram lamp were light sources for excitation. Various glass and interference filters from Schott-Mainz were used to select wavelength for excitation and emission. HNP'B-linear polarizers from Polaroid were used for polarization measurements.

The membranes were formed over a rectangular hole of approx. 1 cm^2 area in a black teflon support. The membrane forming solution contained 2.5 mg phospholipid per ml solvent (a mixture of 10 vol *n*-decane and 1 vol *n*-butanol). Temperature in the cell was 25 ± 2 °C.

Results

1. ANS-fluorescence in solution

To make sure that the observed effects are due to the interaction of ANS and protein with the black lipid membrane we first studied fluorescence changes after adding proteins to an aqueous ANS solution. The effects are always much smaller than those observed with the membrane system at comparable concentrations of salt, ANS and protein.

The fluorescence of ANS solutions was partially quenched on adding cytochrome c. This small quenching could be explained as an inner filter effect of cytochrome c. No effect of the ionic strength on the fluorescence quenching was found. The polarization of the ANS fluorescence was the same with and without protein. For solutions of protein and probe at the concentrations used in the membrane experiments the total quenching (due to the inner filter effect) was about 1%.

Addition of lysozyme to an aqueous ANS solution results only in a small enhancement of fluorescence [4]. A linear increase of the intensity of fluorescence with the concentration of lysozyme (up to 10^{-4} M) was observed. A solution of lysozyme and ANS at the concentrations used in the membrane experiments gave a fluorescence enhancement below 1%.

A small enhancement of the ANS fluorescence around 480 nm was seen after adding protamine sulfate. The effect increased linearly up to a protein concentration of 1.5 mg/ml at constant ANS concentration 4×10^{-5} M. At identical salt and ANS concentrations protamine addition enhanced the ANS fluorescence by maximally 10% in the absence of a membrane while the enhancement was by a factor of 2 to 3 in the presence of a membrane.

2. ANS fluorescence in black lipid membranes

In Fig. 1 the time course of fluorescence is plotted for typical experiments. At time zero the straylight of the aqueous phase was measured. At point 1 a lamella of about 1 cm^2 area was formed on the aperture. Straylight increased by a factor of 2 to 3. Between points 1 and 2 the lamella turned into the final black state with a small decrease in stray light. At point 3 a solution of 10^{-2} M ANS in ethanol (between 1 μl and 60 μl) was added to the aqueous phase (between 10 and 20 ml) on the backward side of the membrane only. Stirring and fluorescence measurements were started. Point 4 was the first measured after addition of ANS, point 5 was the final value reached after about 15 min. The emission maximum of membrane bound ANS lies between 477 and 505 nm after correcting for filter transmission and photomultiplier sensitivity. Most measurements were done at 477 nm. If ANS was added to the thick lamella (point 1') fluorescence followed the pointed line and reached the same point 5 when the membrane was in the black state. Fluorescence stayed now constant for extended time. At point 6

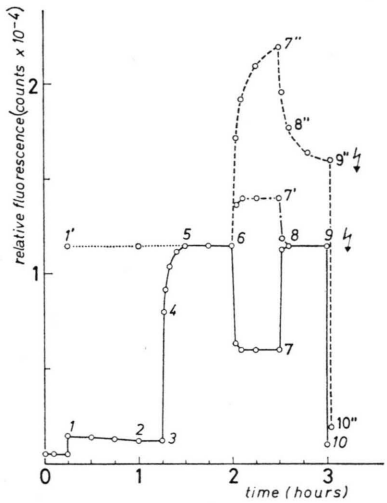

Fig. 1. Fluorescence of ANS bound to black lecithin membranes. (For a more detailed description see text.) Excitation at 365 nm, emission measured at 477 nm. Aqueous phase 10^{-3} M KCl or phosphate buffer, pH 6.3. At point 3 ANS was added to a concentration of 1×10^{-5} M. At point 6 different proteins were added in separate experiments. Full line (point 6 to 7) after addition of 2×10^{-7} M cytochrome c. Line between points 6 and 7' after addition of 1×10^{-7} M lysozyme. Broken line (point 6 to 7") after addition of 1×10^{-7} M protamine. At points 7,7' and 7" the ionic strength was increased from 1 mM to 50 mM with KCl. (Fluorescence of ANS in the aqueous phase was subtracted in all measurements.) ↯ marks destruction of membranes.

the proteins were added under stirring to the same side where ANS was present (separate experiments for each protein). Addition of cytochrome c (oxidised or reduced form) resulted in a fluorescence decrease (points 6 → 7), addition of lysozyme in a small increase (points 6 → 7′), addition of protamine in a big fluorescence increase (points 6 → 7″). If the aqueous phase was 100 mM in KCl instead of 1 mM, no effects were observed in the case of cytochrome c and lysozyme and a small fluorescence increase in the case of protamine. If proteins and ANS were added on different sides of the membrane no fluorescence change occurred. At points 7, 7′ and 7″ the ionic strength of the aqueous phase was increased to 50 mM by adding small amounts of saturated KCl solution under stirring. The fluorescence returned to the original value in the case of cytochrome c and lysozyme (points 8, 9). The effect of protamine was not completely reversed (points 8″, 9″). Finally 10 V were applied across the membranes *via* two silver-silverchloride-electrodes, in order to destroy the membranes (marked by ↯). The fluorescence dropped to a low value (points 10, 10″). Since ANS had only been added to one side, mixing of the two compartments led to variable values of the aqueous phase fluorescence [12]. This was circumvented by a second set of experiments where the two compartments were separated by a 0.2 mm thick glass plate instead of the membrane. ANS was then added on one side only and the aqueous phase fluorescence could be measured accurately.

In Fig. 2 the fluorescence of membrane bound ANS is plotted against the ANS concentration in the aqueous phase in the absence (full line) and presence of constant concentrations of the three proteins. Increasing cytochrome c and lysozyme concentrations to 4×10^{-7} M did not increase the effects shown in Fig. 2. In the case of protamine higher concentrations increased the fluorescence further, but the membranes became unstable. The final fluorescence values were reached within 5 min after increasing the cytochrome or lysozyme concentrations from zero to 2×10^{-7} M or above; they were reached within 15 min after increasing the concentrations from zero to 1×10^{-7} M. In the case of protamine no steady values were reached within 30 min.

The same results were obtained when the pH was set to 6.3 with 1 mM phosphate buffer or to 5.1 with 1 mM citrate buffer.

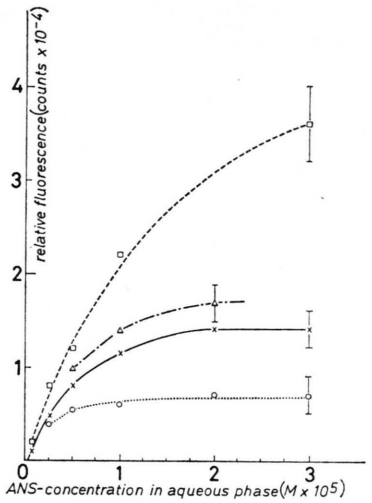

Fig. 2. Fluorescence of ANS bound to black lecithin membranes versus ANS concentration in the aqueous phase (measuring conditions as in Fig. 1). ×—×, without protein; ○···○, with 2×10^{-7} M cytochrome c; △–·–·–△, with 1×10^{-7} M lysozyme; □— —□, with 1×10^{-7} M protamine. Measurements were done 20 min after adding the proteins. The bars on the right side of the curves indicate the mean error for eight experiments.

No change of the membrane conductivity ($\lambda \sim 10^{-8}\,\Omega^{-1}\,\mathrm{cm}^{-2}$) was found after adding cytochrome c and lysozyme. Addition of 1×10^{-7} M protamine increased the conductivity at least by a factor of 3.

The fluorescence polarization (excitation with horizontally polarized light) of ANS bound to membranes was 0.20 ± 0.05. No significant changes were observed after adding proteins.

No significant fluorescence differences between PC- and PI-membranes were found at equal ANS concentrations.

Dansyl-PE was incorporated into PC- and PI-membranes (molar ratio of label to lipid 1:3 in both film-forming solutions). With both lipids the BLM showed the same fluorescence (excitation at 330 nm, emission measured at 505 nm). Addition of 10^{-7} M cytochrome c gave 10 times stronger quenching in the case of PI-membranes than in the case of PC-membranes.

Discussion

The interaction of cytochrome c with lipid dispersions and membrane vesicles has repeatedly been studied, *e. g.* [13–15]. The use of black lipid membranes (BLM) is advantageous because one can easily add substances to both sides of the membrane or apply

a potential difference across the membrane. Yguerabide [16] has suggested the use of hanging spherical membranes for such studies. A further advantage of the BLM method is, that one can measure the fluorescence polarization of an oriented membrane and calculate the orientation of pigment molecules in this membrane [11, 16].

The ANS fluorescence in BLM in the absence of proteins has already been studied with egg phosphatidylcholine and glycerolmonooleate [5]. The results with dierucoylphosphatidylcholine are similar. The fluorescence of a thick film in the presence of ANS was nearly the same as that of the BLM (Fig. 1, points 1′ and 5) in contrast to films containing chlorophyll [17]. This is a good evidence that ANS is always located at the membrane-water interface and not in the hydrocarbon layer. The number of ANS molecules per unit area of the membrane can be calculated by comparing the fluorescence of an aqueous volume of known geometry and ANS concentration with the fluorescence of the illuminated membrane area. Using the quantum yield of 0.004 for ANS in water [4] and 0.20 for ANS in the membrane [5] we obtained $[ANS]_{membrane} = (3 \pm 1) \times 10^{13}$ molecules ANS per cm^2 at the aqueous concentration $[ANS]_{water} = 1.5 \times 10^{-5}$ M and $[ANS]_{membrane} = (4 \pm 2) \times 10^{12}$ molecules ANS per cm^2 at the aqueous concentration $[ANS]_{water} = 2 \times 10^{-6}$ M. A binding constant $K = \frac{[ANS]_{membrane}}{[ANS]_{water}} = (3 \pm 2) \times 10^{-3}$ cm may be calculated.

In our experiments both PC and PI membranes showed the same fluorescence at equal concentrations of ANS in the aqueous phase. This was taken to indicate that membranes from both lipids bind the same number of ANS molecules. However one must keep in mind that the quantum yield of bound ANS may be different for lipids with different polar head groups.

Addition of different proteins to ANS labelled black lipid membranes caused different fluorescence changes (see Figs 1 and 2). By control experiments we excluded that the effects were due to a change of the ANS fluorescence in the aqueous phase. This control is much easier for cytochrome c, lysozyme and protamine than for other proteins (like bovine serum albumin [18]) that enhance fluorescence in water considerably. It has been shown [2] that PI-membranes electrostatically bind a monolayer with 10^{13} cytochrome c molecules per cm^2 and PC-membranes bind at least 20 times less cytochrome c. The present study gives evidence that the same number of ANS molecules is bound to PI- and PC-membranes as discussed above. In both types of membranes half of the ANS fluorescence was quenched maximally by cytochrome c addition at $ANS_{water} = 3 \times 10^{-5}$ M. At $ANS_{water} = 3 \times 10^{-6}$ M cytochrome c did still quench half of the ANS fluorescence in PI-membranes but only 20% of the ANS fluorescence in PC-membranes. Our interpretation of these findings is that at high ANS-concentrations sufficient negative charges are introduced into PC-membranes to bind a monolayer of cytochrome c. At low ANS-concentrations considerably less cytochrome c is bound. This interpretation is supported by experiments with Dansyl-PE [9] in BLM and with 12-(9-anthroyl) stearic acid [14] in lipid dispersions. In both cases strong quenching by cytochrome c was found in the presence of negatively charged lipids (like PI or cardiolipin) and weak quenching when only PC was present. This difference between zwitterionic and negative lipids is also observed at low ANS concentrations but not at high ANS concentrations as described above. The ANS concentration obviously affects the interaction between the lipid membrane and proteins. This is a disadvantage of ANS in addition to the well known facts that it is difficult to decide whether it binds to the lipid or to the protein portion of membranes [3] and its relatively high solubility in water [12]. Similar effects are expected with other charged and water soluble fluorescence markers. Lipid soluble probes or fluorescence-labelled lipids are more reliable for this reason.

Concerning the quenching mechanism, we believe that energy transfer to the nonfluorescent porphyrin ring of cytochrome c occurs, as has been suggested in similar cases [14, 15]. The alternative interpretation that the number of membrane bound ANS molecules changes is unlikely for various reasons. Increasing the cytochrome c concentration above 2×10^{-7} M did not change the fluorescence further. The fluorescence changes during adsorption and desorption of cytochrome c were faster than after adding ANS (see Fig. 1). Lysozyme which is similar in size, shape and charge should be equally bound by negatively charged membranes. No quenching of ANS fluorescence is possible because it does not carry a group absorbing in the visible spectral range. The slight fluorescence increase in the presence of lysozyme may be due to a small polarity change in the

surrounding of bound ANS. The big fluorescence increase after addition of protamine obviously has other reasons. This highly positively charged molecule interacts more strongly with the BLM. Increased ionic strength does not inhibit the interaction completely. The conductivity of the membrane is increased by a factor of 3 or more. If Dansyl-PE was used as a probe, protamine addition did not change the fluorescence. The most likely explanation of the fluorescence increase after adding protamine and ANS to a BLM is binding of additional ANS molecules to the membrane.

The polarization measurements show that ANS mobility and (or) orientation in the lipid membrane are not significantly changed during adsorption of cytochrome c, lysozyme and protamine.

To summarize the main points this study shows that ANS can be used as a probe for binding of proteins to black lipid membranes. The fluorescence of membrane-bound ANS is changed qualitatively similar as fluorescence of ANS in water by addition of three different proteins, but the effects are strongly enhanced. ANS contentrations in water above 3×10^{-6} M can affect the interaction between proteins and lipid membranes which is a disadvantage of this probe.

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