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THE EFFECT OF PHOSPHOLIPASE A UPON THE INTERACTION OF 1-ANILINONAPHTHALENE-8-SULFONATE WITH ERYTHROCYTE MEMBRANES

E. WEIDEKAMM, D. F. H. WALLACH AND H. FISCHER
Max-Planck-Institut für Immunbiologie, Freiburg (Germany)
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SUMMARY

1. Phospholipase A alters the lipid-protein interactions in erythrocyte membranes as reported by the fluorescent probe, 1-anilinonaphthalene-8-sulfonate (ANS).

2. The ANS-binding parameters change markedly following the action of this enzyme.

3. Our polarisation measurements suggest that the probe is bound to membrane proteins before and after phospholipase A treatment.

4. The split products of phospholipase A action can be extracted with fat-free bovine serum albumin, revealing new ANS-binding sites.

INTRODUCTION

Biomembranes mediate numerous critical functions, demanding a high degree of organization among the participating molecules. A real understanding of the interrelationships involved rests upon a precise knowledge of membrane structure, but, to obtain this, the membrane, or at least the functional domain involved, must be intact. For this reason modern spectroscopic methods are being applied increasingly to study membranes under conditions of minimal perturbation. Optical activity measurements, as well as infrared and proton magnetic resonance spectroscopy have been widely applied. They point to hydrophobic interactions between membrane proteins and lipids and are also consistent with a high, overall helicity in the protein moiety[1–5].

In studying the dynamics of certain membrane phenomena, paramagnetic and fluorescent "probes" are being used increasingly[6–10]. The fluorescent "probes" generally employed are small, apolar molecules, which can penetrate to and/or into the membrane. When the physical properties of their microenvironments change, this is reported by characteristic alterations of the fluorescence spectra. Fluorescent "probes" possess the additional advantage that many of their binding parameters can be very sensitively determined on intact membranes, under varied conditions[12–14].

In the present study we use 1-anilinonaphthalene-8-sulfonate (ANS) to provide information as to lipid-protein interactions in erythrocyte membranes, measuring the

Abbreviation: ANS, 1-anilinonaphthalene-8-sulfonate.

number and affinity of its binding sites, as well as its polarisation, as a function of temperature and other environmental variables and after the action of phospholipase A. The data suggest, as do refs. 11, 15–17, that the probe binds principally to membrane proteins.

MATERIALS AND METHODS

Membranes were prepared as in ref. 15 and studied in 0.1 M Tris–HCl, pH 7.4. Normally oriented and inside-out membrane vesicles were obtained as in ref. 18. ANS was purified as in ref. 15. Bovine serum albumin (Behringwerke, Marburg/Lahn, Germany) was defatted when necessary, using activated charcoal[19]. ^{125}I-labelled bovine serum albumin was prepared as in ref. 20. To saturate bovine serum albumin with oleic acid, 7 mg of the protein in 3 ml 0.1 M Tris–HCl, pH 7.4, was covered with a 1 ml heptane layer, containing 1 μmole oleate and 17.5 nmoles [1-^{14}C]oleate (160000 counts/min), incubated at room temperature for 24 h and the aqueous phase isolated. Experiments with ^{125}I-labelled bovine serum albumin indicate that this adsorbs to the membranes, accounting for about 2 % of the protein in the membrane pellet, after 4 washes.

Phospholipase A was purified from the venom of *Naja naja* (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as in ref. 21. 5 μg enzyme protein was added to 100 μg membrane protein in 0.1 M Tris–HCl, pH 7.4 and 1 mM $CaCl_2$ and the mixture incubated at 25° for 30 min. Controls were treated identically, but without enzyme. Phospholipid analyses on treated and untreated membranes were on lipid extracts prepared as in ref. 22, separated by thin-layer chromatography on CM-cellulose–silica gel, using chloroform–methanol–water (65:40:10, by vol.). Lipid components were visualized by exposure to I_2 vapor, scraped off and their phosphorus content determined as in ref. 23.

Removal of the split products of phospholipase A action was as in ref. 24: Treated and control membranes were incubated for 10 min at 25° in 2 % defatted bovine serum albumin in 0.1 M Tris–HCl, pH 7.4, and then washed 4–5 times in the buffer (Spinco L2-65 ultracentrifuge, rotor Ti50, 20000 rev./min, 20 min). Thereafter the supernatants were free of fluorometrically[28] detectable protein. None of the phospholipase A remains membrane associated; all of the initial enzyme activity is recovered in the supernatants and the washed membranes show no phospholipase A activity.

Fluorescence was measured in a Perkin–Elmer spectrophotofluorometer (MPF 2A). For polarisation studies the Perkin–Elmer attachment was used together with ultraviolet filter 25. Anomalies due to light polarization by the diffraction gratings were corrected as in ref. 25. Our polarisation measurements on erythrocyte "ghosts", or vesicles derived therefrom (50 μg protein per ml in 0.1 M Tris–HCl or phosphate, pH 7.4) but without ANS, yielded unrealistic p values at 480 nm, upon excitation at 290–380 nm. This was not seen with solvents alone, but even at narrow bandwidths and with ultraviolet barrier filters in the excitation beam, the values were never less than 0.51. The anomaly was detected only at higher instrumental sensitivities than used for measurement of ANS polarisation. Addition of sucrose to 2 M lowered the backround p value to less than 0.2.

We consider this effect to be due to stray scattered light, polarized by the grat-

ings of the MPF 2A instrument and have not been able to fully obviate it; the phenomenon does not account for the ANS p values reported herein.

Electrophoresis of treated and untreated membranes in sodium dodecyl sulfate polyacrylamide gels was as in ref. 26. ANS binding sites were determined as described by SCATCHARD[27]. The proportion of bound ANS was determined fluorometrically, exciting at 380 nm and reading emission at 480 nm. This approach was confirmed by measurement of free ANS, using Centriflo Membrane Ultrafilters CF50A (Amicon Co., Lexington, MA., U.S.A.) and centrifuging for 15 min at 900 × g. In these experiments, the ANS in the filtrate was determined after addition of 96 % ethanol, exciting at 380 nm, reading fluorescence at 480 nm, and employing a suitable calibration curve.

The protein concentration of membrane suspensions was assayed fluorimetrically[28].

RESULTS

ANS binds to several apolar domains in membranes. The dye fluoresces minimally in water, but does so strongly when membrane associated. This phenomenon, which is accompanied by an emission blue shift of about 70 nm, permits determination of the proportion of ANS molecules bound hydrophobically to the membrane (*eg.* ref. 29).

Fig. 1 shows the fluorescence intensity of the ANS-membrane complexes as a function of ANS concentration; the resulting saturation curve is a measure of the

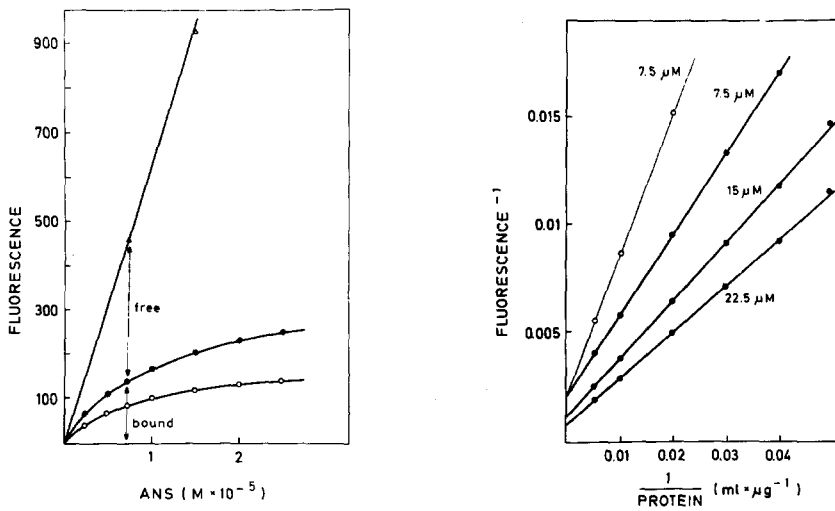

Fig. 1. Titration of erythrocyte ghosts with ANS: 56 μg membrane protein per ml; Tris–HCl (0.1 M, pH 7.4); 1 mM $CaCl_2$. Excitation at 380 nm; emission at 480 nm; excitation and emission bandwidths, 5 nm. Fluorescence in arbitrary units. ●—●, membrane; ○—○, membrane + phospholipase A (3 μg/ml).

Fig. 2. Determination of saturation fluorescence intensity by titration of given ANS concentration with membrane protein. Plot of 1/fluorescence *vs.* 1/membrane concn. for normal and phospholipase A-treated ghosts. Buffer and instrumental conditions as in Fig. 1. ANS concn.: $7.5 \cdot 10^{-6}$ M; $1.5 \cdot 10^{-5}$ M and $2.25 \cdot 10^{-5}$ M. ●—●, membrane; ○—○, membrane + phospholipase A.

bound ANS. In parallel experiments a constant amount of ANS was titrated with increasing amounts of membrane. Using the double reciprocal plot, 1/fluorescence *vs.* 1/membrane concentration, a straight line is obtained (Fig. 2). By extrapolation, the intercept of 1/fluorescence at the ordinate yields the maximal fluorescence (when all the ANS is membrane-bound). The difference between these maximal values and those obtained at the same ANS level (Fig. 1), yields the values for free ANS at each level of the dye. Knowing the proportions of free and bound ANS, we used the Scatchard technique to determine the number of ANS molecules bound per unit mass of membrane protein under various conditions.

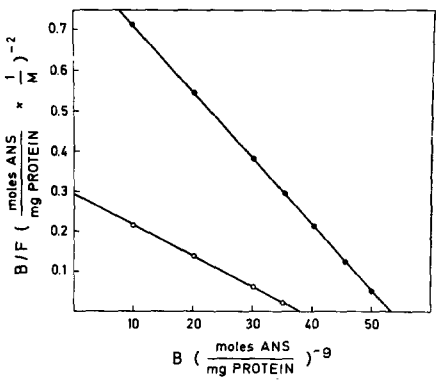

Fig. 3. Scatchard plot for the interaction of ANS with erythrocyte ghosts. Bound ANS, B, (from Fig. 1) in moles ANS bound per mg protein and free ANS, F, (from Fig. 1) in moles/l. ●—●, membrane; ○—○, membrane + phospholipase A.

Fig. 3 illustrates the relation between moles of ANS bound per mg membrane protein (abscissa) and (l/moles free ANS·moles bound ANS/mg membrane protein), (ordinate). The points yield a straight line, intersecting the abscissa at n. The slope of the line gives the average binding constant, K_A for the ANS–membrane complexes. The free energy of binding, ΔF, is obtained from the equation: $\Delta F = RT \ln K_A$. We found identical binding values, using the centrifugal technique. Our values for n, the number of binding sites, K_A and ΔF, computed from six individual experiments, are given in Table I.

TABLE I

ANS–STROMA INTERACTION

Data are mean ± S.D.

	Binding sites (moles ANS/mg protein × 10⁻⁸)	*Binding constant (M^{-1} × 10⁵)*	*Free energy (ΔF) (kcal/mole)*
Erythrocyte membranes	5.3 ± 0.32	1.64 ± 0.17	−7.0 ± 0.73
Erythrocyte membranes after phospholipase A treatment			
without bovine serum albumin	3.8 ± 0.33	0.76 ± 0.11	−6.5 ± 0.94
with bovine serum albumin	7.5 ± 0.42	2.48 ± 0.30	−7.2 ± 0.87

Under our conditions of phospholipase A treatment, 50–70 % of the membrane lecithin was converted to lysolecithin and 70–95 % of the cephalin to lysocephalin, but we find no electrophoretic indication of peptidolysis. Enzyme treatment effects a 50 % decrease in the fluorescence of the ANS–membrane complex; in contrast, similar handling of sonicated lecithin reduces the fluorescence intensity by only 30 %. However, as shown in Fig. 2, the maximal fluorescence of phospholipase A-treated membranes is the same as that of the controls. The diminution of fluorescence intensity is thus not due to a change in quantum yield, but to a decrease in the number of binding sites, n, together with a lowering of the affinity constant and a change in the free energy of association to —6.5 kcal/mole (Table I).

The phospholipase A-induced changes can be reversed by treating the membranes with defatted bovine serum albumin (and correcting for the ANS binding by residual bovine serum albumin). However, our results are not explained by displacement of ANS through enzyme-generated fatty acids, since equilibration of the membranes with oleate-saturated bovine serum albumin yields a maximal fluorescence decrement of only 30 %. Other products of phospholipase A action must therefore be involved (Fig. 4).

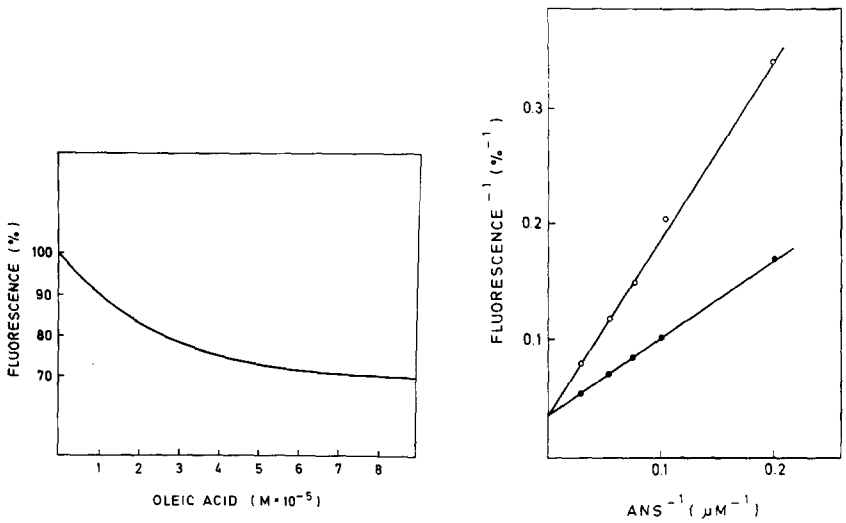

Fig. 4. Decrement of ANS fluorescence upon addition of oleate: The fluorescence without oleate was set at 100%. The protein concentration was 60 μg/ml with $1.5 \cdot 10^{-5}$ M ANS. Inner filter effects (due to oleate turbidity) were corrected. Excitation at 380 nm; emission at 480 nm. Excitation bandwidth, 7 nm; emission bandwidth, 8 nm.

Fig. 5. Plot of 1/(fluorescence decrement at 338 nm) *vs.* 1/ANS concn. Each point was corrected for the absorption of light by ANS at 286 nm and 338 nm at the concentrations employed. The intercepts on the ordinate yield a measure of the maximal energy transfer from membrane tryptophan to bound ANS. Buffer as in Fig. 1. Membrane protein concn., 30 μg/ml; phospholipase A concn., 3 μg/ml. Excitation at 286 nm and emission at 338 nm. Bandwidths, 4 nm. ●—●, membrane; ○—○, membrane + phospholipase A.

As shown in Fig. 5, phospholipase A action does not detectably influence energy transfer from membrane tryptophans to ANS; the quenching limit of tryptophan fluorescence, determined as in ref. 15, is 26 % in both treated and normal membranes.

Kinetically, "inside-out" membrane vesicles show an increase in ANS fluorescence for about 3 min after addition of the dye (Fig. 6), which is in contrast to the normally oriented erythrocyte membrane vesicles. However, after phospholipase A treatment, both membranes show the same kinetic behavior.

The polarisation of membrane-associated ANS changes similarly with temperature as do the ANS complexes of bovine serum albumin and Electroplax membranes[17]. This is illustrated in Fig. 7; unexpectedly, the p values for phospholipase A-treated membranes are greater than those of the controls. Additional information about the location of the ANS binding sites comes from the relation of ANS-polarisation to medium viscosity, varied by adding sucrose to membrane suspensions up to 2 M. A PERRIN[30] plot of $1/p$ *vs.* T/η at 23° (Fig. 8) reveals no viscosity dependence of

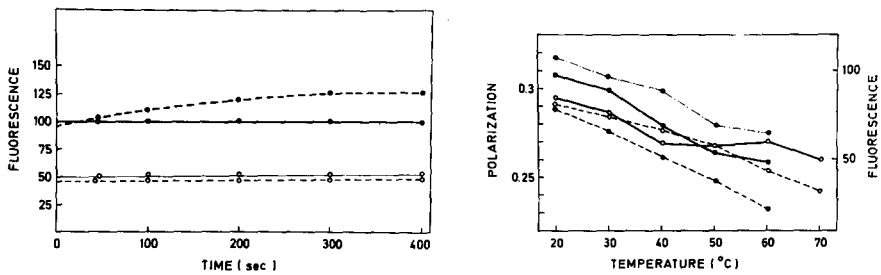

Fig. 6. ANS-binding rates for normally oriented and inverted erythrocyte membrane fragments. Medium as in Fig. 1. Excitation and emission 380 and 480 nm, respectively. Bandwidths, 4 nm. 40 μg membrane protein per ml and 4 μg phospholipase A per ml. ●---●, inverted; ○---○, inverted + phospholipase A; ●—●, normally oriented; ○—○, normally oriented + phospholipase A.

Fig. 7. Variation of fluorescence intensity and polarization with temperature: 35 μg membrane protein and 200 μg fat-free bovine serum albumin per ml. The ANS concentrations for membrane and bovine serum albumin were 17.5 and 2.5 μM, respectively. The membranes and bovine serum albumin were excited at 380 and 378 nm, respectively. Fluorescence emission was read at 480 nm in the case of membranes and 465 nm in the case of bovine serum albumin. The bandwidths were 6 nm. Polarization: ●—●, membrane; ●-·-●, membrane + phospholipase A; ●---●, bovine serum albumin. Fluorescence intensity: ○—○, membrane; ○---○, bovine serum albumin.

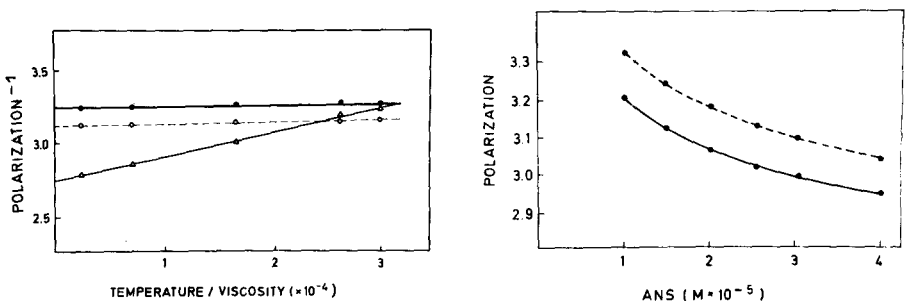

Fig. 8. Double reciprocal Perrin plot relating polarization of bound ANS to medium viscosity at 20°. Instrumental settings as in Fig. 7. Protein, 40 μg/ml, with 18 μM ANS; bovine serum albumin, 10 mg/ml, with 10 μM ANS. ●—●, membrane; ○--○, membrane + phospholipase A; △—△, bovine serum albumin.

Fig. 9. Dependence of ANS polarization upon ANS concentration. 0.1 M Tris–HCl, pH 7.4; membrane concentration, 60 μg/ml; excitation at 380 nm; emission at 480 nm; bandwidths, 8 nm. ●—●, membrane; ●--●, membrane + phospholipase A.

fluorescence polarisation before or after phospholipase A treatment; in contrast, bovine serum albumin showed the relationship described in ref. 17. Polarisation diminishes with rising ANS concentration (Fig. 9), suggesting a concentration-dependent migration of the excited state between binding sites. The polarisation of ANS in sonically dispersed lecithin (0.19) and lysolecithin (0.21), both in 0.1 M Tris–HCl, pH 7.4, is considerably less than in the membranes; there we typically obtain a p value of 0.31, at an ANS concentration of $1.75 \cdot 10^{-5}$ M and a membrane protein concentration of 35 μg/ml.

DISCUSSION

The present data are in accord with our earlier energy transfer studies[15] and indicate that in erythrocyte "ghosts" ANS is associated primarily with the membrane proteins. Such also appears to be the case for the membranes of the Electroplax electric organ[17,31,32] and for sarcoplasmic reticulum[14].

Our studies with phospholipase A suggest that the architecture of some membrane proteins depends upon the nature and state of membrane lipids, and that not all of these two membrane components can change state independently, as suggested by GLASER *et al.*[33]. Rather, our data fit those of GORDON *et al.*[21], whose optical activity spectra indicate that the action of phospholipase A on erythrocyte membranes reduces the overall helicity of the membrane proteins, *i.e.* that the conformations of some peptide linkages in membranes depend upon the state of certain membrane phosphatides. The fact that phospholipase A does not change the quantum yield of bound ANS, but markedly alters its binding parameters, is consistent with this view.

The lysophosphatides and fatty acids generated by the action of phospholipase A remain membrane associated[14,24]. According to HASSELBACH AND HEIMBERG[14], the fluorescence decrement following the action of phospholipase A, is due to displacement of ANS from its normal binding sites by newly formed fatty acids and can be reversed by their extraction with defatted bovine serum albumin. In the case of erythrocyte "ghosts", too, application of this method markedly augments the ANS fluorescence of treated membranes. About 20% of this bovine serum albumin effect is due to its adsorption on the membranes. The major action of bovine serum albumin must, however, be other than that of extracting saturated fatty acids, because oleate-saturated bovine serum albumin increases the fluorescence identically in normal and phospholipase A-treated "ghosts", in an effect, which can be attributed to the binding of ANS by residual, membrane-associated bovine serum albumin. Preliminary studies further indicate that lysolecithin is also not primarily responsible for the fluorescence decrement. Our data are consistent with the argument that phospholipase A action generates new binding sites, which become accessible to ANS after removal of certain split products of enzyme action; however, the substance(s) involved remain to be identified.

We consistently obtain relatively high polarisation values for membrane-bound ANS. The data are in accord with those on Electroplax membranes[17,31,32], but do not fit with the p values previously reported for erythrocyte membranes[29]. The basis for the discrepancy is unclear, but it may relate to our method of membrane preparation, which avoids the action of leukocyte proteases on erythrocyte "ghosts"[26,35]; this matter is under study.

The high p values of the ANS bound to our membrane isolates, and their independence of solvent viscosity, indicate that the probe is situated relatively rigidly, deeply within the membrane; it cannot be exclusively located in a "fluid" lipid phase, as suggested elsewhere[34], nor does it move into such after phospholipase A treatment. Quantitative estimates of probe "mobilities" clearly require measurement of fluorescence life times.

The asymmetry of the erythrocyte membrane demonstrated by other methods[18,35], is also expressed in the different ANS-binding rates of normally oriented and "inside-out" membranes[18]; however, the changes wrought by phospholipase A treatment appear to block the slowly reacting sites.

ANS was originally assumed to be a probe for lipid regions in membranes (*e.g.* refs. 8 and 29). However, our new data support our previously reported energy transfer studies[15], as well as those of BROCKLEHURST *et al.*[36], and the arguments detailed in refs. 14, 15, 17, 31 and 32, that ANS reports primarily from the apolar domains of membrane proteins.

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