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FLUORESCENCE STUDIES OF THE BLOOD PLATELET MEMBRANES ASSOCIATED WITH FIBRINOGEN

M.A. KOWALSKA and C.S. CIERNIEWSKI

Department of Biophysics, Institute of Physiology and Biochemistry, Medical School in Lódź, 90-131, Łódź (Poland)

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To follow microviscosity changes in membranes associated with fibrinogen binding to human platelets, specific fluorescent probes were used and their fluorescence anisotropy was analysed. The degree of fluorescence anisotropy of diphenylhexatriene, anilinonaphthalene sulfonate (ANS) and fluorescamine increased significantly when fibrinogen reacted with its membrane receptors. Fluorescence polarization analyses showed that fibrinogen binding to platelet membranes is accompanied by an increase in the membrane lipid rigidity. On the other hand, changes in the fluorescence anisotropy of membrane tryptophans and *N*-(3-pyrene)maleimide suggest augmented mobility of the membrane proteins. The binding of fibrinogen to the membrane receptors is not accompanied by any change in the fluorescence intensity of ANS attached to the membranes. This may suggest that covering of platelets with fibrinogen molecules does not influence the surface membrane charge.

Introduction

Platelet membrane vesicles contain specific receptor for fibrinogen molecules which, in contrast to the intact platelets, do not require ADP to become active [1]. Isolated platelet membranes may thus serve as a simple system for analysis of the interaction of fibrinogen with the receptors. In principle, mobility of the fibrinogen receptors is controlled by the local microviscosity and plasticity properties of the membrane. They are determined mainly by the composition of the cell surface lipids and their distribution pattern. To follow microviscosity changes of the platelet membranes, the fluorescence polarization analysis of the fluorescent probes may be a very useful and sensitive technique [2–4]. The fluorescent probes can be embedded into different regions of the platelet membranes, i.e., to the surface lipid layer, to the lipid core, at the lipid-protein contacts or attached to the membrane proteins. Thus, any changes in microviscosity of these membrane regions can be monitored by fluorescence properties of the attached probe. In addition, we employed here ANS (1-anilino-8-naphthalene sulfonate), an anionic fluorescent probe, which is known to be an indicator of changes in the membrane charge [5,6]. ANS binds both to the membrane proteins and lipids. It appears to be a specific probe for polar/nonpolar interfaces, particularly at lipid/protein, lipid/water, or lipid/protein/water contacts [7].

Materials and Methods

Platelets, platelet membranes, and fibrinogen preparation. Platelets were isolated from human blood freshly collected into acid-citrate/dextrose by differential centrifugation and gel filtration [8,9].

Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate.

After removal of the platelet-rich plasma by a 20 min centrifugation at $200 \times g$ the platelet were sedimented for 20 min at $1000 \times g$ and washed twice with the modified tyrode buffer (140 mM NaCl/5 mM KCl/10 mM glucose/15 mM Tris-HCl (pH 7.4)). The resulting pellet was resuspended in 1 ml of the platelet-poor plasma and applied on a Sepharose 2B column (1×30 cm), equilibrated with the above buffer. The platelets were counted and its final concentration ranged from $2 \cdot 10^9$ to $5 \cdot 10^9$ cells per ml.

Platelet membranes were isolated according to the method of Barber and Jamieson [10]. In order to avoid proteolytic degradation, all buffers contained 10 mM benzamidine and 10 mM ϵ-aminocaproic acid. Protein composition of the platelet membranes was the same as described previously [11].

Fibrinogen was prepared by cold ethanol precipitation followed by ammonium sulfate fractionation at 26% saturation and 4°C [12]. Protein concentration was determined spectrophotometrically [13].

Labelling of platelets with ANS. 1-Anilino-8-naphthalene sulfonate, magnesium salt (ANS), purchased from Aldrich-Europe, was diluted in the Tris buffer describe above. Platelet suspension ($2 \cdot 10^9$ platelets per ml) was incubated for 1 h at room temperature with 4 vol. of 2.5 mM ANS solution [14]. Free ANS was removed by two cycles of washing with the above buffer.

Labelling of the platelet membranes with fluorescent probes. $4 \cdot 10^{-4}$ M 1,6-diphenyl-1,3,5-hexatriene in dimethylformamide was diliuted 100-fold with a vigorously stirred aqueous solution of Tris-HCl buffer (pH 7.4). After addition of an equal volume of membrane suspension (400 μg membrane protein per ml), the mixture was incubated for 2 h at 37°C [4]. ANS was dissolved in the above buffer and used at a final concentration of 40 μM. Small aliquots of *N*-(3-pyrene)maleimide (Fluka) dissolved in ethanol and fluorescamine (4-phenylspirofuran-2(3H)-1′-phthalan-3,3′-dione) in acetone were added to a final concentration of 50 μM or 500 μM, respectively. The membrane suspensions (200 μg membrane protein per ml) were incubated with these fluorescent probes for 1 h at room temperature.

Fluorescence measurements. Fluorescence measurements were made using a Perkin-Elmer MPF-3L fluorescence spectrophotometer. Excitation and emission wavelengths were: (a) 360 and 440 nm for diphenylhexatriene; (b) 380 and 480 for ANS; (c) 390 and 480 for fluorescamine, (d) 345 and 380 for *N*-(3-pyrene)maleimide, and (e) 295 and 333 nm for protein tryptophan fluorescence, respectively. In each experiment control samples of the fluorescent probe solutions and the unlabelled membranes were examined according to Radda [15], and corrections for the scatter contribution (approx. 2–5% of the total fluorescence) were made. Fluorescence intensities were measured with the analyzer and polarizer oriented parallel and perpendicular to the direction of the polarized excitation beam. From these measurements the fluorescence polarization (P) and anisotropy (r) were calculated according to the equation:

$$P\frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}, \qquad r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where: I_{VV} and I_{VH} are the fluorescence intensities measured with a vertical polarizer and analyzer mounted vertically and horizontally, respectively. $G = I_{HV}/I_{HH}$ is the correction factor [16]. The interrelationship of P and r is obvious; henceforth we employ only the r parameter in this paper.

To study the fluorescent probe mobility, the rotational rate (R) of the probe can be calculated applying the Perrin-Weber equation [17]. Due to technical limitations, investigation of the fluorescence lifetime of the probes in the platelet membranes could not be carried out. For these reasons, observations of the fluorescent probe mobility are expressed in terms of anisotropy. In the case of diphenylhexatriene, the structural parameter of the Perrin equation could not be measured, either [18]. Thus, the microviscosity of the lipid core does not reflect absolute quantities and all the values presented below may serve for comparative purposes only.

ADP and fibrinogen binding assays. Binding of fibrinogen (0.01 μM) to platelet membranes (200 μg proteins per ml) was performed in the presence and absence of ADP (0.15 μM, Sigma), after 20 min incubation [1]. Separately, the effect of ADP was studied.

Results

Microviscosity changes in platelet membranes

In order to detect microviscosity changes in platelet membranes caused by fibrinogen and ADP, fluorescence anisotropy of the fluorescent probes embedded in different regions of the membranes was analyzed (Fig. 1).

Measurement of the fluorescence anisotropy of diphenylhexatriene incorporated in the lipid bilayer of the platelet membranes is assumed to test changes in the fluidity of the membrane lipid core. The early course of fibrinogen binding is associated with increased membrane microviscosity (Table I). Such an increased membrane rigidity usually results in a greater exposure of the membrane proteins [19]. Therefore, this effect may be important for the ADP- and fibrinogen-treated platelets.

The ANS fluorescent probe can be used to monitor the physical state of the protein-lipid interfaces in the membranes. The ANS fluorescence anisotropy was significantly enhanced after the addition of the ADP or fibrinogen to the platelet membranes (Table I).

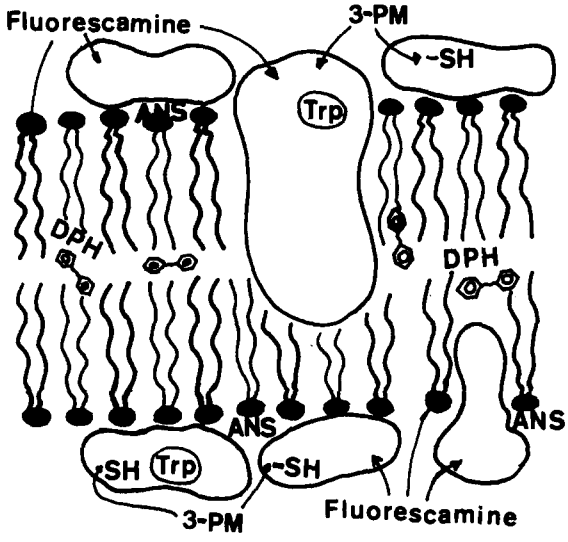

Fig. 1. Schematic representation of a platelet membrane with lipid bilayer and cell surface membrane proteins. Fluorescent probes monitor specific areas of the membrane. DPH is located in deeper portions of the hydrocarbon core, ANS monitors the lipid-protein interfaces, fluorescamine attaches to primary amines on the cell surface, and *N*-(3-pyrene)maleimide is bound to the -SH group of membrane proteins. Additionally, tryptophan (Trp) fluorescence of membrane proteins represents a parameter related to their dynamic properties.

Fluorescamine covalently binds to the primary amines of the cell surfaces, including membrane proteins, glycoproteins and phospholipids such as phosphatidylethanolamine and phosphatidylserine [20,21]. Steady-state fluorescence studies using this probe showed slightly increased anisotropy in the fibrinogen-treated platelet membranes when compared to the control. These changes were insignificant and smaller than those of diphenylhexatriene or ANS (Table I).

N-(3-Pyrene)maleimide is fluorescent when bound to the -SH group of protein, but is not fluorescent in aqueous solutions. It is commonly used in fluorescence polarization studies due to its long lifetime [22,23]. When fibrinogen was bound to the platelet membranes, the fluorescence anisotropy of *N*-(3-pyrene)maleimide indicated an increased rotational mobility as compared to the controls (Table I).

Tryptophan fluorescence of the membrane components might be also used to approach dynamic properties of the membrane proteins. There was no alteration of the fluorescence intensity of tryptophan residues in the membrane complexed with fibrinogen. However, their fluorescence anisotropy was reduced due to increased fluctuational mobility of the membrane proteins and/or their conformational changes (Table I).

The platelet membrane charge.

Fluorescence spectra and polarization of ANS are very sensitive to alteration in the microenvironment of the attached probe. ANS has a very low fluorescence quantum yield in water, but a much higher one in non-polar environments. It might thus be used to follow the membrane charge changes caused by different factors.

In order to evaluate the extent of ANS binding to the human platelet membranes, samples of the membrane suspension containing 150 μg proteins per ml were incubated with increasing concentration of ANS. The emission spectra of the membrane-bound ANS are presented in Fig. 2a. There was only a slight shift of the emission maximum, from 476 to 480 nm, accompanied by the enlargement of the fluorescence intensity when the ANS

TABLE I

FLUORESCENCE ANISOTROPY (r) OF DIFFERENT PROBES BOUND TO THE PLATELET MEMBRANES

Mean ± S.D, $n = 6$.

Fluorescent probes	Control	ADP	Fibrinogen
Diphenylhexatriene	0.176 ± 0.005	0.181 ± 0.012 [a]	0.196 ± 0.002 [b]
ANS	0.154 ± 0.012	0.176 ± 0.012 [a]	0.180 ± 0.007 [b]
Fluorescamine	0.118 ± 0.012	0.127 ± 0.010	0.131 ± 0.011 [a]
N-(3-Pyrene)maleimide	0.087 ± 0.006	0.086 ± 0.006	0.077 ± 0.005 [b]
Tryptophan	0.138 ± 0.005	0.138 ± 0.005	0.120 ± 0.005 [b]

[a] $P < 0.01$ by Student's t-test.
[b] $P < 0.001$ by Student's t-test.

concentration was increassed from 10 to 50 μM. It is noteworthy that neither ADP nor fibrinogen influenced significantly the fluorescence intensity of the membrane-bound ANS. Similarly, when the platelet membranes were replaced by intact human platelets, ADP and fibrinogen did not induce any detectable changes in the fluorescence intensity of the membrane-associated ANS. The latter experiments were performed in the absence of Ca^{2+} to avoid platelet aggregation [24].

The fluorescence intensity of ANS bound to the platelet membranes was enhanced in the presence

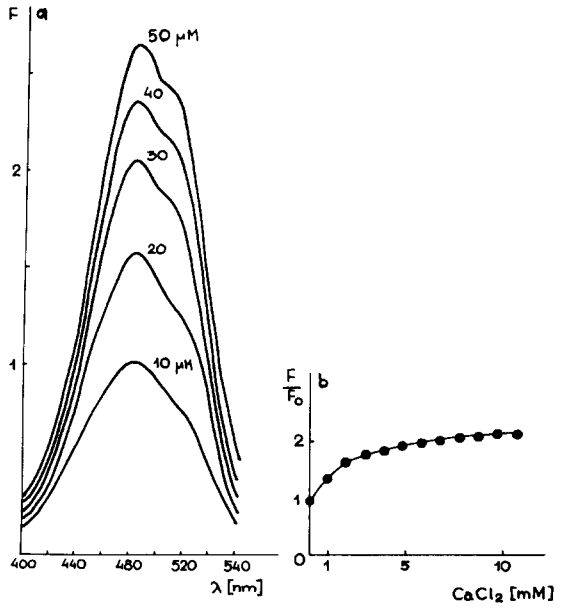

Fig. 2. (a) Fluorescence emission spectra of ANS bound to the platelet membranes at constant membrane concentration (150 μg of protein per ml). $\lambda_{ex} = 380$ nm. ANS concentration varied between 10 and 50 μM. (b) Effect of $CaCl_2$ on the enhancement of ANS fluorescence in the platelet membranes. ANS fluorescence was measured at 480 nm. $\lambda_{ex} = 380$ nm.

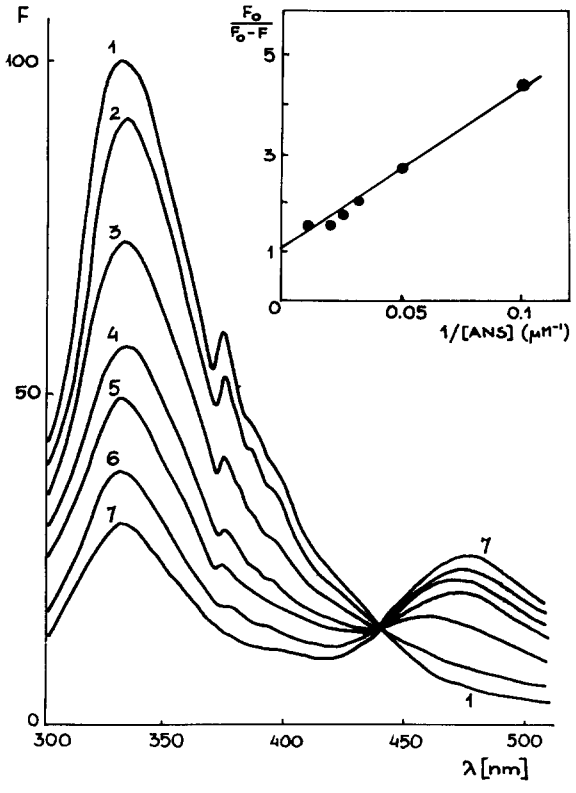

Fig. 3. Tryptophan fluorescence spectra of the platelet membranes in the absence (1) and presence (2–7) of 10, 20, 30, 40, 60 and 80 μM of ANS (membrane concentration 200 μg of protein per ml), $\lambda_{ex} = 295$ nm. Insert represents a double-reciprocal plot of the fluorescence intensity of tryptophan vs. ANS concentration. F_0 and F denote the tryptophan fluorescence in the absence and presence of ANS, respectively.

of Ca^{2+} and reached its maximum at concentration of 7 mM (Fig. 2b). In contrast to other plasma proteins, fibrinogen alone failed to bind the ANS molecules.

Energy transfer studies

Fig. 3 shows the emission spectra of the platelet membranes in the absence (curve 1) and in the presence (curves 2–7) of the increasing amounts of ANS. In the absence of ANS, fluorescence is emitted by tryptophan residues with maximum at 333 nm ($\lambda_{ex} = 295$ nm). Upon addition of increasing amounts of ANS the tryptophan fluorescence band is quenched and a second fluorescence band appears with a maximum at 476 nm. Since free ANS does not fluoresce under these conditions, the new band represents tryptophan-excited ANS fluorescence. Fig. 3 (insert) shows the double-reciprocal plot of decrease in the fluorescence intensity of the tryptophan vs. ANS concentration. The intercept on the ordinate gives the reciprocal maximal transfer efficiency corresponding to a state of complete occupation of all ANS binding sites [25]. From such a plot the maximal transfer efficiency was calculated to be 0.95. There was again no effect of either ADP or fibrinogen on the energy transfer from tryptophans to the ANS attached to platelet membranes.

Discussion

Fluorescence polarization provides a sensitive technique for the evaluation of dynamic properties of biological membranes [2–4,18,19,26,27]. This technique was applied in our study to monitor changes in microviscosity of the platelet membranes associated with fibrinogen binding. As fluorescence probes we used DPH, ANS, pyrene maleimide and fluorescamine. In previous studies they were reported to be highly efficient in measuring microviscosities of cell membranes.

In all the fluorescent probes used, the degree of the fluorescence anisotropy of DPH, ANS and fluorescamine increases significantly when fibrinogen molecules bind to their membrane receptors. Apparently, it relates directly to the microviscosity of the labelled region. These fluorescent probes are attached to different areas of the platelet membranes (Fig. 1). Hence, the microviscosity changes caused by fibrinogen binding appear to be quite significant and are detectable in different areas of the platelet membranes.

Microviscosity in biological membranes is a major physical parameter which controls lateral and rotational mobilities of membrane proteins. The increase in membrane lipid rigidity following fibrinogen binding may be important factor promoting the platelet aggregation. It has been shown that the increased membrane lipid rigidity leads to greater exposure of membrane proteins, including fibrinogen receptors. This might facilitate formation of molecular linkages between neighboring platelets. A dimeric fibrinogen molecule perfectly fits to this model as a bivalent molecule.

In addition to changes in the fluorescence polarization of the probes attached to the lipid layer, the binding of fibrinogen to the platelet membranes causes increased mobility of fluorescent probes linked to membrane -SH groups. It does suggest that binding of fibrinogen molecules to platelet receptors is associated with increased mobility of the platelet membrane proteins. This was further supported by an analysis of the platelet membrane tryptophans.

The fluorescence yield and the emission maximum prove that the microenvironment of ANS molecules attached to the membranes is much less polar than that of the hydrated surfaces. The insignificant displacement of the fluorescence maximum associated with the augmented binding of ANS to the membranes suggests that the probe is in those areas of the membrane showing the same polarity. The energy transfer measurements locate the ANS molecules in the membrane proteins or very close to them. This is supported by the observation that almost 100% of the energy is transferred from tryptophan residues to ANS. Neither ADP nor fibrinogen affected the yield of the energy transfer.

There was also no alteration in the fluorescence intensity of ANS bound to the platelet membranes in the presence of ADP or fibrinogen. This was observed both for the intact platelets and their isolated membranes. It means that the binding of fibrinogen molecules does not change the surface charge of the platelet membranes, and thus, it does not increase the binding efficiency of ANS. This observation does not support the idea that the role

of fibrinogen molecules in platelet aggregation might be to reduce the negative charge of the platelets, and thus to facilitate platelet-platelet interactions.

In contrast to fibrinogen, Ca^{2+} caused an increase in the fluorescence intensity resulting from the more efficient binding of ANS to the platelet membranes. Ca^{2+} complexed with the negatively charged regions of the membrane phospholipid molecules and partially neutralized them. This explains the enlarged number of the ANS binding sites in the platelet membranes.

In summary, our fluorescence polarization analyses showed that the reaction of fibrinogen molecules with the membrane receptors is accompanied by an increase of the membrane lipid rigidity and augmented mobility of the membrane protein components and does not influence the membrane charge.

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