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Fluorescence Anisotropy Changes in Platelet Membranes during Activation†

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ABSTRACT: Dynamic changes in platelet membrane components were evaluated by two fluorescent probes, the anion channel blocker 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) and the membrane-impermeant stachyose derivative of pyrenebutyryl hydrazide (SPBH). Fluorescence anisotropy, r, was measured in intact platelets treated with either fluorophore. Activation of platelets by thrombin, arachidonic acid, and ADP under nonaggregating conditions increased the anisotropy values of DIDS within 60–120 s. A slow return to base-line values occurred after 8–10 min. Thrombin produced an initial transient reduction of r during the first 60 s. Its effect was specific as inactivated enzyme did not induce any changes. The latter could also be prevented by omitting Ca^{2+} from the platelet suspension. Treatment of platelets with SPBH, a fluorophore inserted into the lipid leaflet of membranes, revealed an activation-induced increase of its fluorescence anisotropy during the first 120 s. It was followed by a 6–8 min lasting decline of r when thrombin and ADP were the stimulants. Preexposure of platelets to colchicine did not change significantly the fluorescence anisotropy pattern of either fluorophore, but cytochalasin B inhibited such changes almost completely. The findings are interpreted as demonstrating greater motional freedom in the lipid bilayer but a decrease in this parameter in membrane proteins upon stimulation of platelets.

Platelet aggregation is a complex process (Marcus, 1969) which is initiated by the interaction of certain agents with their specific receptor sites on the platelet surface. This sets in motion a series of events in the course of which the discoid platelet is changed to a contracted spherical cell with many pseudopodial projections. Concomitantly the platelets become sticky and adhere to each other. An internal redistribution of subcellular granules is associated with a change in the state of polymerization of structural membrane proteins and is eventually followed by release of granule contents to the surrounding medium. The conversion of the platelet from a cell with nonadhesive character to one of extreme stickiness is an amazing phenomenon with respect to both the profoundness and the rapidity of the change. We believe that the platelet transformation to a spiny sphere is not solely responsible for the change in adhesivity. Dynamic changes in platelet membrane components have been reported during platelet activation induced primarily by thrombin (Nathan et al., 1979, 1981). In these studies, however, platelets were stimulated by the aggregating agent while being vigorously agitated through continuous stirring. The resultant clumping of platelets makes it virtually impossible to determine whether changes in fluorescence polarization are due to platelet–platelet interaction or due to a positional redistribution of membrane components. For this reason, we have designed our experiments to expose platelets to aggregating agents without con-

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0006-2960/84/0423-0247$01.50/0

comitant aggregation. To study dynamic changes of membrane proteins in response to stimulation by aggregating agents we have used two fluorophores, one binding to membrane protein(s) and the other inserting itself into the lipid matrix of membranes. Our studies give clear evidence of an increase in fluorescence anisotropy during platelet activation when the protein-bound fluorophore was the probe but a decrease of this parameter with the fluorophore distributed in the lipid leaflet of membranes.

Materials and Methods

Preparation of Platelets. Pooled concentrates of plasma-suspended platelets were obtained from the Central Laboratory of the Swiss Red Cross. Red cells were eliminated by centrifugation for 1–2 min at 100*g*. The pH of the platelet suspensions was adjusted to 6.5 with acid–citrate–dextrose (U.S.P.). The platelets were then left at 37 °C for 30 min after which they were filtered through a 10 × 2 cm column of Sepharose CL-2B (Sigma Chemical Co., St. Louis, MO) which was preequilibrated with 0.05 M phosphate buffer, pH 6.8, containing 0.14 M NaCl (PBS). The column was temperature controlled at 37 °C. Generally, 5–6 mL of platelet suspension was filtered at one time. The breakthrough peak of platelets was collected and divided into two or more aliquots.

The platelets that were used for these experiments were morphologically and functionally intact. Both before and after gel filtration, the platelets were discoid as shown by phase microscopy and by a large difference in the optical transmission tracing between stirred and nonstirred platelet suspensions. The functional integrity of the platelets was evidenced by their sensitivity to aggregating agents. Plasma-suspended platelets were irreversibly aggregated by ADP in concentrations of 1–2 μM while gel-filtered platelets responded to 0.02–0.04 unit of bovine thrombin/mL (Parke-Davis Co., NJ) with maximal aggregation. Release of [^{3}H]-5-hydroxytryptamine induced by 0.04 unit of thrombin/mL from prelabeled (Hirschman & Shulman, 1973), gel-filtered platelets ranged from 78 to 84% of the total amount taken up.

These results show that the platelets obtained for our experiments from pooled concentrates were comparable in every respect to those isolated from freshly drawn blood.

Measurement of Fluorescence Polarization. Platelets suspended in PBS, pH 6.8, were treated with 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) (Sigma Chemical Co., St. Louis, MO) dissolved in 0.05 M phosphate buffer, pH 8.0, or with the stachyose derivative of pyrenebutyryl hydrazide (PBH) (Molecular Probes, Inc., Junction City, OR). Their respective concentrations were usually 0.2 mM for DIDS and 0.1 mM for SPBH. Controls were incubated with a volume of these buffers equal to that of the fluorophore solutions added to the test samples. After a 15-min incubation at room temperature, the platelets incubated with SPBH were washed with PBS, pH 6.8, by centrifugation at 1000*g* for 12 min and were then resuspended in PBS, pH 7.3. Platelets that were incubated with DIDS were washed with 0.05 M Tris-HCl[1] buffer, pH 6.8, containing 0.14 M NaCl (TBS). This was repeated with TBS containing 0.5% bovine serum albumin. After this the platelets were resuspended in PBS, pH 7.3. The platelets, whether incubated with DIDS or SPBH, were counted, and their concentration was adjusted to 0.25 × 10^9/mL. Ca^{2+}, 60 μM, apyrase, 0.07 unit/mL, and D-glucose, 1 mg/mL, were added, and the resultant suspensions were left at 37 °C until fluorescence polarization could be measured. This was usually within 60–120 min. DIDS and SPBH at concentrations up to 0.25 and 0.15 mM, respectively, did not inhibit platelet aggregation induced by thrombin or arachidonic acid. Platelets pretreated with 0.4 mM DIDS, however, failed to aggregate with ADP and exhibited marked inhibition of aggregation with thrombin and arachidonic acid.

When the concentration of DIDS was increased from 0.2 to 0.4 mM, the platelets did not show the typical changes in fluorescence anisotropy that were found after stimulation with thrombin and other aggregating agents. The changes that were observed were of lesser degree but, in general, were similar to those obtained with 0.2 mM DIDS. Treatment of platelets with ≤0.15 mM DIDS yielded fluorescence intensities that were too low for reproducible results.

Steady-state fluorescence polarization measurements were made on a Perkin-Elmer fluorospectrophotometer (Model MPF-3L) equipped with a xenon lamp. When DIDS fluorescence was measured, the samples were excited with light of wavelength of 360 nm while emission was measured at 440 nm. On the other hand, SPBH-labeled platelets were excited with light of wavelength 344 nm, and emission was measured at 396 nm. Cutoff filters of 380 nm for the former and 370 nm for the latter fluorophore were placed in the emission light path. The emission light intensity was determined on a digital voltmeter. All readings on test samples were corrected by subtracting the voltmeter readings of control platelet suspensions. These were of identical concentration and preparation as the test samples except for the omission of the respective fluorophore. The light intensities of the fluorophore-containing samples exceeded those of the respective controls by 38–45%.

Evaluation of Fluorescence Studies. Fluorescence intensities were measured at 25 °C parallel and perpendicular to the direction of the polarized excitation beam. Four relative intensities were recorded for each sample, I_{VV}, I_{VH}, I_{HH}, and I_{HV}, whereby the first subscript signifies the direction of the plane polarized excitation light and the second the position of the polarizer through which emission was detected. From these measurements the fluorescence anisotropy, r, was calculated according to the equation

$$r = \frac{(I_{VV} - I_{VH})\sigma}{(I_{VV} + 2I_{VH})\sigma}$$

in which $\sigma = I_{HV}/I_{HH}$.

Total fluorescence intensity $F = I_{\parallel} + 2I_{\perp}$ of DIDS- and SPBH-treated platelets showed a 0.5–2.0% decrease over a 15-min period after addition of platelet activators. This was found to be due to settling of platelets during the time interval of fluorescence measurements. Platelet suspensions that were not treated with activators but simply inverted ×2 at time zero exhibited a similar decrease in fluorescence intensity. Differences in fluorescence anisotropy between base-line and experimental values were evaluated by Student's t test (Goldstein, 1964).

Activation of Platelets. Platelet suspensions were activated by addition of bovine thrombin (Parke-Davis Co., NJ) in concentrations ranging from 0.1 to 2 units/mL, of ADP in concentrations of 5–20 μM, and of arachidonic acid in concentrations of 50 μM. Concentrations of ADP ≥5 μM were required to overcome the degradative activity of apyrase. Aggregating agents were added to both controls and test samples, and platelet suspensions were inverted twice. At this point timing was started. The first fluorescence measurement was made 30 s after inversion of the cuvette containing the platelet suspension and was continued at 2–3-min intervals for

[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

a total of 18 min. Platelets showed no visible aggregation under these conditions. This was confirmed by phase microscopy which failed to show any significant platelet clumping. An occasional (≤2/low power field) small aggregate of ≤5 platelets was seen.

Replacement of the suspension medium of activated platelets by fresh buffer did not show a decrease of the intensity of fluorescence emission when corrections for the loss of platelets due to centrifugation and resuspension were made. This finding which was obtained both with platelets prelabeled with DIDS and SPBH indicates that in our experiments the fluorescent probes are not released from platelets during the activation process.

Polyacrylamide Gel Electrophoresis. Gradient gel electrophoresis was performed according to Clemetson et al. (1979). Platelets treated with DIDS were solubilized in a solution of 0.065 M Tris-HCl, pH 8.0, containing 2 mM EDTA and 2% sodium dodecyl sulfate ($NaDodSO_4$) (solubilizing solution). The protein concentration was adjusted to 1 mg/mL. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. The gradient gels were cut into 0.5 cm wide pieces which were minced and incubated overnight in 0.05 M phosphate buffer, pH 7.0, containing 0.1% $NaDodSO_4$. The fluorescence intensity of the eluates of these fractions was measured at the maximum emission wavelength of DIDS, 445 nm, by using a photon counter attached to a fluorospectrophotometer. Background counts (102 ± 8) of gel pieces treated identically to the gel containing fluorophore-labeled platelets were subtracted.

Preparation of Stachyose Derivative of Pyrenebutyryl Hydrazide. SPBH was prepared essentially according to Cogan & Schachter (1981). After unreacted PBH was removed from the reaction product by thin-layer chromatography on silica gel plates G-100 UV254 (Brinkmann Instruments, Inc.) by using chloroform–methanol (9:1 v/v) as solvent, the plates were thoroughly dried and then developed with 1-butanol–glacial acetic acid–water (4:1:2 v/v/v). The R_f of the fluorescent SPBH product was 0.15. Elution and further purification of the oligosaccharide derivative of PBH were as described by Cogan & Schachter (1981). The pyrene:oligosaccharide ratio of SPBH was 0.87. The carbohydrate content of SPBH was measured by the phenol–sulfuric acid method (Dubois et al., 1956).

Results

Spectral Characteristics of the Fluorophores. Excitation and emission spectra for DIDS and SPBH are shown in Figure 1. In situ, i.e., in the membrane, SPBH showed a slight red shift indicative of its less polar environment there. On the other hand, DIDS did not exhibit any change in its excitation maximum when bound to platelet membranes, suggesting that it localized in a predominantly hydrophilic environment. The observed maxima for free DIDS and SPBH were 361 and 340 nm, respectively, while the membrane-bound fluorophores had maxima at 361 nm for DIDS and 346 nm for SPBH. Emission maxima occurred at 445 nm for DIDS and 379 nm for SPBH.

Distribution and Binding Sites of DIDS in Platelets. The total amount of DIDS incorporated into platelets was estimated from the optical absorbance of $NaDodSO_4$-solubilized platelets. A molar absorbance value $E = 2.65 \times 10^4$ at 360 nm was used for DIDS dissolved in phosphate buffer, pH 8.0. Platelets incubated with 0.2 mM DIDS bound 1.6×10^{-19} mol/cell. DIDS in the lipid fraction of platelets, isolated by chloroform–methanol (2:1 v/v) extraction (Okuma et al., 1971), was 7.5% of total amount of fluorophore incorporated.

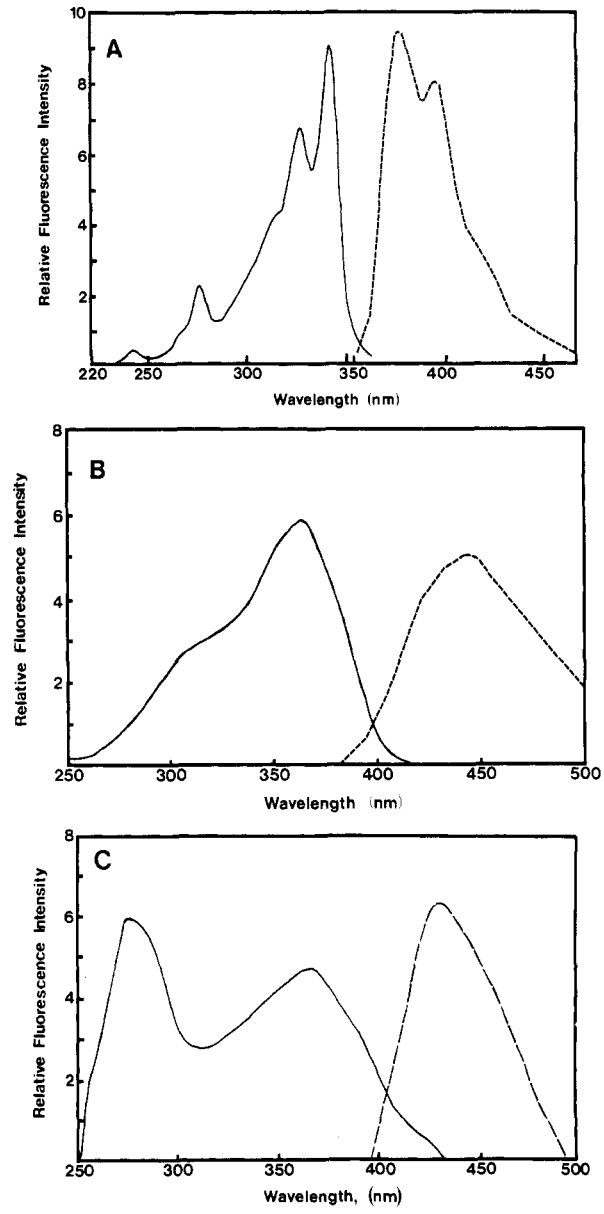

FIGURE 1: (A) Uncorrected excitation (solid line) and emission (dashed line) spectra of SPBH dissolved in PBS, pH 7.3. For recording the excitation spectrum, the fluorescence was monitored at 379 nm. The emission spectrum was measured while exciting the fluorophore at 346 nm. (B) Uncorrected excitation (solid line) and emission (dashed line) spectra of DIDS. The concentrated fluorophore dissolved in 0.05 M phosphate buffer, pH 8.0, was diluted with PBS, pH 7.3. The excitation spectrum was recorded while measuring emission at 445 nm, and the emission spectrum was recorded while exciting DIDS at 361 nm. (C) Uncorrected excitation (solid line) and emission (dashed line) spectra of DIDS-labeled platelets. Excitation and emission spectra were recorded as in (B).

To identify the DIDS binding sites, platelets were treated as for fluorescence studies. After the washing procedure, the platelet pellet was lysed with solubilizing solution and the lysate subjected to polyacrylamide gradient gel electrophoresis. Another portion of the platelet suspension was surface labeled with sodium [^{3}H]borohydride by the periodate borohydride technique (Gahmberg & Anderson, 1977). The fluorescence profile of the gel revealed one major and three minor areas of distinct DIDS fluorescence (Figure 2). The approximate molecular mass of the major protein was 100 000 daltons whereas the three minor polypeptides were estimated to have molecular masses of 204 000, 42 500, and 13 600 daltons, respectively. Together these peaks accounted for 73% of the fluorescence with the major one contributing 37%.

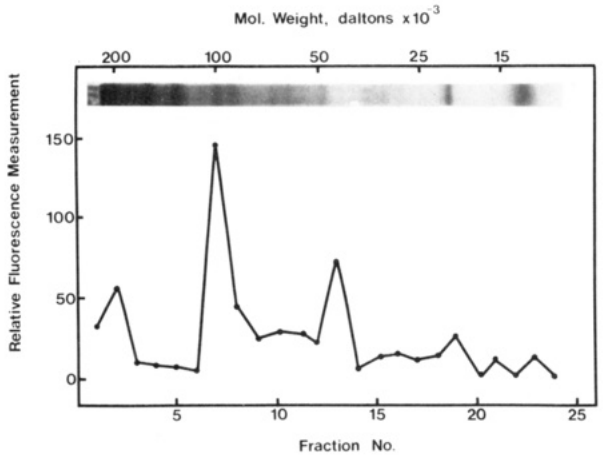

FIGURE 2: Electrophoretic pattern of fluorescent polypeptides derived from DIDS-treated platelets. Suspensions of gel-filtered platelets were incubated with DIDS. After washing with TBS and TBS containing 0.5% bovine serum albumin, the cells were prepared for gradient polyacrylamide electrophoresis. Individual gel fractions were eluted, and fluorescence was measured as described under Materials and Methods. Background counts were subtracted. Fractions are numbered from the cathodal end. A fluorographic tracing of surface-labeled platelets (see text) is shown above the fluorescence profile.

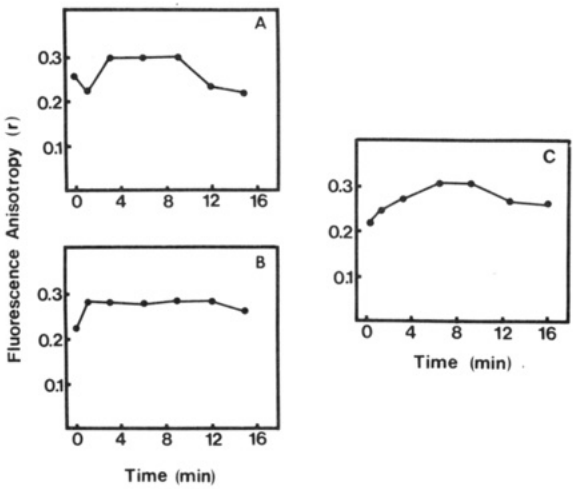

FIGURE 3: Changes in fluorescence anisotropy of DIDS in platelets exposed to thrombin, 1 unit/mL (A), ADP, 10 μM (B), and arachidonic acid, 50 μM (C). Timing was started with the addition of the platelet activators. Details of platelet preparation, probe concentration, and fluorescence measurement are described under Materials and Methods. Each point in (A) and (B) represents the mean of four separate experiments and in (C) of three experiments.

Fluorescence Studies with DIDS. In nonactivated platelets r was found to be 0.22 ± 0.012 (mean ± SD, n = 8). The addition of thrombin in concentrations of 0.5–2.0 units/mL produced a transient decrease of fluorescence anisotropy followed by a rise which persisted for 8–10 min (Figure 3A). The initial drop in the value of r was dependent on platelets having discoid shape. It occurred in all experiments in which thrombin was the stimulant. The decrease was significant ($p < 0.05$ by t test for paired observations). When platelets were subjected to conditions inducive of a shape change, e.g., small doses of ADP or vigorous centrifugation, the decrease in r occurring during the first 30–60 s was not observed when they were subsequently stimulated with thrombin. The increase in fluorescence anisotropy that followed ranged from 20 to 36% above base-line levels (0.28 ± 0.013, mean ± SD, $p < 0.001$). Beyond a certain threshold concentration (≥0.5 unit/mL) the variability in the magnitude of the increase that was observed appeared to be related to the source of the platelets, the donors,

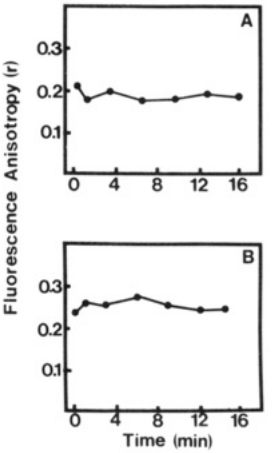

FIGURE 4: Fluorescence anisotropy of DIDS in platelets pretreated for 30 min with 10 μM cytochalasin B and stimulated by addition of thrombin, 1 unit/mL (A), and in platelets stimulated with PMSF-treated thrombin, 0.5 unit/mL (B). Timing was started with addition of thrombin or the thrombin derivative. Experimental procedures are described under Materials and Methods. Each point in (A) represents the mean of three separate experiments and in (B) of two experiments.

rather than the strength of the stimulus. ADP produced a rise in fluorescence anisotropy similar to that of thrombin (0.28 ± 0.010, mean ± SD, $p < 0.001$) (Figure 3B). Arachidonic acid gave slightly higher levels of r (0.29 ± 0.015, mean ± SD, $p < 0.001$) (Figure 3C). ADP and arachidonic acid differed from thrombin in their failure to produce the initial transient decrease in fluorescence anisotropy; 8–10 min after addition of the aggregating agent, there was a return toward the initial anisotropy values. However, the original r values were not always reached within the period of observation (15–18 min).

Platelets contain a marginal bundle of microtubules which appear to be responsible, at least in part, for their discoid shape. Disruption of these structures with colchicine did not significantly affect the thrombin-induced change of fluorescence polarization of DIDS-labeled platelets. On the other hand, cytochalasin B prevented the platelet activation-related increase in r but did not affect the initial drop in fluorescence polarization (Figure 4A).

The omission of Ca^{2+} or the addition of an excess of EDTA to the platelet suspension prevented the thrombin-induced changes in fluorescence anisotropy (0.225 ± 0.015, mean ± SD). Similarly, when thrombin was replaced by phenylmethanesulfonyl fluoride treated (PMSF) thrombin, no change in r occurred (Figure 4B).

Fluorescence Studies with SPBH. The fluorescence anisotropy value of SPBH in normal nonactivated platelets was 0.047 ± 0.003 (mean ± SD, n = 6). When platelets were stimulated by aggregating agents (thrombin, ADP, or arachidonic acid), there was a transient increase in r followed by a decrease, when thrombin or ADP were the activators, which reached a nadir between 6 and 8 min (Figure 5). During the remaining time of observation, there was a slow return toward the initial r values. With most platelet preparations these were obtained after 12–16 min.

Discussion

The two fluorophores used for these experiments labeled membrane components. DIDS which is known to have binding specificity for the anion transport protein of red cells (Cabantchik & Rothstein, 1974) was found to label one major protein in platelets. Its size was estimated to be 100 000

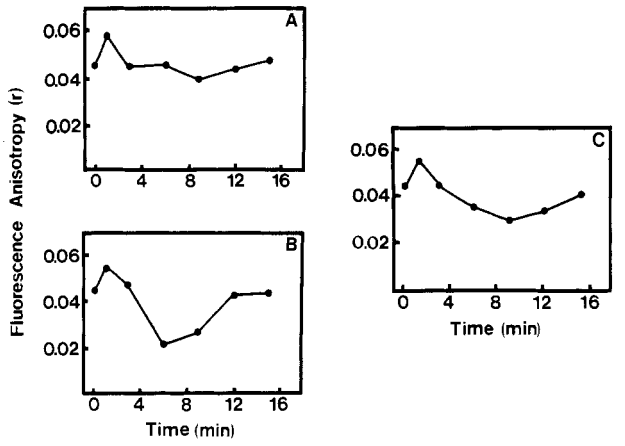

FIGURE 5: Fluorescence anisotropy of SPBH in platelets stimulated with arachidonic acid, 50 μM (A), thrombin 1 unit/mL (B), and ADP, 20 μM (C). Timing was started with addition of the platelet activators. Experimental procedures for preparation of the fluorophore, labeling of the platelets, and fluorescence measurement are given under Materials and Methods. Each point represents the mean of three separate experiments.

daltons. This represents a rough approximation which could be off by as much as ±7000. The extraction of gel pieces of 0.5-cm width for photon counting is the reason for the inability to determine the molecular weight of the primary ligand protein of DIDS more precisely.

Although specific studies to localize SPBH in platelets were not performed, previous studies by Cogan & Schachter (1981) using intact red cells and leaky ghost membranes made it clear that such membrane-impermeant fluorophores were inserted into the lipid matrix. The derivatization of the membrane-permeable fluorescent pyrene moiety with water-soluble oligosaccharide made it possible to insert such probes into the outer leaflet of intact cells (Cogan & Schachter, 1981). The fluorescence polarization values of SPBH obtained with platelets were similar to those observed in red cells. The anisotropy was close to zero, indicative of considerable mobility of the fluorophore in the platelet membrane. The r values of DIDS were relatively high compared to those of SPBH.

The expression "mobility" is used here in its broadest sense, denoting changes in the rate of rotation and/or the extent of movement of the fluorophore in the anisotropic lipid bilayer of platelet membranes. The Perrin equation which has usually been applied to describe the fluorescence anisotropy of such a probe is inadequate when its rotation is hindered (Jähnig, 1979; Heyn, 1979). A better approximation is given by the relation $r = r_\infty + (r_0 - r_\infty)[\rho_c/(\rho_c + \tau)]$ where r_0 is the maximal limiting anisotropy, r_∞ the limiting anisotropy after long time intervals in time-resolved decay experiments, ρ_c the correlation time, and τ the excited-state lifetime. Changes in r_∞ relate to changes in an order parameter. As both components may contribute to changes in r in this and similar applications, we have used the above expression of mobility to denote deviations of r, rather than "fluidity" which has been suggested to assess only changes in ρ_c (Jähnig, 1979; Heyn, 1979).

The increase or decrease of r in fluorophore-treated platelets after addition of platelet activators was not thought to be due to a change in fluorescence lifetime of the probes. Although direct measurements of such lifetimes were not performed in this study, the lack of significant deviation of total fluorescence intensity from initial base-line values argues against a change in lifetime being responsible for the observed changes in fluorescence anisotropy.

Platelet activation under nonaggregating conditions had quite different effects on proteins and lipids of platelet membranes. A rise in the fluorescence anisotropy of protein-bound fluorophores in response to exposure of platelets to aggregating agents has been noted by other investigators by using thrombin as the stimulant and subjecting platelets to continuous agitation (Nathan et al., 1981). Our studies confirm this. In addition, we were able to dissect the initial events following the activation of platelets by thrombin. Presumably because of the omission of stirring and the consequent absence of overt platelet clumping, we could observe that thrombin initially, i.e., within the first 60 s, induces a decrease in the fluorescence anisotropy of DIDS which is followed by a longer lasting increase in r. The changes in anisotropy may be interpreted as a transient increase in the mobility of DIDS-binding protein(s) immediately upon binding of thrombin by platelet membranes, followed by a decrease in mobility lasting for 8–10 min. The other platelet activators that were tested in this study, arachidonic acid and ADP, did not enhance protein mobility during the initial stimulation period. At this time we do not have an explanation for this behavior. The changes in protein mobility are dependent upon a specific interaction of ligand with membrane receptor. This was exemplified by the effect of PMSF–thrombin on fluorescence anisotropy.

Activation of SPBH-treated platelets by thrombin was a virtual mirror image of the fluorescence anisotropy changes in DIDS-treated cells. The initial increase in r after addition of platelet activators was similar to that reported with 1,6-diphenyl-1,3,5-hexatriene (Nathan et al., 1979), but in our studies this effect was transient, disappearing within 120 s. We believe that the different experimental conditions under which platelets were exposed to stimulating agents without aggregation and release are responsible for the different behavior of r over more extended periods of time. The depression of fluorescence polarization that followed the immediate poststimulation rise was also transient, disappearing or at least tending toward base-line values within 12–16 min.

We interpret these findings as indicative of a redistribution of membrane proteins upon interaction of platelets with aggregating agents. After a brief, initial decrease the motional freedom of the lipid matrix is enhanced at least with thrombin and ADP as activating agents. This may facilitate complex formation of membrane proteins. The latter process would lead to a decrease in the mobility of proteins which included the DIDS-ligating moiety. Evidence for such complex formation has been obtained by gel electrophoretic analyses of cross-linked membrane proteins (Howard et al., 1982; Davies & Palek, 1982). The abrogation of thrombin-induced DIDS anisotropy changes in the absence of Ca^{2+} agrees with the observation that complexation of platelet membrane proteins is a Ca^{2+}-dependent process (Howard et al., 1982).

These findings demonstrate that platelet activation is associated with changes in the mobility of membrane proteins and lipids which occur without aggregation of platelets and probably represent one of the early responses of the platelet to stimulation by aggregating agents.

Acknowledgments

We thank Prof. T. Binkert and H. Gysel, Institute of Applied Physics, University of Berne, Switzerland, for the use of their fluorospectrophotometer and their generous help with some of these experiments.

Registry No. ADP, 58-64-0; arachidonic acid, 506-32-1; thrombin, 9002-04-4; calcium, 7440-70-2.

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Enzyme-Bound Intermediates in the Conversion of Glucose 1-Phosphate to Glucose 6-Phosphate by Phosphoglucomutase. Phosphorus NMR Studies†

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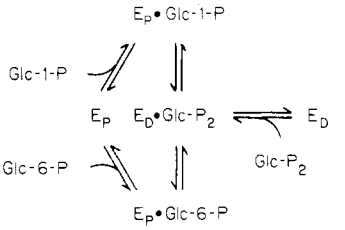
ABSTRACT: The interactions between metal ions and the phospho form of rabbit muscle phosphoglucomutase (EC 2.7.5.1) have been studied by ^{31}P NMR. In the metal-free enzyme, the width at half-height of the ^{31}P signal is 10 ± 1 Hz at 81 MHz. In enzyme-Cd^{2+} complexes, the presence of spin-spin coupling with $^{113}Cd^{2+}$ ($J_{^{113}Cd\text{-}O\text{-}^{31}P}$ = 16 Hz) and the absence of such splitting with $^{114}Cd^{2+}$ indicate that Cd^{2+} binds directly to the enzymic phosphate. The absence of detectable splitting on transfer of the phosphate group to the acceptor hydroxyl group of bound glucose 1-phosphate, or glucose 6-phosphate (to give the $^{113}Cd^{2+}$ complex of the dephosphoenzyme and glucose 1,6-bisphosphate), indicates that this transfer eliminates the direct metal ion-phosphate interaction. The enzyme-catalyzed reaction is slowed sufficiently by the addition of Li^+ to allow studies of three discrete intermediate complexes by NMR techniques: glucose 1-phosphate bound to the phosphoenzyme, glucose 1,6-bisphosphate bound to the dephosphoenzyme (only one complex of this type was observed), and glucose 6-phosphate bound to the phosphoenzyme. Complete assignments of the phosphorus resonances of these intermediates have been made by labeling the phosphate ester group of either the enzyme or the sugar with ^{17}O and by NMR polarization transfer studies. The effect of bound metal ions on these resonances also was determined. A ^{31}P NMR titration study of the Li^+ complex of the dephosphoenzyme with glucose 1,6-bisphosphate and a ^{31}P NMR polarization transfer experiment indicate that β-glucose 1,6-bisphosphate binds to the enzyme less tightly than α-glucose 1,6-bisphosphate. The relative mobilities and solvent accessibility of the phosphate ester groups in the free phosphoenzyme and the above complexes have been investigated by measurements of ^{31}P NMR line widths as a function of magnetic field strength, nuclear Overhauser effects, and spin-lattice relaxation times in ^1H_2O and ^2H_2O. The serine phosphate in the free phosphoenzyme is highly accessible to solvent molecules. Binding of Li^+ does not affect this solvent accessibility. In a ternary complex (phosphoenzyme, glucose 6-phosphate, metal ion), the enzymic phosphate becomes much less accessible and possibly inaccessible to solvent. The phosphate ester group of the substrate also is partially immobilized, but not to as great an extent as the enzymic phosphate. An analysis was conducted of contributions to the line width of the ^{31}P NMR signal of the phosphoenzyme provided by various relaxation mechanisms, including relaxation induced by ^{17}O substitution.

Phosphoglucomutase catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate via a type of ping-pong reaction sequence in which glucose 1,6-bisphosphate is considered as both the first product and second substrate (Ray & Peck, 1972). The active form of the enzyme is phosphorylated at Ser-116 (Ray et al., 1983). The sequence of phosphate transfer steps that occur during a catalytic cycle is shown below.[1]



(1)

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0006-2960/84/0423-0252$01.50/0