173–180, JCSU Press, Bari, Italy.
Hoppe, J., Brunner, J., & Jørgensen, B. B. (1984) *Biochemistry 23*, 5610–5616.
Jap, B. K., Maestre, M. F., Hayward, S. B., & Glaeser, R. M. (1983) *Biophys. J. 43*, 81–89.
Khorana, H. G., Gerber, G. E., Herlihy, W. C., Gray, C. P., Anderegg, R. J., Nihei, K., & Biemann, K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5046–5050.
Klip, A., & Gitler, C. (1974) *Biochem. Biophys. Res. Commun. 60*, 1155–1162.
Lämmli, U. K. (1970) *Nature (London) 227*, 680–685.
Nicholas, R. A. (1984) *Biochemistry 23*, 888–898.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667–678.
Peters, H. M., Feigl, D. M., & Mosher, H. S. (1968) *J. Org. Chem. 33*, 4245–4250.
Ross, A., Radhakrishnan, R., Robson, R. J., & Khorana, H. G. (1982) *J. Biol. Chem. 257*, 4152–4161.
Shapiro, A. L., Viñuela, E., & Maizel, J. B. (1967) *Biochem. Biophys. Res. Commun. 28*, 815–820.
Spiess, M., Brunner, J., & Semenza, G. (1982) *J. Biol. Chem. 257*, 2370–2377.
Stoeckenius, W. (1980) *Acc. Chem. Res. 13*, 337–344.
Takagaki, Y., Gerber, G. E., Nihei, K., & Khorana, H. G. (1980) *J. Biol. Chem. 255*, 1536–1541.
Takagaki, Y., Radhakrishnan, R., Gupta, C. M., & Khorana, H. G. (1983a) *J. Biol. Chem. 258*, 9128–9135.
Takagaki, Y., Radhakrishnan, R., Wirtz, K. W. A., & Khorana, H. G. (1983b) *J. Biol. Chem. 258*, 9136–9142.
Tomasi, M., & Montecucco, C. (1981) *J. Biol. Chem. 256*, 11177–11181.
Trewhella, J., Anderson, S., Fox, R., Gogol, E., Khan, S., Engelman, D., & Zaccai, G. (1983) *Biophys. J. 42*, 233–241.
White, E. H., Perks, H. M., & Roswell, D. F. (1978) *J. Am. Chem. Soc. 100*, 7421–7423.

Thiol-Specific Probes Indicate That the β-Chain of Platelet Glycoprotein Ib Is a Transmembrane Protein with a Reactive Endofacial Sulfhydryl Group†

Efstathia L. Kalomiris‡ and Barry S. Coller*

Departments of Pathology and Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794

Received January 7, 1985

ABSTRACT: We used two membrane-permeable fluorescent reagents, monobromobimane and *N*-[[5-(dimethylamino)-1-naphthalenyl]sulfonyl]aziridine (*N*-dansylaziridine), and one membrane-impermeable fluorescent probe, monobromo(trimethylammonio)bimane, all three of which react selectively with protein thiols, to (1) assess the presence of reactive sulfhydryls in the platelet glycoprotein Ib (GPIb) molecule and (2) establish the topology of any GPIb-reactive thiols in the platelet membrane. Intact platelets were reacted with 1–10 mM monobromobimane or monobromo(trimethylammonio)bimane or 50–100 μM *N*-dansylaziridine for 30–60 min at 37 °C. The platelets were then washed, solubilized in 1% Triton X-100, and analyzed by nonreduced–reduced polyacrylamide gel electrophoresis either directly or indirectly after immunopurification of GPIb. Monobromobimane and *N*-dansylaziridine labeled GPIbβ but not GPIbα in intact platelets. This labeling could be inhibited by pretreating the platelets with either *N*-ethylmaleimide or *p*-(chloromercuri)benzenesulfonic acid, confirming the specificity of these probes for thiol groups. Monobromo(trimethylammonio)bimane, the membrane-impermeable reagent, did not label GPIbβ in intact platelets. However, it did label GPIbβ in sonicated platelets, indicating that the thiol group of GPIbβ occupies an intracellular location. Since the carbohydrate moiety of GPIbβ can be labeled from the outside of intact platelets with membrane-impermeable reagents, we conclude that GPIbβ has a transmembrane orientation.

The mechanism(s) by which platelet membrane events affect intracytoplasmic constituents and the way(s) in which intracytoplasmic constituents modulate platelet membrane events are the subject of intense study. Much work has focused on changes in lipids, calcium, and cyclic nucleotides as transducing agents (Holmsen & Karpatkin, 1983; Lasslo & Quintana, 1984) with growing evidence that a dynamic structural interaction exists between membrane glycoproteins and the cytoskeletal proteins contained within the cytoplasm (Phillips et al., 1980). There is circumstantial evidence to suspect that protein sulfhydryl and disulfide groups may be contributing to these processes since (1) sulfhydryl blocking agents are powerful inhibitors of platelet phospholipid metabolism and platelet aggregation (Harbury & Schrier, 1974; MacIntyre et al., 1977; Silk et al., 1981), (2) platelets can be aggregated by the disulfide reducing agent dithiothreitol (Zucker et al., 1983), (3) the sulfhydryl oxidizing agent diamide induces aggregate formation of cytoskeletal proteins, inhibits the induction of platelet aggregation, and promotes the deaggregation of previously aggregated platelets (Ando & Steiner,

† This investigation was supported, in part, by Grant 19278 from the National Heart, Lung, and Blood Institute, by a grant-in-aid from the American Heart Association, and with funds contributed in part by the American Heart Association, Suffolk County Chapter, NY.

* Address correspondence and reprint requests to this author at the Division of Hematology, State University of New York at Stony Brook, Stony Brook, NY 11794.

‡ This paper contains work performed by E.L.K. in partial fulfillment of the Ph.D. degree in Experimental Pathology. Present address: Department of Medicine (R-38), University of Miami School of Medicine, Miami, FL 33101.

0006-2960/85/0424-5430$01.50/0

1973; Hofmann, B., et al., 1982, 1983; Hofmann, J., et al., 1983), (4) the formation of mixed disulfide bonds, a process shown to play a role in erythocyte membrane–cytoplasm physiology, allows for enormous plasticity since it offers a mechanism for interaction between any two sulfhydryl-containing proteins (Rao, 1979; Flynn et al., 1981; Smith & Palek, 1982, 1983), and (5) recent data indicate that endofacial cysteine residues are required for fatty acid acylation of transmembrane proteins and that the lipid moiety may help anchor the protein to the lipid bilayer (Kaufman et al., 1984; Rose et al., 1984).

Several previous studies reported on the total sulfhydryl and disulfide bond content of platelets (Robey et al., 1979; Ando & Steiner, 1976), and sulfhydryl-containing proteins were identified by radiolabeling platelets with sulfhydryl-specific probes and then separating the proteins by one-dimensional polyacrylamide gel electrophoresis (Nachman & Ferris, 1972). In the present study, we have explored this subject further using new classes of highly fluorescent sulfhydryl-specific probes (Scouten, 1973; Kosower et al., 1978, 1979, 1981) that are available with selective modifications that determine whether they can or cannot penetrate the platelet membrane, thus allowing the discrimination between sulfhydryl groups on the exofacial and endofacial surfaces.

We observed that membrane-impermeant thiol-specific probes did not label GPIbβ[1] in intact platelets but did label GPIbβ after sonication or membrane solubilization. In contrast, membrane-permeant probes labeled GPIbβ in intact platelets. These data indicate that GPIbβ has a reactive thiol on the cytoplasmic side of the membrane. Since GPIbβ has previously been shown to have a carbohydrate-containing segment exposed on the outside of the platelet membrane (Phillips, 1979), our data support a transmembrane orientation for GPIbβ.

Experimental Procedures

Materials. Halide derivatives of 3,4,6,7-tetramethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione and dimethyl-9,10-dioxa-*syn*-bimane [monobromobimane and monobromo(trimethylammonio)bimane] and D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone were obtained from Calbiochem-Behring (La Jolla, CA); *N*-[[5-(dimethylamino)-1-naphthenyl]sulfonyl]aziridine (*N*-dansylaziridine) was from Molecular Probes (Junction City, OR); Basic Fuchsin, RX-5 X-ray film, and Kodak Royal Pan film 4141 were from Eastman Kodak (Rochester NY); Affi-Gel 10 active ester agarose and Affi-Gel 501 were from Bio-Rad Laboratories (Richmond, CA), and prestained high molecular weight protein standards were from Bethesda Research Laboratories (Rockville, MD).

Platelet Preparation. Fresh human blood anticoagulated with citrate/phosphate/dextrose/adenine (CPD, 2.23 g of dextrose/1.84 g of sodium citrate/229 mg of citric acid/115 mg of monosodium phosphate/19.3 mg of adenine per 500 mL of blood) was obtained from the blood bank of the State University of New York at Stony Brook University Hospital from volunteer donors who had not ingested any drugs for at least 2 weeks before donation. Platelet-rich plasma was obtained from 1 unit of whole blood by centrifugation at 3200*g* for 3.5 min at 22 °C. After transfer to a satellite bag, the platelet-rich plasma was made 10 mM in disodium ethylenediaminetetraacetate (Na_2EDTA, 269 mM stock solution, pH 7.4). Contaminating red blood cells were removed by repetitive centrifugations at 1000*g* for 3 min at 22 °C until visual inspection indicated minimal contamination. The platelets were then sedimented at 3200*g* for 10 min at 22 °C, and the pellet washed 3 times with 0.01 M sodium phosphate/0.15 M NaCl, pH 7.4 buffer (PBS) containing 10 mM benzamidine hydrochloride and 10 mM EDTA (PBS/EB).

Platelet Counts. Platelets were counted on an Electrozone/Celloscope particle counter (Particle Data Inc., Elkhart, IN).

Labeling of Platelets with Monobromobimane. The washed platelet pellet recovered from 1 unit of blood was resuspended in PBS/EB buffer that had been made 5–10 mM with monobromobimane (200 mM stock solution dissolved in acetonitrile just before use) and incubated for 30–60 min at 22 or 37 °C. To test the specificity of the reagent, control platelet samples were pretreated with 5–10 mM *N*-ethylmaleimide (NEM), 5 mM reduced glutathione, 5 mM oxidized glutathione, or 5 mM *p*-(chloromercuri)benzenesulfonic acid for 30–60 min at 22 or 37 °C and centrifuged before being resuspended in PBS/EB containing 5 mM monobromobimane and incubated for 1 h at 22 or 37 °C. To identify proteins containing disulfide bonds, platelets were pretreated with 1–5 mM dithiothreitol for 30–60 min at 37 °C to reduce disulfide bonds to free thiols and then centrifuged, resuspended, and reacted with either 5–10 mM monobromobimane or the sequential combination of 5–10 mM NEM and 5–10 mM monobromobimane at 37 °C for 30–60 min. After the reactions were completed, the suspensions (2×10^{12} platelets/L) were then diluted 10-fold with PBS/EB buffer containing 0.2% bovine serum albumin and incubated at 37 °C for 1 h. The platelets were then pelleted and washed once in PBS/EB/0.2% BSA and once in PBS/EB and solubilized in either sodium dodecyl sulfate (SDS) or Triton X-100. For SDS solubilization, platelets were resuspended to a count of 2×10^{12}/L in PBS containing 20 mM NEM, 20 mM EDTA, 20 mM benzamidine hydrochloride, 10 mM mersalyl acid, 10 μM D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone, 50 μg/mL leupeptin, 50 μg/mL 1-chloro-3-(tosylamino)-7-amino-2-heptanone hydrochloride, and 100 units/mL trasylol (lysing buffer). The suspension was made 3% with SDS (30% stock), vortexed briefly, heated to 100 °C for 5 min, and revortexed. Platelets were solubilized in Triton X-100 by resuspending the washed platelets in lysing buffer, adding 0.11 volume of 10% Triton X-100, rocking at 4 °C for 1 h (Ames aliquot mixer, Miles Laboratories, Inc., Elkhart, IN), and centrifuging at 27715*g* at 4 °C for 15 min. The lysates were aliquoted and stored at −80 °C to be analyzed at a later time.

Purification of GPIb. GPIb was extracted from monobromobimane-labeled platelets by resuspending the washed platelet pellet at 5×10^{12} platelets/L in 0.01 M Tris/0.15 M NaCl buffer, pH 7.4 (TS), containing 20 mM EDTA, 20 mM benzamidine hydrochloride, 20 mM NEM, 10 μM D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone, 100 μg/mL soybean trypsin inhibitor, 50 μg/mL leupeptin, and 1% Triton X-100, rocking at 4 °C for 1 h, and removing the cell debris and the membrane fragments by sequential centrifugation at 27715*g* for 15 min at 4 °C and at 105000*g* for 30 min at 4 °C. The supernate was then applied at 3 mL/h to a 0.8 × 3.5 cm column of Affi-Gel 10 to which approximately 4.5 mg of a purified monoclonal antibody specific for

[1] Abbreviations: TS, 0.01 M Tris/0.15 M NaCl, pH 7.4; PBS, 0.01 M phosphate/0.15 M NaCl, pH 7.4; PBS/E, 0.01 M phosphate/0.15 M NaCl/10 mM EDTA, pH 7.4; PBS/EB, 0.01 M phosphate/0.15 M NaCl/10 mM EDTA/10 mM benzamidine, pH 7.4; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; GPIb, glycoprotein Ib; GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa.

GPIb (6D1) had been covalently attached as previously described (Coller et al., 1984). The column was washed sequentially with 3 bed volumes of TS buffer containing 20 mM benzamidine hydrochloride, 20 mM EDTA, 20 mM NEM, 50 μg/mL soybean trypsin inhibitor, and 1% Triton X-100, 10 bed volumes of TS buffer containing 10 mM EDTA, 10 mM NEM, and 0.05% Triton X-100, and 10 bed volumes of 0.01 M Tris/1.5 M NaCl buffer, pH .4, containing 10 mM EDTA, 5 mM NEM, and 0.05% Triton-X 100, pH 7.4. Elution of the bound GPIb was then achieved with a solution containing 0.05 M diethylamine, 0.01 M Tris, 10 mM EDTA, 5 mM NEM, 0.05% NaN_3, and 0.05% Triton X-100, pH 11.5.

Labeling of Platelets with N-Dansylaziridine. Platelets were washed once in PBS/10 mM EDTA (PBS/E) containing 85.7 mM glucose, resuspended at 2.5×10^{12} platelets/L in PBS/E, and reacted with 50–100 μM *N*-dansylaziridine (200 mM stock solution in acetonitrile) in PBS/E for 2 h at 22 °C. Control samples were pretreated with 5 mM NEM, reduced glutathione, or *p*-(chloromercuri)benzenesulfonic acid for 1 h at 22 °C before reaction with 50–100 mM *N*-dansylaziridine for an additional hour. Unreacted *N*-dansylaziridine was removed by incubating the platelets at 37 °C for 2 h in PBS/EB containing 0.2% BSA (200 mL/unit platelets), centrifuging, incubating again in the same buffer overnight at 22 °C, centrifuging again, and washing once in PBS/EB (200 mL of buffer/wash). The final washed pellet was lysed in Triton X-100 as described earlier and analyzed by nonreduced–reduced PAGE.

Labeling of Platelets with Monobromo(trimethylammonio)bimane. Labeling of intact platelets by monobromo(trimethylammonio)bimane was performed as described for monobromobimane labeling. Labeling of sonicated platelets by monobromo(trimethylammonio)bimane was performed by washing 2 units of freshly prepared platelets in PBS/EB, resuspending at 5×10^{12} platelets/L, and dividing the suspension in half. One aliquot was pretreated with 15 mM NEM at 4 °C for 45 min, and then, both were reacted with monobromo(trimethylammonio)bimane for 45 min. The samples were sonicated (output control 7) while on ice 6 times for 30 s with 45-s intervals between bursts to cool (sonifier cell disruptor, Model W140; Heat Systems Ultrasonics, Inc., Plainview, NY). Two-milliliter samples were then layered over 30% sucrose made in PBS/EB with 20 mM NEM (7 mL) and centrifuged at 105000*g* for 60 min at 4 °C. After removal of the supernatant and sucrose layers, the pellet of platelet membrane fragments was then resuspended in lysing buffer and extracted with Triton X-100 as described above. All extracts were aliquoted and stored at −80 °C for further analysis by nonreduced–reduced PAGE.

Reaction of GPIb with Affi-Gel 501. Washed platelets were resuspended in PBS/E buffer, pH 7.4, to a count of approximately 3.42×10^{11} platelets/L. One aliquot was made 20 mM with NEM and a second aliquot 20 mM with monobromobimane. Platelets resuspended in PBS/E buffer served as the control sample. All samples were incubated for 45 min at 4 °C, made 1% with Triton X-100, and solubilized by rocking for 1 h at 4 °C. The cell debris was removed by sequential low- and high-speed centrifugations as described for GPIb purification. One-milliliter aliquots of supernatants from lysates of (1) control, (2) NEM-treated, and (3) monobromobimane-treated platelets were reacted with 1.2 mL of packed gel volume of Affi-Gel 501, an organomercurial agarose that selectively reacts with thiol groups, by rocking at 4 °C for 1 h. The gel was then centrifuged at 3000*g* for 10 min, and the supernatants were removed. An aliquot of each sample before and after reaction with the Affi-Gel 501 was assayed for GPIb content by radioimmunoelectrophoresis as described previously (Coller et al., 1984). To exclude the possibility that the reactive thiol is in the glycocalicin portion of GPIbα, a glycocalicin-rich extract was prepared as described previously (Coller et al., 1984) and used to perform a similar experiment. Three aliquots (1 mL) of glycocalicin-rich extract were diluted 1:1 with PBS containing 20 mM EDTA. One aliquot was made 20 mM with NEM, and a second aliquot was made 20 mM with monobromobimane. All three aliquots were incubated at 4 °C for 2 h. Approximately 0.8 mL of each sample was reacted with 1.2 mL of Affi-Gel 501 for 2 h with rocking at 4 °C. Aliquots of each sample before and after reaction with the Affi-Gel were tested for glycocalicin content by radioimmunoelectrophoresis.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed by the method of Studier (1973) with minor modifications. Two-dimensional nonreduced–reduced SDS–PAGE was carried out by a modification (Nurden et al., 1981) of the procedure described by Phillips & Poh Agin (1977) with minor adjustments on a Protean electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA). The first-dimension electrophoresis of nonreduced samples (100–1000 μg of protein) was done with a 5% acrylamide cylindrical gel with a 3% stacking gel. The second-dimension electrophoresis of the reduced gel was performed with a 10% separating gel and a 3% stacking gel. Electrophoresis was performed at constant voltage.

Fluorescent Photography. Fluorescent pictures of the gels were taken with a Polaroid camera (MP4 Land Camera, Cambridge, MA) fitted with a green filter using transilluminating ultraviolet light (Chromato-Vue transilluminator, Model C-62, Ultraviolet Products Inc., San Gabriel, CA) and Kodak Royal Pan film 4141. The film was developed with the prepackaged chemicals according to the manufacturer's specifications.

Gel Staining. The protein bands were visualized by staining with 0.025% Coomassie brilliant blue R in 25% methanol/10% glacial acetic acid and destaining in 50% methanol/10% acetic acid. The carbohydrate bands were visualized by periodic acid–Schiff staining by the procedure described by Maurer (1971) with minor modifications.

Molecular Weight Estimations. Molecular weight estimations were determined graphically from a standard curve obtained by the electrophoretic mobilities of well-characterized proteins [myosin, 200K; β-galactosidase, 116K (130K); phosphorylase *b*, 92.5K; bovine serum albumin, 68K; ovalbumin, 43K; α-chymotrypsinogen, 25.7K; β-lactoglobulin, 18.4K; cytochrome *c*, 12.3K] relative to the mobility of the tracking dye.

Protein Determinations. Protein determinations were done by the modified Lowry et al. (1951) procedure described by Markwell et al. (1978) with the exception that the final volume was 1.2 mL. The samples were read at 660 nm in a Hitachi spectrophotometer (Model 100-40, Tokyo, Japan).

Results

Labeling of GPIb with Thiol-Specific Reagents. Analysis of platelets reacted with monobromobimane, the membrane-permeant, sulfhydryl-specific probe, revealed that nearly all of the protein bands that stained with Coomassie blue were also fluorescent, indicating the presence of free thiol groups. However, the α-granule proteins fibrinogen and thrombospondin labeled only weakly, if at all (Figure 1). The fluorescence intensity of these proteins was increased considerably when platelets were pretreated with dithiothreitol to

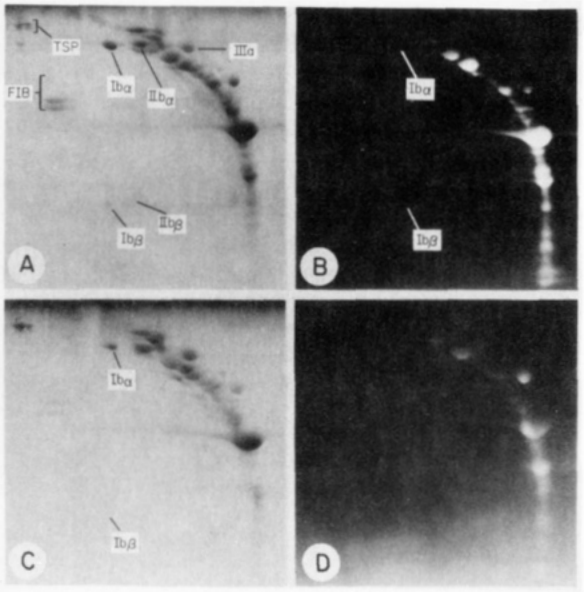

FIGURE 1: Labeling of washed platelets with monobromobimane. Combined periodic acid–Schiff and Coomassie blue staining (A and C) and fluorescent photography (B and D) of two-dimensional (5% unreduced vs. 10% reduced SDS–polyacrylamide) gels of Triton X-100 extracts from platelets that were treated with 10 mM monobromobimane. Platelets were either unpretreated (A and B) or pretreated with 5 mM NEM (C and D). The following proteins are identified: GPIbα, GPIbβ, GPIIbα, GPIIbβ, GPIIIa, fibrinogen (FIB), and thrombospondin (TSP). Note that pretreatment of platelets with NEM inhibits subsequent labeling of GPIbβ with monobromobimane (B vs. D).

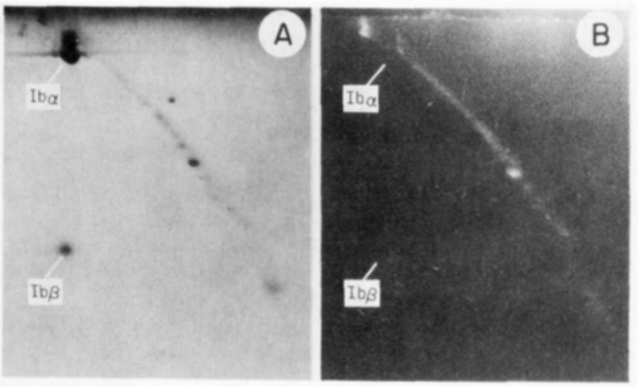

FIGURE 2: Labeling of GPIb with monobromobimane. Platelets were labeled with monobromobimane, and then GPIb was purified from a Triton X-100 extract by affinity chromatography. Combined periodic acid–Schiff and Coomassie blue staining (A) and fluorescent photography (B) of nonreduced–reduced two-dimensional (7% nonreduced vs. 10% reduced SDS–polyacrylamide) gel electrophoresis. Note the fluorescent labeling of GPIbβ and the absence of GPIbα fluorescence.

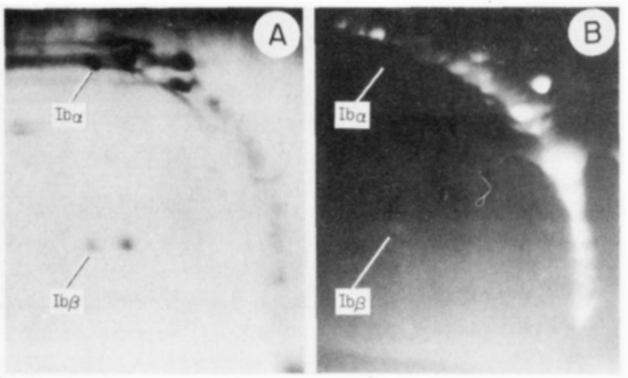

FIGURE 3: Labeling of GPIbβ with *N*-dansylaziridine in washed platelets. Periodic acid–Schiff staining (A) and fluorescent photography (B) of nonreduced–reduced (5% nonreduced vs. 10% reduced SDS–polyacrylamide) gels of Triton X-100 extracts from platelets that were reacted with 100 μM *N*-dansylaziridine. Note absence of labeling of GPIbα by this reagent.

reduce the disulfide bonds (data not shown).

The specificity of the fluorescent probes for reactive sulfhydryl groups was supported by showing that pretreating either intact platelets or platelet lysates with 5–10 mM NEM, a nonfluorescent alkylating reagent with a high degree of specificity for free sulfhydryl groups at neutral pH, significantly reduced the subsequent incorporation of the fluorescent monobromobimane (Figure 1). NEM treatment also inhibited the incorporation of monobromobimane into platelet proteins that were prereduced with dithiothreitol (data not shown). Thus, monobromobiamane appears to be no less specific for thiol groups than NEM.

Analysis of nonreduced–reduced polyacrylamide gels of monobromobimane-treated platelets by both periodic acid–Schiff staining (to identify glycoproteins) and fluorescence revealed no fluorescence in association with the α-chain of GPIb, the α-or β-chains of GPIIb, or GPIIIa. In contrast, the β-chain of GPIb was consistently fluorescent (Figures 1 and 2). Confirmation of the specificity of the reaction with GPIbβ was obtained by showing that preincubation of platelets with either 10 mM NEM (Figure 1) or 5 mM *p*-(chloromercuri)benzenesulfonic acid, another thiol-specific reagent that crosses membranes more slowly than NEM and converts sulfhydryl groups into mercaptides, inhibited labeling of GPIbβ by monobromobimane (data not shown). Additional evidence for the presence of a reactive sulfhydryl group on GPIbβ was obtained by showing that the β-chain also labeled with 50–100 μM *N*-dansylaziridine, another thiol-specific, fluorescent probe that is highly hydrophobic and membrane permeable (Figure 3). Monobromo(trimethylammonio)bimane, a hydrophilic, membrane-impermeant, thiol-specific probe, did not react with GPIbβ or any other platelet components in intact platelets (data not shown). However, it was possible to label GPIbβ with this probe when platelets were solubilized in SDS (data not shown) or sonicated in the presence of monobromo(trimethylammonio)bimane (Figure 4). Labeling of GPIbβ by monobromo(trimethylammonio)bimane was prevented when platelets were pretreated with 5–10 mM NEM (Figure 4) before sonication and reaction with 10 mM monobromo(trimethylammonio)bimane.

The observation that GPIb has a reactive thiol was substantiated by the finding that GPIb formed covalent mercaptide bonds with sulfhydryl-reactive groups on Affi-Gel 501 when platelets were solubilized in PBS/E buffer containing 1% Triton X-100 (Figure 5A, wells 3 and 4). However, GPIb failed to react with Affi-Gel 501 when the platelets were pretreated with either 20 mM NEM or 20 mM monobromobimane to block reactive thiol groups in GPIb (Figure 5A, wells 1, 2, 5, and 6). Further evidence that the reactive thiol is confined to the β-chain of GPIb was obtained by showing that glycocalicin, a fragment encompassing nearly the entire α-chain of GPIb, failed to react with Affi-Gel 501 (Figure 5B).

Discussion

Our study provides evidence to support the presence of an endofacial sulfhydryl group on GPIbβ and additional data on the thiol content of other platelet components. Labeling of platelets with monobromobimane and *N*-dansylaziridine, the thiol-specific membrane-permeable probes, indicates that nearly all of the cytoplasmic proteins have free thiol groups,

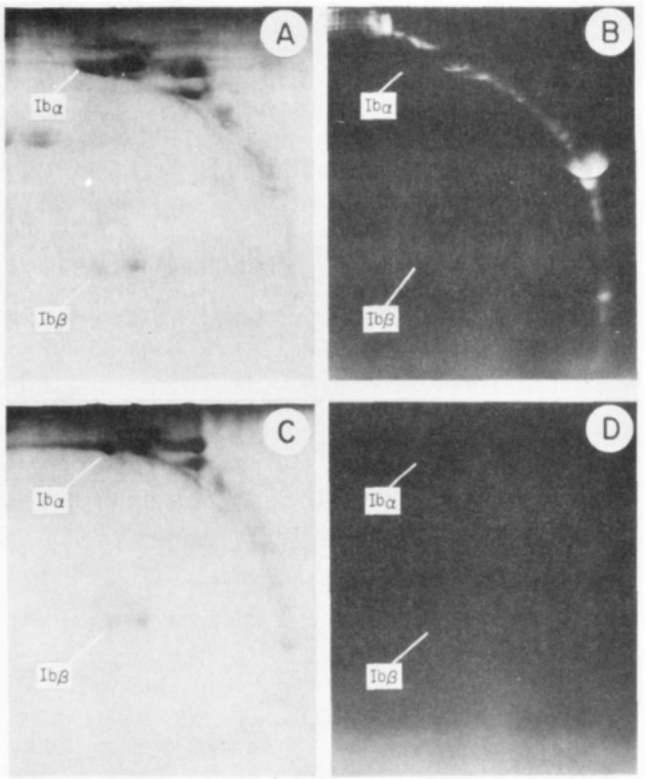

FIGURE 4: Labeling of GPIbβ by monobromo(trimethylammonio)-bimane after platelet sonication. Periodic acid–Schiff staining (A and C) and fluorescent photography (B and D) of nonreduced–reduced (5% unreduced vs. 10% reduced SDS–polyacrylamide) gels of Triton X-100 extracts from sonicated platelets. The platelets were first incubated for 45 min at 4 °C in the presence (C and D) or absence (A and B) of 15 mM NEM, then made 15 mM in monobromo(trimethylammonio)bimane, sonicated, allowed to react 45 min, centrifuged, extracted in Triton X-100, and prepared for SDS–PAGE. Note that NEM inhibited labeling of GPIbβ by monobromo(trimethylammonio)bimane (B vs. D).

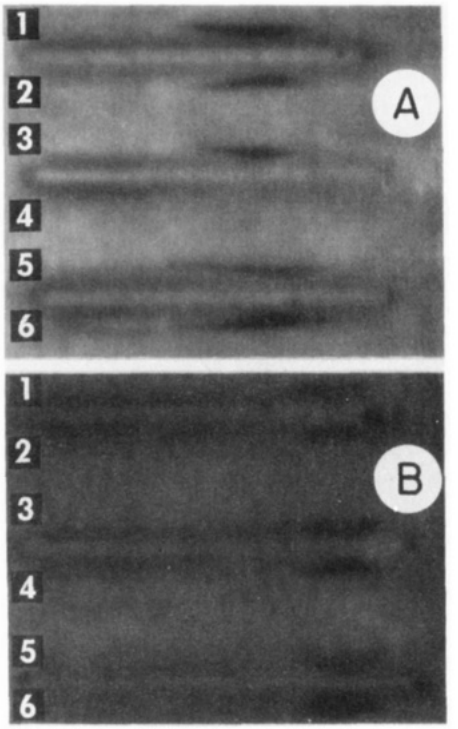

FIGURE 5: Radioimunoelectrophoresis of platelet extracts and glycocalicin-rich extracts. (A) Radioimmunoelectrophoresis of Triton X-100 extracts from NEM-treated platelets (Wells 1 and 2), untreated platelets (wells 3 and 4), and monobromobimane-treated platelets (wells 5 and 6). Wells 1, 3, and 5 contain samples before adsorption of proteins by Affi-Gel 501, and wells 2, 4, and 6 contain samples after adsorption. (B) Radioimmunoelectrophoresis of glycocalicin-rich extracts treated with NEM (wells 1 and 2), untreated extracts (wells 3 and 4), and monobromobimane-treated extracts (wells 5 and 6). Wells 1, 3, and 5 contain samples before adsorption of proteins by Affi-Gel 501, and wells 2, 4, and 6 contain samples after adsorption. Note that native GPIb binds to the Affi-Gel 501, whereas glycocalicin does not. The specificity of the binding to reactive thiol groups was confirmed by blocking the binding with NEM monobromobimane.

a result in keeping with the high concentration of the reducing agent glutathione known to be present in the cytoplasm of platelets (Karpatkin & Strick, 1972). The high intensity of fluorescence of the actin band is consistent with the finding by others that muscle actin contains five free thiol groups that occupy physiologically important domains in the molecule (Lin, 1978). Other proteins associated with the platelet cytoskeleton, including the myosin heavy chain and actin binding protein (filamin), also were highly fluorescent, indicating the potential for interactions that involve mixed disulfide bond formation. As expected, α-granule proteins contain mostly disulfide bonds and thus showed increased fluorescence labeling with monobromobimane when the platelets were prereduced with dithiothreitol (Counts et al., 1978; Smith et al., 1982; Wagner & Hynes, 1980; Garcia Pardo et al., 1983; Mosher & Johnson, 1983).

Among the major membrane glycoproteins, only the β-chain of GPIb labeled consistently with the membrane-permeant, thiol-specific probe monobromobimane. Confirmation of the specificity of this reaction was obtained by finding comparable labeling with another fluorescent, membrane-permeable probe, *N*-dansylaziridine. Furthermore, the nonfluorescent, thiol-specific agents NEM and *p*-(chloromercuri)benzenesulfonic acid were able to block the reaction of the fluorescent probes with GPIbβ.

On the contrary, GPIbβ did not react with monobromo-(trimethylammonio)bimane, the hydrophilic, membrane-impermeable probe, when the labeling was performed with intact platelets. However, GPIbβ reacted with this reagent when the platelets were solubilized or sonicated in the presence of monobromo(trimethylammonio)bimane. The failure of the membrane-impermeant monobromo(trimethylammonio)-bimane to label GPIbβ in intact platelets indicates that the reactive thiol is most likely in an endofacial location. Moreover, since the carbohydrate–containing portion of GPIbβ is located on the exterior of the platelet (Phillips, 1979), a corollary of our finding is that GPIbβ must completely span the platelet membrane.

GPIb clearly plays an important role in platelet physiology since patients with the Bernard-Soulier syndrome, whose platelets lack this glycoprotein, have a severe bleeding disorder (Evensen et al., 1974). It is well established that GPIb serves as a receptor to which von Willebrand factor binds when platelets are treated with the antibiotic ristocetin (Zucker et al., 1977; Moake et al., 1980; Coller et al., 1983). In addition, in ex vivo models of platelet interaction with the subendothelial surface of blood vessels, GPIb appears to be crucial for the initial adhesion of platelets (Weiss et al., 1974). Thrombin binds to GPIb (Hagen et al., 1982), and GPIb may contribute, either directly or indirectly, to the binding of drug-dependent antibodies (Kunicki et al., 1981) and other antibodies that bind to platelets via the Fc portion of the molecule (Moore et al., 1978). Much of the research on GPIb has focused on the larger α-chain and its proteolytic fragment, glycocalicin (Okumura et al., 1976; Phillips & Jakabova, 1977), which we recently demonstrated circulates in normal plasma (Coller et al., 1984).

There is considerable circumstantial evidence that GPIb may interact with other platelet proteins, especially cytoskeletal elements, as an integral part of its function: (1) von Willebrand factor dependent platelet function is inhibited by increasing platelet cAMP, and this cAMP effect can be abolished by pretreating platelets with agents that disrupt the cytoskeleton (Coller, 1981); (2) von Willebrand factor dependent platelet function is enhanced by some agents that disrupt the cytoskeleton (Coller, 1982); (3) protein cross-linking studies suggest an interaction between GPIb and several other platelet proteins, including GPIa and actin (Rotman, 1982; Jung & Moroi, 1983); (4) affinity chromatography studies suggest an interaction between GPIb and a M_r 17 000–20 000 glycoprotein (Coller et al., 1983; Berndt et al., 1983); (5) immunoelectrophoretic studies show GPIb to be present in several arcs of differing mobility, which is thought to reflect the complexing of GPIb with other proteins (Solum et al., 1983); (6) association between GPIb and an unidentified M_r 70 000 protein has been described with two-dimensional electrophoresis that separates proteins by p*I* and molecular weight (Meyer & Herrman, 1984); (7) the reducing agent dithiothreitol can alter the Triton X-114 solubility characteristics of GPIb, presumably reflecting differences in association with the "cytoskeleton" (Zucker et al., 1983). Our identification of a reactive thiol on the endofacial portion of GPIb raises the possibility that some of the above interactions may be mediated by mixed disulfide formation. Since GPIbβ appears to be transmembrane, the strong interactions between GPIb and the cytoskeletal components may be mediated by the integral and transmembrane domains of GPIbβ. It is also interesting to speculate on whether the endofacial sulfhydryl group(s) on GPIb serve(s) as a site of attachment for a fatty acid, as has been shown for other transmembrane proteins with endofacial sulfhydryl groups (Kaufman et al., 1984; Rose et al., 1984), and if so, what the function of such a group might be.

ACKNOWLEDGMENTS

We are indebted to Dr. Dennis Galanakis of the University Hospital Blood Bank, Stony Brook, NY, for special arrangements to obtain platelets for these studies and thank Dr. Sidonie Morrison of the Division of Hematology for helpful suggestions.

REFERENCES

Ando, Y., & Steiner, M. (1973) *Biochim. Biophys. Acta 311*, 38–44.

Ando, Y., & Steiner, M. (1976) *Biochim. Biophys. Acta 419*, 51–62.

Berndt, M. C., Gregory, C., Chong, B. H., Zola, H., & Castadi, P. A. (1983) *Blood 62*, 800–807.

Coller, B. S. (1981) *Blood 57*, 846–855.

Coller, B. S. (1982) *Blood 60*, 731–743.

Coller, B. S., Peerscke, E. I., Scudder, L. E., & Sullivan, C. A. (1983) *Blood 61*, 99–110.

Coller, B. S., Kalomiris, E. L., Steinberg, M., & Scudder, L. E. (1984) *J. Clin. Invest. 73*, 793–799.

Counts, R. B., Paskell, S. L., & Elgee, S. K. (1978) *J. Clin. Invest. 62*, 702–709.

Flynn, T. P., Johnson, G. J., & Allen, D. M. (1981) *Blood 57*, 59–65.

Garcia-Pardo, A., Pearlstein, E., & Frangione, B. (1983) *J. Biol. Chem. 258*, 12670–12674.

George, J. N., Nurden, A. T., & Phillips, D. R. (1984) *N. Engl. J. Med. 311*, 1084–1098.

Hagen, I., Brosstad, F., Gogstad, G. O., Korsmo, R., & Solum, N. O. (1982) *Thromb. Res. 27*, 549–554.

Harbury, C. B., & Schrier, S. L. (1974) *Thromb. Diath. Haemorrh. 31*, 469–484.

Hofmann, B., Hofman, J., & Till, U. (1982) *Acta Biol. Med. Germ., Suppl. 41*, K19–K24.

Hofmann, B., Danz, R., Hofmann, J., Percarmona, G., Bosia, A., Meyer, M., Losche, W., & Till, U. (1983) *Biomed. Biochim. Acta 42*, 489–501.

Hofmann, J., Losche, W., Hofmann, B., Arese, P., Bosia, A., Pescarmona, G., & Till, U. (1983) *Biomed. Biochim. Acta 42*, 479–487.

Holmsen, H., & Karpatkin, S. (1983) *Hematology* (Williams, W. J., Beutler, E., Erslev, A. J., & Lichtman, M. A., Eds.) pp 1149–1176, McGraw-Hill, New York.

Jung, S. M., & Moroi, M. (1983) *Biochim. Biophys. Acta 761*, 151–162.

Karpatkin, S., & Strick, N. (1972) *J. Clin. Invest. 51*, 1235–1243.

Kaufman, J. F., Krangel, M. S., & Strominger, J. L. (1984) *J. Biol. Chem. 259*, 7230–7238.

Kosower, E. M., Bernstein, J., Coldberg, I., Pazhenchevsky, B., & Goldstein, E. (1978) *J. Am. Chem. Soc. 101*, 1620–1621.

Kosower, N. S., Kosower, E. M., Newton, G. L., & Ranney, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3382–3386.

Kosower, N. S., Kosower, E. M., Zipser, Y., Faltin, Z., & Shomrat, R. (1981) *Biochim. Biophys. Acta 640*, 748–759.

Koufos, A., & Sagone, A. L. (1980) *Blood 55*, 835–840.

Kunicki, T. J., Russel, N., Nurden, A. T., Aster, R. H., & Caen, J. P. (1981) *J. Immunol. 126*, 398–402.

Kurantsin-Mills, J., & Lessin, L. S. (1981) *Biochim. Biophys. Acta 641*, 129–137.

Lasslo, A., & Quintana, R. P. (1984) *Blood Platelet Function and Medicinal Chemistry* (Lasslo, A., Ed.) pp 229–324, Elsevier Biomedical Press, New York, Amsterdam, and Oxford.

Lin, T.-I. (1978) *Arch. Biochem. Biophys. 185*, 285–299.

Lowry, O. H., Rosebrough, N. R., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

MacIntyre, D. E., Grainge, C. A., Drummond, A. H., & Gordon, J. L. (1977) *Biochem. Pharmacol. 26*, 319–323.

Markwell, H. R., Haas, S. M., Bierer, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.

Maurer, H. R. (1971) *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis*, de Grueter, Berlin and New York.

Meyer, M., & Herrman, F. H. (1984) *Thromb. Res. 35*, 359–364.

Moake, J. L., Olson, J. D., Troll, J. H., Tang, S. S., Funicella, T., & Peterson, D. M. (1980) *Thromb. Res. 19*, 21–27.

Mosher, D. F., & Johnson, R. B. (1983) *J. Biol. Chem. 258*, 6595–6601.

Nachman, R. L., & Ferris, B. (1972) *J. Biol. Chem. 247*, 4468–4475.

Nurden, A. T., Dupuis, D., Kunicki, T. J., & Caen, J. P. (1981) *J. Clin. Invest. 67*, 1431–1440.

Okumura, T., Lombart, C., & Jamieson, G. A. (1976) *J. Biol. Chem. 251*, 5950–5955.

Phillips, D. R. (1979) *Thromb. Haemostasis 42*, 1638–1651.

Phillips, D. R., & Jakabova, M. (1977) *J. Biol. Chem. 252*, 5602–5605.

Phillips, D. R., & Poh Agin, P. (1977) *J. Biol. Chem. 252*, 2121–2126.

Phillips, D. R., Jennings, L. K., & Edwards, H. H. (1980) *J. Biol. Chem. 86*, 77-86.
Rao, A. (1979) *J. Biol. Chem. 254*, 3503-3511.
Robey, F. A., Jamieson, G. A., & Hunt, J. B. (1979) *J. Biol. Chem. 254*, 1010-1012.
Rose, J. K., Adams, G. A., & Gallione, C. J. (1984) *Proc. Natl. Acad Sci. U.S.A. 81*, 2050-2054.
Rotman, A., Heldman, J., & Linder, S. (1982) *Biochemistry 21*, 1713-1719.
Scouten, W. H., Lubcher, R., & Baughman, W. (1973) *Biochim. Biophys. Acta 336*, 421-426.
Silk, S. T., Kenneth, T. H., & Marcus, A. J. (1981) *Biochemistry 20*, 391-397.
Smith, D. E., Mosher, D. F., Johnson, R. B., & Furcht, L. T. (1982) *J. Biol. Chem. 257*, 5831-5838.
Smith, D. K., & Palek, J. (1982) *Nature (London) 297*, 424-425.
Smith, D. K., & Palek, J. (1983) *Blood* 62, 1190-1196.
Solum, N. O., Olsen, T. M., Gogstad, G. O., Hagen, I., & Brosstad, F. (1983) *Biochim. Biophys. Acta 729*, 53-61.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237-248.
Wagner, D. D., & Hynes, R. O. (1980) *J. Biol. Chem. 255*, 4304-4312.
Weiss, H. J., Tschopp, T. B., Baumgartner, H. R., Sussman, I. I., Johnson, M. M., & Egan, J. J. (1974) *Am. J. Med. 57*, 920-925.
Zucker, M. B., Kim, S.-J., McPherson, J., & Grant, R. A. (1977) *Br. J. Haematol. 35*, 535-549.
Zucker, M. B., Varon, D., Masiello, N. C., & Karpatkin, S. (1983) *Thromb. Haemostasis 50*, 848-851.

Fusogenic Capacities of Divalent Cations and Effect of Liposome Size†

Joe Bentz*,‡ and Nejat Düzgüneş§

Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, and Cancer Research Institute, School of Medicine, University of California, San Francisco, California 94143

Received January 4, 1985

ABSTRACT: The initial kinetics of divalent cation (Ca^{2+}, Ba^{2+}, Sr^{2+}) induced fusion of phosphatidylserine (PS) liposomes, LUV, is examined to obtain the fusion rate constant, f_{11}, for two apposed liposomes as a function of bound divalent cation. The aggregation of dimers is rendered very rapid by having Mg^{2+} in the electrolyte, so that their subsequent fusion is rate limiting to the overall reaction. In this way the fusion kinetics are observed directly. The bound Mg^{2+}, which by itself is unable to induce the PS LUV to fuse, is shown to affect only the aggregation kinetics when the other divalent cations are present. There is a threshold amount of bound divalent cation below which the fusion rate constant f_{11} is small and above which it rapidly increases with bound divalent cation. These threshold amounts increase in the sequence $Ca^{2+} < Ba^{2+} < Sr^{2+}$, which is the same as found previously for sonicated PS liposomes, SUV. While Mg^{2+} cannot induce fusion of the LUV and much more bound Sr^{2+} is required to reach the fusion threshold, for Ca^{2+} and Ba^{2+} the threshold is the same for PS SUV and LUV. The fusion rate constant for PS liposomes clearly depends upon the amount and identity of bound divalent cation and the size of the liposomes. However, for Ca^{2+} and Ba^{2+}, this size dependence manifests itself only in the rate of increase of f_{11} with bound divalent cation, rather than in any greater intrinsic instability of the PS SUV. The destabilization of PS LUV by Mn^{2+} and Ni^{2+} is shown to be qualitatively distinct from that induced by the alkaline earth metals.

Recent studies on the fusion of phosphatidylserine (PS)[1] liposomes induced by divalent cations have elucidated the basic criteria that any proposed molecular mechanism must satisfy [for reviews, see Nir et al. (1983a) and Düzgüneş & Papahadjopoulos (1983)]. Basic to any study of liposome fusion is recognizing that the first step of the overall fusion process is given by the mass action reaction:

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \xrightarrow{f_{11}} F_2 \qquad (1)$$

where V_1 denotes the liposome, V_2 denotes the dimer aggregate, and F_2 denotes the fused doublet, i.e., when the bilayers have merged and the encapsulated contents of the liposomes have mixed.

Thus, to evaluate the effect of environmental parameters on the fusion rate constant, f_{11}, it is necessary to monitor fusion at early times so that higher order aggregates do not interfere and, of equal importance, to know that the rate-limiting step for the overall reaction is the fusion reaction itself. Recently, we have established the theoretical and experimental methodology necessary to obtain these rate constants (Wilschut et al., 1980, 1981; Nir et al., 1980b, 1982, 1983b; Bentz et al., 1983a,b, 1985). However, we have also developed a simple method for accelerating the aggregation rate and slowing down the fusion rate, so that we can observe the value of f_{11} directly. In Bentz et al. (1983b), we used PS SUV with high Na^+ and Li^+ concentrations (300-500 mM) to accelerate aggregation (Bentz & Nir, 1981a,b; Nir et al., 1981) and to keep the amounts of bound divalent cation low by direct competition of the divalent and monovalent cations for binding to the PS

† This investigation was supported by Research Grants GM-31506 (J.B.) and GM-28117 (N.D.) from the National Institutes of Health and a Grant-in-Aid from the American Heart Association with funds contributed in part by the California Affiliate (N.D.).

‡ Departments of Pharmacy and Pharmaceutical Chemistry.
§ Department of Pharmaceutical Chemistry and Cancer Research Institute.

[1] Abbreviations: DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetate; CF, carboxyfluorescein; LUV, large unilamellar vesicle, diameter ~150 nm; MLV, multilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SUV, small unilamellar vesicle, diameter ~30 nm.

0006-2960/85/0424-5436$01.50/0