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The involvement of cytoskeleton in the regulation of transbilayer movement of phospholipids in human blood platelets

Paul Comfurius, Edouard M. Bevers and Robert F.A. Zwaal *

Laboratory of Biochemistry, University of Limburg, P.O. Box 616, Maastricht, 6200 MD (The Netherlands)

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Activation of human platelets by different activators resulted in a different extent of degradation of the cytoskeletal proteins actin-binding protein and myosin, as well as of the non-cytoskeletal protein P235. The highest extent of proteolysis was observed with Ca-ionophore A23187 and decreased on going from A23187 > collagen plus thrombin > collagen > thrombin = ADP. The same order of potency has been found previously ((1983) Biochim. Biophys. Acta 736, 57–66) for the ability of platelet activators to induce exposure of aminophospholipids in the outer leaflet of the platelet plasma membrane, and to stimulate platelets to become procoagulant. Degradation of cytoskeletal proteins as a result of platelet stimulation by collagen plus thrombin was prevented in the presence of dibutyryl cAMP or EDTA but not in the presence of aspirin. This also runs in parallel with platelet procoagulant activity. Moreover, platelets from a patient with a partial deficiency in platelet procoagulant activity revealed a diminished extent of degradation of cytoskeletal proteins upon platelet stimulation with collagen plus thrombin. It is concluded that alterations in cytoskeletal organization upon platelet stimulation may lead to alterations in the orientation of (amino)phospholipids in the plasma membrane, and may therefore play a regulatory role in the expression of platelet procoagulant activity.

The asymmetric distribution of phospholipids between the inner and outer layer of the platelet plasma membrane is disturbed when platelets are treated with calcium ionophore A23187, SH-oxidizing agent diamide, or when platelets are stimulated by the combined action of collagen and thrombin [1,2]. These treatments result in an increased exposure of aminophospholipids in the outer leaflet of the plasma membrane, presumably resulting from an induced transbilayer movement of phospholipids. Consequently, these platelets become able to enhance conversion of prothrombin to thrombin by a complex of coagulation factors Xa and Va, and of factor X to factor Xa by a complex of coagulation factors IXa and VIIIa [1–3]. Both catalytic effects are caused by increased complex formation of coagulation factors and negatively charged phospholipid headgroups provided by the increased exposure of phosphatidylserine [4,5].

The non-random orientation of phospholipids in resting platelets resembles that in red cells [6–9]. The origin of this asymmetry and the mechanisms responsible for its regulation are still open to conjecture, but recent studies with red cells have indicated that interactions between the major cytoskeletal protein spectrin and anionic phosphatidylserine may contribute to the maintenance of the orientation of this phospholipid in the inner leaflet of the membrane bilayer [10–13]. Oxidation of spectrin SH-groups by diamide [10], or decou-

* To whom corresponce should be addressed.

pling of spectrin from the bilayer in spicules from irreversible sickle cells [13], results in increased exposure of aminophospholipids (including phosphatidylserine) at the outer surface of the erythrocyte. Also in platelets, treatment with diamide has been shown to produce extensive cross-linking of cytoskeletal proteins (including actin-binding protein, heavy chain myosin and actin) [14,15], and this may result in a dramatic reorientation of aminophospholipids by a mechanism similar to that operating in diamide treated red cells [2,12]. Another distinct possibility to enhance transbilayer movement of phospholipids in platelets is by alteration of the cytoskeleton structure upon activation. In this respect, it is of interest that cytoskeletal organization differs between platelets activated by thrombin or by calcium ionophore [16,17]. Platelets contain a calcium-dependent protease [18,19] that produces almost complete breakdown of actin-binding protein (as well as of the non-cytoskeletal protein P235) when platelets are activated by A23187, whereas activation by thrombin does not result in degradation of cytoskeletal proteins as judged from one-dimensional polyacrylamide gel electrophoresis [16]. In a recent study using two-dimensional polyacrylamide gel electrophoresis, Fox et al. [20] were able to show limited calcium-dependent proteolysis of actin-binding protein upon stimulation of platelets with thrombin.

In view of the finding that the amount of phosphatidylserine exposed at the platelet outer surface depends on the activation procedure [2], we were interested to study whether this would correlate with calcium-dependent proteolysis of cytoskeletal proteins. For this purpose, we used one-dimensional polyacrylamide gel electrophoresis for convenient comparison between the gels. Moreover, two of the known products of calcium-dependent proteolysis (M_r, 48000 and 135000) are not found on two-dimensional gels, presumably because they have isoelectric points beyond the range of the Ampholines usually employed [20]. On one-dimensional gels, however, the degradation fragment of M_r 100000 is usually not observed, perhaps because it co-migrates with α-actinin (M_r 105000) [19].

Human platelets were isolated from freshly drawn blood and washed by centrifugation as described previously [2]. All steps in the washing procedure were carried out in polystyrene tubes at room temperature. Prior to the activation procedure, washed platelets were resuspended at a concentration of $2 \cdot 10^7$ platelets/ml in Hepes buffer (pH 7.5), containing 137 mM NaCl, 2.68 mM KCl, 10 mM Hepes, 1.7 mM $MgCl_2$ and 5 mM glucose. Stirred platelet suspensions were made 3 mM in $CaCl_2$ (unless otherwise stated) and activated with different stimulators at 37°C. After a 5 min activation period, platelets were directly spun down at $1000 \times g$ for 2 min and prepared for electrophoretic analysis as indicated in the legend of Fig. 1. As described previously [2,3], all platelet agonists produced similar aggregation and dense body release, while platelet lysis remained below 2%.

Fig. 1. shows the protein patterns of control platelets, and platelets activated for 5 min with thrombin, collagen, collagen plus thrombin, calcium ionophore A23187, and ADP. On the basis of their apparent molecular weights and by comparison with previous studies [16–20], actin-binding protein, P235, heavy chain myosin, α-actinin and G-actin were identified. In concordance with previous investigators using a similar one-dimensional polyacrylamide gel electrophoresis [16,17], A23187 produced virtual complete breakdown of actin-binding protein and protein P235 and the formation of four degradation products of M_r 190000, 135000, 93000 and 48000 (Fig. 1, lane E). Evidence has been obtained that calcium-dependent proteolysis of actin-binding protein results in the formation of fragments M_r 200000 and 93000, while protein P235 is degraded to fragments with M_r 200000 and 48000 [21]. Treatment of platelets with A23187 also revealed an essentially complete breakdown of heavy chain myosin, an effect which has not been recognized before. In this respect it is of interest to mention that platelet myosin was shown to be cleaved by endogenous proteases during myosin isolation, to give separable rod (M_r = 130000) and head (M_r = 100000) polypeptide [22]. Therefore, it is possible that the degradation fragment M_r = 135000 observed in ionophore-treated platelets represents the rod fraction of myosin.

In contrast to the effect of A23187, platelet activation by thrombin (even at concentrations of 20 nM) produced no visible calcium-dependent

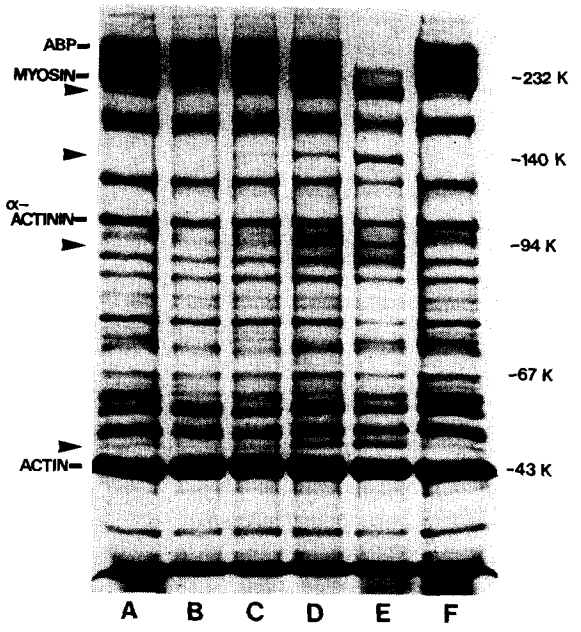

Fig. 1. Protein patterns from activated platelets. Lane A, non-stimulated platelets, lane B, stimulated with 2 nM thrombin; lane C, stimulated with 10 μg/ml collagen (Hormon Chemie, Munich); lane D, stimulated with 2 nM thrombin plus 10 μg/ml collagen; lane E, stimulated with 1 μM calcium-ionophore A23187; lane F, stimulated with 10 μM ADP. Platelet activations were carried out as described in the text. Platelet pellets were dissolved in 2% SDS (w/v), 5% β-mercaptoethanol (v/v) and 1 mM EDTA. Electrophoresis was carried out as described by Laemmli [30] using 7.5% polyacrylamide gels with a 4% stacking gel. Gels were stained with Coomassie blue. The cytoplasmic protein P235 is located between actin-binding protein (ABP) and myosin. The arrowheads designate the four major degradation products. Indicated on the right side of the patterns are the molecular weight standards (M_r: $\times 10^{-3}$ catalase (232), lactate dehydrogenase (140), phosphorylase B (94), bovine serum albumin (67) and ovalbumin (43).

proteolysis (Fig. 1, lane B), although limited breakdown has been shown to appear on two-dimensional gels [20]. Stimulation of platelets by collagen seemed to be somewhat more effective than thrombin in that minor formation of the four degradation fragments (M_r = 190 000, 135 000, 93 000 and 48 000) occurred (Fig 1, lane C). No change in the extent of degradation was observed when collagen concentration ranged from 4 to 40 μg/ml or when the activation time period varied from 2 to 15 min. Simultaneous activation of platelets with collagen plus thrombin produced much more polypeptide degradation (Fig. 1, lane D) than treatment of platelets with either of the two stimulants separately. It is evident that activation by collagen plus thrombin resulted in substantial formation of the same four degradation products as observed upon platelet activation by ionophore. Also, a decrease in actin-binding protein, P235 and presumably also in heavy chain myosin is apparent in gels of collagen plus thrombin activated platelets. In general, the degradation patterns were highly reproducible, provided that platelet activation was performed at concentrations below 10^8 platelets/ml under stirring to produce aggregation. At higher platelet concentrations, the appearance of the degradation fragments was usually less reproducible, particularly in the preparations activated by collagen or by collagen plus thrombin. It is well known that the higher the platelet concentration the larger are the platelet aggregates formed. It is therefore conceivable that this will reduce the fraction of platelets in direct contact with collagen, as more platelets will be aggregated by released ADP. Activation of platelets with ADP in the presence of extracellular calcium did not produce any of the proteolysis fragments (Fig. 1, lane F).

Fig. 2 shows that formation of the four degradation fragments induced by activation of platelets with collagen plus thrombin was essentially the same irrespective of whether platelets were activated for 1 or for 15 min (Fig. 2, lanes B and C). However, when the platelets were treated first with dibutyryl cAMP (Fig. 2, lane D) or when extra-cellular calcium is replaced by EDTA (Fig. 2, lane E) none of the degradation fragments were formed. Pretreatment of the platelets with aspirin did not prevent the formation of the four degradation fragments when platelets were subsequently activated by collagen plus thrombin (Fig. 2, lane F). We have previously shown that appearance of phosphatidylserine at the platelet outer surface as induced by platelet stimulation with the combined action of collagen plus thrombin is abolished in the presence of dibutyryl cAMP or EDTA, but is not prevented in the presence of aspirin [23].

Fig. 3 shows the protein composition of the apparent cytoskeletons of activated platelets, obtained in the Triton-insoluble residue. The main proteins in these preparations concern actin-binding protein (M_r 250 000), heavy chain myosin (M_r

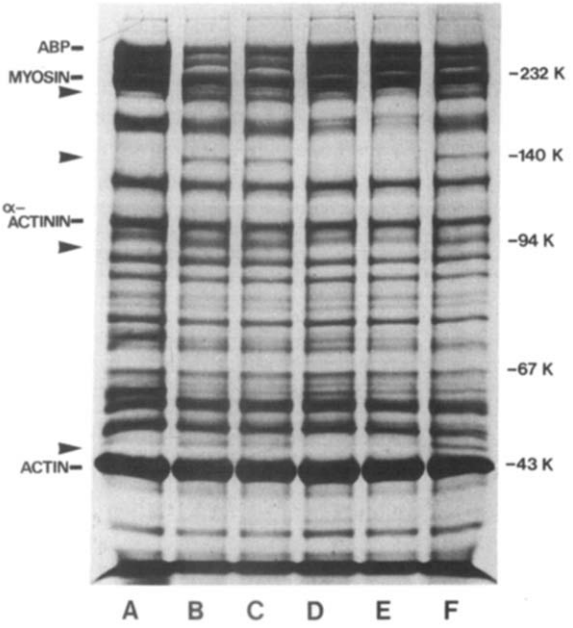

Fig. 2. Effect of different platelet function inhibitors on protein patterns from collagen plus thrombin activated platelets. Lane A, non-stimulated platelets; lane B, platelets stimulated with collagen (10 μg/ml) plus thrombin (2 nM) for one min; lane C, for 15 min; lanes D, E, F, preincubated for 5 min with inhibitors followed by a 5 min stimulation with collagen (10 μg/ml) plus thrombin (2 nM); lane D, dibutyryl cAMP (3 mM); lane E, 1 mM EDTA in the absence of calcium; lane F, aspirin (1 mg/ml). Further experimental details are described in the legend of Fig. 1.

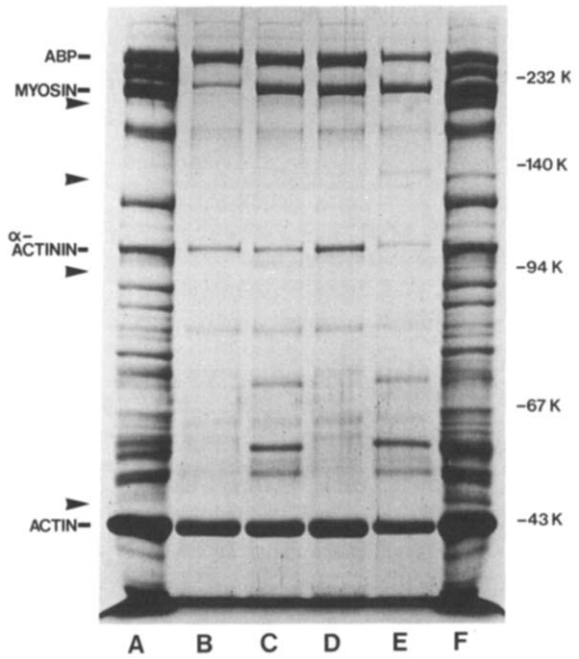

Fig. 3. Protein patterns of platelet cytoskeletons. Cytoskeletons from (activated) platelets were prepared as Triton-insoluble residues according to Rosenberg et al. [31]. Lane A, total protein pattern of non-stimulated platelets; lane B, cytoskeleton of non-stimulated platelets; lane C, cytoskeleton after thrombin (2 nM) stimulation; lane D, cytoskeleton after stimulation with collagen (10 μg/ml); lane E, cytoskeleton after stimulation with collagen (10 μg/ml) plus thrombin (2 nM); lane F, total platelet protein pattern after stimulation with collagen (10 μg/ml) plus thrombin (2 nM). For further experimental details see Fig. 1.

200 000), α-actinin (M_r 105 000) and actin (M_r 43 000), which is in agreement with previous investigators [22]. In addition, it was confirmed that a prominent polypeptide (M_r 56 000) is recovered in the cytoskeleton of thrombin-activated platelets [24]. Yields of cytoskeleton were negligible with ionophore treated platelets which may reflect extensive calcium-dependent proteolysis of cytoskeletal proteins. Relative to heavy chain myosin, the largest reduction of actin-binding protein was observed with platelets activated by collagen plus thrombin (Fig. 3, lane E), apart from ionophore treated platelets of which not enough material could be collected. Of the four degradation products observed in protein patterns of the platelet preparations activated by collagen plus thrombin (Fig. 3, lane F), only the polypeptide of M_r 135 000 was (presumably partly) recovered in the corresponding Triton-insoluble residue (Fig. 3, lane E). We did not observe that the highest molecular weight fragment (M_r 190 000) remained associated with the cytoskeleton, as did Truglia and Stracher [19] after treatment of platelet preparations with highly purified calcium-dependent protease from platelets. The reasons for this discrepancy are not known, but could easily be due to differences in experimental conditions.

The results clearly indicate that the extent of calcium-dependent proteolysis upon platelet activation depends on the activator: A23187 > collagen + thrombin > collagen > thrombin = ADP. It is remarkable that both the amount of phosphatidylserine exposed at the outer surface and the concordant ability of platelets to stimulate prothrombinase activity is similarly dependent on the platelet activator (Fig. 4). In addition, we recently

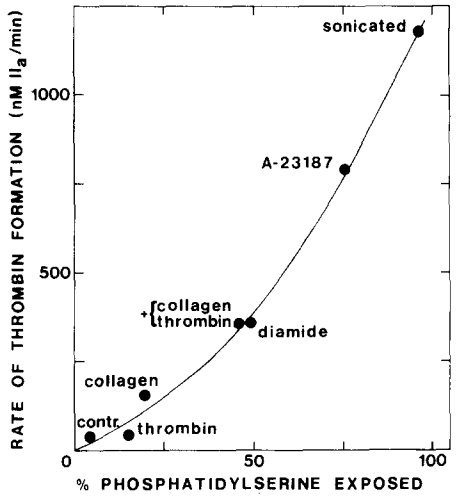

Fig. 4. Relation between prothrombin converting activity and exposure of phosphatidylserine in activated human blood platelets. Each point represents a different platelet treatment as indicated. Contr., non-stimulated platelet. This plot is a graphic representation of data from Ref. 2.

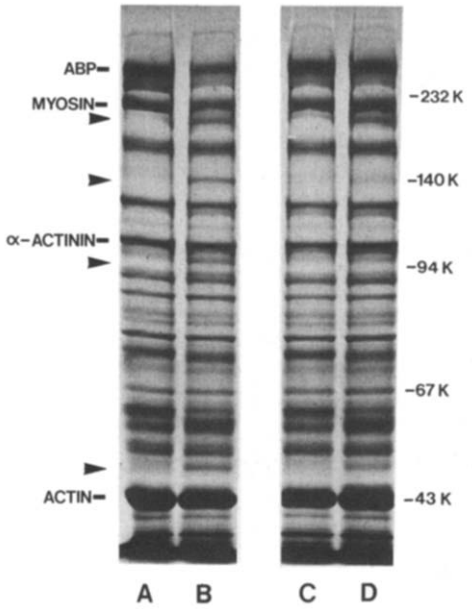

Fig. 5. Platelet protein patterns from a patient with a bleeding disorder. Lanes A and B are from a control donor, lanes C and D from the patient. Lanes A and C, non-stimulated platelets; lanes B and D, platelets stimulated with collagen (10 μg/ml) plus thrombin (2 nM). Further experimental details are given in Fig. 1.

studied platelets from a patient earlier described by Weiss et al. [25] to have a deficiency of platelet procoagulant activity. These platelets appeared to have a reduced ability to promote both prothrombin- and factor X-activation after stimulation by collagen plus thrombin, and this was accompanied by reduced exposure of phosphatidylserine at the platelet outer surface. It is of interest that these platelets also revealed a diminished calcium-dependent proteolysis upon stimulation with collagen plus thrombin (Fig. 5, compare lane D with B). In particular, the formation of degradation fragments M_r 135 000 and 93 000 was found to be strongly reduced compared to identically activated normal platelets. Treatment of the patient's platelets with Ca-ionophore A23187 produced the same extent of proteolysis as found with ionophore-treated normal cells. This strongly suggests that the patient's platelets are not (partly) deficient in Ca^{2+}-dependent protease, but that they have a decreased ability to raise the cytoplasmic calcium concentration upon activation. Also, it is likely that activation of normal platelets by collagen plus thrombin produces a higher increase in cytoplasmic calcium concentration than activation by either of the two agonists separately. Dibutyryl cAMP prevents Ca^{2+}-dependent proteolysis by suppressing cytoplasmic calcium concentrations. The inhibitory effect of EDTA strongly indicates that extracellular calcium has to be taken up in order to attain cytoplasmic calcium levels, sufficiently high to stimulate Ca^{2+}-dependent protease activity.

It is conceivable that degradation of cytoskeletal proteins is accompanied by alterations in cytoskeletal organization which result in a detachment of the cytoskeleton from the interior half of the lipid bilayer membrane. In so far as direct interactions between cytoskeletal proteins and phosphatidylserine exist in platelets (which has not yet been demonstrated), a decoupling might facilitate transbilayer movement of phosphatidylserine similar to that postulated for red cell membranes [12,13]. It should be emphasized, however, that loss of phospholipid asymmetry does not automatically have to occur upon decoupling of cytoskeleton from the membrane. It has been demonstrated that spectrin-free microvesicles obtained from ionophore-treated red cells retain their asymmetric phospholipid distribution, unless incubated

for 16 h at 37°C [26]. It is therefore more likely that decoupling of the cytoskeleton from the lipid bilayer is a prerequisite for rapid transbilayer movement to occur, the rate of which would be dependent on structural alterations in the membrane bilayer itself to form sites that allow for transbilayer reorientation [27–29].

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References

1 Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 429–436
2 Bevers, E.M., Comfurius, P. and Zwaal, R.F.A. (1983) Biochim. Biophys. Acta 736, 57–66
3 Rosing, J., Van Rijn, J.L.M.L., Bevers, E.M., Van Dieijen, G., Comfurius, P. and Zwaal, R.F.A. (1985) Blood, in the press
4 Rosing, J., Tans, G., Govers-Riemslag, J.W.P., Zwaal, R.F.A. and Hemker, H.C. (1980) J. Biol. Chem. 255, 274–283
5 Van Dieijen, G., Tans, G., Rosing, J. and Hemker, H.C. (1981) J. Biol. Chem. 256, 3433–3442
6 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–95
7 Chap, H., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146–164
8 Zwaal, R.F.A. (1978) Biochim. Biophys. Acta 515, 163–205
9 Schick, P.K. (1979) Semin. Hematol. 16, 221–233
10 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
11 Mombers, C., De Gier, J., Demel, R.A. and Van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 603, 52–62
12 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352
13 Franck, P.F.H., Bevers, E.M., Lubin, B.H., Comfurius, P., Chiu, D.T.-Y., Op den Kamp, J.A.F., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–189
14 Davies, G.E. and Palek, J. (1982) Blood 59, 502–513
15 Bosia, A., Spangenberg, P., Lösche, W., Arese, P, and Till, U. (1983) Thromb. Res. 30, 137–142
16 White, G.C. (1980) Biochim. Biophys. Acta 631, 130–138
17 McGowan, E.B., Yeo, K.-T. and Detwiler, T.C. (1983) Arch. Biochem. Biophys. 227, 287–301
18 Phillips, D.R. and Jakábová, M. (1977) J. Biol. Chem. 252, 5602–5605
19 Truglia, J.A. and Stracher, A. (1981) Biochem. Biophys. Res. Commun. 100, 814–822
20 Fox, J.E.B., Reynolds, C.C. and Phillips, D.R. (1983) J. Biol. Chem. 258, 9973–9981
21 Fox, J.E.B., Goll, D.E., Reynolds, C.C. and Phillips, D.R. (1985) J. Biol. Chem. 260, 1060–1066
22 Adelstein, R.S. Pollard, T.D. and Kuehl, W.M. (1971) Proc. Natl. Acad. Sci. USA 68, 2703–2707
23 Zwaal, R.F.A., Comfurius, P., Hemker, H.C. and Bevers, E.M. (1984) Haemostasis 14, 320–324
24 Fox, J.E.B. and Phillips, D.R. (1983) Semin. Hematol. 20, 243–260
25 Weiss, H.J., Vicic, W.J., Lages, B.A. and Rogers, J. (1979) Am. J. Med. 67, 206–213
26 Raval, P.J. and Allan, D. (1984) Biochim. Biophys. Acta 772, 192–196
27 Van Deenen, L.L.M. (1981) FEBS Lett. 123, 3–15
28 Comfurius, P., Bevers, E.M. and Zwaal, R.F.A. (1983) Biochem. Biophys. Res. Commun. 117, 803–808
29 Dressler, V., Haest, C.W.M., Plasa, G., Deuticke, B. and Erusalimsky, J.D. (1984) Biochim. Biophys. Acta 775, 189–196
30 Laemmli, U.K. (1970) Nature 227, 680–685
31 Rosenberg, S., Stracher, A. and Lucas, R.C. (1981) J. Cell. Biol. 91, 201–211