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DISSOCIATION OF THE GLYCOPROTEIN IIb-IIIa COMPLEX IN ISOLATED HUMAN PLATELET MEMBRANES

DEPENDENCE OF pH AND DIVALENT CATIONS

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The platelet membrane glycoproteins IIb and IIIa normally exist as a complex which forms a predominant immunoprecipitate after crossed immunoelectrophoresis of Triton-X-100-solubilized platelets. Dissociation of the complex occurs by solubilization in the presence of EDTA or EGTA at pH 8.7 and is readily verified by crossed immunoelectrophoresis. Incubations of isolated membranes with EDTA or EGTA at various pH levels were performed. Removal of the chelators and solubilization showed no dissociation of the glycoprotein IIb-IIIa complex in membranes incubated at pH below 8.0. At pH above 8.0 a dissociation which increased with increasing pH was seen. Under these conditions, dissociation appears to take place already in the intact membranes. The tendency of the glycoprotein IIb-IIIa complex to become dissociated with EDTA or EGTA at increasing pH seems to be due to increased chelating capacity of the chelators concomitant with a decreased chelating capacity of glycoprotein IIb and IIIa. The divalent cations Ca^{2+} and Mg^{2+}, but not Cu^{2+}, Zn^{2+}, Mn^{2+} or Sr^{2+}, in molar concentrations below that of EGTA were able to prevent the dissociation of the glycoprotein IIb-IIIa complex by the chelator at pH 9.0, indicating that Ca^{2+} as well as Mg^{2+} can be used to keep the complex together. In some experiments it was possible to reverse the dissociation in the membranes after removal of EDTA. At pH 7.5 reassociation occurred within 15 min whether divalent cations were added or not. At pH 9.0, reassociation occurred within 2 h provided Ca^{2+} was present. The tendency of glycoprotein IIb and IIIa to form a complex thus appeared to be most pronounced over the physiological pH range and to be a rapid process in platelet membranes under such conditions.

Introduction

Platelet membrane glycoproteins are considered important for normal platelet function. Two major platelet glycoproteins, glycoproteins IIb and IIIa, have been demonstrated in the plasma membrane [1] as well as in the α-granule membranes [2,3]. Using crossed immunoelectrophoresis, Hagen et al. [1,4] showed that these two glycoproteins were present as a complex after solubilization of the platelets or the platelet membranes in a buffer containing Triton X-100. This has been confirmed by Kunicki et al. [5]. Upon solubilization of isolated membranes, the area covered by the immunoprecipitate representing the glycoprotein complex is occasionally decreased concomitant with the appearance of two new immunoprecipitates [1,5,6]. Shulman and Karpatkin [7] suggested that this represented a splitting of the glycoprotein complex and that this process was due to proteolysis. Later it was demonstrated that this actually represented a dissociation of the complex into glycoproteins IIb and IIIa [5,8]. It has also been observed that addition of an excess of Ca^{2+}, but

not of Mg^{2+}, was able to reconstitute the complex after prolonged incubation [5]. Karpatkin et al. [8] showed that Ca^{2+}, but not Mg^{2+}, was able to prevent dissociation of the complex when added in excess to EDTA or EGTA prior to solubilization of isolated membranes. It is therefore assumed that the complex formation between the glycoproteins IIb and IIIa is dependent on Ca^{2+}. In a recent study, Nachman and Leung [9] have isolated glycoproteins IIb and IIIa and found that a mixture of these proteins were able to bind fibrinogen in the presence of Ca^{2+}.

In this study we present observations that indicate that the complex exists in the isolated membrane and can be dissociated prior to solubilization, that pH is an important factor in this process, and that dissociation can be rapidly reversed in the physiological pH range. It seems probable that Mg^{2+} as well as Ca^{2+} can be used to keep the glycoprotein IIb-IIIa complex together.

Materials and Methods

Chemicals. Agarose type HSA was from Litex, Glostrup, Denmark. ADP and Triton X-100 was from Sigma Chemical Company, St. Louis, U.S.A.

Platelets. Platelets were isolated and washed as described elsewhere [2].

Platelet membranes. These were isolated as described previously [2]. The membranes were routinely washed with 0.27 M sucrose prior to the experiments.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed as described by Hagen et al. [1] using membranes or platelets solubilized in 0.038 M Tris/0.1 M glycine buffer (pH 8.7) containing 1% Triton X-100. The antibodies were against whole platelets or glycoprotein IIb/IIIa.

Dissociation/reassociation experiments. Platelet membranes were suspended to about 0.5 mg/ml protein in media containing 0.154 M NaCl and 20 mM Tris-HCl buffer ranging in pH from 7.0 to 9.0. Dissociation experiments were performed using 2 mM EDTA in the suspensions and incubation for 30 min. The membranes were then sedimented at $100000 \times g_{av}$ for 90 min and washed once in 0.27 M sucrose followed by solubilization and electroimmunochemical analyses, or incubation in a new medium to study reassociation. After each incubation, the membranes were routinely washed in 0.27 M sucrose to avoid the influence of residual compounds on the complex formation or dissociation. For each step in a series of incubations to study the dissociation/reassociation phenomenon, aliquots were removed for analyses by crossed immunoelectrophoresis. All incubations were performed at 20°C.

Antibodies. Rabbit antibodies to whole human platelets were prepared as described by Hagen et al. [1]. An antiserum directed against the glycoprotein IIb-IIIa complex was obtained as follows. After crossed immunoelectrophoresis of platelet membranes against whole platelet antibodies and washing of the immunoplates, the agarose containing the easily visible immunoprecipitate corresponding to the glycoprotein IIb-IIIa complex was cut out, suspended in 0.154 M NaCl by ultrasonication, and injected into rabbits. When the antiserum then obtained was used in crossed immunoelectrophoresis of solubilized platelets, a single immunoprecipitate was formed (Fig. 1A). If the antiserum was included in the intermediate gel during crossed immunoelectrophoresis of solubilized platelets against anti-platelet antibodies, only the glycoprotein IIb-IIIa complex was retarded in the second-dimension electrophoresis (not shown). Thus, precipitating antibodies present in this rabbit antiserum are directed against the glycoprotein IIb-IIIa complex as the only protein entity in platelets. The glycoprotein IIb-IIIa complex may be dissociated into its subunits by solubilization of platelets or platelet membranes at pH 8.7 in the presence of EDTA [5,8]. Crossed immunoelectrophoresis with antiserum to whole platelets shows this as a disappearance of immunoprecipitate 16 (representing the glycoprotein IIb-IIIa complex) and a concomitant appearance of two new immunoprecipitates [1,5–8]. Occasionally, partial dissociation was seen as shown in Fig. 1B. Also, when the antiserum directed against the glycoprotein IIb-IIIa complex was used to analyze membranes with a partly dissociated complex, immunoprecipitates representing the complex as well as the subunits, glycoproteins IIb and IIIa, were seen (Fig. 1C). This shows that this antiserum contains antibodies directed against antigenic determinants in glycoprotein IIb as well as in glycoprotein IIIa.

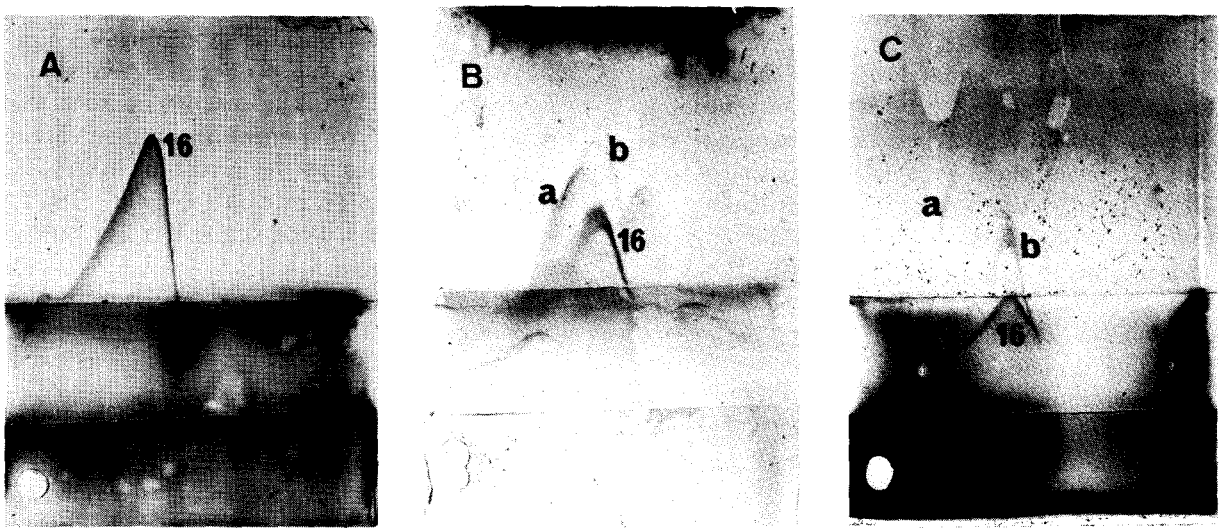

Fig. 1. Crossed immunoelectrophoresis of: (A) solubilized whole platelets against antibodies specific to glycoprotein IIb/IIIa; (B) isolated platelet membranes washed with 2 mM EDTA at pH 7.5, sedimented and solubilized without EDTA. Antibodies against whole platelets; (C) isolated platelet membranes treated as in B against antibodies specific to glycoprotein IIb-IIIa. Membranes were suspended in 0.154 M NaCl/20 mM Tris-HCl (pH 7.5)/2 mM EDTA to about 0.5 mg protein/ml, incubated at 20°C for 30 min, sedimented at $100000 \times g_{av}$ for 90 min and finally solubilized in 0.038 M Tris/0.1 M glycine buffer (pH 8.7) containing 1% Triton X-100. Platelets were solubilized in the same medium. About 60 μg platelet protein and 25 μg membrane protein were applied to 1% agarose gels. Electrophoresis in the first dimension was performed at 10 V/cm for 1 h. An antibody-free gel was inserted between the first-dimension gel and the gel containing the antibodies (400–600 μg protein/ml) to obtain good resolution. The second-dimension electrophoresis was performed overnight at 2 V/cm. The electrode buffer was 0.038 M Tris/0.1 M glycine (pH 8.7). All agarose gels contained this buffer and 0.5% Triton X-100. Immunoprecipitate 16 represents the glycoprotein IIb-IIIa complex and the immunoprecipitates a and b represent the glycoproteins IIIa and IIb, respectively.

Since the glycoprotein IIb/IIIa-specific antibodies showed weak immunoprecipitates with the dissociated glycoproteins IIb and IIIa, the polyspecific whole platelet antibodies were used in the experiments, whereas the glycoprotein IIb/IIIa antibodies were used to confirm the identities of immunoprecipitates related to glycoproteins IIb and IIIa. This was performed by always making two parallel immunoelectrophoreses, both having anti-platelet antibodies in the second-dimension gels and one having the glycoprotein IIb/IIIa antibodies in the intermediate gel. Immunoprecipitates related to glycoprotein IIb and/or glycoprotein IIIa were then lowered after the second dimension electrophoresis compared to the control.

Measurement of Ca^{2+} and Mg^{2+}. Ca^{2+} and Mg^{2+} in the membranes were measured using atomic absorption spectrophotometry. The samples were treated according to a routinely used procedure for serum analyses by suspending the membranes in 0.27 M sucrose to 2.0 mg protein/ml followed by the addition of 10% trichloroacetic acid/25 mM $LaCl_3$, incubation for 18 h at 4°C and centrifugation. The metal ions were analyzed in the supernatant using a Perkin Elmer 5000 Atomic Absorption Spectrophotometer.

Results

1. Effect of divalent cations on dissociation with EDTA or EGTA

The ability of the metal chelators EDTA and EGTA to dissociate the glycoprotein IIb-IIIa complex was investigated in order to decide whether the dissociation was due to the binding of the chelators per se or by the chelation of divalent

cations. Furthermore, the possible cation specificity was investigated.

Since the experimental design and the interpretation of the results depended on the concentrations of divalent cations in the membrane preparation, Ca^{2+} and Mg^{2+} were measured using atomic absorption spectrophotometry. Other cations are unlikely to contribute to the total amounts of divalent cations in platelet material. The concentrations of Ca^{2+} and Mg^{2+} were measured to 0.040–0.048 μmol/mg protein and 0.017–0.024 μmol/mg protein, respectively. This corresponds to concentrations of 0.020–0.024 mM Ca^{2+} and 0.009–0.012 mM Mg^{2+} in the final membrane suspension containing 0.5 mg protein/ml. The amounts of divalent cations present in the membrane preparations therefore appear to be negligible. This conclusion is supported by the finding that 0.1 mM of either EGTA or EDTA was sufficient to dissociate the glycoprotein IIb-IIIa complex completely at pH 8.7 and 0.5 mg membrane protein/ml.

Isolated platelet membranes were suspended in Tris/glycine buffer (pH 8.7) and a series of aliquots were withdrawn from the suspension and added one of the metal ions Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} or Sr^{2+} to various concentrations in the range 0–1.1 mM and then added EGTA to 1.0 mM. Suspensions with no EGTA and 1.0 mM of the various metal ions served as controls. To the suspensions was finally added Triton X-100 to 1% and they were analyzed by crossed immunoelectrophoresis. The controls showed an intact immunoprecipitate 16 (glycoprotein IIb-IIIa complex). In the presence of EGTA, a similar intact immunoprecipitate 16 was obtained with the concentrations of Ca^{2+} or Mg^{2+} down to 0.6 mM (metal ion : EGTA ratio above 6 : 10). Below these concentrations of Ca^{2+} or Mg^{2+} the glycoprotein IIb-IIIa complex was dissociated which was shown as a disappearance of immunoprecipitate 16 and a concomitant appearance of two new immunoprecipitates representing the glycoproteins IIb and IIIa, respectively. In contrast, the other metal ions could not prevent dissociation of the complex when they were present in concentrations below that of the EGTA. Ca^{2+} and Mg^{2+} therefore behave markedly different from the other divalent cations in their ability to prevent the dissociation of the glycoprotein IIb-IIIa complex in the presence of EGTA. It was not possible to distinguish Ca^{2+} and Mg^{2+} in this respect. Similar results were obtained using EDTA. The experiments show that there is a specific requirement for divalent cations in the maintenance of the glycoprotein IIb-IIIa complex. Furthermore, with Ca^{2+} or Mg^{2+}, EGTA can be present in a certain molar excess without causing dissociation of the glycoprotein IIb-IIIa complex, showing that the free chelator as such does not provoke the dissociation process.

2. The effect of chelators prior to membrane solubilization

Membranes were suspended in Tris-buffered NaCl (pH 7.5) and divided into three portions. The first and the second portion were added EDTA to 2 mM and the membranes were sedimented. The membranes of the first portion were solubilized directly in Tris/glycine buffer (pH 8.7) containing 1% Triton X-100, while those of the second portion were washed by resuspension in 0.27 M sucrose and centrifuged prior to solubilization as above. The third portion served as control with no addition of EDTA. When analyzed by crossed immunoelectrophoresis the control membranes showed an intact immunoprecipitate 16 (glycoprotein IIb-IIIa complex). The membranes solubilized after sucrose washing of EDTA-treated membranes also showed an intact glycoprotein IIb-IIIa complex (Fig. 2B) while the membranes which were solubilized directly after EDTA treatment (no sucrose washing) showed dissociation, or partial dissociation of the glycoprotein IIb-IIIa complex as shown in Fig. 1B. It thus appears that residual EDTA in the membrane preparations may cause dissociation of the glycoprotein IIb-IIIa complex upon membrane solubilization.

3. Effect of pH on dissociation with EDTA or EGTA

Isolated platelet membranes were suspended in Tris-buffered NaCl containing 2 mM EDTA at pH values ranging from 7.0 to 9.0 and incubated for 30 min at 20°C. Thereafter, the membranes were sedimented, washed once by resuspension in 0.27 M sucrose, centrifuged and finally solubilized in Tris/glycine buffer (pH 8.7) containing 1% Triton X-100. When these membrane preparations were analyzed by crossed immunoelectrophoresis

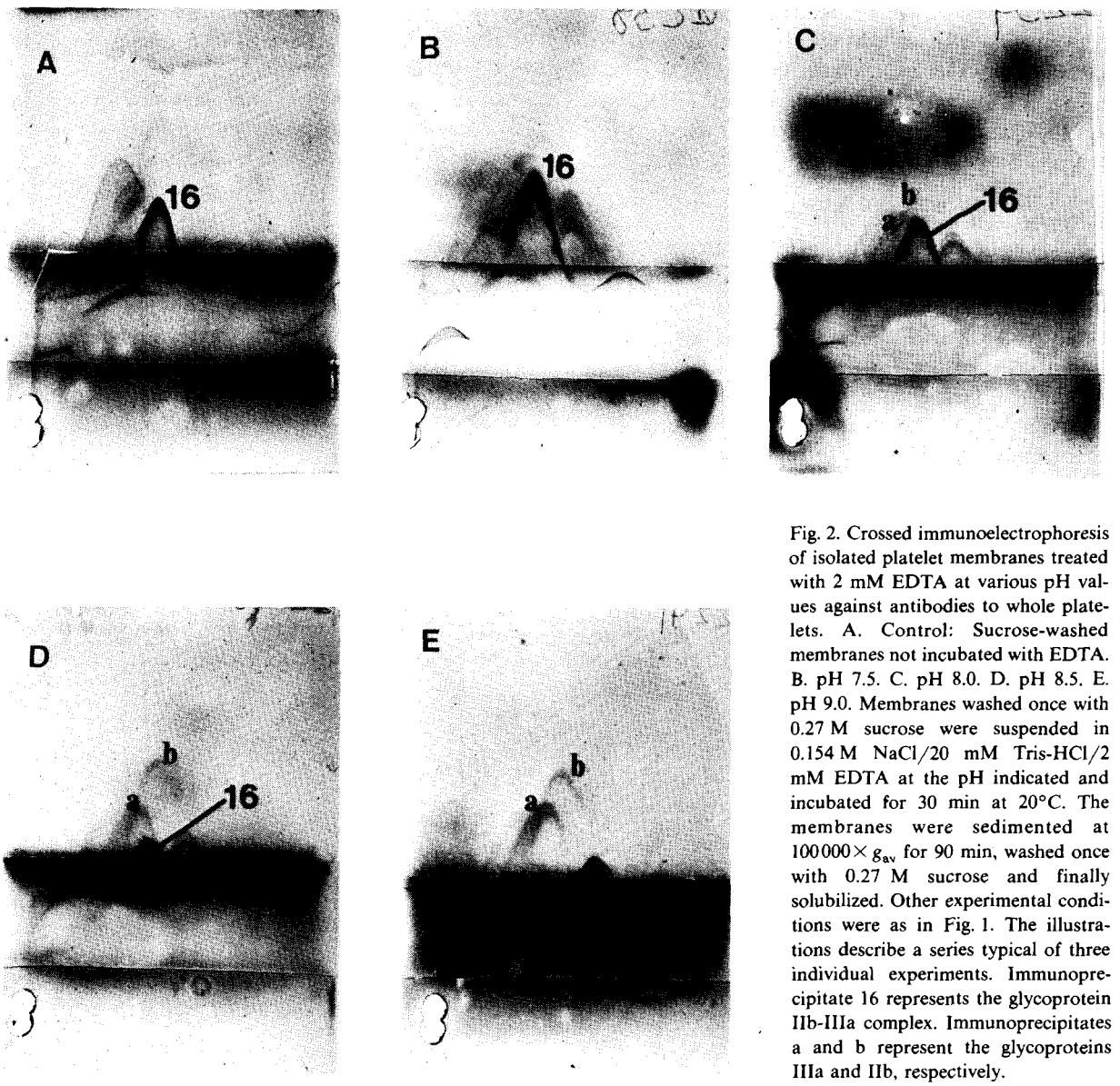

Fig. 2. Crossed immunoelectrophoresis of isolated platelet membranes treated with 2 mM EDTA at various pH values against antibodies to whole platelets. A. Control: Sucrose-washed membranes not incubated with EDTA. B. pH 7.5. C. pH 8.0. D. pH 8.5. E. pH 9.0. Membranes washed once with 0.27 M sucrose were suspended in 0.154 M NaCl/20 mM Tris-HCl/2 mM EDTA at the pH indicated and incubated for 30 min at 20°C. The membranes were sedimented at $100000 \times g_{av}$ for 90 min, washed once with 0.27 M sucrose and finally solubilized. Other experimental conditions were as in Fig. 1. The illustrations describe a series typical of three individual experiments. Immunoprecipitate 16 represents the glycoprotein IIb-IIIa complex. Immunoprecipitates a and b represent the glycoproteins IIIa and IIb, respectively.

(Fig. 2) a gradual disappearance of immunoprecipitate 16 (glycoprotein IIb-IIIa complex) and a concomitant appearance of the precipitates representing the glycoproteins IIb (b) and IIIa (a) was seen with the increasing pH used in the incubation of membranes with EDTA. Similar results were obtained using EGTA. Concomitant incubations of membrane suspensions in buffers without EDTA did not produce dissociation at any pH value, from which it appears that the effect depends on the presence of EDTA or EGTA. The effect of the increased pH is therefore interpreted as being due to the increase in chelating capacity of EDTA as well as EGTA (the formation constant of EDTA-metal complexes at pH 9.0 is 27-times that at pH 7.5). However, to investigate whether the more efficient metal chelation by EDTA was the only cause of the pH-dependent dissociation, membranes were incubated with increasing EDTA concentrations at pH 7.5. At this pH, a concentration

of 54 mM EDTA will chelate the same amount of a given cation as 2 mM EDTA at pH 9.0. No dissociation was seen, even at 100 mM EDTA and pH 7.5, from which it appears that additional factors like pH-dependent alterations in the protein conformation may also play a role.

4. Reassociation experiments

A series of experiments was performed with the intention of obtaining reassociation of the glycoprotein IIb-IIIa complex after this had been dissociated by EDTA. The glycoproteins IIb and IIIa present as separate entities after solubilization of platelets in Tris/glycine buffer (pH 8.7)/1% Triton X-100 in the presence of EDTA have been reported to reassociate by the addition of Ca^{2+} to the extract [5]. For some reason we were not able to obtain such reassociation at any of the temperatures, 4, 20 or 37°C, even if the Triton-solubilized material were incubated with an excess of Ca^{2+} for several days. However, in three out of seven experiments using non-solubilized, isolated membranes, reassociation was obtained as described in the following. When reassociation occurred, a set of results similar to those shown schematically in Fig. 3 was clearly reproduced. Membranes were incubated at pH 9.0 in the presence of 2 mM EDTA to dissociate the glycoprotein IIb-IIIa complex, and then incubated in various media as illustrated schematically in Fig. 3. After each incubation, aliquots were solubilized and analyzed by crossed immunoelectrophoresis to ascertain whether or not the glycoprotein IIb-IIIa complex was dissociated. To remove compounds which might influence the next treatment, the membranes were routinely washed with 0.27 M sucrose after each incubation. When membranes containing dissociated glycoproteins IIb and IIIa were incubated at pH 7.5, reassociation was seen after 15 min whether divalent cations had been added to the medium or not. The presence of 2 mM EDTA, but not 0.1 mM EDTA, prevented the reassociation, which indicates that divalent cations were involved. The incubation medium inevitably contained small amounts of divalent cations which might have been sufficient to reconstitute the com-

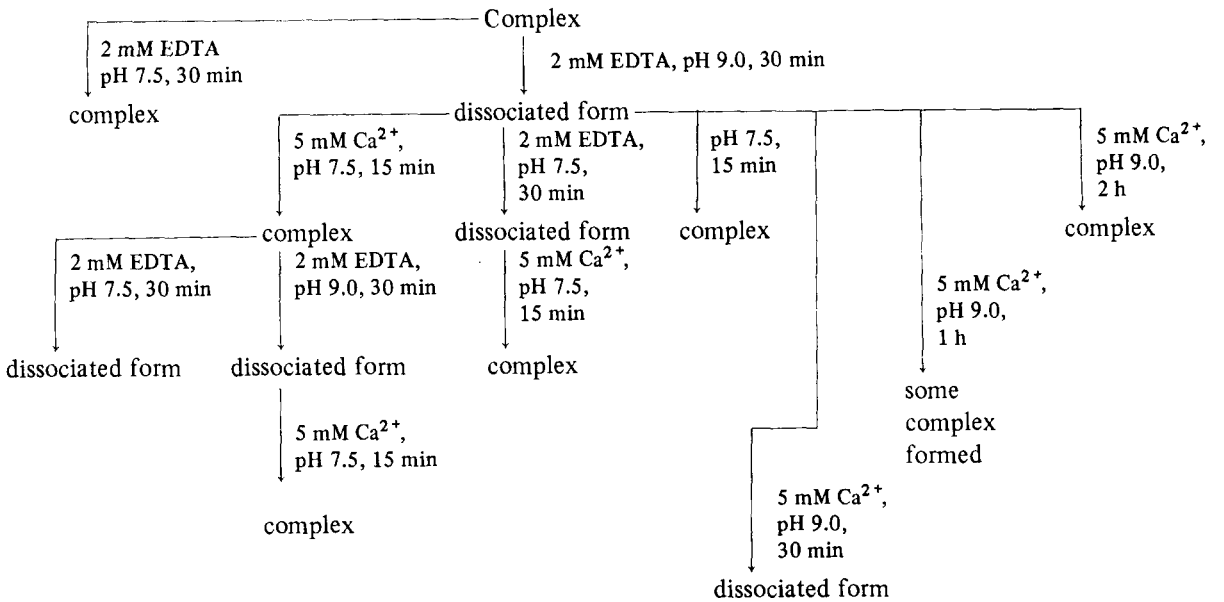

Fig. 3. Appearance of glycoproteins IIb and IIIa after crossed immunoelectrophoresis of isolated platelet membranes incubated in various media. Isolated platelet membranes were suspended to 0.5 mg protein/ml in 0.154 M NaCl/20 mM Tris-HCl with the indicated pH and content of Ca^{2+} or EDTA. After each incubation at 20°C for the period indicated, the membranes were sedimented at $100000 \times g_{av}$ for 90 min and washed once in 0.27 M sucrose before new incubation or analysis by crossed immunoelectrophoresis. 'Complex' designates the glycoprotein IIb-IIIa complex and 'dissociated form' the separate glycoproteins IIb and IIIa as observed in crossed immunoelectrophoresis.

plex. The reassociated glycoprotein IIb-IIIa complex could be dissociated again with 2 mM EDTA at pH 9.0 and once more reassociated at pH 7.5 (Fig. 4).

When membranes containing the dissociated glycoproteins IIb and IIIa were incubated at pH 9.0 after removal of EDTA, a slow reassociation occurred which was completed within 2 h of incubation provided Ca^{2+} was present (Fig. 5). Samples with Mg^{2+} showed no similar reassociation of the glycoprotein IIb-IIIa complex. It appears that reassociation proceeds much more slowly at pH

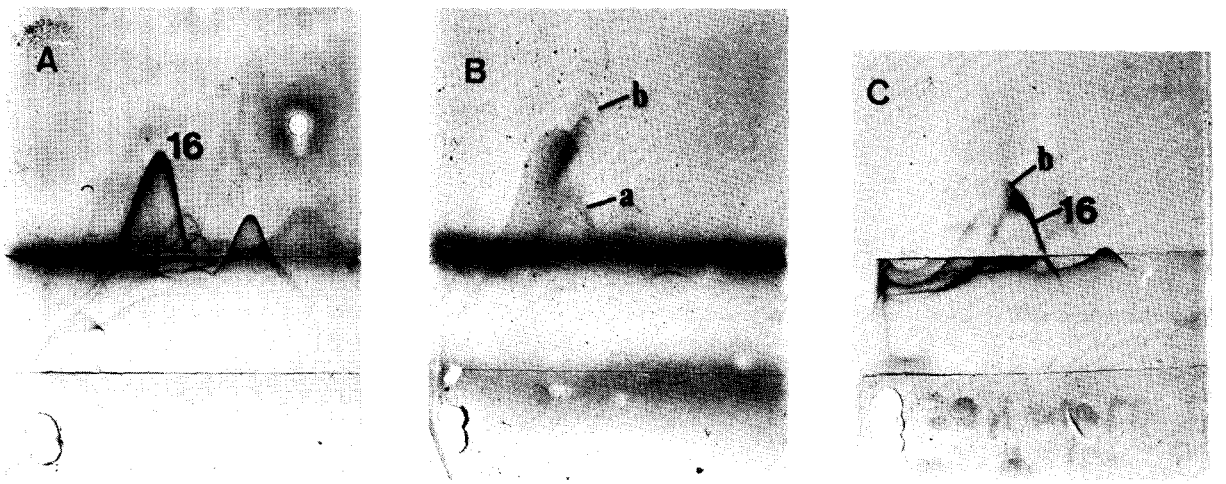

Fig. 4. Reversibility of dissociation of the glycoprotein IIb-IIIa complex. Crossed immunoelectrophoresis of solubilized aliquots of membrane suspensions after each of the following steps. A. After the platelet membranes had been washed once with 0.27 M sucrose. B. After these membranes had been suspended in 0.154 M NaCl/20 mM Tris-HCl (pH 9.0)/2 mM EDTA and incubated for 30 min at 20°C, sedimented and washed in 0.27 M sucrose. C. After these membranes had been incubated further with 0.154 M NaCl/20 mM Tris-HCl (pH 7.5)/5 mM $CaCl_2$ for 30 min at 20°C, sedimented and washed with 0.27 M sucrose. D. After the membranes had been incubated with 2 mM EDTA at pH 9.0 again. E. After the membranes had been incubated once more with 5 mM $CaCl_2$ (pH 7.5). The illustrations describes a series which was observed in three out of seven experiments. Other experimental conditions were as in Fig. 1. Immunoprecipitate 16 represents the glycoprotein IIb-IIIa complex and the immunoprecipitates a and b represent the glycoproteins IIIa and IIb, respectively.

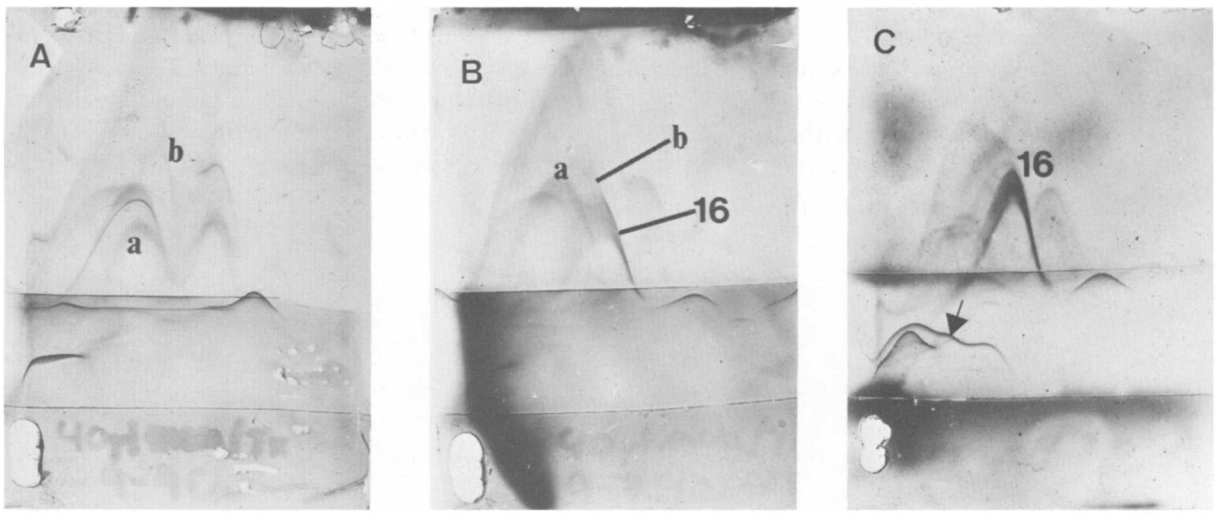

Fig. 5. Time-dependent reassociation of the glycoprotein IIb-IIIa complex at pH 9.0. Isolated membranes were suspended in 0.154 M NaCl/20 mM Tris-HCl/ 2 mM EDTA at pH 9.0, incubated at 20°C for 30 min, sedimented, washed in 0.27 M sucrose and incubated with 0.154 M NaCl/20 mM Tris-HCl/5 mM $CaCl_2$ at pH 9.0 for (A) 30 min (B) 1 h; (C) 2 h. Experimental conditions were as in Fig. 1. Immunoprecipitate 16 represents the glycoprotein IIb-IIIa complex and the immunoprecipitates a and b represent the glycoproteins IIIa and IIb, respectively. The arrow in C indicates an artificial immunoprecipitate.

9.0 than at pH 7.5 and that the presence of Ca^{2+} is essential.

Since reassociation did not occur at all in more experiments it appears that factors that were not under control influenced this process. It should also be noted that when reassociation did occur, this was not always complete, i.e., crossed immunoelectrophoresis showed immunoprecipitates representing the separate glycoproteins IIb and IIIa in addition to the major glycoprotein IIb-IIIa complex precipitate (Fig. 4C). In such cases, further reassociation did not occur even after prolonged incubation. The addition of ADP, human fibrinogen, platelet-soluble cytoplasm, protease inhibitors (iodoacetamide, *N*-ethylmaleimide, *para*-chloromercuribenzoate or leupeptin) or combination of these substances were not able to ensure reassociation.

Discussion

Previous studies have demonstrated that the predominant antigen seen after crossed immunoelectrophoresis of solubilized platelet membranes contains the glycoproteins IIb and IIIa [1,4,5]. Furthermore, the disappearance of this antigen and the concomitant appearance of two new immunoprecipitates [5,6] in the immunoelectrophoresis patterns obtained with extracts that were prepared in the presence of EDTA, has been shown to represent a dissociation of the complex into the glycoproteins IIb and IIIa [5]. The behaviour of the antigens a and b seen in the present study was identical to those representing glycoprotein IIIa and glycoprotein IIb, respectively, in the study performed by Kunicki et al. [5]. We therefore conclude that the immunoprecipitates a and b correspond to glycoprotein IIIa and glycoprotein IIb.

It has been suggested that the formation of the glycoprotein IIb-IIIa complex is dependent on Ca^{2+} [5,6,8,9]. Although we were not able to obtain reassociation in Triton X-100-solubilized material as described by Kunicki et al. [5], a calcium-induced reassociation was observed in intact membranes at pH 9.0. Thus, we were able to confirm the finding of Kunicki et al. [5] that Ca^{2+} is essential for such reassociation, at least at higher pH values. However, the addition of Ca^{2+} in excess to EDTA or EGTA may induce the libera-

tion of cations which bind more weakly to the chelators than Ca^{2+}, e.g., Mg^{2+}. It can therefore not be excluded that also Mg^{2+} may be involved in the reassociation of the glycoprotein IIb-IIIa complex. Karpatkin et al. [8] have reported that Ca^{2+}, but not Mg^{2+}, can prevent dissociation of the glycoprotein IIb-IIIa complex by EDTA or EGTA. We were not able to confirm this result and find it most probable that Ca^{2+} as well as Mg^{2+} can be used to prevent such dissociation. This suggestion is based on the finding that both Ca^{2+} and Mg^{2+} prevented dissociation of the glycoprotein IIb-IIIa complex in a manner different from that obtained with four other divalent cations, and that no difference was seen between Ca^{2+} and Mg^{2+} in this respect. Since reassociation of the complex in membranes at pH 9.0 was possible only in the presence of an excess of Ca^{2+}, it is possible that the metal specificity varies with pH and also that this may be different for reassociation of the complex and the maintenance of the complex already formed. The dependence of cations at physiological pH was difficult to investigate with respect to reassociation, probably because the proteins bind metal ions very strongly under such conditions and that small amounts of contaminating divalent cations present in the media might be sufficient to restore the metal-protein complexes.

Theoretically, the glycoproteins IIb and IIIa and divalent cations may be organized in several ways in the isolated membranes. First, the glycoproteins may be present as a complex containing the metal ion(s). Second, the glycoproteins IIb and IIIa may exist as separate entities having the metal ion(s) bound to one or both of the glycoproteins. In this case the complex may be formed during or after membrane solubilization. Alternatively, the metal ions may be liberated from another source in the membranes upon solubilization, leading to complex formation. However, the separate location of the glycoproteins IIb and IIIa in the isolated membranes seems less likely for the reasons listed below. (1) Membranes treated with EDTA in such a way that the glycoprotein IIb-IIIa complex was dissociated demonstrated a very slow reassociation of this complex at pH 9.0 or pH 8.7 when incubated with an excess of Ca^{2+}. This slow complex formation is in sharp contrast to the situation which existed when membranes that were not pretreated with EDTA or EGTA were solubilized. Such solubilization was routinely performed at pH 8.7 and the glycoproteins IIb and IIIa were always immediately present quantitatively complexed. (2) When the glycoproteins IIb and IIIa occur as dissociated after solubilization in the presence of EDTA, the reported reassociation in solution after introduction of an excess Ca^{2+} at pH 8.7 [5] proceeds even more slowly than upon incubation of membranes, showing that a reassociation after solubilization of the membranes is most unlikely. The most probable explanation therefore seems to be that the glycoproteins IIb and IIIa are closely associated in a complex containing divalent cations in the isolated membranes. However, this does not necessarily mean that the complex exists in the membrane of the intact, unstimulated platelet, as discussed later.

The dissociation of the glycoprotein IIb-IIIa complex by EDTA or EGTA seems to be influenced by pH in two ways. First, the chelating capacity of EDTA and EGTA is markedly increased by increasing the pH in the medium. Second, there seems to be a reduced metal-binding property of the glycoprotein IIb-IIIa complex itself at higher pH, since the complex is not dissociated after introduction of a chelating capacity at pH 7.5 equivalent to that which dissociates the complex at pH 9.0. The observation that membranes washed at pH 7.4 in the presence of EDTA followed by sedimentation and direct solubilization at pH 8.7 in the absence of EDTA gives the pattern of dissociated complexes [5,6] may be explained by the presence of residual EDTA. An intermediate washing with isotonic sucrose seems to prevent this dissociation.

The fact that reassociation of the glycoprotein IIb-IIIa complex was not always observed indicates that at least one factor additional to the presence or absence of divalent cations regulates this process. Furthermore, while the complex required EDTA and pH above 8.0 to be dissociated the first time, the reassociated complex was readily dissociated at pH 7.5 in the presence of EDTA. It has been suggested that proteolysis is involved in the dissociation of the complex [7], but no effect of protease inhibitors could be observed. Similarly, other factors (ADP, human fibrinogen, platelet-

soluble cytoplasm) were investigated without having any detectable effect on the dissociation/reassociation of the glycoprotein IIb- IIIa complex. It is probable that the formation of the glycoprotein IIb-IIIa complex represents an important event in activation of platelets and that this in some way is kept under metabolic control. Such control systems may influence the variable occurrence of reassociation of the glycoprotein IIb-IIIa complex in isolated platelet membranes.

In conclusion, the formation of the glycoprotein IIb-IIIa complex seems to be a rapid process in isolated platelet membranes at physiological ionic strength and pH. Even if the glycoproteins seem to be complexed in such membranes it is still a question how they are organized in the membranes of the circulating platelets. Polley et al. [11] have given ultrastructural evidence for the idea that the glycoproteins IIb and IIIa are separate entities in the membrane of the unstimulated platelet but that they rapidly form complexes in clusters when the platelets are stimulated with thrombin. Several studies have provided evidence for glycoprotein IIb and glycoprotein IIIa being involved in the binding of fibrinogen to the platelet surface [1,4,9,12–15]. The work performed by Nachman and Leung [9] and results obtained in our laboratory (unpublished results) strongly indicate that the glycoprotein IIb-IIIa complex constitutes the fibrinogen-receptor itself. Binding of fibrinogen to stimulated platelets correlates well to platelet aggregation [16] and it has been suggested that the dimeric fibrinogen molecule may constitute a bridge between two fibrinogen receptors on adjacent platelets [17]. This mechanism and a possible formation of the glycoprotein IIb-IIIa complex as a prerequisite for fibrinogen binding to the platelet surface may be the molecular explanation of platelet aggregation.

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