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Calcium and temperature regulation of the stability of the human platelet integrin GPIIb/IIIa in solution: an analytical ultracentrifugation study

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Abstract. The human platelet integrin GPIIb/IIIa (228 kDa), a Ca-dependent heterodimer formed by the α_{IIb} subunit (GPIIb, 136 kDa) and the β_3 subunit (GPIIIa, 92 kDa), serves as the fibrinogen receptor at the surface of activated platelets. The degree of dissociation of the GPIIb/IIIa heterodimer (s°_{20}*, 8.9 S) into its constituent glycoproteins (GPIIb, 5.8 S; and GPIIIa, 3.9 S) has been assessed by analytical ultracentrifugation in Triton X100 buffers, and its Ca^{2+}- and temperature-dependence correlated with Ca^{2+}-binding to GPIIb/IIIa and its temperature dependence. At 21 °C half-maximal dissociation of GPIIb/IIIa occurs at $5.5 \pm 2.5 \times 10^{-8}$ M Ca^{2+}, very close to the dissociation constant of the high affinity Ca-binding site of GPIIb/IIIa (Kd_1 $8 \pm 3 \times 10^{-8}$ M) (Rivas and González-Rodríguez, 1991) and much lower than the Kd of the 3.4 medium affinity Ca-binding sites (Kd_2 $4 \pm 1.5 \times 10^{-5}$ M), which seems to demonstrate that the stability of the heterodimer in solution at room temperature is regulated by the degree of saturation of the high-affinity Ca-binding site. At 4 °C, the stability of the heterodimer is apparently Ca^{2+}-independent, while at room and physiological temperatures (15–37 °C) the degree of dissociation of the heterodimer is regulated by the degree of dissociation of the high- and medium-affinity Ca-binding sites, respectively. On increasing the Ca^{2+} concentration up to 1×10^{-4} M after dissociation in Triton X100 solutions, the reconstitution of the GPIIb/IIIa heterodimer depends on the time and temperature at which the dissociated heterodimer was maintained, being almost complete within the first 5–10 min at 37 °C and within the first 1–2 h at 21 °C. After this time, a time- and temperature-dependent irreversible autoassociation of GPIIb (covalent) and GPIIIa (non-covalent) occurs, which hinders both the isolation of permanently stable monomers of GPIIb and GPIIIa and the reconstitution of the GPIIb/IIIa heterodimer in Triton X100 solutions.

Key words: Integrin GPIIb/IIIa – Platelet fibrinogen receptor – Ca^{2+}- and temperature-regulation – Analytical ultracentrifugation

Introduction

Human platelet integrin GPIIb/IIIa (228 kDa) is a Ca^{2+}-dependent heterodimer, which is formed by the bitopic membrane glycoproteins IIb (GPIIb or α_{IIb}, 136 kDa) and IIIa (GPIIb or β_3, 92 kDa) and serves as the receptor for fibrinogen and other adhesive proteins at the surface of activated platelets (Kieffer and Phillips 1990; Rivas et al. 1991 b). Ca^{2+} has been shown to be required for maintaining the stability of this heterodimer (Kunicki et al. 1981; Jennings and Phillips 1982), for the binding of adhesive proteins and RGD peptides to this receptor (Plow and Ginsberg 1989; Steiner et al. 1989), and for platelet aggregation (Born and Cross 1964; Bennett and Vilaire 1979). It has also been shown by indirect and direct methods that Ca^{2+} binds to GPIIb/IIIa (Brass and Shattil 1984; Rivas and González Rodríguez 1991). However, there are apparently strong discrepancies in the literature on the Ca^{2+} concentration required to maintain GPIIb associated to GPIIIa in the GPIIb/IIIa heterodimer, both in solution and in the natural membrane (Fujimura and Phillips 1983; Brass et al. 1985; Fitzgerald and Phillips 1985; Steiner et al. 1989).

We report here that while at room and physiological temperature the dissociation of GPIIb/IIIa into its constituent glycoproteins in solution is Ca^{2+}-dependent, at 4 °C the stability of the heterodimer is Ca^{2+}-independent. Thus, in the temperature range from 15 up to 37 °C there is a good correlation between the degree of Ca^{2+}-dependent dissociation of GPIIb/IIIa, assessed here by analytical ultracentrifugation, and the degree of unsaturation of

Abbreviations: GPIIb, GPIIIa, and GPIIb/IIIa, glycoprotein IIb, IIIa, and the heterodimer formed by them, respectively; s°_{20}*, the sedimentation coefficient of the glycoprotein-detergent complexes determined at 20 °C, after extrapolation to zero-glycoprotein concentration

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the high- and/or medium-affinity Ca-binding sites of GPIIb/IIIa, determined elsewhere (Rivas and González-Rodríguez 1991).

Materials and methods

Materials

GPIIb, GPIIIa, and GPIIb/IIIa were isolated from the plasma membrane fraction of human platelets, as described elsewhere (Eirin et al. 1986; Rivas et al. 1991 a).

Analytical methods

Glycoprotein concentration was calculated from protein determination by the method of Markwell et al. (1978), as described before (Eirín et al. 1986). SDS/polyacrylamide gel electrophoresis was done according to Laemmli (1970). Total calcium concentration was determined by atomic absorption spectrometry (Perkin Elmer model 303) using calcium standards prepared according to Forstner and Manery (1971).

Size-exclusion chromatography

Size-exclusion chromatography was used both for equilibration of GPIIb/IIIa at a given Triton X100 concentration (Rivas et al. 1991 b) and for analysis of the reversibility of the Ca^{2+}-dependent dissociation of GPIIb/IIIa. 1 ml samples of GPIIb/IIIa (2–6 mg/ml) in 50 mM *Tris*/HCl/0.2% (w/v) Triton X100, pH 7.4 buffer, at a given calcium and EGTA concentration, were loaded onto a Sephacryl S300 column (135 cm × 1 cm) equilibrated in the same buffer containing 1×10^{-4} M Ca^{2+} at 21 ± 1 °C, and eluted at 6 ml/h. The column was calibrated using globular proteins, as described elsewhere (Rivas et al. 1991 b).

Determination by analytical ultracentrifugation of the isotherms of Ca^{2+}-dependent dissociation of GPIIb/IIIa

Samples of GPIIb/IIIa (2 mg/ml) were equilibrated in 50 mM *Tris*/HCl/0.2% Triton X100/0.1 mM Ca^{2+}, pH 7.4 buffer and the free Ca^{2+} in each sample adjusted to the required concentration by addition of EGTA, calculated according to Fabiato (1981), to account for the influence of pH, temperature and ionic strength in each of them on the equilibrium constants of the chelating agent. Then the samples were subjected to sedimentation velocity measurements, at 60 000 rpm and at a given temperature, in a Beckman model E ultracentrifuge, using Schlieren optics. The percentage of free GPIIb and GPIIIa subunits and GPIIb/IIIa heterodimer, sedimenting at a given free Ca^{2+} concentration and temperature and at a given centrifugation time after reaching top speed, was determined by integration of the area under the Schlieren peak of each species, after projecting the Schlieren profile in a Nikon profile projector. The data was fitted to the expression $F = K[Ca^{2+}]/(1 + K[Ca^{2+}])$ using a non-linear least-squares procedure based on the Nelder-Head algorithm (Press et al. 1986), where F is the mass fraction of the undissociated heterodimer, $F = [\text{GPIIb/IIIa}]/[\text{GPIIb/IIIa}]_T$, [GPIIb/IIIa] and $[\text{GPIIb/IIIa}]_T$ are the concentrations of the undissociated and total GPIIb/IIIa heterodimer, respectively, and K is the Ca^{2+}-association constant of the heterodimer.

Results

Ca^{2+}- and temperature-dependence of the dissociation in solution of GPIIb/IIIa into its constituent subunits. Dissociation reversibility

The sedimentation coefficients of GPIIb (s°_{20} * 5.9 S) and GPIIIa (3.9 S), in 50 mM *Tris*/HCL/0.2% (w/v) Triton X100, pH 7.4 buffer, differ quite significantly from that of the GPIIb/IIIa heterodimer (8.9 S) (Rivas et al. 1991 b). That led us to choose analytical ultracentrifugation as the most convenient technique for the quick and easy separation of the subunits from the heterodimer. Thus, we were able to measure the isotherms of the Ca^{2+}-dependent dissociation of GPIIb/IIIa at different temperatures and to study the time- and temperature-dependence of the reversibility of this dissociation.

After 30 min of incubation at 21 °C of pure GPIIb/IIIa (2 mg/ml) at different Ca^{2+} concentrations, from 10^{-3} M to 10^{-9} M, the Schlieren patterns of sedimentation, carried out at 21 °C, showed a fast moving peak of 8.9 S, which corresponds to the GPIIb/IIIa heterodimer. As the Ca^{2+} concentration decreases, a slower moving peak of about 5 S, which corresponds to the unresolved mixture of the dissociated subunits, GPIIb and GPIIIa, was apparent, whose area gradually increases at the expense of that of the 8.9 S peak (Fig. 1). Quantitative analysis of the areas of both Schlieren peaks, at a fixed time after reaching top speed, provided the equilibrium concentrations of the subunits and the heterodimer, at the different Ca^{2+} concentrations, from where the isotherm at 21 °C of the Ca^{2+}-dependent dissociation of GPIIb/IIIa (average of 4 experiments) was constructed (Fig. 2). Theoretically, a limitation in our quantitative estimates of the undissociated GPIIb/IIIa heterodimer could have arisen from the decrease of the area under the fast Schlieren peak (s°_{20} * 8.9 S), due to the inverse concentration gradient of dissociated GPIIb and GPIIIa, the slow moving species (s°_{20} * 3.9 S and 5.9 S, respectively), at the fast sedimentation boundary. In practice, however, three experimental characteristics in our system – the low glycoprotein concentration used in our experiments (2 mg/ml) the fairly large difference between the sedimentation coefficient of the undissociated heterodimer and those of the dissociated subunits; and the very low concentration-dependence of the sedimentation coefficient of the three species, particularly that of GPIIb/IIIa, in the experimental buffer (Rivas et al. 1991 b) – make it reasonable for us to ignore those very weak Johnston-Ogston effects (see, for exam-

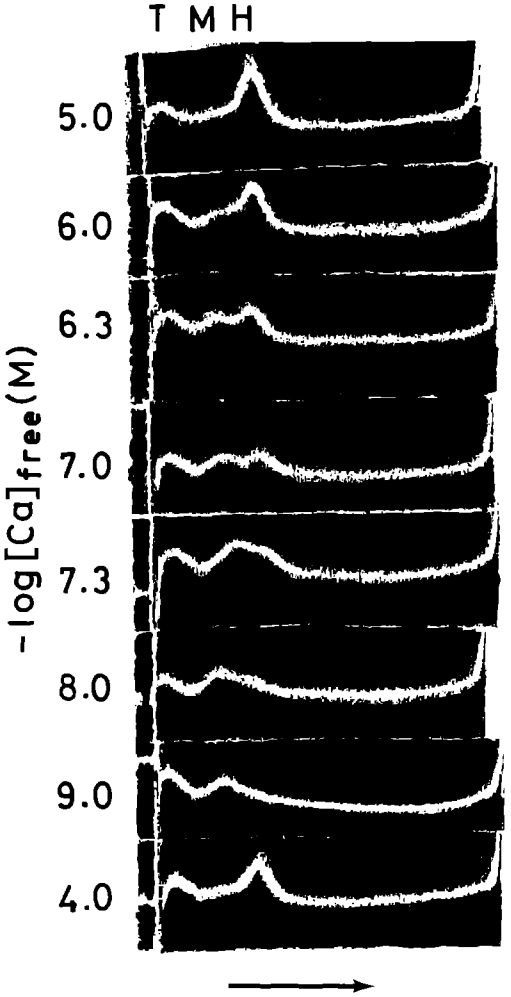

Fig. 1. Sedimentation velocity pattern of the Ca^{2+}-dependent dissociation of the GPIIb/IIIa heterodimer (2 mg/ml) in 50 mM *Tris*/HCl/0.2% (w/v) Triton X100/pH 7.4, at 21 °C, as a function of the Ca^{2+} concentration. The Schlieren profiles were taken at 21 °C and at 24 min after reaching top speed (60 000 r.p.m.), as indicated in the Methods section. The arrow indicates the direction of sedimentation. T, M, and H indicate the position of the Schlieren peaks for the Triton X100 micelles, the mixture of dissociated GPIIb and GPIIIa (5–6 S), and the undissociated GPIIb/IIIa heterodimer (8.9 S), respectively. The Schlieren profile at the bottom of the figure corresponds to the GPIIb/IIIa heterodimer reformed at 1×10^{-4} M Ca^{2+}, after being dissociated at 1×10^{-9} M Ca^{2+} for 1 h at 21 °C

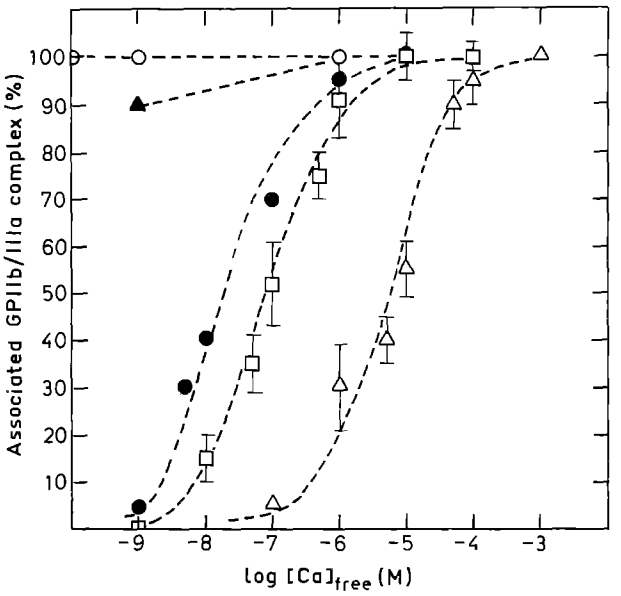

Fig. 2. Isotherms of the Ca^{2+}-dependent dissociation of the GPIIb/IIIa heterodimer at various temperatures: 4 °C (○); 10 °C (▲); 15 °C (●); 21 °C (□); 35 °C (△). The percentage of undissociated GPIIb/IIIa was determined by analytical ultracentrifugation from the areas under the Schlieren pattern for the monomer (GPIIb and GPIIIa) and the heterodimer GPIIb/IIIa fractions, as indicated in the Methods section

ple, Bowen 1970) and, therefore, rely on our quantitative estimates.

Thus, at 21 °C GPIIb/IIIa is fully undissociated at 1×10^{-5} M Ca^{2+}, half-dissociated at $5.5 \pm 1.5 \times 10^{-8}$ M Ca^{2+}, and fully dissociated at 1×10^{-9} M Ca^{2+}. The isotherm at 15 °C (a single experiment) shows that the half-maximal dissociation of GPIIb/IIIa at this temperature takes place at 1.6×10^{-8} M. However when the incubation and the sedimentation analyses were done at 4 °C, the heterodimer remains undissociated, even at 1×10^{-10} M Ca^{2+} (5 experiments) (Fig. 2). On the other hand, the isotherm at 35 °C (average of 4 experiments) shows that the half-maximal dissociation of GPIIb/IIIa occurs at $7.5 \pm 4.5 \times 10^{-6}$ M Ca^{2+} (Fig. 2).

Temperature- and time-dependence of the Ca^{2+}-dependent dissociation of GPIIb/IIIa

On bringing the Ca^{2+} concentration back to 1×10^{-4} M, the reversibility of the dissociation of GPIIb/IIIa was assessed by the sedimentation pattern and found to be dependent on the temperature and the time of dissociation. Thus, the heterodimer (8.9 S) is fully reformed after dissociation at 21 °C and 1×10^{-9} M Ca^{2+}, provided that the incubation time is not longer than 1 h (Schlieren profile at the bottom of Fig. 1). At longer incubation times the dissociation becomes gradually irreversible, being fully irreversible after 10–12 h. At 37° and 1×10^{-9} M Ca^{2+} the dissociation of GPIIb/IIIa is already fully irreversible after 15 min of incubation.

Nature of the products of irreversible dissociation of GPIIb/IIIa

To ascertain further the nature of the reformed heterodimer and the products of irreversible dissociation of GPIIb/IIIa, they were subjected to size-exclusion chromatographic analysis at 21 °C (see Methods), and the chromatographic fractions obtained subjected to sedimentation velocity and electrophoretic analysis. GPIIb/IIIa reformed at 1×10^{-4} M Ca^{2+} (from GPIIb/IIIa dissociated at 1×10^{-9} M Ca^{2+} either at 21 or 37 °C for no longer than 60 or 5 min, respectively) showed an elution pattern (Fig. 3 A) where the major fraction (Kp 0,13, s°_{20} * 8.9 S) comprises about 90% of the loaded protein and whose electrophoretic analysis proved a 1/1 GPIIb/IIIa

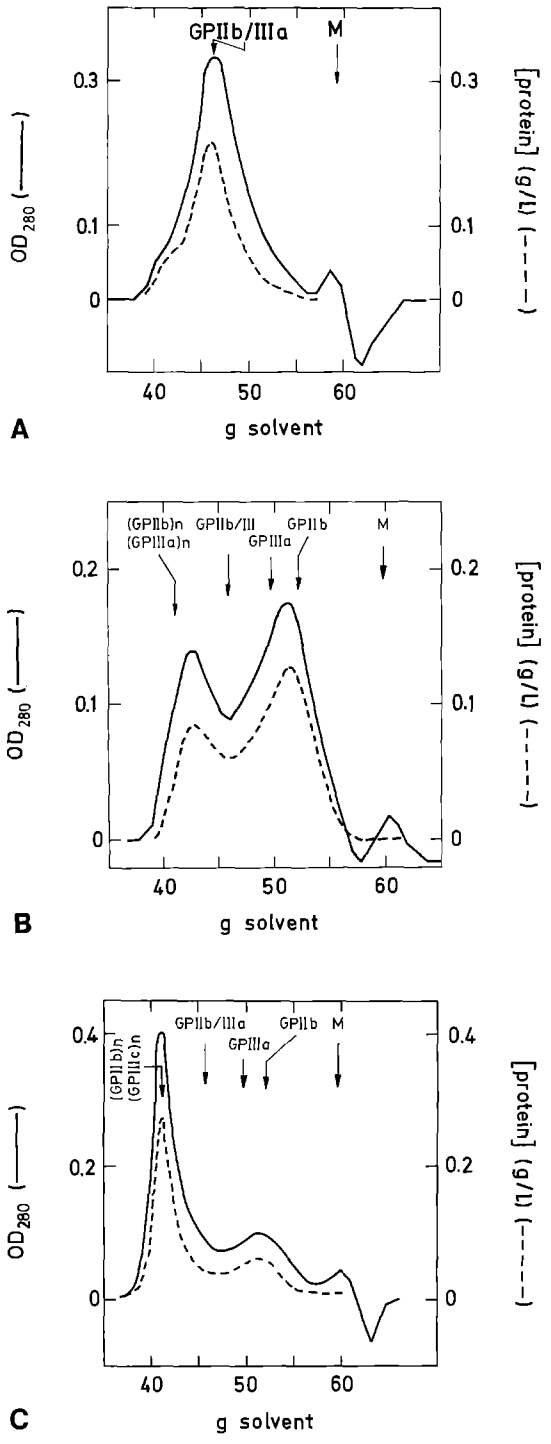

Fig. 3A–C. Size-exclusion chromatographic analysis (at 21 °C) of the degree of reversibility of the Ca^{2+}-dependent dissociation of GPIIb/IIIa and its dependence on the time and temperature at which the dissociated heterodimer has been maintained. In all cases, 1 ml (2 mg protein/ml) of sample was loaded into a Sephacryl column (135 cm × 1 cm) equilibrated in 50 mM *Tris*/HCl 0.2% (w/v) Triton X100/0.1 mM Cl_2Ca/pH 7.4 as indicated in the Methods section. **A** chromatographic analysis of GPIIb/IIIa reconstituted after increasing the Ca^{2+} concentration up to 1×10^{-4} M in solutions of the heterodimer which had been maintained dissociated for 5–10 min at 37 °C or 1 h at 21 °C. **B** and **C**, analysis of the products of irreversible dissociation of GPIIb/IIIa after raising the Ca^{2+} concentration up to 1×10^{-4} M in solutions of the heterodimer which had been maintained dissociated for 3 h and 12 h, respectively, at 21 °C. M, Triton X100 micelles. $(GPIIb)_n$ and $(GPIIIa)_n$, the fractions of homopolymers of GPIIb and GPIIIa, respectively

molar ratio, as expected for the undissociated heterodimer (Rivas et al. 1991 b).

When GPIIb/IIIa was dissociated at 1×10^{-9} M either at 21 or 37 °C and $[Ca^{2+}]$ increased up to 1×10^{-4} M after more than 120 and 15 min, respectively, the chromatographic patterns obtained are similar to those of pure GPIIb and GPIIIa in Triton X-100 buffers (Rivas et al. 1991 b), with a heterogenous large molecular-size fraction (Kp 0.10, 10–12 S), and a monomer fraction (Kp 0.21, 5–6 S) characteristic of the mixture of GPIIb and GPIIIa (Fig. 3 B). Electrophoretic analysis shows that the major component of the large molecular-size fraction is the non covalent aggregate of GPIIIa, $(GPIIIa)_n$, being the covalent aggregate of GPIIb, $(GPIIb)_n$, the minor component. In contrast, the monomer fraction is made up mainly of GPIIb monomer and, to a lesser extent, some GPIIIa monomer. When the dissociation time is prolonged further, the aggregates fraction gets sharper, its Kp decreases to 0.07, and its area increases at the expense of the monomeric fraction, becoming the major fraction in samples subjected to 10–12 h of dissociation time (Fig. 3 C). The increase in ionic strength and/or pH did not affect this chromatographic pattern.

As seen above, we have not been able to maintain permanently (for more than 2 h at room temperature) stable monomers of GPIIb and GPIIIa after dissociation of GPIIb/IIIa in Triton buffers, and therefore we have not been able to isolate those monomers. Thus, attempts were made to reconstitute the GPIIb/IIIa heterodimer using GPIIb and GPIIIa monomers isolated in SDS (Eirin et al. 1986; Rivas et al. 1991 a). So far we have always failed, in spite of the multiple modifications made in the reconstitution buffer, such as in the ionic strength, pH, Triton X-100, lipid, and Ca^{2+} concentrations, and temperature. In all cases we obtained a major fraction composed mainly of non-covalent aggregates of GPIIIa and, to a lesser extent, covalent aggregates of GPIIb, and a minor fraction composed of GPIIb monomers and, to a lesser extent, GPIIIa monomers, as seen above for GPIIb/IIIa dissociated for long periods (Fig. 2 B and C).

Discussion

In this work we have determined the isotherms (at 4, 15, 21 and 35 °C) of dissociation of the pure GPIIb/IIIa heterodimer in solution as a function of the Ca^{2+} concentration, by assessing the degree of dissociation through sedimentation velocity using analytical ultracentrifugation. We have also investigated the degree of reversibility of this Ca^{2+} -dependent dissociation and its dependence on the time and temperature at which the dissociated heterodimer has been maintained. Now, we will compare and discuss these results in relation with the Ca-binding isotherms of GPIIb/IIIa in solution at different temperatures, the molecular properties of GPIIb, GPIIIa, and GPIIb/IIIa in solution, and previous studies on the GPIIb/IIIa dissociation where criteria or techniques different from those used here were employed.

At 21 °C the half-maximal dissociation of GPIIb/IIIa takes place at $5.5 \pm 2.5 \times 10^{-8}$ M Ca^{2+}, which is very

close to the dissociation constant of the high-affinity Ca-binding site of GPIIb/IIIa in solution (Kd_1 8×10^{-8} M) (Rivas and González-Rodríguez 1991). This Ca^{2+} concentration is almost half the Ca^{2+} concentration obtained by Steiner et al. (1989) using density gradient centrifugation at 25 °C, ten times lower than the Ca^{2+} concentration (4×10^{-7} M) at which half-maxima binding of a GPIIb/IIIa-specific monoclonal antibody to GPIIb/IIIa occurs at 25 °C (Brass et al. 1985), and much lower than the Kd of the 3.4 medium-affinity Ca-binding sites (Kd_2 4×10^{-5} M). All this seems to demonstrate that the stability of the GPIIb/IIIa heterodimer in solution at room temperature is regulated by the degree of saturation of its high-affinity Ca binding site.

At 15 °C the half-maximal dissociation of GPIIb/IIIa takes place at 1.6×10^{-8} M Ca^{2+}, about a three-fold lower concentration than that found at 21 °C. However, at 4 °C the heterodimer remains undissociated even at 1×10^{-10} M Ca^{2+}, in spite of the fact that the high-affinity site does not appreciably change its Ca-affinity (Kd_1 6×10^{-8} M) compared with that at 21 °C (Kd_1 8×10^{-8} M) (Rivas and González-Rodríguez 1991). This shows that the stability of the heterodimer at 4 °C is Ca^{2+}-independent, and explains the inability of the chelating agents to dissociate GPIIb/IIIa at this temperature in solution, as observed before (Rosa et al. 1984), and the ability of Fitzgerald and Phillips (1985) to assess the degree of dissociation of GPIIb/IIIa in intact platelet after lysis and solubilization at 4 °C in Triton X-100 buffers containing EDTA.

At 35 °C the half-maximal dissociation of GPIIb/IIIa occurs at $7.5 \pm 4.5 \times 10^{-6}$ M Ca^{2+} concentration, slightly lower than that estimated by the GPIIb/IIIa susceptibility to thrombin attack and by sucrose-density gradient centrifugation at 37 °C (Fujimura and Phillips 1983), and about three-fold lower than the Kd determined for the four medium-affinity Ca-binding sites determined by equilibrium dialysis at 37 °C (Rivas and González-Rodríguez 1991). The accuracy of the binding data at 37 °C did not let us decide whether the high-affinity Ca-binding site determined at 4 and 21 °C becomes structurally lost at physiological temperatures or whether its affinity decreases to the point of becoming indistinguishable from the medium-affinity sites. If the latter interpretation is the correct one, this would imply that the high-affinity Ca-binding site still regulates the stability of the GPIIb/IIIa heterodimer at 37 °C in solution.

We have found that on increasing the Ca^{2+} concentration up to 1×10^{-4} M at room and physiological temperatures, the reconstitution of the GPIIb/IIIa heterodimer depends on the time and temperature at which the dissociated heterodimer was maintained. Thus, while at 21 °C the dissociation is fully reversible within the first two hours after dissociation, at 37 °C the reformation of the heterodimer is complete only within the first 5–10 min after dissociation, in agreement with observations made by Fitzgerald and Phillips (1985) and Shattil et al. (1985) in intact platelets at 37 °C. These authors had also pointed out the formation of high-molecular mass polymers during prolonged incubation of intact platelets with EDTA at 37 °C, but they were not able to ascertain whether this involved auto- or heteroassociation of GPIIb and GPIIIa. Here we have isolated these polymers by size-exclusion chromatography after prolonged incubation of pure GPIIb/IIIa at both 21 and 37 °C and at Ca^{2+} concentration low enough to maintain the heterodimer fully dissociated. Further sedimentation velocity and electrophoretic analysis demonstrated the presence of disulphide-bonded homopolymers of GPIIb and non-covalent homopolymers of GPIIIa, similar in size heterogeneity and, most probably, in the mechanism of autoassociation, as those found in Triton X-100 solutions of pure GPIIb and GPIIIa (Rivas et al. 1991b). Therefore, both the dissociation of the GPIIb/IIIa heterodimer and the irreversible autoassociation of GPIIb and GPIIIa seem to be the most probable mechanism of the irreversible loss of aggregability and reduced Ca^{2+} binding capacity, repeatedly observed in platelets subjected to prolonged incubation with EDTA at 37 °C (Zucker and Grant 1978; Peerschke et al. 1980; Shattil et al. 1985; Pidard et al. 1986). Our observations are equally relevant in explaining our inability to reconstitute the GPIIb/IIIa heterodimer from the isolated constituent glycoproteins. On the one hand, the time and temperature-dependent irreversible autoassociation of GPIIb and GPIIIa in Triton X-100 solutions hinders the isolation of stable monomers of GPIIb and GPIIIa in this detergent. On the other hand, when the monomers of GPIIb and GPIIIa obtained in SDS buffers are transferred to Triton X-100 solutions they autoaggregate, which again impedes the reconstitution of GPIIb/IIIa from its isolated constituent glycoproteins.

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