Eur Biophys J (1991) 19:335–345

European Biophysics Journal

Molecular characterization of the human platelet integrin GPIIb/IIIa and its constituent glycoproteins

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Received October 29, 1990/Accepted in revised form January 18, 1991

Abstract. Human platelet plasma membrane glycoproteins IIb (GPIIb) and IIIa (GPIIIa) form a Ca^{2+}-dependent heterodimer, the integrin GPIIb/IIIa, which serves as the receptor for fibrinogen and other adhesive proteins at the surface of activated platelets. Below the critical micellar concentration of Triton X100 (TtX), the three glycoproteins do not bind appreciably to TtX and form association products of large size. The size-exclusion chromatographic patterns of GPIIb, GPIIIa and GPIIb/IIIa have been obtained at 0.2% TtX, and the molecular properties of the association products and monomer fractions have been determined by analysis of the detergent bound to the glycoproteins, laser-light scattering, sedimentation velocity, and electron microscopy (TEM). The monomer of the GPIIb-TtX complex was identified by the molecular mass (M) of the glycoprotein moiety (125 ± 15 kDa), the molecular size (9.5 ± 1.5 nm $\times$ 11 ± 1.5 nm) and globular shape observed by TEM. It has a molecular mass (M^*) of 197 ± 20 kDa, a sedimentation coefficient $s^{\circ}_{20}{}^*$ of 5.8 ± 0.1 S, a Stokes radius R_s^* of 6.8 ± 0.4 nm, and a frictional ratio f^*/f^*_{min} of 1.7 ± 0.14. The $(GPIIb)_n$-TtX complexes are disulphide-bonded size-heterogeneous association products of GPIIb, tetramers being the smallest species found. GPIIIa has a greater propensity to self-associate than GPIIb, this tendency being lower below 1 mg GPIIIa/ml, 0.1 mM Ca^{2+}, pH 9.0. The $(GPIIIa)_n$-TtX complexes are noncovalent size-heterogeneous association products of GPIIIa, tetramers being the smallest form observed. The monomer of the GPIIIa-TtX complex was identified by the 103 ± 15 kDa M determined for the glycoprotein moiety, and the 9 ± 1.5 nm $\times$ 10 ± 1.5 nm size and globular shape observed by TEM. It has a M^* of 136 ± 15 kDa, a $s^{\circ}_{20}{}^*$ of 3.9 ± 0.3 S, a R_s^* of 6.4 ± 0.5 nm, a f^*/f^*_{min} of 1.9 ± 0.3, and, when stored at pH 7.4, has a certain tendency to form filamentous association products (20–70 nm $\times$ 2–5 nm), as observed by TEM. The GPIIb/IIIa-TtX complex in 0.2% TtX/0.1 mM Ca^{2+} elutes as a single monomeric fraction, as deduced from the 210 ± 15 kDa M determined for its glycoprotein moiety and the 12 ± 1.5 nm $\times$ 14 ± 1.5 nm size of the globular forms observed by TEM. The GPIIb/IIIa-TtX complex has a M^* of 315 ± 20 kDa, a $s^{\circ}_{20}{}^*$ of 8.9 ± 0.2 S, a R_s^* of 7.4 ± 0.2 nm, a f^*/f^*_{min} of 1.5 ± 0.12, and appears in the electron micrographs in multiple forms (filled globular, empty oval, head-two-tails, and bilobular shapes), which on lowering the TtX concentration tend to self-associate, forming abundant rosettes below the TtX critical micellar concentration.

Key words: GPIIb, GPIIIa, and GPIIb/IIIa heterodimer – Platelet fibrinogen receptor – Molecular mass, size, shape and self-association

Introduction

Glycoproteins IIb (GPIIb) and IIIa (GPIIIa) are the two major bitopic glycoproteins of the platelet surface, where they form the α and β subunits, respectively, of the integrin GPIIb/IIIa, the Ca^{2+}-dependent heterodimer which serves as the receptor for fibrinogen and other adhesive proteins upon platelet activation (Nachman and Leung 1982; Bennett et al. 1982; Ruggeri et al. 1983; Plow et al. 1985; Thiagarajan and Kelly 1988; Phillips et al. 1988). GPIIb, the two-chain 136 kDa α_{IIb} subunit, and GPIIIa, the single-chain 92 kD β_3 subunit, are the integrin subunits whose covalent structure, molecular properties and functional topography are better known today (Nurden et al. 1986; Hynes 1987; Rouslahti and Pierschbacher 1987; Usobiaga et al. 1987; Calvete et al. 1989; Calvete et al. 1991 a, b, c; D'Souza et al. 1990; Heidenreich et al. 1990; Zimrin et al. 1990; Rivas and González-Rodríguez 1991).

Abbreviations: GPIIb, GPIIIa and GPIIb/IIIa, glycoproteins IIb, IIIa and the heterodimer formed between them; GPIIbα and GPIIbβ, the α- and β-chains of GPIIb; c.m.c., critical micellar concentration; TtX, Triton X-100; SDS, sodium dodecyl sulphate; superscript (*) attached to a symbol means a property referred to the glycoprotein-detergent complexes

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The molecular mass of pure GPIIb and GPIIIa and of the isolated chains of the former, and the hydrodynamic properties and molecular dimensions of their complexes with SDS have been determined before, using rigorous procedures (Usobiaga et al. 1987). The same has been done for GPIIb, GPIIIa and GPIIb/IIIa in 0.05% Triton X100 (TtX) solution (Jennings and Phillips, 1982; Carrell et al. 1985) and for GPIIb/IIIa in 0.1% TtX (Parise et al. 1987) by a combination of gel filtration, sucrose density gradient centrifugation, and electron microscopy (TEM). However, these studies left unanswered several important questions: e.g., the TtX bound to these glycoproteins; the influence of the concentration of TtX, Ca^{2+}, and glycoprotein on the size-exclusion chromatographic patterns of these glycoproteins in TtX solutions and on the degree of self-association; the disagreement between the molecular mass determined for GPIIb and GPIIIa (Usobiaga et al. 1987) and those determined for GPIIb/IIIa (Jennings and Phillips 1982; Parise et al. 1987); and the lack of agreement between the molecular and hydrodynamic properties determined for GPIIIa (and the filamentous structure) and the molecular dimensions determined for the monomer of GPIIIa under the same conditions by TEM (Jennings and Phillips 1982; Carrell et al. 1985). Furthermore, in the course of reconstitution experiments of GPIIb/IIIa from its constituent glycoproteins, we attempted to isolate GPIIb and GPIIIa by size-exclusion chromatography in TtX buffers, after dissociation of GPIIb/IIIa at room temperature by lowering the Ca^{2+} concentration below $1 \times 10^{-9} M$ (Rivas and González-Rodríguez 1991), and we observed very unexpected elution patterns for the two glycoproteins. All this prompted us to initiate the present investigation where, after establishing the size-exclusion chromatographic patterns of pure GPIIb, GPIIIa and GPIIb/IIIa under different solubilization conditions, we study the isolated monomer and association product fractions for the three glycoproteins by determining the TtX bound (via chemical analysis), the glycoprotein molecular mass (by means of laser-light scattering), the hydrodynamic properties of the glycoprotein-TtX complexes (by analytical centrifugation), and the molecular shape and dimensions of these complexes (with the aid of TEM). Our results are compared with one another and discussed in relation to molecular information already available for these glycoproteins, both in SDS and in TtX solutions, to deduce a more clear and complete picture.

Materials and methods

Chemicals and biochemicals

Eosin-5-isothiocyanate was from Molecular Probes (U.S.A.).Triton X100 (TtX) was from Merck, and whenever necessary was purified according to Ashani and Catravas (1980). Sephacryl S 300 and DEAE Sephacel were from Pharmacia (Sweden). All chemicals and biochemicals were of analytical or chromatographic grade. GPIIb, GPIIIa and GPIIb/IIIa were prepared as described elsewhere (Eirin et al. 1986; Rivas et al. 1991).

Analytical methods

Glycoprotein concentration was calculated from protein determination by the method of Markwell et al. (1978) and by amino acid and sugar analyses of the glycoproteins (Eirin et al. 1986). TtX was determined according to Garewall (1973), after evaporation of the samples in a Speed Vac Concentrator (Savant, USA). Analysis of oxidant contaminations in TtX solutions were done as described by Chang and Bock (1980). SDS/polyacrylamide gel electrophoresis was done according to Laemmli (1970). The critical micellar concentration of TtX in buffers was determined by the fluorimetric procedure of Chattopadhyay and London (1984).

Size-exclusion chromatography of the glycoproteins. Triton binding

Glycoprotein samples (1–6 mg/ml) in 50 m*M* *Tris*/HCl/0.2% (w/v) TtX/0.1 m*M* $CaCl_2$/pH 7.4 were pre-equilibrated at a given TtX concentration by retaining them in a DEAE-Sephacel column (4 cm × 1 cm) which was subsequently eluted with the same buffer containing 0.5 *M* NaCl and the required TtX concentration. The pre-equilibrated samples were loaded in an analytical Sephacryl S 300 column (135 cm × 1 cm), equilibrated at 21 ± 1 °C in 50 m*M* *Tris*/HCl with, TtX, Ca^{2+} or EDTA, and pH as required (*Tris*/TtX buffer), and eluted at 6 ml/h. This column was calibrated using globular proteins (ferritin, human IgG, bovine serum albumin, ovalbumin, cytochrome *C*) of known molecular mass and Stokes radius (R_s), which bind less than 0.04 mg TtX/mg protein (Helenius and Simons 1972; Makino et al. 1973; Clarke 1975). The partition coefficient Kp is defined as $Kp = (V_p - V_0)/(V_t - V_0)$, where V_0, V_t, and V_p are the total exclusion volume, the total inclusion volume, and the elution volume of the protein under consideration, respectively. The optical absorption at 280 nm, and the TtX and protein content of every fraction along the elution profile were determined, from which the TtX bound to the glycoprotein at equilibrium could be calculated, after subtraction of the TtX in the elution buffer.

Sedimentation velocity measurements

Glycoprotein samples (0.1–2 mg/ml) that had been equilibrated in a *Tris*/0.2% TtX buffer by size exclusion chromatography, as described above, were subjected to analytical ultracentrifugation at 20 ± 1 °C in a Beckman model E. Sedimentation coefficients of the glycoprotein-TtX complexes ($s^{\circ}_{20}{}^{*}$) were determined from the sedimentation patterns using either Schlieren optics at glycoprotein concentrations above 0.6 mg/ml or 530 nm absorption at concentrations below 0.6 mg/ml. For the latter measurements, glycoproteins were labelled with eosin-5-isothiocyanate, as described previously (Usobiaga et al. 1987), but now using 50 m*M* *Tris*/HCl/0.01% TtX/pH 8.0, and, once freed of unbound dye, equilibrated in the required *Tris*/0.2% TtX buffer. Labelled glyco-

proteins did not show any appreciable change in the amount of TtX bound or in their electrophoretic, chromatographic or sedimentation behavior, as compared with unlabelled samples.

The Stokes radii (R_s^*) of the glycoprotein-TtX complexes were calculated according to Tanford et al. (1974) using the expression $R_s^* = M^*/(1-\bar{v}_p^* d_{20})/S_{20}^{\circ *} N 6\pi\eta_{20}$, (Eq. (1)), where $M^* = M(1+\delta_d)$, is the molar mass of the glycoprotein-TtX complex, obtained from the molar mass of the glycoprotein moiety (M) determined before (Usobiaga et al. 1987), and the bound detergent (δ_d) determined here; $\bar{v}_p^* = (\bar{v}_p + \delta_d \bar{v}_d)/(1+\delta_d)$, is the partial specific volume of the glycoprotein-detergent complex, obtained from $\bar{v}_p$, the partial specific volume of the glycoprotein moiety (Usobiaga et al. 1987), δ_d, and $\bar{v}_d$, the partial specific volume of the detergent (Tanford et al. 1974); $s_{20}^{\circ *}$ is determined as indicated above; N is Avogadro's number; and d_{20} (1.01 ± 0.01 g cm^{-3}) and η_{20} ($10.2 \pm 0.1 \times 10^{-3}$ P) are the density and viscosity of the equilibrium buffer used in the size-exclusion column, respectively, measured at 20 °C, using an Anton Paar DMA O2D precision densimeter and a Ubbelohde viscometer.

The frictional ratios of the glycoprotein-TtX complexes were calculated using the expression of $f^*/f_{min}^* = R_s^*/R_{min}^*$, where R_s^* has been defined above, and $R_{min}^* = (3M^* \bar{v}_p^*/4\pi N)^{1/3}$ (Tanford and Reynolds 1976).

Size-exclusion chromatography coupled to laser-light scattering

To determine the molecular mass of the glycoprotein species in the different fractions coming out of the size-exclusion column in a reliable and economical way, a peristaltic pump, a Millipore MF 0.22 µm filter, a Chromatix type KMX-6 low-angle (6–7°) laser (He-Ne, 2 mW, λ 633 nm)-light scattering photometer, a differential refractometer (Spectra-Physics SP 6040), and a UV detector were arranged in series at the outlet of a Sephacryl S 300 column (135 cm × 1 cm), as described before (Maezawa et al. 1983). All measurements were performed at 21 ± 1 °C, and samples and buffers were clarified using a Millipore 0.1 µm filter.

As discussed elsewhere (Maezawa et al. 1983; Hayashi et al. 1989), the molar mass (M) of the glycoproteins and the scattered light intensity are related by the following expressions:

$$M = K_1 (\text{Output})_{LS}/(dn^*/dc)(\text{Output})_{RI}, \quad (2)$$

where

$$(dn^*/dc) = K_2 \varepsilon (\text{Output})_{RI}/(\text{Output})_{UV}, \quad (3)$$

therefore

$$M = K_3 (\text{Output})_{LS}(\text{Output})_{UV}/\varepsilon (\text{Output})_{RI}^2, \quad (4)$$

where (dn^*/dc) is the refractive index increment of the glycoprotein-TtX complex, $(\text{Output})_{LS}$, $(\text{Output})_{RI}$, and $(\text{Output})_{UV}$ are the outputs of the light scattering photometer, the refractometer, and the UV detector, ε is the extinction coefficient of the protein under consideration, and K_1, K_2 and K_3 are instrumental constants which were determined using standard proteins of known M, dn/dc, and ε, and which do not bind TtX appreciably. In our case, ovalbumin (M 44 000 g mol^{-1}, dn/dc 0.189 ml g^{-1}, ε 0.67 ml mg^{-1} cm^{-1}), bovine serum albumin (M 68 000, dn/dc 0.188, ε 0.67), IgG (M 150 000, dn/dc 0.191, $\varepsilon = 1.12$) (Fasman 1976; Hayashi et al. 1983; Maezawa et al. 1983). The values of dn^*/dc and ε determined for the glycoproteins studied here in 0.2% TtX were: GPIIb (0.248 ± 0.07 ml g^{-1}, 1.88 ± 0.17 ml mg^{-1} cm^{-1}), GPIIIa (0.253 ± 0.07; 1.93 ± 0.21), and GPIIb/IIIa (0.243 ± 0.01; 1.84 ± 0.12).

Electron microscopy of rotary shadowed samples

Samples of every glycoprotein were prepared for TEM observation by rotary shadowing at very low angle. Glycoproteins (0.2 mg/ml) in 50 m*M* *Tris*/HCl/0.1 m*M* $CaCl_2$/0.1% TtX/pH 7.4 buffer were diluted at a final concentration of 20 µg/ml using a 2/1 (v/v) buffer/glycerol mixture. Occasionally, either the TtX or the Ca^{2+} concentration or the pH of the buffer were modified as indicated, according to the aims of the experiment. About 50 µl of the diluted glycoprotein were sprayed at a 50 cm distance onto 1 cm^2 piece of freshly cleaved mica, which was immediately transferred into a BAE 300 Balzers unit and dried for 30 min at 10^{-5}–10^{-6} Torr. The dehydrated samples were further rotary shadowed with a 0.5–0.6 nm thick platinum/carbon film at an angle of 5°, and backed with a 15 nm thick carbon layer. Replicas were floated on water and mounted on grids. TEM observations were carried out in a Siemens Elmiskop 1 A at 80 KV and micrographs were taken at a 40 000 × magnification. Molecular dimensions were measured using a Nikon profile projector.

Results

Molecular analysis of the fraction of pure GPIIb in Triton solution isolated by size-exclusion chromatography

When pure GPIIb (1.5 mg/ml) was subjected to size exclusion chromatography on an analytical Sephacryl S 300 column (135 cm × 1 cm) equilibrated in *Tris*/0.2% TtX buffer, with 0.1 m*M* Ca^{2+} or, alternatively, 1 m*M* EDTA, two fractions were obtained (Fig. 1 A), a minor one (5–10% of the loaded glycoprotein) with a Kp of 0.07 ± 0.01, and a major one (90–95% of the loaded glycoprotein) with a Kp of 0.25 ± 0.01 in 0.1 m*M* Ca^{2+} and of 0.23 ± 0.01 in 1 m*M* EDTA (Fig. 2 A). Upon rechromatography of the isolated fractions in the same conditions, each fraction was eluted as a single entity with the same Kp as before. The proportion of the minor fraction depends on the solubilization conditions. So, on increasing the concentration of GPIIb from 1 mg/ml up to 5 mg/ml, the percentage of this fraction increases up to 20–25% of the glycoprotein loaded into the column (Fig. 1 B). Ca^{2+} concentration, pH, and ionic strength, do not seem to influence this percentage. However, solu-

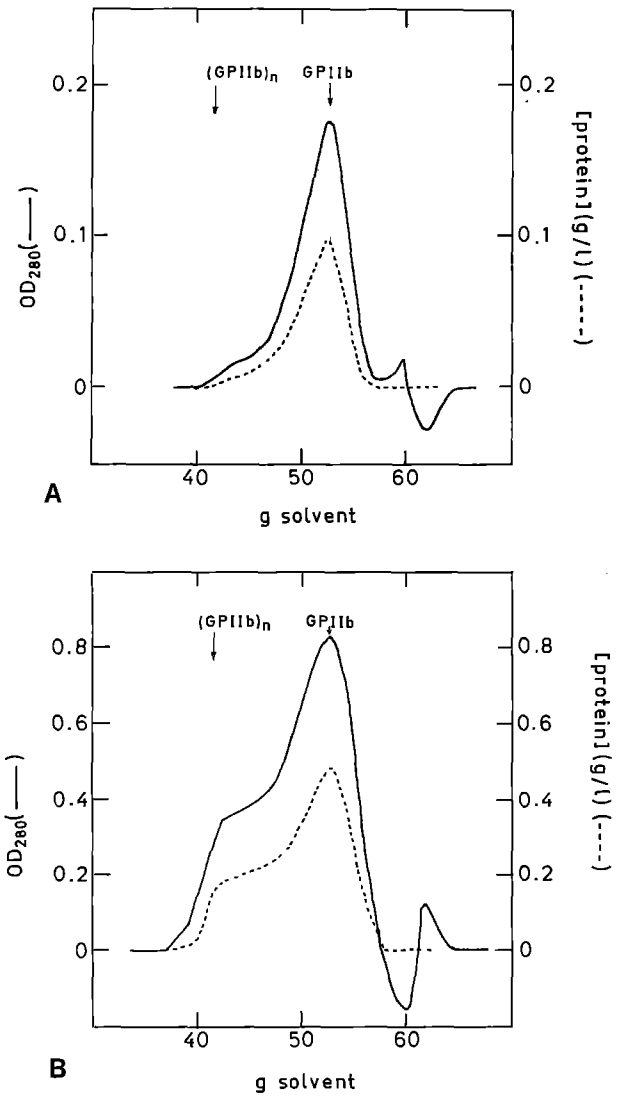

Fig. 1 A, B. Influence of the glycoprotein concentration on the size-exclusion chromatographic pattern of pure GPIIb in equilibrium buffer (50 m*M* *Tris*/HCl/0.1 m*M* $CaCl_2$/0.2% Triton X100/0.02% NaN_3/pH 7.4). GPIIb was dissolved in equilibrium buffer at 1.5 (**A**) and 5 mg (**B**) of glycoprotein/ml, adding an excess of detergent equivalent to the Triton X100 bound to the glycoprotein at saturation (see Fig. 3). 1 ml of the glycoprotein solution was loaded on a Sephacryl S300 column (135 cm × 1 cm) in equilibrium buffer. Abbreviations: GPIIb, the fraction of the GPIIb monomer; $(GPIIb)_n$, the fraction of the GPIIb association products; column fractions are expressed in grams of solvent

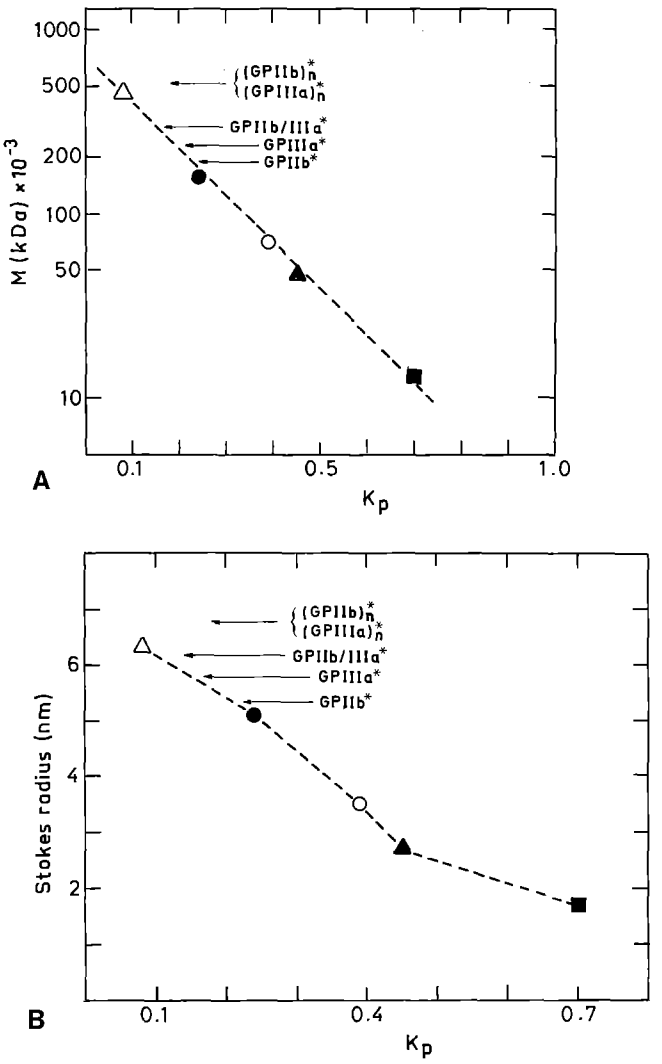

Fig. 2. Size exclusion chromatographic determination of the molecular masses (M^*) (**A**) and Stokes radii (R^*) (**B**) of the complexes (*) of Triton X100 with GPIIb, GPIIIa, GPIIb/IIIa, $(GPIIb)_n$, and $(GPIIIa)_n$, as described in the Methods section. The following standard proteins, whose M and R values were taken from the literature as indicated in the Methods section, were used for column calibration: ferritin (△); IgG (●); bovine serum albumin (○); ovalbumin (▲); cytochrome c (■). The M^* and R^* values for the glycoprotein-Triton complexes are given in Table 1

bilization of GPIIb in guanidine HCl increased this percentage up to 60%.

When the TtX concentration in the column buffer was varied from 0 to 0.5% (w/v), and the detergent bound to the glycoprotein determined (see Methods section), the isotherms of TtX binding to GPIIb in the two fractions were obtained (Fig. 3 A). At a TtX concentration below the c.m.c. (0.016%) no detergent bound to the glycoprotein, which eluted with the total exclusion volume of the column. At 0.2% TtX, the detergent binding in both fractions, Kp 0.07 and 0.25, reached saturation and was 0.29 ± 0.07 and 0.44 ± 0.08 mg of TtX per mg of GPIIb, respectively (Table 1).

From the Kp of 0.25 ± 0.01 and the calibration of the Sephacryl column, we estimated a molecular mass of 180 ± 20 kDa and an R_s^* of 5.3 ± 0.5 nm (in 0.1 m*M* Ca^{2+}) and 5.5 ± 0.5 nm (in 1 m*M* EDTA) for the GPIIb-TtX complex in this fraction (Fig. 2 B). This molecular mass is very close to the 197 ± 20 kDa calculated from the 136 kDa determined before for GPIIb (Usobiaga et al. 1987) and the 0.44 ± 0.08 mg of TtX bound per mg of glycoprotein in this fraction determined here. Therefore this fraction was tentatively identified with the monomer of GPIIb. Further support for this identification comes from the following observations: the 125 ± 15 kDa molecular mass determined for the glycoprotein moiety by flow laser-light scattering; and the 5.8 ± 0.1 S sedimentation coefficient, determined by analytical centrifugation in *Tris*/0.2% TtX/0.1 m*M* Ca^{2+} (Fig. 4), which

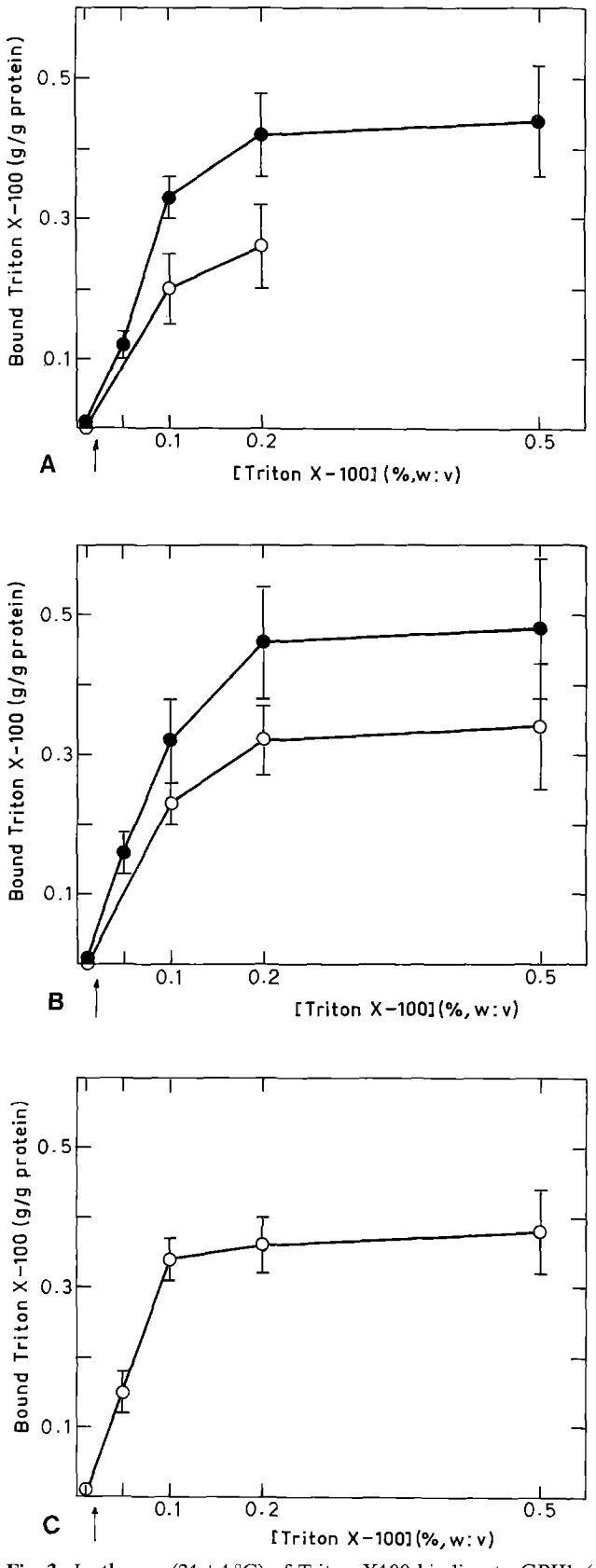

Fig. 3. Isotherms (21 ± 1 °C) of Triton X100 binding to GPIIb (**A**, curve – ● –), $(GPIIb)_n$ (**A**, curve – ○ –), GPIIIa (**B**, curve – ● –), $(GPIIIa)_n$ (**B**, curve – ○ –), and GPIIb/IIIa (**C**), were determined after equilibration by size-exclusion chromatography, as described in the Methods section. The arrow points to the Triton X100 critical micellar concentration determined as described in the Methods section

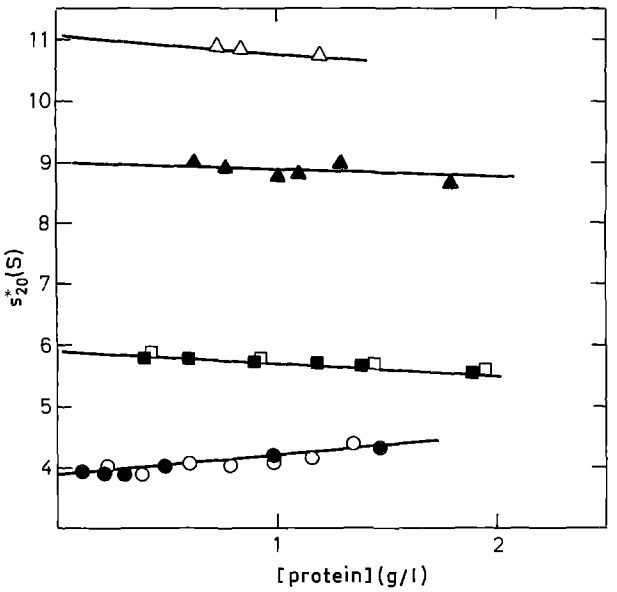

Fig. 4. Glycoprotein concentration dependence of the sedimentation coefficient of the GPIIb (– ■ –, – □ –), $(GPIIb)_n$ (– △ –), GPIIIa (– ● –, – ○ –), and GPIIb/IIIa (– ▲ –), Triton X100 complexes in 50 m*M* *Tris*/HCl/0.1 m*M* $CaCl_2$ (– ● –, – ■ –) or 1 m*M* EDTA (– ○ –, – □ –)/0.2% Triton X100/pH 7.4 (or pH 9.0 for GPIIIa), determined by analytical ultracentrifugation at 20 ± 1 °C

increased to 5.9 ± 0.1 S in the same buffer containing 1 m*M* EDTA instead of Ca^{2+}. Using (1) and the sedimentation coefficient and molecular mass of the GPIIb-TtX monomer, an R_s^* of 6.8 ± 0.4 nm and an f^*/f^*_{min} of 1.7 ± 0.14 were calculated (Table 1), which are slightly higher than the same parameters estimated above from the Kp determined by size-exclusion chromatography.

From the Kp of 0.07 ± 0.1 of the large-size fraction, a molecular mass of about 450–500 kDa was estimated, which could correspond to a mixture of dimers (347 kDa) and trimers (526 kDa) of GPIIb-TtX complexes, as calculated from the molecular mass of GPIIb (Usobiaga et al. 1987) and the TtX bound to the glycoprotein in this fraction (0.29 ± 0.07 mg/mg) determined here. Flow laser-light scattering detected several species (> 570 kDa) in this fraction, the smallest of which would correspond to a tetramer of GPIIb. Therefore, from now on, this GPIIb association product fraction will be called $(GPIIb)_n$. When this fraction was subjected to SDS/7% polyacrylamide gel electrophoresis, the glycoprotein did not enter the separating gel. However, upon reduction with 1% 2-mercaptoethanol two bands were seen which correspond to the α- and β-chains of GPIIb, and point to the presence in this fraction of disulphide bonded association products of GPIIb. To find out which cysteine residues were involved, $(GPIIb)_n$ was reduced with increasing concentrations of dithioerythritol for various periods of time. At 25-fold molar excess of reductant over the theoretical half-cysteines in GPIIb and incubation times of 5 h, $(GPIIb)_n$ started to break down into two bands, corresponding again to the α- and β-chains of GPIIb; no intact GPIIb was liberated, which indicates that the disulphide bonds cross-linking the association products are cleaved at the same molar excess of reductant as that required to cleave the single interchain disulphide bond of GPIIb.

Table 1. Summary of the molecular properties of GPIIb, GPIIIa, GPIIb/IIIa and their complexes with detergent

Glycoprotein and detergent (a)	Ref.	Property										
		M (kDa) (b)	δd (g/g) (d)	M^* (kDa) (e)	(f)	$s^{\circ}_{20}{}^*$ (S)	R^*_{min} (nm) (g)	R^*_s (nm) (h)	(i)	R^* (nm) (j)	f^*/f^*_{min} (k)	f/f_0 (l)
GPIIb												
0.2% TtX	(A)	125	0.44	197	180	5.8	3.9	5.3	6.8	5.3	1.7	–
0.05% TtX	(B)	–	0.12	152	125	4.7	–	6.1	–	4.5	–	1.7
0.1% SDS	(C)	136	1.22	302	–	5.3	–	–	7.5	–	1.6	–
GPIIIa												
0.2% TtX	(A)	103	0.48	136	250	3.9	3.5	5.8	6.4	4.8	1.9	–
0.05% TtX	(B)	–	0.16	107	93	3.2	–	6.7	–	–	–	2.1
0.1% SDS	(C)	92	1.05	189	–	4.7	–	–	6.2	–	1.6	–
GPIIb/IIIa												
0.2% TtX	(A)	210 (228) (c)	0.38	315	310	8.9	4.6	6.2	7.4	6.5	1.5	–
0.05% TtX	(B)	–	0.15	262	265	8.6	–	7.1	–	–	–	1.5
0.1% TtX	(D)	–	0.30	296	297	8.4	–	7.4	–	–	–	–

* Property referred to the glycoprotein-detergent complexes
(a) Detergent concentration in equilibrium buffer
Ref.: (A), this work; (B), Jennings and Phillips (1982) and Carrel et al. (1985); (C), Usobiaga et al. (1987); (D) Parise et al. (1987)
(b) Determined by laser-light scattering (A) and sedimentation equilibrium (C)
(c) Calculated from the molecular masses of the glycoprotein components taken from (C)
(d) (g bound detergent/g glycoprotein) taken from the Triton X100 (A) and SDS (C) binding isotherms
(e) $M^* = M\,(1+\delta d)$, calculated using δd values from the previous column and the following M values: GPIIb (136 kDa); GPIIIa (92 kDa); GPIIb/IIIa (228 kDa)
(f) Determined using hydrodynamic methods: size-exclusion chromatography (A) and a combination of size-exclusion chromatography and density gradient sedimentation (B and D)
(g) $R^*_{min} = (3\,\bar{v}^*_p\,M^*/4\pi N)^{1/3}$, where $\bar{v}^*_p$ was calculated as indicated in the Methods section, and M^* was calculated as in (e)
(h) R^*_s calculated using size-exclusion chromatographic data
(i) $R^*_s = M^*\,(1-\bar{v}^*_p\,d_{20})/s^{\circ}_{20}{}^*\,N\,6\pi\eta_{20})$ as indicated in the Methods section; M^* calculated as indicated in (e)
(j) R^* estimated from the globular structures seen by electron microscopy
(k) $f^*/f^*_{min} = R^*_s/R^*_{min}$, using R^*_s calculated as indicated in (i)
(l) f/f_0, taken from Ref. (B), where it was calculated assuming a hydration of 0.2 g H_2O/g glycoprotein

Molecular analysis of the fractions of pure GPIIIa in Triton solutions isolated by size-exclusion chromatography

As with GPIIb, when GPIIIa was subjected to size-exclusion chromatography on Sephacryl S 300 in *Tris*/TtX/Ca^{2+} buffer, two fractions were obtained, with Kp of 0.07 ± 0.01 and 0.18 ± 0.01, (Fig. 5). The glycoprotein content in the large-size fraction is very dependent on the glycoprotein concentration in the loaded sample (30% at 1.5 mg/ml and 80% at 5 mg/ml), and on the solubilization and elution conditions, highest at 0.1 m*M* Ca^{2+}/pH 7.4 > 1 m*M* EDTA/pH 7.4 > 0.1 m*M* Ca^{2+}/pH 9.0, being independent of the ionic strength. When the TtX concentration in the column buffer was varied from 0 up to 0.5% (w/v) and the TtX bound to the glycoprotein determined in the two fractions (Fig. 3B), below the c.m.c. no detergent binding was measurable and the glycoprotein was eluted with the void volume. At 0.2% TtX, the binding in both fractions (Kd 0.07 and 0.18) reached saturation and was 0.34 ± 0.05 and 0.48 ± 0.10 mg TtX/mg GPIIIa, respectively (Table 1).

A molecular mass of 250 ± 20 kDa and an R^*_s of 5.8 ± 0.6 nm from the Kp of 0.18 ± 0.01 was estimated for the GPIIIa-TtX complex eluted in this fraction. This molecular mass is much higher than the 136 ± 20 kDa calculated from the 92 kDa determined earlier for GPIIIa (Usobiaga et al. 1987) and the 0.48 ± 0.10 mg of TtX/mg GPIIIa determined here. This could imply that this GPIIIa fraction is in a dimer form. However a molecular mass of 103 ± 15 kDa and a sedimentation coefficient of 3.9 ± 0.3 S were determined for GPIIIa in this fraction in *Tris*/TtX/Ca^{2+}/pH 9.0 buffer by flow laser-light scattering and analytical centrifugation, respectively. Using (1) and the molecular mass and sedimentation coefficient of the GPIIIa-TtX complex, a R^*_s of 6.4 ± 0.5 nm and a f^*/f^*_{min} of 1.9 ± 0.3 were calculated. All these data led us to identify this fraction with the monomer of GPIIIa.

From the Kp of 0.07 ± 0.01 of the large-size fraction of GPIIIa, a molecular mass of 450–500 kDa was estimated, which could be a tetramer of the GPIIIa-TtX complex, if we take into consideration the molecular mass of GPIIIa and the TtX bound to GPIIIa in this fraction. However flow laser light-scattering analysis of this fraction shows that it is even more heterogeneous than the $(GPIIb)_n$ fraction referred to above, the smallest species being a tetramer. When $(GPIIIa)_n$ was subjected to SDS/7% polyacrylamide gel electrophoresis, both in reduced and non-reduced conditions, a single band corresponding to the monomer of GPIIIa was observed. Therefore the self-association of GPIIIa is non-covalent, contrary to what happens with GPIIb.

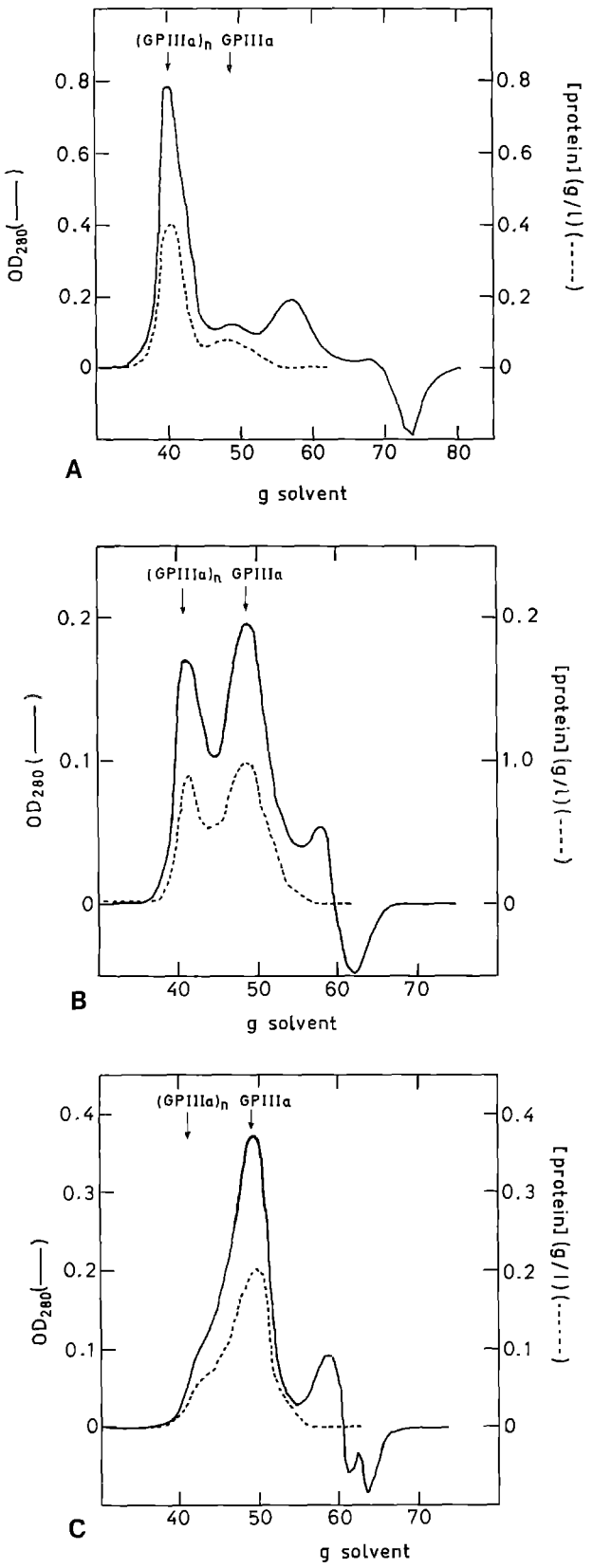

Fig. 5. Influence of the glycoprotein concentration and pH on the size-exclusion chromatographic pattern of pure GPIIIa in equilibrium buffer (50 m*M* *Tris*/HCl/0.2% Triton X100/0.1 m*M* Cl_2Ca/0.02% NaN_3/pH 7.4 (**A** and **B**) or 9.0 (**C**). GPIIIa was dissolved in equilibrium buffer at 4 mg (**A**) and 2 mg (**B** and **C**) of glycoprotein/ml, adding an excess of detergent equivalent to the Triton X100 bound to the glycoprotein at saturation (see Fig. 3). 1 ml of the glycoprotein solution was loaded on a Sephacryl S300 column (135 cm × 1 cm) in equilibrium buffer. Abbreviations: GPIIIa, the fraction of the GPIIIa monomer; $(GPIIIa)_n$, the fraction of the GPIIIa association products; column fractions are expressed in grams of solvent

Molecular analysis of pure GPIIb/IIIa in Triton solution isolated by size-exclusion chromatography

When pure GPIIb/IIIa was subjected to size-exclusion chromatography on Sephacryl S 300 in *Tris*/TtX/Ca^{2+} buffer, a single homogeneous fraction eluted with a Kp of 0.13 ± 0.01. Analysis by SDS/7% polyacrylamide gel electrophoresis showed two bands corresponding to GPIIb and GPIIIa, which after densitometry, using pure GPIIb and GPIIIa as standards, gave a 1 to 1 molar ratio. At Ca^{2+} concentrations in the nM region, GPIIb/IIIa starts to dissociate into its constituent glycoproteins, and the elution pattern changes completely (Rivas, Usobiaga and González-Rodríguez, unpublished results), due to the superposition of the elution pattern of undissociated GPIIb/IIIa and the patterns of dissociated GPIIb and GPIIIa described above. The TtX binding isotherm (Fig. 3C) again shows no TtX binding to GPIIb/IIIa below the c.m.c., when the heterodimer is fully excluded. Above the c.m.c., TtX binding to GPIIb/IIIa starts to be measurable and reaches saturation at 0.2% (w/v), where the TtX bound is 0.38 ± 0.06 mg/mg GPIIb/IIIa.

From the Kp of 0.13 ± 0.01 a molecular mass of 310 ± 10 kDa and an R_s^* of 6.2 ± 0.3 nm were estimated, in good agreement with the 315 ± 20 kDa calculated from the molecular mass of the individual glycoproteins and the TtX bound by GPIIb/IIIa. This fraction was tentatively identified with the monomer of GPIIb/IIIa. Definitive confirmation came from the 210 ± 15 kDa molecular mass of the glycoprotein moiety and 8.9 ± 0.2 S sedimentation coefficient of the glycoprotein-TtX complex determined by flow laser-light scattering and analytical centrifugation, respectively, in this fraction. Using (1) and the molecular mass and sedimentation coefficient of the GPIIb/IIIa-TtX complex, a R_s^* of 7.4 ± 0.2 nm and a f^*/f^*_{min} of 1.5 ± 0.12 were calculated (Table 1).

Electron microscopy of the rotary shadowed monomer fraction of GPIIb, GPIIIa and GPIIb/IIIa in Triton solution

Figure 6A shows the monomer fraction of GPIIb in *Tris*/TtX/Ca^{2+} buffer, where globular structures of 9.5 ± 1.5 nm × 11 ± 1.5 nm (after subtraction of the platinum/carbon shell estimated to be about 3–3.5 nm) are preponderant, in good agreement with the R_s^* estimated by size-exclusion chromatography and lower than that calculated using hydrodynamic parameters of the GPIIb-TtX complex (Table 1). This confirms once more the monomeric structure of GPIIb in this fraction. Occasionally, some aggregates of larger size are also seen (see inset).

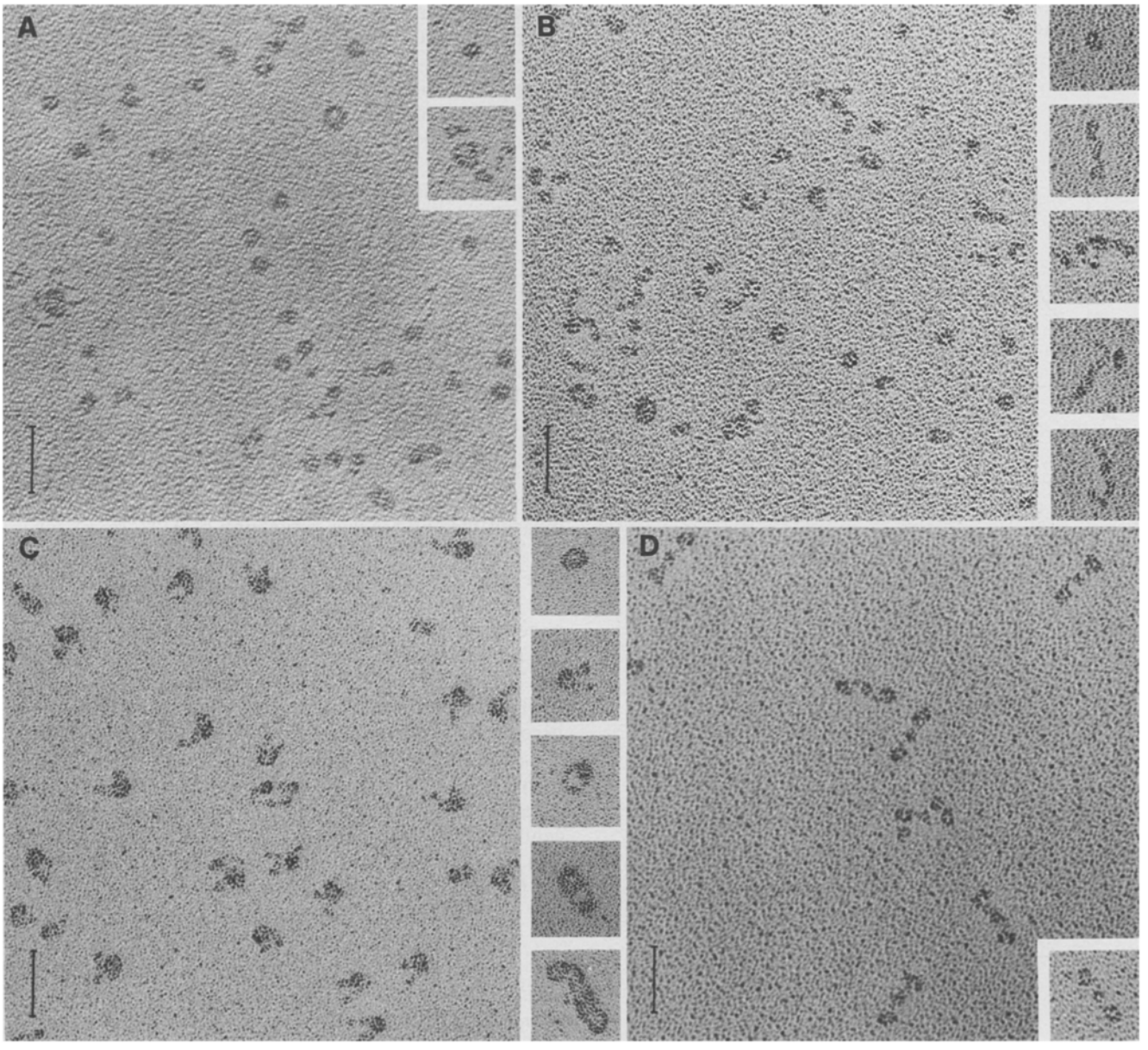

Fig. 6. Electron micrographs of the monomer fractions of GPIIb (**A**), GPIIIa (**B**), GPIIb/IIIa (**C**), and fibrinogen (**D**), in 50 m*M* *Tris*/HCl/0.1 m*M* $CaCl_2$/0.1% Triton X100 pH 7.4. The glycerol-spray technique and the Pt/C rotary shadowing of the glycoproteins were performed as described in the Methods section. At the right-hand side of each micrograph a gallery of images showing single monomers (**A**, **B**, **C**, and **D**) different monomer shapes (**C**) and association products (**A**, **B**, and **C**) of each glycoprotein can be observed. Fibrinogen is presented for comparison with its physiological platelet receptor: the GPIIb/IIIa heterodimer. Magnification 200 000×. (Bar = 50 nm)

The appearance of GPIIIa in the electron micrographs depends on the history of the sample and the solubilization buffer. GPIIIa from the monomer fraction isolated in *Tris*/TtX/Ca^{2+}/pH 9.0 shows mainly globular structures of 9 ± 1.5 nm × 10 ± 1.5 nm, smaller than those expected from the R_s^* calculated from the molecular mass and sedimentation coefficient of the GPIIIa-TtX complex (Fig. 6B and Table 1). However, GPIIIa that had been stored frozen at pH 7.4 shows 20–75 nm long and 3–5 nm wide filamentous structures as the major species, the globular structures now becoming a minority (see inset). Occasionally, strings of 3–5 beads are seen, with the individual beads resembling the characteristics of the individual monomers (see inset). These findings confirm, once more, the monomeric nature of this chromatographic fraction of GPIIIa.

GPIIb/IIIa displays in the electron micrographs a great variety of shapes, within the same preparation (Fig. 6C) and from one preparation to the next (inset); this made it difficult to measure the dimensions of GPIIb/IIIa, and demanded these dimensions be referred to each particular shape of GPIIb/IIIa observed. The filled globular shapes have 12 ± 1.4 mm × 14 ± 1.5 nm dimensions, slightly larger than those estimated by size-exclusion chromatography, and smaller than those calculated from hydrodynamic measurements (Table 1). The

empty oval shapes have 20 ± 2 nm $\times$ 24 ± 2 nm overall dimensions, a head of 12 ± 1.5 nm $\times$ 14 ± 1.5 nm, and an oval hole of 9 ± 2 nm $\times$ 10.5 ± 2 nm, asymmetrically arranged and surrounded by arms 3–5 nm wide. The head-two-tails form, with a total length of 21–28 nm, and a distance between the ends of both tails up to 25 nm, has a head of 12 ± 1.5 nm $\times$ 15 ± 1.5 nm, from where two tails (10–14 nm long and 3–5 nm wide) emerge from the same side. Occasionally, bilobular shapes of 23–25 nm total length, sometimes in pairs, are seen with a large lobule of 11 nm $\times$ 15 nm and a small lobule of 10–14 nm $\times$ 10 nm. Finally, on lowering the TtX concentration below 0.1%, aggregates of 2–12 head-two-tails forms in line are observed more often. When the TtX concentration is decreased below the c.m.c., aggregates of 3–6 monomers are abundant, having a rosette shape with the tails joining in the middle and the heads at the periphery.

Discussion

In the present work, a combination of size-exclusion chromatography, analytical determination of bound TtX, laser-light scattering, analytical ultracentrifugation, and TEM, were used for the characterization of the molecular mass, size and shape, hydrodynamic properties, and self-association of GPIIb, GPIIIa, GPIIb/IIIa, and their complexes with TtX in solution.

The TtX bound to the three glycoproteins at detergent concentrations below the c.m.c. (0.016%) is undetectable by the analytical procedure used here, and the glycoproteins self-associate in aggregates of large size. At 0.2% TtX, the detergent bound to the three glycoproteins reached saturation, and, therefore, all our molecular and hydrodynamic measurements were done at this TtX concentration. Jennings and Phillips (1982) state that GPIIb, GPIIIa and GPIIb/IIIa do not bind TtX at 0.05, the detergent concentration at which they do their hydrodynamic measurements, and Carrell et al. (1985) their electron microscopic studies. However, at this concentration we found a TtX binding to GPIIb, GPIIIa and GPIIb/IIIa of 0.12, 0.16 and 0.15 mg/mg of glycoprotein, respectively. Therefore their measurements, done supposedly on the glycoproteins, correspond in fact to the glycoprotein-detergent complexes. The TtX bound to GPIIb/IIIa at 0.1% TtX was determined by Carrell et al. (1985), and agrees with our isotherm within experimental errors.

GPIIb in 0.2% TtX solutions exists mainly as a monomer, as shown by the size-exclusion chromatography pattern, the agreement between the molecular mass of the glycoprotein moiety determined here by laser-light scattering (125 ± 15 kDa), and that determined earlier (136 kDa) (Usobiaga et al. 1987), and the molecular size (9.5 ± 1.5 nm $\times$ 11 ± 1.5 nm) and globular shape observed by TEM. The GPIIb-TtX complex, which binds 0.44 ± 0.08 mg TtX/mg GPIIb, has a R_s^* of 6.8 ± 0.4 nm as calculated from its molecular mass [$136\,(1+0.44) = 197$ kDa] and sedimentation coefficient ($5.8 + 0.1$ S), and a f^*/f^*_{min} ratio of 1.7 ± 0.14, unexpectedly, the same value as that for the f/f_0 ratio calculated by Jennings and Phillips (1982) for GPIIb in 0.05% TtX, assuming a hydration of 0.2 mg/mg GPIIb. However, size-exclusion chromatography measurements, from which a good estimate of the molecular mass of this complex was obtained (180 kDa), provided a R_s^* of 5.3 ± 0.5 nm, the same value as that determined for the average radius of the globular structures seen in the electron micrographs, and from which an f^*/f^*_{min} of 1.4 is obtained, which is more compatible with a globular shape (Table 1). Given that both asymmetry and hydration contribute to the f^*/f^*_{min} ratio, that TtX has a high degree of hydration (up to 1.5 ± 0.4 mg/mg TtX) (Yedgar et al. 1974; Robson and Dennis 1977), that the GPIIb-TtX complex has a globular shape in the electron micrographs, and that the differences in conformation and hydration between GPIIb in solution and GPIIb after evacuation, prior to metal shadowing, are unknown, it is not possible to ascertain the contributions of asymmetry and hydration to the f^*/f^*_{min} and to reconcile the high f^*/f^*_{min} ratio with the globular structures observed by TEM, without further measurements. The slightly lower dimensions found by Carrell et al. (1985) for GPIIb (8 nm $\times$ 10 nm) in their electron micrographs, compared with those measured here (9.5 nm $\times$ 11 nm) can easily be accounted for by the differences in TtX bound to GPIIb in 0.05 and 0.2% TtX solutions. The same reasoning plus the obvious limitations of their technique compared with ours (Tanford and Reynolds 1976) can explain the lower sedimentation coefficient (4.7 S) determined for GPIIb in 0.05% TtX by Jennings and Phillips (1982) compared with ours (5.8 S).

The covalent association products of GPIIb, $(GPIIb)_n$, which have not been reported so far, are heterogeneous in size, the tetramers being the smallest species found. For the moment we do not know the disulphide bond cross-linking pattern, whether it is a regular or a random array of α- and β-chains; we only know that part of the hydrophobic surfaces of the monomer are not available now, as deduced from the lower TtX bound to $(GPIIb)_n$, compared with that bound to the monomer.

GPIIIa in 0.2% TtX/pH 7.4 solutions has a much greater tendency to self-associate than GPIIb, this tendency being lower at glycoprotein concentrations below 1 mg/ml, 0.1 mM Ca^{2+}, pH 9.0. GPIIIa association products are non-covalent and heterogeneous in size, the tetramer being the smallest species found. The monomeric fraction was unequivocally identified by the agreement between the molecular mass of the glycoprotein moiety, determined here by laser-light scattering (103 ± 15 kDa), and that determined before (92 kDa) (Usobiaga et al. 1987), and by the molecular size (9 ± 1.5 nm $\times$ 10 ± 1.5 nm) and globular shape observed by TEM. The GPIIIa-TtX complex, which binds 0.48 ± 0.10 mg of detergent/mg GPIIIa, has a R_s^* of 6.4 ± 0.5 nm, as calculated from its molecular mass [$92\,(1+0.48) = 136$ kDa] and sedimentation coefficient (3.9 ± 0.3 S), and a f^*/f^*_{min} ratio of 1.9 ± 0.3, close to the f/f_0 ratio of 2.1 calculated by Jennings and Phillips (1982) for GPIIIa in 0.05% TtX, assuming a hydration of 0.2 mg/mg GPIIIa. The difference between the $s^{\circ}_{20}{}^*$ determined here for the GPIIIa-TtX complex and that determined by Jennings and Phillips can easily be accounted for by the same reasons as those

given above for the $s^{\circ}_{20}{}^{*}$ of the GPIIb-TtX complex, while the high frictional ratio calculated for the GPIIIa-TtX complex by these authors, compared with that calculated here, is partly due to the exceedingly high R_s^* determined by size-exclusion chromatography, as a result of the anomalous chromatographic behavior of the GPIIIa monomer mentioned above. However, our main disagreement is with Carrell et al. (1985). At that time, and probably misled by the apparent similarity, in the electron micrographs, between the tails and the head of the GPIIb/IIIa heterodimer and GPIIIa and GPIIb, respectively, they concluded that the 20–30 nm long filamentous structures seen in GPIIIa electron micrographs were the monomers of GPIIIa, contrary to what we found here. This conclusion was reached in spite of the following observations: "often the isolated GPIIIa appeared shorter and thicker, sometimes almost globular structures were observed", and "GPIIIa often exists in forms that sediment faster than 3.2 S" (Carrels et al. 1985). Here we unequivocally identify the nearly globular structures (9 nm × 10 nm) seen in the electron micrographs with the GPIIIa monomers. As discussed above for GPIIb, it is neither possible to reconcile these globular structures with the high fractional ratio calculated, not to ascertain the contributions of the molecular asymmetry and hydration to this ratio, without further measurements.

Contrary to what was observed with isolated GPIIb and GPIIIa, the GPIIb/IIIa heterodimer in *Tris*/0.2% TtX/0.1 m*M* Ca^{2+} buffer eluted from the column as a single fraction without tendency to self-associate, as deduced from the agreement between the molecular mass of the glycoprotein moiety (210 ± 15 kDa), determined by laser-light scattering, and that expected from the molecular mass of the individual glycoprotein components (136 + 92 = 228 kDa), determined before (Usobiaga et al. 1987). The GPIIb/IIIa-TtX complex which binds 0.38 ± 0.06 mg TtX/mg GPIIb/IIIa, has a molecular mass of 310 kDa, as estimated by size-exclusion chromatography, in good agreement with that expected from the molecular mass of the heterodimer and the TtX bound to it [228 (1 + 0.38) = 315 kDa], and slightly higher than the 297 kDa determined by Parise et al. (1987) in 0.1% TtX. The R_s^* of 7.4 ± 0.2 nm, calculated from the molecular mass of the GPIIb/IIIa-TtX complex and its sedimentation coefficient (8.9 S) determined here, is the same as that obtained by Parise et al. (1987) in 0.1% TtX, slightly higher than the average radius of 6.5 nm measured for the globular forms of GPIIb/IIIa (12 ± 1.5 nm × 14 ± 1.5 nm) in the electron micrographs, and higher than the 6.2 nm estimated by size-exclusion chromatography. The f^*/f^*_{min} for this complex ranges from 1.5 ± 0.12, as obtained using the R_s^* calculated from hydrodynamic measurements, down to 1.3 obtained using the R_s^* estimated from size-exclusion chromatography, and could be easily accounted for by the non globular shapes observed in the electron micrographs (Fig. 6C). The dimensions found by Carrell et al. (1985) for the oblong head of GPIIb/IIIa (8 nm × 10 nm) are in good agreement with those for the same structure of the fibronectin receptor (8 nm × 12–15 nm) measured by Nermut et al. (1988), and are lower than those measured here (12 nm × 14 nm). These differences cannot be accounted for only by the difference between the TtX bound to GPIIb/IIIa in 0.05 and 0.1% TtX solutions, as was the case for GPIIb and GPIIIa. Further TEM studies are required together with image analysis (Frank 1989) and the use of monoclonal antibodies whose epitopes are well assigned (Calvete et al. 1990a, b), to deal with the problem of the multiple molecular orientations and the identification of molecular domains, and to be able to do a three dimensional reconstruction of the heterodimer image closer to reality.

Rotational diffusion measurements of GPIIb, GPIIIa and GPIIb/IIIa in 0.2% TtX solutions (Lillo, Rivas, Acuña, González-Rodríguez, unpublished work), indicated that the three glycoproteins are not compact structures, but rather flexible, which will have to be taken into account in further hydrodynamic studies to ascertain the asymmetry and hydration of these glycoproteins.

Acknowledgements. We want to thank Prof. K. Razi Naqvi for reading the manuscript, Mr. L. Company, Mrs. C. Martín de Loeches, Mrs. G. Pinillos, and Mr. J. M. Sánchez for technical assistance, Mr. F. Romera for typing the manuscript, the Servicio de Microscopía Electrónica of the Centro de Investigaciones Biológicas (C.S.I.C., Madrid) for free access to the shadowing chamber, and the Blood Banks of Centro Ramón y Cajal, La Paz, and Doce de Octubre (Madrid) for providing us with outdated platelet concentrates. This work was supported by the Secretaría de Estado para Universidades e Investigación (ID 87077 SEUI; PM-022 SEUI).

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