Eur Biophys J (1987) 14: 211–218

European Biophysics Journal

Molecular characterization of human platelet glycoproteins IIIa and IIb and the subunits of the latter

P. Usobiaga, J. J. Calvete, J. L. Saíz, M. T. Eirín, and J. González-Rodríguez*

Instituto de Química Física, C.S.I.C., Serrano, 119, E-28006 Madrid, Spain

Received August 21, 1985/Accepted in revised form August 1, 1986

Abstract. Sedimentation equilibrium and low-angle laser-light scattering were used to determine the molar mass of the glycoprotein moieties in the complexes of sodium dodecyl sulphate with the human platelet membrane glycoproteins IIb (GPIIb), IIIa (GPIIIa), and the α (GPIIbα) and β (GPIIbβ) subunits of GPIIb. The values obtained by both procedures, except those for GPIIb, agree within experimental error with those calculated from their chemical composition: GPIIbα (114,000 g mol^{-1}), GPIIbβ (22,200 g mol^{-1}), and GPIIIa (91,500 g mol^{-1}). The molar mass of GPIIb determined by light scattering (142,000 g mol^{-1}) and sedimentation equilibrium at different solvent densities (134,000 g mol^{-1}) also agree, within experimental error, with the values calculated either from its chemical composition (136,500 g mol^{-1}) or from the sum of the molar masses of its subunits. However the molar mass determined by sedimentation equilibrium at constant solvent density, is consistently underestimated (116,000 g mol^{-1}).

High-performance size-exclusion chromatography in sodium dodecyl sulphate solutions overestimates the molar mass of these glycoproteins and their Stokes radii, and therefore the maximal frictional ratios derived from them.

Key words: Molar mass, size, shape, platelet glycoproteins, sedimentation equilibrium, laser-light scattering

* To whom offprint requests should be sent

Abbreviations: GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa; GPIIbα and GPIIbβ, α and β subunits of GPIIb, respectively; CM-GPIIbα, CM-GPIIbβ, and CM-GPIIIa, totally reduced and carboxymethylated forms of GPIIbα, GPIIbβ, and GPIIIa, respectively; SDS, sodium dodecyl sulphate; eosin-ITC, eosin-5-isothiocyanate

Introduction

Human platelet glycoproteins IIIa (GPIIIa) and IIb (GPIIb) have been isolated and chemically characterized in several laboratories (Leung et al. 1981; McEver et al. 1982; Jennings and Phillips 1982; Eirín et al. 1986). The α and β subunits of GPIIb may be isolated from the pure glycoprotein under reducing conditions (Calvete and González-Rodríguez 1986). The weight percentage of sugar is 15.7% for GPIIb, 16.4% for GPIIbα, 10.2% for GPIIbβ and 12.5% for GPIIIa. Acetyl neuraminic acid amounts to 30%, 30%, 28% and 15% of the total sugar weight of GPIIb, GPIIbα, GPIIbβ and GPIIIa, respectively. Apparent molecular masses and hydrodynamic properties of these glycoproteins have been measured by SDS polyacrylamide-gel electrophoresis, gel filtration and sucrose density gradients (Jennings and Phillips 1982). Limitations of these procedures for accurate molecular chracterization have been discussed by Tanford and Reynolds (1976), and the use of theoretically more rigorous procedures, such as analytical ultracentrifugation (Tanford et al. 1974; Reynolds and Tanford 1976), or any of the several scattering procedures available (Sardet et al. 1976; Takagi et al. 1980), has been recommended. In particular, estimates of the molecular masses of GPIIb, GPIIbα, GPIIbβ, and GPIIIa by SDS polyacrylamide-gel electrophoresis vary over a wide range (George et al. 1981), depending on sample preparation and the electrophoretic system used. Consequently, more rigorous procedures for the molecular characterization of these glycoproteins were called for.

In the present paper we determine the molar masses of GPIIb, GPIIIa, and the α and β subunits of GPIIb, by independent measurements of sedimentation equilibrium and laser-light scattering. At the same time the gross conformation of the glycoproteins is obtained by high-performance size-exclu-

sion chromatography, and the molecular mass and gross conformation of the glycoprotein-SDS complexes in solution are obtained by sedimentation velocity and sedimentation equilibrium data, together with the analytical determination of the detergent bound to the glycoproteins. Finally, our results are compared with previous data already available for these glycoproteins.

Materials and methods

Chemicals and biochemicals

SDS was from Fluka (Switzerland). Eosin-ITC was from Molecular Probes (USA). Water was from a Milli Q water purification system (Millipore, USA). Sephadex-50, Sephacryl-200 and 300 from Pharmacia (Sweden). TSK-GEL SW analytical (7.5 × 600 mm) and TSK-GEL SWG preparative (21.5 × 600 mm) columns from Toyo Soda (Tokyo, Japan). All chemicals and biochemicals were of analytical or chromatographic grade.

The preparation of the platelet glycoproteins will be outlined here very briefly. GPIIb and GPIIIa were isolated from human platelet plasma membrane after solubilization with detergents, by zonal centrifugation in a sucrose density gradient, followed by preparative high-performance size-exclusion chromatography on tandem TSK-G4000SWG-3000SWG columns eluted at 0.2 ml/min, with elution buffer: 0.1 *M* sodium phosphate, 1 m*M* EDTA, 0.025% sodium azide, 0.1% SDS, pH 6.8 (Eirín et al. 1986). GPIIbα and GPIIbβ were prepared from pure GPIIb by reduction with a five-fold molar excess of 1,4-dithioerythritol over theoretical half-cystines (an average of 2.3 ± 0.4 half-cystines are reduced per molecule of GPIIbα) followed by carboxymethylation with a two-fold molar excess of iodoacetate over reducing agent. Finally, the two subunits were isolated by size-exclusion chromatography on Sephacryl 200 or by analytical chromatography on two TSK-G4000SW and one TSK-G3000SW columns in series with elution at 0.05 ml/min in the same buffer (Calvete and González-Rodríguez 1986). Fully reduced and carboxylmethylated subunits, CM-GPIIbα and CM-GPIIbβ, were prepared from pure GPIIb with a 150 fold molar excess of 1,4-dithioerythritol, followed by carboxymethylation and isolation of the subunits as above. CM-GPIIIa was prepared by reduction of pure GPIIIa with a 100 fold molar excess of 1,4-dithioerythritol over theoretical half-cystines followed by carboxymethylation, as above.

Analytical methods

Glycoprotein concentration was calculated from the protein concentration determined by a modification (Markwell et al. 1978) of the method of Lowry et al. (1951) and from the amino acid and sugar composition of the glycoproteins (Eirín et al. 1986; Calvete and González-Rodríguez 1986). SDS was analyzed by the procedure of Sokoloff and Frigon (1981). The SDS gel electrophoretic analysis was according to Laemmli (1970). Analytical high-performance size-exclusion chromatography was on two TSK-G4000SW and one TSK-G3000SW columns in series eluted at 0.05 ml/min with elution buffer as above.

The partial specific volumes of GPIIIa, GPIIb, GPIIbα and GPIIbβ were calculated from their amino acid and sugar composition (Eirín et al. 1986; Calvete and González-Rodríguez 1986). The following sugar compositions, expressed as residues (of galactose, mannose, N-acetylglucosamine, N-acetyl galactosamine and N-acetylneuraminate, respectively) per molecule of glycoprotein, were used: GPIIIa (9, 31, 17, 0, 6); GPIIb (28, 23, 28, 10, 22); GPIIbα (25, 13, 24, 10, 20); and GPIIbβ (2, 3, 4, 0, 2).

Glycoprotein labelling with eosin-ITC

0.2 to 1 mg of eosin-ITC in 1 ml of 20 m*M* sodium bicarbonate, 0.05% SDS, pH 8.5, were added to 1 to 5 mg of pure glycoprotein in 1 ml of the same solution. The mixture was left in darkness at room temperature for 2 h. The unbound dye was separated from the labelled glycoprotein by passage through a Sephadex 50 column (26 × 1.6 cm) equilibrated with 20 m*M* phosphate, 0.05% SDS, pH 6.8. The labelled glycoprotein solutions were concentrated to 0.25 ml, using Amicon membranes, SDS added to a final concentration of 1%, and filtered (0.45 µm, Millipore). Before their use for analytical ultracentrifugation and analysis of glycoprotein-bound SDS, the labelled glycoproteins were subjected to analytical chromatography for equilibration with elution buffer (0.1% SDS) and analysis of their integrity and monodispersity, the chromatographic fractions being further analyzed electrophoretically.

The degree of labelling with eosin was determined spectrophotometrically, using an $\varepsilon_{530} = 8.5 \pm 0.5 \times 10^4\ M^{-1}\ cm^{-1}$; the ratio was from 2 to 4 moles eosin per molecule glycoprotein. Labelled glycoproteins did not show any appreciable change in the amount of bound SDS or their electrophoretic, chromatographic or sedimentation behavior, as compared with unlabelled samples.

Analytical ultracentrifugation

Sedimentation experiments were carried out in a Beckman model E analytical ultracentrifuge equipped with a photoelectric scanner, using Schlieren and absorption optics. Sedimentation velocity experiments were done at 60,000 rpm and at 20 °C.

Meniscus depletion sedimentation equilibrium experiments (Yphantis 1964), both at constant and at different solvent densities, were at 18,000 to 22,000 rpm for GPIIIa and CM-GPIIIa, 15,000 to 18,000 rpm for GPIIb, GPIIbα and CM-GPIIbα, and 40,000 rpm for CM-GPIIbβ, using absorption optics at 530 nm to monitor glycoprotein concentration (c) over radial position (r) in the sedimentation cell. In all the experiments the plots of log c vs. r^2 were linear, and approximately the upper half of the absorption patterns was horizontal.

The molar mass (M) of the glycoproteins excluding detergent and other solvent components was obtained, according to Tanford et al. (1974), as

$$M(1-\phi'\varrho) = M[(1-\bar{v}\varrho) + \delta_D(1-\bar{v}_D\varrho)], \quad (1)$$

where: $M(1-\phi'\varrho) = (2RT/\omega^2)(d\ln c/dr^2)$ is the experimental parameter; ω, angular velocity; ϕ', effective partial specific volume of the glycoprotein at constant chemical potential of the solvent; $\bar{v}$, true partial specific volume of the glycoprotein; $\bar{v}_D$, partial specific volume of the detergent when bound to the glycoprotein; ϱ, solvent density; and δ_D, g of bound SDS per g of glycoprotein. Glycoprotein molar masses were also determined by meniscus depletion sedimentation equilibrium without using the data on the bound detergent, by measuring $M(1-\phi'\varrho)$ as a function of ϱ, according to Reynolds and Tanford (1976). In the latter procedure, labelled glycoprotein solutions equilibrated in elution buffer (0.1% SDS) in H_2O were diluted with the same buffer in D_2O. The solvent densities were calculated from the measured densities of the original buffers. All analytical centrifugation data were subjected to least-squares analyses.

Laser-light scattering

12 to 15 mg of unlabelled pure glycoprotein were subjected to preparative chromatography in a TSK-SWG 4000-3000 column at 0.2 ml/min and elution buffer (0.1% SDS), to obtain a true Donnan equilibrium, and successive 2 ml fractions along the glycoprotein peak taken for measurements of glycoprotein concentration, specific refractive index increments and laser-light scattering. Glycoprotein concentration was determined both by absorption at 280 nm, using the extinction coefficients given in Table 2, and as described above; there was a good correlation between the two procedures for the four glycoproteins. Differential indices of refraction were measured with a Zeiss Jena L13 interferometer, calibrated for the 632.8 nm light of the He-Ne laser used by the scattering photometer; values given in Table 2. Light scattering measurements were made in a Chromatix type KMX-6 low-angle (6°–7°) laser-light scattering photometer. Refractive indices were measured in an Abbe refractometer. All measurements were performed at 21 °C.

The glycoprotein molar masses were determined according to Takagi et al. (1980) by the following equation:

$$\left(\frac{2\pi^2(1+\cos^2\theta)}{N\lambda^4}\right)\left(\frac{dn^*}{dc}\right)^2\frac{n_0^2 c}{\bar{R}_\theta} = \frac{1}{M} + 2B^*(1+\delta_D)^2 c, \quad (2)$$

where: $\bar{R}_\theta = [(I_\theta/I_0)_{\text{solution}} - (I_\theta/I_0)_{\text{solvent}}](\sigma' l')^{-1}$, is the excess Rayleigh factor; I_0, incident beam intensity; I_θ, scattered light intensity; σ', solid angle of collection of scattered light; l', equivalent light path; n_0, solvent refractive index; (dn^*/dc), refractive index increment of the glycoprotein-SDS complexes; θ, scattering angle; N, Avogadro's number; λ, light wavelength; B^*, second virial coefficient of the glycoprotein-SDS complexes; and c, δ_D and M are the same as defined above. $k = [2\pi^2(1+\cos^2\theta)/N\lambda^4]$ is the instrument constant, 4.079×10^{-6} cm^{-4} · mol in our case. The scattering analysis at low angles was done by measuring $\bar{R}_\theta$ as a function of c, and extrapolating the reciprocal of the molar masses $[(dn^*/dc)^2 n_0^2 k c/\bar{R}_\theta]$ to zero concentration to obtain $1/M$ by a least-squares fit.

Other procedures

Solvent viscosities and densities were measured with an Ostwald viscometer and an Anton Paar DMA 02D precision densimeter, respectively. Critical micellar concentrations were measured fluorimetrically according to De Vendittis et al. (1981) using an SLM 8000 spectrofluorimeter.

The following results were found for elution buffer (0.1% SDS): $\varrho_{20\,°C} = 1.01059 \pm 5 \times 10^{-5}$ g cm^{-3}; $\eta_{20\,°C} = 10.44 \times 10^{-3}$ P; and critical micellar concentration, 0.025% SDS (w/v) at 20 °C.

Results

Determination of the SDS bound to the glycoproteins

The amount of detergent bound to the glycoproteins was determined by equilibration of the glycoproteins

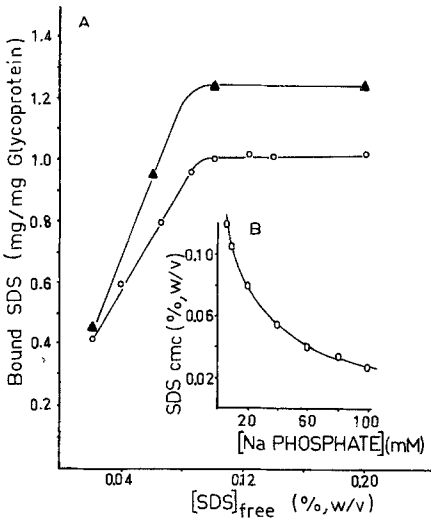

Fig. 1. Binding isotherms of SDS to GPIIb (—▲—) and GPIIIa (—○—) equilibrated at 21 ± 1 °C in elution buffer at various SDS concentrations by analytical high-performance size-exclusion chromatography, on two TSK-G4000SW and one TSK-G3000SW columns in series, as described in *Methods*. The inset shows the critical micellar concentration (cmc) of SDS in elution buffer (0.1% SDS) as a function of the sodium phosphate concentration

in elution buffer at different SDS concentrations (from 0.02 to 0.2%) and differential measurement of the protein-bound detergent in complexes isolated by analytical chromatography. Figure 1 shows that the amount of bound SDS reaches a maximum of 1.22 ± 0.05 and 1.05 ± 0.05 mg per mg (for GPIIb and GPIIIa, respectively) at 0.1% of SDS and it remains constant up to at least 0.2% of SDS. Additional binding data (at an SDS concentration of 0.1%) is given in Table 1; GPIIb and GPIIbα are the glycoprotein species which bind the most SDS.

Partial specific volumes of the glycoproteins and their SDS complexes

The partial specific volumes of the glycoproteins (Table 1) were calculated on the basis of the partial specific volume (Cohn and Edsall 1943; Gibbons 1971) and the weight percentage (Eirín et al. 1986; Calvete and González-Rodríguez 1986) of each amino acid and sugar residue (see analytical methods). The partial specific volume of the glycoprotein complexes with SDS were calculated in the same way, using $\bar{v}$ and δ_D for each glycoprotein (Table 1) and the partial specific volume of SDS (Tanford et al. 1974).

Table 1. Values of some physical properties of GPIIb, GPIIbα, CM-GPIIbα, CM-GPIIb β, GPIIIa and CM-GPIIIa, obtained by sedimentation equilibrium

Property	$\bar{v}$ ($cm^3 g^{-1}$) [a]	$\delta_D \left(\frac{\text{mg SDS}}{\text{mg glycoprotein}}\right)$ [b]	S^{0*} (S) [c]	$M(1-\phi'\varrho)$ ($g\,mol^{-1}$) [d]	M ($g\,mol^{-1}$) [d]	M ($g\,mol^{-1}$) [e]	M ($g\,mol^{-1}$) [a]
Glycoprotein							
GPIIb	0.700	1.22 ± 0.05	5.3 ± 0.15	51,300 ± 1,400	116,600 ± 4,600	134,000 ± 15,000	136,500
GPIIbα	0.701	1.31 ± 0.05	4.4 ± 0.1	44,900 ± 700	99,800 ± 2,900	–	114,000
CM-GPIIbα	0.701	0.95 ± 0.05	5.1 ± 0.1	45,300 ± 1,100	111,000 ± 4,400	–	114,000
CM-GPIIbβ	0.713	1.02 ± 0.05	2.1 ± 0.1	8,500 ± 150	21,000 ± 800	–	22,200
CM-GPIIIa	0.712	1.05 ± 0.05	4.7 ± 0.05	36,300 ± 400	88,400 ± 2,000	–	91,500
GPIIIa	0.712	1.05 ± 0.05	4.7 ± 0.05	36,700 ± 600	90,100 ± 2,800	84,600 ± 7,000	91,500

[a] Calculated from the chemical composition (Eirín et al. 1986; Calvete and González-Rodríguez 1986)
[b] Analytically determined
[c] This property refers to the corresponding glycoprotein-SDS complexes
[d] Calculated from sedimentation equilibrium data at constant solvent density. Standard deviations in M reflect the error in $M(1-\phi'\varrho)$ and in the SDS binding measurements
[e] Calculated from sedimentation equilibrium data at different solvent densities

Sedimentation velocity measurements

Eosin-labelled glycoproteins were subjected to analytical ultracentrifugation, and their sedimentation coefficients (Table 1) determined from the sedimentation patterns monitored by absorption at 530 nm, free of any scattering artifact from the SDS micelles. It is remarkable that whereas GPIIIa and CM-GPIIIa have the same sedimentation coefficient, the total reduction and carboxymethylation of GPIIbα increases the sedimentation coefficient from 4.4 to 5.1 *S*.

Sedimentation equilibrium measurements

The molar masses of CM-GPIIbα, CM-GPIIbβ, GPIIIa and CM-GPIIIa (Table 1) obtained at constant solvent density by extrapolation to infinite dilution (Fig. 2), are in good agreement with the values calculated from their amino acid and sugar composition (Eirín et al. 1986; Calvete and González-Rodríguez 1986). However, the molar mass found for GPIIb was 15% lower than the values calculated both from its chemical composition or from the sum of the molar masses of CM-GPIIbα and CM-GPIIbβ determined by sedimentation equilibrium. Equally underestimated was the value obtained for GPIIbα, which is 11% lower than the value found for CM-GPIIbα.

Because of these discrepancies, we carried out sedimentation equilibrium experiments in elution buffer (0.1% SDS) in H_2O-D_2O mixtures of increasing density. The plots of $M(1-\phi'\varrho)$ as a function of ϱ for GPIIb and GPIIIa (Fig. 3), extrapolated to $\varrho = 1/\bar{v}_D$, give a molar mass for GPIIb significantly higher ($134{,}000 \pm 15{,}000$ g mol^{-1}) than that obtained by sedimentation equilibrium at constant density (Table 1), and very close to the expected value, although with an uncertainty three times higher. However, the value found for GPIIIa ($84{,}600 \pm 7{,}000$ g mol^{-1}) agrees, within experimental error, with the molar mass determined at constant density (Table 1).

Laser-light scattering measurements

The molar masses of the unlabelled glycoproteins equilibrated in elution buffer were also determined by light scattering (Fig. 4). The values obtained (Table 2) are in good agreement with the molar masses of eosin-labelled CM-GPIIbα, CM-GPIIbβ, and GPIIIa found by sedimentation equilibrium at constant solvent density, and with the molar mass of GPIIb found by sedimentation equilibrium at varying solvent densities.

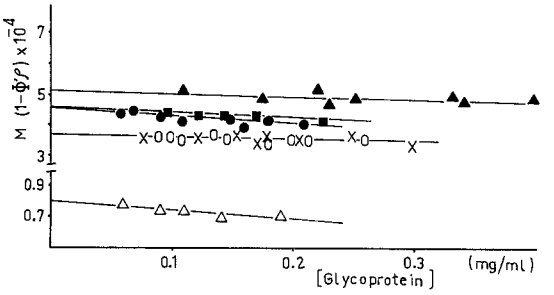

Fig. 2. Glycoprotein concentration dependency of the term $M(1-\phi'\varrho)$ determined by sedimentation equilibrium at constant solvent density for GPIIb (—▲—), CM-GPIIbα (—●—), GPIIbα (—■—), CM-GPIIbβ (—△—), GPIIIa (—○—) and CM-GPIIIa (—×—)

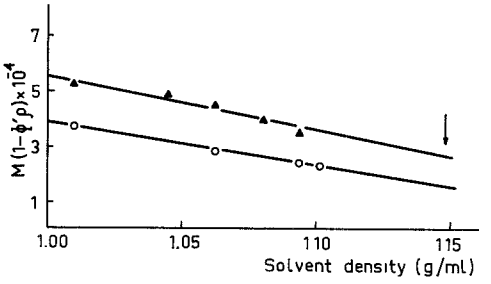

Fig. 3. Results for GPIIb (—▲—) and GPIIIa (—○—) of the sedimentation equilibria as a function of solvent density, in elution buffer (0.1% SDS) in H_2O-D_2O mixtures. The *arrow* indicates the point at which $\varrho = 1/\bar{v}_D$

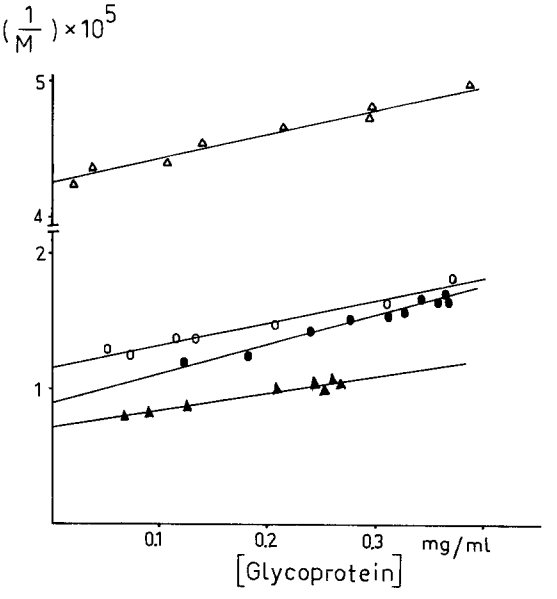

Fig. 4. Glycoprotein concentration dependency of the reciprocal of the molar masses determined by laser-light scattering for GPIIb (—▲—), CM-GPIIbα (—●—), CM-GPIIbβ (—△—) and GPIIIa (—○—)

Table 2. Values of some physical properties of GPIIb, GPIIbα, GPIIbβ, GPIIIa, and their SDS complexes obtained by laser-light scattering

Property	GPIIb	CM-GPIIbα	CM-GPIIbβ	GPIIIa
ε_{280} (ml mg^{-1} cm^{-1})*	1.26 ± 0.03	1.00 ± 0.04	1.21 ± 0.02	1.00 ± 0.04
dn/dc*	0.441 ± 0.04	0.483 ± 0.014	0.271 ± 0.014	0.398 ± 0.05
B (ml mol g^{-2})*	1.3×10^{-6}	3.2×10^{-6}	2.1×10^{-6}	1.2×10^{-6}
M (g mol^{-1})	142,600 ± 5,700	113,800 ± 5,000	23,500 ± 400	83,000 ± 4,700

* Property referred to the glycoprotein-SDS complexes in elution buffer (0.1% SDS)

Table 3. Stokes radii, frictional ratios of GPIIb, GPIIbα, GPIIbβ and GPIIIa and their SDS complexes determined by high-performance size-exclusion chromatography and analytical centrifugation

Property	GPIIb	GPIIbα	CM-GPIIbα	CM-GPIIbβ	GPIIIa	CM-GPIIIa
$R_{min\,(nm)}$ [a]	3.4	3.2	3.2	1.8	3.0	3.0
R_s [b]	5.2	5.1	4.8	2.4	4.3	4.8
(f/f_{min}) [b]	1.5	1.6	1.5	1.3	1.4	1.6
R^*_{min} [c]	4.6	4.0	4.1	2.4	3.9	3.9
R^*_s [b]	10.7	10.3	10.0	4.8	8.9	10.0
R^*_s [d]	7.5	7.7	6.8	3.1	6.2	6.1
(f/f_{min})* [d]	1.6	1.9	1.7	1.3	1.6	1.6

* Property referred to the glycoprotein-SDS complexes in elution buffer (0.1% SDS)

[a] Determined using $\bar{v}$ given in Table 1 and the following molar masses (g mol^{-1}): GPIIb (136,500); GPIIbα (114,000); GPIIbβ (22,200); and GPIIIa (91,500)

[b] Calculated from chromatographic data

[c] Calculated using the same molar masses as in [a] and the δ_D values given in Table 1

[d] Calculated from analytical centrifugation data

Stokes radii and frictional ratios of the glycoproteins and their complexes with SDS

The apparent Stokes radii of the glycoproteins (R_s) and their complexes with detergent (R^*_s), were determined by chromatography in analytical TSK-GEL SW columns and elution buffer, using for calibration proteins of known R_s and R^*_s as shown in Fig. 5. It is assumed in deriving R_s that the presence of SDS has the same relative effect upon the glycoprotein component as upon the calibration proteins. If we calculate the minimum radius of the glycoproteins (R_{min}) and of their complexes (R^*_{min}) as $R_{min} = (3M\bar{v}/4\pi N)^{1/3}$ and $R^*_{min} = (3M^*(\bar{v}+\delta_D\bar{v}_D)/4\pi N)^{1/3}$, where M and M^* are the molar masses of the glycoproteins and their complexes with SDS, respectively, and the rest of the terms are as defined above, then we can obtain their maximum frictional ratios (Table 3), and the degree of departure of their shapes from that of a spherical globular particle.

The Stokes radii of the glycoprotein-SDS complexes were more rigorously calculated from the frictional coefficient, f^*, obtained from analytical centrifugation data, by the expression (Tanford et al. 1974):

$$s^* = M(1-\phi'\varrho)/Nf^*, \quad (3)$$

where s^* and $M(1-\phi'\varrho)$ have already been determined (Table 1), and $f^* = 6\pi\eta R^*_s$. Table 3 summarizes the R^*_s values obtained for the complexes of GPIIb, GPIIbα, CM-GPIIbα, CM-GPIIbβ, CM-GPIIIa and GPIIIa with SDS, which together with the R^*_{min} allow us to calculate their maximum frictional ratios.

Discussion

The chromatographic method was found to be superior to the commonly used equilibrium dialysis through semipermeable membranes for equilibration of aqueous protein solutions at SDS concentrations above their critical micellar concentration. It is quick and reproducible, and demonstrates the homogeneity, integrity and state of aggregation of the protein-SDS complexes. Also, when labelled proteins are used, as in our case, chromatography establishes the absence of free dye. The labelling of proteins with an optical probe having a high extinction coefficient in the visible region, such as eosin, permits the monitoring of the ultracentrifugation experiments by light absorption in the visible region. This avoids the scattering at 280 nm due to the detergent micelles, which is particularly undesirable in meniscus depletion sedimentation equilibrium.

The good agreement between the molar masses of GPIIb, CM-GPIIbα, CM-GPIIbβ, and GPIIIa found by sedimentation equilibrium, laser-light scattering, and chemical composition gives confi-

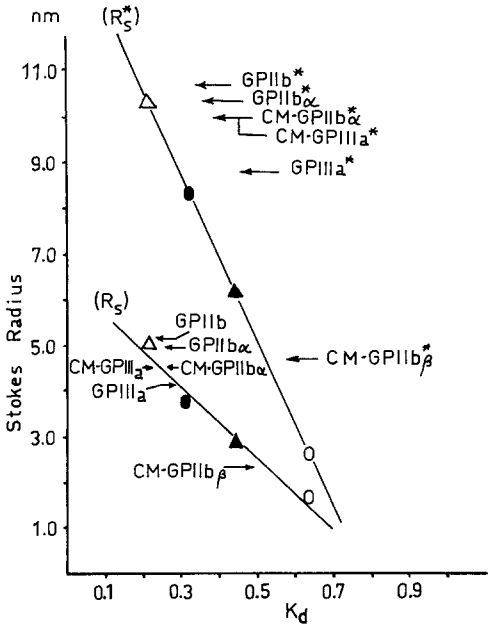

Fig. 5. High-performance size-exclusion chromatography determination of the Stokes radii of GPIIb, CM-GPIIbα, CM-GPIIbβ, GPIIIa, SDS complexes (R*) and calculation of the Stokes radii of the glycoproteins (R_s). Two TSK-G4000SW and one TSK-G3000SW columns in series and elution buffer (0.1% SDS) were used as described in *Methods*. The following proteins were used for calibration: Immunoglobulin G (△) ($R_s = 5.1$ nm, $R_s^* = 10.3$); Bovine serum albumin (●) ($R_s = 3.5$, $R_s^* = 8.4$); Ovalbumin (▲) ($R_s = 2.7$, $R_s^* = 6.1$); Cytochrome c (○) ($R_s = 1.7$, $R_s^* = 2.6$). All these values were taken from Tanford et al. (1974), except R_s^* for IgG* which was calculated by us from s^{0*} and $M(1-\phi'\varrho)$ determined by analytical ultracentrifugation. Values of the Stokes radii for the glycoproteins and their complexes with SDS are given in Table 3

dence in the values obtained. More divergent results were found for the molar masses of GPIIb and GPIIbα obtained by sedimentation equilibrium at constant solvent density, which deserve a special discussion. The log c vs. r^2 plots were linear in all the experiments, and the samples were checked, before and after each experiment (sedimentation velocity, analytical chromatography and SDS gel electrophoresis) for glycoprotein integrity, homogeneity, state of aggregation, and absence of free dye. Therefore, the apparently low values determined for GPIIb and GPIIbα at constant solvent density point towards complicating factors other than heterogeneity of the sedimenting glycoprotein.

Protein molar mass values, obtained by sedimentation equilibrium in SDS, lower than those expected from the chemical composition or determined by other physical measurements, have been reported before from different laboratories, both for water soluble and membrane proteins (Nakae et al. 1979; Tokunaga et al. 1979; Miyake et al. 1978; Kameyama et al. 1982). The possibility of preferential interactions of the glycoprotein-SDS complexes with counterions has been suggested as the cause of these molar mass underestimations (Kameyama et al. 1982; Tanford 1961; Yphantis and Roark 1972). However, this does not seem generally to be the case, since the molar masses of GPIIIa, CM-GPIIIa, CM-GPIIbα, and CM-GPIIbβ agree with those found by other procedures. The molar masses which are apparently underestimated are those for the two glycoprotein species which bind more SDS, i.e. GPIIb and GPIIbα. Cooperative binding of SDS to these two glycoproteins does not seem likely either, since the [SDS] exceeds the critical micellar concentration, and this type of binding is not observed in the binding isotherm of GPIIb, at least up to 0.2% SDS. Possible sources of error exist in several parameters eg. true $\bar{v}$ of the glycoproteins, $\bar{v}_D$ of the detergent when bound to the glycoproteins, and δ_D of the glycoprotein-SDS complexes at sedimentation equilibrium, and additional experiments will be necessary to explain these discrepancies.

In addition to the wide range of apparent molecular mass data available in the literature for these glycoproteins determined by SDS electrophoresis, Jennings and Phillips (1982), from the Stokes radius and the sedimentation coefficient, calculated an apparent molecular mass of 125,000 ± 13,000 Da for GPIIb, which is in the same lower range as the value found here by sedimentation equilibrium at constant density, and a molecular mass of 93,000 ± 12,000 Da for GPIIIa, which agrees with the value found here. These agreements are somewhat unexpected, considering the limitations of gel filtration and density gradient centrifugation used by these authors, and the higher value used by them for the partial specific volume of the glycoproteins. However the values of R_s and f/f_0 given by Jenning and Phillips (1982) are significantly different from those in Table 3.

If we compare the Stokes radii and frictional ratios obtained for the glycoprotein-SDS complexes by analytical centrifugation with the same parameters obtained by high-performance size-exclusion chromatography (Table 3), it is readily seen that chromatography overestimates them. The same effect is observed in the parameters for the glycoprotein moieties of the complexes calculated from chromatographic data. In spite of this and of the use of a R_{min} calculated for the anhydrous glycoproteins (compared with the R_0 calculated by Jennings and Phillips (1982) assuming a hydration of 0.2 g per g of glycoprotein) the maximum frictional ratios that we obtained are significantly lower than the values given by these authors, the disagreement being particularly striking for GPIIIa (Table 3). The Stokes radius given for this glycoprotein by Jennings and Phillips (1982) is much larger than the one we

obtained here, and even larger than the Stokes radius we determined by analytical ultracentrifugation for the GPIIIa-SDS complex.

The overestimation of the R_s^* of the glycoprotein-SDS complexes obtained by size-exclusion chromatography on TSK-GEL SW columns is most probably due to electrostatic interactions between the complexes and the column matrix. The pI of the glycoproteins is about 5.5 (McGregor et al. 1981) and the charge of the bound SDS imparts a highly negative charge to the glycoprotein-SDS complexes. Also, it is known that there are residual silanol groups at the surface of the silica matrix of the TSK-GEL SW columns, which bear weak negative charges (Regnier 1983). The higher sialic acid contents and the higher bound SDS for GPIIb and GPIIbα compared with GPIIbβ and GPIIIa may explain why the complexes of those glycoproteins diviate most from the ideal size-exclusion behaviour.

Acknowledgements. We thank Dr. A. U. Acuña for his advice on the ultracentrifuge optics and detectors, Dr. C. Gutiérrez for assistance with the English, the Blood Banks of Centro Ramón y Cajal and La Paz (Madrid) for providing us with outdated platelet concentrates, Mrs. C. Martin de Loeches for technical assistance and Mrs. M. L. Ruíz Pineda for typing the manuscript. This work was supported by the Comisión Asesora de Investigación Científica y Técnica, Spain, under Project N° 613/131.

References

Calvete JJ, González-Rodríguez J (1986) Isolation and biochemical characterization of the α and β subunits of glycoprotein IIb from human platelet plasma membrane. Biochem J 240:155–161

Cohn EJ, Edsall JT (1943) Proteins, amino acids and peptides. Reinhold, New York, pp 157–161 and 370–375

De Vendittis E, Palumbo G, Parlato G, Bocchini V (1981) A fluorimetric method for the estimation of the critical micelle concentration of surfactants. Anal Biochem 115: 278–286

Eirín MT, Calvete JJ, González-Rodríguez J (1986) New isolation procedure and further biochemical characterization of glycoproteins IIb and IIIa from human platelet plasma membrane. Biochem J 240:147–153

George JN, Nurden AT, Phillips DR (1981) Recommendation of the International Committe on Thrombosis and Haemostasis concerning platelet membrane glycoprotein nomenclature. Thromb Haemost 46:764–765

Gibbons RA (1972) Physico-chemical methods for the determination of the purity, molecular size and shape of glycoproteins. In: Gottschalk A (ed) Glycoproteins: Their composition, structure and function. Elsevier, Amsterdam, pp 31–140

Jennings LK, Phillips DR (1982) Purification of glycoproteins IIb and IIIa from human platelet plasma membranes and characterization of a calcium-dependent glycoprotein IIb-IIIa complex. J Biol Chem 257:10458–10466

Kameyama K, Nakae T, Takagi T (1982) Estimation of molecular weights of membrane proteins in the presence of SDS by low-angle laser-light scattering combined with high-performance porous silica gel chromatography. Biochim Biophys Acta 706:19–26

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Leung LLK, Kinoshita T, Nachman RL (1981) Isolation, purification and partial characterization of platelet membrane glycoproteins IIb and IIIa. J Biol Chem 256: 1994–1997

Lowry OH, Rosebrough NJ, Farr AL, Randal RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Markwell MAK, Haas SM, Bieber LL, Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87:206–210

McEver RP, Baenziger JU, Majerus PW (1982) Isolation and structural characterization of the polypeptide subunits of membrane glycoprotein IIb-IIIa from human platelets. Blood 59:80–85

McGregor JL, Clemetson KJ, James E, Capitanio A, Greenland T, Lüscher EF, Dechavanne M (1981) Glycoprotein of platelet membranes from Glanzmann's thrombasthenia. A comparison with normal using carbohydrate-specific or protein-specific labelling techniques and high-resolution two-dimensional gel electrophoresis. Eur J Biochem 116: 379–388

Miyake J, Ochiai-Yanagi S, Kasumi T, Takagi T (1978) Isolation of a membrane protein from R. rubrum chromatophores and its abnormal behavior in SDS-polyacrylamide gel electrophoresis due to a high binding capacity for SDS. J Biochem 83:1679–1686

Nakae T, Ishii J, Tokunaga M (1979) Subunit structure of functional porin oligomers that form permeability channels in the outer membrane of Escherichia coli. J Biol Chem 254:1457–1461

Regnier FE (1983) High-performance liquid chromatography of proteins. Methods Enzymol 91:137–190

Reynolds JA, Tanford C (1976) Determination of molecular weight of the protein moiety in protein-detergent complexes without direct knowledge of detergent binding. Proc Natl Acad Sci USA 73:4467–4470

Sardet C, Tardieu A, Luzzati V (1976) Shape and size of bovine rhodopsin: a small-angle X-ray scattering of a rhodopsin-detergent complex. J Mol Biol 105:383–407

Sokoloff RL, Frigon RP (1981) Rapid spectrophotometric assay of dodecyl sulfate using acridine orange. Anal Biochem 118:138–141

Takagi T, Miyake J, Nashima T (1980) Assessment study on the use of the low angle laser light scattering technique for the estimation of molecular weight of the polypeptide forming a complex with sodium dodecyl sulfate. Biochim Biophys Acta 626:5–14

Tanford C (1961) Physical chemistry of macromolecules. Wiley, New York, pp 265–270

Tanford C, Reynolds JA (1976) Characterization of membrane proteins in detergent solutions. Biochim Biophys Acta 457: 133–170

Tanford C, Nozaki Y, Reynolds JA, Makino S (1974) Molecular characterization of proteins in detergent solutions. Biochemistry 13:2369–2376

Tokunaga M, Tokunaga H, Okajima Y, Nakae T (1979) Characterization of porins from the outer membrane of Salmonella typhimurium 2. Physical properties of the functional oligomeric aggregates. Eur J Biochem 95:441–448

Yphantis DA (1964) Equilibrium ultracentrifugation of dilute solutions. Biochemistry 3:297–317

Yphantis DA, Roark DE (1972) Equilibrium centrifugation of nonideal systems. Molecular weight moments for removing the effects of nonideality. Biochemistry 11:2925–2934