Biochem J. (1986) 240, 147–153 (Printed in Great Britain)

New isolation procedure and further biochemical characterization of glycoproteins IIb and IIIa from human platelet plasma membrane

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We describe a new procedure for isolation of glycoproteins IIb (GPIIb) and IIIa (GPIIIa) from human platelet plasma membrane with high yields (2.7 mg of GPIIb and 3.3 mg of GPIIIa per 100 mg of starting platelet membrane proteins), equivalent to a recovery of 35% and 55% respectively of the total GPIIb and GPIIIa of the membrane. The procedure involves Triton X-100 differential extraction of platelet membranes, SDS solubilization of the 4%-Triton X-100 supernatant, zonal centrifugation in a sucrose density gradient, and preparative high-performance size-exclusion chromatography. The weight percentage of sugar is 15.7% for GPIIb and 12.5% for GPIIIa. Neuraminic acid is present in both glycoproteins, representing 30% and 15% respectively of the total sugar weight of GPIIb and GPIIIa. Mannose, galactose and glucosamine account for 45%, 13% and 28% respectively of the sugars of GPIIIa, whereas galactosamine was not detected. Mannose, galactose, glucosamine and galactosamine represent 17%, 21%, 24% and 10% respectively of the sugar content of GPIIb. The molar percentages of half-cystine and methionine are 4-fold and 2-fold higher respectively in GPIIIa than in GPIIb. From the amino acid and sugar compositions we confirmed the acidic nature of both glycoproteins. The M_r values obtained, 136500 for GPIIb and 91500 for GPIIIa, are in very good agreement with those obtained by physical methods. The apparent lack of free thiol groups in both glycoproteins indicates that the tertiary structure of GPIIIa is maintained by 21 intrachain disulphide bonds, and that there are eight intrachain and interchain disulphide groups in GPIIb.

INTRODUCTION

Several laboratories have already isolated GPIIb and GPIIIa to different degrees of purity and by different procedures and have biochemically characterized them to some extent. Leung *et al.* (1981) combined lentil lectin affinity chromatography with electrophoretic elution from SDS/polyacrylamide gels. McEver *et al.* (1982) separated GPIIb from GPIIIa by preparative SDS/polyacrylamide-gel electrophoresis, after isolation of the GPIIb–GPIIIa complex by immunoaffinity chromatography with a specific monoclonal antibody. Their amino acid and partial sugar compositions confirmed the finding by Leung *et al.* (1981) that the peptide maps of the two glycoproteins are completely different. Jennings & Phillips (1982) purified GPIIb and, partially, GPIIIa by gel filtration in Triton X-100 buffers, with or without urea or guanidinium chloride. They calculated from hydrodynamic data the M_r values of GPIIb, GPIIIa and the GPIIb–GPIIIa complex, and studied the role of Ca^{2+} in the formation of the heterodimer. Finally, Newman & Kahn (1983) combined ion-exchange chromatography with analytical h.p.s.e.c. to isolate GPIIb and GPIIIa in the presence of SDS.

Several laboratories (McEver *et al.*, 1980, 1983; Bennett *et al.*, 1983; McGregor *et al.*, 1983; Di Minno *et al.*, 1983; Pidard *et al.*, 1983; Melero & González-Rodríguez, 1984) have prepared monoclonal antibodies against the GPIIb–GPIIIa complex or the individual glycoproteins, which allowed differentiation of several structural and functional areas in these glycoproteins and in the complex, and to quantify the amount of GPIIb and GPIIIa at the surface and in the whole platelet (Calvete *et al.*, 1986). In the present paper we describe a procedure for purification of GPIIb and GPIIIa with improved yields, in the scale of tens of milligrams, and we also report the complete amino acid and sugar compositions and the numbers of disulphide bonds per molecule in these glycoproteins.

MATERIALS AND METHODS

Materials

Water was from a Milli Q water-purification system (Millipore, Bedford, MA, U.S.A.). All chemicals and biochemicals were of analytical or chromatographic grade. Protein standards were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TSK-SW analytical (7.5 mm × 600 mm) and preparative (21.5 mm × 600 mm) chromatographic columns were from Toyo Soda (Tokyo, Japan), the Techsil 10 C_{18} column (5 mm × 250 mm) was from HPLC Technology (Macclesfield, Cheshire, U.K.), Dowex 50W X4 was from Fluka (Buchs, Switzerland), and C_{18} Sep-Pak was from Waters Associates (Milford, MA, U.S.A.).

Aqueous buffers were as follows: solubilization buffer, 0.02 M-Tris/HCl/0.15 M-NaCl/5 mM-EDTA, pH 7.4; zonal-centrifugation buffer, 0.01 M-sodium phosphate/

Abbreviations used: GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa; h.p.s.e.c., high-performance size-exclusion liquid chromatography; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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5 mM-EDTA/0.025% NaN_3/0.1% SDS, pH 7.0; h.p.s.e.c. buffer, 0.1 M-sodium phosphate/1 mM-EDTA/0.025% NaN_3/0.1% SDS, pH 6.8.

Platelet concentrates (72 h after blood collection) were obtained from the blood banks of Centro Ramon y Cajal and La Paz (Madrid, Spain).

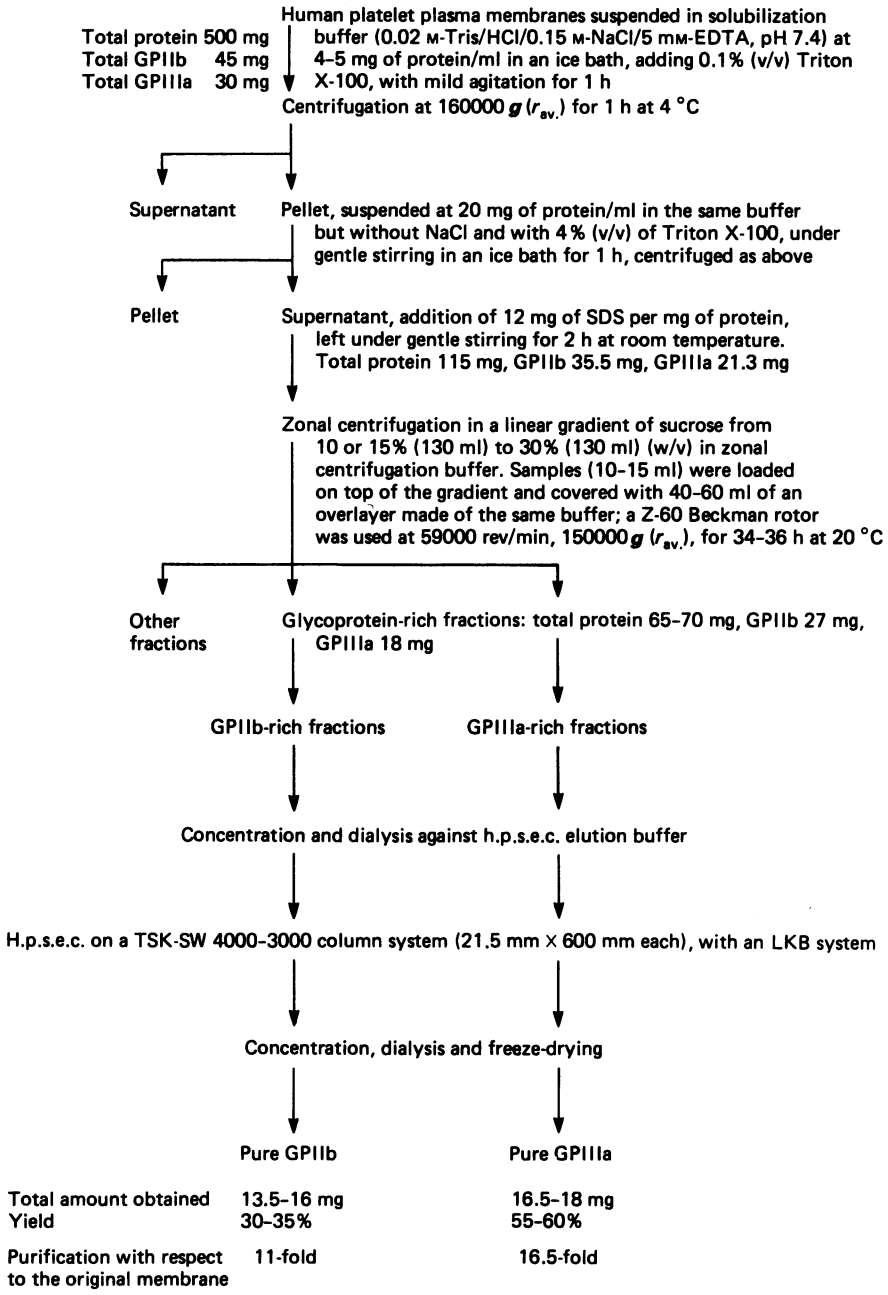
Analytical methods

Phosphorus was assayed by the method of Bartlett (1959), with KH_2PO_4 as standard. Constant dry weights were obtained under vacuum in a Cahn model 1000 electrobalance.

Protein concentration was determined by the Folin procedure (Lowry *et al.*, 1951; Markwell *et al.*, 1978), with bovine serum albumin as standard, by using $a_{278} = 65.8$ litre·g^{-1}·cm^{-1} for calculation of the standard concentration.

Sialic acid was determined after hydrolysis of dry samples in 0.05 M-H_2SO_4 at 80 °C for 1 h by the fluorimetric method of Hammond & Papermaster (1976), with *N*-acetylneuraminic acid as standard.

Individual hexoses were determined as their dansylhydrazones by h.p.l.c. (Alpenfels, 1981) after hydrolysis of dry samples (10–20 nmol of each sugar) in 2 M-HCl at 100 °C for 2 h, drying of the hydrolysate under vacuum, passage through Dowex 50W X4 (Boas, 1953), treatment with dansylhydrazine and passage through a C_{18} Sep-Pak. Maltose was used as internal standard. A Waters h.p.l.c.



Scheme. 1. Isolation of GPIIb and GPIIIa

For full experimental details see the text. Protein concentration was determined as described by Markwell *et al.* (1978). Individual glycoproteins were determined by gel electrophoresis and enzyme immunoassay (Calvete *et al.*, 1986).

system was used and sugars were separated in a Techsil 10 C_{18} column by isocratic elution with 18% (w/w) acetonitrile/water. Fluorescence detection was done in a Perkin–Elmer MPF 3 spectrofluorimeter equipped with a 15 μl flow cell.

Individual hexosamines were analysed as their dansyl derivatives by h.p.l.c. (Hjerpe *et al.*, 1980), by using the same system, column and elution procedure as above, and after hydrolysis of dry samples in 4 M-HCl at 100 °C for 4 h, drying of the hydrolysate under vacuum, passage through Dowex 50W X4, treatment with dansyl chloride and passage through a C_{18} Sep-Pak. Mannosamine was used as internal standard.

Amino acid analysis of dry samples containing 50–100 μg of protein were carried out after hydrolysis of the samples resuspended in 6 M-HCl in sealed ampoules that had been evacuated three times down to 1.3 mPa. After 24 h, 48 h and 72 h hydrolysis at 105 °C, samples were dried under vacuum at 40 °C, dissolved in sample buffer and loaded on an LKB model 3201 amino acid analyser. Tryptophan was determined by the spectrophotometric method of Edelhoch (1967) as modified by Koziarz *et al.* (1978) for samples containing SDS.

The number of free thiol groups was determined by the method of Ellman (1959) with 100–200 μg of glycoprotein solubilized in 2% SDS and a 5–10-fold molar excess of 5,5′-dithiobis-(2-nitrobenzoic acid) with respect to the glycoprotein half-cystine residues determined by amino acid analysis. The use of other denaturing agents or a larger excess of 5,5′-dithiobis-(2-nitrobenzoic acid) did not change the results. Disulphide bonds were determined by the method of Zahler & Cleland (1968), with 25–200 μg of glycoprotein and a 5–200-fold molar excess of dithioerythritol. Performic acid oxidation of glycoproteins was carried out as described by Hirs (1967). Cysteine and cystine were determined as cysteic acid, after hydrolysis of the oxidized samples.

Phosphodiesterase (EC 3.1.4.1) activity was assayed by the method of Koerner & Sainsheimer (1957) at pH 5.5 at 37 °C, by monitoring the liberation of *p*-nitrophenol at 410 nm and using $\epsilon_{410} = 18000\ M^{-1} \cdot cm^{-1}$.

SDS/polyacrylamide-slab-gel electrophoresis was performed by the procedure of Laemmli (1970). A linear 7–12% polyacrylamide gradient was the resolving gel, with a 4.5% polyacrylamide stacking gel. Samples in 3% (w/v) SDS were reduced with 1% (v/v) 2-mercaptoethanol at 100 °C for 2 min. Gels were stained with Coomassie Blue (Fairbanks *et al.*, 1971) or with periodic acid/Schiff reagent (Zacharius *et al.*, 1969). Microdensitometric scans of the gels were obtained with a Joyce–Loebl model M KIIIC double-beam microdensitometer.

GPIIb and GPIIIa were individually quantified in solution, in subcellular fractions and at the surface of intact platelets by enzyme immunoassay and by gel-electrophoretic analysis, as described elsewhere (Calvete *et al.*, 1986).

Isolation of GPIIb and GPIIIa

Platelets were purified from platelet concentrates, and the plasma membranes isolated by the glycerol lysis technique, as described by Barber & Jamieson (1970). Yields were 1.4 ± 0.2 mg of plasma-membrane proteins per blood unit and the phosphodiesterase activity was 0.245 mol of *p*-nitrophenol/h per mg of protein. The procedure that we have used to isolate GPIIb and GPIIIa is outlined in Scheme 1.

Platelet subcellular fractionation

Human platelets from outdated platelet concentrates (72 h after blood collection) were subjected to hypoosmotic lysis after intracellular loading with glycerol (Barber & Jamieson, 1970). The lysate was loaded on top of a discontinuous 30–50% (w/v) sucrose gradient, and centrifuged at 85000 *g* ($r_{av.}$ 123 mm) for 3 h at 4 °C in a swing-out rotor (Calvete *et al.*, 1986). After being washed by differential centrifugation, the four particulate fractions (FI, FII, FIII and FIV), obtained on top of the 30%, 40%, 45% and 50% sucrose layers, were used for isolation of GPIIb and GPIIIa by the same procedure as that outlined above for the isolation of these glycoproteins from the plasma-membrane fraction prepared as described by Barber & Jamieson (1970).

RESULTS

Isolation of GPIIb and GPIIIa from platelet plasma membrane

The procedure begins with the extraction of the platelet membranes with Triton X-100 as described by Jennings & Phillips (1982). The 4%-Triton X-100 supernatant (see the purification scheme) contains 23% of the total protein and about 70% of the GPIIb and GPIIIa of the starting membrane, as estimated by gel electrophoresis and enzyme immunoassay. The chemical and electrophoretic analyses of the fractions obtained by density-gradient centrifugation in a zonal rotor show that the two glycoproteins are paradoxically distributed in a band in the heaviest fractions (Fig. 1). At the lower-density edge of this band more than 50% of the protein is GPIIb, whereas at the higher-density edge more than 80% of the protein is GPIIIa, both glycoprotein fractions being slightly contaminated with actin and nearly free of myosin. Finally, GPIIb and GPIIIa were isolated by h.p.s.e.c. on preparative TSK-SW gel columns with yields of about 2.7 mg and 3.3 mg respectively per 100 mg of the initial membrane proteins (Fig. 2). Purified GPIIb and GPIIIa were identified by their electrophoretic and chromatographic behaviour, as compared with the behaviour of these glycoproteins in the SDS/polyacrylamide-gel electrophoretic and analytical h.p.s.e.c. elution patterns of platelet plasma membrane solubilized in SDS.

Characterization of GPIIIa

In Table 1 we show the amino acid composition of GPIIIa obtained by us and by McEver *et al.* (1982) and Jennings & Phillips (1982). All the results are in agreement, except for methionine, for which our values are the highest. We have also determined the number of tryptophan residues in the molecule. More than half of the amino acid residues are polar, and therefore this protein cannot be considered a protein of low polarity (Capaldi & Vanderkooi, 1972). Although not included in the total amino acids, ammonia is also given as its contribution derives from aminated dicarboxylic amino acids, aminated sugars and tryptophan. The contribution from the remaining amino acids was corrected by extrapolation to time zero from three hydrolysis times. The sugar analysis of GPIIIa gives 9 mol of sugar per 100 mol of amino acids, i.e. 12.5% of the total weight of the glycoprotein, in close agreement with McEver *et al.* (1982). In addition, we have also measured the sialic acid

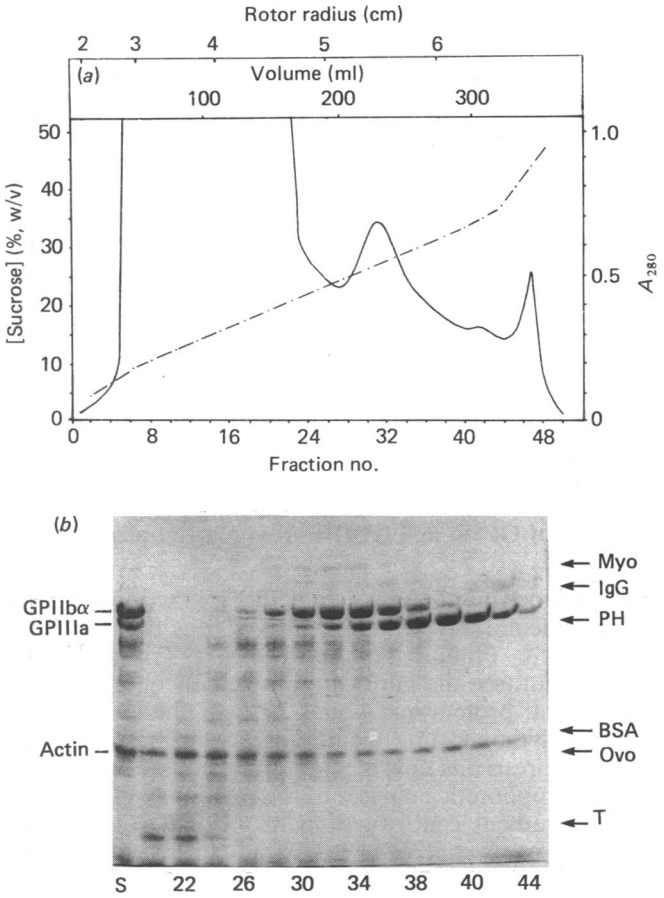


Fig. 1. Isolation of the platelet membrane glycoprotein fraction by zonal centrifugation

(*a*) 280 nm-absorption profile (——) of a 4%-Triton X-100 supernatant (106 mg of protein) of platelet plasma membranes, subjected to zonal centrifugation in a linear 15–30% (w/v) sucrose gradient (—·—) at 150000 *g* ($r_{av.}$) for 36 h at 20 °C. (*b*) Electrophoretic analysis of the reduced fractions of the density gradient in (*a*), done in a 7–12% polyacrylamide gradient by the procedure of Laemmli (1970) and stained with Coomassie Blue. Key: S, 4%-Triton X-100 supernatant; Myo, myosin; IgG, immunoglobulin G; PH, phosphorylase *b*; BSA, bovine serum albumin; Ovo, ovalbumin; T, trypsin.

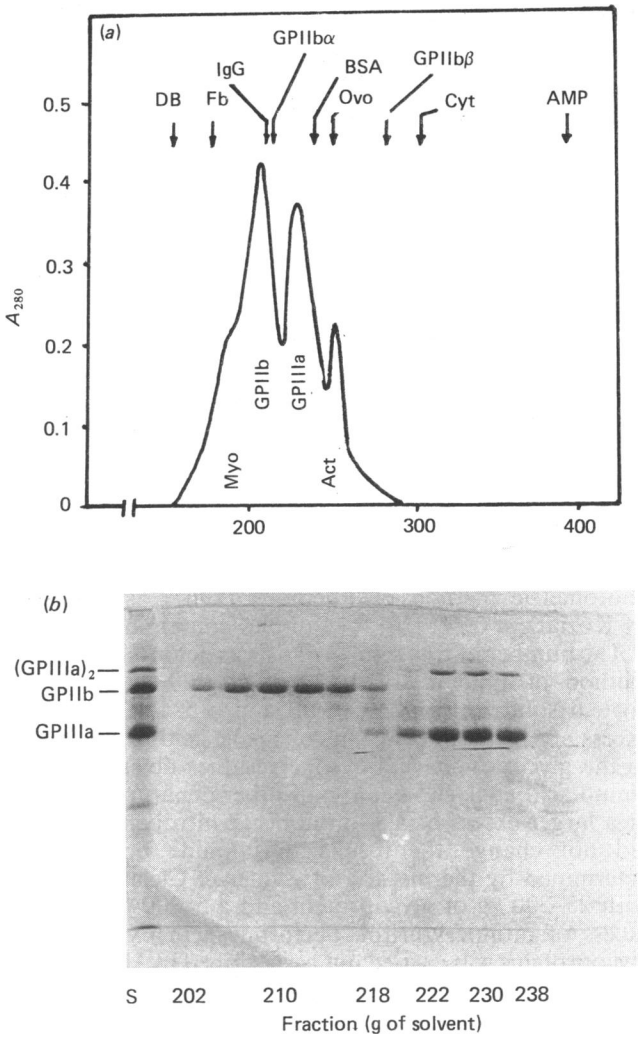


Fig. 2. Isolation of GPIIb and GPIIIa by h.p.s.e.c.

(*a*) H.p.s.e.c. elution profile at 280 nm of 25 mg (in 1.5 ml of h.p.s.e.c. elution buffer) of protein, from the glycoprotein band of a sucrose-density-gradient fractionation of a Triton X-100 supernatant of platelet membranes, applied to a TSK-SWG 4000 column in series with a TSK-SWG 3000 column. Elution and monitoring were effected in an LKB h.p.l.c. system, at 0.2 ml/min. Key: DB, Dextran Blue; Fb, fibrinogen; Act, actin; Cyt, cytochrome *c*; AMP, adenosine monophosphate; other abbreviations as in Fig. 1. (*b*) Electrophoretic analysis of the non-reduced fractions of the column profile referred to in (*a*), done as in Fig. 1. S, original sample from the glycoprotein band of a density-gradient fractionation. $(GPIIIa)_2$, dimer of GPIIIa. Column fractions are expressed in grams of solvent.

content of the molecule. Mannose represents about 50% of the molar content of sugar, the resting being 14.6% galactose, 27% glucosamine and 9.5% neuraminic acid. The mannose content, obtained by h.p.l.c. analysis of the dansylhydrazone derivative, may be lower than the actual content (McEver *et al.*, 1982; Eirín, 1980). There are reasons to believe that this technique gives lower recoveries of mannose (Alpenfels *et al.*, 1982; Calvete, 1985).

From the amino acid and sugar compositions we calculated (Hoy *et al.*, 1974) an M_r value of 91500 for GPIIIa, in good agreement with the values obtained by gel electrophoresis and exclusion chromatography for non-reduced samples of the glycoprotein (Table 2). Phosphorus was not detected by the method used in a 500 μg sample of GPIIIa.

No free thiol groups in GPIIIa could be titrated with 5,5′-dithiobis-(2-nitrobenzoic acid) under different denaturing conditions. However, when the molecule was previously reduced with dithioerythritol, titratable thiol groups appeared, depending on the amount of reducing agent and the time of reduction. In Fig. 3 we show a plot of the titratable thiol groups versus molar excess of reducing agent with respect to the half-cystine residues determined by amino acid analysis. In the conditions used, it is necessary to reach a high molar excess of reducing agent to titrate all the 41.5 thiol groups per molecule, i.e. half a thiol group less than the 42 half-cystine residues per molecule of GPIIIa found by amino acid analysis, for an M_r of 91500. In Fig 3 inset we show the

Table 1. Amino table and sugar compositions of GPIIIa and GPIIb

(a) The present work, an average of ten to 12 determinations with four to six different preparations; (b) from McEver *et al.* (1982); (c) from Jennings & Phillips (1982). Abbreviations: n.d., not determined; N.D., not detected.

	Composition							
	(mol/100 mol of total amino acids)							
	GPIIIa			GPIIb			(residues/molecule)*	
Residue	(a)	(b)	(c)	(a)	(b)	(c)	GPIIIa	GPIIb
Asp	11.44	12.39	10.47	9.01	10.35	8.84	82	95
Thr	5.10	6.70	5.80	4.57	4.99	4.25	37	48
Ser	8.03	8.34	9.22	8.19	8.23	9.06	58	86
Glu	11.06	12.90	11.86	11.69	14.34	12.48	80	123
Pro	4.56	2.91	5.66	6.57	4.24	6.89	33	69
Gly	7.32	9.10	9.52	10.02	9.85	10.50	53	106
Ala	5.33	5.56	6.05	7.37	7.98	7.32	33	78
Cys	5.97	6.45	n.d.	1.44	2.62	n.d.	43	15
Val	5.63	4.93	6.84	6.69	6.23	7.99	41	71
Met	4.06	0.76	1.85	2.06	0.37	1.27	29	22
Ile	4.63	3.03	4.39	2.95	2.24	2.81	33	31
Leu	8.91	8.34	9.30	11.69	10.47	11.21	64	123
Tyr	2.93	2.40	3.22	1.83	2.37	2.78	21	19
Phe	3.25	4.55	3.95	3.28	4.61	3.45	23	35
His	1.25	1.26	1.73	1.94	2.12	2.19	9	21
Lys	5.58	5.81	6.08	4.00	3.86	4.15	40	42
NH_3†	10.59	—	—	12.47	—	—	76	131
Arg	4.23	4.55	4.39	5.34	5.11	4.82	30	56
Trp	0.72	n.d.	n.d.	1.34	n.d.	n.d.	5	14
Gal	1.31	1.26	n.d.	2.67	2.12	n.d.	9	28
Man	4.37	6.07	n.d.	2.18	2.62	n.d.	31	23
GlcNAc	2.20	2.40	n.d.	2.41	2.72	n.d.	17	28
GalNAc	N.D.	n.d.	n.d.	0.85	n.d.	n.d.	—	10
NeuAc	0.85	n.d.	n.d.	2.08	n.d.	n.d.	6	22

* Assuming M_r 91500 for GPIIIa and M_r 136500 for GPIIb.
† Not included in the total amino acids.

dependence of the number of titratable thiol groups on the reduction time when a 73 molar excess of dithioerythritol is used. If the reduction temperature is raised to 60 °C, 50% of the thiol groups are titrated in 10 min, and 90% in 30 min.

Characterization of GPIIb

In Table 1 we give the amino acid composition of GPIIb. This protein contains nearly twice as much dicarboxylic amino acid residues as basic amino acid residues, similarly to GPIIIa. In contrast, the molar percentage of half-cystine in GPIIb is about 4-fold lower than in GPIIIa, and that of tryptophan 2-fold higher. The molar percentage of half-cystine in GPIIb is about half that determined by McEver *et al.* (1982), and the molar percentage of methionine is higher than those found by Jennings & Phillips (1982) and McEver *et al.* (1982).

Mannose is not the major sugar, and sialic acid doubles its molar percentage with respect to GPIIIa. There is some galactosamine, which is absent from GPIIIa, and the total weight percentage of sugar in GPIIb is 15.7%, slightly higher than that in GPIIIa. The discrepancy between our weight percentage for GPIIb and that reported by McEver *et al.* (1982) is due to sialic acid and galactosamine, which were not determined by those authors.

From the amino acid and sugar compositions we calculate (Hoy *et al.*, 1974) an M_r of 136500 for GPIIb, which is in good agreement with the values obtained by gel electrophoresis and in evident disagreement with the apparent M_r determined by h.p.s.e.c. for non-reduced GPIIb (Table 2).

Again, no free thiol groups were titratable in GPIIb with 5,5'-dithiobis-(2-nitrobenzoic acid), as in GPIIIa. However, when the molecule was reduced with dithioerythritol before titration, thiol groups appeared in a proportion that depended on the amount of reducing agent and the time of reduction. In Fig. 3 we show a plot of the titratable thiol groups versus molar excess of reducing agent with respect to the half-cystine residues determined by amino acid analysis. A molar excess of dithioerythritol even higher than in the case of GPIIIa was needed to titrate all the 14.4 thiol groups per molecule, i.e., nearly 1 thiol group less than the number of half-cystine residues obtained by amino acid analysis, for an M_r of 136500.

Isolation and amino acid and sugar compositions of GPIIb and GPIIIa from platelet subcellular fractions

GPIIb and GPIIIa were also isolated from the fractions (FI, FII, FIII and FIV), obtained after subcellular fractionation of the platelet homogenates by sucrose-density-gradient centrifugation, by the same

Table 2. Apparent M_r values of GPIIb and GPIIIa determined by gel electrophoresis and by size-exclusion chromatography and from the amino acid and sugar composition

For reduction conditions used for the preparation of reduced GPIIIa see the Materials and methods section. Reduced and carboxymethylated GPIIIa was prepared in 0.15 M-Tris/HCl/1 mM-EDTA/2% SDS, pH 8.0, by reduction of GPIIIa with a 100-fold molar excess of dithioerythritol over theoretical half-cystine residues in the glycoprotein, for 2 h at room temperature, followed by carboxymethylation with a 2-fold molar excess of iodoacetate over reductant.

	Apparent M_r			
Procedure	Non-reduced GPIIb	Non-reduced GPIIIa	Reduced GPIIIa	Reduced and carboxymethylated GPIIIa
Gel electrophoresis (Laemmli, 1970)	130000	97000	105000	128000
Size-exclusion chromatography on:				
TSK-SW 4000–3000 analytical columns	190000	94000	—	120000
Sephacryl 300	170000	84000	—	—
Amino acid and sugar analysis	136500	91500	—	—

procedure described above for their isolation from the plasma-membrane fraction. The isolated glycoproteins from the subcellular fractions of two different homogenates were subjected to amino acid (after a single hydrolysis time) and sugar analysis, it being observed that the compositions of GPIIb and GPIIIa are very similar among the different subcellular fractions.

DISCUSSION

We present an improved method of purification of platelet plasma-membrane glycoproteins GPIIb and GPIIIa, which consists in solubilization of the membranes with Triton X-100 in two steps (Jennings & Phillips, 1982), followed by zonal centrifugation and h.p.s.e.c. in the presence of SDS. The yields of GPIIb and GPIIIa are 8-fold and 5-fold higher respectively than those reported by Jennings & Phillips (1982) and 3.5-fold higher than those obtained by McEver *et al.* (1982), and higher than the yields obtained by our own earlier procedures (Eirín, 1980; Melero & González-Rodríguez, 1984; Calvete 1985). In fact, we recover 30–35% and 55–60% respectively of the total GPIIb and GPIIIa of the membrane (Calvete *et al.*, 1986).

The paradoxical distribution of these proteins along the sucrose density gradient in zonal centrifugation cannot be explained either by their bound SDS or by their molecular masses and hydrodynamic parameters (Usobiaga *et al.*, 1986). The presence of dimers in concentrated solutions of pure GPIIIa, observed by h.p.s.e.c. and SDS/polyacrylamide-gel electrophoresis (Fig. 2), as well as in platelet membranes solubilized in SDS and analysed by immuno-electroblotting, might be related with this abnormal pattern. A similar paradoxical distribution was also observed by Jennings & Phillips (1982) in size-exclusion chromatography with a Triton X-100/urea buffer, and it was interpreted as being due to a larger molecular asymmetry of GPIIIa compared with that of GPIIb. However, these glycoproteins were distributed according to their size in sucrose density gradients containing Triton X-100 and EDTA (Jennings & Phillips, 1982; Pidard *et al.*, 1982).

The chemical compositions of GPIIb and GPIIIa and their molecular properties determined by physical methods (Usobiaga *et al.*, 1986) are in very good agreement and independent of the procedure used for their isolation, confirming their homogeneity. In the same way, GPIIb and GPIIIa isolated from different platelet subcellular fractions are apparently identical (Calvete *et al.*, 1986).

Although GPIIb and GPIIIa are integrated membrane proteins (Nachman *et al.*, 1973; Phillips & Agin, 1974), the low percentage of non-polar amino acids present in their composition indicates that only a small proportion on their protein moieties should actually be within the hydrophobic part of the bilayer and facing it, as already suggested by Jennings & Phillips (1982). The amino acid and sugar compositions are also in agreement with the acidic nature of the two glycoproteins, as indicated by their isoelectric points of about 5.5 (Clemetson *et al.*, 1979). The previous work by Nachman & Ferris (1972) and McGregor *et al.* (1981) on the presence of sialic acid in GPIIb and GPIIIa is confirmed here, GPIIb being the more sialylated of the two. Galactosamine was only found in GPIIb, which allows us to predict that the sugar chains in GPIIIa are *N*-linked only through *N*-acetylglucosamine.

The information concerning the sulphur amino acids confirms earlier suggestions on the electrophoretic behaviour of these glycoproteins (Phillips & Agin, 1977). The very high half-cystine content of GPIIIa and the apparent lack of free thiol groups suggest that the tertiary structure of GPIIIa is maintained by 21 intrachain disulphide bonds. In contrast, the half-cystine content of GPIIb is less than one-third of that of GPIIIa, which together with the lack of free thiol groups suggest that eight intrachain and interchain disulphide bonds maintain the structure of GPIIb.

Antisera and monoclonal antibodies prepared by animal immunization with GPIIb and GPIIIa isolated in the presence of SDS are able to recognize these glycoproteins in their native state in isolated membrane and in intact platelets, as well as in membranes solubilized with non-ionic detergents, and to inhibit

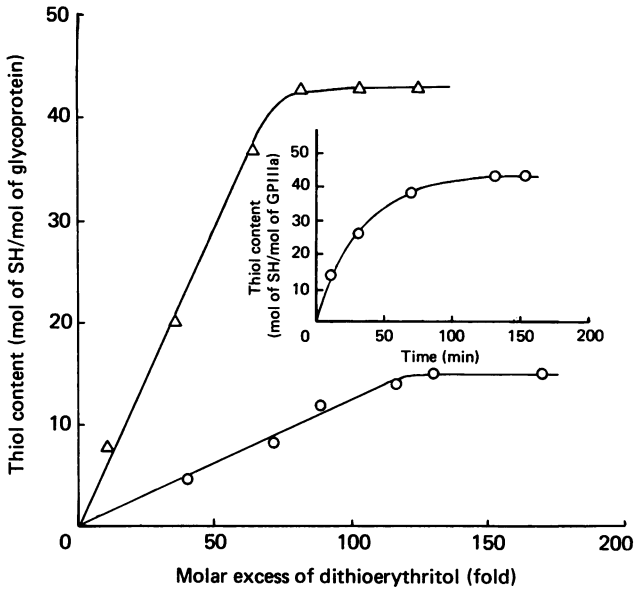


Fig. 3. Quantification of disulphide bonds in GPIIIa and GPIIb

The main Figure shows the dependence of the titratable thiol groups in GPIIIa (△) and GPIIb (○) on the molar excess of reducing agent. To samples of glycoprotein in 0.5 ml of 0.01 M-Tris/HCl/0.04% SDS, pH 9.0, increasing amounts of dithioerythritol were added; dithioerythritol was in a 5–200-fold molar excess with respect to the expected half-cystine content of the glycoprotein in each sample, as determined by amino acid analysis. After 120 min at room temperature the pH was lowered to 8.1 and $NaAsO_2$ was added. After 5 min monothiols were determined by addition of 5,5′-dithiobis-(2-nitrobenzoic acid) and monitoring was done at 412 nm. The inset shows the dependence of the titratable thiol groups on the reduction time when a 73-fold molar excess of dithioerythritol, over the expected half-cystine content, is added to GPIIIa at room temperature.

platelet aggregation induced by physiological effectors (Melero & González-Rodríguez, 1984; Calvete *et al.*, 1986; J. González-Rodríguez & J. Navarro, unpublished work). However, an assessment of the degree of irreversible denaturation of these glycoproteins isolated in the presence of SDS remains to be done.

We thank Dr. Saíz for the computer calculation of the M_r values of the glycoproteins from the biochemical composition Dr. C. Gutiérrez for polishing the English, and Professor R. Marco for suggestions on the presentation of the manuscript. We also thank Mrs. M. L. Ruíz Pineda for typing the manuscript and Mrs. C. Martín de Loeches for technical assistance. This work was supported by the Comisión Asesora de Investigación Científica y Técnica of Spain (Project no. 222).

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Received 7 April 1986/9 June 1986; accepted 23 July 1986