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# New procedures for glycophorin A purification with high yield and high purity

Sylvie Cochet[a], Gisèle Volet[b], Jean-Pierre Cartron[a], Olivier Bertrand[a,*]

[a]*INSERM U 76 GIP INTS 6 Rue Alexandre Cabanel 75739 Paris Cedex 15, France*
[b]*Laboratoire de Recherche sur les Polymères, CNRS, Université d'Evry, 2–8 rue Henry Dunant, 94320 Thiais, France*

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**Abstract**

Glycophorin A (GPA) is the major glycoprotein of the human erythrocyte membrane. It is known to form, in SDS gels as well as in a membrane environment, homodimers, and also heterodimers with the homologous molecule Glycophorin B (GPB). It is shown in this report that the propensity of GPA to dimerize with GPB precludes satisfactory preparation with high yield of pure GPA using classical techniques including SEC and RPLC. It was demonstrated using multiple angle light scattering that GPA is eluted from RPLC columns as dimers. A convenient procedure was devised which allowed us to get pure GPA with high yield. This procedure consists of selectively blocking GPA–GPB heterodimer formation by selective modification of Cysteine 50 of GPB before RPLC. 

*Keywords:* Glycophorins; Membrane proteins

## 1. Introduction

Glycophorin A, (GPA) carries the MN blood group antigens. GPA is the major glycoprotein of the human erythrocyte membrane and accounts for 80% of PAS positive material in SDS–PAGE of an erythrocyte membrane proteins extract (reviews in [1,2]). It was the first membrane protein to be fully sequenced [3]. The 3D structure of its helical transmembrane domain was established by NMR [4]. Glycophorin A is known to dimerize either as homodimer A2 or as heterodimer AB with Glycophorin B (GPB) a protein which is highly homologous to GPA. Numerous studies were devoted to the GPA propensity to homodimerize and it was conclusively demonstrated that dimerisation depends on the conservation of a defined sequence motif at the dimerisation interface [4–8].

Since GPA seems to be one of the best known membrane proteins describing new procedures for its purification does not seem to be an issue. However, as will be shown below, classical purification techniques are tedious, have low yield and do not result in a product of high purity essentially because of the propensity of GPA to dimerize with GPB. Since we are interested in studying interactions of glycophorins reconstituted in liposomes with other membrane proteins of the human erythrocytes, we

*Corresponding author. Tel.: +33-1-4449-3042; fax: +33-1-4306-5019.

*E-mail address:* obertran@infobiogen.fr (O. Bertrand).

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searched for alternative purification procedures ending with a product of the required purity. Reversed-phase liquid chromatography was found to be efficient for this purpose, but setting adequate conditions for separation of this membrane protein was not straightforward as detailed in this report.

## 2. Experimental

### 2.1. Materials

Human blood was obtained from Etablissement de Transfusion Sanguine Sud-Est Francilien (Rungis, France). Chemicals were bought from Sigma (St Louis, MO, USA) Merck (Darmstadt, Germany), Calbiochem (San Diego, CA, USA) or Carlo Erba (Milan, Italy). Empigen BB (*N*-dodecyl-*N*,*N*-dimethylglycine) was acquired as a 30% (w:v) solution in 1.2 *M* NaCl from Albright and Wilson (Warley, West Midlands, UK). Chromatography columns were purchased from Pharmacia (Uppsala, Sweden), Vydac (Hesperia, CA, USA) or from Waters (Milford, MA, USA). Chromatography equipments were from Pharmacia or Gilson (Villiers le Bel, France).

### 2.2. Extraction of crude glycophorins from red cells membranes

Membranes are routinely prepared from red cells by hypotonic lysis and extensive washings performed in a hollow fiber filtration cartridge [9]. When needed (see below) inside out vesicles (IOV) were prepared as follows: membranes were washed by centrifugation in 0.3 m*M* sodium phosphate 0.1 m*M* EDTA buffer pH 8.0 containing 0.2 m*M* phenyl methane sulfonyl fluoride (PMSF), then suspended in the same buffer (one volume of membrane for 10 volumes of buffer) and incubated at 37°C for 30 min. IOV were recovered by centrifugation at 200 000 *g* for 30 min in a Beckman 50Ti rotor.

Crude glycophorins preparative procedure starts with red cell membranes washed in 5 m*M* phosphate buffer pH 8.0: membranes are suspended in an equal volume of the same buffer supplemented with 1.2 *M* NaCl (0.6 *M* NaCl final concentration was found adequate after trials which will be fully described below in Results). To this suspension, 9 volumes of chloroform methanol mixture (2:1 v:v) are added. The suspension is shaken for 30 min at room temperature and then left to stand in the cold room overnight. The aqueous phase is recovered and clarified by centrifugation for 30 min at 40 000 *g* in a JA 20 Beckman rotor. The clarified supernatant is dialyzed against several changes of 5 m*M* ammonium carbonate pH 8.3 and finally lyophilized.

### 2.3. Gel filtration of crude glycophorins

Gel filtration experiments were performed on a Superose 12 PC 3.2-mm internal diameter 30-cm high Pharmacia column operated in a Pharmacia Smart System apparatus. Flow-rate was 40 μl/min and the column was operated at 23°C. Mobile phases contained 10 m*M* phosphate buffer pH 7.4 and 150 m*M* NaCl (PBS) supplemented with either lauryl dimethylaminoxide, (LDAO, 0.1% w:v) or sodium dodecyl sulfate (SDS. 0.1% w:v). Phosphate buffer pH 7.4 (10 m*M*), containing 166 m*M* NaCl and Empigen BB (4% w:v), was also used as mobile phase. The injected sample was 20 μl of a 5 mg/ml crude glycophorin solution in the equilibrating mobile phase.

### 2.4. Carboxymethylation of crude glycophorins and of purified GPA

The optimized preparative protocol is as follows. Lyophilized crude glycophorins are dissolved at 1 mg/ml in 0.1 *M* Tris, 5 *M* Guanidine hydrochloride buffer pH 8.6 and β-mercaptoethanol is added to 20 m*M* final concentration. The solution is incubated under nitrogen for 2 h at 37°C. An adequate volume of a 1 *M* iodoacetic acid solution in 0.1 *M* Tris, 5 *M* Guanidine pH 8.6 is then added to the glycophorin solution to make final iodoacetic acid concentration 25 m*M*. The reaction is allowed to proceed for 2 h at 37°C and 100 μmol of β-mercaptoethanol per milliliter of solution are added. The solution is further incubated overnight at 37°C. It is then injected on the reverse phase chromatography column after filtration through a 0.22-μm pore diameter filter.

Another protocol for carboxymethylation of crude glycophorins has been evaluated: it differed from the former one by the concentration of iodoacetic acid which was 100 m*M* and by the fact that no further

incubation with β-mercaptoethanol after the carboxymethylation reaction was performed.

RPLC-purified GPA was carboxymethylated as follows: either it was incubated using the 0.1 *M* iodoacetic acid carboxymethylation conditions given above or it was incubated for 2 h in 0.1 *M* iodoacetic acid dissolved in 5 *M* Guanidine HCl pH 3.2 [10]. Carboxymethylated GPA solutions were thereafter dialyzed against water prior to be analyzed by SDS–PAGE.

### *2.5. Reversed-phase liquid chromatography of crude glycophorins*

Some scouting experiments to evaluate chromatography conditions were performed using Vydac $C_4$ and $C_{18}$, 0.46-cm I.D., 25-cm long RPLC columns (operated at 1 ml/min). Trials have also been carried out using the Hypersil $C_1$, 0.46-cm id, 25-cm long column marketed by Waters. However, most of the experiments have been realized on Vydac $C_4$ and $C_{18}$, 1-cm I.D., 25-cm long columns operated at a 4-ml/min flow-rate. Different mobile phases compositions have been tried (given in the Results section) but suitable conditions to chromatograph glycophorins are as follows: initial and final mobile phase contain 0.1% trifluoroacetic acid (TFA) in water (A, initial mobile phase) and in 70% acetonitrile water (B, final mobile phase). Usual sample loading corresponds to 12 mg crude or carboxymethylated glycophorins. Conditions for sample preparation have been given above and/or will be indicated in the Results section. After completion of sample loading the column is rinsed with A and then developed with a 60 min linear gradient from 0 to 100% B, followed by isocratic rinse with B. Absorbance of effluent is routinely monitored at 206 and 280 nm.

### *2.6. Multiple angle laser light scattering analysis of GPA oligomerisation status*

The oligomerisation status of purified GPA eluted from the RPLC column was evaluated using a multiple angle laser light scattering detector (Mini-Dawn, Wyatt Technology Corporation, Santa Barbara, CA., USA) and a UV detector (UV 100, Spectra-Physics Analytical Inc., Fremont, CA, USA) connected to a 0.46-cm I.D., 25-cm long Vydac $C_4$ column. The mobile phase system was the same as for the preparative procedure above. The column was equilibrated in mobile phase A and was loaded with RPLC purified GPA dissolved in water. A linear gradient to 100% B in 60 min was used. Absolute molecular mass of eluted glycophorin was computed from detectors output using software associated to the instrument. The value of refractive index increment ($d_n/d_c$) was measured by dissolving lyophilized purified GPA in water or in water acetonitrile mixtures and injecting solutions in a differential refractometer (R 401, Waters Corporation, Milford, MA, USA). Refractometer calibration was checked using aqueous solutions of bovine serum albumin.

### *2.7. SDS–PAGE and Western Blot analysis*

SDS–PAGE was performed in 10% or 12.5% (w/v) acrylamide Tris glycine [11] mini-gels using a Novex apparatus (San Francisco, CA, USA).

Samples from the RPLC fractions were either dried down in a Speed Vac apparatus (Emeryville CA, USA) or neutralized by addition of 1 *M* Tris pH 12 solution (20 μl neutralizing solution for 100 μl fraction aliquot). The composition of all samples was similar to that given by Laemmli [11] but sample buffer concentrate was formulated so that the final SDS concentration was 2% (w:v). All samples were heated at 80°C for 15 min prior to being loaded onto the gels. Silver nitrate gels staining was performed as essentially described in [12]. Western blot analysis was performed using nitrocellulose membranes and the murine monoclonal antibody 3F4 which recognizes both GPA and GPB [13], (a gift from Dr. D. Blanchard ETS, Nantes, France), murine E3 clone purchased from Sigma which recognizes only GPA [14], the anti-Band 3 antibody (clone BIII-136 purchased from Sigma), anti-spectrin, anti-ankyrin rabbit antisera, gifts from Dr. D. Dhermy (Inserm U409 Paris France). An anti-p55 antibody was also used, it is an in house prepared antiserum against a synthetic peptide (corresponding to the Gln28-Met-47sequence). The buffer for incubation of membranes with antibodies was 10 m*M* Tris, pH 7.4 containing 5% skim milk (w:v) and 150 m*M* NaCl. Secondary antibodies were, as needed, anti-mouse

IgG or anti-rabbit IgG peroxydase-tagged antibodies (Biosys, Compiègne, France).

Revelation of Immunoblots was performed with the ECL chemiluminescent system from Amersham (Bucks, UK). They were exposed for a few minutes to X-ray film (Biomax MR, Kodak, Rochester, NY, USA).

## 3. Results and discussion

### *3.1. Preparation of crude glycophorins*

The extraction of erythrocytes membranes through chloroform–methanol is an established method for glycophorin purification since it was described more than 35 years ago [15]. Aqueous phase contains glycophorins in soluble form. Organic phase contains lipids extracted from membrane and at the interface accumulate precipitated denatured proteins. SDS–PAGE of aliquots of aqueous phase obtained after extraction by Chloroform–methanol mixture are shown in Fig. 1.

Sample loaded in lane 1 was an aliquot of the aqueous phase generated by extraction from ghosts suspended in a low ionic strength buffer with no salt added (as originally described in [15]). It shows large amounts of spectrin and band 3 (identities of these bands suspected from their electrophoretic migration was confirmed by western blotting, which showed also the presence of ankyrin and p55).

It is in fact not surprising that glycophorins extracted at low ionic strength may be contaminated by proteins which constitute the red cell cytoskeleton since glycophorin C is known to constitute a major anchorage point of the cytoskeleton to the membrane, GPA is associated to Band 3 which itself is linked to skeleton via its interaction with ankyrin (review in [1,2]). One may think that extraction of glycophorins which are recovered in soluble form in the aqueous phase because of their heavily glycosylated extra cellular domain may drag to the aqueous phase skeletal proteins as well as membrane proteins attached to them and protect them from denaturation with organic solvent.

Contamination of glycophorins by spectrin and other elements from the membrane skeleton might be reduced to rather low amounts if starting material for extraction is not red cell membranes but inside out

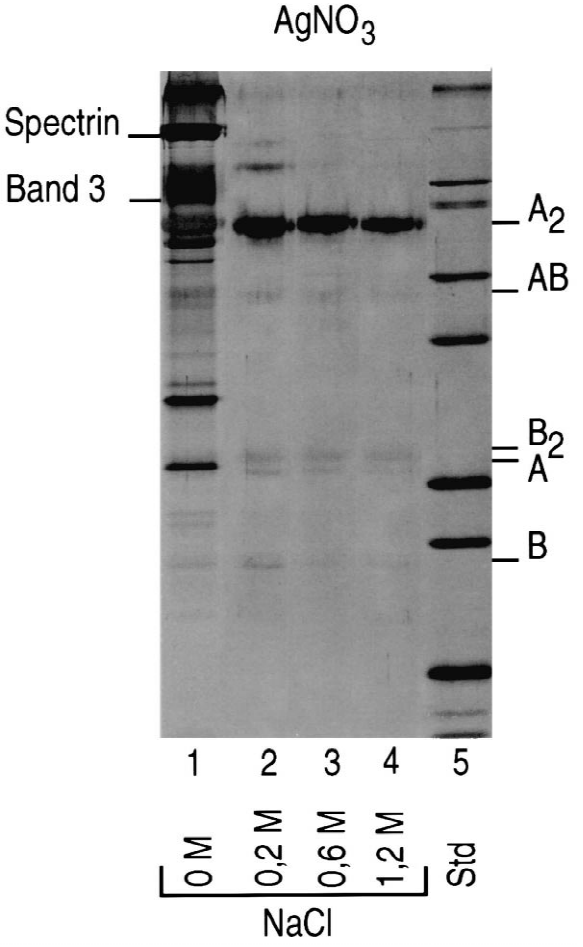


Fig. 1. Analysis by SDS–PAGE of extraction conditions of crude glycophorins: 12.5% acrylamide gel was stained with silver nitrate. Lanes 1–4 were loaded with aliquots of aqueous phases obtained by extracting red cell membranes suspended in 5 m*M* phosphate pH 8.0 buffer containing NaCl at the concentrations indicated on bottom of the gel. Lane marked Std was loaded with a molecular mass standard mixture ($M_r$ of main bands are from top to bottom: 116 000; 97 400; 66 300; 55 400; 36 500; 31 000). Positions of spectrin, band 3, GPA homodimers (A2), GPB homodimers (B2), GPA–GPB heterodimers (AB), GPA monomer (A) and GPB (B) are indicated.

vesicles (IOV) produced by low ionic strength extraction of spectrin (not shown). Such a method was also used by others, e.g. in [16]. However, the preparation of IOV is rather tedious and needs high speed centrifugation (see Methods).

The addition of salt to the buffer used to suspend red cells membranes before organic solvent extrac-

tion was explored as an easy alternative procedure to get minimal contamination by other proteins linked to the skeleton. Indeed, lanes 2–4 of Fig. 1 demonstrate that a glycophorins preparation of increased purity is extracted in presence of salt. A molarity of 0.6 *M* was chosen for preparative purposes.

### *3.2. Size exclusion chromatography of crude glycophorins*

A chromatogram obtained by injecting crude glycophorins in a high-performance Superose 12 column is shown in Fig. 2. The peaks are identified by roman numerals. The insert shows SDS–PAGE analysis of the six fractions collected as indicated at the bottom of the chromatogram. GPA was present in the fractions 2–6 as bands corresponding to A monomer, A2 dimer and AB heterodimer (obvious in fractions 4 and 5). GPB was present in fractions 4–7 as bands corresponding to AB heterodimer (fractions 4 and 5) B dimer and B monomer (fractions 4–6). Glycophorins C and D were in fractions 5–7. To summarize, while peak II of the chromatogram (fractions 2–5) contained a large amount of GPA, it

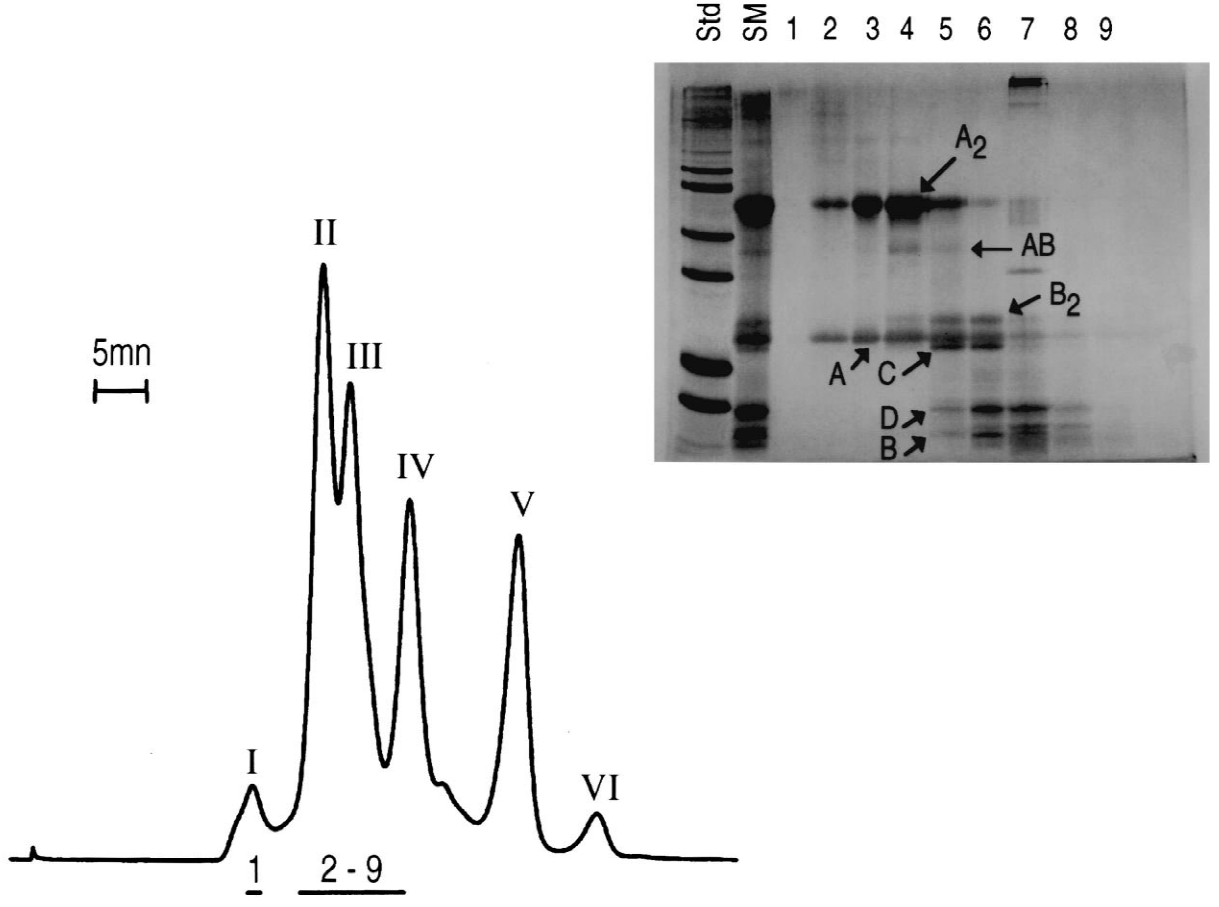


Fig. 2. Gel filtration on a Superose 12 column of the crude glycophorin preparation: The column was equilibrated in 10 m*M* Phosphate buffer containing 166 m*M* NaCl and 4% (w:v) Empigen BB. Flow-rate was 40 μl/min. Sample was 20 μl crude glycophorins solution in the mobile phase. Collected fractions are indicated at the bottom of the chromatogram and SDS–10% acrylamide gel is shown in the insert. Lanes marked SM and Std were respectively loaded with crude glycophorins and with molecular mass standards ($M_r$ of main bands are from top to bottom: 116 000; 97 400; 66 300; 55 400; 36 500; 31 000). Position of A2 and AB dimers are indicated as well as locations of monomeric GPA (A), GPB (B), GPC (C) and GPD (D).

is not totally free of GPB. Peak III contained mostly small molecular mass bands, some of them unidentified. Peaks IV–VI did not contain proteic material. The results of Fig. 2 have been obtained using Empigen BB. Other detergents listed in Methods gave identical results with regard to peak positions and distribution of protein species in peaks. Of particular interest is the result obtained with SDS which even though it is considered as able to induce dissociation of many protein complexes does not preclude dimerisation of glycophorins (notice that dimer also survives to SDS–PAGE).

These results, which have been obtained with a small particle size, high-performance support, are not supposed to be better on more usual gel filtration media. Gel filtration was formerly claimed to adequately purify glycophorin A but fractions purity was checked by PAS staining of SDS gel, a technique which was not sensitive enough to show GPB contamination of GPA [15,17].

### *3.3. Reversed-phase chromatography of crude glycophorins*

Fig. 3 shows a chromatogram recorded after injection of crude glycophorins on the $C_4$ Vydac column operated with a 0.1% TFA containing mobile phase. A large peak (corresponding to fractions 1–6 indicated at the bottom of the chromatogram) was eluted at ca. 80% of the gradient. It contained both GPA and GPB as demonstrated by the SDS–PAGE of collected fractions (insert). Gel shows A2 homodimer, and some more highly polymerized material, some A monomer and AB heterodimer. Identities of these bands were confirmed by western blotting. Late

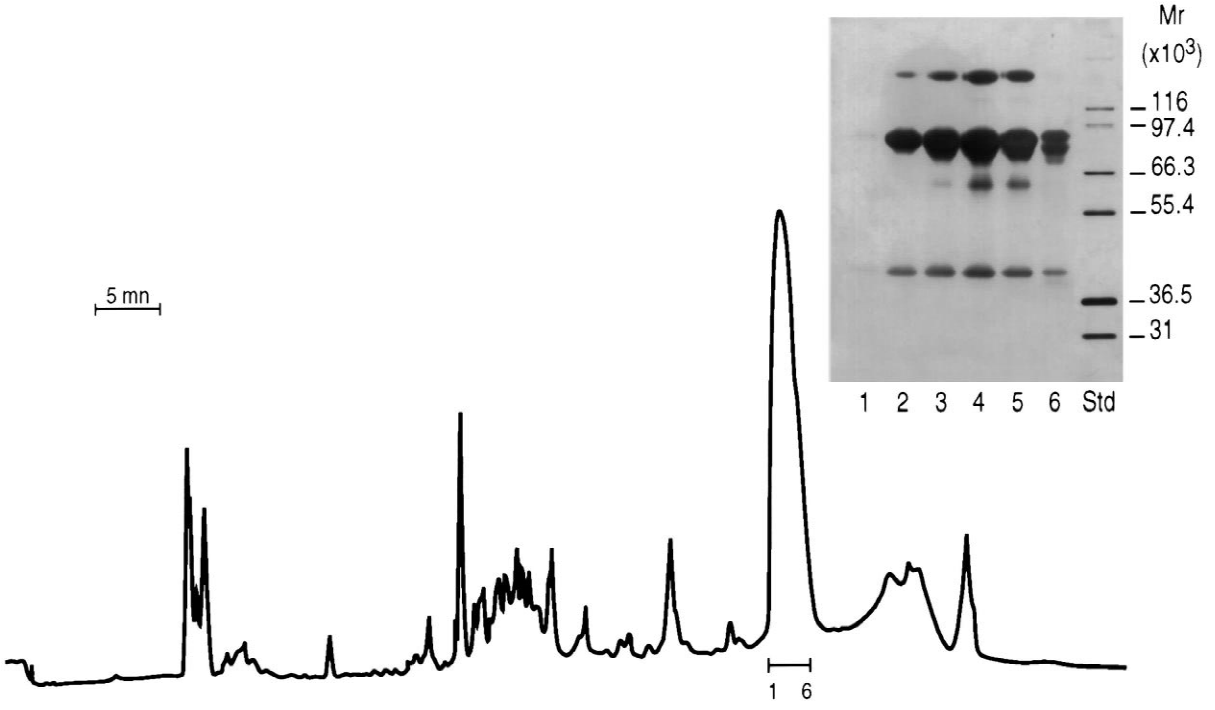


Fig. 3. Reversed-phase liquid chromatography of crude glycophorins on a $C_4$ column: 12 mg crude glycophorins were dissolved in 12 ml distilled water filtered through a 0.22 μm filter and injected onto the 1-cm I.D., 25-cm long $C_4$ Vydac column equilibrated in 0.1% trifluoroacetic acid in water. Recorder tracing obtained after sample loading and column rinsing is shown (see text for details on the acetonitrile gradient). Selected fractions (0.5 min) collected during the run were analyzed on a 10% acrylamide SDS–PAGE (insert, fraction numbers are indicated at the bottoms of the gel and of the recorder tracing). Lane marked Std was loaded with a standard molecular masses mixture ($M_r$ values are indicated). Gel was silver stained.

eluting fractions (on lanes 5 and 6) distinctly show doublets in place of the single banded usual A2 and A bands. Western blot demonstrated that both bands of these doublets contained GPA. When fractions containing either only the usual single-band A2 dimer or dimers with doublet bands were submitted to neuraminidase digestion, migrations of end products were undistinguishable (not shown). This suggests strongly that the doublets in late fraction of the GPA peak are due to incomplete sialylation of GPA. Worth of notice doublet bands are observed with some batches of crude glycophorins and not with others. The presence of doublets was not related to sample treatment before electrophoresis (drying in a Speed Vac apparatus or neutralisation) and was also observed when mobile phase did not contain TFA but 50 m*M* ammonium acetate pH 6.0. Hence, the doublet cannot be attributed to some hydrolytic effect of mobile phase on the oligosaccharidic side chains. It is assumed that presence of the doublet reflects some heterogenity in the glycophorin starting material. Progressive desialylation of glycophorins may occur with aging of red cells [18].

RPLC fell short to comply with our primary goal of obtaining high purity GPA with reasonably high yield. Again, purity might be obtained only with major sacrifice in yield by discarding GPB containing fractions. Several alterations of the mobile phase conditions have been tried in order to get a better separation of GPA and GPB. No adequate separation between A2 and AB was obtained using a more shallow gradient: some peak broadening was observed and indeed GPB was more enriched in late eluting fractions taken across the peak (not shown), but the result still fell far from satisfactory. Increasing TFA concentration to 1% did result in delayed elution of the large glycophorin peak (which was centered at about 95% of the gradient) but again, fractions showed a similar pattern of A2 and AB dimers. When instead of TFA, the mobile phase contained 50 m*M* ammonium acetate pH 6, separation was not better either. Use of $C_{18}$ columns in place of $C_4$ did not afford better separation: large GPA/GPB peak was eluted later only. Trials made with the least hydrophobic column Hypersil $C_1$ using either TFA–acetonitrile or TFA–*n*-propanol/*n*-butanol mobile phases [20] were also unsuccessful: elution of GPA from this column was obtained early in the gradient but again it was contaminated with GPB.

Looking at the SDS–PAGE results in Fig. 3 one might wonder if the presence of A2 and AB dimers on SDS–PAGE meant that GPA and GPB were eluted as dimers from the column or if dimers were formed during sample preparation for electrophoresis. It is well established that the amount of dimer on gel is governed by glycophorin concentration in the sample for electrophoresis; SDS concentration in sample buffer and the temperature of incubation of samples prior to electrophoresis are the other factors which may influence dimer amount [19,21]. The question was not only of formal interest since would glycophorins be eluted as dimers, one might imagine to preclude GPB contamination of GPA by making selectively impossible AB heterodimer formation without impairing A2 formation. GPA dimerisation has been studied in great details using molecular biology techniques, it has been demonstrated that introduction of mutation in residues $L_{75}I_{76}$, $G_{79}V_{80}$, $G_{83}V_{84}$, $T_{87}$ of GPA might impair dimerisation e.g. even such a seemingly benign mutation of $G_{83}$ to alanine makes dimerisation impossible because it interferes with close packing of the two helices of the A2 dimer [4,5,8]. It is most likely that AB heterodimer formation is due to the same close packing necessary for A2 formation. When the sequences of the homologous GPA and GPB transmembrane domains are aligned the position of cysteine 50 of GPB corresponds to glycine 79 in the transmembrane domain of GPA (Fig. 4). We reasoned that deliberate modification of GPB cysteine 50 might selectively impair formation of AB heterodimers (GPA does not contain cysteines). One might also suppose that deliberate introduction of a polar modification like that one brought by iodoacetic acid modification of cysteine would in any case modify the polarity of GPB trans-membrane domain and hence would selectively move elution position of GPB in $C_4$ column effluent.

### *3.4. Malls detection shows that purified Glycophorin A is eluted from RPLC column as a dimer*

The refractive index increment was measured for glycophorin dissolved in water or in 80% acetonitrile

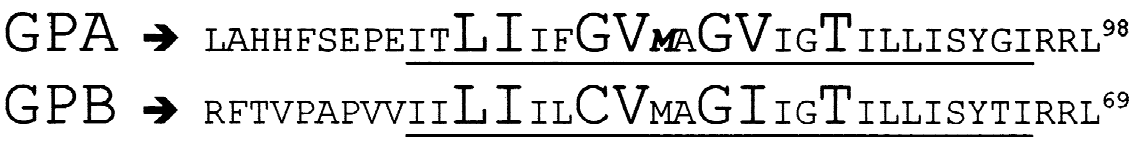


Fig. 4. Alignment of transmembrane domains of GPA and GPB: Transmembrane helical domain of GPA as described in [7] is underlined. Homologous region of GPB is aligned. Important residues which constitute dimer interface [8] are marked with bigger and bold types. Methionine 81 of GPA is shown in bold italics.

containing mobile phase B. The same value of 0.175 ml/g was determined in every case. So, the small variation of mobile phase composition during peak elution was not likely to influence computed molecular mass. As expected, purified GPA was eluted as a single peak from the analytical column connected to the Malls apparatus. Data were collected at 1-s intervals corresponding to slice volumes of 16.7 μl. The average molecular weight calculated in the slices corresponding to 90% of the optical density peak was 83 000±8 000 g/mol. The small standard deviation allows us to conclude that the peak corresponds to the elution of a monodisperse molecular species. Moreover, it can be confidently assumed that GPA is eluted as a dimer from the 0.46-cm I.D. RPLC column. Hence, one can safely infer that the same situation prevails with the preparative 10-mm I.D. preparative column which is packed with the same support.

### *3.5. Chemical modification of cysteine 50 of GPB in crude glycophorins permits easy GPA purification through RPLC*

The crude glycophorins were carboxymethylated as described in Methods. The chromatogram obtained by direct loading of the carboxymethylation mixture is shown on Fig. 5. The same crude glycophorins sample which was used (without modification) for the chromatogram of Fig. 3 was used also after carboxymethylation for the chromatogram of Fig. 5. The settings for the UV detector were identical for Fig. 3 and Fig. 5. One may notice that the large peak corresponding to the glycophorin dimer is similar in size and position in both figures. SDS–PAGE analysis of the collected fractions demonstrates that adequate purity of GPA was obtained after carboxymethylation: fractions 3–5 (Fig. 5) contain pure GPA, as no contamination through GPB was observed in these fractions even by western blotting, (Fig. 6, panel A, lane 4). Hence the selective modification of GPB stands as an efficient procedure to obtain pure GPA.

Fraction 2 (Fig. 5) corresponds to a peak which is absent from non-treated material (compare Figs. 3 and 5). While fraction 1 contains non-identified low molecular mass proteins, fraction 2 contains a protein band with an apparent molecular mass slightly lower that GPA. Data in Fig. 6 support strongly that this band is indeed carboxymethylated GPA: when purified GPA was carboxymethylated either at acidic or alkaline pH it generated a band with identical migration to the band in fraction 2 (compare lanes 1, 2 and 3 in Fig. 6, panel B), which clearly cannot be ascribed to A monomer due to the usual dimer monomer equilibrium (compare lanes 2 and 3, panel B with lane 4, panel B which contains unmodified purified GPA). The fraction 2 band is recognized by the E3 monoclonal, which is GPA-specific (lane 1 in Fig. 6, panel C). Moreover when carboxymethylation of crude glycophorins was performed with higher iodoacetic concentration (0.1 *M*) and when no incubation with β-mercaptoethanol to destroy excess iodoacetic acid was used (see Methods), we observed a decreased height of the glycophorin dimers peak associated to a large peak with the same elution position as fraction 2 in Fig. 5 (not shown).

GPA does not contain cysteine but methionines might be modified by carboxymethylation [19]. Clearly, the GPA in fraction 2 (Fig. 5) may correspond to GPA carboxymethylated on Met 81. Carboxymethylation of methionine 81 was proven to interfere with dimerisation of GPA [10] and led once to the erroneous conclusion that methionine side chain was present at the interface of the dimer [10]. It was later established that this was not the case [4]. SDS is known to stabilize helix formation [22]. Met 79 carboxymethylation (as polar mutations in the

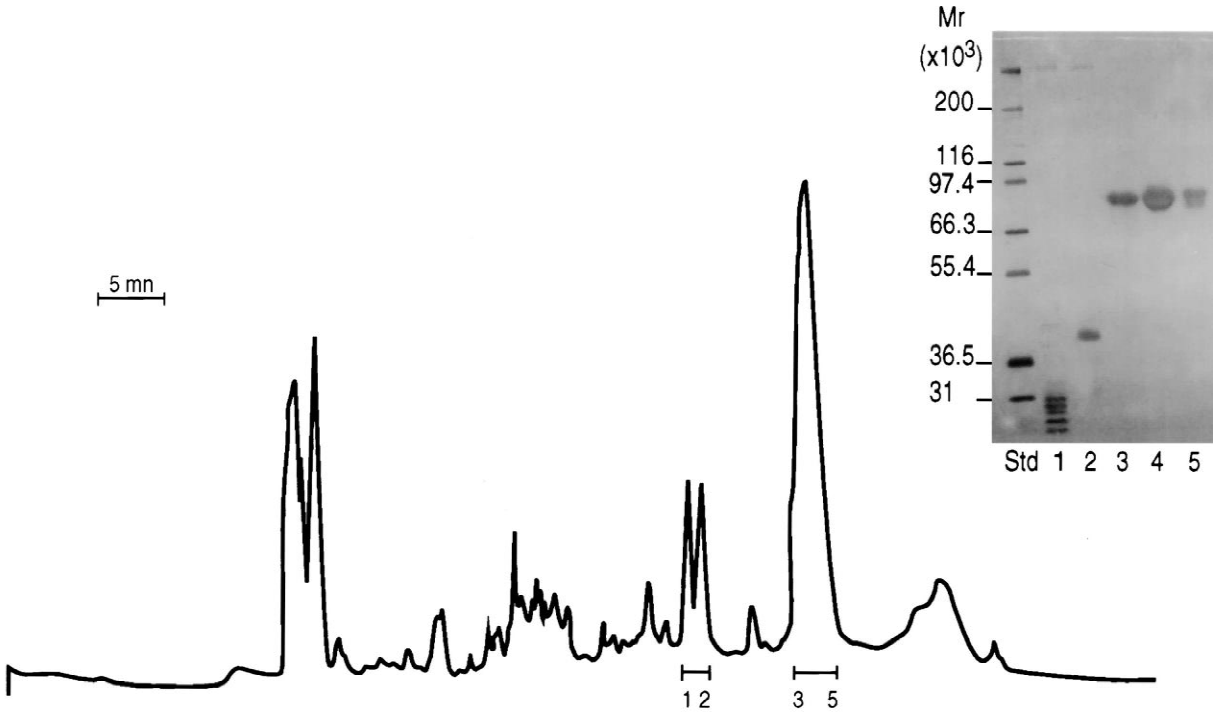


Fig. 5. Reversed-phase chromatography of carboxymethylated crude glycophorins on a $C_4$ preparative column: Carboxymethylation mixture containing 12 mg glycophorins was filtered through a 0.22-μm filter and injected onto the 1-cm I.D., 25-cm long $C_4$ Vydac column equilibrated in 0.1% trifluoroacetic acid in water. Recorder tracing obtained after sample loading and column rinsing is shown (see text for details on the acetonitrile gradient). Selected fractions (1 min) collected during the run were analyzed on a 10% acrylamide SDS–PAGE (insert, fraction numbers are indicated at the bottoms of the gel and of the recorder tracing). Lane marked Std was loaded with a standard molecular masses mixture ($M_r$ values are indicated). Gel was silver stained.

GPA helix) are likely to impair somewhat SDS binding, resulting in a decrease in dimerisation, which depends on adequate secondary structure in each monomer [4].

As emphasized elsewhere [23], one should not neglect the possibility of unwanted modifications of methionines during carboxymethylation of cysteine residues. The main factor which may favour unwanted carboxymethylation of methionines is a great excess of iodoacetic acid. Hence, we deliberately used a reasonable iodoacetic acid concentration (final concentration of iodoacetic acid added to a solution containing already 20 m*M* β-mercaptoethanol was 25 m*M*). Moreover, after incubation with iodoacetic acid for cysteine modification, we added β-mercaptoethanol to the reaction mixture to destroy excess iodoacetic acid and also to reverse carboxymethylation of methionine residues if any were present [24]. In doing so, the amount of unwanted modified GPA was maintained at a fairly low level (notice that fraction 2 of Fig. 5 corresponds to a tiny peak and that the area of the dimers peak, i.e. fractions 3–5, which is contributed mostly by GPA dimer, is not appreciably reduced — compare with Fig. 3). Another strategy might have been to use a more selective modification reagent for cysteine residues.

## 4. General comments and conclusions

This report describes an easy, straightforward procedure to purify significant amounts of GPA by reversed-phase chromatography.

Simple but effective modifications of organic solvent extraction conditions have been described for crude glycophorins preparation. More importantly,

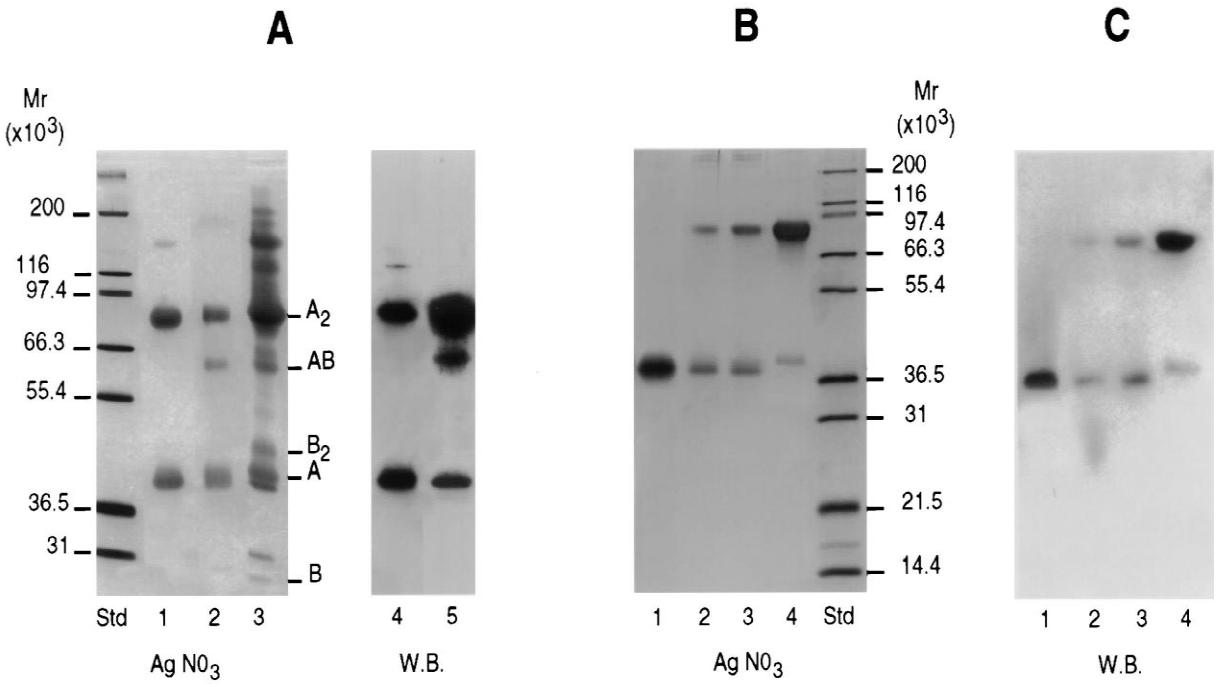


Fig. 6. Analysis by SDS–PAGE and western blotting of glycophorins samples: SDS gels were run in 10% (panel A) and 12.5% acrylamide gels (panels B and C). Panel A: analysis of aliquots of GPA dimer peak obtained after RPLC performed in conditions of Fig. 5 (lanes 1 and 4) and of GPA dimer peak contaminated with GPA–GPB heterodimer obtained after RPLC performed in conditions of Fig. 3 (lanes 2 and 5). Lane 3 was loaded with an aliquot of crude glycophorins and lane marked Std with a standard molecular weight mixture. Revelation used silver nitrate or western blot with E3 antibody which recognizes GPA. Positions of GPA dimer (A2), GPA–GPB heterodimer (AB) of B dimer (B2) and of A and B monomers are indicated. Panel B was stained with silver nitrate. Lane marked Std was loaded with a standard molecular weight mixture. Lane 1 was loaded with an aliquot of fraction 2 of Fig. 5 while lanes 2 and 3 were loaded with respectively acid and alkaline pH carboxymethylated purified GPA. Lane 4 was loaded with purified GPA obtained by chromatography in conditions used for Fig. 5. Panel C was revealed by western blotting performed with the E3 anti-GPA antibody. Lanes 1–4 were loaded with same samples as in lanes 1–4 of panel B.

RPLC, which is established as a method of choice for separation of soluble proteins and peptides, was shown to be an efficient technique for separation of GPA, a membrane protein. Problems frequently associated with RPLC of membrane proteins or membrane proteins fragments are poor solubility, poor recovery, carry over with elution of some material in blank runs performed without sample injection [19,25] but none of them was observed with GPA. This is clearly attributable to the large glycosylated extracellular domain of the protein. RPLC of the isolated GPA transmembrane domain obtained through proteolytic digestion [8] or by synthesis [19] is clearly more difficult.

Interestingly, glycophorin A was eluted as a dimer from the RPLC column. Several factors might contribute to the formation of glycophorin dimers during RPLC experiments: (i) acetonitrile is known to be a strong helix inducer [26] and dimer formation requires that transmembrane domains of both monomers are helical [4,5,7,8], (ii) contact with the stationary phase, which might be seen as embedding into an organic layer, might possibly also favour helix formation [26]; in this regard it has been shown using NMR after deuterium exchange that a helix might be a conserved structural motif in one protein bound to an RPLC support [27] and (iii) an increase in protein concentration due to retention on reversed-phase support should probably drive the dimerisation equilibrium towards dimer formation, as it occurs in solution [20,21]. Moreover, clearly, when hydrophobic interactions stabilize tertiary and quaternary

structure (e.g. helix dimerisation due to Leucine zippers motives [28]), contact with the RPLC support may disrupt tertiary and quaternary structures [26]. However, dimerisation of glycophorins is not driven by hydrophobic contacts but by the exquisite spatial complementarity of interfaces allowing van der Waals contacts [4]. In any case, it has been shown in this report that conservation of the glycophorin dimers' quaternary structure during RPLC offered an easy way to prepare pure GPA homodimer: selective chemical modification of GPB was used to impede GPA–GPB heterodimer formation and hence, GPA homodimer was recovered free of GPA–GPB heterodimer.

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