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Quantitative adsorption of platelet glycoprotein G (thrombin-sensitive protein, thrombospondin) to barium citrate

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Glycoprotein G (thrombin-sensitive protein, thrombospondin) is a high-M_r calcium-sensitive protein secreted by activated platelets. We observed that this protein was precipitated with barium citrate, and this property was used to purify glycoprotein G. The simple and rapid purification procedure consisted of barium citrate adsorption followed by heparin–agarose affinity chromatography. Unlike other calcium-sensitive proteins that are precipitated by barium citrate, glycoprotein G does not contain γ-carboxyglutamic acid. The ability of glycoprotein G to bind to both heparin and barium citrate is consistent with this protein possessing clusters of positive and negative charges.

Activated platelets (Baenziger *et al.*, 1972; Hagen, 1975; Hagen *et al.*, 1976), cultured endothelial cells (McPherson *et al.*, 1981; Mosher *et al.*, 1982) and cultured fibroblasts (Raugi *et al.*, 1982; Jaffe *et al.*, 1983) secrete a high-M_r glycoprotein, referred to as 'thrombin-sensitive protein' (Baenziger *et al.*, 1971, 1972), 'glycoprotein G*' (GPG) (George, 1978), or 'thrombospondin' (Lawler *et al.*, 1978; Margossian *et al.*, 1981). This protein has an approximate M_r of 450000 (Lawler *et al.*, 1978). It consists of three apparently identical disulphide-linked subunits of M_r 150000–185000 (Lawler *et al.*, 1978; Margossian *et al.*, 1981). GPG exhibits calcium-induced changes in conformation (Lawler *et al.*, 1982), and it binds to platelets in a calcium-dependent manner (Phillips *et al.*, 1980). We report here that GPG, like certain calcium-binding coagulation proteins from plasma [for a review, see Magnusson (1971)], adsorbs to precipitates of barium citrate, and we describe herein a simple and rapid purification of GPG based on this property.

The ability of certain coagulation proteins to adsorb to barium citrate precipitates, as well as their ability to bond to platelets, has been attributed to clusters of γ-carboxyglutamate residues in these proteins [for a review, see Stenflo (1978)]. In contrast, we have detected no γ-carboxyglutamic acid in hydrolysates of GPG.

Abbreviations used: GPG, glycoprotein G; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate.

* None of these names is definitive and consistent with current knowledge about the function or properties of this protein. In the present paper we use the name 'glycoprotein G' (George, 1978) because it implies the least incorrect information and because we do not wish to add greater confusion by introducing what would seem to us a more appropriate name.

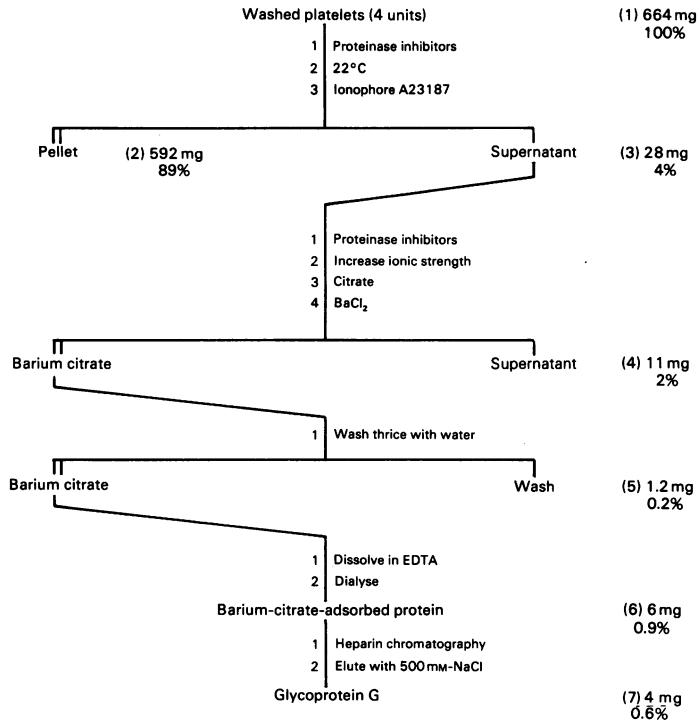
Materials and methods

Platelets

Human platelets were collected by centrifugation of 2 to 4 units of fresh platelet concentrates obtained from the New York Blood Center, for 20 min at 2000 ***g***. The platelets were washed three times with 136 mM-NaCl/25 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/0.8 mM-sodium citrate by suspension in 50 ml per unit of platelets and centrifugation at 2000 ***g*** for 20 min. They were finally suspended in 6 ml (per unit of platelets) of this solution, also containing 5 mM-glucose.

Secreted proteins

The platelet suspension was made 50 μM with respect to leupeptin (obtained from Dr. A. Stracher of this Department) and 1 mM with respect to PMSF (Sigma). After warming to room temperature, the platelets were activated by addition of the ionophore A23187 (Calbiochem) to a final concentration of 3 μM (0.6 μl of 5 mM-A23187 in dimethyl sulphoxide/ml of platelet suspension). After 2.5 min, the platelet suspensions were cooled in an ice/water



Scheme 1. *Purification of GPG*

The numbers in parentheses indicate the electrophoretic lanes in Fig. 2, where each sample was analysed. Details of the procedure are described in the text. For each of the numbered samples, the total protein and yield are indicated.

bath for 5 min before centrifugation at 4°C for 20 min at 2000 *g*. The supernatant solution was then centrifuged at 12000 *g* for 45 min to remove any residual particulate material.

Purification of GPG

The purification is outlined in Scheme 1. This purification began with 4 units of washed platelets that were activated with 3 μM-A23187, followed by preparation of the supernatant solution as described above. All subsequent procedures were at 4°C. To the 25 ml of supernatant solution containing secreted platelet proteins, the following additions were made: 12 μl of 100 mM-leupeptin and 100 μl of 250 mM-PMSF (to ensure inhibition of proteinases); 18.3 ml of 1 M-NaCl (to increase ionic strength, thereby minimizing precipitation of myosin and other cytoskeletal proteins); and 17.3 ml of 120 mM-trisodium citrate. This was followed by 12.1 ml of 1 M-$BaCl_2$ added dropwise over a period of 30 min with vigorous stirring (to form the barium citrate precipitate). Stirring was continued for an additional 30 min before centrifugation of the suspension at 2000 *g* for 20 min. The precipitate was washed three times with 15 ml of water, and it was dissolved in the minimum volume (about 15 ml) of 500 mM-EDTA, pH 7.6. After addition of 10 μl of 100 mM-leupeptin and 50 μl of 250 mM-PMSF, the solution was dialysed overnight against two changes of 2 litres of a solution containing 200 mM-NaCl, 55 mM-sodium acetate, pH 6.0, 5 mM-EDTA and 100 μM-PMSF. The retained material was finally chromatographed on a heparin–agarose affinity column essentially as described by Lawler *et al.* (1978), a commercial preparation of heparin–agarose (Affi-gel Heparin; Bio-Rad, Rockville Centre, NY, U.S.A.), containing 1 ($\pm$0.2) mg of heparin/ml of gel, being used. The dialysed material (15–20 ml) was applied to a column (0.9 cm $\times$ 15 cm) of heparin–agarose. The column was washed with the sample buffer solution until the A_{278} of the effluent returned to zero. GPG was then eluted with a linear gradient of NaCl or with a stepwise increase to 500 mM-NaCl (the concentration of the other components of the solution being kept constant). The gradient elution has shown no apparent improvement over the step elution, which we now use routinely, obtaining over 80% of the GPG in 2.5 ml.

Purified GPG (8 mg) was precipitated with 10% (w/v) trichloroacetic acid, resuspended in 2 M-KOH (Hauschka, 1977) and sealed in thin-walled Pyrex tubes by the method of Hugli & Moore (1972).

Hydrolysis and neutralization of the sample were performed as described by Hauschka (1977). A portion of the hydrolysate equivalent to 2mg of GPG was loaded on to a 0.9cm x 69cm Beckman AA-15 column and developed on a Beckman 120 C analyser by using the buffers described in the short acidic program of Hauschka (1977). For standards we used γ-carboxyglutamate (Sigma) and a mixture of γ-carboxyglutamate with a standard amino acid mixture (Pierce). As a positive control we analysed the protein adsorbed by barium citrate from a Cohn Fraction II paste kindly supplied by Dr. John W. Fenton, II, Division of Laboratories and Research, New York State Department of Health, Albany, NY, U.S.A. (Fenton *et al.*, 1977). Analysis of this material revealed 4.3 residues of γ-carboxyglutamate per 100 residues of glutamate. γ-Carboxyglutamate was identified by comparison of elution profiles with that of the standard and by loss on hydrolysis in 6M-HCl.

Other analytical procedures

Protein was assayed by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (Sigma) as standard. Polyacrylamide-gel electrophoresis in the presence of SDS was essentially as described by Laemmli & Favre (1973). Samples were heated at 100°C for 2–3min in 2% (w/v) SDS, in either the absence (non-reduced) or presence (reduced) of 35mM-dithiothreitol or 5% (v/v) 2-mercaptoethanol. The gels were electrophoresed overnight at 7mA/gel, and they were stained with Coomassie Brilliant Blue.

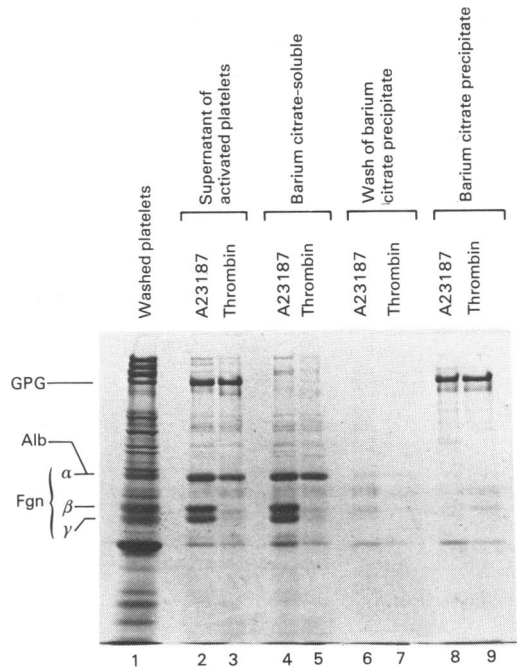


Fig. 1. *Precipitation of GPG by barium citrate*
Washed platelets (lane 1) were activated with 2.5μM-A23187 or 23nM-thrombin. After 10min, the platelets were removed by centrifugation. To 1ml of the supernatant solutions (lanes 2 and 3) at 4°C was added 0.2 ml of 120mM-trisodium citrate, and then 0.12ml of 1M-$BaCl_2$ was added slowly with constant stirring. The precipitate was collected by centrifugation and wshed once with cold water. The precipitate was dissolved in the original volume of 1ml and dialysed overnight against 62.5mM-Tris/HCl/5mM-EDTA, pH6.8. Samples were made 2% with respect to SDS and 5% with respect to 2-mercaptoethanol before electrophoresis through an 8% polyacrylamide gel. Abbreviations used: Alb, albumin; Fgn, fibrinogen.

Results and discussion

Barium citrate precipitation of GPG

Barium citrate precipitated most of the GPG from the supernatant solution of either thrombin- or ionophore-activated platelets (Fig. 1). No other secreted protein was quantitatively precipitated. There was a trace of fibrinogen in the precipitate obtained from ionophore-activated platelets, and there were cytoskeletal proteins (actin and myosin) in each, presumably due to some platelet lysis. Similar results were obtained with precipitation by calcium oxalate.

Purification of GPG

The published method for GPG purification is based on gel-filtration chromatography and heparin–agarose affinity chromatography (Lawler *et al.*, 1978; Lawler & Slayter, 1981). This method is successful when the starting material is the supernatant solution of platelets activated by thrombin, since thrombin also catalyses the polymerization of fibrinogen, the major contaminant in the supernatant solution. Since GPG is a substrate for thrombin (Lawler & Slayter, 1981; Lawler *et al.*, 1982), and since thrombin apparently forms a complex with GPG (K. J. Danishefsky & T. C. Detwiler, unpublished work), it is preferable to begin with the supernatant solution of platelets activated by non-proteolytic agonists, such as the ionophore A23187, otherwise fibrinogen is a very troublesome contaminant. We have found that precipitation with barium citrate is more effective than either of the above chromatographic procedures for removal of fibrinogen. We have been able to purify GPG from A23187-activated platelets by using barium citrate precipitation in combination with either heparin affinity chromatography or gel-filtration chromatography. The more effective and the more rapid

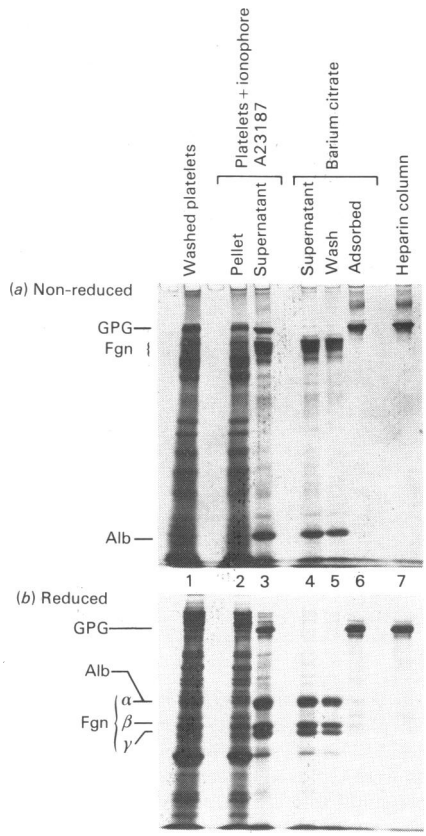


Fig. 2. *Analysis of GPG purification*
GPG was purified as described in the Materials and methods section and in Scheme 1. At each step in the purification a portion of the solution was made 2% with respect to SDS and incubated at 100°C in (*a*) the absence (non-reduced) or (*b*) the presence (reduced) of 35mM-dithiothreitol and electrophoresed on a 6% (*a*) or an 8% (b) polyacrylamide gel. The sample loads (μg of protein) were: lane 1, 133; lane 2, 113; lane 3, 44; lane 4, 18; lane 5, 7; lane 6, 20; and lane 7, 15. Lane numbers correspond to numbered samples in Scheme 1. For abbreviations, see Fig. 1.

method involves barium citrate precipitation followed by heparin affinity chromatography, as described in the Materials and methods section.

Gel electrophoresis of samples from a typical preparation gave the results shown in Fig. 2. The supernatant solution of ionophore-activated platelets (Fig. 2, lane 3) contained the secreted platelet proteins (GPG, fibrinogen, albumin and the smaller proteins that migrated to the dye front on these gels), as well as contaminants apparently due to trace lysis of platelets. The barium citrate precipitate (lane 6) contained essentially all of the GPG, but only small amounts of the other proteins, which were recovered in the barium citrate supernatant solution and the wash of the precipitate (lanes 4 and 5). Chromatography of the barium citrate-adsorbed protein on a heparin–agarose column with elution by 500mM-NaCl led to further purification of GPG. The final product (Fig. 2, lane 7) was not entirely homogeneous. In the non-reduced gel there was material that did not pass through the stacking gel, and in the reduced gel there was material of lower apparent M_r, as seen by others (Lawler *et al.*, 1978). Both of these appear to increase on storage, suggesting that they represent GPG that has aggregated (see in non-reduced gels) and been partially degraded (seen as material with lower M_r in reduced gels). The amount of aggregated protein was not changed by incubation for 30min at 37°C with either 20mM-EDTA or 20mM-$CaCl_2$. To lessen the possibility of aggregates of GPG due to thiol/disulphide exchange, much of the purification was done at pH6, where disulphide exchange should be much less than at neutral pH; this decreases the amount of protein that, on non-reduced gels, does not pass through the stacking gel.

This purification procedure can be completed in 2 days with an overnight dialysis between. It is carried out in the continuous presence of proteinase inhibitors. The yield of GPG is routinely 0.5–1mg of GPG per unit of platelet concentrate, a value consistent with the yields reported by Lawler *et al.* (1978). The product is electrophoretically similar to GPG in whole platelets or in the supernatant solution of activated platelets (Figs. 1 and 2).

Measurement of γ-carboxyglutamate in GPG

Three properties of GPG are similar to those of γ-carboxyglutamate-containing coagulation proteins: (1) it is precipitated by barium salts, (2) it undergoes calcium-dependent conformational changes, and (3) it binds to platelets by a calcium-dependent mechanism. We therefore analysed for γ-carboxyglutamate in an alkaline hydrolysate of GPG. None was detected, and, on the basis of the sensitivity of our amino acid analyser, we conclude that there was less than 1 γ-carboxyglutamate residue per 200 glutamate residues, which corresponds to less than one residue per subunit.

Conclusions

Although the function of GPG is not known, physical and chemical properties of this protein have been described. GPG is secreted by activated platelets (Baenziger *et al.*, 1972; Hagen, 1975; Hagen *et al.*, 1976), and it has been reported to bind to platelets in the presence of calcium, but not in its absence (Phillips *et al.*, 1980) and to associate with fibrinogen (Leung & Nachman, 1982). An apparently identical protein is synthesized and secreted by

cultured endothelial cells (McPherson *et al.*, 1981; Mosher *et al.*, 1982) and cultured fibroblasts (Raugi *et al.*, 1982; Jaffe *et al.*, 1983), and it has been reported to be incorporated into the extracellular matrix of these cells (Raugi *et al.*, 1982; Jaffe *et al.*, 1983). It has been shown that calcium ions have a marked effect on the conformation and physical properties of GPG (Lawler *et al.*, 1982). The calcium-sensitive structure may also be responsible for adsorption of this protein to barium citrate. The binding of several coagulation proteins (such as prothrombin, protein C, and Factors VII, IX, and X) to barium citrate is apparently mediated through clusters of γ-carboxyglutamic acid residues (Stenflo & Ganrot, 1972; Nelsestuen & Suttie, 1972), which also make up the sites for calcium binding. Since GPG contains no γ-carboxyglutamic acid, its ability to adsorb to barium citrate must be due to some other clusters of negative charge. Domains of negative charge are consistent with the fact that GPG is a very acidic protein, with a pI of 4.7 (Lawler *et al.*, 1978), but GPG also contains domains of positive change, as demonstrated by its binding to the polyanion heparin (Lawler *et al.*, 1978; Lawler & Slayter, 1981).

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