Identification and characterization of glycoproteins after extraction of bovine chromaffin-granule membranes with lithium di-iodosalicylate

Purification of glycoprotein II from the soluble fraction

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Chromaffin-granule membranes were separated into insoluble and soluble fractions after extraction with lithium di-iodosalicylate (LDIS). These fractions were characterized by one- and two-dimensional gel electrophoresis, and glycoproteins were detected after electroblotting with peroxidase-labelled concanavalin A and wheat-germ agglutinin (WGA). The LDIS-insoluble fraction contained components identified as glycoproteins III, H, J and K (carboxypeptidase H). Microsequence analysis indicated that component J is an *N*-terminally extended form of glycoprotein K. A major glycoprotein, GpII (*M*_r 80000–100000), present in the LDIS-soluble fraction was purified by affinity chromatography on WGA–Sephrose. This was characterized by one- and two-dimensional gel electrophoresis with Coomassie Blue staining, by amino acid analysis and automated *N*-terminal sequence analysis. Extraction of chromaffin-granule membranes with LDIS is a simple and rapid procedure that facilitates studies concerned with the structure and function of membrane glycoproteins from these and other secretory granules.

INTRODUCTION

Chromaffin granules are subcellular organelles of the adrenal medulla with specialized functions for the biosynthesis, storage and release of catecholamines. The adrenal medulla also secretes peptides and proteins (e.g. enkephalins and chromogranins), so chromaffin granules must share some functions in common with both endocrine and exocrine secretory granules as well as neurotransmitter-storing vesicles [for a recent review see Winkler *et al.* (1986)].

A variety of one- and two-dimensional gel-electrophoresis techniques have been used to analyse chromaffin-granule membrane components. In some cases, proteins of known function, such as dopamine (3,4-dihydroxyphenethylamine) β -hydroxylase, cytochrome *b*₅₅₉ and subunits of the H⁺-translocating ATPase, have been identified (Hörtnagl *et al.*, 1972; Apps *et al.*, 1980; Percy & Apps, 1986). Five glycoproteins were identified by Huber *et al.* (1979) and, more recently, 20 different glycosylated components were identified by a combination of two-dimensional gel electrophoresis and lectin binding (Gavine *et al.*, 1984). Analysis of chromaffin-granule membrane proteins is simplified by phase separation in Triton X-114, and distinct differences were found in glycoproteins fractionating in the P1, P2 and S2 fractions (Pryde & Phillips, 1986).

Dopamine β -hydroxylase (D β H, GpI) and glycoprotein components II to V and H, J, and K are the main glycoproteins identified by two-dimensional gel electrophoresis with lectin staining (Pryde & Phillips, 1986; Winkler *et al.*, 1986). Sequential lectin affinity chromatography has been used to purify D β H, GpII and GpIII (Fischer-Colbrie *et al.*, 1982). However, this method has the disadvantage that lectins, particularly concanavalin A (Con A), have limited stability in SDS. We have developed a simple procedure based on the extraction of chromaffin-granule membranes with lithium di-iodosalicylate (LDIS), a reagent used to extract glycoporphin A from

erythrocytes (Marchesi & Andrews, 1971). GpII, which has recently been shown to be present in both endocrine and exocrine tissues (Obendorf *et al.*, 1988), was isolated by this procedure in a form suitable for structural analysis.

MATERIALS AND METHODS

Preparation of chromaffin granules and extraction of chromaffin-granule membranes

Chromaffin granules were prepared from bovine adrenal medulla as described by Bartlett & Smith (1974), except that all sucrose solutions were buffered with 10 mM-Hepes/NaOH, pH 7.0 (Phillips, 1974) and that the final purification of chromaffin granules involved sedimentation through a 1.7 M-sucrose cushion (Beckman 70Ti, 50000 rev./min, 60 min, 170000 *g*_{av.}) at 4 °C. Chromaffin granules were lysed by suspension in 5 mM-Tris/succinate, pH 5.9, followed by freezing and thawing and the membranes recovered by centrifugation (Beckman 70Ti, 37000 rev./min, 30 min, 100000 *g*_{av.}) at 4 °C. Chromaffin-granule membranes were washed three times by centrifugation and finally resuspended (3–4 mg of protein/ml) in 5 mM-Tris/succinate, pH 5.9, and stored at –70 °C. Protein concentration was determined as described by Markwell *et al.* (1978).

The procedure for extraction of chromaffin-granule membranes with LDIS was based on that used by Marchesi & Andrews (1971). Chromaffin-granule membranes were pelleted and resuspended (4 mg of protein/ml) in ice-cold 0.25 M-LDIS in 50 mM-Tris/HCl, pH 7.6, and stirred on ice for 30 min. The suspension was diluted with 2 vol. of ice-cold water, stirred on ice for a further 10 min and centrifuged (Beckman 70Ti, 37000 rev./min, 30 min, 100000 *g*_{av.}) at 4 °C. The pellet (LDIS-insoluble fraction) was redissolved in 1% SDS (1 ml/10 mg of membrane protein extracted). The supernatant (LDIS-soluble

Abbreviations used: LDIS, lithium di-iodosalicylate; Gp, glycoprotein; Con A, concanavalin A; WGA, wheat-germ agglutinin; TBS, Tris-buffered saline; PVDF, poly(vinyl difluoride); D β H, dopamine β -hydroxylase (GpI).

material) was treated by stirring on ice for 15 min with an equal volume of 50 % phenol (the phenol had been equilibrated with 0.1 M-Tris/HCl, pH 8.0) and centrifuged (SS-34 rotor, 7000 rev./min, 30 min, 4000 $g_{av.}$) at 4 °C. The aqueous fraction was dialysed extensively against water and freeze-dried.

One- and two-dimensional gel electrophoresis and electroblotting

One-dimensional gel electrophoresis was carried out using 10 % polyacrylamide gels (10 cm × 8 cm) run in SDS using the buffer system of Laemmli (1970). The isoelectric-focusing gels for two-dimensional electrophoresis (O'Farrell, 1975) were cast in tubes (7.5 cm × 1.5 mm) containing 4 % acrylamide, 9.2 M-urea, 2 % Nonidet P40 and 2.8 % Ampholines (pH 3.5–10, 5–7, 3.5–5, 2.5–5; Pharmacia-LKB; 2:2:1:2, by vol.). Electrofocusing gels were pre-run for 10 min at 200, 300 and 400 V. Samples were applied at the basic (negative) end of the gels and run for 2 h at 1000 V. These gels were calibrated using carbonic anhydrase II (pI 5.9), soya-bean trypsin inhibitor (pI 4.6) and Methyl Red (pI 3.8). After treatment with 0.0625 M-Tris/HCl (pH 6.8)/2 % SDS/10 % (v/v) glycerol/5 % (v/v) 2-mercaptoethanol for 10 min, first-dimension gels were applied directly to the top of the second-dimension stacking gel.

After electrophoresis, one- or two-dimension gels either were stained with Coomassie Blue or the proteins electroblotted on to nitrocellulose (0.22 μ m; Schleicher and Schüll) in 25 mM-Tris/192 M-glycine/20 % methanol at 5 V/cm for 2 h. Before the detection of glycoproteins the nitrocellulose was incubated overnight with 5 % BSA (Sigma; fraction V) in Tris-buffered saline (TBS; 20 mM-Tris/HCl/0.5 M-NaCl/0.05 % Tween 20, pH 7.4). The nitrocellulose was then incubated with a mixture of horseradish-peroxidase-labelled Con A and wheat-germ agglutinin (WGA) (Sigma; at 2 μ g and 1 μ g/ml respectively) for 8 h at room temperature on a shaking platform. The nitrocellulose was then washed briefly with water and then by two 10 min washes in TBS. Peroxidase labelling was detected with diaminobenzidine (0.2 mg/ml)/0.005 % H_2O_2 in TBS without Tween 20.

Purification of GpII

Chromaffin-granule membranes (48 mg of protein) were extracted with LDIS, and the soluble fraction was dialysed extensively against water. The sample (64 ml) was made 0.1 % (w/v) with respect to SDS and diluted with an equal volume of 0.1 M-sodium phosphate (pH 7.0)/0.4 M-NaCl and pumped (7 ml/h) on to a column (1 cm × 5 cm) of WGA-Sepharose equilibrated with 0.05 M-sodium phosphate (pH 7.0)/0.2 M-NaCl. The column was washed with 10 vol. of buffer containing 0.05 % SDS (20 ml/h) and eluted with 30 ml of 0.3 M-N-acetylglucosamine in 0.05 M-sodium phosphate (pH 7.0)/0.2 M-NaCl. The eluted material was dialysed extensively against water and freeze-dried.

Preparation of WGA-Sepharose

WGA was extracted from wheat-germ and purified using N-acetylglucosamine-Sepharose essentially as described (Vretblad, 1976). WGA (200 mg) (5 mg/ml in 0.1 M-NaHCO₃/0.2 M-NaCl/0.1 M-N-acetylglucosamine) was combined with 40 g of CNBr-activated Sepharose CL-4B (March *et al.*, 1974) and mixed by rotation for 15 h at 4 °C. The WGA-Sepharose was washed with 0.1 M-NaHCO₃/0.2 M-NaCl and incubated with 1 M-ethanolamine/HCl, pH 9.0, for 1 h at room temperature. Finally the WGA-Sepharose (5 mg of WGA/ml of gel) was washed with 500 ml of 0.05 M-sodium phosphate (pH 7.0)/0.2 M-NaCl and stored in 0.05 M-sodium phosphate (pH 7.0)/0.2 M-NaCl containing 0.02 % (w/v) NaN₃.

Protein chemical characterization of glycoproteins II, J and K

Reduction and alkylation of GpII was performed by dissolving the protein (approx. 200 μ g) in 1.0 ml of 6 M-guanidinium chloride/0.3 M-Tris (pH 8.5)/2 mM-EDTA and adding dithiothreitol (25 μ mol; Calbiochem). The sample was incubated for 3 h at 37 °C, and then 75 μ mol of 4-vinylpyridine (Sigma) was added, followed by incubation for 4 h at room temperature. The reduced and S-pyridyethylated sample was then dialysed extensively against 0.05 M-NH₄HCO₃ and freeze-dried. Samples of reduced and alkylated GpII were subjected to gas-phase hydrolysis and amino acid analysis essentially as described by Bidlingmeyer *et al.* (1984). Sequence analysis was performed using a gas-phase sequencer (470A) equipped with an on-line amino acid phenylthiohydantoin derivative analyser (120A) and chemicals and the program (03RPTH) supplied by the manufacturer (Applied Biosystems). Samples of GpII were also sequenced after electroblotting samples of the LDIS-soluble fraction to poly(vinyl difluoride) (PVDF) membranes (Matsudaira, 1987). Components J and K were characterized by microsequence analysis after electroblotting to PVDF membranes. Aliquots (100 μ l) of the LDIS-insoluble fraction [solubilized in 0.1 % (w/v) SDS] were precipitated with 9 vol. of ethanol at –20 °C before electrophoresis. PVDF membranes were stained with Coomassie Blue, and spots corresponding to components J and K were cut out and stored at –20 °C before sequence analysis. Material obtained from three or four two-dimensional gels was used for sequence analysis.

RESULTS

The identification and characterization of chromaffin-granule membrane glycoproteins is complicated by their relative poor staining with Coomassie Blue and the predominance of D β H and other membrane components in most extracts. We were interested in developing a simple procedure to overcome some of these problems.

Extraction of chromaffin granule membranes with LDIS

In preliminary experiments, membranes were extracted with various concentrations of LDIS (0.05–0.3 M) and separated into soluble and insoluble fractions by centrifugation. Most of the proteins in the soluble fraction were precipitated by treatment with 50 % phenol, and the resulting aqueous fraction (termed 'LDIS-soluble') was dialysed extensively against water and freeze-dried. The LDIS-insoluble fraction was solubilized with 1 % SDS before subsequent analyses. Fig. 1 shows the results of extraction of membranes with 0.25 M-LDIS and analysis of the fractions by SDS/PAGE with Coomassie Blue staining or after electroblotting to nitrocellulose membranes after reaction with peroxidase-labelled Con A and WGA. This concentration of LDIS gave a distinct set of components in the two fractions compared with whole membranes and was used for all subsequent experiments. A major glycoprotein (M_r 37000) was retained in the LDIS-insoluble fraction along with several other glycoprotein bands (50000–70000 M_r range). The major lectin-binding glycoproteins in the LDIS-soluble fraction corresponded to 80–100 kDa and 44 kDa components.

Identification of LDIS-insoluble and LDIS-soluble glycoproteins by two-dimensional gel electrophoresis

It is well established that chromaffin-granule membranes contain glycoprotein components identified as D β H, and H, J, K, II, III, IV and V (Pryde & Phillips, 1986; Winkler *et al.*, 1986). It appears that most of GPIII and components H, J and K are retained in the LDIS-insoluble fraction. The soluble fraction

Fig. 1. Extraction of chromaffin-granule membranes with LDIS

Chromaffin-granule membranes (15 μ g of protein, lanes 1 and 4) and samples representing 1/100th of the LDIS-insoluble (lanes 2 and 5) and LDIS-soluble (lanes 3 and 6) fractions from an extract of chromaffin membranes (19 mg of protein) were run on an SDS/10 % polyacrylamide gel. Lanes 1–3, staining with Coomassie Blue; lanes 4–6, staining of electroblotted glycoproteins with peroxidase-labelled Con A and WGA.

Table 1. N-Terminal sequence analysis of GpJ and GpK

Samples of the LDIS-insoluble fraction were subjected to two-dimensional gel electrophoresis followed by electroblotting to PVDF membranes. Spots corresponding to GpJ and GpK, revealed by Coomassie Blue staining, were cut out and subjected to sequence analysis. The recovery of amino acid phenylthiohydantoin derivatives in the sequence runs corresponded to 10 and 20 pmol of GpK and GpJ respectively. (), unidentified residue.

Cycle	Sequence	
	GpK	GpJ
1	()	()
2	Pro	Gly
3	Gln	Pro
4	Glu	Gly
5	Asp	Gly
6	Gly	Pro
7	Ile	Val
8	Ser	Ala
9	Phe	Gly
10	Gly	Ala
11		Gly
12		Arg
13		Arg
14		()
15		Arg
16		Pro
17		Gln
18		Glu
19		Asp
20		Gly
21		Ile
22		Ser

contains GPII, GPIV and GPV and a small amount of GPIII also extracted by the LDIS treatment. Most of the D β H is extracted with 0.25 M-LDIS, but is not soluble after treatment with phenol.

Characterization of glycoprotein components J and K by microsequence analysis after electroblotting from two-dimensional gels

GPJ and GPK are known to represent carboxypeptidase H.

Fig. 2. Analysis of glycoproteins in LDIS extracts of chromaffin-granule membranes by two-dimensional gel electrophoresis

Chromaffin-granule membranes (200 μ g of protein, a) and samples representing 1/50th of the LDIS-insoluble (b) and LDIS-soluble (c) fractions from an extract of chromaffin-granule membranes (19 mg of protein) were subjected to two-dimensional gel electrophoresis. The electroblotted glycoproteins were detected with peroxidase-labelled Con A and WGA.

These glycoproteins were characterized by N-terminal sequence analysis after electroblotting to PVDF membranes (Table 1). The N-terminal sequence of GpK corresponds to the N-terminal sequence reported for this carboxypeptidase (Fricker *et al.*, 1986). Sequence analysis of GpJ enabled identification of 20 out of 22 of the N-terminal residues (Table 1). Residues 15–22 corresponded to the N-terminal sequence of GpK, indicating

Fig. 3. Comparison of the LDIS-soluble fraction before and after chromatography on WGA-Sepharose by two-dimensional gel electrophoresis

Samples of the LDIS-soluble fraction (1/500th of the extract from 48 mg of membrane protein, *a*) and 1/30th of the material recovered after chromatography of the LDIS-soluble fraction on WGA-Sepharose (*b*) were subjected to two-dimensional gel electrophoresis followed by staining with Coomassie Blue.

that this component represents an *N*-terminally extended form of the carboxypeptidase.

Isolation and characterization of glycoprotein II from the LDIS-soluble fraction

The aqueous fraction from LDIS-extracted membranes is enriched in several glycoproteins (Figs. 1 and 2c). A glycoprotein was purified from this fraction by affinity chromatography on WGA-Sepharose in the presence of SDS (Figs. 3a and 3b). Most of the Coomassie Blue-staining material in the LDIS-soluble material before lectin affinity chromatography is due to chromogranin A-related material (results not shown). Comparison of the two-dimensional gel of the purified component, stained with Coomassie Blue in Fig. 3(b) with the result presented in Fig. 2 indicated that the purified component was GpII. Analysis of this preparation, at a high protein loading, by SDS/PAGE with Coomassie Blue staining showed that a high degree of purity had been achieved (Fig. 4). The major component detected corresponded to a broad band of 80–100 kDa. Similar results were obtained after electroblotting of the sample to nitrocellulose and staining with WGA- and Con A-labelled peroxidase (results not shown). The amino acid composition of

Fig. 4. Analysis of the GpII preparation by SDS/polyacrylamide-gel electrophoresis

A sample containing approx. 15 μ g of protein was run on a 10% acrylamide gel (10 cm $\times$ 8 cm $\times$ 0.7 cm) in the presence of SDS and stained with Coomassie Blue. The positions of M_r marker proteins, β -galactosidase (116000), phosphorylase (97000), BSA (66000), ovalbumin (44000) and carbonic anhydrase (36000), are indicated.

Table 2. Amino acid composition of GpII

Samples of *S*-pyridylethylated GpII (approx. 3.5 μ g) were subjected to gas-phase hydrolysis with 5.7 M-HCl (110 $^{\circ}$ C, 24 h, *in vacuo*). Amino acids present in the hydrolysates were identified by reverse-phase h.p.l.c. as phenylthiocarbamylamino acid derivatives. The result is the average of three determinations. Tryptophan was not determined.

Amino acid	Composition (residues/100 residues)
Asp	11.23
Glu	8.76
Ser	9.21
Gly	10.07
His	1.11
Arg	3.92
Thr	7.47
Ala	8.09
Pro	4.46
Tyr	1.57
Val	7.07
Met	1.34
Cys	4.19
Ile	4.00
Leu	8.69
Phe	4.84
Lys	3.96

reduced and *S*-pyridylethylated GpII indicates that this protein is rich in acidic amino acids and cysteine residues (Table 2). Automated *N*-terminal sequence analysis of GpII revealed two sequences in similar amounts: (Val,Leu)-(Phe,Glu)-(Val,Leu)-(Val,Gly)-(Lys,Leu). In a separate experiment the two components of GpII resolved by two-dimensional electrophoresis (Fig. 3) were electroblotted to PVDF membranes. The upper band had the sequence Val-Phe-Val- and the lower band Leu-Glu-Leu-, which agreed with the two sequences present before electroblotting. The sequence Val-Phe-Val-Val-Lys was also obtained from the upper portion of a GpII band after electroblotting from an SDS/polyacrylamide gel to a PVDF membrane.

DISCUSSION

The chromaffin-granule membrane contains a number of glycoproteins, for most of which there is little structural or functional information. Fractionation of membrane components by phase separation with Triton X-114 greatly simplifies the electrophoretic analysis of membrane components (Pryde & Phillips, 1986). Although distinct differences are seen in the glycoproteins present in the various phases, use of these fractions as a starting point for further purification is complicated by the presence of detergent or large amounts of other membrane components. Extraction of chromaffin-granule membranes with LDIS was found to separate glycoproteins into two distinct groups, one of which appears to be resistant to solubilization. A number of glycoproteins in the LDIS-soluble fraction remain in solution after phenol extraction and dialysis, as was found for glycophorin A extracted from red blood cells (Marchesi & Andrews, 1971).

Approx. 25% of the membrane protein is not solubilized by treatment with 0.25 M-LDIS. Interestingly, this insoluble fraction contains several glycoproteins, which have been identified as components H, J, K and III (Pryde & Phillips, 1986; Winkler *et al.*, 1986). Immunochemical analysis has identified components J and K as carboxypeptidase H, an enzyme involved in the processing of proenkephalin (Laslop *et al.*, 1986), which also has a soluble form (Fricker & Snyder, 1982). In the present study this identification was confirmed and extended by direct sequence analysis. GpK was shown to be an *N*-terminally extended form of carboxypeptidase H which has not been previously identified. The additional sequence does not appear sufficiently hydrophobic to account for membrane attachment.

GpIII also has membrane and soluble forms which have been isolated (Fischer-Colbrie *et al.*, 1982, 1984), and to date no differences have been observed between the membrane and soluble forms of these proteins. It will be interesting to determine whether GpIII and components J and K are associated in the membrane. Membrane GpIII has been purified by sequential lectin affinity chromatography in the presence of SDS (Fischer-Colbrie *et al.*, 1982). We have purified GpIII from the LDIS-insoluble fraction by detergent solubilization followed by affinity chromatography on WGA-Sepharose and reverse-phase h.p.i.c. (results not shown).

GpII is a heterogeneous membrane component with a pI of 4.2–4.7 and an M_r of 80000–100000 (Pryde & Phillips, 1986; Winkler *et al.*, 1986) which is present in both endocrine and exocrine secretory vesicles (Obendorf *et al.*, 1988). Although GpII is known to contain a large amount of carbohydrate with the major sugars being galactose, *N*-acetylglucosamine and sialic acid (Fischer-Colbrie *et al.*, 1982), this glycoprotein has not been characterized previously by amino acid analysis or *N*-terminal sequence analysis. It is of interest that, whereas SDS was not required for the solubility of GpII, attempts to purify GpII on WGA-Sepharose in the absence of SDS resulted in substantial contamination of this glycoprotein with chromogranin A. Presumably these components are extracted in a soluble, but aggregated, form, resulting in co-purification in the absence of SDS. The heterogeneity of this glycoprotein has always been attributed to variations in the number of sialic acid residues.

However, sequence analysis revealed *N*-terminal sequence heterogeneity. It is noteworthy that topographical studies indicated that part of GpII is exposed on the outside of chromaffin granules and can be degraded by exogenous proteinases (Abbs & Phillips, 1980).

The LDIS extraction procedure appears to be well suited to the extraction and isolation of chromaffin-granule membrane glycoproteins. It is hoped that this will facilitate further characterization of GpII, including the full sequence determination and the probing of its function in endocrine and exocrine secretory vesicles. This technique will also be useful in comparing glycoproteins present in secretory vesicles prepared from a range of endocrine and exocrine tissues.

This work was supported by a project grant from the Medical Research Council of New Zealand. We gratefully acknowledge the assistance of Mr. Mark Devonald during an early stage of this work. D.J.P. is the recipient of a Postgraduate Scholarship from the Medical Research Council of New Zealand.

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