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Cytochrome b_{-245} from human neutrophils is a glycoprotein

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Cytochrome b_{-245} is a glycoprotein. It runs as a broad band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and its apparent M_r varies with the concentration of acrylamide. It stained positively with Schiff reagent and with silver stains after oxidation with periodic acid. It preferentially bound the lectin of *Phaseolus vulgaris* (type III), and cleavage of carbohydrate with endoglycosidase F resulted in a sharp band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an apparent M_r of 55000. G.l.c. analysis of carbohydrate showed this to account for about 15% of the M_r and *N*-acetylglucosamine and galactose to be the major sugars.

Cytochrome b_{-245} is an integral component of the microbicidal oxidase system of phagocytic leukocytes (Segal & Jones, 1978; Segal *et al.*, 1981). We purified this cytochrome from neutrophils of patients with chronic granulocytic leukaemia (Harper *et al.*, 1984). Despite precautions to minimize proteolysis, the purified cytochrome ran as a broad band with an apparent M_r of 68000–78000 on SDS/polyacrylamide-gel electrophoresis. The ratio of haem to protein was 18 nmol/mg, considerably greater than the theoretical maximum of 13 nmol/mg calculated from an M_r of 78000. These anomalies are well recognized with glycoproteins, and the present studies were conducted to examine the possibility that the cytochrome contained a significant amount of carbohydrate.

Experimental

Materials and solutions

Nitrocellulose paper (pore size 0.45 μm) was from Anderman and Co. (Kingston upon Thames, Surrey, U.K.). Bovine serum albumin (fraction V, powder), concanavalin A, horseradish peroxidase, lectins from *Arachis hypogaea*, *Glycine max* (type VI) and *Phaseolus vulgaris* (type III), rabbit antibodies to these lectins, periodate/Schiff stain, *N*-acetyl-β-glucosaminidase (EC 3.2.1.30; from *Aspergillus niger*; 48 units/mg of protein), neuraminidase (EC 3.2.1.18; type X; 160 units/mg of protein), β-galactosidase (EC 3.2.1.23; grade V; 18 units/mg of protein) and other chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.). Endoglycosidase D (EC 3.2.1.–), Gelbond and goat anti-rabbit IgG–horseradish peroxidase conjugate were from Miles Scientific (Slough, Berks., U.K.). Endoglycosidase F (EC 3.2.1.–) was from New England Nuclear (Boston, MA, U.S.A.; 18 units/mg of protein). Reactivials (100 μl) were purchased from Pierce and Warriner, Chester, U.K.

Purification and measurement of cytochrome b_{-245}

The cytochrome was purified by Triton N101 extraction and chromatography on heparin–agarose as described by Harper *et al.* (1984), and it was quantified by reduced-minus-oxidized spectroscopy, by using $\Delta(\varepsilon_{559}-\varepsilon_{540}) = 21.6\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ (Cross *et al.*, 1982). Protein was measured as described by Markwell *et al.* (1978) on samples after overnight precipitation with trichloroacetic acid in the presence of sodium deoxycholate (Bensadoun & Weinstein, 1976).

Electrophoresis, transfer of proteins to nitrocellulose and lectin binding

Analytical SDS/polyacrylamide-gel electrophoresis was as described previously (Harper *et al.*, 1984) on gradient slab gels of 7–15% acrylamide and linear gels of 5, 10 and 12.5% acrylamide. Gels were stained for carbohydrate with periodate/Schiff reagent (Glossman & Neville, 1971) and by silver staining (Dubray & Bezard, 1982). Controls were done in the absence of periodate oxidation.

Abbreviations used : SDS, sodium dodecyl sulphate; TBS, Tris-buffered saline [50 mM-Tris/HCl (pH 7.4)/0.2 M-NaCl].

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Immediately after separation by SDS/polyacrylamide-gel electrophoresis the proteins were transferred to nitrocellulose sheets, as described by Towbin *et al.* (1979), with 0.1% SDS added to the electrode buffer (Erickson *et al.*, 1982), by using a water-cooled Atto blotting apparatus (Genetic Research Instrumentation, Bishops Stortford, Herts., U.K.) at a current of 0.5A for 3h. Nitrocellulose blots were stained for protein with Amido Black (Schaffner & Weismann, 1973).

For the detection of lectin-binding proteins, the nitrocellulose sheets were processed in sealed plastic bags at 37°C. They were first washed in TBS for 30min before blocking of the binding sites with albumin in TBS for 30min. They were then overlaid with the lectins (25μg/ml) in TBS containing albumin and agitated continuously for 2h. The sheets were then washed with TBS and blocked with albumin as above. The blots incubated with the lectins from *Phaseolus vulgaris*, *Arachis hypogaea* and *Glycine max* were then incubated for 2h with the appropriate antibody at a dilution of 1:500 in TBS containing albumin. They were then washed with TBS and then with TBS containing albumin, and then incubated for 2h with the goat anti-rabbit IgG–horseradish peroxidase conjugate (diluted 1:1000 in TBS containing albumin). The blots were then washed in TBS before reaction with the peroxidase substrate 4-chloro-1-naphthol (Hawkes, 1982). Bound concanavalin A was identified by the binding of horseradish peroxidase (50μg/ml, in TBS containing albumin; Hawkes, 1982). After incubation for 2h, the blot was washed with TBS and made to react with the peroxidase substrate. Albumin was used at a concentration of 3% (w/v).

Removal of carbohydrate

Attempts were made to remove the carbohydrate from the cytochrome: (*a*) by chemical methods, by treating a sample (1nmol) with alkaline $NaBH_4$ (Ogata & Lloyd, 1982) and trifluoromethanesulphonic acid (Edge *et al.*, 1981); (*b*) enzymically, by incubating the cytochrome with exo- and endo-glycosidases. A sample (0.5nmol) was incubated with a mixture of the exoglycosidases neuraminidase (10 munits), β-galactosidase (60 munits) and *N*-acetyl-β-glucosaminidase (100 munits) for 1h at 37°C in sodium acetate (50mM, pH5.0) containing Triton N101 (1%). Endoglycosidase D was used as described by Koide *et al.* (1977), and endoglycosidase F as described by the manufacturers (NENZYMES technical data sheet), with SDS (0.1%) and 2-mercaptoethanol (1%, v/v) in the reaction mixture and Triton N101 (1%, w/v) instead of Nonidet P-40. Where incubation with endoglycosidase D and/or F was preceded by incubation with exoglycosidases, the mixture was then dialysed for 14h at 4°C against the appropriate buffer.

Determination of carbohydrate and amino acids

Carbohydrate was determined by g.l.c. (Chaplin, 1982) in (1) samples of the purified cytochrome and (2) the 76000–88000-M_r band eluted from the gel after SDS/polyacrylamide-gel electrophoresis.

(1) To samples of purified cytochrome (1.0ml; 6–10μM; 500–1000μg of protein/ml) were added sodium deoxycholate (250μl of 10mg/ml) and trichloroacetic acid (200μl of 100%, w/v). After 2h on ice samples were centrifuged at 7000*g* for 10min in Reactivials. The precipitates were washed in toluene (100μl) and then acetone

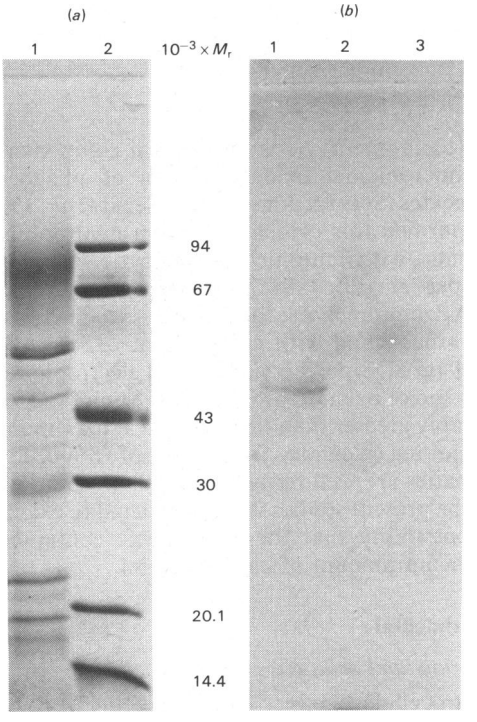

Fig. 1. *Coomassie Blue and periodate/Schiff stains of cytochrome b_{-245} after SDS/polyacrylamide-gel electrophoresis*

See the text for experimental details. (*a*) Stained with Coomassie Blue. Track 1, cytochrome b_{-245} (0.24nmol, 20μg of protein). Track 2, M_r markers are indicated at the right of the Figure; in order of increasing M_r these are lactalbumin (6.1μg), trypsin inhibitor (4μg), carbonic anhydrase (4.2μg), ovalbumin (7.4μg), albumin (4.2μg) and phosphorylase *b* (3.2μg). (*b*) Stained with periodate/Schiff reagent. Track 1, horseradish peroxidase (20μg); track 2, cytochrome b_{-245} (0.48nmol, 40μg of protein); track 3, M_r markers as above.

(100 μl) to remove Triton N101 and contaminating lipids before analysis.

After hydrolysis in HCl (6M, containing 0.3% phenol) at 110°C for 24h, the amino acid composition of duplicate samples was analysed on a Rank Hilger Chromaspek J180 amino acid analyser.

(2) Samples of the cytochrome (about 0.75nmol) were electrophoresed on a 7.5–15%-acrylamide gel as described above, except that the gel was poured on Gelbond to facilitate location of the cytochrome, which was accomplished by cutting out a section of the gel and staining it for protein. The band containing the cytochrome was then excised, transferred to a small dialysis bag (1cm in diameter) containing electrode buffer (250 μl), placed in the blotting apparatus and electrophoresed out of the polyacrylamide under exactly the same conditions as used for blotting. Samples of the eluted protein were then prepared for carbohydrate analysis by trichloroacetic acid precipitation as described above, except that precipitates were not washed with toluene.

Results

Electrophoretic mobility of the cytochrome

Initial studies revealed that the cytochrome ran as a broad band on SDS/polyacrylamide-gel electrophoresis, with an apparent M_r of about 68000–78000 (Harper *et al.*, 1984). Those studies were conducted with gradient gels of 5–15% acrylamide. In the present study with 7.5–15%-acrylamide gels, the cytochrome moved with an apparent M_r of 76000–88000. These anomalous results could in part have ~~been due~~ to differences in the gradients, because on gels of 5, 10 and 12.5% acrylamide the cytochrome ran as a broad band with apparent M_r values of 72000–84000, 76000–86000 and 80000–90000 respectively.

Staining for carbohydrate

(*a*) *With periodate/Schiff stain.* A positive reaction of the 76000–88000-M_r band was observed if more than 0.25nmol of the cytochrome was loaded (Fig. 1). The glycoprotein M_r marker ovalbumin did not give a positive stain at the concentration

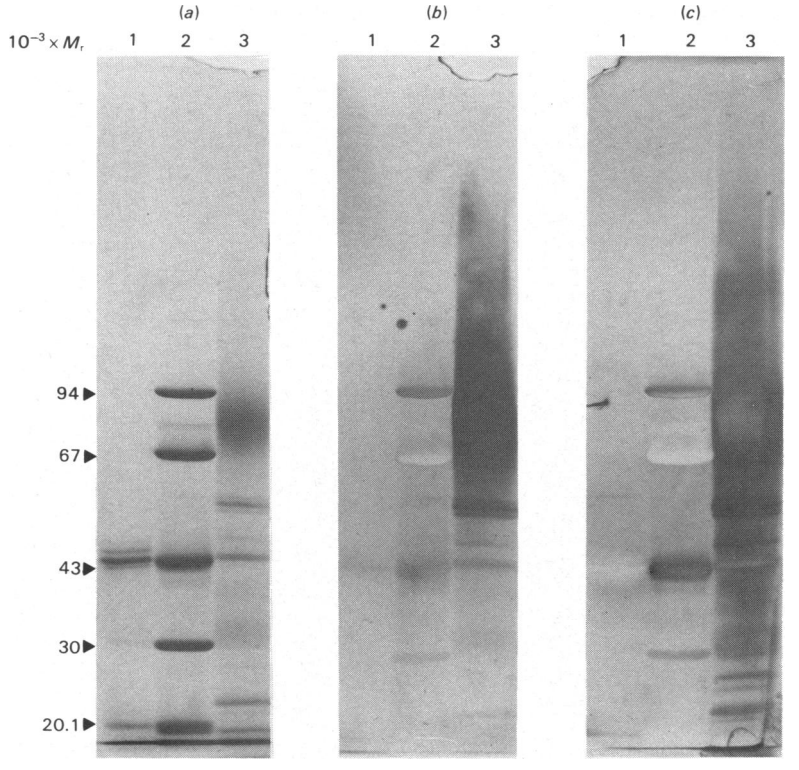

Fig. 2. *Coomassie Blue* (*a*), *periodate/silver* (*b*) *and silver* (*c*) *stains of cytochrome* b_{-245} *after SDS/polyacrylamide-gel electrophoresis*

See the text for experimental details. Tracks 1, horseradish peroxidase (12.5 μg); tracks 2, M_r markers as for Fig. 1; tracks 3, cytochrome b_{-245} (0.5nmol, 21 μg of protein).

loaded (7.35 μg). No staining of the cytochrome or horseradish peroxidase was observed if the periodate oxidation step was omitted.

(*b*) *With periodate/silver stain.* The bands of the cytochrome, horseradish peroxidase and ovalbumin all gave a very strong reaction with the silver stain, but only after periodate oxidation (Fig. 2). As described previously (Dubray & Bezard, 1982), the 94000-M_r (phosphorylase *b*) and 30000-M_r (carbonic anhydrase) markers stained positively with the periodate/silver stains, but unlike them we found a positive reaction without periodate oxidation.

Lectin binding

The broad band of the cytochrome reacted with the type III lectin from *Phaseolus vulgaris*, but not with those from *Glycine max* or *Arachis hypogaea*, or with concanavalin A. Some of the other protein-staining bands reacted with the other lectins; for example the bands of M_r 57000 and 30000 reacted with all four lectins (Fig. 3).

G.l.c. analysis of carbohydrate

The major carbohydrates present in the purified cytochrome eluted from heparin–agarose and in that extracted from the band of polyacrylamide are shown in Table 1. The same sugars were present in both samples in similar relative proportions, except for xylose and glucose, which were probably contaminants. On the basis of the amino acid analysis of the most pure material prepared by column chromatography (Table 1), the carbohydrate content was determined as 15%.

Amino acid analysis

The amino acid composition of the sample eluted from the heparin–agarose column is shown in Table 1.

Removal of carbohydrate, and apparent M_r of apoprotein

Endoglycosidase F changed the electrophoretic mobility of the cytochrome from that of a broad band with an apparent M_r of between 76000 and 88000 to a sharp band of M_r 55000 (Fig. 4). Intermediate stages in this modification were seen, with banding with a periodicity of about 11000. The exoglycosidases and endoglycosidase D had little or no effect, separately or together. The results of the chemical modifications were not easily identifiable, because they caused the cytochrome to aggregate and to fail to enter the gel.

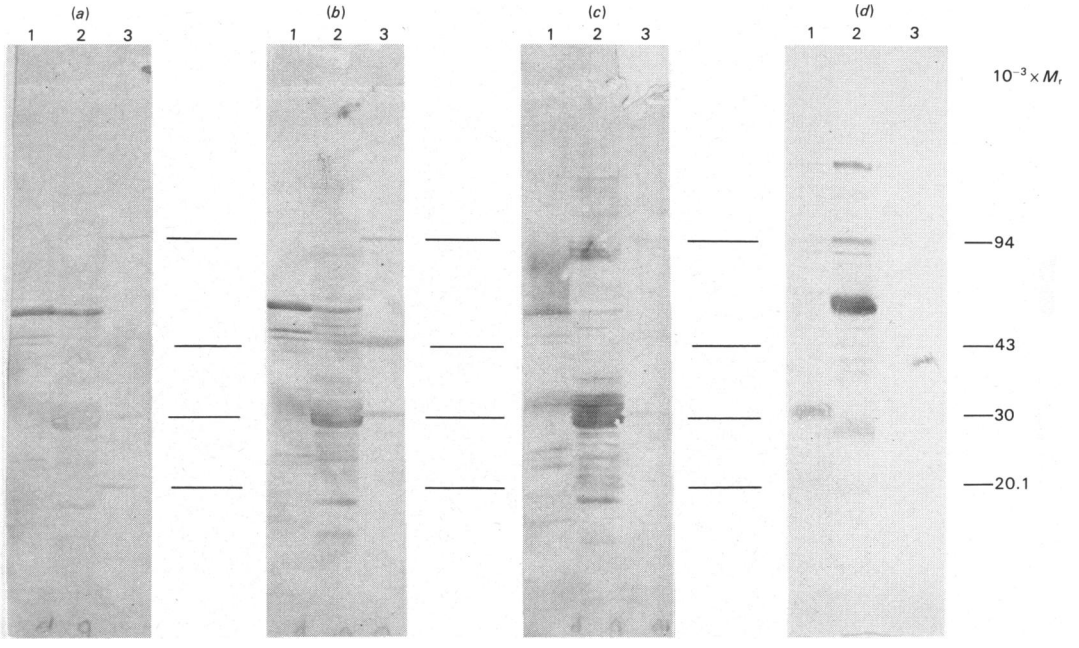

Fig. 3. *Lectin binding to cytochrome b_{-245} after separation by SDS/polyacrylamide-gel electrophoresis and blotting on to nitrocellulose*

See the text for experimental details. (*a*) *Glycine max* type VI lectin; (*b*) *Arachis hypogaea* lectin; (*c*) *Phaseolus vulgaris* type III lectin; (*d*) concanavalin A. Tracks 1, cytochrome b_{-245} (0.6nmol, 40μg of protein); tracks 2, pelleted neutrophil organelles (165μg of protein; approx. 13pmol of cytochrome b_{-245}); tracks 3, M_r markers (as for legend to Fig. 1); the M_r values of those markers that bind the lectins are indicated by the Figure.

Table 1. *Analysis of carbohydrate and amino acid contents of cytochrome* b_{-245}
See the text for experimental details.

	Composition of cytochrome from heparin–agarose (nmol/55 μg of protein)*	Composition of 76000–88000-M_r band from SDS/polyacrylamide-gel electrophoresis (μg/sample)†
Sugars		
Fucose	3.9	0.015
Xylose‡	0.7	<0.001
Mannose	6.5	0.038
Galactose	15.1	0.064
Glucose‡	5.8	0.457
N-Acetylgalactosamine	0.2	<0.010
N-Acetylglucosamine	19.0	0.137
N-Acetylneuraminic acid	2.1	0.017
Amino acids		
Methionine	8.67	
Aspartic acid	41.04	
Threonine	25.74	
Serine	29.16	
Glutamic acid	45.39	
Glycine	47.22	
Alanine	46.31	
Valine	36.08	
Isoleucine	30.46	
Leucine	51.52	
Tyrosine	17.31	
Phenylalanine	28.22	
Histidine	24.76	
Lysine	28.37	
Arginine	28.90	
Proline	7.25	
Cysteine	1.11	

* Each value is an average of four replicates.
† Each value is an average of two replicates.
‡ Probable impurities.

Discussion

The properties of cytochrome b_{-245} are consistent with those of other glycoproteins. The difficulties in determining the M_r by SDS/polyacrylamide-gel electrophoresis (Segrest & Jackson, 1972; Frank & Rodbard, 1975; Leach *et al.*, 1980; Poduslo, 1981), together with the appearance of some as broad bands (Fairbanks *et al.*, 1971; Papermaster & Dreyer, 1974; Frank & Rodbard, 1975), have long been observed. In addition, determination of the protein concentration of glycoproteins by the Lowry technique is inaccurate, because the absorption coefficient falls with increasing carbohydrate, but not in a simple proportion (Maddy *et al.*, 1972).

The identification of cytochrome b_{-245} as a glycoprotein was established by its positive staining with periodate/Schiff reagent, g.l.c. analysis for sugars, lectin binding and decreased M_r after cleavage with endoglycosidase F. Our initial inability to stain the cytochrome with periodate/Schiff reagent (Harper *et al.*, 1984) was probably due to the fact that we loaded too little material and that the Schiff reagent may have been improperly stored. The binding of the lectin from *Phaseolus vulgaris* was consistent with the determinations by g.l.c. analysis that the major sugars present in both the initial preparation of purified cytochrome and the band eluted from acrylamide were *N*-acetylglucosamine and galactose. The lack of *N*-acetylgalactosamine residues is reflected in the lack of binding to lectins from *Arachis hypogaea* or *Glycine max*. It therefore appears likely that the cytochrome contains only *N*-linked high-lactosamine complex-type oligosaccharide moieties.

The establishment of the probable M_r of the apoprotein as 55000 should be important in the identification of the cytochrome in hybrid-selected translation experiments aimed at cloning the gene.

The observation that this cytochrome is so heavily glycosylated is consistent with its location in the plasma membrane (Segal & Jones, 1979; Garcia & Segal, 1984). Whether its carbohydrate components function as receptors and play a role in

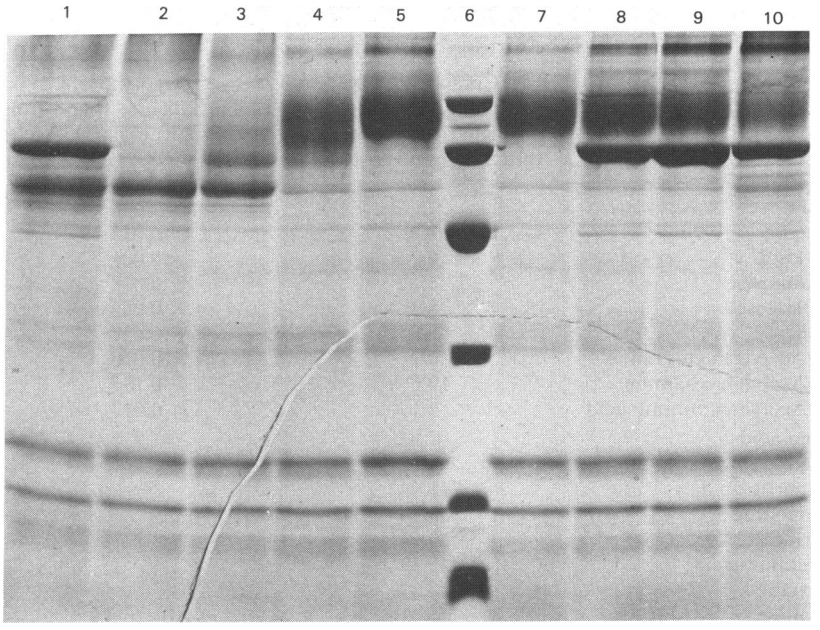

Fig. 4. *Endoglycosidase treatment of cytochrome* b_{-245}

See the text for experimental details. Tracks 5 and 7, untreated pure cytochrome (0.5nmol); tracks 4, 3 and 2, cytochrome after incubation at 37°C for 0, 1 and 14h respectively with endoglycosidase F; tracks 8, 9 and 10, as for tracks 4–2, but with endoglycosidase D; track 1, as for track 2, but with both endoglycosidases; track 6, markers of M_r ($\times 10^{-3}$) 94, 67, 43, 30, 20.1 and 14.4. The heavy band of M_r 67000 seen in the tracks (8–10) containing endoglycosidase D is albumin, present as carrier protein.

activation of the electron-transport chain remains to be established.

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