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Study of the glycosylation of apolipoprotein H

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Abstract

Apolipoprotein H is a single chain polypeptide composed of 326 amino acids highly glycosylated. Its carbohydrate content is approximately 19% of the molecular weight. We show that it is rich in sialic acid linked α(2–6) to galactose or *N*-acetylgalactosamine. Sialic acid is not α(2–3) linked to galactose. Galactose is β(1–4) linked to *N*-acetylglucosamine and β(1–3) linked to *N*-acetylgalactosamine. Carbohydrate O-linked chains (mainly sialic acid) are α(2–6) linked to galactose or *N*-acetylgalactosamine. Galactose is also organised in O-linked chains and β(1–4) linked to *N*-acetylglucosamine and β(1–3) linked to acetylgalactosamine. Concanavalin A lectin was used to isolate two groups of apolipoprotein H molecules bearing biantennary and truncated hybrids and high mannose and hybrid oligosaccharides. Apolipoprotein H fails to bind lysine–Sepharose. Our results thus show that it presents truncated hybrid or hybrid-type carbohydrate chains which bear few unmasked mannose residues as a terminal sugar. Biochemical analysis of carbohydrate structures conducted on single isoforms separated through IEF revealed that no specific carbohydrate complex is bound to a single isoform.

Keywords: Apolipoprotein H; β2-Glycoprotein I; Affinity chromatography; Concanavalin A; Glycosylation; Lectin

1. Introduction

Apolipoprotein H (apoH), also known as β2-glycoprotein I, is a 43–50 kDa plasma glycoprotein. It is a single chain polypeptide of 326 amino acids (Lozier et al., 1984) and a structural component of chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Polz and Kostner, 1979). It binds to platelet membranes, interacts with negatively charged macromolecular structures including heparin, DNA, phospholipids, modulates the activity of adenylate cyclase and may be involved in blood coagulation

Abbreviations: AAA, *Aleuria aurantia* agglutinin; ACA, *Amaranthus caudatus* agglutinin; apoH, apolipoprotein H; DSA, *Datura stramonium* agglutinin; EDTA, ethylenediaminetetraacetic acid; GNA, *Galanthus nivalis* agglutinin; HDL, high density lipoproteins; IEF, isoelectrofocussing; LDL, low density lipoproteins; MAA, *Maackia amurensis* agglutinin; PHA, *Phaseolus vulgaris* agglutinin; PNA, peanut agglutinin; SDS-PAGE, sodium dodecyl sulfate polyacrilamide gel electrophoresis; SNA, *Sambucus nigra* agglutinin; TBS, Tris-buffer saline; VLDL, very low density lipoproteins.

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since it inhibits the prothrombinase activity on human platelets (Nakaya et al., 1980; Schousboe, 1985; Nimpf et al., 1986). It is also thought to be involved in haemostasis since it displays anticoagulant properties in vitro (McNeil et al., 1990). Compared to other human plasma proteins apoH has a high content of cystein (6.2%) and proline (8.3%) and is highly glycosylated (Lozier et al., 1984).

ApoH, displays a high degree of polymorphism (Sepehrnia et al., 1989). According to Kamboh et al. (1988), it has two kinds of polymorphic variations: a post-synthetic polymorphism consisting of multiple isoforms due to differences in the number of terminal sialic acid moieties and a genetic polymorphism revealing structurally different isoforms with different isoelectric points. Glycosylation of apoH accounts for about 19% of its molecular weight. The five reported glucosamine-attached oligosaccharide side chains are composed of galactose, mannose, *N*-acetylglucosamine, fucose, and *N*-acetylneuraminic acid (Kamboh et al., 1988).

ApoH is composed of five repeating domains of about 60 amino acids each with a conserved pattern of cysteine residues (Steinkasserer et al., 1991). Carbohydrates are mainly linked to asparagine residues in the carbohydrate acceptor sequence Asn-X-Ser/Thr (Lennarz, 1980). The oligosaccharides are attached to asparagine residues at positions 143, 164, 169, 174, and 234 (1). At positions 174 and 234 the acceptor sequence is Asn-Trp-Ser/Thr (Lozier et al., 1984).

ApoH existed as 40% β-sheet, 30% β-turn and 30% random coil (Walsh et al., 1990), but after deglycosylation the primary structure of the polypeptide chain is maintained and the secondary structure alters to form a greater number of β-turns, accompanied by reduction in random coil structures (David et al., 1994).

Some studies (Kamboh et al., 1988; Steinkasserer et al., 1991) have described the main oligosaccharides forming the glycosylated chains, whereas apoH's inner structures have not been investigated. This gap should be filled as glycosylation is a very important process that regulates the structure and biological functions of proteins.

In diabetes, glycation of proteins is a non-enzymatic process due to hyperglycaemia and is regarded as one of the factors contributing to its severity (West, 1982). Glycation of apolipoproteins generates free radicals and increases the oxidative damage (Lyons, 1992). Glycosylated or glycoxidised lipoproteins may be immunogenic and potent stimulators of foam-cell formation and increase the risk of atherosclerosis (Lyons, 1992).

Lectins are proteins whose specific binding to the moieties of carbohydrate is used to identify their structures. Moreover, affinity chromatography of glycoproteins on immobilised lectins, such as Concanavalin A (Con A), is a useful oligosaccharide fractionation method (Baenziger and Fiete, 1979; Cummings and Kornfeld, 1982). N-Linked oligosaccharide structures interact with Con A according to their branching properties as follows: triantennary, tetraantennary, and bisecting oligosaccharides do not bind to Con A, biantennary and truncated hybrids bind weakly, and high mannose and hybrid oligosaccharides bind firmly.

In this paper, the micro-heterogeneity of glycosylations characterising the structure of apoH is illustrated by describing their types of glycosylations and distribution on apoH, and among the N-linked and O-linked chains and among the main isoforms, as well as their influence on apoH phenotype and the patterns of Con A elution of apoH isolated from human plasma. Biochemical analysis of glycosylations could clarify the aspects of apoH metabolism that are still poorly understood (Wurm et al., 1982; McNeil et al., 1990; Vlachoyiannopoulos et al., 1992; Hunt et al., 1993; Cassader et al., 1994).

2. Materials and methods

2.1. Materials

Tris, urea, Tween 20, acrylamide and *N*,*N*′-methylene-bis-acrylamide, analytical grade, were purchased from Bio-Rad (Richmond, CA). Gel Bond and ampholytes (pH 4–6.5 and pH 6.5–9) were obtained from LKB (Bromma, Sweden); nitrocellulose sheets from Bio-Rad (Milan, Italy).

Polyclonal rabbit antibody to apoH was kindly supplied by Behring (Scoppito, Italy). Goat anti-rabbit IgG (alkaline phosphatase conjugated) and the alkaline phosphatase conjugate substrate kit were purchased from Bio-Rad. Molecular weight standards (low M.W.) were from Bio-Rad. DIG Glycan Differentiation Kit and lectins digoxigenin-labelled were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Cyanogen-bromide-activated Sepharose 6B was bought from Pharmacia (Fine Chemicals, Piscataway, NJ). Triethylamine, $NaClO_4$, and acetonitrile were purchased from Sigma (Milan, Italy). A C_{18}- Reverse Phase Chromatography column was bought from Beckman (Milan, Italy).

2.2. Isolation and purification of apoH

ApoH was isolated from human plasma and purified through a combination of affinity chromatography and continuos elution electrophoresis as previously described (Gambino et al., 1996). Briefly, rabbit anti-apoH antibodies (22 mg) were immobilised on a cyanogen-bromide-activated Sepharose 6B in 5 ml total volume and packed into a 0.7×13.5 cm column equilibrated with 0.1 M phosphate buffer, 0.3 M NaCl, 0.05% NaN_3, pH 7.6 (buffer A) at a flow rate of 0.3 ml/min. Absorbance was monitored at 280 nm. Normal plasma samples (5 ml per run) were diluted into 40 ml buffer A and applied to the column at a flow rate of 0.17 ml/min. The column was washed with buffer A at 0.3 ml/min until the absorbance reached the baseline, then equilibrated at 0.3 ml/min for 1 h with a lower ionic strength buffer composed of 0.01 M phosphate buffer, 0.15 M NaCl, 0.05% NaN_3, pH 6.8. The apoH bound to the column was eluted with 0.1 M glycine, 0.05% NaN_3, pH 2.5. Sample fractions were collected every minute and the pH was immediately adjusted with 60 μl 0.5 M phosphate buffer pH 7.6. Eluates from four loaded column were pooled and concentrated with Centriprep-10 concentrators (Amicon Inc., Beverly, MA) at 3000 rpm and 20°C in a Beckman-J6B centrifuge (Beckman Instruments, Palo Alto, CA) to a final volume of about 1 ml. The purity of the sample was assessed by SDS-PAGE electrophoresis using a 12%-acrylamide gel. The gel was then silver stained. As can be seen in Fig. 1 (lane 1), the approximately 50 kDa band corresponding to apoH was accompanied by higher-weight bands whose presence showed that further purification was required.

2.3. Continuous-elution electrophoresis

Continuous-elution electrophoresis of the proteins (about 2.5 mg) was therefore performed with a Prep-Cell (Bio-Rad Laboratories, Milan, Italy) through a cylindrical gel (gel tube inside diameter 37 mm; gel length 50 mm) composed of 8.5% acrylamide, 2.7% *N*,*N*′-methylene-bis-acrylamide, and 0.1% SDS. The buffers were 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.4. Separation was achieved at 40 mA in about 8 h. Samples collected every minute with a tube collector were dot-blotted with specific anti-apoH antibody (data not shown). The samples containing

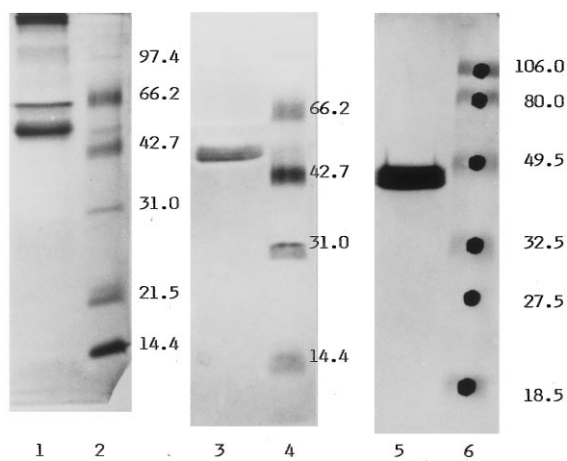

Fig. 1. SDS-PAGE gel electrophoresis of apoH in the two purification steps: lane 1: silver-stained 12% SDS-PAGE gel of eluted apoH fractions from affinity chromatography column; lane 3: silver-stained 12% SDS-PAGE gel of purified apoH by continuous elution electrophoresis; lane 5: Western blot of duplicate gel of lane 3 stained with specific apoH antiserum, using a two-antibody method. In lane 2, molecular mass markers are (from top to bottom): 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa. In lane 4, molecular mass markers are (from top to bottom): 66.2, 42.7, 31.0, 14.4 kDa. Prestained molecular mass marked with black points (lane 6) are (from top to bottom): 106.0, 80.0, 49.5, 32.5, 27.5, 18.5 kDa.

apoH were pooled, dialysed in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4, and subjected in duplicate to 12% SDS-PAGE. One gel was silver stained (Fig. 1, lane 3), the other was immunoblotted with the specific antibodies (Fig. 1, lane 5). These lanes show a single, approximately 50 kDa band corresponding to the pure protein. As judged by SDS-PAGE purity was 98–100%. The isolated protein was also subjected to 12% SDS-PAGE after reduction with DTT (data not shown). There was an apparent increase in the molecular weight of the reduced form as reported by others (McNally et al., 1993). The recovery of pure apoH from the protein mixture eluted from the column in the first purification step was 52%. The final yield from 20 ml starting plasma was about 34% as in other studies (McNally et al., 1993).

2.4. Reverse-phase chromatography

The separated apoH was examined by reverse-phase chromatography on a C_{18} IP Beckman column (4.6 × 250 mm) connected to a Beckman HPLC system. Ten micrograms were dissolved in 0.1 M H_3PO_4, 0.02 M triethylamine, 0.05 M $NaClO_4$, pH 3 (buffer A). Buffer B was acetonitrile. The analysis was performed in a 20–70% acetonitrile gradient for 35 min at 1 ml/min. Absorbance was measured at 214 nm. Analysis of the N-terminal region with a Model 475A Gas-Phase Sequencer (Applied Biosystems, Foster City, CA) showed that its ten amino acids were those published for apoH by Steinkasserer et al. (1991), namely Gly-Arg-Thr-X-Pro-Lys-Pro-Asp-Asp-Leu.

2.5. SDS-PAGE

To analyse the purity, or the type of glycosylation, apoH was subjected to SDS-PAGE electrophoresis using a 12% acrylamide gel in a Bio-Rad Mini Protean II apparatus under non-reducing conditions. The buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.

2.6. Western blot

ApoH was then blotted after SDS-PAGE electrophoresis in 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3 at 295 mA for 100 min. The nitrocellulose membrane was then tested with the specific antibody, and the bands were visualised with a second, alkaline phosphatase labelled anti-rabbit IgG antibody or tested with specific lectins.

2.7. Glycoprotein detection

The specific binding of lectins to carbohydrate moieties was used to identify these structures. The lectins applied were conjugated with the steroid hapten digoxigenin for immunological detection of its binding. Lectins that selectively recognise the terminal sugars are used to differentiate carbohydrate structures by identifying their carbohydrate chains (DIG Glycan Differentiation Kit). All filters were incubated overnight in blocking solution at 4°C. They were then washed once in 10 mM Tris–HCL, 150 mM NaCl, 0.01% Thimerosal, pH 7.6 (TBS) and twice in lectin buffer, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ in TBS. Digoxigenin-labelled lectins were diluted in lectin buffer and the filters were incubated at room temperature for 1.5 h by gentle agitation. When this incubation was completed nitrocellulose membranes were washed three times in TBS at room temperature with gentle agitation to remove unbound lectins. The lectins used were: GNA (*Galanthus nivalis* agglutinin), SNA (*Sambucus nigra* agglutinin), MAA (*Maackia amurensis* agglutinin), PNA (peanut agglutinin), DSA (*Datura stramonium* agglutinin), ACA (*Amaranthus caudatus* agglutinin), AAA (*Aleuria aurantia* agglutinin), PHA (*Phaseolus vulgaris* agglutinin). GNA recognises terminal mannose, α(1–3), α(1–6) or α(1–2) linked to mannose. SNA recognises sialic acid linked α(2–6) to galactose. MAA recognises sialic acid linked α(2–3) to galactose. PNA recognises the core disaccharide galactose β(1–3) *N*-acetylgalactosamine. DSA recognises Gal-β(1–4) *N*-acetylglucosamine in complex and hybrid N-glycans, in O-glycans and *N*-acetylglucosamine in O-glycans. ACA shows a high specifi-

city for the α-anomer of the disaccharide unit Gal-β(1–3) *N*-acetylgalactosamine-α-Ser/Thr. AAA binds specifically to α(1–6) linked fucose residues in complex N-glycan structures. PHA binds preferentially to the β(1–6) linked lactosamine branch of complex N-glycans. Nitrocellulose membranes were then incubated with polyclonal sheep anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase for 1.5 h at room temperature by gentle agitation and washed three times in TBS. Bands were visualised by the immuno-blot assay kit of Bio-Rad.

2.8. Deglycosylation of apoH

Four micrograms of apoH in 100 μl were denatured in 0.2% SDS (w/v) by boiling for 2 min at 100°C. Then, N-glycosidase F incubation buffer (50 mM phosphate, 50 mM EDTA, nonidet-P 40 1%) was added. These mixtures were again boiled for 2 min, cooled down to 37°C, the enzyme added (4 units of N-glycosidase F in 200 μl) and incubated for 18 h at 37°C. Fifty microlitres (about 1 μg of protein) were loaded onto one lane of an SDS-gel. Detection of glycosylations was performed as described above. To rule out non-specific proteolytic digestions due to the long incubation at 37°C, apoH was also incubated without N-glycosidase F.

2.9. Isoelectric focussing and Western blotting

Isoelectric focussing electrophoresis (IEF) was carried out in a Bio-Phoresis TM horizontal electrophoresis cell (Bio-Rad), using a model 3000/300 power unit. The polyacrylamide gel, T = 5%, C = 3%, contained 2% ampholytes (pH 4–6.5 and 6.5–9, 3.75:1, v/v) and 3 M urea. Cathode and anode solutions were 100 mM NaOH and 40 mM glutamic acid, respectively. After pre-focussing at 500 constant V for 35 min, 1 μg of apoH was applied onto each lane. Electrophoresis was performed at 10 constant W for 3 h. After IEF, the proteins were transferred overnight at room temperature onto a 0.45-μm pore size nitrocellulose membrane by simple diffusion and the nitrocellulose was then incubated with specific anti-apoH antibody or with lectins as described above.

2.10. Concanavalin A lectin affinity chromatography

A lectin column (2.5 ml of Con A–Sepharose, Sigma) was equilibrated with a buffer containing 10 mM Tris–HCl, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.01% Thimerosal, pH 8.0 (Con A buffer) at a flow rate of 0.3 ml/min. Approximately 55 μg of apoH diluted in 1 ml were applied to the column at a flow rate of 0.1 ml/min to allow sample interaction with the lectin. After this incubation, Con A buffer was added at a flow rate of 0.2 ml/min for 60 min in order to wash away unbound sample. Weakly bound apoH was eluted from the Con A column with 10 mM α-D-methylglucopyranoside (Sigma) at a flow rate of 0.3 ml/min for 60 min and firmly bound apoH was subsequently eluted with 300 mM α-D-methylmannopyranoside (Sigma). Eluates containing unbound, weakly bound and firmly bound apoH were collected into 0.6 ml fractions. When unbound fractions were pooled and reloaded on the column, no bound material was recovered. Similarly, when bound fractions were pooled, dialysed, and reloaded, neither unbound nor weakly bound material was found, and bound materials were eluted in the initial proportion. The same profile was obtained when other samples of apoH were run through the column.

2.11. Protein dot-blotting of Con A fractions

Rapid screening of Con A fractions was conducted in the Bio-Dot micro-filtration apparatus (Bio-Rad). A sheet of nitrocellulose was clamped between the gasket and the 96-well sample template. Fifty microlitres of sample were allowed to filter through the membrane. After the antigen was immobilised, the nitrocellulose was incubated in 4% albumin blocking solution, then incubated with rabbit IgG anti-apoH (Behring) at a 1/2000 dilution at room temperature for 1.5 h, and next with goat IgG anti-rabbit IgG labelled with alkaline phosphatase at a 1/6000 dilution (Sigma). Dots are visualised with the procedure contained in the Immun-Blot Assay kit (Bio-Rad).

2.12. ELISA of Con A fractions

ApoH in both plasma and column fractions was measured by ELISA. We analysed the fractions that were positive in the dot-blotting assay, since this is not suitable for a quantification of very low sample levels. The observation of very low traces of apoH in only one of the three positive fractions suggested that non-specific absorption of antibody may occur on the nitrocellulose. This overestimation due to the enzymatic reaction could mimic a large sample amount.

2.13. Lysine–Sepharose

A lysine–Sepharose column (2.5 ml, Sigma) was equilibrated with 0.1 M phosphate buffer, pH 7.4 and 77 μg of human purified apoH was applied in a 700 μl volume at 0.25 ml/min. The column was then extensively washed with 0.2 M 6-amino-*n*-caproic acid in 0.1 M phosphate buffer, pH 7.4. Eluates containing unbound and bound were collected into 0.3 ml fractions.

3. Results

The technique currently used for the purification and isolation of apoH requires three or more chromatographic steps. Our procedure is much simpler and avoids all precipitating steps that could lead to protein loss. We found that pure apoH could not be obtained with a single affinity chromatography step. Higher molecular weight proteins, in fact, co-eluted with apoH (Fig. 1, lane 1). They were also recognised, albeit with a weaker signal, by the anti-apoH antibodies in a Western blot analysis (data not shown), probably because of the polyclonality of the antibody binding apoH in the column, or because apoH formed indirect bonds with other proteins due to its strong electric charge, as reported by other authors (McNally et al., 1993). Subsequent purification by means of continuous-elution electrophoresis, on the other hand, gave 98–100% pure apoH (Fig. 1, lanes 3 and 5). The purified material displayed only one band reacting with the antisera, suggesting that our procedure included conditions for dissociation of the apoH complexes in plasma or, better still, was selective for free apoH. The final yield after the two purification steps was in agreement with that reported by McNally et al. (1993), who observed binding of apoH to many biological substances by hydrophilic and other interactions.

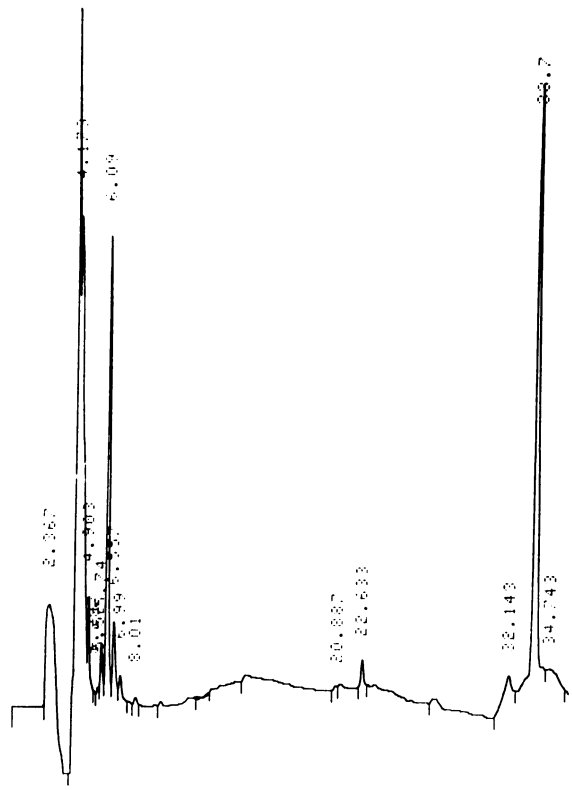

Fig. 2. Chromatographic analysis of apoH performed in HPLC. The pure protein eluted as a single peak with a retention time of 33.7 min, using a 20–70% acetonitrile gradient for 35 min at 1 ml/min. The first peaks of the chromatogram are due to the solvent.

By reverse-phase chromatography on a C_{18} IP Beckman column, the pure protein eluted as a single peak with a retention time of 33.7 min, when a 20–70% acetonitrile gradient was used for 35 min at 1 ml/min. (Fig. 2).

Undigested apoH subjected to lectin analysis gave positive glycan bands with SNA and DSA as shown from different pictures. SNA indicates sialic acid, terminally linked α(2–6) to galactose

or *N*-acetylgalactosamine (Fig. 3, lane 3). DSA indicates galactose-β(1–4) *N*-acetylglucosamine (Fig. 4, lane 3). The reaction with PNA was less positive, but still appreciable (Fig. 5, lane 2). This reaction indicates galactose-β(1–3) *N*-acetylgalactosamine. A very weak reaction with GNA indicates mannose, terminally linked α(1–6 or 1–3 or 1–2) (Fig. 6, lane 2). The reaction with MAA was absolutely negative indicating no sialic acid was terminally linked α(2–3) to galactose. The reactions with ACA and PHA were negative. A bare band was observed with AAA. To differentiate N- and O-linked chains, the carbohydrate residues in the Asn-linked chains were removed with the enzyme N-glycosidase F and apoH was then probed with the same lectins as described above. N-Glycosidase F cleaves all types of asparagine bound N-glycans provided both the amino-group and the carboxyl-group are present in a peptide linkage and that the oligosaccharide has the minimum length as the chitobiose core unit (Tarentino et al., 1985; Chu, 1986). After the enzymatic N-deglycosylation the mass of apoH was substantially reduced as assessed by the increased electrophoretic mobility. Fig. 3, lane 2, showed a 38, 37, 32.5, and a 30.5 kDa band after probing apoH with SNA. The 32.5 kDa band was the main band after N-deglycosylation. This reaction indicates sialic acid, terminally linked α(2–6) to galactose or *N*-acetylgalactosamine in O-glycan structure. The doublet 37 and 38 kDa bands could be N-glycanase proteins. Fig. 4, lane 2, showed that

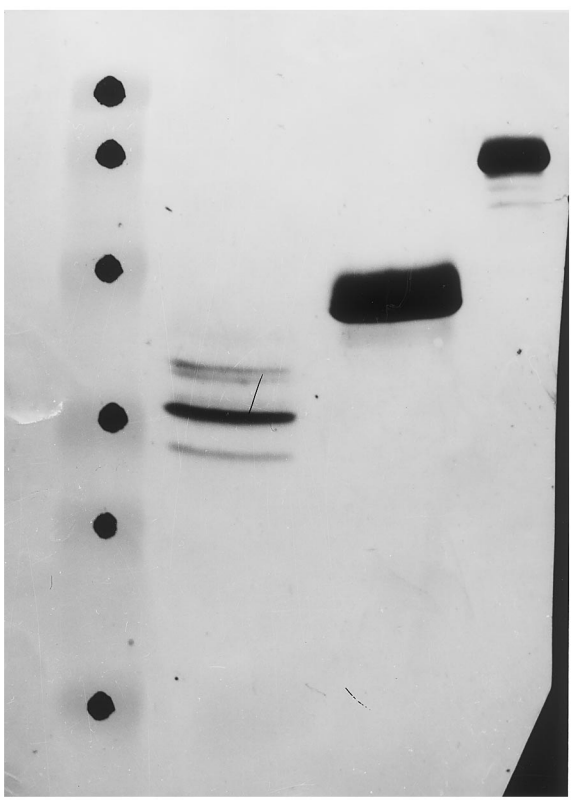

Fig. 3. Western blot of apoH probed with SNA lectin (from left to right): lane 1: prestained molecular mass markers marked with black points are (from top to bottom): 106.0, 80.0, 49.5, 27.5, 18.5 kDa; lane 2: apoH after digestion with N-glycosidase F; lane 3: native apoH; lane 4: transferrin, positive control glycoprotein.

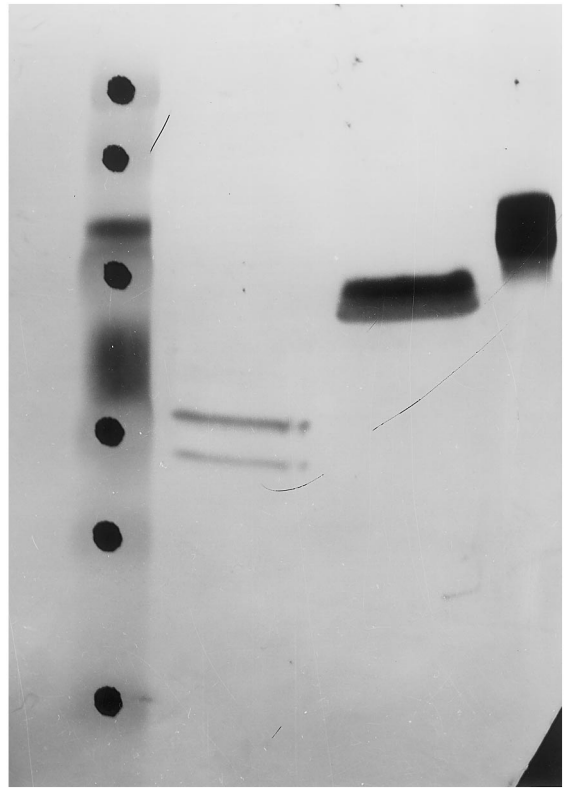

Fig. 4. Western blot of apoH probed with DSA lectin (from left to right): lane 1: prestained molecular mass markers marked with black points are (from top to bottom): 106.0, 80.0, 49.5, 27.5, 18.5 kDa; lane 2: apoH after digestion with N-glycosidase F; lane 3: native apoH; lane 4: fetuin, positive control glycoprotein.

after probing N-digested apoH with DSA lectin a 32.5 and a 30 kDa band appeared. Here, too, the upper band was more intense. ApoH presented galactose-β(1–4) *N*-acetylglucosamine structures in *O*-glycans. Fig. 5, lane 1, showed N-deglycosylated apoH probed with PNA. ApoH displays a 32.5 and a 30.5 kDa band and the upper band is again predominant. This picture indicated galactose-β(1–3) *N*-acetylgalactosamine structures in *O*-glycans. Fig. 6, lane 1, showed that digestion with N-glycosidase F produced a barely detectable 32.5 kDa band. This very weak reaction confirmed the presence of mainly *O*-glycosidically-linked 'high mannose' in apoH.

Glycosylations were also analysed on isoforms separated by IEF. Fig. 7, lane 1, showed small amounts of terminally linked mannose on the three major isoforms (APOH*1, APOH*2 and APOH*3). Lanes 2 and 5 showed that sialic acid, terminally linked α(2–6) to galactose or *N*-acetylgalactosamine and galactose-β(1–4) *N*-acetylglucosamine were distributed among all major isoforms (APOH*1, APOH*2, APOH*3 and APOH*4) and on a minor isoform. Lane 3 demonstrated the presence of galactose-β(1–3) *N*-acetylglucosamine on the major isoforms. Lane 4 showed sialic acid is not terminally linked α(2–3) to galactose. The IEF phenotypes for deglycosylated and native apoH are shown in Fig. 8 (lanes 1, 2, 3, 4, 5). The banding patterns for these two samples were very similar. Four major bands were present in all samples and several minor bands in

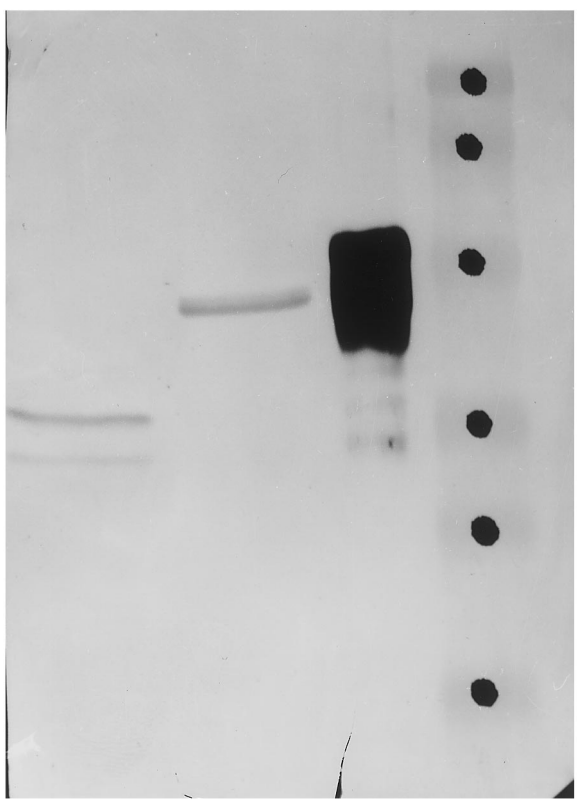

Fig. 5. Western blot of apoH probed with PNA lectin (from left to right): lane 1: apoH after digestion with N-glycosidase F; lane 2: native apoH; lane 3: asialofetuin, positive control glycoprotein; lane 4: prestained molecular mass markers marked with black points are (from top to bottom): 106.0, 80.0, 49.5, 27.5, 18.5 kDa.

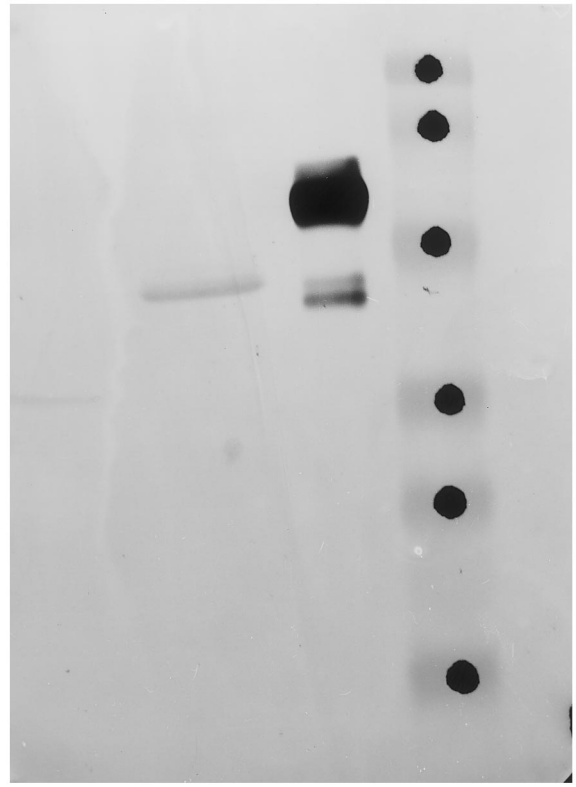

Fig. 6. Western blot of apoH probed with GNA lectin (from left to right): lane 1: apoH after digestion with N-glycosidase F; lane 2: native apoH; lane 3: carboxypeptidase Y, positive control glycoprotein; lane 4: prestained molecular mass markers marked with black points are (from top to bottom): 106.0, 80.0, 49.5, 27.5, 18.5 kDa.

native apoH. N-Deglycosylation did not alter the banding pattern for these two samples significantly and the phenotype was still detectable. Comparison of lanes 1 and 2 with lanes 3–5 indicated that four isoforms were shifted by N-glycosidase F treatment.

Dot-blotting analysis mirrors the chromatographic pattern of apoH eluted from Con A column. ApoH interacted with the Con A lectin. Detectable amounts of protein were not found in the first chromatographic fractions, suggesting that apoH is not eluted with Con A buffer. After adding a buffer with low sugar concentration (10 mM glucoside) a large amount of apoH was recovered. These molecules of apoH weakly bound to the lectin. When a higher sugar concentration (300 mM mannoside) was added, most of the sample eluted as firmly bound apoH. Column fractions positive in the dot-blotting assay were tested for their content of apoH by ELISA. The unbound fraction contains less than 0.1% of the total amount. Weakly bound and firmly bound

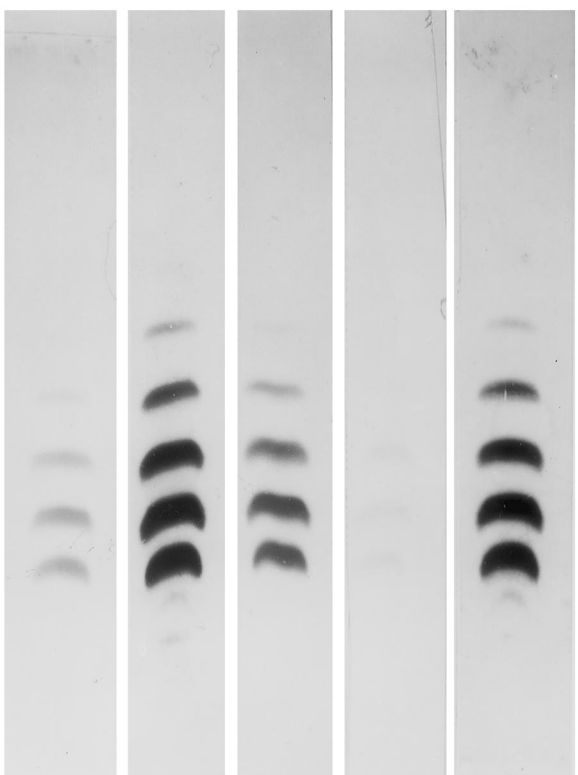

Fig. 7. Oligosaccharides analysis of apoH isoforms separated trough IEF. Purified apoH was subjected to IEF in 5% acrylamide gels with pH 4–8 ampholytes as described in Section 2. From left to right: lane 1: isoforms probed with GNA-lectin; lane 2: isoforms probed with SNA-lectin; lane 3: isoforms probed with PNA-lectin; lane 4: isoforms probed with MAA-lectin; lane 5: isoforms probed with DSA-lectin. Anode is at top; cathode is at bottom.

Fig. 8. Analysis of apoH isoforms separated by IEF. Purified apoH was subjected to IEF in 5% acrylamide gels with pH 4–8 ampholytes and probed with specific antibody. From left to right: lanes 1 and 2: apoH after digestion with N-glycosidase F; lanes 3, 4, and 5: native apoH at different concentrations. Anode is at the top; cathode is at the bottom. The banding patterns for these two samples were very similar. Four major bands were present in all samples and several minor bands in native apoH. N-Deglycosylation did not alter the banding pattern for these two samples significantly and the phenotype was still detectable. Comparison of lanes 1 and 2 with the native sample (lanes 3, 4, and 5) indicated that there were at least four different isoforms shifting with N-glycosidase F treatment.

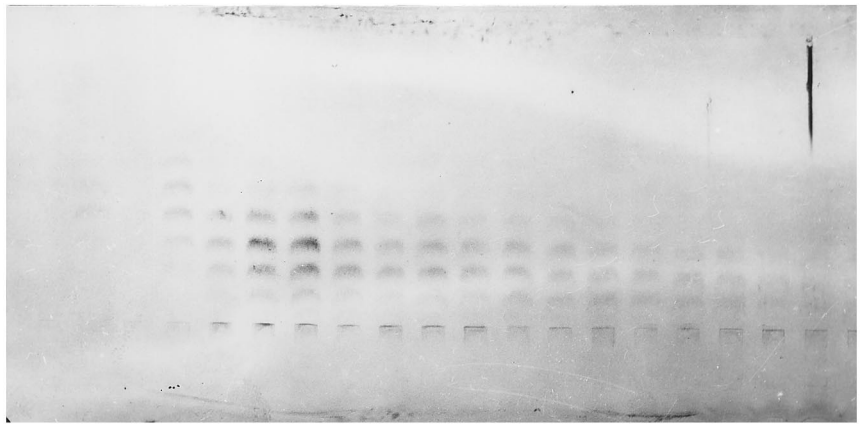

Fig. 9. Analysis of apoH isoforms separated by IEF followed by Western blot: Con A fractions were subjected to IEF in 5% acrylamide gels with pH 4–8 ampholytes, transferred to nitrocellulose and probed with specific antibody. From left to right: unbound fractions (lane 1). Weakly bound fractions contained molecules of apoH having a predominance of more acidic isoforms (lanes 2–16). Anode is at top; cathode is at bottom. The unbound fraction (lane 1) contains no isoforms whereas weakly bound fractions contain molecules of apoH having a predominance of more acidic isoforms (lanes 2–16).

apoH correspond to 18 and 82%. Final recovery ranges from 84 to 101%.

The IEF results of Con A fractions are illustrated in Figs. 9 and 10. The unbound fraction (Fig. 9, lane 1) contains no isoforms. The weakly bound fractions contain apoH molecules with predominantly more acidic isoforms (Fig. 9, lanes 2–16), and the firmly bound fractions contain molecules with predominantly cathodic isoforms (Fig. 10, lanes 8–13). Isoforms APOH*3 and APOH*4 are mostly represented in the molecules of apoH binding weakly to the lectin. Isoforms APOH*1, APOH*2, and APOH*3 are mostly present in the firmly binding fractions (Kamboh et al., 1988).

In the Western blot, weakly bound and firmly bound fractions migrated with an apparent molecular weight of approximately 50 kDa, demonstrating that no proteolytic cleavage had occurred during chromatography. Unbound fractions did not contain apoH (data not shown).

The ability of apoH to bind lysine–Sepharose was also investigated. No interaction between apoH and the lysine–Sepharose was detected. ApoH eluted in the unbound fractions. Samples were subjected to SDS-PAGE followed by Western blotting. No traces of apoH were found in the bound fractions. Glycosylations may thus impair the ability of apoH to bind to lysine–Sepharose.

4. Discussion

The structural and functional integrity of many proteins relies on specific co- and post-translational protein-modification reactions. Asparagine-linked protein glycosylation may serve many diverse roles. Some proteins require N-linked oligosaccharides to maintain proper function (Joao et al., 1992; Rudd et al., 1994) or to be correctly targeted (Pfeffer and Rothman, 1987). N-linked glycosylation occurs cotranslationally (Kiely et al., 1976; Bergman and Kuehl, 1978) and may affect the course of protein folding. Glycosylation serves a vital role in the folding and assembly of viable proteins (Marquardt and Helenius, 1992). It can alter the conformational profile of a polypeptide and allow it to sample conformational space not originally accessible to it. Glycosylation could serve to funnel the nascent polypeptide structure through a particular pathway for folding. In the absence of glycosylation, specific folded intermediates would be inaccessible, and the outcome would be a delinquent protein product.

In apoH, carbohydrates are mainly linked to asparagine residues in the carbohydrate acceptor sequence Asn-X-Ser/Thr (Lennarz, 1980). The oligosaccharides are attached to asparagine

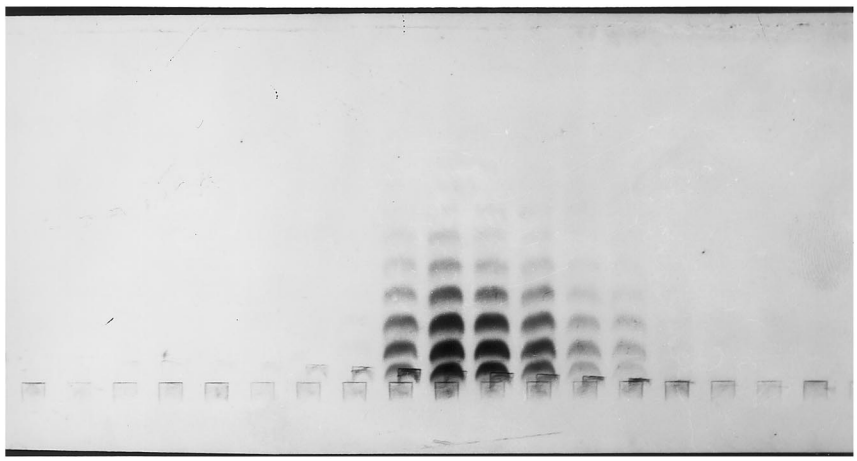

Fig. 10. Analysis of apoH isoforms separated by IEF followed by Western blot: Con A fractions were subjected to IEF in 5% acrylamide gels with pH 4–8 ampholytes, transferred to nitrocellulose and probed with specific antibody. From left to right: firmly bound fractions contained molecules of apoH having a predominance of more cathodic isoforms (lanes 8–13). Anode is at top; cathode is at bottom.

residues at positions 143, 164, 169, 174, and 234 (Lozier et al., 1984). At positions 174 and 234, the acceptor sequence is Asn-Trp-Ser/Thr. This sequence is very rare among glycoproteins (Lozier et al., 1984). ApoH has also O-linked glycosylation.

Our results showed that apoH is rich in sialic acid linked α(2–6) to galactose or *N*-acetylgalactosamine (Gambino et al., 1997a). Sialic acid is not α(2–3) linked to galactose. Galactose is β(1–4) linked to *N*-acetylglucosamine and β(1–3) linked to *N*-acetylgalactosamine. High-mannose N-glycan chains are barely detectable. ApoH is not particularly rich in fucose. To assess whether glycosylations are N-linked or O-linked, apoH was deglycosylated with N-glycosidase F, an enzyme that cleaves all types of asparagine bound N-glycans and thus allows O-linked chains to be detected. After N-deglycosylation the molecular weight of apoH is substantially reduced. The main band is 32.5 kDa. Carbohydrates on apoH are mainly organised in N-linked structures, bound to asparagine residues (Lozier et al., 1984; Gambino et al., 1997a). However, carbohydrate O-linked chains which are bound to serin or threonin are mainly represented by sialic acid α(2–6) linked to galactose or *N*-acetylgalactosamine (Gambino et al., 1997a). Galactose is also organised in O-linked chains and it is β(1–4) linked to *N*-acetylglucosamine and β(1–3) linked to acetylgalactosamine. Galactose-β(1–4) *N*-acetylglucosamine was found in certain mucins while the disaccharide galactose-β(1–3) *N*-acetylgalactosamine usually forms the core unit of O-glycans. Mannose is organised in N-linked structure. (Gambino et al., 1997a). Biochemical analysis of carbohydrate structures conducted on single isoforms separated through IEF reveal that no specific carbohydrate complex is bound to a single isoform. Each type of glycosylation is shared among all isoforms (Gambino et al., 1997a). Since the more acidic isoforms have a higher molecular weight than the more basic isoforms (our observation), they are likely to have more glycosylations.

N-glycosylations do not seem to influence the phenotype of glycoprotein, even if oligosaccharide chains can carry positive or negative charges. After enzymatic N-deglycosylation, in fact, it overlaps that for a glycosylated apoH (Fig. 8). The phenotype of apoH is not dependent on the presence of N-linked carbohydrate chains. However, we have to check whether the O-linked carbohydrate chains that remain after deglycosylation affect the resulting phenotype. Treatment with neuroaminidase alone alters the isoelectric point of glycoprotein. Terminal desialylation may

alter the oligosaccharide charge to mass ratio and thus affect the electrophoretic analysis.

Glycosylation is a very important process since it regulates the structure and the biological functions of proteins. Asparagine glycosylation is very important for the appropriate folding and assembly of intact proteins. Moreover, in some pathologies such as diabetes, glycosylation of proteins is a non-enzymatic process and regarded as one of the factors contributing to its severity of the disease (West, 1982; Lyons, 1992; Cassader et al., 1997). ApoH is rich in lysine residues representing potential sites of non-enzymatic glycation when serum glucose levels are too high. This process of glycation causes functional impairment of proteins and this could be true for apoH. Though the role of apoH in lipid metabolism is still uncertain, recent investigations of the possible relation between plasma apoH levels and increased plasma lipids and the thrombotic risk could explain the increased atherosclerotic risk in diabetic patients (Cassader et al., 1997). Glycation of apolipoproteins generates free radicals and increases oxidative damage, while glycosylated or glycoxidised lipoproteins may be immunogenic and potent stimulators of foam-cell formation and increase the risk of atherosclerosis (Lyons, 1992). Further studies will be needed to evaluate any functional impairments due to non-enzymatic glycation.

We have also studied the molecular structure of the carbohydrate moiety of apoH. Lectin affinity chromatography was used to analyse apoH according to the characteristics of its carbohydrate chain inner to sialic acid residues (Gambino et al., 1997b). Con A has the advantage of sialyl residues not being recognised. It allowed us to isolate two groups of apoH molecules bearing biantennary and truncated hybrids and high mannose and hybrid oligosaccharides. We were able to avoid column overloading since just 55 μg of apoH was applied to 2.5 ml of gel. Most of apoH bound both weakly and firmly to the column. Some material was found only in one of the unbound fractions, but its concentration was extremely low when assayed in an ELISA method. Since the unbound fraction contains less than 0.1% of total apoH applied, this amount can be considered insignificant. After isoelectrofocusing electrophoresis only a few, acidic bands are barely detectable. Isoelectrofocusing of other apoH forms fractionated on Con A demonstrates that weakly bound fractions have more acidic isoforms than firmly fractions and may thus contain more negatively charged molecules. Biantennary and truncated hybrid rather than high mannose-rich or hybrid carbohydrate chains may bear more sialic acid residues and so modify the glycoprotein overall charge (Baenziger and Fiete, 1979; Cummings and Kornfeld, 1982). Sialic acid is a negatively charged terminal sugar which covers penultimate galactose residues. Firmly bound molecules have a predominance of cathodic, less negatively charged isoforms and could be mainly organised in hybrid carbohydrate chains bearing fewer sialic acid residues, rather than in high mannose oligosaccharide structures. On a molecular bases this heterogeneity indicates that each isoform may contain different glycosylation patterns (Sardanons et al., 1987; Green and Baenziger, 1988a,b; Papandreou et al., 1993a,b). Heterogeneity of apoH molecular size and isoelectric point is due to the oligosaccharide units and the degree of sialylation. However, its physiological significance has not been adequately investigated. When the Con A fractions were subsequently subjected to SDS-PAGE, they resolved into an approximately 50 kDa band. Apparent molecular weights were not significantly different, suggesting that lectin chromatography isolates two classes of apoH molecules. The difference between weakly and firmly bound molecules lies in their carbohydrate organisation.

We have here used Con A affinity chromatography to show the high degree of heterogeneity in apoH's glycosylated structure and its different glycosylation. ApoH molecules bear either hybrid structures or biantennary and truncated hybrid structures. High mannose and hybrid structures bear more mannose residues than biantennary and truncated hybrid structure. Since this method allows fractionation of molecules differing in the extent of carbohydrate branching irrespective of the sialyl residues, we can conclude that mannose residues are masked with other sugars such as galactose-β(1–4) *N*-acetylglucosamine, galactose-β(1–3) *N*-acetylgalactosamine and sialic acid

linked α(2–6) to galactose or to *N*-acetylgalactosamine (our observations), or probably capped with sulphated residues (Papandreou et al., 1993b). Thus, according to our results apoH presents truncated hybrid or hybrid-type carbohydrate chains which bear few unmasked mannose residues as terminal sugar.

The failure of apoH to bind lysine–Sepharose could be due to the high degree of glycosylations which might affect its ligand properties. Additionally, if its interaction with lysine–Sepharose mirrors its binding behaviour to fibrin, it has no affinity. Thus, this mechanism, which would localise apoH to regions of lesion development could potentially be neutralised by glycosylation.

The expected significance of the study is that information on glycosylation is needed to understand key processes involved in the regulation of the biological functions of proteins. Asparagine glycosylation is very important for the appropriate folding and assembly of intact proteins. The potential of N-linked oligosaccharides for structural variation is not confined to their chain-terminating sugars. Carbohydrate branching also affects the biological activity of glycoproteins by inducing variations in their tertiary structure (Papandreou et al., 1993a). Moreover, high amounts of sialic acid regulate the blood circulation of glycoproteins by protecting them from hepatic galactose receptor (Morell et al., 1971; Ashwell and Harford, 1982). Thus, further experiments get necessary to understand whether apoH is involved into a similar physiological mechanism.

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