Qualitative analysis of the carbohydrate composition of apolipoprotein H.

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Qualitative Analysis of the Carbohydrate Composition of Apolipoprotein H

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The specific binding of digoxigenin-labeled lectins to carbohydrate moieties is used to characterize the carbohydrate chains bound to apolipoprotein H. Our results show that apolipoprotein H is rich in sialic acid linked $\alpha(2-6)$ to galactose or N-acetylgalactosamine. Sialic acid is not $\alpha(2-3)$-linked to galactose. Galactose is $\beta(1-4)$-linked to N-acetylglucosamine and $\beta(1-3)$-linked to N-acetylgalactosamine. High-mannose N-glycan chains are barely detectable. After N-glycosidase F treatment the molecular weight is substantially reduced. The main band is 32,500 daltons. Carbohydrate O-linked chains, which are mainly represented by sialic acid, are $\alpha(2-6)$-linked to galactose or N-acetylgalactosamine. Galactose is also organized in O-linked chains and it is $\beta(1-4)$-linked to N-acetylglucosamine and $\beta(1-3)$-linked to acetylgalactosamine. Biochemical analysis of carbohydrate structures reveals that no specific carbohydrate complex is bound to a single isoform.

KEY WORDS: Apolipoprotein H; β2-glycoprotein I; lectin affinity chromatography; glycosylation.

1. INTRODUCTION

Apolipoprotein H (apoH)[4] is a plasma glycoprotein which has a molecular weight ranging from 43,000 to 50,000 daltons. It is a structural component of chylomicron, 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 heparin, modulates the activity of adenylate cyclase, and may be involved in blood coagulation, since it inhibits the prothrombinase activity on human platelets (Nakaya *et al.*, 1980; Schousboe, 1985; Nimpf *et al.*, 1986). Compared to other human plasma proteins, apoH has a high content of cystein (6.2%) and proline (8.3%) and it is highly glycosylated (Lozier *et al.*, 1984).

ApoH, also known as β2-glycoprotein I, exhibits a high degree of polymorphism (Sephernia *et al.*, 1989). According to Kamboh *et al.* (1988), apoH has two kinds of polymorphic variants: a postsynthetic 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 reported five glucosamine-attached oligosaccharide side chains are composed of galactose, mannose, N-acetylglucosamine, fucose, and N-acetylneuraminic acid (Kamboh *et al.*, 1988).

In some pathologies such as diabetes, glycation

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[4] Abbreviations: apoH, apolipoprotein H; HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEF, isoelectrofocusing; TBS, tris-buffer saline; EDTA, ethylenediaminetetraacetic acid; 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.

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of proteins occurs as a nonenzymatic process resulting from the high plasma glucose levels (West, 1982). Nonenzymatic glycosylation is thought to be one of the factors contributing to the severity of the disease (West, 1982). Glycation of apolipoproteins can generate free radicals, increasing oxidative damage (Lyons, 1992). Glycated or glycoxidized lipoproteins may be immunogenic and potent stimulators of foam-cell formation and increase the risk of developing atherosclerotic arterial disease (Lyons, 1992).

The aim of this work is to study the types of glycosylations distributed on apoH, how the glycosylations are shared among N-linked and O-linked chains, the distribution of different glycosylations among the main isoforms, and the influence of glycosylations on apoH phenotype. The biochemical analysis of glycations could allow us to clarify those aspects about the metabolism of apoH which are still poorly understood (Wurm *et al.*, 1982; Cassader *et al.*, 1994; Vlachoyiannopoulos *et al.*, 1992; McNeil *et al.*, 1990; Hunt *et al.*, 1993).

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) and 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 digoxigenin-labeled lectins were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany).

2.2. Isolation and Purification of ApoH

ApoH was isolated from human plasma and purified through a combination of affinity chromatography and continuous elution electrophoresis as previously described (Gambino *et al.*, 1996).

2.3. SDS-PAGE

In order to analyze the type of glycosylation, apoH was subjected to 12% SDS-PAGE electrophoresis in a Bio-Rad Mini Protean II apparatus under nonreducing conditions. Electrophoresis buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, *p*H 8.3.

2.4. Western Blot

ApoH was then blotted after SDS-PAGE electrophoresis in 25 mM Tris, 192 mM glycine, and 20% methanol, *p*H 8.3, at 295 mA for 100 min. With this method carbohydrate moieties of glycoproteins bound to nitrocellulose can be characterized.

2.5. Glycoprotein Detection

The specific binding of lectins to carbohydrate moieties is used to identify these structures. The lectins applied are conjugated with the steroid hapten digoxigenin, which enables immunological detection of the bound lectins. When differentiating between carbohydrate structures, lectins which selectively recognize the terminal sugars are used, thus allowing the carbohydrate chain to be identified (DIG Glycan Differentiation Kit). All filters are incubated overnight in blocking solution at 4°C. They are then washed once in 10 mM Tris-HCl, 150 mM NaCl, and 0.01% Thimerosal *p*H 7.6 (TBS) and twice in lectin buffer, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ in TBS. Digoxigen-labeled lectins are diluted in lectin buffer and the filters are incubated at room temperature for 1.5 hr by gentle agitation. When this incubation is completed nitrocellulose membranes are washed three times in TBS at room temperature by gentle agitation to remove unbound lectins. Lectins used are 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), and PHA (*Phaseolus vulgaris* agglutinin). GNA recgonizes terminal mannose, $\alpha(1-3)$, $\alpha(1-6)$- or $\alpha(1-2)$-linked to mannose. SNA recognizes sialic acid linked $\alpha(2-6)$ to galactose. MAA recognizes sialic acid linked

α(2–3) to galactose. PNA recognizes the core disaccharide galactose β(1–3) N-acetylgalactosamine. DSA recognizes Gal-β(1–4) N-acetylglucosamine in complex and hybrid N-glycans and in O-glycans and n-acetylglucosamine in O-glycans (Crowley *et al.*, 1984). ACA shows a high specificity 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 are then incubated with polyclonal sheep anti-dioxigenin Fab fragments, conjugated with alkaline phosphatase for 1.5 hr at room temperature by gentle agitation, and washed three times in TBS. Bands are visualized by the immunoblot assay kit of Bio-Rad.

2.6. Deglycosylation of ApoH

Four μg of apoH in 100 μl was denatured in 0.2% SDS (w/v) by boiling for 2 min. 100 μl buffer for N-glycosidase F incubation (50 mM phosphate, 50 mM EDTA, Nonidet-P 40 1%) was added. These mixtures were again boiled for 2 min, cooled to 37°C, the enzyme added (4 units N-glycosidase F in 200 μl) and incubated for 18 hr at 37°C. Then 50 μl (about 1 μg protein) was loaded into one lane of an SDS gel. Detection of glycation was performed as described above. In order to exclude any aspecific proteolytic digestions due to a long incubation at 37°C, apoH was also incubated without N-glycosidase F.

2.7. Isoelectric Focusing and Western Blotting

Isoelectric focusing 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 (*p*H 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 prefocusing at 500 constant volts for 35 min, 1 μg apoH was applied onto each lane. Electrophoresis was performed at 10 constant watts for 3 hr. After IEF was performed, the proteins were transferred overnight at room temperature onto 0.45-μm-pore size nitrocellulose membrane by simple diffusion. After transfer, the nitrocellulose was incubated with specific antibody anti-apoH or with lectins as described above.

3. RESULTS

Undigested apoH gave positive glycan bands with SNA and DSA. SNA indicates sialic acid, terminally linked α(2–6) to galactose or N-acetylgalactosamine (Fig. 1, lane 3). DSA indicates galactose–β(1–4) N-acetylglucosamine (Fig. 2, lane 3). The reaction with PNA was less positive than the previous ones, but it was still appreciable (Fig. 3, lane 2). A positive reaction with PNA 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. 4, lane 2). The reaction with MAA was absolutely negative, indicating no sialic acid was terminally linked α(2–3) to galactose. The reactions with

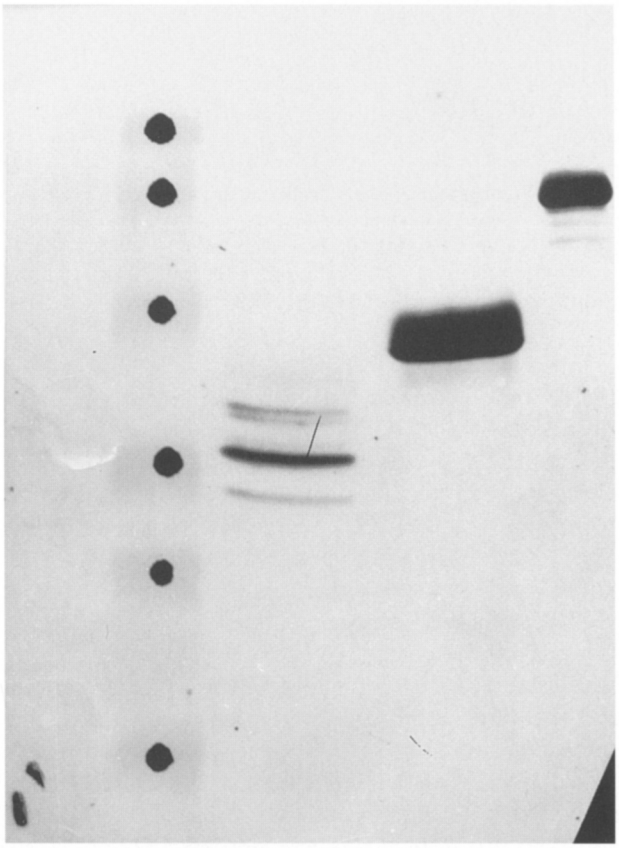

Fig. 1. Western blot of apoH probed with SNA lectin. From left to right: Lane 1; prestained molecular mass markers at (from top to bottom) 106.0, 80.0, 49.5, 27.5, and 18.5 kDa; lane 2, apoH after digestion with N-glycosidase F; lane 3, native apoH; lane 4, transferrin, positive control glycoprotein.

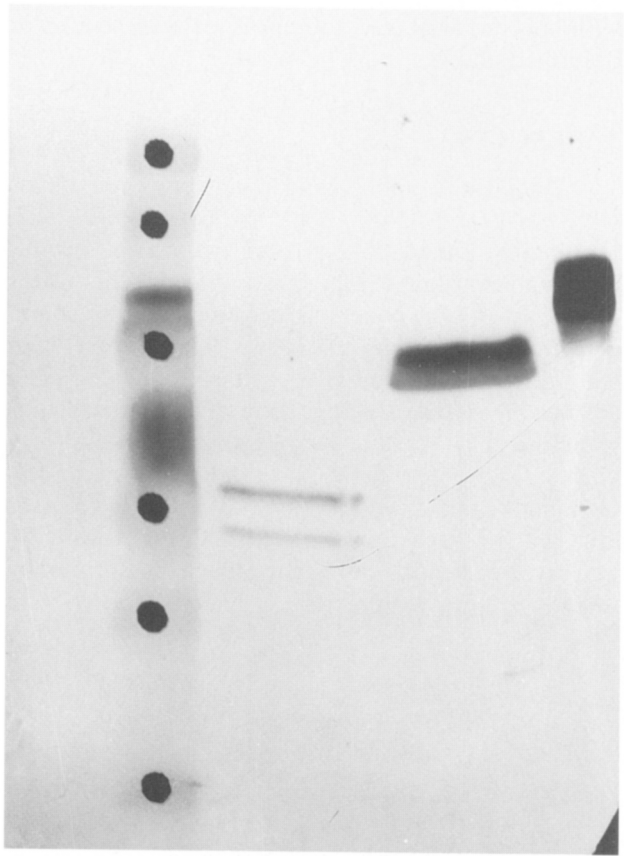

Fig. 2. Western blot of apoH probed with DSA lectin. From left to right. Lane 1, prestained molecular mass markers at (from top to bottom) 106.0, 80.0, 49.5, 27.5, and 18.5 kDa, lane 2, apoH after digestion with N-glycosidase F; lane 3, native apoH; lane 4, fetuin, positive control glycoprotein.

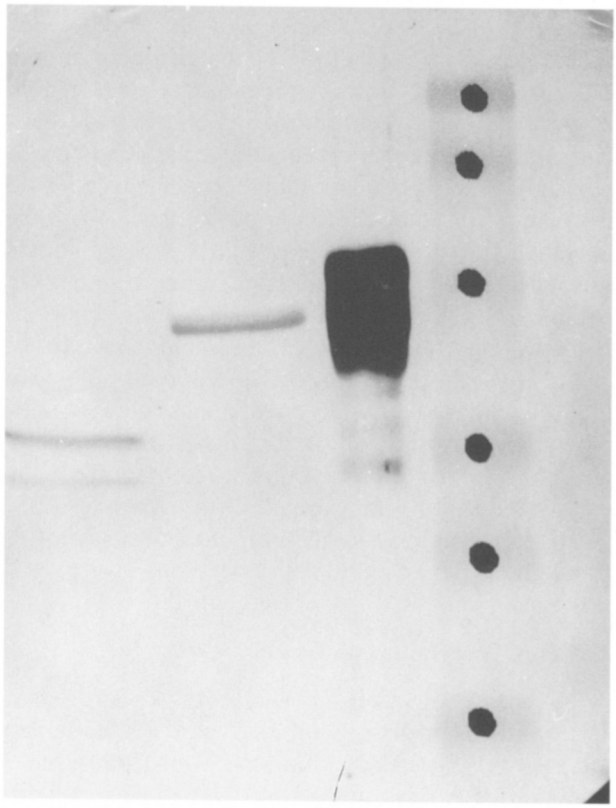

Fig. 3. Western blot of apoH probed with PNA Lectin. From left to right: Lane 1, apoH after digestion with N-glycosidase F, lane 2, native apoH, lane 3, asialofetuin, positive control glycoprotein; lane 4, prestained molecular mass markers at (from top to bottom) 106.0, 80.0, 49.5, 27.5, and 18.5 kDa.

ACA and PHA were negative. A bare band was observed with AAA (Fig. 5, lane 2). For differentiating between N- and O-Linked chains the carbohydrate residues in the Asn-linked chains were removed with the enzyme N-glycosidase F and after the enzymatic digestion apoH was probed with the same lectins as described above. N-Glycosidase F cleaves all types of asparagine-bound N-glycans provided that the amino group as well as the carboxyl group are present in a peptide linkage and that the oligosaccharide has the minimum length of the chitobiose core unit (Tarentino *et al.*, 1985; Chu, 1986). After the enzymatic N-deglycation the mass of apoH was substantially reduced as assessed by the increased electrophoretic mobility.

Figure 1, lane 2, shows four bands after probing apoH with SNA. The molecular weights of these bands are 38,000, 37,000, 32,500, and 30,500 daltons, respectively. After N-deglycosylation a main band of 32,500 resulted. This reaction indicates sialic acid, terminally linked $\alpha(2\text{–}6)$ to galactose or N-acetylgalactosamine in O-glycan structure. The doublet bands at approximately 37,000 and 38,000 daltons could be N-glycanase proteins. Figures 2, lane 2, shows that after probing N-digested apoH with DSA lectin two bands of 32,500 and 30,000 daltons appear. Also in this case the most intense band is the upper one. ApoH presented galactose-$\beta(1\text{–}4)$ N-acetylglucosamine structures in O-glycans. Figure 3, lane 1, shows N-deglycosylated apoH probed with PNA. ApoH showed two bands whose molecular weights were always 32,500 and 30,500 daltons, respectively. The upper band was always predominant. This indicated galactose-$\beta(1\text{–}3)$ N-acetylgalactosamine in O-glycans structure. Figure 4, lane 1, shows that digestion with N-glycosidase F produces a barely detectable band of 32,500 daltons. This very weak

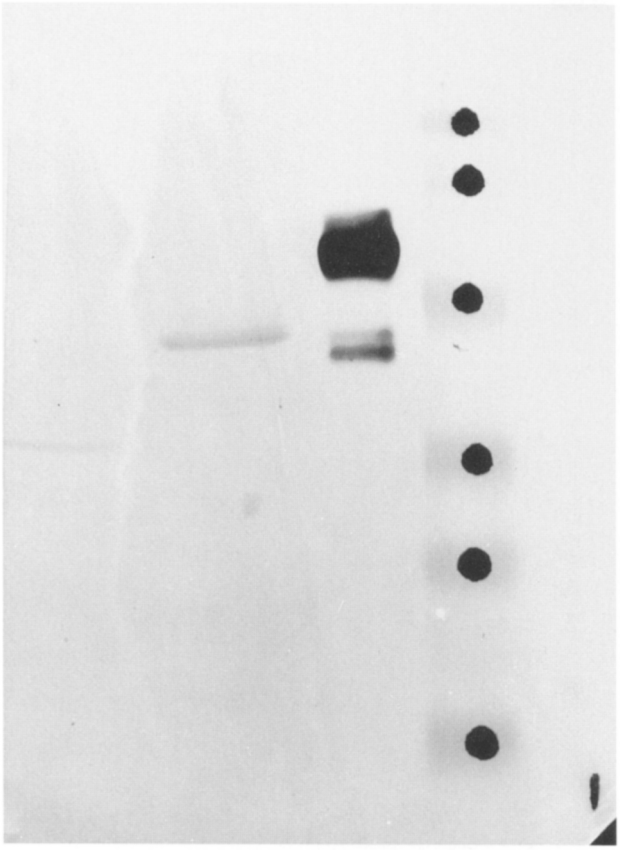

Fig. 4. Western blot of apoH probed with GNA lectin. From left to right: Lane 1, apoH after digestion with N-glycosidase F; lane 2, native apoH; lane 3, carboxypeptidase Y, positive control glycoprotein, lane 4, prestained molecular mass markers at (from top to bottom) 106.0, 80.0, 49.5, 27.5, and 18.5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

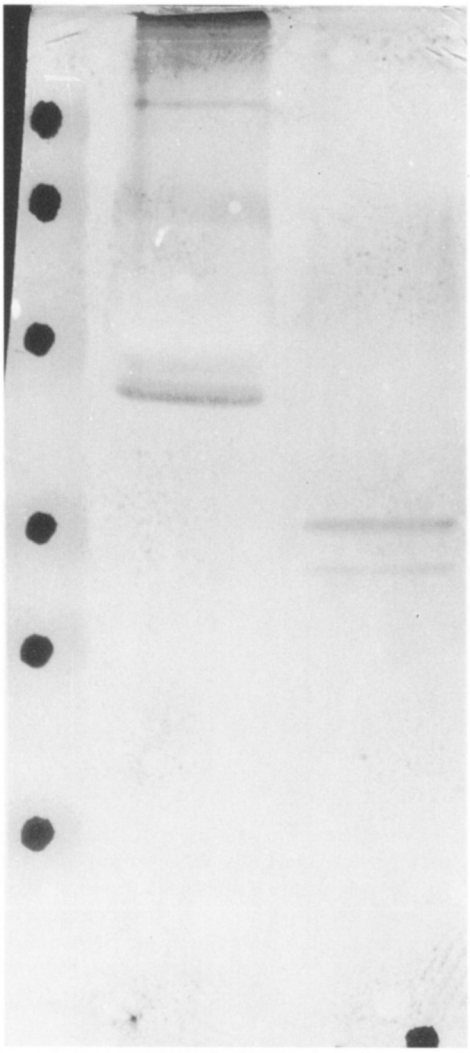

Fig. 5. Western blot of apoH probed with AAA lectin. From left to right: Lane 1, prestained molecular mass markers at (from top to bottom) 106.0, 80.0, 49.5, 27.5, and 18.5 kDa; lane 2, native apoH; lane 3, apoH after digestion with N-glycosidase F.

reaction confirmed the presence of mainly O-glycosidically linked "high mannose" in apoH. Figure 5, lane 3, shows that also after enzymatic N-digestion the reaction was still positive.

The same analysis of glycosylations was carried out on isoforms separated through IEF. Figure 6, lane 1, shows that terminally linked mannose was present in a small amount in the three major isoforms (APOH*1, APOH*2, and APOH*3). Lanes 2 and 5 show that sialic acid terminally linked $\alpha(2\text{–}6)$ to galactose or N-acetylgalactosamine and galactose-$\beta(1\text{–}4)$ N-acetylglucosamine were distributed among all major isoforms (APOH*1, APOH*2, APOH*3, and APOH*4) and in a minor isoform. Lane 3 proves the presence of galactose-$\beta(1\text{–}3)$ N-acetylglucosamine on the most important isoforms of apoH. lane 4 shows that sialic acid is not terminally linked $\alpha(2\text{–}3)$ to galactose.

The IEF phenotypes for deglycosylated and native apoH are shown in Fig. 7 (lanes 1 and 2 and lanes 3–5, respectively). The banding patterns for these two samples are very similar. Four major bands are present in all samples and several minor bands in native apoH. N-Deglycosylation did alter the banding pattern for these two samples significantly, so that the phenotype was still detectable. Comparison of lanes 1 and 2 with the native sample (lanes 3–5) indicates that there are at least four different isoforms shifting with N-glycosidase F treatment.

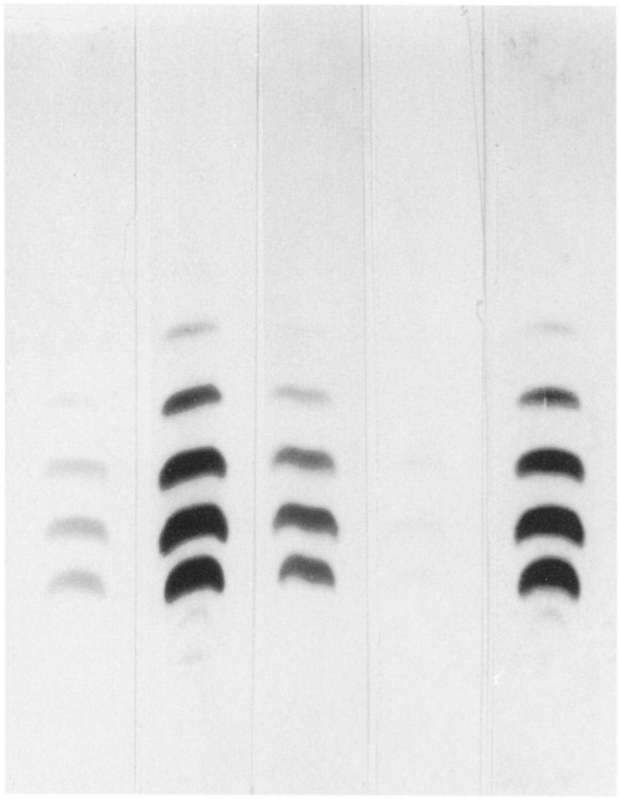

Fig. 6. Oligosaccharide analysis of apoH isoforms separated through IEF. Purified apoH was subjected to denatured IEF in 5% acrylamide gels with *p*H 4–8 ampholytes as described in Section 2. From left ro right: Lane 1, isoforms probed with GNA-lectin; lane 2, isoforms probed with SNA-lectin; lane 3, 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.

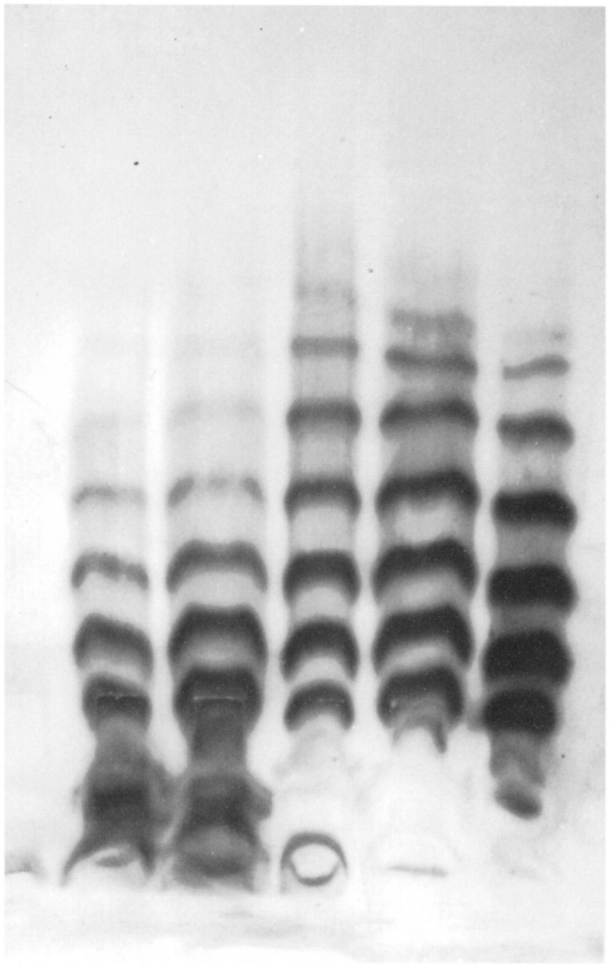

Fig. 7. Analysis of apoH isoforms separated through IEF. Purified apoH was subjected to denaturated IEF in 5% acrylamide gels with *p*H 4–8 ampholytes and probed with specific antibody. From left to right: Lanes 1 and 2, apoH after digestion with N-glycosidase F; lanes 3–5, native apoH at different concentrations. Anode is at top; cathode is at bottom.

4. DISCUSSION

The structural and functional integrity of many proteins relies on specific co- and posttranslational 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 (Bergman and Kuehl, 1978; Kiely *et al.*, 1976) and has the potential to affect the course of protein folding. Glycosylation serves a vital role in the folding and assembly of viable proteins (Marquardt and Helenius, 1992). Glycosylation can alter the conformational profile of a polypeptide and allow it to sample conformational space not originally accessible to it. The glycosylation event 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 residues at positions 143, 164, 169, 174, and 234 (Lozier *et al.*, 1984). At asparagine-174 and asparagine-234 the acceptor sequence is Asn-Trp-Ser/Thr. This sequence is very rare among glycoproteins (Lozier *et al.*, 1984). ApoH also has O-linked glycosylation.

Our results showed that apoH is rich in sialic acid linked $\alpha(2-6)$ to galactose or N-acetylgalactosamine. Sialic acid is not $\alpha(2-3)$-linked to galactose. Galactose is $\beta(1-4)$-linked to N-acetylglucosamine and $\beta(1-3)$-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 the enzyme N-glycosidase F. N-Glycosidase F cleaves all types of asparagine-bound N-glycans, allowing O-linked chains to be detected. After N-deglycosylation the molecular weight of apoH is substantially reduced. The main band is 32,500 daltons. Carbohydrates on apoH are mainly organized in N-linked structures bound to asparagine residues, as published previously (Lozier *et al.*, 1984). However, carbohydrate O-linked chains which are bound to serin or threonin are mainly represented by sialic acid $\alpha(2-6)$-linked to galactose or N-acetylgalactosamine. Galactose is also organized in O-linked chains and it is $\beta(1-4)$-linked to N-acetylglucosamine and $\beta(1-3)$ linked to acetylgalactosamine. Galactose-$\beta(1-4)$ N-acetylglucosamine was found in certain mucins, while the disaccharide galactose-$\beta(1-3)$ N-acetylgalactosamine usually forms the core unit of O-glycans. Mannose is organized in an N-linked structure.

Biochemical analysis of carbohydrate structures conducted on single isoforms separated through IEF reveals that no specific carbohydrate complex is bound to a single isoform. Each type of glycosylation is shared among all isoforms. Since the molecular weight of the most acidic isoforms is higher than the molecular weight of the most basic isoforms (our observation), it is likely that acidic isoforms have more glycosylations.

N-Glycosylations do not seem to influence the phenotype of the glycoprotein even if oligosaccharide chains can carry positive or negative charges. As a matter of fact, after enzymatic N-deglycosylation the phenotype overlaps that for a glycated apoH (Fig. 7). 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 left on the molecule after deglycosylation have some effects on the resulting phenotype. On the other hand, treatment with neuroaminidase alone alters the isoelectric point of the glycoprotein. Terminal desialylation may alter the oligosaccharide charge-to-mass ratio and therefore affect the electrophoretic analysis.

Glycosylation is a very important process since it is able to regulate the structure and the biological functions of proteins. In particular, asparagine glycosylation is very important for the appropriate folding and assembly of intact proteins. Moreover, in some pathologies, such as diabetes, glycation of proteins occurs as a nonenzymatic process. ApoH is rich in lysine residues, representing potential sites of nonenzymatic glycosylation when serum glucose levels are too high. This process of glycosylation causes a functional impairment of proteins and it could also occur for apoH. Nonenzymatic glycosylation is thought to be one of the factors contributing to the severity of the disease (Lyons, 1992; West, 1982). Glycation of apolipoproteins can generate free radicals, increasing oxidative damage, and glycated or glycoxidized lipoproteins may be immunogenic and potent stimulators of foam-cell formation and increase the risk of developing atherosclerotic arterial disease (Lyons, 1992).

Further studies will be needed to evaluate any functional impairments due to nonenzymatic glycation.

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