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# EVIDENCE FOR UNIFORMITY OF THE CARBOHYDRATE CHAINS IN INDIVIDUAL GLYCOPROTEIN MOLECULAR VARIANTS

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SUMMARY.- Pooled and alkylated $\alpha_1$-acid glycoprotein was fractionated on a Con A-Sepharose column into two fractions : Con A-non reactive and Con A-reactive. The carbohydrate moiety from the $\alpha_1$-acid glycoprotein Con A-reactive variant, obtained by hydrazinolysis and quantitative re-N-acetylation, contains only identical two-branched oligosaccharide chains. From the present work on $\alpha_1$-acid glycoprotein and from previous studies on $\alpha_1$-fetoprotein, one can assume that glycoprotein glycosylation occurs uniformly along each polypeptide chain giving it identical oligosaccharide units at each glycosylation site.

## INTRODUCTION

Most of glycoproteins exhibit molecular heterogeneity upon lectin-affinity experiments, indicating variations in their carbohydrate moieties (1). However, structural studies of asparagine-linked glycans have been performed generally using the glycopeptides obtained by exhaustive enzymic digestion of unfractionated, heterogeneous molecular populations of the glycoprotein studied. Then, if more than one glycosylation site and several glycan structures are characterized for a given microheterogeneous glycoprotein, it remains to determine (i) whether the different glycan units are unevenly distributed on each individual molecular variant of the glycoprotein or, on the contrary, (ii) whether each of these individual molecular variants carries only one type of carbohydrate chain.

From our previous studies on rat alpha-fetoprotein (AFP) (2, 3), evidence was obtained which favours the second possibility : the Con A-reactive AFP variant contains two identical Con A-reactive, two-branched glycan units and the Con A-non reactive AFP variant possesses two other identical Con A-non reactive, two-branched glycan units with an additional N-acetylglucosamine residue on the β-mannose.

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In order to extend this observation to less simple heterogeneous glycoproteins than rat AFP (6 % carbohydrate content), we have dealt in the present work with the study of human $\alpha_1$-acid glycoprotein (AGP). This glycoprotein contains about 43 % carbohydrate distributed onto five glycosylation sites (4, 5) as bi, tri and tetra-branched glycan structures (6). Fractionation of AGP on Con A-Sepharose and characterization of the glycans of the Con A-reactive variant of AGP have indicated that this variant at least contains only one type (two-branched) of glycan unit. These results lead us to consider the concept of the glycan-uniformity of individual glycoprotein molecular variant in the fondamental process of protein glycosylation.

## MATERIAL AND METHODS

*Glycoprotein isolation.* Human AGP was isolated from a pool of normal human plasma by a method described by Bürgi and Schmid (7).

*Reduction and alkylation of the glycoprotein.* Human AGP was reduced and alkylated with 0.1 mCi of iodo [$^{14}$C] acetamide (specific activity 53 mCi/mmole) according to the method of Crestfield *et al.* (8).

*Affinity chromatography.* $\alpha_1$-acid glycoprotein was chromatographed on Con A-Sepharose column as described for rat $\alpha_1$-fetoprotein (9). Some commercial Con A-linked agarose or Sepharose display different glycoprotein affinity patterns (10). We used commercial Con A coupled with Sepharose 4B previously activated according to the CNBr method of March *et al.* (11). The $\alpha_1$-acid glycoprotein elution profile was obtained by rocket immuno-electrophoresis of each fraction on anti-AGP impregnated agarose plates (12) (anti-AGP was purchased from Immuno, Diagnostic Division, Belgium). Glycoproteins were also analysed by crossed-affino-immuno-electrophoresis with free Con A in the first dimension gel as previously described (13, 14).

*Exhaustive hydrazinolysis of the glycoprotein.* $\alpha_1$-acid glycoprotein or its Concanavalin A-affinity variants were subjected to hydrazinolysis as previously described (15, 16). Human $\alpha_1$-acid glycoprotein (1 to 5 mg) was treated with distilled hydrazine (0.5 ml) in screw-cap vials at 100°C for 30 hours. The reaction mixture was then evaporated under a nitrogen stream. The dry residue was dissolved in 1 ml of 20 % acetic acid previously cooled to 4°C. The mixture was then desalted on a Biogel P 6 column (1.5 x 30 cm). and purified de-N-acetylated glycans were re-N-acetylated with $^{14}$C acetic anhydride (17).

*Thin layer chromatography.* A thin layer chromatographic system was used for the separation of glycoprotein-derived oligosaccharides (2).

*Carbohydrate analysis.* The molar ratio of monosaccharides was estimated by gas liquid chromatography according to Zanetta *et al.* (18) after methanolysis with methanol-0.5 M HCl at 80° for 20 hours (19). Total carbohydrates were determined by classical colorimetric methods (20).

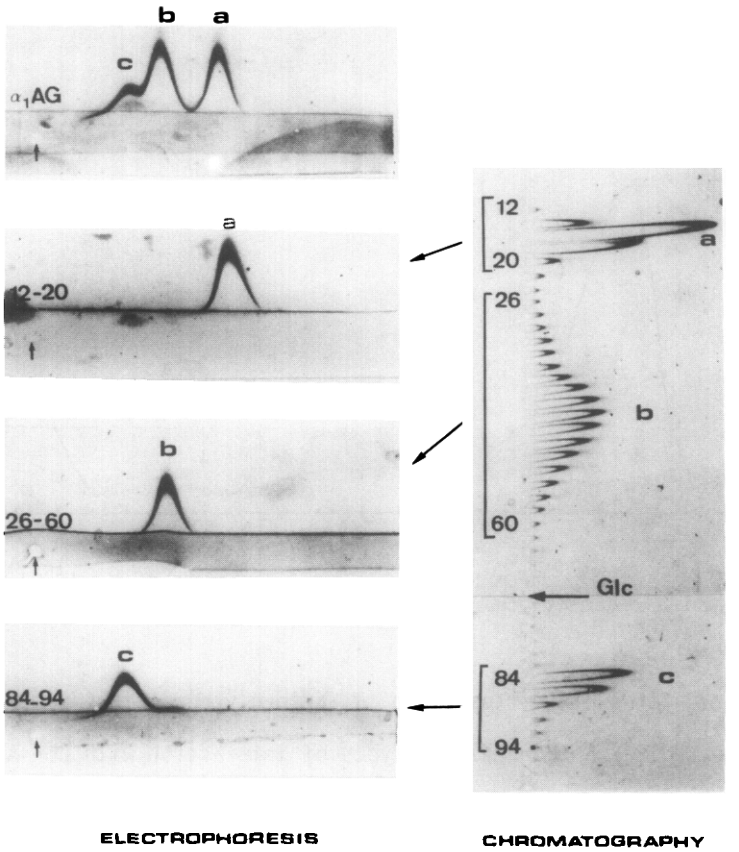


Figure 1.- Right : Elution pattern of human AGP on Con A-Sepharose column. Peaks a (fractions 12-20) and b (fractions 26-60) were eluted with 0.05 M Tris-HCl (pH 7.6) 1 M NaCl/1 mM $MgCl_2$/1 mM $MnCl_2$/1 mM $CaCl_2$. Addition of 0.15 M methyl-α-D-glucose resulted in the elution of the Con A-reactive fraction c. Left : Affino-immuno-electrophoresis sequential analysis of the chromatographic fractions eluted from the Con A-Sepharose column.

## RESULTS

*$\alpha_1$-Acid glycoprotein heterogeneity on Con A-Sepharose.* Upon affinity chromatography on Con A-Sepharose, $\alpha_1$-acid glycoprotein can be resolved into three fractions : (a) Con A-non reactive, (b) Con A-weakly reactive, (c) Con A-reactive. Fractions a and b are eluted with the equilibration buffer and c is specifically desorbed by addition of 0.15 M methylglucoside. As shown in figure 1 the same Con A affinity pattern is obtained by affino-immuno-electrophoresis using free Con A in the first dimension gel. The percentage of each molecular form is approximately : peak a 40.6 %, peak b 43.7 %, and peak c 15.6 %.

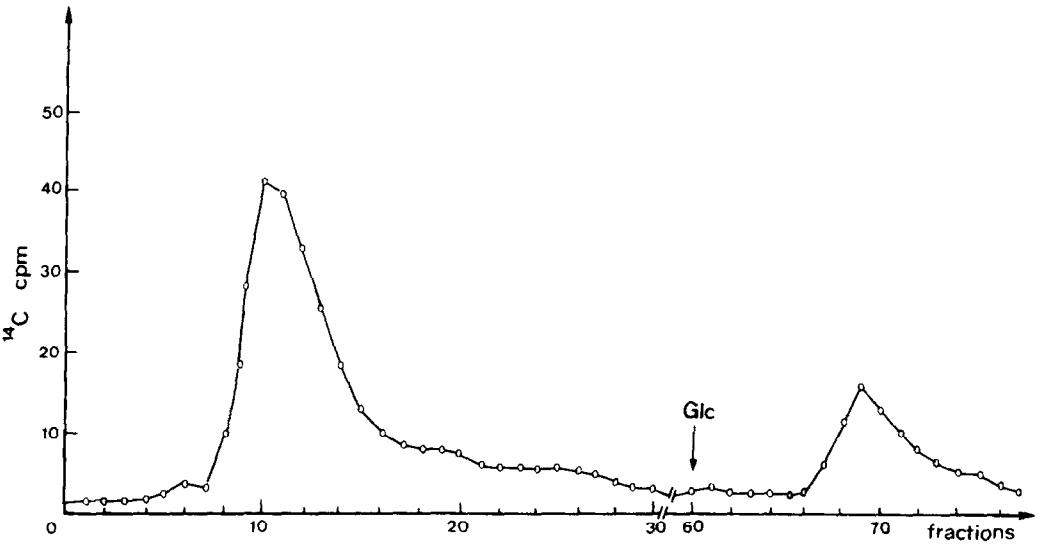


Figure 2.- Affinity chromatography on Con A-Sepharose of the [$^{14}$C] S-carboxymethylated $\alpha_1$-acid glycoprotein. Elution conditions were the same as detailed in figure 1.

As for rat $\alpha_1$-fetoprotein, the three $\alpha_1$-acid glycoprotein Con A affinity fractions reflect different degrees or types of interaction with Con A (9). After S-carboxymethylation of AGP only two peaks are obtained (Fig. 2), the Con A-non reactive peak, and the Con A-reactive peak ; the Con A-weakly reactive fraction completely disappears. So, we can assume that the presence of this fraction is due to hydrophobic interaction and/or special conformation of the polypeptide backbone. To avoid these non sugar-specific interactions of AGP with Con A we have studied only the labeled S-carboxymethylated Con A-non reactive and reactive AGP variants. The carbohydrate molar ratios of the two Con A fractions (non reactive and reactive), calculated on the basis of three mannose residues per glycans, were found to be very different (Table I). The Con A-non reactive $\alpha_1$-acid glycoprotein variants possess a molar composition of the tetra-branched oligosaccharide chain whereas the Con A-reactive fraction exhibits a two-branched carbohydrate composition.

*Oligosaccharides isolation.* The carbohydrate chains from the two S-carboxymethylated $\alpha_1$-acid glycoprotein Con A variants were obtained by exhaustive hydrazinolysis followed by quantitative re-N-acetylation. The hydrazinolysis

Table I.- Molar ratios of monosaccharides present in S-carboxymethylated AGP Con A fractions and in their derived glycans.

| Monosaccharides | S-carboxymethylated AGP | | Glycans from | |
|---|---|---|---|---|
| | Con A non reactive Peak 1 | Con A reactive Peak 2 | Peak 1 | Peak 2 |
| Man[a] | 3.0 | 3.0 | 3.0 | 3.0 |
| Gal | 4.3 | 2.4 | 4.0 | 2.1 |
| GlcNAc | 6.3 | 4.4 | 6.4 | 3.9 |
| Fuc | 0.8 | 0 | 0.7 | 0 |
| NANA | 4.3 | 2.0 | 4.1 | 1.7 |

[a]Molar ratio on the basis of 3 moles of mannose per carbohydrate side chain.

procedure breaks the N-glycosyl asparagine amide linkage and quantitatively releases oligosaccharide units (21). During this treatment acetamido linkages of N-acetylglucosamine and N-acetylneuraminic acid residues are also cleaved. Because of low quantities of material generally used, oligosaccharides chains were quantitatively re-N-acetylated with [$^{14}$C] acetic anhydride and characterized by thin layer chromatography.

As expected, thin layer chromatography of the glycans obtained from unfractionated $\alpha_1$-acid glycoprotein reveals (Fig. 3) a very complex pattern corresponding to the mixture of the different oligosaccharide chains described by others (6). The Con A-non reactive AGP fraction presents the same complex pattern of glycans as the unfractionated glycoprotein except for the fastest moving glycan (Fig. 3) which is absent. This fast moving component is, in fact, recovered as the unique one in the pattern of the Con A-reactive $\alpha_1$-acid glycoprotein fraction.

As shown in figure 3, this fast moving oligosaccharide and the two-branched oligosaccharide chain of rat AFP possess an identical migration rate.

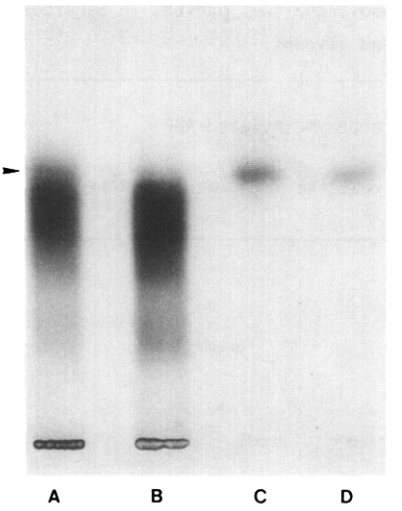


Figure 3.- Thin layer chromatography of glycans which derived from S-carboxymethylated AGP (A) and their 2 Con A fractions : non reactive (B) and reactive (C). (D) Rat AFP two-branched glycan having the same structure than the human AGP two-branched oligosaccharide chain (6).

Moreover the carbohydrate molar ratio of this glycan (Table I), calculated on the basis of 3 mannose residues per glycan, confirmed the carbohydrate analysis made on the Con A affinity variant of intact $\alpha_1$-acid glycoprotein.

## DISCUSSION

In order to determine the specificity of glycosyltransferase system which catalyse the successive steps of the biosynthesis of the carbohydrate chains of glycoproteins, it is necessary to establish the precise chemical structure of glycans and their localization at each glycosylation site. The presence of an unique two-branched oligosaccharide chain at each carbohydrate site of the Con A reactive $\alpha_1$-acid glycoprotein variant and the absence of this type of glycan in the Con A-non reactive molecule was additional support for the following general concept : each glycoprotein variant contains identical complex type asparagine linked oligosaccharide chains at each glycosylation site of the molecule.

It is now established that biosynthesis of asparagine linked oligosaccharide "first involves the *en bloc* transfer of a high mannose oligosaccharide

chain from a lipid carrier to the nascent polypeptide chain in the rough endoplasmic reticulum, followed in the Golgi apparatus by the processing of the oligosaccharide to the mature complex type carbohydrate chain" (22-24, for a review see 25). It has been shown that in most glycoproteins, glycosylation of N-asparagine glycans occurs in β-turn area (26). Yet recent preliminary results, concerning the fact that a high mannose oligosaccharide chain was not processed into a mature complex type carbohydrate chain, could be explained by the fact that some particular β-turns become less accessible due to the folding of the polypeptide chain (Aubert, J.P., Helbecque, N. and Loucheux-Lefebvre, M.H., personal communication). This observation could explain the coexistence of high mannose oligosaccharide chain with complex type asparagine linked oligosaccharide chains in glycoproteins (27).

From the results presented here, one can expect that other glycoprotein variants may possess the same oligosaccharide uniformity along the polypeptide chain as that described for rat $\alpha_1$-fetoprotein. This concept allows the simplest view of the process of maturation of the carbohydrate chain and assumption that each carbohydrate site of the polypeptide chain would be processed by the same defined glycosyltransferase system. Since molecular microheterogeneity in oligosaccharide chains exists, it can be speculated that it will be possible to find the corresponding microheterogeneous glycosyltransferase systems. It still remains unknown if these different enzymic systems which catalyse the successive steps of addition of monosaccharide units are present either in well defined areas within the same Golgi apparatus or in different types of cells and tissues as has been demonstrated for specific sialyltransferases (25).

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