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Fractionation of the genetic variants of human α_1-acid glycoprotein in the native form by chromatography on an immobilized copper(II) affinity adsorbent

Heterogeneity of the separate variants by isoelectrofocusing and by concanavalin A affinity chromatography

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ABSTRACT

Fractionation of the three main genetic variants (F1, S and A) of human α_1-acid glycoprotein (AAG), in their native (sialylated) form, by chromatography on immobilized copper(II) affinity adsorbent was investigated. This chromatographic method had been previously developed to fractionate the desialylated protein variants. For that purpose, the three main AAG phenotypes samples (F1S/A, F1/A and S/A), which had been previously isolated from individual human plasma samples, and an AAG sample from commercial source (a mixture of the phenotypes) were used in the native form. Affinity chromatography of these different samples on an iminodiacetate Sepharose–copper(II) gel at pH 7 resolved two protein peaks, irrespective of the origin of the native AAG sample used. The unbound peak 1 was found to consist of the F1, the S or both variants, depending on the phenotype of the AAG sample used in the chromatography. The bound peak 2 was found to consist of the A variant in a pure form. The fractionation results obtained with native AAG were found to be the same as those originally yielded by the desialylated protein. However, comparison of the interactions of native and desialylated AAG with immobilized copper(II) ions, using an affinity chromatographic method and a non-chromatographic equilibrium binding technique, respectively, showed that desialylation increased the non-specific interactions of the protein with immobilized copper(II) ions. The AAG variants were not fractionated when affinity chromatography was performed using immobilized zinc, nickel or cobalt(II) ions, instead of copper. After purification of each variant in the sialylated form (F1, S and A), their respective heterogeneity was studied by analytical isoelectrofocusing with carrier ampholytes in the pH range 2.5–4.5. In addition, the lectin-binding behaviour of the separate sialylated AAG variants was investigated by affinity chromatography on immobilized concanavalin A.

INTRODUCTION

Human α_1-acid glycoprotein (AAG) is a plasma protein with a peptide chain of 181 amino acids and characterized by an unusually high carbohydrate content (up to 45% of the total molecular mass) and a large number of sialyl residues [1]. The AAG primary structure is complicated by an important heterogeneity, which is found both in its amino acid composition and in its car-

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bohydrate moiety. The heterogeneity in the amino acid composition is revealed when the desialylated form of AAG is analysed by isoelectrofocusing (IEF) [2]. Three main variants are distinguished by their electrophoretic migration, one "fast" and two "slow" bands, corresponding to the F1, S and A variants, respectively. This polymorphism is generated by the presence of two different genes, A and B/B', which code for AAG in humans [3], and by the presence of several allelic forms of the two genes in the human population [4]. Genetic studies have shown that the A variant (encoded by the AAG B/B' gene) differs from variants F1 and S (encoded by two alleles of the same gene, AAG A) by 22 amino acid substitutions [5]. Three main AAG phenotypes are observed in the human population, roughly 50% with F1S/A, 35% with F1/A and 15% with S/A, according to the concentrations of the F1, S and A variants in desialylated plasma [4].

The heterogeneity in the carbohydrate moiety is revealed when native AAG is analysed by IEF [6]. The protein shows an isoelectric point (p*I*) heterogeneity in the number of bands revealed (five, six, seven or eight). This heterogeneity appears to be genetically determined and is due to different linkages of the terminal sialic acid residues [1]. These different linkages yield the polymorphic forms of AAG. No clear correlation had been established between the polymorphic forms and the variants of AAG.

Human AAG also exhibits heterogeneous lectin-binding behaviour [7]. However, this heterogeneity, which concerns structural differences in the glycan chains, is not genetically determined.

The reason for the genetic polymorphism of AAG is still unknown. It has been suggested that this polymorphism may be related to the existence of different functional forms of the protein. However, the separate variants of AAG were needed to test this hypothesis.

We had previously developed a fractionation method for desialylated AAG variants by chromatography on an immobilized affinity copper(II) adsorbent [8]. We have now investigated the possibility of using this chromatographic method for the separation of the AAG variants in the native (sialylated) form. For that purpose, we separately purified the three main AAG phenotypes (F1S/A, F1/A and S/A) from individual human plasma [9] and then used these native AAG samples in affinity chromatography on immobilized copper(II) ions. The chromatographic fractionation was compared with the electrophoretic heterogeneity of the three main AAG phenotypes samples, as visualized by analytical IEF.

The quantitative affinity relationships of immobilized copper(II) ions with native AAG were also determined by using a non-chromatographic protocol, previously described by Hutchens and Yip [10], and the results were compared with those obtained with desialylated AAG.

In addition, the heterogeneity on IEF and the lectin-binding behaviour of the AAG variants, after their fractionation in the native form, were studied.

EXPERIMENTAL

Human AAG (from Cohn fraction VI), *Clostridium perfringens* neuraminidase type X, ultrapure urea, copper(II), zinc(II), nickel(II) and cobalt(II) chloride (ACS reagents) and methyl-α-D-glucopyranoside were from Sigma (St. Louis, MO, USA). Immobiline (pK_a 4.6 and 9.3), Ampholine carrier ampholytes (in the pH range 2.5–4.5), acrylamide, GelBond PAG films and chelating Sepharose Fast Flow (45–165 μm mean particle size, 22–30 μmol Cu^{2+} per ml gel) were from Pharmacia LKB (Uppsala, Sweden). N,N'-Methylenebisacrylamide (bis), ammonium persulphate, N,N,N',N'-tetramethylethylenediamine, Coomassie Brilliant Blue R-250, 2-mercaptoethanol and imidazole were obtained from Merck (Darmstadt, Germany). Concanavalin A immobilized to Ultrogel AcA 22 (40–60 μm mean particle size, 3–4 mg immobilized lectin per ml gel) was from IBF Biotechnics (Villeneuve-la-Garenne, France), and YM 10 membrane filters from Amicon (Danvers, MA, USA). All other reagents were of grade A or analytical-reagent grade and were purchased from local suppliers.

Purification of individual AAG samples with different phenotypes

Samples of the three main AAG phenotypes, F1S/A, F1/A and S/A, were purified separately from individual human plasma samples, previously phenotyped for AAG, as previously described [9], using a one-step chromatographic procedure on Cibacron Blue F3G-A immobilized to Sephadex G-100. The AAG preparations all appeared homogeneous after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoelectrophoresis against anti-whole human plasma.

Experiments with immobilized metal ion to iminodiacetate (IDA)–Sepharose

Equilibration of iminodiacetate (IDA)–Sepharose with copper(II), zinc(II), nickel(II) or cobalt (II) chloride was as described by Hutchens and Yip [10].

Affinity chromatography of native (sialylated) AAG on immobilized metal affinity adsorbent

This was performed at room temperature (20–23°C), using small columns of IDA–Sepharose gel loaded with the appropriate metal, M [IDA–M(II) gel] (1.5 cm I.D., bed volume 10 ml) and equilibrated in a buffer (pH 7.0) of 20 m*M* sodium phosphate, containing 0.5 *M* NaCl (equilibration buffer E). Two types of AAG samples were used: individually purified AAG samples, consisting of F1S/A, F1/A or S/A phenotype, and a commercial AAG sample, *i.e.* a mixture of the phenotypes that contains almost equal proportions of the F1, S and A variants. The AAG samples were all dissolved in buffer E (5–7 mg in 1.5 ml) and applied to the columns at a linear flow-rate of 10 ml/h. Fractions of 1.25 ml were collected, and their respective absorbances were measured spectrophotometrically at 280 nm. Next, several volumes of buffer E were applied to remove the unbound variant(s); a second elution buffer, also pH 7.0, consisting of 20 m*M* imidazole in buffer E, was applied to remove the bound variant(s).

After completion of chromatography, the appropriate peak fractions were collected and concentrated on a YM 10 membrane filter. They were then dialysed against deionized water and finally lyophilized before studying by analytical IEF.

Non-chromatographic equilibrium binding experiments

The quantitative affinity relationships of immobilized copper(II) ions with native (sialylated) AAG were determined in a batch mode, as already described [8], using a non-chromatographic protocol previously validated by Hutchens and Yip [10]. IDA–Sepharose gel, loaded with copper(II) ions and equilibrated in buffer E, was allowed to settle in a 10-ml graduated cylinder to a constant final bed volume (at least 45 min). A homogeneous gel suspension of IDA–Cu(II) gel (50%, v/v) was prepared in buffer E, and 50-μl aliquots (25 μl of gel) were dispensed into small incubation tubes containing 100 μl of native AAG solution in buffer E, at ten different concentrations (1–30 μM). The final volume in the tubes was adjusted to 300 μl with buffer E. After incubation for 30 min at room temperature, the unbound (free) protein and the ligand-bound protein were separated by centrifugation using a table-top centrifuge (30 s at 200 *g*). A 150-μl volume of the clear supernatant was sampled from each tube for spectrophotometric determinations at 278 nm of the unbound protein concentrations.

Equilibrium-binding control experiments were performed using commercial AAG after its desialylation with neuraminidase. Desialylation was as described by Eap *et al.* [11].

The determination of equilibrium concentrations of unbound (free) protein ([P]) in the supernatant and ligand-bound protein ([PL]) at each concentration, and the calculation of the binding parameters (apparent association constant of AAG for immobilized Cu(II) ions and maximum IDA–Cu(II) gel protein-binding capacity) were as described previously [8].

Isoelectrofocusing

An LKB 2117 Multiphor II electrophoresis apparatus equipped with a 2297 Macrodrive 5 con-

stant-power supply, was used for the IEF experiments.

Analytical IEF on an immobilized pH 4.4–5.4 polyacrylamide gel gradient

Prior to analytical IEF, the AAG samples were desialylated with neuraminidase as follows. A 15-μl volume of each AAG sample (5 mg of protein per ml of deionized water) was added with 60 μl of neuraminidase (1 U/ml) in a 5 m*M* sodium acetate buffer (pH 5.5) containing 0.9 m*M* $CaCl_2$ and 15.4 m*M* NaCl. The mixture was then incubated for 24 h at 37°C and then directly used in analytical IEF. The IEF was performed as previously described by Eap *et al.* [2], in an immobilized pH 4.4–5.4 polyacrylamide gel gradient (0.5 mm thick) supplemented with 8 *M* urea and 2% (v/v) 2-mercaptoethanol. The desialylated variants in the gel were detected by staining with Coomassie Brilliant Blue R-250.

Analytical IEF with carrier ampholytes in the pH range 2.5–4.5

Thin-layer polyacrylamide gel slabs (0.5 mm thick) with a pH gradient of 2.5–4.5, were prepared according to ref. 12. The IEF was carried out at 10°C for 4 h. The AAG variants in the native (sialylated) form (20–40 μg in deionized water) were applied at the cathodic end of the gel using small pieces of filter paper, which were removed after 1 h of focusing. The gels were fixed, stained with Coomassie Brilliant Blue R-250 and preserved according to ref. 12, with a modification of the staining period, which was lengthened from 10 to 45 min.

Concanavalin A affinity chromatography

Concanavalin A immobilized to Ultrogel AcA 22 and equilibrated in a 10 m*M* sodium acetate buffer (pH 6.0), containing 0.25 *M* NaCl, 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$, was packed into small glass columns (1.5 cm I.D.) up to a final bed volume of 10 ml. The native AAG variants or unfractionated protein were dissolved separately (*ca.* 6 mg each) in 2.0 ml of the sodium acetate buffer and applied to the affinity columns at a linear flow-rate of 4 ml/h. Chromatography was performed at 4°C. Fractions (1.25 ml) were collected and their respective absorbances were measured spectrophotometrically at 280 nm. After penetration of the AAG sample solution into the affinity gel, chromatography was stopped for 30 min to allow for satisfactory interaction between concanavalin A and the glyco conjugate. The affinity gel was washed with the sodium acetate buffer (pH 6.0) until the absorbance of the effluent at 280 nm was zero. The adsorbed protein material was then eluted by adding 100 m*M* methyl-α-D-glucopyranoside in the sodium acetate buffer. After completion of chromatography, the appropriate peak fractions were pooled, concentrated on a YM 10 membrane filter, and then dialysed against deionized water.

RESULTS AND DISCUSSION

Interactions of native (sialylated) AAG with immobilized copper(II) ions

We had previously shown that affinity chromatography on immobilized copper(II) ions could successfully be used to purify the three main variants (F1, S and A) of desialylated AAG [8]. Here we investigated the possibility of using this method to fractionate the variants in their native (sialylated) form. For that purpose, the interactions of native AAG with copper(II) ions immobilized to an IDA–Sepharose [IDA–Cu(II) gel] were studied using two different methods, an analytical chromatographic method and a non-chromatographic protocol. The results were systematically compared with those obtained with desialylated AAG in control binding experiments.

Affinity chromatography on immobilized copper(II) ions

Fig. 1 illustrates the elution profiles obtained by affinity chromatography of native (sialylated) AAG samples on IDA–Cu(II) gel equilibrated with a 20 m*M* sodium phosphate buffer (pH 7) containing 0.5 *M* NaCl (buffer E). The native AAG samples used were from a commercial AAG sample (a mixture of the phenotypes that contains almost equal proportions of the F1, S and A variants) and from individually purified AAG samples with the F1S/A, F1/A and S/A

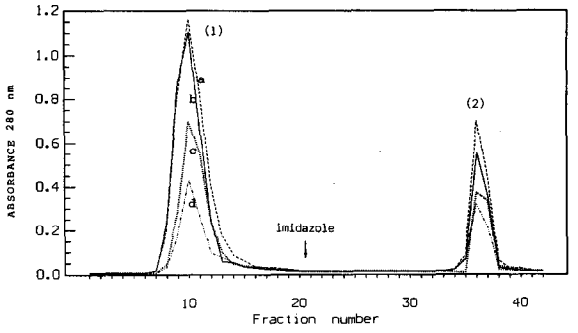

Fig. 1. Elution of the sialylated AAG variants by affinity chromatography of native AAG samples on IDA–Cu(II) gel at pH 7. The affinity gel was packed into small glass columns (1.5 cm I.D.) up to a final bed volume of 10 ml. Native commercial AAG (c) or individually purified AAG samples with the F1S/A (b) F1/A (d) or S/A phenotype (a) were dissolved in buffer E (5–7 mg in 1.5 ml) and applied to the columns equilibrated with the same buffer at room temperature (20–23°C). The flow-rate was 10 ml/h. Fractions of 1.25 ml were collected and their respective absorbances were measured spectrophotometrically at 280 nm. After several volumes of buffer E had been applied to remove unbound protein(s), 20 m*M* imidazole was introduced (indicated by an arrow) to elute high-affinity protein(s). The total protein recovery was *ca.* 95% in all experiments. Peaks 1 and 2 from the different chromatographic experiments were separately pooled, concentrated and then dialysed against deionized water and finally lyophilized.

phenotypes. Samples of the three main AAG phenotypes had previously been isolated from individual human plasma, as already described [9]. Fig.1 shows that, irrespective of the native AAG sample used, the chromatography resolved only two protein peaks. Peak 1 was "unbound" and was found to elute with several volumes of the equilibration buffer. The "bound" peak 2 was eluted after the addition of 20 m*M* imidazole to buffer E, in order to elute the high-affinity proteins. The elution volumes measured for peaks 1 and 2, respectively, were very similar in the different chromatographic experiments.

The total recovery of native AAG measured in peaks 1 and 2 was *ca.* 95% in all the experiments. Integration showed that peaks 1 and 2, respectively, consisted of *ca.* 70 and 30% of commercial AAG, 75 and 25% of F1S/A AAG, 65 and 35% of F1/A AAG and 65 and 35% of S/A AAG.

The variants of the different chromatographic peaks were characterized by analytical IEF on an immobilized pH 4.4–5.4 polyacrylamide gel gradient, after desialylation of a small amount of each peak with neuraminidase. The electrophoretic patterns are shown in Fig. 2. This figure also shows the electrophoretic patterns of the different AAG samples used in affinity chromatography, which were obtained after desialylation of small amounts of these samples. Depending on the native AAG sample used, peak 1 was found to consist of only the F1 (F1/A AAG) or the S (S/A AAG) variant, or of a mixture of the F1 and S variants (F1S/A AAG and commercial AAG). However, in all AAG samples, peak 2 signified the A variant in a pure form.

It was concluded that the sialylated A variant was the fraction of native AAG that was strongly bound to IDA–Cu(II) gel. Conversely, depending on the phenotype, the sialylated F1, the sialylated S or both variants were unbound, showing no significant binding affinity for immobilized copper(II) ions.

The chromatographic recoveries of the variants, as calculated by integration of peaks 1 and 2 (see earlier), were in agreement with the proportion of each variant in the different AAG samples, which were: F1 50%, S 23% and A 27% for commercial AAG; F1 55%, S 20% and A 25% for F1S/A AAG; F1 61% and A 39% for F1/A AAG; S 63% and A 37% for S/A AAG (as found by scanning the gel shown in Fig. 2).

The fractionation results achieved here with native AAG were the same as those obtained originally with the desialylated protein [8]. This demonstrated the possibility of chromatography on immobilized copper(II) affinity adsorbent for the separation of the AAG variants either in the native (sialylated) or the desialylated form.

We had previously proposed that the observed heterogeneity on binding to immobilized copper(II) ions between the desialylated variants of AAG could involve differences in the surface structure of their amino acids, the desialylated A variant having a more favourable surface topography for binding to transition metal ions than the desialylated F1 and S variants [8]. Indeed, the A variant and the F1 and/or S variants are encoded by two different genes and differ by numer-

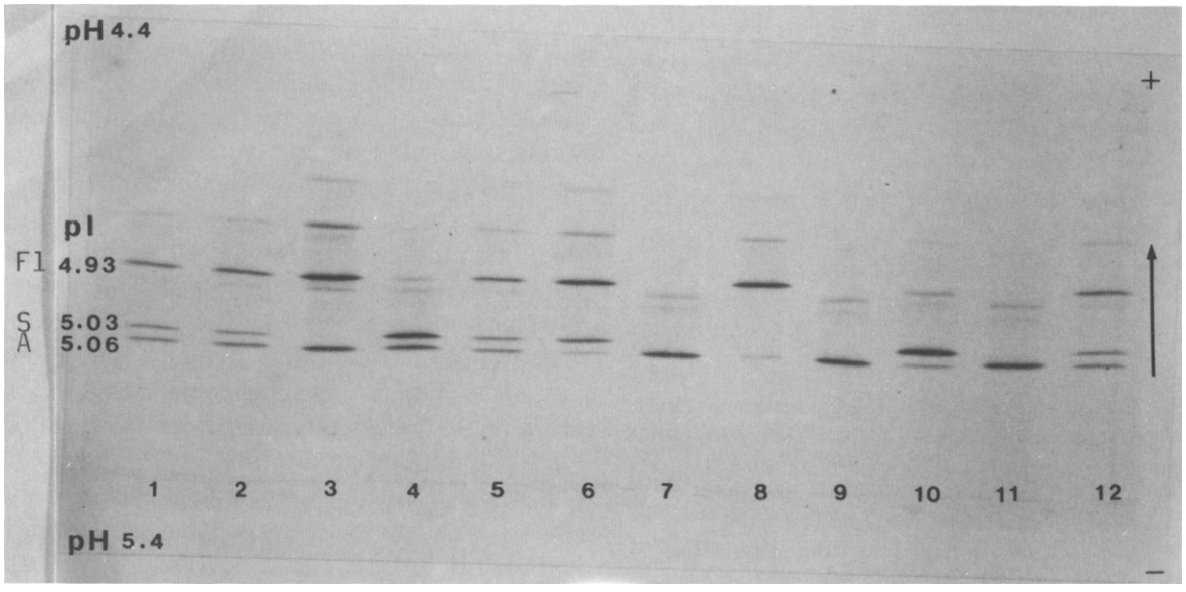

Fig. 2. Electrophoretic patterns on immobilized pH 4.4–5.4 polyacrylamide (4.85%, w/v) gel gradient with 8 *M* urea and 2% (v/v) 2-mercaptoethanol, of the isolated peaks 1 and 2, after affinity chromatography on IDA–Cu(II) gel of different native AAG samples. Prior to analytical IEF, small amounts of the different peaks (1 and 2) and of the AAG samples used in the chromatography were desialylated with neuraminidase as described in Experimental. Lanes 1, 5 and 12, commercial AAG (20 μg). Lanes 2, 3 and 4, individually purified AAG samples with the F1S/A, F1/A and S/A phenotype, respectively (each 20 μg). Lane 6, peak 1 (variants F1 and S, 20 μg) and lane 7, peak 2 (variant A, 20 μg), fractionated by affinity chromatography of commercial native AAG or of native F1S/A AAG. Lane 8, peak 1 (variant F1, 20 μg) and lane 9, peak 2 (variant A, 20 μg), fractionated by affinity chromatography of native F1/A AAG. Lane 10, peak 1 (variant S, 20 μg) and lane 11, peak 2 (variant A, 20 μg), fractionated by affinity chromatography from native S/A AAG. The desialylated variants were detected in the gel by staining with Coomassie Brilliant Blue R-250. The pH scale and the approximate isoelectric point (p*I*) value for each variant are indicated. The faint, more anodic bands that were also observed after staining with Coomassie Brilliant Blue indicated the presence of a small proportion of incompletely desialylated protein [less than 5% in all samples, except in F1/A AAG (lane 3) where it was 15%].

ous substitutions in the peptide chain [1,5]. The differences in primary structure could therefore involve surface structure differences between the variants.

Furthermore, as the fractionation results achieved here with native AAG were similar to those previously obtained with desialylated AAG, this indicated that the role of the sialic acid residues in the surface topography of the variants for binding to immobilized copper(II) ions seems to be minor. Nevertheless, a comparison of the elution profiles obtained by affinity chromatography of native and desialylated commercial AAG on columns of IDA–Cu(II) gel equilibrated in buffer E showed the existence of differences (Fig. 3). It was observed that the "unbound" peak 1′ of desialylated AAG, which signified the F1 and S variants, was eluted from the column with a larger volume of buffer E and that its elution was retarded compared with the "unbound" peak 1 of native AAG. However, no differences in elution were found between the "bound" peaks 2′ and 2 (the A variant) of desialylated and native AAG, respectively, probably because of the use of a strong competitor in buffer E. These results suggested that desialylation of AAG favoured its non-specific interaction with the affinity gel. This suggestion was further supported by the equilibrium-binding study of native and desialylated AAG to immobilized copper(II) ions.

Non-chromatographic equilibrium binding experiments

The quantitative affinity relationships of immobilized copper(II) ions with AAG were determined in a batch mode, using a commercial AAG

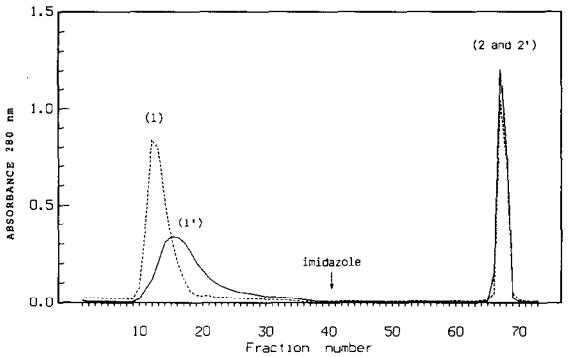

Fig. 3. Elution of the variants by affinity chromatography of native and desialylated AAG samples from commercial source on IDA–Cu(II) gel at pH 7. Native (-------) and desialylated (———) AAG were dissolved in buffer E (*ca.* 18 mg in 4 ml) and applied to columns of IDA–Cu(II) gel (1.5 cm I.D.; bed volume 40 ml) equilibrated with the same buffer at room temperature (20–23°C). The flow-rate was 20 ml/h and fractions of 4 ml were collected. All other experimental details were as in the legend of Fig. 1. The total protein recovery exceeded 90% in all experiments. Peaks 1 and 2 were from native AAG, and peaks 1′ and 2′ were from desialylated AAG.

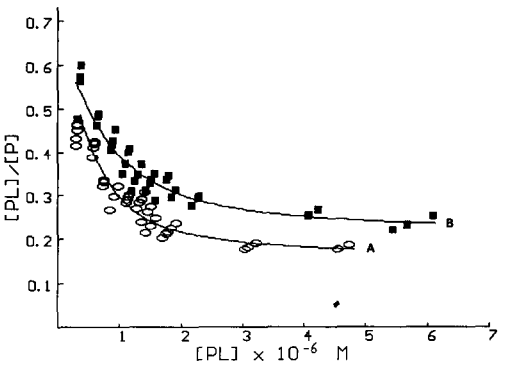

Fig. 4. Scatchard plots of equilibrium binding analyses for the interactions of native and desialylated commercial AAG with immobilized copper(II) ions. A 25-μl volume of IDA–Cu(II) gel was allowed to interact (30 min at 20–23°C) with various AAG concentrations in total reaction volume of 300 μl. The reaction buffer was 20 m*M* sodium phosphate (pH 7.0) containing 0.5 *M* NaCl (buffer E). To determine the immobilized ligand-bound protein concentrations, the equilibrium concentrations of unbound protein, [P], were subtracted from the total protein used. The plots obtained for the interaction of native (1–30 μM) and desialylated (1–30 μM) AAG with the IDA–Cu(II) gel are shown in curves A and B, respectively. The curves were analysed by non-linear regression with a one-class binding site model plus non-specific binding (NSB). The calculated values of the apparent equilibrium association constants (K_a), maximum IDA–Cu(II) gel protein-binding capacities, and NSB are given in Table I.

sample in the native form and after its desialylation with neuraminidase. The two Scatchard plots presented in Fig. 4 show the results from the equilibrium binding analyses of native and desialylated AAG interactions with IDA–Cu(II) gel at pH 7.0. The Scatchard plots (A and B) were representative (n = 41 and n = 42, respectively) of native (curve A) and desialylated AAG (curve B), and were obtained in total reaction volumes of 300 μl, with a 25-μl IDA–Cu(II) gel volume. Both proteins had a high binding affinity for immobilized copper(II) ions. The one class of high-affinity binding sites, plus non-specific binding (NSB), model was used to achieve an adequately fit to the experimental results.

The calculated values for the apparent association constant (K_a) of native and desialylated AAG for immobilized copper(II) ions and the maximum IDA–Cu(II) gel protein-binding capacities, as a function of the protein, are shown in Table I. The NSB values calculated for each protein are also given in Table I.

Although the high-affinity K_a value of native AAG ($0.8 \cdot 10^6$ l/mol) was slightly higher than that calculated for desialylated AAG ($0.6 \cdot 10^6$ l/mol), the differences were found to be non-significant. However, the NSB calculated value of desialylated AAG (0.21) was significantly higher than that calculated for native AAG (0.15), indicating that desialylation increased the non-specific interactions of AAG with the IDA–Cu(II) gel.

The binding results agreed with the chromatographic results shown in Fig. 3. The high-affinity binding could correspond to the (sialylated or desialylated) A variant, which was strongly bound by the IDA–Cu(II) gel affinity column and the NSB could be attributed to the (sialylated or desialylated) F1 and S variants, which were not bound by the same columns. One possible explanation for the increased NSB value of desialylated AAG, compared with that of native AAG, is that removal of the sialic acids, and relaxation of

TABLE I

APPARENT ASSOCIATION CONSTANTS OF NATIVE AND DESIALYLATED COMMERCIAL AAG FOR IMMOBILIZED COPPER(II) IONS AND IDA–Cu(II) GEL PROTEIN-BINDING CAPACITIES

k is the apparent intrinsic association constant of the high affinity sites; NSB is non-specific binding; L is the gel-binding capacity (amount of protein bound per ml of gel). The confidence interval of each calculated value is indicated in parentheses.

Binding parameters	Native AAG	Desialylated AAG
k (l/mol)	$0.79 \pm 0.26 \cdot 10^6$	$0.60 \pm 0.25 \cdot 10^6$
	(0.26–1.32)	(0.10–1.10)
L (nmol protein bound/ml IDA–Cu(II) gel)	7.97 ± 0.96	9.29 ± 1.67
NSB	(6.02–9.91)	(5.92–12.66)
	0.154 ± 0.004	0.209 ± 0.006
	(0.146–0.162)	(0.196–0.221)

the constraints due to mutual repulsion of these negatively charged residues, might have slightly changed the conformation of the variants, but without significantly altering their behaviour in binding to IDA–Cu(II) gel.

The fractionation results obtained here with native AAG showed that it is possible to separate all combinations of the native AAG variants, except the F1S mixture.

In other experiments, we found that the chromatographic fractionation was not improved by performing affinity chromatography on IDA–Cu(II) gel at pH values higher or lower than 7 (results not shown). Furthermore, all attempts to separate the variants using immobilized zinc, nickel or cobalt(II) ions, instead of copper, failed. Indeed, the chromatography of native commercial AAG with small columns of IDA–Zn(II), –Ni(II) or –Co(II) gel, equilibrated at pH 7, showed that all the variants were eluted with several volumes of the column equilibration buffer, indicating that the variants had no significant binding affinity for these metals (results not shown).

Analytical IEF of the native F1, S and A variants with carrier ampholytes

Having individually purified small amounts of the F1, S and A variants in the native form, we studied the microheterogeneity of each native (sialylated) variant by analytical IEF on a polyacrylamide gel gradient with carrier Ampholytes in the pH range 2.5–4.5. The electrophoretic patterns are shown in Fig. 5. All the native variants appeared to be very heterogeneous, consisting of several sub-populations that focused at pH 3.2–3.7. However, there were differences between the polymorphic patterns of the sialylated variants. The native F1 and S variants seemed to be more heterogeneous than the A variant. Six to seven bands of different intensity were visible for the F1 and S variants, whereas the A variant consisted of five bands. It was also observed that the bands corresponding to the native A variant (lane 1 in Fig. 5) were poorly stained compared with those corresponding to the F1 and S variants, even though the amount of the A variant loaded on the gel was twice that of the F1 and S variants. There was also a distinction between the average pI values of the native variants. Variant F1 focused at pH 3.2–3.6 with four major bands, whereas variants S and A were slightly less acidic and focused at pH 3.4–3.7 with three major bands.

The average pI values of the native variants were found to follow those of the desialylated variants. As shown in Fig. 2, the desialylated F1 variant had a lower pI value than the desialylated S and A variants. These results seemed to demonstrate that there is a correlation between the na-

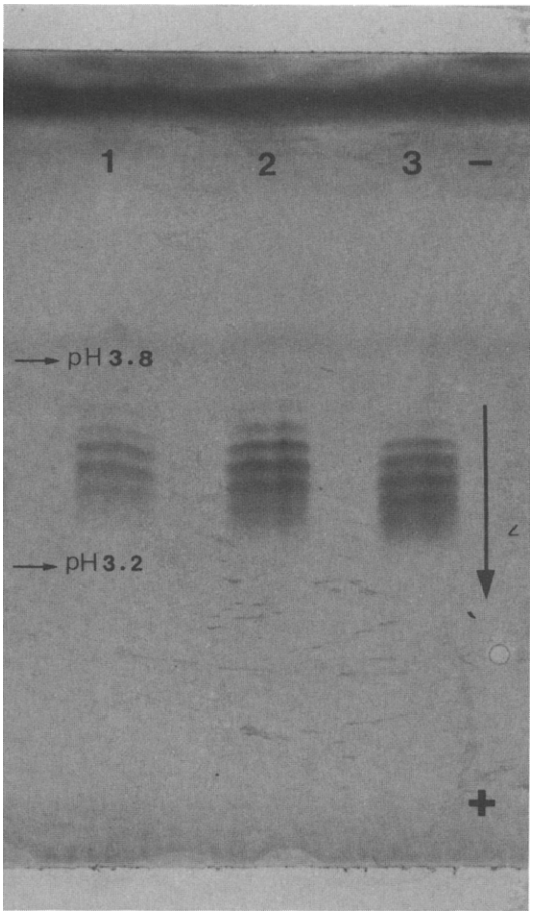

Fig. 5. Analytical IEF of the F1, S and A variants, after their purification in the native form, on a polyacrylamide (6.06%, w/v) gel gradient with carrier ampholytes in the pH range 2.5–4.5. Lanes 1, 2 and 3 were the native (sialylated) A (40 μg), S (20 μg) and F1 (20 μg) variants, respectively. The variants were detected in the gel by staining with Coomassie Brilliant Blue R-250. The pH scale is indicated.

tive and desialylated forms of the AAG variants. This finding can be grouped with the previous results of Montiel *et al.* [13], which showed a correlation between the microheterogeneity of native AAG in untreated plasma, as visualized by analytical IEF with carrier ampholytes, and the phenotype F1S/A, F1/A or S/A of the protein.

Concanavalin A affinity chromatography

This was performed using a pure A variant sample and the F1S variant mixture, which were separated by affinity chromatography of native commercial AAG on immobilized copper(II) ions. The behaviour of the separate variants on binding to immobilized concanavalin A was compared with that of unfractionated commercial AAG, used as a control. Fig. 6 illustrates that irrespective of the origin of the sample, the chromatographic elution profiles showed three peaks. The first two peaks (A and B) were eluted by washing the column with the equilibration buffer, 10 m*M* sodium acetate buffer (pH 6.0) containing 0.25 *M* NaCl, 1 m*M* $MnCl_2$, 1 m*M* $MgCl_2$ and 1 m*M* $CaCl_2$, and corresponded to concanavalin A non-reactive and weakly reactive material, re-

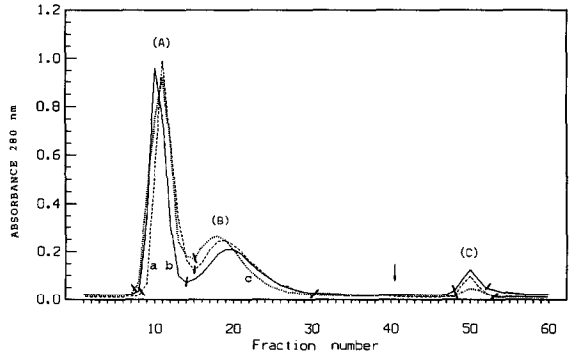

Fig. 6. Elution of the separate A variant and F1S variant mixture, in the native form, and of native commercial AAG by affinity chromatography on concanavalin A immobilized to Ultrogel AcA 22. The affinity gel was packed into small glass columns (1.5 cm I.D.) to a final bed volume of 10 ml, and equilibrated with 10 m*M* sodium acetate buffer (pH 6.0) containing 0.25 *M* NaCl, 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$. The A variant (b), F1S variant mixture (a) and commercial AAG (c) were dissolved in the column equilibration buffer (*ca.* 6 mg in 1.5 ml) and applied to the columns at 4°C. The flow-rate was 4 ml/h, and fractions of 1.25 ml were collected. After penetration of the AAG sample solution into the affinity gel, the chromatography was stopped for 30 min to allow for satisfactory interaction between concanavalin A and the glyco conjugate. The elution profile was followed by measuring the absorbance at 280 nm. The concanavalin A non-reactive (peak A) and weakly reactive (peak B) materials were eluted by washing the column with acetate buffer (pH 6.0). The concanavalin A reactive material (peak C) was desorbed after using of 0.1 *M* methyl-α-D-glucopyranoside (indicated by an arrow). The total protein recovery exceeded 90% in all experiments. Peaks A, B and C for each chromatographic experiment were separately pooled, as indicated. The relative proportions of the concanavalin A non-reactive, weakly reactive and reactive materials of each variant and of commercial AAG are given in Table II.

TABLE II

RELATIVE PROPORTIONS OF CONCANAVALIN A REACTIVE, WEAKLY REACTIVE AND NON-REACTIVE MATERIALS BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED CONCANAVALIN A IN THE NATIVE A VARIANT AND F1S VARIANT MIXTURE AND IN NATIVE COMMERCIAL AAG

Values are the mean of three experiments.

	Concanavalin A reactive material (%)	Concanavalin A weakly reactive material (%)	Concanavalin A non-reactive material (%)
Purified A variant	8.2	37.8	54
F1S variant mixture	2.4	41	56.6
Unfractionated commercial AAG	5.7	39.5	54.8

spectively. The concanavalin A reactive material (peak C) was desorbed from the column by addition of 0.1 *M* methyl-α-D-glucopyranoside. The elution volumes measured for the non-reactive, weakly reactive and reactive peaks were very similar in the various chromatographic experiments.

The total recovery of the separate variants and of unfractionated AAG, which was measured in peaks A, B and C, exceeded 90% in all chromatographic experiments. The respective proportions of the chromatographic peaks for each separate variant and for AAG are given in Table II. The A variant sample and the F1S variant mixture were found to contain similar proportions of concanavalin A non-reactive and weakly reactive materials, which represented, moreover, the major part of both samples. However, the proportion of concanavalin A reactive material in the A variant sample (*ca.* 8%) was significantly higher than that found in the F1S mixture (*ca.* 2%). The proportions of the concanavalin A non-reactive, weakly reactive and reactive materials in unfractionated AAG were found to be intermediate between those found for the separate A variant and the F1S mixture, given that the commercial protein contains *ca.* 70% of the F1 and S variants and 30% of the A variant. Our results with unfractionated AAG concurred with those previously reported by Nicollet *et al.* [14] which showed that AAG from plasma of normal subjects contained *ca.* 55% concanavalin A non-reactive, *ca.* 41% weakly reactive and *ca.* 4% reactive materials, as determined by crossed immunoaffinity electrophoresis.

The AAG heterogeneity in binding to immobilized concanavalin A concerns its carbohydrate structure. Five glycosylation sites have been localized on the polypeptide chain of AAG. These sites carry carbohydrate structures with various terminal sialic acid groupings (bi-, tri- and tetra-antennary) [15]. The specificity of concanavalin A binding properties requires the C-3, C-4 and C-6 hydroxyl groups of the α-D-mannopyranosyl units. The α-D-mannopyranosyl units in the tri- and tetra-antennary carbohydrate structures have fewer of the C-4 and C-6 hydroxyl groups available than the units in the bi-antennary carbohydrate structure [15]. Thus, the tri- and tetra-antennary structures might have less affinity for concanavalin A than the bi-antennary structure.

The increased proportion of concanavalin A reactive material in the A variant sample compared with that in the F1S mixture, seems to indicate that the A variant is enriched in bi-antennary structures. Furthermore, this finding agreed with the less acidic average p*I* value of the A variant, as found on IEF with carrier ampholytes, given that the bi-antennary chain contains intrinsically less sialic acid residues than the tri- and tetra-antennary chains. Carbohydrate analysis would be necessary to confirm this.

CONCLUSION

We showed that, like the desialylated variants, the variants in the native (sialylated) form exhibit very heterogeneous behaviour on binding to immobilized copper(II) affinity adsorbent, and this heterogeneity was used for their subsequent separation. Practically speaking, the affinity chromatographic method on immobilized copper(II) ions makes it possible to purify small amounts of the three AAG variants, F1, S and A, from individually purified AAG samples with the F1/A and S/A phenotypes. Large amounts of a pure A variant sample and of a mixture of the F1 and S variants are obtained when commercial AAG is used. As variant A and the mixture of variants F1 and S correspond to the two gene products of human AAG, their separation in the native state will greatly help investigations of the structure and function of each variant, with respect to its genetic origin.

In addition to differences in their amino acid compositions, which seem to be responsible for the heterogeneity on binding to immobilized copper(II) ions, the AAG variants also seem to show differences in their carbohydrate structures. Indeed, the variants were found to exhibit different polymorphic patterns on analytical IEF. Also, some differences were observed between the reactivity of the A variant and of the F1S variant mixture towards immobilized concanavalin A.

AAG has been shown to have an immunomodulatory activity [16], in which its carbohydrate moiety seems to play an important role. In particular, the concanavalin A non-reactive (enriched in tri- and tetra-antennary carbohydrate structures) and reactive AAG (enriched in bi-antennary structures) seem to have significantly different modulatory properties on thymocytes and macrophages [17]. It would therefore be interesting to investigate if these differences are related to the existence of different immunomodulative properties between the A variant and the F1 and/or S variant, the two gene products of AAG.

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