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Journal of Chromatography B, 688 (1997) 35–46

JOURNAL OF CHROMATOGRAPHY B

Abnormal microheterogeneity detected in one commercial α_1-acid glycoprotein preparation using chromatography on immobilized metal affinity adsorbent and on hydroxyapatite

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Received 21 March 1996; revised 4 June 1996; accepted 7 June 1996

Abstract

The study of one commercial preparation of human α_1-acid glycoprotein (AAG) by isoelectric focusing and by different chromatographic methods, previously developed to purify and fractionate the genetic variants of AAG, revealed an abnormal heterogeneity for this preparation. In addition to the three main variants (F1, S and A) of AAG normally present, this preparation contained five other AAG variants (called here Σ, α, β, δ and γ), accounting for ca. 40% of the total. As it is very unlikely that the latter variants are rare AAG variants, the abnormal heterogeneity of this AAG preparation is most probably due to structural alterations occuring during the large scale isolation. The α and the Σ, β, δ and γ variants could correspond to altered forms of the A and the F1 and S variants, respectively, because of their similar retention behaviour on immobilized copper(II) ions and their similar drug binding properties. However, the elution of the variants from the immobilized metal affinity column suggested that Σ, α, β, δ and γ were desialylated. Chromatography on hydroxyapatite enabled the separation of the F1, S and A variants from the the Σ, α, β, δ and γ variants. The inability of the latter variants to bind to hydroxyapatite suggested that the structural alterations might involve acidic amino acid residues. This proposal agreed with the isoelectric focusing study of variants Σ, α, β, δ and γ. Since the different separation methods used were able to resolve the variants of this AAG, this protocol could be used for characterization of commercial AAG proteins.

Keywords: Metal affinity adsorbent; Hydroxyapatite; Genetic variants; α_1-Acid glycoprotein

1. Introduction

A case of protein heterogeneity with significant effects on plasma drug transport is human α_1-acid glycoprotein (AAG). This heavily glycosylated protein is the main plasma carrier for basic drugs and binds a variety of other ligands, for a review see Ref. [1]. AAG presents a genetic polymorphism [2,3]. Various alleles corresponding to two loci have been reported; the first locus has two main variants (F1 and S), while the second is mainly monomorphic (the A variant). Human AAG genes have been cloned and sequenced [4] and, in accordance with the existence of two loci postulated by population polymorphism studies, two different genes (AAG-A and AAG-B/B′) have been found in a human haploid genome. A study using transgenic mice [5] has shown that variant F1 is encoded by the AAG-A gene, and the A variant is encoded by the AAG-B/B′ gene. The AAG variants are demonstrated in

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PII S0378-4347(96)00260-5

desialylated samples using polyacrylamide gel isoelectric focusing (IEF) and identified according to their electrophoretic mobility [2,3]. Depending on the presence of two or three of the variants F1, S and A in plasma, three phenotypes are most frequently observed for AAG in the human population, F1*S/A, F1/A and S/A, (ditto).

The recent development of several methodologies has allowed the fractionation and study of the drug binding properties of the individual AAG variants, according to their separate genetic origin [6–10]. Because large amounts of each AAG variant were needed for the study of binding properties, the variants have been fractionated on a preparative scale from commercial AAG, i.e. a mixture of similar proportions of the three main, F1, S and A, variants of AAG. The existence of functional (drug-binding) heterogeneity between the genetic variants has been demonstrated [11–13] and may explain the variations in the plasma binding of drugs to AAG between individuals [14,15].

Studying the variants of a commercially available AAG preparation by analytical IEF, we noticed that this AAG exhibited an abnormal microheterogeneity compared with other commercial AAG preparations studied by the same method. Therefore, we decided to further characterize the variants of this peculiar AAG preparation by different chromatographic methods previously developed in the laboratory. The drug binding properties of the individual variants of this AAG were also investigated, after separation by chromatography. The results were compared with those obtained in control experiments with two other available AAG preparations from the same commercial source as the former one.

2. Experimental

2.1. Material

Human AAG (from Cohn fraction VI; batch no. 113H9308, 90H9317 and 13H9336), goat anti-rabbit IgG conjugated with alkaline phosphatase, rabbit antiserum to whole human serum, copper(II) chloride (ACS reagent), urea ultrapure and warfarin were from Sigma (St. Louis, MO, USA). Rabbit immunoglobulins to human AAG were from Dakopatts (Glostrup, Denmark). *Clostridium perfringens* neuraminidase was from Boehringer (Mannheim, Germany). Immobiline DryPlate (in the pH range 4.5–5.4) and chelating Sepharose Fast Flow (45–165 μm mean particle size, 22–30 μmol Cu(II) per ml gel) were from Pharmacia LKB (Uppsala, Sweden). Biogel HT hydroxylapatite for chromatography and Econocolumns were from Bio-Rad Laboratories (Richmond, CA, USA). Acrylamide and N,N′-methylenebisacrylamide were from Serva (Heidelberg, Germany) and Agarose A37 was from Industrie Biologique Française (Villeneuve-la-Garenne, France). Nitrocellulose membranes (0.45 μm) were from Sartorius (Göttingen, Germany). Imidazole, ammonium persulphate, N,N,N′,N′-tetramethylenediamine, Coomassie Brilliant Blue R-250 and 2-mercaptoethanol were obtained from Merck (Darmstadt, Germany). YM 10 membrane filters were from Amicon (Danvers, MA, USA). Imipramine hydrochloride was given by Ciba Geigy (Rueil-Malmaison, France). [^{3}H]Imipramine (21 Ci/mmol, 925 GBq/mmol) and [^{14}C]warfarin (46 mCi/mmol, 1.70 GBq/mmol) were purchased from Amersham International (Buckinghamshire, UK). All other reagents were of analytical-reagent grade and were purchased from local suppliers.

2.2. Affinity chromatography on an immobilized copper(II) affinity adsorbent and chromatography on hydroxyapatite

These two methods have been described in detail previously [7–9]. Accordingly, they are briefly outlined in this section. Chromatography was performed at room temperature (20–23°C), using small columns of iminodiacetate–Sepharose gel loaded with copper(II) ions [IDA–Cu(II) gel] or of Bio-Gel HT hydroxylapatite (0.7 cm I.D., bed volume 8 ml in both cases). The IDA–Cu(II) gel columns were equilibrated with a 20 m*M* sodium phosphate buffer, pH 7.0, containing 0.5 *M* NaCl, and the hydroxyapatite columns with a 10 m*M* sodium phosphate buffer, pH 7.0, containing 0.1 *M* NaCl. The commercial AAG samples were dissolved in the appropriate equilibration buffer (ca. 5 mg in 0.5–0.8 ml) and separately applied to the columns at a flow-rate of 8 ml/h. Fractions of 1 ml were collected, and their respective absorbances were measured spectrophoto-

metrically at 280 nm. Elution of AAG from the IDA–Cu(II) gel columns was carried out by washing the gel with several bed volumes of the equilibration buffer to remove the non-bound AAG variants, and then with several volumes of a second (pH 7) elution buffer, consisting of 20 m*M* imidazole in the equilibration buffer, to remove the high-affinity variants [8,9]. Elution of AAG from the hydroxyapatite columns was performed by washing the columns successively with the equilibration buffer and with a 20 m*M* sodium phosphate buffer, pH 7.0, containing 0.1 *M* NaCl [7]. After completion of chromatography, the appropriate peak fractions were collected and concentrated on a YM10 membrane filter. They were then dialyzed against deionized water and finally lyophilized.

2.3. Sodium-dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoelectrophoresis

SDS–PAGE was performed using 8×8 cm slabs and a 12% polyacrylamide resolving gel/4% polyacrylamide stacking gel, according to the method of Laemmli [16]. Immunoelectrophoresis was performed using 1% agarose gel, according to the method of Grabar and Williams [17].

2.4. Isoelectric focusing (IEF)

IEF was run with a LKB 2117 Multiphor II electrophoresis apparatus equipped with a 2297 Macrodrive 5 constant-power supply.

Prior to analytical IEF, the AAG samples were desialylated with neuraminidase, as described previously [7,9]. The IEF was performed in an immobilized pH 4.5–5.4 polyacrylamide gel gradient (0.5 mm thick) supplemented with 8 *M* urea and 2% (v/v) 2-mercaptoethanol, as essentially described by Eap and Baumann [18]. The desialylated AAG variants were either detected in the gel by staining with Coomassie Brilliant Blue R-250 or were probed, after blotting onto nitrocellulose membrane, with rabbit immunoglobulins to human AAG, with the use of an alkaline phosphatase-linked secondary antibody system, 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitroblue tetrazolium (Merck) to visualize the AAG bands [18]. The relative proportion of each AAG band in the gel or membrane was determined by scanning with an LKB 2202 Ultroscan laser densitometer.

2.5. Determination of AAG concentration

The specific assay of AAG was carried out by an immunonephelometric method, with a Beckman assay kit and nephelometer analyzer (Model 7571 ARRAY TM Protein System; Beckman Instruments, Fullerton, CA, USA). To calculate the molar concentration of AAG, a molecular mass of 40 000 was assumed [1].

2.6. Equilibrium dialysis

Binding experiments were performed by equilibrium dialysis at 4°C, as described previously [19], in dialysis cells with a total volume of 200 μl. All solutions were prepared in 0.067 *M* sodium/potassium phosphate buffer, pH 7.4, containing 50 mg gelatin/l, in order to avoid adsorption of ligand to dialysis membrane or cells [19]. The different AAG samples used in the equilibrium dialysis studies were treated with charcoal at pH 7.4, as described previously [11], in order to remove endogeneous ligands which might be bound to the proteins. The concentrations of the protein solutions were 10 μ*M*. Each ligand was used at different concentrations (3–150 μ*M*), with a constant amount of radioactivity (0.49–5.34 kBq, depending on the ligand). Dialysis time was 22 h with gentle shaking. No significant binding to the dialysis cells and membrane (Visking 18/32; Union Carbide Corp., Chicago, IL, USA) was observed.

At equilibrium, the concentrations of labelled ligand were measured in 50-μl samples from each compartment by liquid scintillation counting (Packard tricarb 2200 CA, Packard IC, Downers Grove, IL, USA). Free (F) and bound (B) concentrations of the labelled ligands were calculated.

2.7. Evaluation of the binding data

The data obtained at equilibrium (B and F) were fitted to the following equation:

$$B/P_t = [nKF/(1 + KF)] + nK'F \quad (1)$$

where n and K denote, respectively, the number of binding sites and the association constant for the specific protein; nK' denotes the non-specific binding, i.e. non-saturable binding (NSB); P_t is the concentration of the protein.

To calculate the binding parameters, the data were subjected to non-linear regression analysis (least-squares criterion used with a Gauss–Newton algorithm), using MicroPharm^C (Micropharm Version 4.0; Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France) [20]. The binding parameters, n, K and nK', were estimated with their standard deviations from experimental values from two triplicate experiments.

3. Results

For a better knowledge of further reading, the commercial AAG preparation with an abnormal microheterogeneity (Sigma; no. 113H9308) was called 'AAG-9308'. Two other commercially available AAG preparations (Sigma; no. 90H9317 and 13H9336) were used in control experiments for this study. As the results yielded by these latter preparations were similar to those obtained previously with other commercial (Sigma) AAG preparations, these were indiscriminately called 'control AAG'.

3.1. SDS–PAGE and immunoelectrophoresis

Both AAG-9308 and control AAG, reduced or non-reduced with 5% (v/v) 2-mercaptoethanol, showed only one protein band corresponding to the molecular mass of AAG (ca. 40 000) on SDS–PAGE (data not shown). The purity of each AAG preparation was further confirmed using immunoelectrophoresis. Both AAG-9308 and control AAG showed a single arc-like immunoprecipitate with a rabbit antiserum to whole human serum and a strong reactivity with rabbit immunoglobulins to human AAG (data not shown).

3.2. Analytical IEF on an immobilized pH 4.5–5.4 polyacrylamide gel gradient

The variants of AAG-9308 and control AAG are illustrated by the electrophoretic patterns shown in Fig. 1, as obtained by analytical IEF and after desialylation of a small amount of each sample. Detection of the variants was made by staining with Coomassie Brilliant Blue R-250 (Fig. 1A) and by immunoblotting with two antibody steps (Fig. 1B), respectively. Reference to Fig. 1A and B shows that control AAG (lane 1) consisted of a mixture of the three main AAG variants, F1, S and A. The relative scanned proportions of these variants were ca. 45% F1, 29% S and 26% A. However, analytical IEF of AAG-9308 (lane 2 in Fig. 1A and B, respectively) revealed the presence of six different AAG variants: three major variants with electrophoretic mobilities corresponding respectively to those of the F1, S and A variants and three more cathodic minor variants which were called respectively α, β and γ. The relative scanned proportions of these variants were ca. 29% F1, 22% S and 33% A and 5% α, 8% β and 3% γ. Furthermore, a comparison between the proportions of the F1, S and A variants in AAG-9308 and control AAG, as calculated from the total of these three variants in each sample, showed a 20% decrease of variant F1 and a 50% increase of variant A in AAG-9308 compared to control AAG. The proportions of variant S in AAG-9308 and control AAG were similar.

3.3. Affinity chromatography on immobilized copper(II) ions

We have demonstrated the possibility of chromatography on immobilized copper(II) affinity adsorbent for the fractionation of the AAG variants, either from individual AAG samples with different phenotypes or from commercial AAG preparations [7–10]. In this method, the A variant was found to strongly bind to immobilized copper(II) ions and was separated from the F1 and S variants, which showed no significant binding affinity for the transition metal ions.

In order to further characterize the variants of AAG-9308, their interactions with copper(II) ions immobilized to an iminodiacetate–Sepharose [IDA–Cu(II) gel] were studied by chromatography (Fig. 2). The results were compared with those obtained with control AAG in similar chromatographic experiments. The elution profiles shown in Fig. 2 indicate that, irrespective of the AAG sample used, affinity

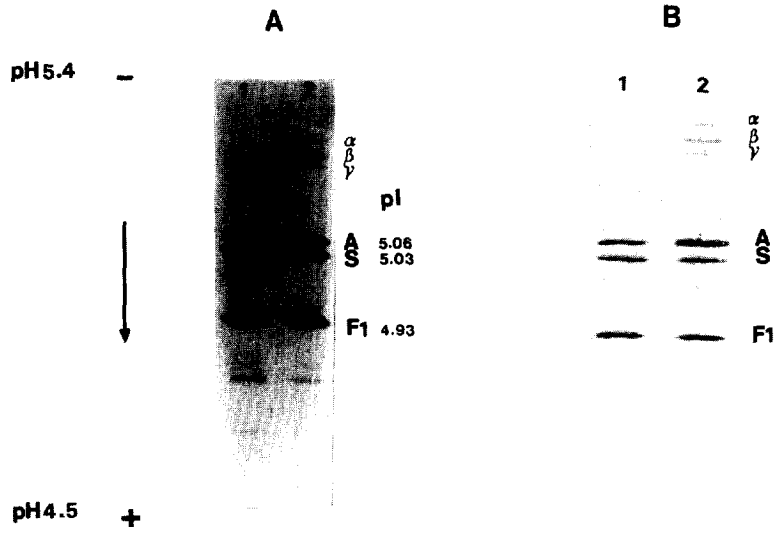

Fig. 1. Electrophoretic patterns on a 4.5–5.4 immobilized pH-gradient with 8 *M* urea and 2% (v/v) 2-mercaptoethanol of AAG-9308 and control AAG. Prior to IEF, small amounts of AAG-9308 and control AAG were desialylated with neuraminidase. Detection of the desialylated AAG variants was made in (A) by staining with Coomassie Brilliant Blue R-250 and in (B) by immunoblotting with two antibody steps. In (A) and (B), lanes 1 and 2: control AAG and AAG-9308, respectively [each 20 μg in (A) and each 0.8 μg in (B)]. The approximate isoelectric point (p*I*) values for the F1, S and A variants and the pH scale are indicated. The AAG bands corresponding respectively to the α, β and γ variants 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 [lanes 1 and 2 in (A)].

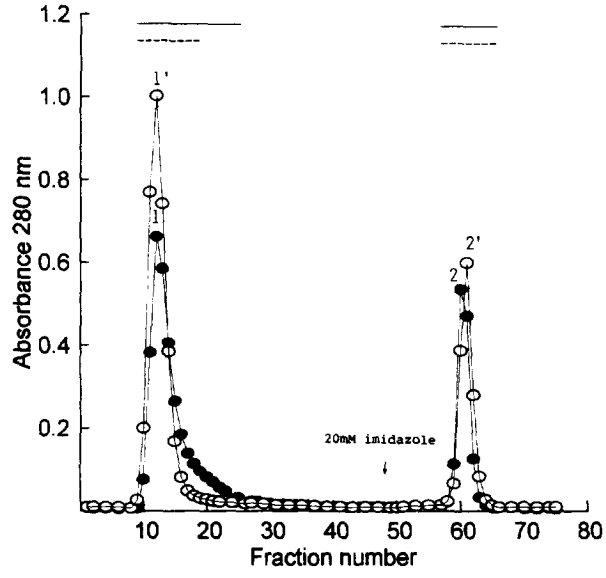

Fig. 2. Elution of the AAG variants by affinity chromatography of AAG-9308 and control-AAG samples on IDA–Cu(II) gel at pH 7. The non-bound variants of AAG-9308 (●) and of control AAG (○) (peaks 1 and 1′, respectively) were eluted with the equilibration buffer (i.e. 20 m*M* sodium phosphate buffer, pH 7.0, with 0.5 *M* NaCl). The high-affinity variants of AAG-9308 and control AAG (peaks 2 and 2′, respectively) were eluted after 20 m*M* imidazole had been introduced (indicated by an arrow). The horizontal lines above each trace (---, AAG-9308; —, control AAG) indicate where fractions were collected. All other details are described in Section 2.

chromatography resolved only two protein peaks. The non-bound variants of peak 1 of AAG-9308 and of peak 1′ of control AAG were eluted with several volumes of the equilibration buffer. The bound variants of peak 2 of AAG-9308 and of peak 2′ of control AAG were eluted after adding 20 m*M* imidazole. However, a comparison of the elution profiles showed that the non-bound variants of peak 1 of AAG-9308 were eluted from the column with a larger volume of the equilibration buffer and that their elution was retarded compared with the non-bound variants of peak 1′ of control AAG. No differences in elution were found between the bound variants of peaks 2 and 2′ of AAG-9308 and control AAG, respectively, probably because of the use of a strong competitor in the equilibration buffer. The total recovery of AAG measured in the non-bound and bound fractions was ca. 95% in all the experiments. Integration showed that the non-bound and bound fractions, respectively, consisted of 78 and 22% of AAG-9308 and 73 and 27% of control AAG.

The variants of the different chromatographic peaks were characterized by analytical IEF, after their desialylation (Fig. 3). Peak 1′ of control AAG (lane 2 in Fig. 3) consisted of a mixture of the F1 and S variants, their relative scanned proportions being 60 and 36%, respectively. Peak 1′ also contained 4% of variant A. However, peak 2′ of control AAG (lane 1 in Fig. 3) signified the A variant in a pure form. These fractionation results agreed with those obtained previously with several other commercial (Sigma) AAG preparations [8–11]. However, the fractionation results obtained with AAG-9308 were very different. Peak 1 of AAG-9308 (lane 5 in Fig. 3) was very heterogeneous, consisting of a mixture of five different variants: the two minor β and γ variants and three major variants corresponding respectively to F1, S and to another variant with an electrophoretic mobility nearly identical to that of the A variant. However, unlike variant A, this variant, which was called Σ, exhibited no significant binding affinity for immobilized copper(II) ions. The relative scanned proportions of variants F1, S, Σ, β and γ in peak 1 of AAG-9308 were ca. 35, 26, 25, 11 and 3%, respectively. Peak 2 of AAG-9308 (lane 4 in Fig. 3) was also heterogeneous, consisting of a mixture of the A and α variants (ca. 80 and 20%, respectively).

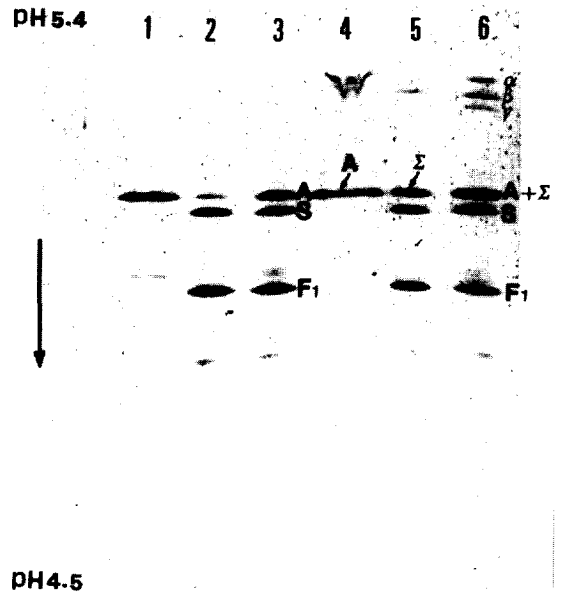

Fig. 3. Electrophoretic patterns on a 4.5–5.4 immobilized pH-gradient with 8*M* urea and 2% (v/v) 2-mercaptoethanol of the different variants isolated after affinity chromatography on IDA–Cu(II) gel of AAG-9308 and control AAG samples. Prior to IEF, small amounts of the different variants and of the AAG samples were desialylated with neuraminidase. Lane 3: control AAG (20 μg). Lanes 2 and · · the variants from peaks 1′ (variants F1 and S, 20 μg) and 2′ (variant A, 20 μg) isolated after affinity chromatography of control AAG. Lane 6: AAG-9308 (20 μg). Lanes 5 and 4: the variants from peaks 1 (variants F1, S, Σ, β and γ, 20 μg) and 2 (variants α and A, 20 μg) isolated after affinity chromatography of AAG-9308. The desialylated variants were detected in the gel by staining with Coomassie Brilliant Blue R-250. The protein bands corresponding respectively to the Σ, α, β and γ variants are indicated. All other details are described in the legend to Fig. 1.

In other experiments, individual fractions of peak 1 of AAG-9308 (fractions no. 17 to 23 in Fig. 2), for which a delayed elution from the IDA–Cu(II) gel column was observed, were characterized by analytical IEF. The results showed that these fractions were enriched in the Σ, β and γ variants (data not shown).

3.4. Chromatography on hydroxyapatite

We have recently developed a separate two-step purification method for human AAG from individual plasma [7]. Chromatography on hydroxyapatite was the second step and its conditions for AAG frac-

tionation were first optimized with commercial (Sigma) AAG. A step-wise gradient of (pH 7) sodium phosphate buffers, ranging between 10 and 40 m*M*, was used to elute AAG from the hydroxyapatite column. Irrespective of the source (commercial or individual) of AAG used in chromatography, AAG was always eluted from the column with the 20 m*M* phosphate buffer. These results agreed with those reported by previous investigators for the elution of AAG from a HPLC hydroxyapatite column [21].

Therefore, the adsorption behaviour of AAG-9308 on hydroxyapatite was studied by chromatography and the results were compared with those obtained with control AAG in similar chromatographic experiments (Fig. 4). The chromatography on hydroxyapatite of AAG-9308 resolved two different peaks (peaks I and II). Peak I was not adsorbed and was eluted with the equilibration buffer (i.e. 10 m*M* sodium phosphate buffer, pH 7.0, with 0.1 *M* NaCl). Desorption of peak II was achieved with a (pH 7) 20 m*M* phosphate buffer with 0.1 *M* NaCl. However, the hydroxyapatite chromatographic profile of control AAG showed only one protein peak (peak 0 in Fig. 4), which was eluted with the 20 m*M* sodium phosphate buffer. The total yield of AAG measured in peaks I and II of AAG-9308 or in peak 0 of control AAG was 93%. Integration showed that peaks I and II, respectively, consisted of 36 and 64% of AAG-9308.

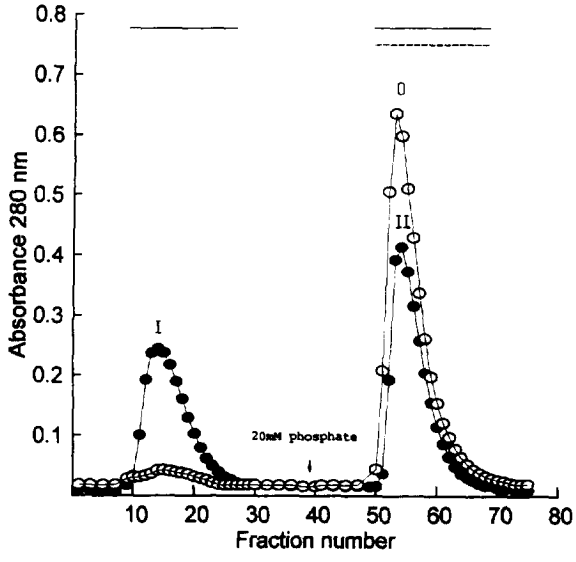

Fig. 4. Elution of the AAG variants by chromatography on hydroxyapatite of AAG-9308 and control AAG samples at pH 7. The non-bound variants of AAG-9308 (●) (peak I) were removed from the column with the equilibration buffer (i.e. 10 m*M* phosphate buffer, pH 7.0, with 0.1 *M* NaCl). The variants of AAG-9308 and control AAG (○) adsorbed on hydroxyapatite (peaks II and 0, respectively), were eluted with a 20 m*M* phosphate buffer, pH 7.0, containing 0.1 *M* NaCl (indicated by an arrow). The horizontal line(s) above each trace (—, AAG-9308; ---, control AAG) indicate(s) where fractions were collected. All other details are described in Section 2.

Fig. 5 illustrates the variants of the different peaks, as obtained by analytical IEF and after desialylation of a small amount of the variants from each peak. This figure also shows the IEF patterns of AAG-9308 and control AAG used in the chromatography (lanes 4 and 11, respectively), which were obtained after desialylation of a small amount of each AAG sample. Peak II of AAG-9308 and peak 0 of control AAG (lanes 7 and 3 in Fig. 5, respectively) eluted with the 20 m*M* phosphate buffer, exhibited similar electrophoretic patterns. Both peaks consisted of a mixture of the F1, S and A variants. Furthermore, the relative scanned proportions of the F1, S and A variants in peak II of AAG-9308 (40, 34 and 26%, respectively) were similar to those of the F1, S and A variants in peak 0 of control AAG or in control AAG (45% F1, 29% S and 26% A). However, peak I of AAG-9308 (lane 10 in Fig. 5) eluted with the 10 m*M* phosphate buffer, consisted of a mixture of five different variants: two major variants, one likely corresponding to Σ and the other to β, and three minor variants corresponding respectively to α, γ and to another variant which was called δ and having an electrophoretic mobility nearly identical to that of the S variant. The relative scanned proportions of variants Σ, β, α, γ and δ in peak I were 46, 25, 11, 9 and 9%, respectively. In other analytical IEF experiments followed by immunoblotting, the Σ, β, α, γ and δ variants were all identified as AAG variants (data not shown).

We have also investigated the retention behaviour on hydroxyapatite of AAG-9308 and of control AAG after their desialylation with neuraminidase (data not shown). The fractionation results obtained with the desialylated proteins were similar to those yielded by the non-treated proteins, but a comparison of the elution profiles indicated some differences. The

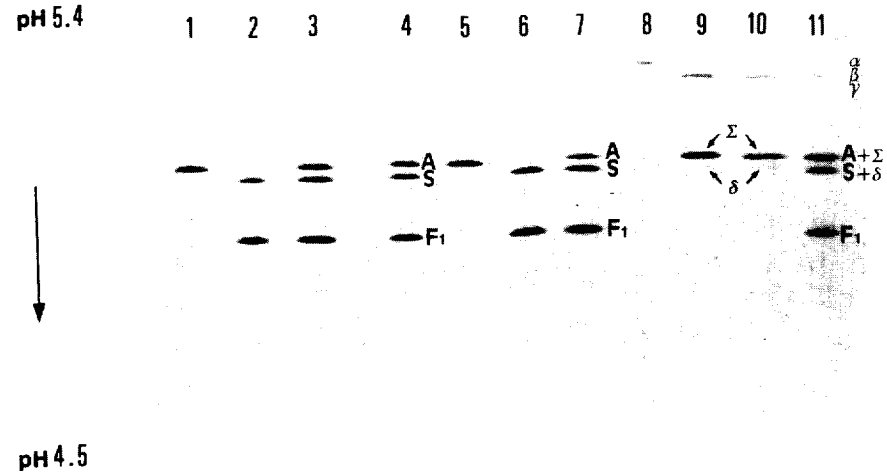

Fig. 5. Electrophoretic patterns on a 4.5–5.4 immobilized pH-gradient with 8*M* urea and 2% (v/v) 2-mercaptoethanol of the different AAG variants from the peaks isolated after chromatography on hydroxyapatite and after combined chromatography on hydroxyapatite and IDA–Cu(II) gel. Prior to IEF, small amounts of the variants from the different peaks and of the AAG samples were desialylated with neuraminidase. Lanes 4 and 11: control AAG and AAG-9308, respectively (each 20 μg). Lanes 3, 7 and 10 refer to the variants isolated after chromatography on hydroxyapatite (see Fig. 4): lane 3, the variants from peak 0 of control AAG (variants F1, S and A; 20μg); lanes 10 and 7, the variants from peak I (variants Σ, α, β, γ and δ; 20 μg) and from peak II (variants F1, S and A; 20 μg) of AAG-9308. Lanes 1, 2, 5, 6, 8 and 9 refer to the variants isolated after combined chromatography on hydroxyapatite and IDA–Cu(II) gel (see Fig. 6): lanes 2 and 1, fractions 0,1 (variants F1 and S; 20 μg) and 0,2 (variant A; 20 μg) from peak 0 of control AAG; lanes 9 and 8, fractions I,1 (variants Σ, β, γ and δ; 20 μg) and I,2 (variant α; 10 μg) from peak I of AAG-9308; lanes 6 and 5, fractions II,1 (variants F1 and S; 20 μg) and II,2 (variant A; 20 μg) from peak II of AAG-9308. All other details are as described in the legend to Fig. 1.

respective bound variants of desialylated AAG-9308 and control AAG (i.e. the F1, S and A variants) were eluted with a much larger volume of the 20 m*M* phosphate buffer and their elution was retarded compared to the bound variants of the non-treated proteins. However, only slight differences in elution were found between the respective non-bound variants of the neuraminidase-treated and the non-treated AAG-9308 (i.e. the Σ, β, α, γ and δ variants) eluted with the 10 m*M* phosphate buffer.

3.5. Affinity chromatography on IDA–Cu(II) gel of the AAG fractions isolated after chromatography on hydroxyapatite

The variants from peaks I and II of AAG-9308 and from peak 0 of control AAG, which were isolated after chromatography on hydroxyapatite, were then separately studied by affinity chromatography on IDA–Cu(II) gel. The elution profiles are shown in Fig. 6. Irrespective of the AAG peak studied, affinity chromatography resolved only two protein fractions, a non-bound fraction eluted with the equilibration buffer and a bound fraction eluted after adding 20 m*M* imidazole. However, it was observed that the elution of the non-bound fraction from peak I of AAG-9308 (i.e. fraction I,1 in Fig. 6) was significantly retarded, as compared with the non-bound fraction from peak II of AAG-9308 (i.e. fraction II,1) or from peak 0 of control AAG (i.e. fraction 0,1). The total recovery of AAG measured in the non-bound and bound fractions exceeded 90% in all experiments. Integration showed that the non-bound and the bound fractions consisted respectively of 85 and 15% of peak I and 70 and 30% of peak II of AAG-9308, and of 71 and 29% of peak 0 of control AAG.

Characterization of the different chromatographic fractions by analytical IEF, after their desialylation (Fig. 5), revealed that the non-bound fraction from peak II of AAG-9308 or from peak 0 of control AAG (lanes 6 and 2, respectively) consisted of a mixture of the F1 and S variants (ca. 53% F1 and 44% S for the fraction from peak II, and 58% F1 and 38% S for that from peak 0). The respective non-bound fractions from peaks II and 0 also contained a

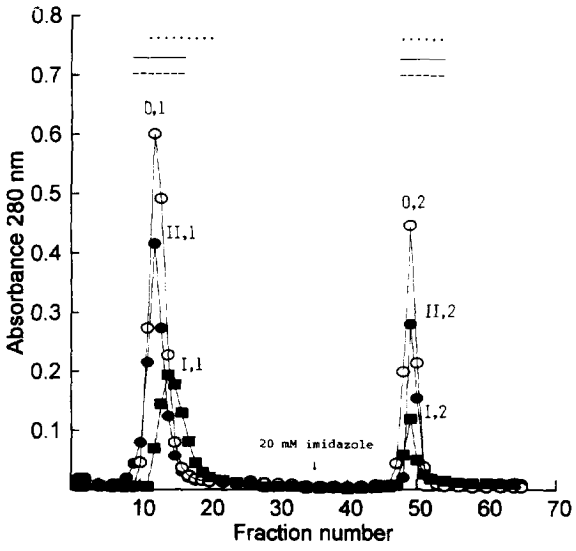

Fig. 6. Affinity chromatography on IDA–Cu(II) gel of the AAG variants from peaks I and II of AAG-9308 and from peak 0 of control AAG, previously isolated by chromatography on hydroxyapatite. The horizontal lines above each trace (....., peak I and —, peak II of AAG-9308; ---, peak 0 of control AAG (see Fig. 4)) indicate where fractions were collected. Fractions 0,1, I,1 and II,1 correspond respectively to the non-bound variants of peak 0 of control AAG (○) and of peaks I (■) and II (●) of AAG-9308. Fractions 0,2, I,2 and II,2 correspond, respectively, to the high-affinity variants of peak 0 of control AAG and of peaks I and II of AAG-9308. The variants of these different fractions are illustrated in Fig. 5. All other details are described in the legend to Fig. 2.

trace amount (3 and 4%) of variant A (i.e. for unknown reasons, a small amount of variant A, never exceeding 5% of the total, has always been found to co-elute with variants F1 and S in the non-bound AAG fraction to IDA–Cu(II) gel [11,13]). However, the bound fraction from peak II or peak 0 (lanes 5 and 1, respectively, in Fig. 5) signified the A variant in a pure form. Besides, the non-bound fraction from peak I of AAG-9308 (lane 9 in Fig. 5) consisted of a mixture of the Σ, β, γ and δ variants (ca. 50% Σ, 30% β, 10% γ and 10% δ), while the bound fraction from peak I (lane 8) contained only the α variant.

It is notable that the same final fractionation results were achieved when AAG-9308 and control AAG were first used in affinity chromatography on IDA–Cu(II) gel and then in chromatography on hydroxyapatite (data not shown).

3.6. Individual drug binding properties of the separate variants of AAG-9308

The binding of two different drugs, warfarin and imipramine, to the F1S variant mixture, the A variant and the Σβγδ variant mixture of AAG-9308, isolated after combined chromatography on hydroxyapatite and IDA–Cu(II) gel (i.e. fractions II,1, II,2 and I,1, respectively, in Fig. 6), was studied by equilibrium dialysis. The drug binding properties of variant α of AAG-9308 (i.e. fraction I,2 in Fig. 6) were not studied because of the small quantities recovered after chromatography. The calculated values of the binding results for imipramine and warfarin are summarized in Table 1.

The warfarin and imipramine binding study of the F1S variant mixture showed that the F1S mixture exhibited a high binding affinity for this acidic drug, but a low affinity for imipramine. Conversely, the A variant strongly bound imipramine, but had no significant binding affinity for warfarin. The results for the binding of warfarin and imipramine to the Σβγδ variant mixture were similar to those obtained

Table 1
Binding parameters of warfarin and imipramine to the separate F1S variant mixture, the A variant and the Σβγδ variant mixture of AAG-9308

Ligand	Warfarin			Imipramine		
	n	k $\times 10^6$ (l mol^{-1})	NSB $\times 10^6$ (l mol^{-1})	n	k $\times 10^6$ (l mol^{-1})	NSB $\times 10^6$ (l mol^{-1})
F1S variant mixture	0.64±0.02	3.14±0.73	0.009±0.001	0.87±0.17	0.064±0.015	0.042±0.005
Variant A	–	–	0.0094±0.0007	0.86±0.03	1.76±0.31	0.013±0.001
Σβγδ variant mixture	0.68±0.02	3.01±0.49	0.011±0.001	1.27±0.22	0.084±0.021	0.017±0.003

Equilibrium dialyses were carried out at 4°C, as described in Section 2. n and k are, respectively, the number of binding sites and the association constant for the specific protein. NSB (l mol^{-1}) is non-specific binding. The binding parameters are given with their standard deviations.

for the binding of these two drugs to the F1S variant mixture.

Data for imipramine and warfarin binding to the separate A and the F1S variants of AAG-9308, agreed with those previously determined for the separate A and the F1S variants of another commercial (Sigma) AAG preparation (no. 29F9314) [11]. It should be noted that the small, non-integral number of sites (n~0.6) determined for the binding of warfarin to the F1S variant mixture [11] is likely due to stereoselective binding of this drug, which is a racemate, and not to differences in binding between variants F1 and S. These two variants exhibit similar binding properties towards warfarin [11]. As previously suggested, the differences in amino acid primary structure, and thus in conformation, between the A variant and the F1 and S variants may be the likely sources of their differences in drug-binding [11–13] and also in binding to immobilized copper(II) ions [7–9]. The A variant and the F1 and S variants correspond to the proteins of the two different genes coding for AAG in humans [4,5] and should differ by at least 22 amino acid substitutions in the peptide chain, among a total of 181 residues.

4. Discussion

There is an increasing evidence that the structural microheterogeneity of AAG is related to the existence of different functional forms of the protein [11–13,22–24]. The key to studying the AAG microheterogeneity is to isolate this glycoprotein from plasma without inducing any structural changes. In this respect, successful isolation procedures have recently been developed [6,7,25–28] but their application on a preparative scale to purify large amounts of plasma AAG is laborious and time-consuming, explaining why commercial AAG has often been used in studies dealing with the role of the microheterogeneity of AAG in the biology of this glycoprotein.

The study of a commercial (Sigma) AAG preparation, AAG-9308, by different chromatographic methods and by analytical IEF has revealed that this preparation consisted of a mixture of at least eight different variants, i.e. the three main variants of AAG, F1, S and A, and five other variants called Σ, α, β, γ and δ and representing about 40% of the total. This heterogeneity is quite abnormal[1]. The AAG system is controlled in humans by two different highly polymorphic genes [4], resulting in the existence of many genetic variants for AAG in the human population [2,3]. Although generally, the AAG phenotypes have only two or three of these variants (F1, S and A), all others are rare variants. Accordingly, AAG isolated from a large pool of plasma, e.g. commercial AAG, should essentially consist of a mixture of the F1, S and A variants. This composition is consistent with that observed for the two commercial (Sigma) AAG preparations used as controls in this study and also for several other commercial (Sigma or Behring) AAG preparations previously analyzed by the same chromatographic and/or electrophoretic methods as the ones used in the present study [6–13,18,22,23,29]. Thus, it seems very unlikely that the Σ, α, β, γ and δ variants of AAG-9308 are rare AAG variants. Moreover, the study of different rare AAG variants by hydroxyapatite chromatography has shown that, like the F1, S and A variants, the rare variants bound to hydroxyapatite [7], whereas the Σ, α, β, γ and δ variants were not adsorbed on the column. Human AAG is an acidic protein [30] and, as such, its retention on hydroxyapatite is controlled by the ionic state of the column [31]. When operated at pH 7.0 after equilibration with phosphate buffer, the hydroxyapatite column has a surface which can be regarded as negative and acidic proteins should bind almost exclusively by the complexation of their amino acid carboxyl groups to calcium sites on the column, the carboxyls forming clusters of [hydoxyapatiteCa - - OOC-Protein] [31]. Therefore, the inability of the Σ, α, β, γ and δ variants to bind to hydroxyapatite

[1] After Sigma Chemical Co. had been informed of the results shown in this study, the company proposed to exchange the human AAG preparation no. 113H9308 for a similar amount (~10 g) of a new AAG preparation. It was agreed with the company to test this new preparation beforehand in order to study its composition into variants. We have recently tested a sample from the new AAG preparation (no. 125H9329) using the same chromatographic and electrophoretic methods as the ones used in the present study and following the same protocol. According to the results, the F1, S and A variants were the only variants present in this new AAG preparation.

might reflect an insufficient density of carboxyls to generate a cluster complementary to the calcium atoms immobilized in the hydroxyapatite crystals.

Affinity chromatography on IDA–Cu(II) gel of the AAG-9308 variants has shown that, like the F1 and S variants, Σ, β, γ and δ had no significant binding affinity for the immobilized metal, whereas like the A variant, α strongly bound to the affinity gel. We have recently shown that AAG variants exhibiting a very high degree of primary sequence homology (i.e. variants encoded by different alleles of the same gene) had similar retention behaviours on immobilized copper(II) ions [7]. Therefore, the fractionation results yielded by the AAG-9308 variants suggest that α may be structurally related to A, and Σ, β, γ and δ to F1 and S. Moreover, this proposal is supported by the existence of similar drug binding properties between the Σβγδ and the F1S variant mixtures of AAG-9308.

Considering all these data, it is suggested that the α and the Σ, β, γ and δ variants may correspond to altered forms of the A and the F1 and S variants, respectively. If this assumption is correct, both the polypeptide and the carbohydrate moieties of the Σ, α, β, γ and δ variants should be altered. Indeed, analytical IEF of the desialylated α and the Σ, β, γ and δ variants showed that their electrophoretic mobilities were more cathodic than those of the desialylated A and the F1 and S variants, respectively, possibly meaning that the former variants had less amino acid carboxyl groups in the peptide chain than the latter ones (i.e. the net charge differences between the desialylated AAG variants must be due to their polypeptide moieties, as their carbohydrate moieties carry no electric charge [30]). It was observed that the elution of the Σ, β, γ and δ variants from the IDA–Cu(II) gel column was retarded compared to the F1 and S variants, suggesting that non-specific interactions of the former variants with the affinity gel were increased. We have previously reported that desialylation of AAG favoured its non-specific interactions with immobilized copper(II) ions, but without altering significantly its behaviour in binding to the affinity gel [9]. Thus, one possible explanation for the delayed elution of the Σ, β, γ and δ variants from the IDA–Cu(II) gel column is that these variants were desialylated. Moreover, this suggestion was supported by the chromatographic study of AAG-9308 on hydroxyapatite, before and after its treatment with neuraminidase. The chromatographic results achieved with the non-treated variants were similar to those yielded by the desialylated variants, indicating that the role of the sialic acid residues in the surface topography of the variants for binding to hydroxyapatite seems to be minor. However, it was observed that desialylation retarded the elution of the bound F1, S and A variants of AAG-9308 (and also that of the bound F1, S and A variants of control AAG) from hydroxyapatite, but did not retard the elution of the non-bound Σ, α, β, γ and δ variants of AAG-9308, possibly meaning that the latter variants were already desialylated before their treatment with neuraminidase. Nevertheless, the existence of such structural alterations, if any, did not seem to impair the binding of drugs to the Σ, β, γ and δ variants or the binding of the α variant to immobilized Cu(II) ions.

On examining the literature, one notices that the stability and denaturation of AAG of commercial source is hardly discussed, although some investigators have reported that the methods used to isolate and purify AAG on a commercial scale may result in desialylation [25] or changes in protein structure [32]. The commercial AAG preparations used in this study were isolated from Cohn Fraction VI. Previous investigators [33] have reported the association of an inactive form of plasma neuraminidase with glycoproteins in commercial preparations of human Fraction VI. The Cohn Fraction VI apparently developed the neuraminidase activity during preparation or storage. Finally, AAG appears to be less stable than other common globular proteins, due to a loosely folded region in its peptide chain [34], and this may further increase the risks of structural alterations for this protein during preparation or storage. These data and those shown in the present study point out the relevance for checking for possible alterations of the structure and the microheterogeneity of AAG in commercial preparations of the protein. Furthermore, as far as the genetic variants of AAG are concerned, simple chromatographic methods, such as chromatography on hydroxyapatite and chromatography on immobilized copper(II) ions, proved to be efficient methods for checking for the presence of irregular variants in AAG preparations.

Acknowledgments

This research was supported in part by research grants from Réseau de Pharmacologie Clinique (Ministère de la Santé) and Direction de la Recherche de l'Education Nationale (France). The laboratory is associated with the University of Paris XII (DRED EA 427).

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