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Evidence for differences in the binding of drugs to the two main genetic variants of human α_1-acid glycoprotein

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1 Human α_1- acid glycoprotein (AAG), a plasma transport protein, has three main genetic variants, F1, S and A. Native commercial AAG (a mixture of almost equal proportions of these three variants) has been separated by chromatography into variants which correspond to the proteins of the two genes which code for AAG in humans: the A variant and a mixture of the F1 and S variants (60% F1 and 40% S). Their binding properties towards imipramine, warfarin and mifepristone were studied by equilibrium dialysis.

2 The F1S variant mixture strongly bound warfarin and mifepristone with an affinity of 1.89 and 2.06 $\times$ 10^6 l mol^{-1}, respectively, but had a low affinity for imipramine. Conversely, the A variant strongly bound imipramine with an affinity of 0.98 $\times$ 10^6 l mol^{-1}. The low degree of binding of warfarin and mifepristone to the A variant sample was explained by the presence of protein contaminants in this sample. These results indicate specific drug transport roles for each variant, with respect to its separate genetic origin.

3 Control binding experiments performed with (unfractionated) commercial AAG and with AAG isolated from individuals with either the F1/A or S/A phenotypes, agreed with these findings. The results for the binding of warfarin and mifepristone by the AAG samples were similar to those obtained with the F1S mixture: the mean high-affinity association constant of the AAG samples for each drug was of the same order as that of the F1S mixture; the decrease in the number of binding sites of the AAG samples, as compared with the F1S mixture, was explained by the smaller proportion of variants F1 and/or S in these samples. Conversely, results of the imipramine binding study with the AAG samples concurred with those for the binding of this basic drug by the A variant, with respect to the proportion of the A variant in these samples.

Keywords α_1-acid glycoprotein genetic variants drug-binding imipramine warfarin mifepristone

Introduction

Human α_1-acid glycoprotein (AAG) has been identified as the main plasma transport protein for basic drugs [1] and binds a variety of other ligands, including progesterone [2], and acidic drugs, such as warfarin [3].

AAG has genetic variants and a variable glycan chain composition in its five glycosylation sites [4]. The AAG variants differ in several amino acid substitutions in the peptide chain. The amino acid polymorphism is generated by the presence of two different genes coding for AAG in humans [5]. However, the multiple allelic forms of the two genes in the human population result in the existence of at least 70–80 genetic variants for AAG, [6]. Although generally, the AAG phenotypes have only two or three of these variants, all other variants account for less than 1% of the population. These variants are revealed when the desialylated form of AAG is analyzed on isoelectrofocusing [7]. The three main AAG variants are designated F1, S and A, depending upon their electrophoretic migration. Three main phenotypes are observed for AAG in the human population, F1S/A, F1/A and S/A,

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depending on the presence of two or three of the variants F1, S and A in plasma. The AAG phenotype and the concentrations of the variants in the plasma of individuals are genetically determined. The respective frequencies of the three main AAG phenotypes in the population are 50% for F1S/A, 35% for F1/A and 15% for S/A [6].

The aim of the investigation was to determine whether the AAG genetic polymorphism is related to drug-binding differences between the variants. Several attempts have been made [8–10], but no direct evidence found for the existence of differences between the drug-binding properties of the variants, essentially because the separate variants were not pure.

We have recently developed a method for the fractionation of the AAG variants by chromatography on an immobilized metal affinity adsorbent (IMAC) [11, 12]. This method can be used to purify small amounts of the F1, S and A variants from individually purified AAG samples with the F1/A and S/A phenotypes. However, because of the relatively low affinities of drugs for human AAG (10^4–10^6 l mol^{-1}), large amounts of each variant are needed for the study of binding properties. Thus, using this IMAC method on a preparative scale, we purified large amounts of the A variant and the F1S variant mixture from commercial AAG. As variant A and the mixture of variants F1 and S correspond to the two gene products of human AAG [6, 13], their separation allowed us to then investigate the drug binding properties of each AAG variant, with respect to its genetic origin.

The drug-binding studies were performed with three different drugs: a basic drug, imipramine; an acidic drug, warfarin and a steroid antagonist, mifepristone (RU486). These drugs have already been shown to bind strongly to human AAG [3, 14, 15]. Control binding experiments for this study were performed with (unfractionated) commercial AAG and individual AAG samples with the F1/A and S/A phenotypes, previously isolated from individual plasma by chromatography on immobilized Cibacron Blue F3G-A [16].

These results were presented in part at the International Symposium on Blood Binding and Drug Transfer, Basel, Switzerland, 18–19 September 1992.

Methods

Human plasma samples

Blood samples (≈ 40 ml) were obtained from healthy subjects who gave their written consent. Blood was collected from the antecubital vein into glass collecting tubes containing sodium citrate. Blood was centrifuged immediately at 500 *g* for 20 min and the plasma fractions were frozen at −20° C until use. Selection of the individual plasma samples containing AAG with the F1/A or S/A phenotype was performed after AAG phenotyping, as described by Eap & Baumann [7].

Purification of individual AAG samples with the F1/A and S/A phenotypes

This was carried out by a one-step chromatographic procedure on Cibacron Blue F3G-A immobilized to Sephadex G-100, as already described [16]. The AAG preparations all appeared to be homogeneous after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoelectrophoresis against anti-whole human plasma.

Separation of the genetic variants of human AAG

The fractionation of human AAG was performed as described previously [11], by immobilized metal ion-affinity chromatography (IMAC), with copper (II) ions immobilized on to an iminodiacetate Sepharose [IDA-CU(II)] gel. The fractionation method was developed originally for desialylated AAG, but the same results were achieved with native (sialylated) AAG [12]. The variants in the native form were separated from a commercial preparation of AAG (from Cohn fraction VI; Sigma Chemical Co., St Louis, MO, USA; n°29F9 314). The commercial preparation was used without further purification and it consisted of a mixture of almost equal proportions of the F1, S and A variants. Its use in affinity chromatography on IDA-Cu(II) gel resolved two different variant fractions: the A variant in a pure form and a mixture of the F1 and S variants. On a preparative scale, the IMAC method was used to purify large amounts of the A variant (≈ 27 mg) and of a mixture of the F1 and S variants (≈ 63 mg) from about 110 mg of native commercial AAG (recovered proteins exceeded 90%).

Isoelectrofocusing (IEF)

The microheterogeneity of the commercial AAG and individual F1/A and S/A AAG samples, and the composition of the A variant and F1S variant mixture were checked by analytical isoelectric focusing (IEF), as follows. Prior to electrophoresis, 0.25 units of neuraminidase (type X from *Clostridium perfringens*: Sigma Chemical Co.) were added to small amounts (approximately 2 mg) of the different AAG samples in 2 ml of a 5 mM sodium acetate buffer, pH 5.5. The mixtures were incubated for 24 h at 37° C with gentle stirring, and then run on an immobilized pH 4.4–5.4 polyacrylamide gel gradient supplemented with 8 M urea and 2% (v/v) 2-mercaptoethanol, as described previously [7]. Detection of the desialyated variants in the gel was by staining with Coomassie Brillant Blue R-250. The relative proportion of each protein band in the gel was determined by scanning with an LKB Ultroscan laser densitometer.

AAG concentration of all samples

The specific assay of AAG was carried out by an immunonephelometric method, with a Behring assay kit and nephelometer analyzer (Model BNA 100). To calculate the molar concentration of the protein, a molecular mass (Mr) value of 40,000 was assumed.

Charcoal treatment at neutral pH

For some experiments, the A variant and the F1S variant mixture used in the equilibrium-dialysis studies were treated with activated charcoal at neutral pH [17]. The variant solutions (with a concentration not exceeding 1

mg ml^{-1}) were incubated for 30 min at 37° C, under gentle stirring, with Norit A charcoal (5 mg ml^{-1} of solution; Sigma Chemical Co.) at pH 7.4. The mixtures were then centrifuged for 30 min at 4° C and 5,000 *g*. The supernatants were collected and then filtered on Millex-GV 0.22 μm filters (Millipore Corp., Bedford, MA, USA).

Buffers

All solutions were prepared in phosphate buffer at pH 7.4 ($Na_2HPO_4 \cdot 12H_2O$, 0.067 M; KH_2PO_4, 0.067 M), containing 50 mg gelatin l^{-1}. Gelatin in buffer was used to avoid adsorption of ligand to dialysis membranes without significantly binding the ligand itself [17]. When necessary, the drugs were dissolved initially in a small volume of ethanol (grade A; Merck, Darmstadt, Germany). The final proportion of ethanol in these drug solutions did not exceed 2%.

Ligands

Warfarin was purchased from Sigma Chemical Co. and [^{14}C]-warfarin (specific activity 46 mCi $mmol^{-1}$, 170 GBq $mmol^{-1}$) was from Amersham France (Les Ullis, France). Imipramine chlorhydrate was a gift from CIBA GEIGY (Rueil Malmaison, France) and [^{3}H]-imipramine (25 Ci $mmol^{-1}$, 925 GBq $mmol^{-1}$) was from Amersham France. Mifepristone (RU486) and [^{3}H]-mifepristone (38.4 Ci $mmol^{-1}$, 1420.8 GBq $mmol^{-1}$) were generous gifts from the Roussel Uclaf laboratories (Romainville, France). The radiochemical purity of the drugs was greater than 98% as determined by t.l.c.

Equilibrium dialysis

Binding experiments were performed by equilibrium microdialysis, as described previously [17], in dialysis cells with a total volume of 200 μl. The concentrations of the protein solutions were between 1 and 15 μM, depending on the ligand. Each ligand was used at different concentrations (0.2–200 μM), with a constant amount of radioactivity)[0.49–5.34] × 10^6 Bq, depending on the ligand). Experiments were carried out at 4° C and pH 7.4 for 20–24 h, depending on the ligand, with gentle shaking. No significant binding to the dialysis membrane was observed (Visking 18/32; Union Carbide Corp., Chicago, IL, USA).

Evaluation of the binding data

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

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

$$B = \sum_{j=1}^{m} \frac{N_j Ka_j F}{1 + Ka_j F} = \sum_{j=1}^{m} \frac{n_j R Ka_j F}{1 + Ka_j F} \quad (1)$$

Where N_j, n_j, Ka_j denote, respectively, the molar binding site concentration of the jth class, the number of binding sites and the association constant of the specific protein; R denotes the concentration of the protein.

To calculate the parameters *K*a and n, the binding data were subjected to non-linear least-squares regression analysis, using the MicroPharm program [18]. This uses commercially available software (Micropharm Version 2.5; Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France) and estimates parameters with their standard deviations (number of binding sites and intrinsic association constant for each class of sites, and non-specific binding (NSB), i.e. non-saturable binding) using an iterative Gauss-Newton algorithm. The parameters were estimated from experimental values of 2–3 triplicate experiments. One-class and two-class binding-sites models, with and without non-specific binding, were used to fit the experimental data. The best-fit model was established by analysis of variance (*F*-test) [19].

Results

The variants of the different AAG samples used in the binding studies are illustrated by the IEF patterns shown in Figure 1. The relative scanned proportions of each variant in the different samples are listed in Table 1. The F1S variant mixture (lane 2 in Figure 1) contained 4% of variant A. The A variant (lane 1 in Figure 1) had 6 and 9% of two minor protein contaminants which were different from the F1 and S variants.

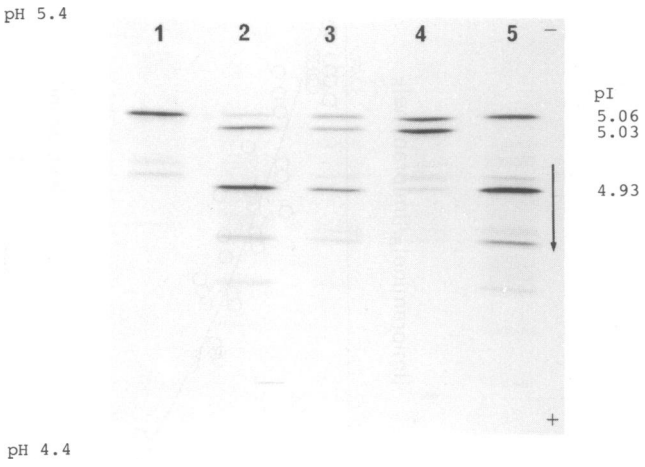


Figure 1 Analytical isoelectrofocusing (IEF) of the different AAG samples on immobilized pH 4.4–5.4 polyacrylamide (4.85% w/v) gel gradient with 8 M urea and 2% (v/v) 2-mercaptoethanol. Tracks 1 and 2: the A variant (20 μg) and the F1S variant mixture (20 μg), respectively, after their chromatographic separation from commercial AAG; track 3: commercial AAG (20 μg); tracks 4 and 5: individually purified AAG samples with the F1/A and S/A phenotypes, respectively (20 μg each). All other experimental details are described in the Methods. The pH scale and the approximate isoelectric point (pI) value for each variant are indicated. The several faint protein bands observed after staining on the anodic side of the gel (lanes 2, 3 and 5), indicated the presence of a small proportion of incompletely desialylated AAG (between 5 and 15% of the total, depending on the AAG sample).

Table 1 Relative proportions of the variants in the different AAG samples used in the binding studies

	*Relative % in AAG sample**		
	Variant F1	*Variant S*	*Variant A*
FIS mixture	57 ± 2	39 ± 2	4 ± 2
A variant**	–	–	100 ± 2
Commercial AAG***	41 ± 2	31 ± 2	28 ± 2
Individual F1/A AAG***	61 ± 2	–	39 ± 2
Individual S/A AAG***	–	63 ± 2	37 ± 2

*Relative proportions of the variants in the AAG samples were determined by scanning the gel shown in Figure 1.
**The A variant sample did not contain any detectable F1 or S variant and the relative percentage of variant A was 100%. However, the absolute percentage of variant A was only 85%, the remainder being minor protein contaminants representing 15% of the total.
***The proportion of the minor protein contaminants in the commercial and individual AAG samples was about 4%.

Individual drug-binding properties of the A variant and of the F1S variant mixture in the native form

The calculated values of the binding results for imipramine, warfarin and mifepristone are shown in Table 2 and Scatchard plots for binding to the A variant and the F1S mixture are shown in Figure 2.

The A variant was found to have a high binding affinity for imipramine (upper Scatchard plot in Figure 2a). The A variant had two classes of binding sites, approximately one high-affinity site with an association

Table 2 Binding parameters for the separate A variant and the F1S variant mixture with respect of imipramine, warfarin and mifepristone. n_1, n_2, k_1 and k_2 are the numbers of equivalent sites and the apparent association constants for the high-affinity and low-affinity classes of binding sites, respectively. NSB (l mol^{-1}) is non-specific binding. The binding parameters are given with their standard deviations

Ligand	*Variant A*	*F1S variant mixture*
Imipramine		
n_1	0.71 ± 0.04	1.22 ± 0.17
k_1 (l mol^{-1})	0.98 ± 0.10 × 10^6	3.71 ± 0.60 × 10^4
n_2	1.72 ± 0.07	–
k_2 (l mol^{-1})	1.86 ± 0.26 × 10^4	–
NSB	–	0.011 ± 0.001
Warfarin		
n_1	0.17 ± 0.01	0.58 ± 0.02
k_1 (l mol^{-1})	0.26 ± 0.06 × 10^6	1.89 ± 0.14 × 10^6
n_2	–	0.52 ± 0.02
k_2 (l mol^{-1})	–	1.59 ± 0.29 × 10^4
NSB	0.0030 ± 0.0001	–
Mifepristone		
n_1	0.21 ± 0.01	2.21 ± 0.02
k_1 (l mol^{-1})	2.18 ± 0.18 × 10^6	2.06 ± 0.06 × 10^6
NSB	0.012 ± 0.001	–

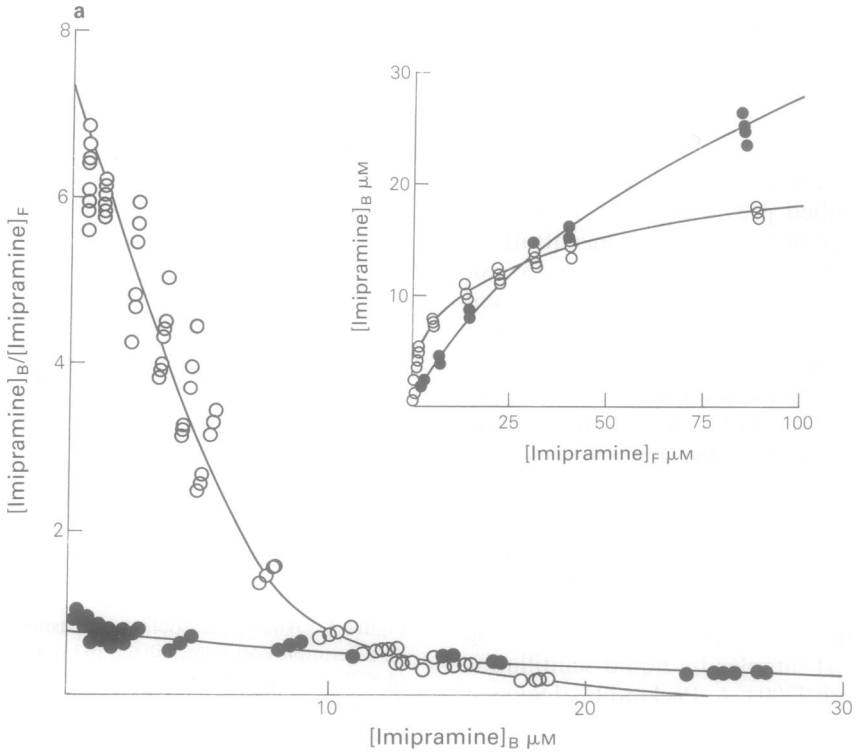
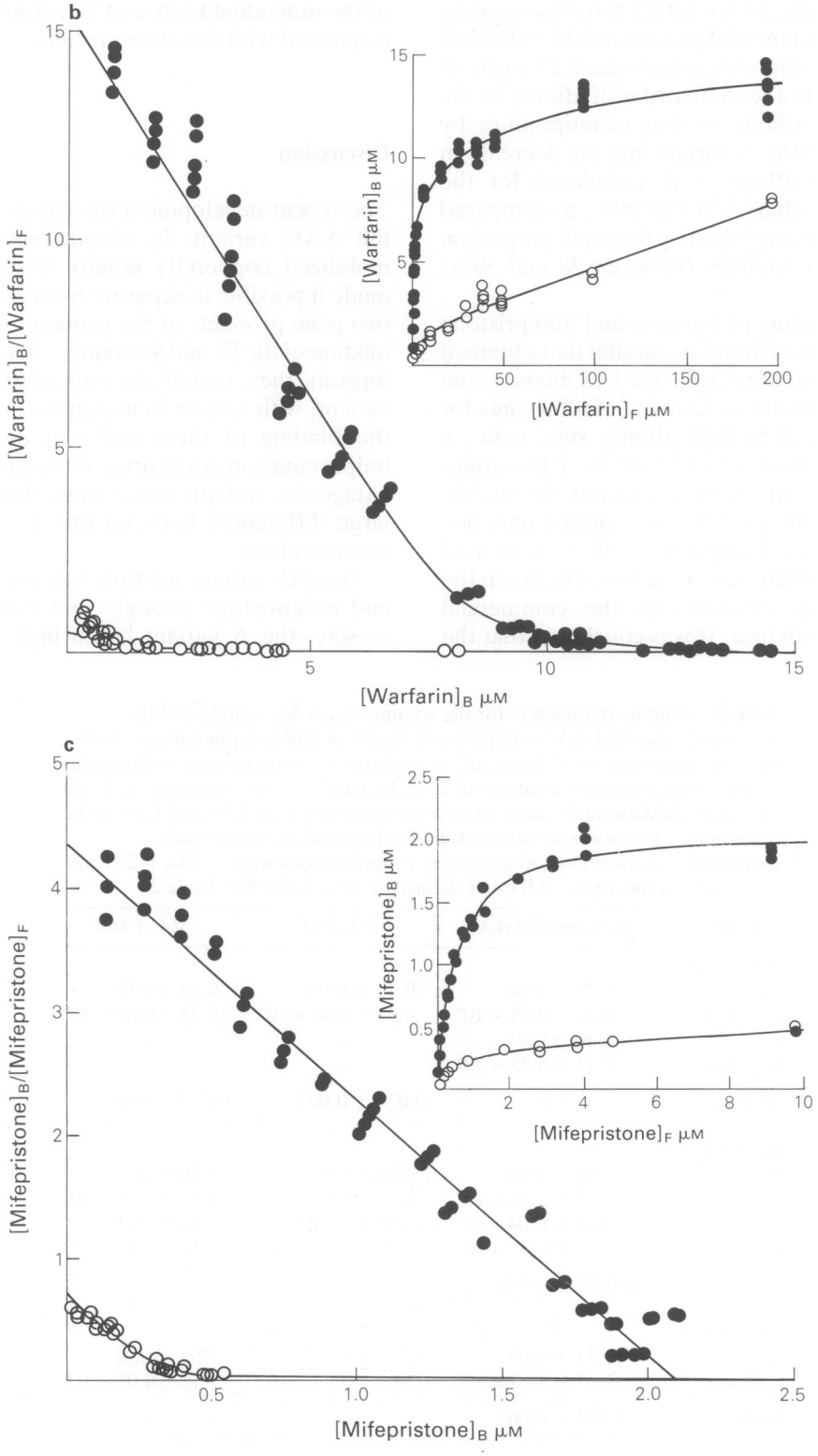


Figure 2 Scatchard plots of the binding of imipramine (a), warfarin (b) and mifepristone (c) by the separate A variant and the F1S variant mixture.
Equilibrium dialyses were carried out at 4° C in 0.067 M sodium/potassium phosphate buffer, pH 7.4, containing 50 mg l^{-1} gelatin for 22–24 h. Imipramine, warfarin and mifepristone concentrations were 1–100, 1–200 and 0.2–20 μM, respectively. In the binding experiments with imipramine, warfarin and mifepristone, the A variant (○○) was used at concentrations of 11.5, 10.2 and 1.5 μM, respectively, and the F1S mixture (●●) was used at concentrations of 14.3, 13.7 and 0.95 μM, respectively. B and F denote the bound and free drug concentrations, respectively. The experimental points correspond to 2–3 triplicate binding experiments. The inset in each figure is the binding curve. The binding parameters calculated from the curves are shown in Table 2.

constant of 0.98×10^6 l mol^{-1} and approximately two sites of lower affinity (Table 2). The warfarin and mifepristone binding study of the A variant sample (lower Scatchard plots in Figure 2b,c, respectively) showed that variant A strongly bound these two drugs, but with a small number of binding sites (0.17 sites for warfarin and 0.21 for mifepristone). Furthermore, the binding of warfarin and mifepristone to the A variant sample was not modified significantly after treatment of this sample by charcoal at neutral pH (data not shown).

The results obtained with the F1S variant mixture were very different from those obtained with the A variant. The F1S mixture had a weaker binding affinity for imipramine than the A variant (lower Scatchard plot in Figure 2a). The association constant of the F1S variant mixture for imipramine was 25-fold less than that of the A variant, with one binding site. Conversely, the F1S variant mixture exhibited a high binding affinity for both warfarin and mifepristone (upper Scatchard plots in Figure 2b and c). The F1S mixture had two classes of binding sites for warfarin, 0.58 sites with an affinity of 1.89×10^6 l mol^{-1} and 0.52 sites with an affinity of 1.59×10^4 l mol^{-1}. The binding of warfarin to the F1S mixture was not modified significantly after charcoal



treatment of the mixture at neutral pH (data not shown). For mifepristone, the F1S variant mixture had 2.21 binding sites with an apparent association constant of 2.06×10^6 l mol^{-1}.

Equilibrium binding control experiments with unfractionated AAG

These were performed with commercial AAG and individual AAG samples with the F1/A and S/A phenotypes. The values of the binding results for imipramine, warfarin and mifepristone are shown in Table 3.

Data for imipramine binding to the commercial and individual AAG samples agreed with that for the separate AAG variants. The commercial protein and the individual F1/A and S/A AAG samples had between 0.27 and 0.49 high-affinity sites, with the same order of affinity as the A variant. The high-affinity binding of imipramine by AAG was ascribed to the A variant and the decrease in the number of high-affinity sites calculated for the commercial and individual AAG samples, as compared with the A variant, was explained by the small proportion of variant A in these samples (between 28 and 39%; Table 1).

In contrast, the binding of warfarin and mifepristone to commercial AAG was found to parallel that obtained with the F1S variant mixture. Like the F1S mixture, the commercial protein had two classes of binding sites for warfarin: there were 0.50 high-affinity sites with an association constant similar to that of the F1S variant mixture; lower-affinity sites were also found. Again, like the F1S mixture, commercial AAG exhibited only one class of binding sites for mifepristone: there were 1.54 sites and only small differences were found between the respective association constants of the commercial protein and the F1S mixture. It was concluded that the high-affinity binding of warfarin and mifepristone by AAG was essentially due to the F1 and S variants in the protein. Indeed, considering all the data, it is clear that in AAG the contribution of the A variant to the high-affinity binding of these two drugs is very small. The decrease in the respective number of high-affinity sites of commercial AAG for warfarin and mifepristone, as compared to the F1S mixture, was explained by the smaller proportion of variants F1 and S in commercial AAG (72%; Table 1).

With respect to the proportion of variant F1 in F1/A AAG and that of variant S in S/A AAG (Table 1), the results of the binding study of warfarin and mifepristone to the individual F1/A and S/A AAG samples (Table 3) concurred with the above results.

Discussion

The recent development of a fractionation method for the AAG variants by chromatography on to an immobilized copper(II) affinity adsorbent [11, 12] has made it possible to separate from commercial AAG the two gene products of the protein—the A variant and a mixture of the F1 and S variants—in their native/sialylated form and then, to study the drug binding properties of each variant, with respect to its genetic origin. The results for the binding of three different drugs—a basic drug, imipramine; an acidic drug, warfarin and a progesterone antagonist, mifepristone—have shown the existence of large differences between the A variant and the F1S variant mixture.

The F1S variant mixture was found to bind warfarin and mifepristone strongly, but not imipramine. Conversely, the A variant had a high binding affinity for

Table 3 Binding parameters for the commercial AAG sample and the individually purified AAG samples with the F1/A and S/A phenotypes. In the binding experiments with imipramine, warfarin and mifepristone, commercial AAG was used at concentrations of 13.7, 13.1 and 0.91 μM, respectively. Each individual AAG sample was used at concentrations of 13, 12.5 and 1 μM in the imipramine, warfarin and mifepristone binding studies, respectively. Imipramine, warfarin and mifepristone concentrations were 1–100, 1–200 and 0.2–20 μM, respectively. All other details are as described in Table 2

Ligand	*Commercial AAG*	*F1/A AAG*	*S/A AAG*
Imipramine			
n_1	0.27 ± 0.04	0.40 ± 0.05	0.49 ± 0.08
k_1 (l mol^{-1})	$1.31 \pm 0.53 \times 10^6$	$0.31 \pm 0.06 \times 10^6$	$0.25 \pm 0.07 \times 10^6$
n_2	2.40 ± 0.08	–	–
k_2 (l mol^{-1})	$2.11 \pm 0.25 \times 10^4$	–	–
NSB	–	0.026 ± 0.001	0.023 ± 0.002
Warfarin			
n_1	0.50 ± 0.05	0.28 ± 0.04	0.20 ± 0.03
k_1 (l mol^{-1})	$1.95 \pm 0.34 \times 10^6$	$1.33 \pm 0.24 \times 10^6$	$2.59 \pm 0.75 \times 10^6$
n_2	0.25 ± 0.04	0.35 ± 0.02	0.36 ± 0.03
k_2 (l mol^{-1})	$1.03 \pm 0.67 \times 10^5$	$0.92 \pm 0.19 \times 10^5$	$2.16 \pm 0.41 \times 10^5$
NSB	0.0006 ± 0.0002	–	–
Mifepristone			
n_1	1.54 ± 0.03	1.31 ± 0.02	1.09 ± 0.01
k_1 (l mol^{-1})	$2.67 \pm 0.14 \times 10^6$	$2.01 \pm 0.09 \times 10^6$	$2.17 \pm 0.08 \times 10^6$
NSB	0.011 ± 0.005	–	–

imipramine. Warfarin and mifepristone were also found to bind strongly to the A variant, but the small number of binding sites found posed a problem. It is unlikely that the 0.17 and 0.21 high-affinity sites for warfarin and mifepristone, respectively, were due to the presence of a large proportion of denatured A variant in the sample, because of the (approximately) one (1) high-affinity site calculated for the binding of imipramine by the A variant sample. Furthermore, the finding that the binding of warfarin and mifepristone to the A variant sample was not modified after charcoal treatment of this sample, did not concur with the presence of (an) endogenous inhibitor(s) at the A variant site(s) for these two drugs. Charcoal allows the removal of hydrophobic polycyclic ligands [20]. Nevertheless, it is possible that endogeneous inhibitor(s) of the A variant, if any, were not removable by charcoal or that they were tightly bound to this variant.

On the other hand, analytical IEF of the A variant sample (lane 1 in Figure 1) revealed two contaminating sub-bands, representing a total proportion of 15% (Table 1). These sub-bands had an electrophoretic mobility close to but distinct from that of the F1 variant. These protein contaminants seem to be co-purified with AAG and their presence was always seen after analytical IEF of commercial and individual AAG samples (lanes 3 to 5 in Figure 1). These contaminants have not been identified, but their presence in AAG has been reported by others [6, 9]. The two contaminants are always found to co-elute with the A variant, after fractionation of AAG. Therefore, it is possible that the small number of binding sites of warfarin and mifepristone on the A variant sample were due to the binding of these two drugs to the contaminants. If so, the A variant had no significant binding affinity for warfarin and mifepristone.

This study is the first to show clear differences in drug-binding between the two separate gene products of human AAG. Nevertheless, previous investigations have suggested the existence of such differences [8–10]. We had already demonstrated the existence of differences between the A variant and the F1S variants, with respect to their binding properties for immobilized copper(II) ions [11, 12].

The differences in drug-binding between the A variant and the F1S variant mixture appear to be related to their separate genetic origin and, thus, to differences in their amino acid primary structure. The two different genes which code for AAG in humans, code for two proteins differing by 22 amino acid substitutions [5]. Genetic characterization of the AAG variants [6, 13] has shown that variants F1 and S are encoded by two alleles of the same gene and should differ by only a few amino acids. The A variant is encoded by the other gene of AAG and should differ from variants F1 and S by more amino acids (at least 22). This may explain why the A variant and the F1S mixture exhibit very different binding properties towards both the different drugs tested and immobilized copper(II) ions.

Furthermore, as variants F1 and S have very few amino acid substitutions in their primary sequences, this suggests that F1 and S could exhibit similar drug binding properties, as shown in their similar behaviour on immobilized copper(II) ions [11]. The study of warfarin and mifepristone binding to F1/A and S/A AAG supports this proposal. Indeed, small differences were observed between the binding parameters of F1/A and S/A AAG for warfarin and mifepristone, respectively (Table 3). Given the similar proportions of the F1 variant in F1/A AAG and the S variant in S/A AAG (Table 1) and the A variant's small contribution in these samples to the high-affinity binding of warfarin and mifepristone, it is concluded that variants F1 and S have similar binding properties towards these two drugs.

The explanation of only 0.6, and not one, high-affinity sites for the binding of warfarin to the F1S mixture, posed a problem. As shown above, it is unlikely that this small number of sites was due to the existence of differences in warfarin binding between variants F1 and S. Furthermore, it was found that the binding of warfarin to the F1S mixture was not modified by charcoal treatment of the mixture at neutral pH, and this did not support the possibility that inhibitor(s) were present at the F1 and S sites for warfarin. Finally, as warfarin is a racemate it is possible that F1 and S recognize specifically only one of the two enantiomers. Although this proposal needs to be further confirmed, it seems likely because stereoselective binding of warfarin has been reported with respect to human serum albumin [21] and stereoselective binding interactions have been demonstrated for AAG [22, 23].

Variable binding of individual drugs to AAG has been reported [24]. This has been explained by experimental factors and the different physicochemical properties of AAG itself. The latter proposal is supported by the study of imipramine binding by commercial and individual AAG samples. The mean high-affinity association constant of both F1/A and S/A AAG for imipramine was lower than that of commercial AAG (Table 3). An explanation for this could be the different origins of these samples (from individual plasma and from pooled biological samples (plasma, serum and/or urine)). The different AAG isolation methods used could also contribute to these differences: commercial AAG (from Cohn fraction VI) is isolated by successive ethanolic precipitations, which probably 'strip' the protein of endogeneous ligands, whereas the individual AAG samples were isolated by a single-step chromatographic procedure under 'mild' conditions [16].

However, having shown that the A variant and the F1S variant mixture do exhibit different drug binding properties, particular attention should now be paid to the microheterogeneity of AAG and to the role of this microheterogeneity in drug-binding, especially when comparing binding data between different sources of AAG. The existence of differences in drug-binding between the genetic variants of AAG could explain why some authors report a small or non-integral number (between 0.2 and 0.7) of high-affinity sites for the binding of basic drugs, such as methadone [25], chlorpromazine [26], propranolol and oxprenolol [27], disopyramide [28] and amitriptyline [9], by commercial AAG, and for the binding of acidic drugs, such as indomethacin and sulphinpyrazone [29], phenylbutazone [3] and warfarin [15]. It should be noted that these studies used commercial AAG preparations (Sigma or Behring) in which the proportions of the F1, S and A variants are similar (F. Hervé, unpublished results).

Given that human AAG plays an important role in

the plasma binding of basic drugs [1], the question of the possible clinical consequences of our results with respect to the separate AAG variants arises. It is conceivable that the functional differences between the genetic variants of AAG could explain differences in drug-binding between individuals. This possibility is supported by the results of investigations suggesting a genetic contribution to the interindividual differences observed in the plasma binding of amitriptyline [30] and methadone [31]. Furthermore, AAG is an acute-phase reactant, the plasma concentration of which increases in association with various inflammatory states [24]. Studies on the expression of the genes coding for human AAG in transgenic mice [32] have shown that, during the acute phase response, the mRNA encoded by the first gene, as expressed by Dente *et al.* [5], is increased, whereas the second gene is not inducible. The functional heterogeneity of AAG could, therefore, also result in alterations in the binding of drugs during disease states.

Finally, the high-affinity binding of imipramine to the A variant only, and that of warfarin and mifepristone to the F1S mixture, contradicts the general assumption that the AAG molecule has a wide and flexible ligand binding area. In terms of both specificity and localization, our results indicate that human AAG has at least two separate sites for its ligands: the first site(s) would be carried by the A variant and be specific for basic drugs, such as imipramine; the second site(s) would be carried by the F1 and/or S variants and be specific for acidic drugs, such as warfarin, and for the steroid antagonist, mifepristone.

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