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Specific ligand binding on genetic variants of human α_1-acid glycoprotein studied by circular dichroism spectroscopy

Ilona Fitos*, Júlia Visy, Ferenc Zsila, Zsolt Bikádi, György Mády, Miklós Simonyi

Department of Molecular Pharmacology, Institute of Chemistry, Chemical Research Center, Hungarian Academy of Sciences, P.O. Box 17, Budapest H-1525, Hungary

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Abstract

Human α_1-acid glycoprotein displays genetic polymorphism. Different drug binding properties of the two main genetic products (F1-S and A variants) have been demonstrated. In search for specific circular dichroism (CD) probes, dicumarol and acridine orange were found to specifically bind to the F1-S and A variants, respectively. Dicumarol binding to the F1-S variant produced induced Cotton effects originating from the favored chiral conformation of the bound label. Acridine orange gave induced biphasic Cotton effects due to chiral intermolecular exciton interaction between label molecules bound to the A variant. Displacement of the CD probes by specific marker ligands was demonstrated. The induced CD spectrum of dicumarol was found to change sign in the presence of imipramine, as a manifestation of high-affinity ternary complex formation on the F1-S variant.

Keywords: α_1-Acid glycoprotein; Genetic variants; Induced circular dichroism; Dicumarol; Acridine orange; Imipramine

1. Introduction

Human α_1-acid glycoprotein (AGP), also known as orosomucoid (ORM), is a minor, acute phase component of blood plasma. Results proving its diverse biological activity have been reviewed recently [1–3]. AGP plays an important role in the plasma protein binding of several, preferably basic drug molecules and endogenous substances [4,5]. The AGP molecule consists of a single polypeptide chain of 183 amino acids and of five asparaginyl-linked glycans. Native AGP isolated from plasma is not homogeneous; beside the high heterogeneity of glycans, the protein part shows genetic polymorphism. AGP of most individuals exists as a mixture of two or three main genetic variants (i.e. A variant and F1 and/or S variants), which can be separated by isoelectric focusing of desialylated AGP [6]. Pooled commercial AGP contains the three variants in similar proportions, but the interindividual variability is high. Concerning native human AGP composition the relative occurrence of the three main phenotypes in the population was found to be about 50% for F1 + S + A, 35% for F1 + A and 15% for S + A [7]. The variants are encoded by two different genes which have 22 codon substitutions [8,9]. The F1 and S variants (i.e. ORM1) are encoded by the alleles of the same gene and differ by less than five amino acids, while the A variant (i.e. ORM2) is the product of the other gene. The multiple allelic forms of the two genes result in the existence of numerous minor variants [6,10].

The different drug binding properties of the genetic variants of AGP were first demonstrated by Eap *et al.* [11,12]. Systematic study on this subject was carried out by Hervé and co-workers. They developed a chromatographic method by which the two main gene products of native AGP, i.e. the A variant and the mixture of the F1 and S variants (F1-S) can be separated [13]. Drug binding studies were performed with these two fractions of native commercial AGP [14–16]. It was demonstrated that various drug molecules have different selectivities in binding affinities for the genetic variants, ranging from the lack of selectivity to the total preference of one of the variants. In binding competition experiments performed by dialysis, radioactive imipramine and warfarin were chosen as high-affinity selective marker ligands for the A variant and the F1-S variant mixture, respectively. Since the majority of

* Corresponding author. Tel.: +36-1325-7900; fax: +36-1325-9188.
E-mail address: fitosi@chemres.hu (I. Fitos).
Abbreviations: AGP, α_1-acid glycoprotein; CD, circular dichroism; AO, acridine orange; AODB, acridine orange-10-dodecyl bromide.

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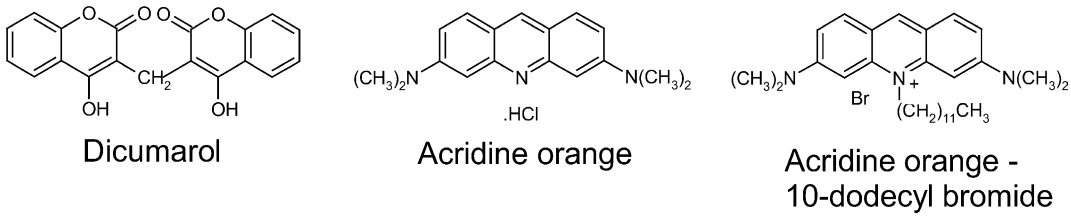

Fig. 1. Chemical structures of ligands.

the published AGP drug binding results relate to the mixture of the variants, structure–binding relationships at the molecular level are not well understood.

The present work was undertaken to find marker ligands which provoke selective induced CD signal when bound to one of the AGP variants. This methodological approach can be successfully used to characterize specific drug binding sites on human serum albumin [17]. On the basis of previous studies performed with native AGP, two ligands were selected. The binding of the basic dye acridine orange-10-dodecyl bromide (AODB) was reported [18] to produce intense biphasic CD signal. The binding of the slightly acidic drug dicumarol to commercial AGP was also found to bring about characteristic polyphasic CD signal [19]. This latter system has unique feature, i.e. the induced CD spectrum of bound dicumarol can be reversed in the presence of imipramine or some other basic tricyclic drugs [19–21], which was explained by positive cooperative interaction. We investigated the interactions of these ligands (Fig. 1) with the separated F1-S and A genetic variants of AGP.

2. Materials and methods

2.1. Ligands

Dicumarol was purchased from Janssen Chimica, acridine orange·HCl (AO·HCl) and AODB from Fluka Chemie, *rac*-disopyramide phosphate and dipyridamole from Sigma Chemical and [^{3}H]imipramine (777 GBq/mmol) from Amersham Pharmacia Biotech. Imipramine·HCl was obtained from EGIS Pharmaceuticals Ltd.

Stock solutions of 3–6 mM were prepared in buffer or in ethanol. Dicumarol was dissolved in small amount of 0.1 N NaOH and diluted with buffer.

2.2. AGP variants

Human AGP purchased from Sigma Chemical was separated at room temperature following the method of Hervé *et al*. [13]. This separation produces about 70% F1-S variant mixture (contaminated with about 4% A variant) and about 30% of pure A variant. A chromatographic column was filled with 50 mL of Chelating-Sepharose Fast Flow gel (Amersham Pharmacia Biotech), and its iminodiacetate functional groups were complexed by copper(II) ions. Samples of 30 mg AGP were applied. The slightly retained F1-S fraction was eluted by buffer, while the strongly retained A fraction was eluted by 20 mM imidazole-containing buffer. The fractions were concentrated, washed by ultrafiltration (in Amicon cells with YM-10 filters) and lyophilized. The yields were about 17 and 7 mg of F1-S and A variants, respectively. The gel was regenerated by EDTA.

In binding studies, AGP variants were dissolved in physiological Ringer buffer, pH 7.4. Protein concentrations were calculated by assuming a molecular mass value of 44,000. Experiments performed with the products of three separation runs gave identical results.

2.3. CD measurements

CD and ultraviolet-visible (UV-Vis) spectra were recorded on a Jasco J-715 spectropolarimeter at 25°, in a rectangular cell with 10 mm path length, equipped with magnetic stirring. The spectra were accumulated three times with a bandwidth of 1.0 nm. Induced CD spectra were obtained as the difference of spectra of ligand(s)–AGP mixture and that of AGP solution alone and ellipticities were expressed in millidegrees. Ligands were added in small aliquots of 3–6 mM stock solutions to 2 mL protein solutions (10–30 μM). Dilution factors were negligible (maximum 1%).

2.4. Equilibrium dialysis

The binding experiments were carried out in a Dianorm-type dialyzer using macro 1 cells and Diachem 10,000 kDa cut-off high permeability membranes. Dialysis of solutions containing the protein and ligand(s) vs. buffer (Ringer, pH 7.4) was performed for 6 hr at 25°, in duplicate. Non-specific ligand binding was measured by dialysis of protein-free ligand solution.

In case of [^{3}H]imipramine ligand (labeled imipramine solution), the concentrations were determined according to the radioactivity. Liquid scintillation countings were performed from the pre-equilibrium solution filled into the protein-containing side of the cell (c_{total}), as well as from both sides of the membrane after the equilibrium was settled. The protein-free side of the cell referred to the free ligand (c_{free}), while the other side gave the sum of the free and bound concentrations ($c = c_{free} + c_{bound} = c_{total} - c_{free}$). Free (unbound) ligand fraction was calculated by taking the concentration ratio of the two compartments (c_{free}/c).

The loss of imipramine by adsorption on the membrane was supposed not to influence the free fraction values.

In case of dicumarol binding, the ligand concentrations were measured by high performance liquid chromatographic (HPLC) analysis of protein-free solutions (c_{total} and c_{free}) and c was calculated. Adsorption loss (about 10%) was taken into account in c_{total} values. HPLC analysis was performed on Chiral-AGP (ChromTech Ltd.) column, using mobile phase of 0.01 M phosphate buffer, pH 7.0, with 12% acetonitrile. The system was composed of a Jasco PU-980 pump with a Rheodyne 7125 injector (20 μL loop), a Jasco 975 UV-Vis detector at 315 nm and Borwin software.

2.5. Calculation of CD spectrum

Dicumarol model was constructed and energy-minimized by semiempirical (AM1) quantum mechanical method with Gaussian 98 program package [22]. Rotational strengths and wavelengths of electronic transitions were calculated by CNDO/S-CI method using DZDO program [23] on a Silicon Graphics Octane workstation under Irix 6.5 operation system. On the basis of these results, non-linear regression analysis were performed to reconstruct the experimental spectrum by Gaussian components.

3. Results

3.1. Binding of dicumarol to the genetic variants of AGP

3.1.1. CD measurements

The induced CD spectra of dicumarol in the presence of F1-S or A genetic variants of AGP can be seen in Fig. 2. The characteristic polyphasic extrinsic Cotton effect which can be obtained for the binding of dicumarol to native AGP (negative maxima at 274 and 304 nm, positive maximum at 328 nm) could be detected in the presence of the F1-S variant, only. The difference CD spectrum recorded for the A variant showed baseline character. The difference UV spectra were identical for both variants.

The signal on the F1-S fraction was tested in some binding interaction experiments. In the presence of *rac*-disopyramide, which is a selective high-affinity ligand of the A variant [15,16,24], the induced CD spectrum of dicumarol was unchanged. Inhibition effect of dipyridamole, a high-affinity ligand of the F1-S variant [15,16], could not be demonstrated because of its own intensive induced negative CD signal around 300 nm. In case of imipramine, which was shown [16] to have an affinity selectivity factor of 11.8 in favor of the A variant, the interaction study on the F1-S variant exhibited results similar to those found with native AGP [19–21]. Upon the addition of increasing amount of imipramine to the solution of dicumarol and F1-S variant, the intensity of the Cotton effect decreased and even reversed signs. The change in the UV spectra were not considerable. Figure 3 shows the inversion of the induced CD spectrum of bound dicumarol in the presence of imipramine added in equimolar concentration. Plot of induced ellipticities at 328 nm vs. imipramine to AGP ratios shows (Fig. 4) that imipramine added at a concentration about five times less than AGP is sufficient to suspend the CD signal, and the maximal intensity of the reversed induced ellipticity is about 2-fold. The change of bound ligand concentrations during this simultaneous binding was determined by dialysis.

3.1.2. Binding study by equilibrium dialysis

The different binding of dicumarol to the genetic variants was measured by a direct binding method. Table 1

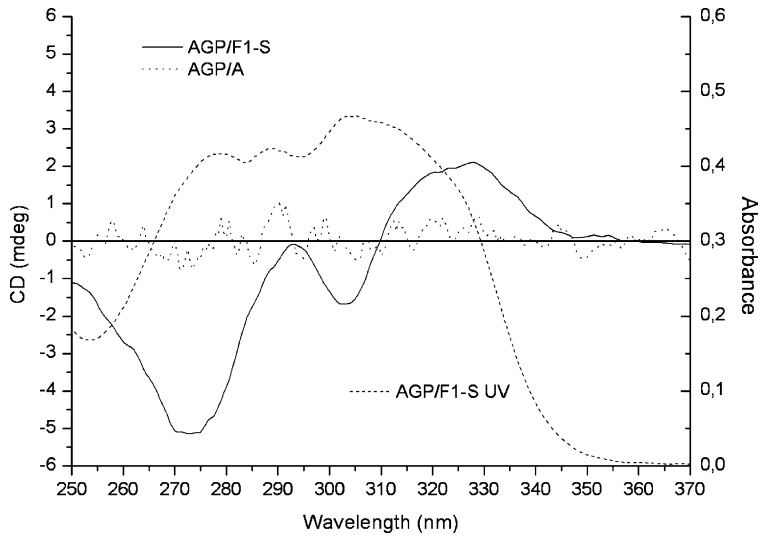

Fig. 2. Induced CD and difference UV spectra of dicumarol in the presence of F1-S and A genetic variants of AGP, in Ringer buffer, pH 7.4, at 25°, in 10 mm cell; $c_{dicumarol} = c_{AGP} = 30$ μM.

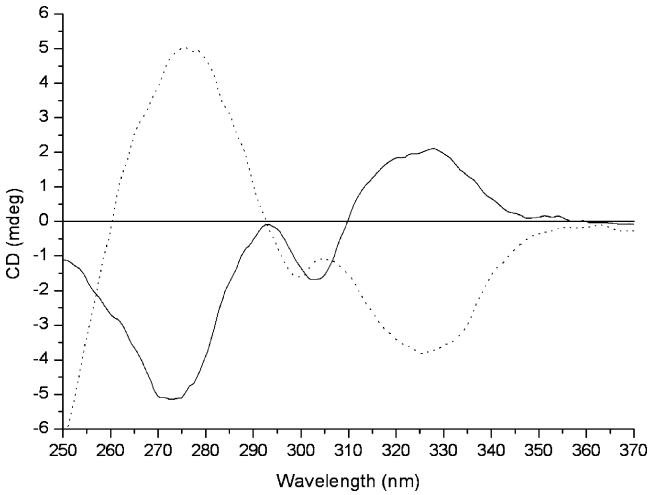

Fig. 3. Effect of imipramine on the induced CD spectra of dicumarol in the presence of F1-S genetic variant of AGP, in Ringer buffer, pH 7.4, at 25°, in 10 mm cell; without imipramine (solid line), with 30 μM imipramine (dotted line); $c_{dicumarol} = c_{AGP} = 30$ μM.

shows the degree of binding when solutions of 20 μM dicumarol and 20 μM AGP variant were dialyzed against buffer. In case of F1-S variant, which produced the induced CD spectra, 44 ± 2% of dicumarol was bound. Dicumarol binding to the A variant was weaker, but not negligible (19 ± 2%). These data suggest an affinity selectivity factor of about four. It means that the lack of induced CD is not the consequence of lack of binding. In the presence of imipramine, the binding degree of dicumarol to the A variant did not show significant change (16 ± 2%), while its binding to the F1-S variant was considerably increased (61 ± 2%), corresponding to about three times higher affinity constant.

Table 1
Equilibrium dialysis data for the binding of dicumarol to AGP genetic variants in the absence and in the presence of imipramine

AGP variant	$c_{imipramine}$[a] (μM)	$c_{dicumarol}$ (μM)	Bound dicumarol (%)
F1-S	None	11.4	44 ± 2
F1-S	40	13.0	61 ±2
A	None	9.9	19 ± 2
A	20	9.8	16 ±2

Pre-dialysis concentrations of dicumarol and AGP variants were 20 μM (Ringer buffer, pH 7.4, 25°).
[a]Pre-dialysis concentration.

Table 2
Equilibrium dialysis data for the binding of [^{3}H]imipramine to F1-S variant of AGP in the presence of dicumarol

$c_{dicumarol}$[a] (μM)	$c_{imipramine}$ (μM)	Bound imipramine (%)
None	9.7	33 ± 2
10	10.7	50 ±2
20	11.4	58 ±2
40	11.8	63 ±1

Pre-dialysis concentrations of imipramine and AGP were 20 μM (Ringer buffer, pH 7.4, 25°).
[a]Pre-dialysis concentration.

In the inverse experimental setup the binding of imipramine was measured to the F1-S variant in the presence of dicumarol. Results in Table 2 indicate enhancement of bound imipramine from 33 up to 63%, corresponding to about five times higher affinity constant. It is to be noted that the applied AGP separation method produces the F1-S fraction contaminated with about 4% of A variant. Since the binding affinity of imipramine to the A variant is known to be 11.8 times higher [16], the determined binding degree of imipramine to the F1-S variant can be somewhat

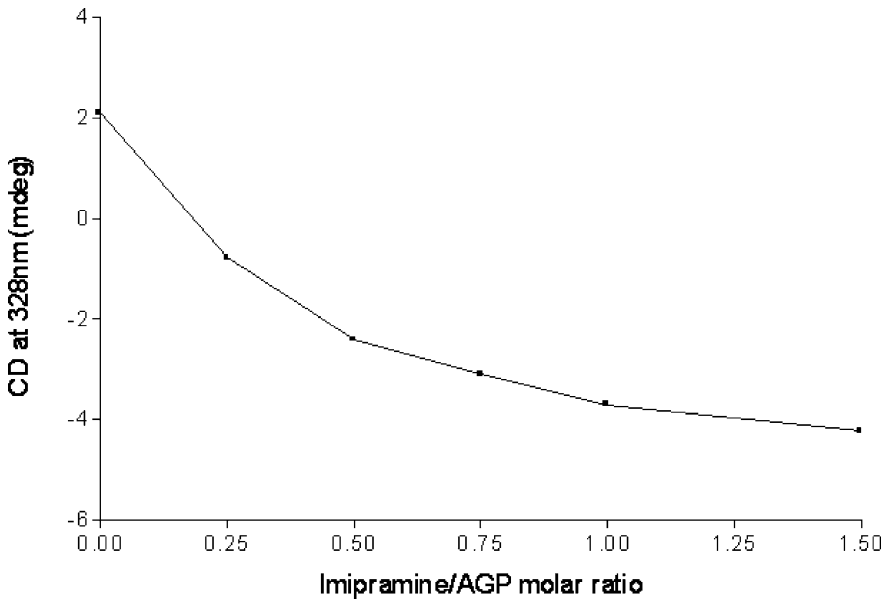

Fig. 4. Change of induced ellipticity of dicumarol bound to F1-S genetic variant of AGP by the addition of imipramine, in Ringer buffer, pH 7.4, at 25°, in 10 mm cell; $c_{dicumarol} = c_{AGP} = 30$ μM; $c_{imipramine} = 7.5$–45 μM.

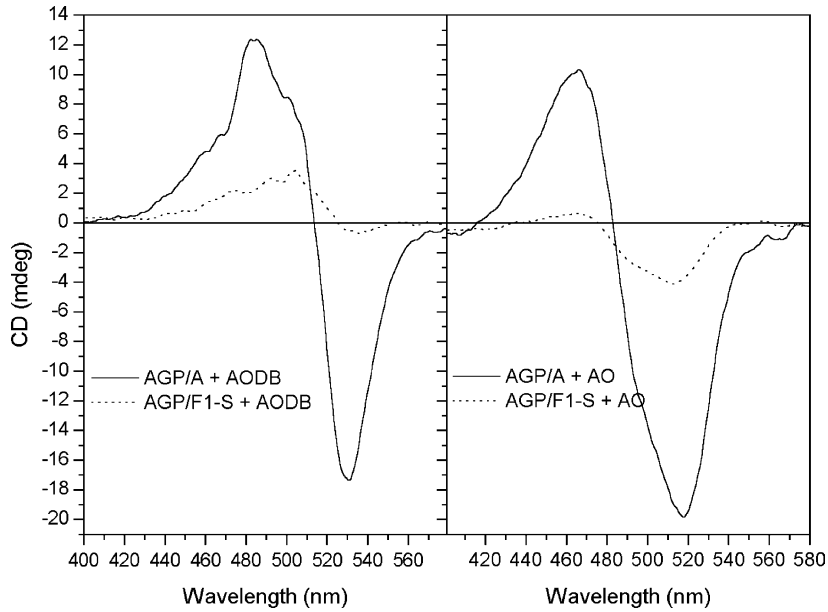

Fig. 5. Induced CD spectra of acridine orange-10-dodecyl bromide (AODB) and acridine orange (AO) in the presence of F1-S and A genetic variants of AGP, in Ringer buffer, pH 7.4, at 25°, in 10 mm cell; $c_{AODB} = c_{AO} = c_{AGP} = 30$ μM.

overestimated. Nevertheless, the measured considerable enhancement by dicumarol should be related to the F1-S variant. The mutual enhancement during simultaneous binding of dicumarol and imipramine (or analogues) found on native AGP [19–21] can be attributed to the F1-S variant. The actual concentrations in the equilibrium dialysis experiments were not the same as those in the CD studies, therefore, the molar ellipticity value of the ternary complex cannot be calculated. However, the double maximal intensity of the inverted CD spectra (Fig. 4) seems to be in accordance with the increased bound ligand concentrations.

3.2. Binding of AO to the genetic variants of AGP by CD measurements

The induced CD spectra of AODB and AO ligands in the presence of F1-S and A genetic variants of AGP (ligand to protein ratios were 1:1) are shown in Fig. 5. It can be observed that the characteristic biphasic signal reported for the interaction of AODB with native AGP [18] can be detected with the A variant only. The intensity of the induced CD on the F1-S variant is smaller and the negative Cotton effect at 530 nm is practically missing (the F1-S fraction can contain about 4% of the A variant). It is

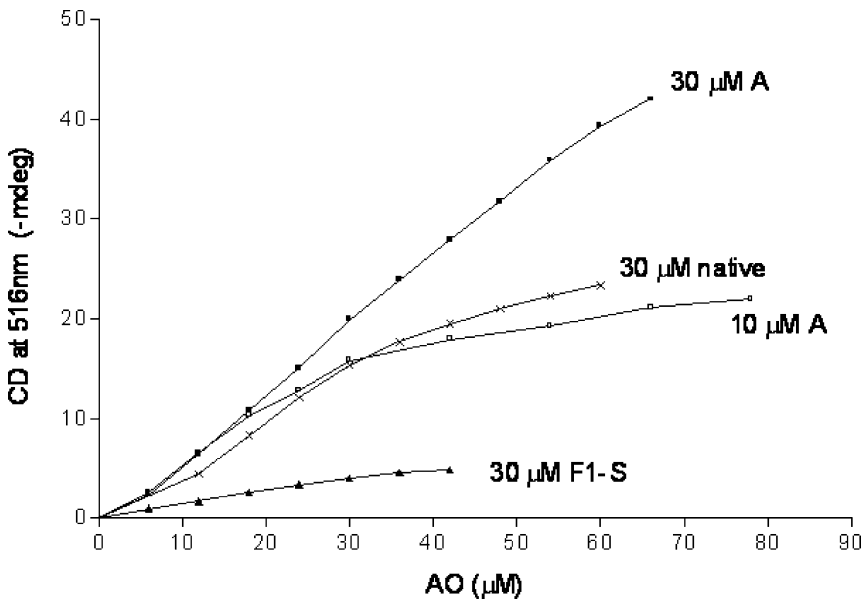

Fig. 6. Comparison of CD titration curves of AGP variants with acridine orange (AO). Plots of induced ellipticities against AO concentrations in different AGP (native, A and F1-S variants) solutions, in Ringer buffer, pH 7.4, at 25°, in 10 mm cell.

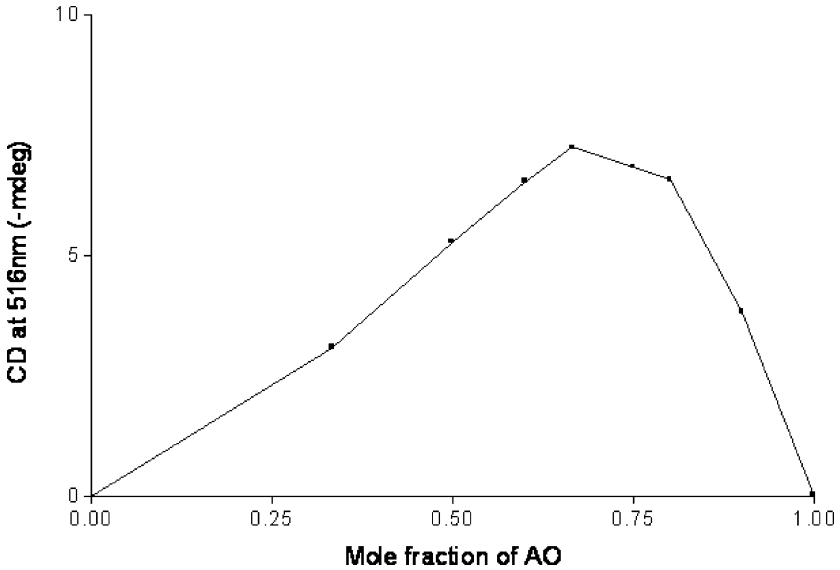

Fig. 7. Job's plot of induced ellipticities for the interaction of acridine orange (AO) and A variant of AGP, in Ringer buffer, pH 7.4, at 25°, in 10 mm cell; $c_{AO} + c_{AGP} = 30\ \mu M$.

interesting that similarly reduced and deformed induced CD of AODB could be detected on native AGP by the addition of protein denaturants, which was explained by the conformational change of AGP [18]. The water-soluble analogue AO ligand showed similar selectivity between the AGP variants. In the presence of the A variant, biphasic induced CD signal of high intensity was found, while with the F1-S variant the induced CD exhibited only a negative peak at 516 nm, substantially weaker than with the A variant.

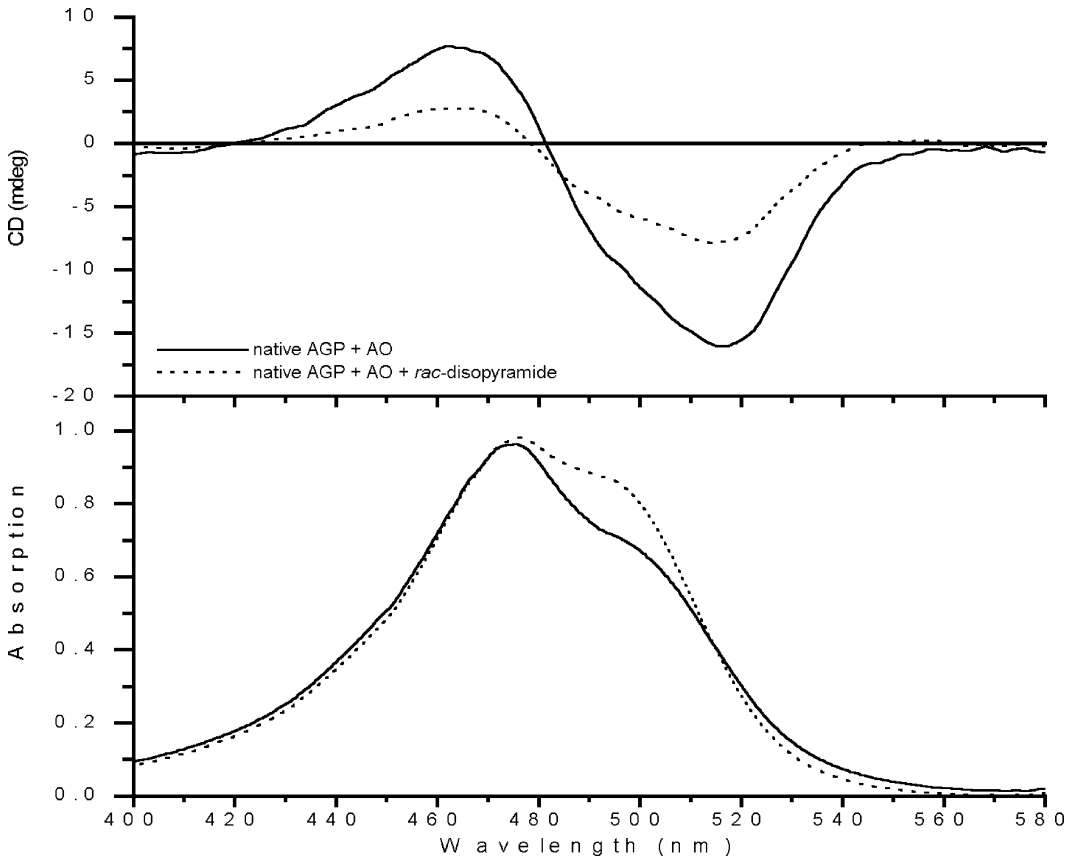

Fig. 8. Effect of *rac*-disopyramide (DIS) on the CD and UV-Vis spectra for the interaction of acridine orange (AO) and native AGP, in Ringer buffer, pH 7.4, at 25°, in 10 mm cell; $c_{AO} = c_{DIS} = c_{AGP} = 30\ \mu M$.

Titration experiments with AO ligand were performed in solutions of both genetic variants as well as of unseparated native AGP. It was found that the difference between the variants is even more considerable at higher ligand to protein ratios. While the signal obtained with the F1-S variant is getting saturated at 1:1 ratio, the induced CD with the A variant is drastically enhanced, maintaining the about 2-fold intensity ratio between the negative peak at 516 nm and the positive peak at 461 nm, with isobestic point at 483 nm. Figure 6 presents the comparison of induced ellipticities at 516 nm during titration of different AGP solutions. It can be observed that the induced CD of 30 μM native AGP is similar to that of 10 μM A variant, in accordance with the about 30% population of this variant in the natural protein. From the titration data, binding parameters (N: number of binding sites, K: association equilibrium binding constant) of $N = 3$, $K \approx 5 \times 10^5\ M^{-1}$ and $N = 1$, $K \approx 3 \times 10^5\ M^{-1}$ can be evaluated for the A variant and for native AGP, respectively. While the difference between the N values is significant, the K values could be determined with less certainty. In order to determine the maximum number of binding sites, Job's plot was constructed for the interaction of A protein variant with AO ligand, by keeping the sum of protein and ligand concentrations constant. Plot on Fig. 7 indicates maximal induced CD at ligand mole fraction of 0.67, corresponding to two bound ligand molecules on a protein. Nevertheless, the extrapolation of the ascending and descending intensity values gives inflexion point at mole fraction of about 0.75, corresponding to 3:1 stoichometry.

The selectively induced CD signal of AO on the A variant was tested in binding interaction experiments. Figure 8 shows the considerable inhibition of the induced CD of AO on native AGP in the presence of equimolar *rac*-disopyramide, which has high affinity for the A variant. The visible absorption spectra also indicate the displacement by increased absorbance at 492 nm, belonging to free monomeric AO molecules [25]. No significant spectral change could be detected in similar interaction study with dipyridamole (selective high affinity ligand of the F1-S variant).

4. Discussion

4.1. Selective interaction of dicumarol with F1-S genetic variant

Warfarin was found to bind selectively to the F1-S variant of AGP [16]. The structurally related compound dicumarol could be expected to prefer this variant, and it was confirmed by the results obtained here. While the binding of dicumarol to the A variant is weak and non-specific, the binding of dicumarol to the F1-S genetic variant is highly specific. The induced characteristic polyphasic Cotton effects reported for native AGP can be attributed to this fraction, amounting to about 70% of the native AGP. This is in agreement with the binding site number of 0.6 found previously in CD binding studies performed with native AGP [19]. This induced CD of dicumarol can be used for selective binding interaction studies, provided that the intrinsic or extrinsic Cotton effects of the other ligands are negligible.

It was shown that in the presence of imipramine the induced CD of dicumarol bound to the F1-S variant is inverted and it is accompanied with mutually enhanced binding of both ligands. This phenomenon detected previously with native AGP was explained by ternary complex formation on a wide and flexible drug binding area, where the binding sites of acidic and basic drugs are partially overlapping [19–21]. Now it was proved that this interaction takes place on the F1-S variant, which is known [16] to be the low-affinity isoprotein in the AGP binding of imipramine. It also means that in the presence of dicumarol the preference of A variant in the binding of imipramine to native AGP is less pronounced.

The induced polyphasic CD and its inversion can be obtained only with coumarin compounds containing two hydroxycoumarin moieties, with some steric limitations in the connecting position [20,21]. The extrinsic Cotton effects are probably originating from chiral conformations of bound dicumarol. There are NMR and IR evidences for the formation of intramolecular hydrogen bonds between the carbonyl and hydroxyl groups of the adjacent rings of dicumarol in chloroform [26]. In buffer solution at pH 7.4 dicumarol exists in different protonated forms according to its pK_a values of 6.10 and 8.05, and its binding to AGP was characterized as mainly hydrophobic interaction [19]. Calculations were performed to see whether the polyphasic induced CD could be explained by intramolecular exciton coupling between the isolated chromophores of dicumarol. Figure 9 shows the couplets obtained for the two intramolecularly hydrogen-bonded chiral conformers M and P. Deprotonation of one hydroxyl group shifts the couplets to higher wavelengths. The experimental induced CD spectrum of dicumarol bound to AGP shows negative maximum at 274 nm which can be assigned to the neutral form of the P conformer, while the positive maximum can be assigned to its partially deprotonated form. The fitted spectrum reconstructs the experimental one as the sum of 58% neutral and 42% partially deprotonated forms of the P conformer, being in excess for bound dicumarol. The inversion of the two main Cotton effects in the presence of imipramine (Fig. 3.) obviously indicates the preference of M conformer, resulting from the simultaneous localization of dicumarol and imipramine at the flexible binding site of the F1-S genetic variant. Although this preliminary approach neglected the intermolecular interaction of dicumarol with the protein, the preferred binding of one chiral conformation of dicumarol gives a possible explanation for the induced CD. The phenomenon shows analogy with bilirubin binding to human serum albumin [27].

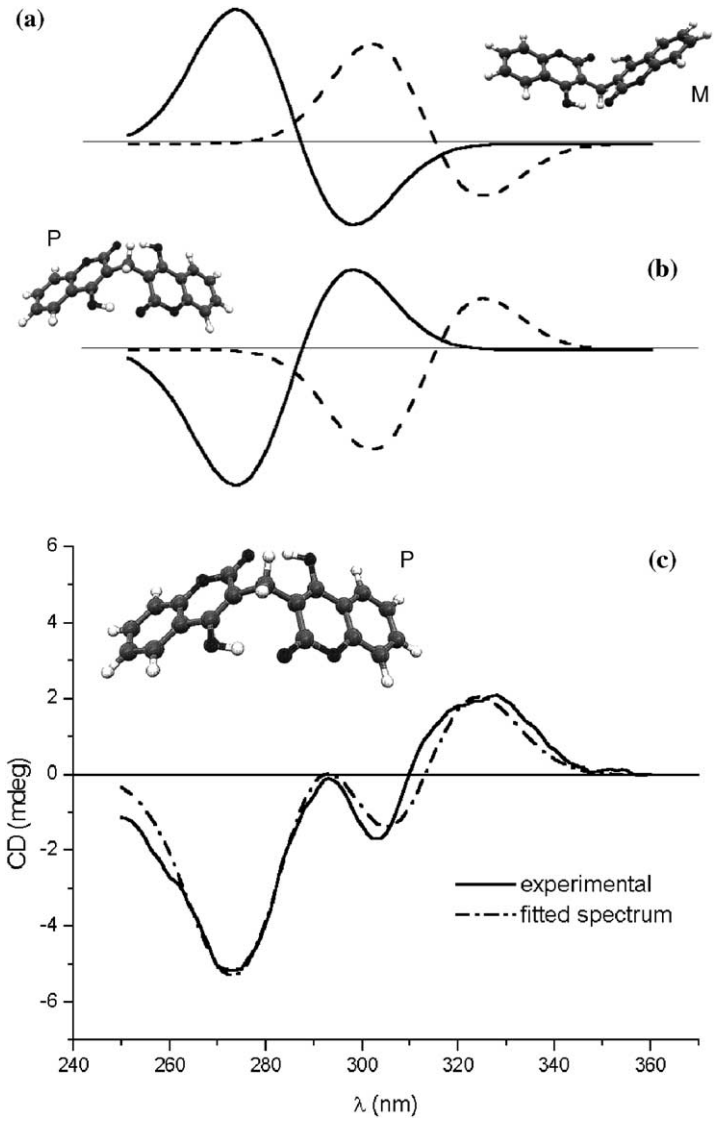

Fig. 9. Reconstruction of the induced CD spectrum of dicumarol bound to AGP by Gaussian components of the favored conformation (c). The two chiral conformations of intramolecularly hydrogen-bonded structures (M in spectrum a and P in spectrum b) correspond to calculated couplets for neutral form (solid line) and partially deprotonated form (dashed line) of the molecule.

4.2. Selective interaction of AO with the A genetic variant

AO was found to induce a selective and an intense biphasic CD signal when bound to the A genetic variant. These CD bands are in the visible region (400–550 nm), where the protein and most drugs are transparent. Therefore, AO can conveniently be used as a CD probe. The positive CD signal at about 461 nm is recommended for selective quantitative binding competition studies, where the contribution of the F1-S variant is negligible. In this way, it is possible to obtain binding information for the A genetic variant, without the necessity of separation of the native AGP.

AO is an extensively used staining agent in molecular biology. Self-aggregation of the dye molecules in aqueous solution is also a well-known process [25,28]. Induced optical activities of the dye upon binding to helical polypeptides [29] or to DNA [30] were first reported in 1961. The main source of the induced biphasic CD was explained by exciton-type interaction between two or more bound ligand molecules [31,32]. The induced exciton-type CD signal found for the interaction of AO and AODB ligands with the A genetic variant of AGP must also be the manifestation of the chiral exciton interaction between at least two dye molecules bound to the protein in left-handed helical arrangement. Further spectroscopic studies are needed to clarify the structure of the complex.

4.3. Ligand specificity of the genetic variants

Drug molecules show very different selectivities in their binding affinity for the main genetic variants of AGP. Three-dimensional structure of AGP has not been reported, yet. Recently, a computer docking of progesterone binding

was reported [33]. The tertiary structure of AGP was constructed on the basis of homologous lipocalins and molecular modeling was combined with Raman vibrational spectroscopy. This model did not see significant structural difference between the two genetic variants. Indeed, the binding of progesterone to the variants was found to exhibit practically equal binding constants [15,16]. Specific ligand binding to the A variant could be described by a simplified haptophoric model, while that approach was not successful for the F1-S variant [16]. This suggests that the binding site on the A variant preferably accommodates ligands having basic nitrogen atom and at least two rings with limited intermolecular distances. In order to get information about the binding interaction on the F1-S variant, a new probe was developed which selectively quenches the native fluorescence of the protein [34]. Its application to the binding of local anesthetics gave the conclusion that this binding site is a broad hydrophobic surface or flexible pocket with hydrogen bonding abilities and with overall positive charge at physiological pH [35].

The results obtained in this study are in accordance with the above models. AO, which is selective for the A variant, possesses the required positively charged nitrogen and tricyclic skeleton. The binding of dicumarol in preferred chiral conformation occurs exclusively on the flexible F-1S variant, allowing a cooperative interaction with the low-affinity binding site of imipramine.

The selective drug binding on genetic variants of AGP may be of clinical significance, resulting in interindividual differences and in increased risk of binding interactions. Further differences can be found in the binding properties of closely related phenotypes [36].

Acknowledgments

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