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Bioorganic & Medicinal Chemistry 12 (2004) 3239–3245
Bioorganic & Medicinal Chemistry

Induced circular dichroism spectra reveal binding of the antiinflammatory curcumin to human α_1-acid glycoprotein

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Received 13 January 2004; accepted 30 March 2004
Available online 8 May 2004

Abstract—This paper reports the first experimental evidence on binding of the plant derived curcumin molecule to human α_1-acid glycoprotein (AGP), an acute phase protein in blood. Oppositely signed induced circular dichroism (CD) bands measured in the visible spectral region in pH 7.4 phosphate buffer indicate that the protein binds this natural polyphenol molecule in a left-handed chiral conformation. Decreasing of the intrinsic fluorescence of AGP upon addition of curcumin confirmed the binding to take place. Fluorescence quenching titration curve of AGP allowed to calculate the association constant of the ligand ($K_a = 4 \times 10^4\ M^{-1}$). Modification of near UV CD spectrum of the protein suggests that curcumin induces changes in the tertiary structure of AGP, which leads to the decrease of binding affinity. By using *rac*-warfarin and amitriptyline, selective high affinity ligands of F1–S and A genetic variants of AGP, CD displacement experiments showed that curcumin is able to bind to both variants. Molecular docking calculations performed on curcumin–AGP and warfarin–AGP complexes suggest the existence of two alternative binding sites for curcumin; either at the open end of the central hydrophobic cavity or in a surface cleft of the protein.

1. Introduction

The major yellow ingredient of the powdered rhizome of *Curcuma longa* L. (turmeric) is a polyphenol molecule called curcumin (diferuloylmethane).[1] In Asia, it has commonly been used as a colouring and flavouring spice in food products. Curcumin has also been traditionally used to treat many inflammatory disorders and for wound healing for centuries. Besides its extensive usage in herbal medicine, it receives growing attention in the modern pharmacology as well due to its beneficial effects including strong antiinflammatory activity and chemopreventive properties against various human malignancies.[2–4] Although curcumin is reported to interact directly with several cellular proteins,[2] molecular mechanisms of these interactions are poorly understood.

AGP, also known as orosomucoid, is a glycoprotein of 183 residues with an extremely high carbohydrate content accounting for 45% of its mass.[5] Due to sialic acid components (10–14%) the protein is negatively charged at physiological pH. On the basis of electrophoretic migration, one fast and two slow bands can be distinguished in commercial AGP samples corresponding to the genetic variants F1, S and A, respectively.[6,7] Proportion of each variant are approximately F1 50%, S 23% and A 27%. There is a difference of at least 22 amino acid residues between the (F1–S) and A variants while F1 and S forms differ by only a few residues. AGP is classified as one of the positive acute phase proteins[8] of which normal plasma level (between 50 and 100 mg/100 mL) can rapidly increase up to three- or fourfold in response to acute inflammation.[9] Although its precise biological function is unclear, AGP was suggested to have both antiinflammatory effects and a role in immunomodulation.[5,8] In the clinical practice AGP is a valuable diagnostic and prognostic tool since its structure and serum level are substantially modified in inflammatory diseases and different malignancies. As an important plasma protein beside serum albumin, AGP binds and transports a number of endogenous and exogenous compounds including various basic and neutral drugs influencing their pharmacokinetics and pharmacodynamics.[9,10]

Unfortunately, three dimensional X-ray structure of AGP is unknown and despite the vast literature data

Keywords: α_1-Acid glycoprotein; Curcumin; Circular dichroism spectroscopy; Induced chirality; Warfarin.
Abbreviations: AGP, α_1-acid glycoprotein; CD, circular dichroism; CE, Cotton effect; HSA, human serum albumin; *L*/*P*, ligand/protein molar ratio
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doi:10.1016/j.bmc.2004.03.074

on small molecule–AGP interactions, the topography of binding sites and the binding mechanisms are not well understood.[7]

Serum level of AGP greatly increases during inflammatory and immunological processes. At the same time curcumin has pronounced antiinflammatory activity.[2,3] Since AGP binding can significantly affect pharmacokinetic/pharmacodynamic properties of drugs[5,9,10] we considered the possibility that curcumin might interact with AGP. Circular dichroism (CD) and electronic absorption spectroscopy methods are proved to be sensitive tool to study the interaction of curcumin with proteins.[11–13] By using these techniques, here we report that AGP is able to bind the curcumin molecule. Extrinsic CD spectrum of the curcumin–AGP complex was used to investigate the binding of curcumin to the genetic variants of AGP through CD displacement experiments. Additionally, based on the recently published 3D molecular model of AGP[14] obtained by homology modeling, docking calculations were performed to map the possible curcumin binding sites of AGP.

2. Materials and methods

2.1. Materials

Human AGP purified from Cohn fraction VI (catalog no G-9885, lot no 081K7604), curcumin (catalog no C-7727, lot no 119H4704) and *rac*-warfarin were obtained from Sigma and used as supplied. Amitriptyline·HCl was a gift from EGIS Pharmaceuticals Ltd (Budapest, Hungary). Double distilled water and HPLC grade ethanol (Chemolab, Hungary) were used. All other chemicals were of analytical grade.

2.2. Preparation of AGP solution

For spectroscopic sample preparation, AGP was dissolved in a pH 7.4 0.07 M phosphate buffer solution. According to the manufacturer, purity of the AGP sample was 99% by agarose electrophoresis. Molar concentration of AGP was calculated on the basis of a molecular mass of 40,000.

2.3. Preparation of curcumin stock solution

Curcumin was dissolved in 100% ethanol; the concentration was measured by determining light absorption at the λ_{max} ($\varepsilon_{429nm} = 55,000\ M^{-1}\ cm^{-1}$).[1]

2.4. Circular dichroism and UV–vis absorption spectroscopy

CD and UV/vis spectra were recorded between 250 and 580 nm on a Jasco J-715 spectropolarimeter at 15 ± 0.2 °C under a nitrogen flow. Temperature control was provided by a Peltier thermostat equipped with magnetic stirring. Cuvettes of 0.2 cm path length (Hellma, USA) were used in the near-UV (250–330 nm) and 1.0 cm path length in the visible regions (330–580 nm), respectively. Each spectrum was signal-averaged at least three times with a bandwidth of 1.0 nm and a resolution of 0.2 nm at a scan speed of 100 nm/min. Spectra were smoothed with Spectra Analysis Software, version 1.53.00 (JASCO). Induced CD is defined as the CD of curcumin–AGP mixture minus the CD of AGP alone at the same wavelengths and is expressed as ellipticity in millidegrees.

2.5. CD/UV–vis titration of AGP with curcumin

The following procedure was used: 2 mL of 1.3×10^{-4} M protein solution was placed in a rectangular cuvette with 1 cm optical path length and small amounts of the ligand stock solution (2.3×10^{-3} M) was added with an automatic pipette in 10 μL aliquots to achieve *L*/*P* molar ratios from 0.1 to 0.7. Ethanol added with the ligand never exceeded 4 % (v/v).

2.6. Measurements of CD and UV/vis spectra of curcumin–AGP solutions in the presence of specific marker ligands

Stock solutions of the ligands were prepared as follows and added stepwise in μL volumes to the curcumin–AGP solutions: 3.3×10^{-3} M amitriptyline in pH 7.4 phosphate buffer and 6.3×10^{-3} M *rac*-warfarin in 0.1 M NaOH + pH 7.4 phosphate buffer. Concentration of AGP was 1×10^{-4} M ($c_{curcumin} = 2.5 \times 10^{-3}$ M, $L/P = 0.26$). Molar ratios of marker ligands/AGP (m/AGP) and marker ligands/curcumin (m/curcumin) were changed as follows: amitriptyline, m/AGP 0.3–1.4, m/curcumin 1.3–5.3; *rac*-warfarin, m/AGP 0.3–1.3, m/curcumin 1.3–5.0.

2.7. Fluorescence quenching of AGP with curcumin and calculation of the association constant of the complex

AGP solution (2 mL 8.6×10^{-6} M) was prepared in a 1 cm rectangular cell by 0.07 M pH 7.4 phosphate buffer. Ethanolic curcumin solution (2.3×10^{-3} M) in μL volumes were consecutively added to the cuvette placed in the sample chamber of the Jasco J-715 spectropolarimeter. Sample solution was excited between 250 and 350 nm with 0.2 nm wavelength steps. Total fluorescence intensity was collected at each wavelength by a Hamamatsu H5784 type photomultiplier detector mounted on a right angle to the light source.

In the sample solution, initial and final concentrations of AGP and curcumin were 8.6×10^{-6}–8.0×10^{-6} M and 2.1×10^{-7}–1.5×10^{-4} M, respectively. [curcumin]/[AGP] molar ratio was varied between 0.02 and 18.6. During the fluorescence measurements, ethanol concentration did not exceed 10 v/v%. Control experiments performed with AGP and EtOH proved the effect of the organic

solvent to be undetectable on the intrinsic fluorescence of the protein.

The stereospecific interaction between a ligand (L) and its primary site on the protein (P) may be quantified by the association constant (K_a):

$$L + P \leftrightharpoons LP; \quad K_a \leftrightharpoons [LP]/[L][P] \tag{1}$$

It is evident that

$$[L] = c_L - [LP] \tag{2}$$

and

$$[P] = c_P - [LP] \tag{3}$$

where c_L and c_P mean the total concentrations of the ligand and protein, respectively.

Assuming that the curcumin–AGP complex (1:1 stoichiometry) is responsible for the fluorescence quenching of the protein, it can be written that

$$Fq_{283.5nm} \quad (\%) = k[LP] \tag{4}$$

where Fq is the percent of the fluorescence quenching of AGP measured at 283.5 nm and k is a constant.

Using Eqs. (1)–(4), we obtain

$$Fq(\%) = \frac{k}{2}(c_P + c_L + K_a^{-1} - \sqrt{(c_P + c_L + K_a^{-1})^2 - 4c_P c_L}) \tag{5}$$

In order to calculate the optimal values of k and K_a, nonlinear regression analysis method was applied (NLREG® statistical analysis programme, version 3.4).

2.8. Molecular modeling calculations

Geometry optimization of curcumin and warfarin molecules were carried out by GAUSSIAN 98 program using AM1 semiempirical method. AUTODOCK 3.0 program package[15] was used for mapping the energetically most favourable binding of neutral curcumin and warfarin to AGP. Published 3D molecular model of the F1–S genetic variant of AGP generated by template alignment was obtained from Kopecky.[14] Gasteiger–Huckel partial charges were applied both for ligands and protein. Solvation parameters were added to the protein coordinate file and the ligand torsions were defined using the 'Addsol' and 'Autotors' utilities, respectively. The atomic affinity grids were prepared with 0.375 Å spacing by the Autogrid programme for the whole protein target. Random starting positions, orientations and torsions (for flexible bonds) were used for the ligands; each docking run consisted of 100 cycles.

3. Results and discussion

The diarylheptanoid type curcumin contains two ferulic acid residues joined by a methylene bridge (Fig. 1). Since the keto-enol tautomerism of the β-diketone moiety, curcumin molecules exist in solution as intramolecularly hydrogen-bonded enols,[16–18] which allows π-conjugation between the two feruloyl parts resulting in the light absorption to occur in the visible spectral region.[16] Accordingly, curcumin solutions exhibit strong yellow colour, that is, the main absorption band is centred at 429 nm in ethanol. Curcumin derivatives bearing a bulky substituent on the central methylene carbon atom are colourless, since they absorb light in the near-ultraviolet.[18]

Due to the lack of a centre of asymmetry, free curcumin molecules show optical activity neither in water nor in organic solutions. However, induced CD band(s) can be measured when the molecules bind to a chiral host, that is, proteins.[11] In our recent papers[12,13] we demonstrated that curcumin binds to a hydrophobic pocket of human serum albumin (HSA) in bent, right-handed chiral conformation accompanied by the appearance of a characteristic, exciton type induced CD spectrum. Using the CD spectroscopic method again, curcumin was consecutively added to the solution of native human AGP (mixture of the three main genetic variants F1, S and A) prepared in pH 7.4 phosphate buffer. According to the spectral position of the slightly broadened absorption band of curcumin at 428 nm, two oppositely signed Cotton effects (CE) appear with a zero crossover point at 432 nm (Fig. 2). The long-wavelength negative band (472 nm) is three-times more intense than the positive one at 414 nm. These opposite bands are typical for excited state interaction, called exciton coupling, between two proximal chromophores created here by mutual rotations of the two feruloyl moieties around the central methylene group.[12,13] The exciton-coupling theory predicts that AGP-bound curcumin molecule has (M)-helicity (or left-handed chirality) since the CD spectrum shows longer wavelength negative and shorter wavelength positive bands. In other words there is negative dihedral angle between the rotated parts of curcumin molecule. The relatively small induced CD values ($\Delta\varepsilon$) might suggest that the binding site of AGP slightly prefers the left-handed chiral conformation of

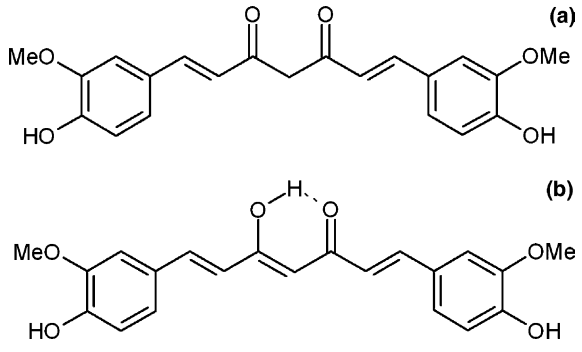

Figure 1. Chemical structures of keto (a) and enol (b) forms of curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)1,6-heptadiene-3,5-dione).

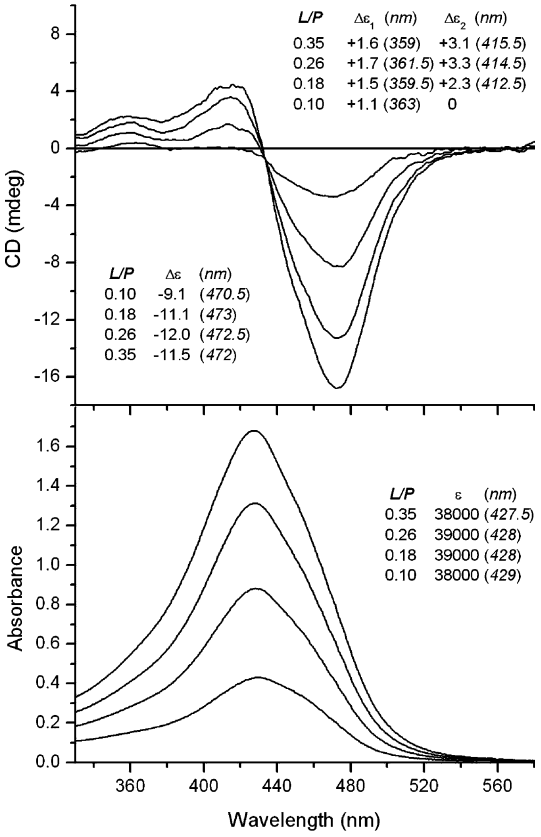

Figure 2. Induced circular dichroism and UV/vis spectra of curcumin–AGP complex in pH 7.4 phosphate buffer solution at different ligand/protein ratios (cell length 1 cm, $c_{AGP} = 1.3 \times 10^{-4}$ M, $T = 15$ °C). Values and wavelength positions of molar absorption and circular dichroic absorption coefficients ($M^{-1}\,cm^{-1}$) are indicated.

curcumin. Probably, this might be a large and flexible site rather than a small pocket, which would hold its ligand in a well-defined stereochemistry. Furthermore, the unequal intensities of CEs refer to that still significant orbital overlap exists between the interacting feruloyl parts. So, the angle between long axes of feruloic moieties is larger than −90°. The small positive CD band around 360 nm could come from the asymmetrically perturbed $n \rightarrow \pi^*$ transition of protein-bound curcumin molecule.

Notably, magnitudes of CD bands increase with the increasing concentration of curcumin but only until the value of 0.35 ligand/protein molar ratio (*L*/*P*) is achieved. Above this, surprisingly, the spectral amplitudes decrease upon further addition of curcumin (Fig. 3). Additionally, CD spectra show temperature dependence; they are unchanged at 15 °C, but above this value band amplitudes begin to decrease in time. Spectral position of the slightly broadened absorption band is the same as found in ethanol (Fig. 2).

Taken together, these spectroscopic data suggest that:

(a) Curcumin binds to AGP. In the resulting complex the ligand molecule is held in a chiral conformation.
(b) A single curcumin molecule is responsible for the induced CD spectrum.

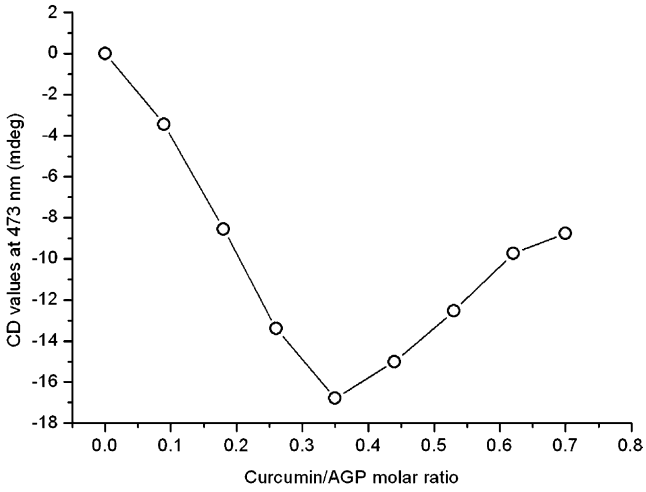

Figure 3. Plot of induced CD value of curcumin measured at 473 nm against curcumin/AGP molar ratios (experimental conditions are as in Fig. 2).

(c) The binding is weak and probably occurs at the outer surface of AGP rather than in its central hydrophobic cavity.

It is important to note that above pH 7.0 in aqueous environment, curcumin is unstable and undergoes rapid decomposition.[2] Indeed, without AGP in pH 7.4 phosphate buffer at 15 °C, the absorption band of curcumin decreases and shifts to shorter wavelengths (416.5 nm) indicating fast hydrolytic degradation (data not shown). Contrary to this, no such phenomenon was observed in the presence of AGP suggesting that the protein may provide protection against this process.

It is quite unusual that above a given concentration further addition of curcumin decreases the amplitudes of its own CD bands. To study this phenomenon, the near-UV CD spectrum of AGP was taken with and without added curcumin (*L*/*P* = 0.7). The aromatic contributions in proteins (tyrosine, phenylalanine, tryptophan) are very sensitive to even minor perturbations of the tertiary structure and such subtle changes frequently occur in the course of ligand binding. CD spectra plotted in Figure 4 demonstrate the alteration of tertiary AGP structure upon curcumin binding. A possible explanation to the decreasing induced CD activity is that above 0.35 *L*/*P* value a second curcumin molecule starts to bind to AGP causing changes in the tertiary structure of AGP, which allosterically affects the binding environment of the first molecule in such a way that this molecule either loses its chiral conformation or dissociates from the protein binding site.

For further confirmation of binding of curcumin to AGP and to calculate the association constant of the complex, fluorescence spectroscopic measurement was performed. AGP contains three Trp residues, one at the surface and two embedded in the protein matrix, which participates to the intrinsic fluorescence of the molecule. Addition of curcumin to the AGP decreased the Trp residues fluorescence indicating the binding of the ligand

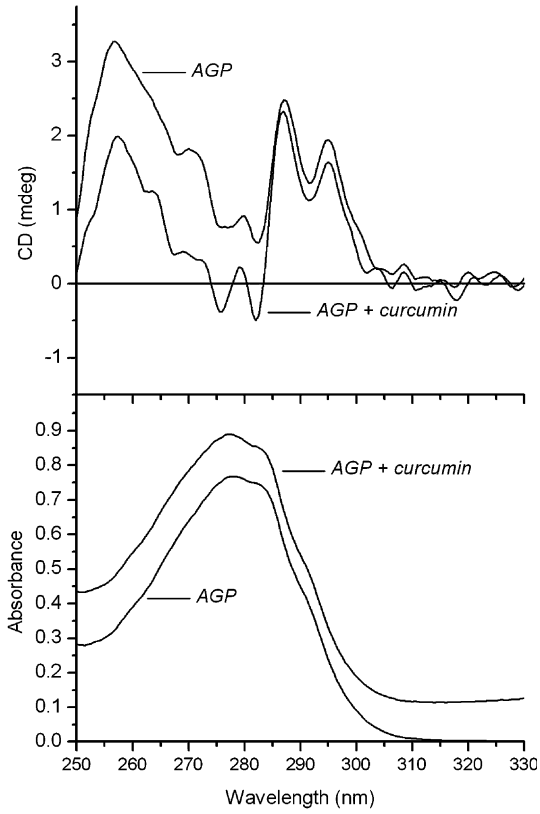

Figure 4. Near-UV CD and absorption spectra of AGP in the presence and absence of curcumin (cell length 1 cm, $c_{AGP} = 1.3 \times 10^{-4}$ M, $c_{curcumin} = 8.7 \times 10^{-5}$ M, $T = 15$ °C).

to the protein host (Fig. 5). Total fluorescence intensity quenching was only achieved with high excess of curcumin due to the different spatial localizations of the aromatic residues; obviously, curcumin quenches most

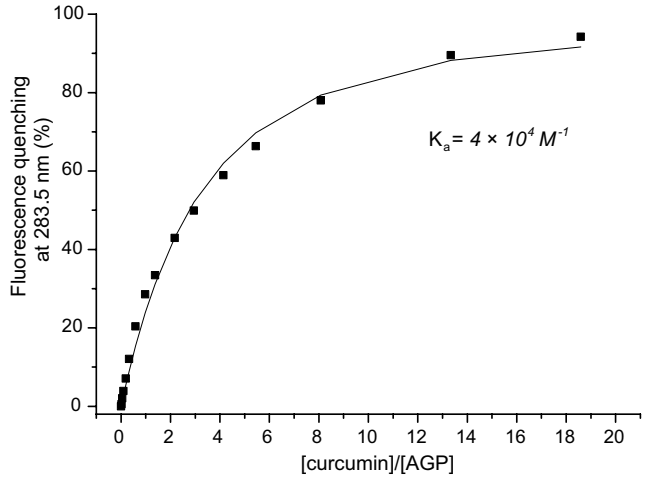

Figure 5. Titration of AGP with curcumin as followed by the decrease in fluorescence intensity of the protein. The titration system consisted of 2 mL of 8.5×10^{-6} M AGP in 0.07 M phosphate buffer, pH 7.4 (cell length 1 cm, $T = 15$ °C). Total fluorescence intensities measured at 283.5 nm are plotted versus the *L*/*P* ratio. (■): the experimental data. (—): the result of the curve fitting-procedure (nonlinear regression analysis with 1:1 stoichiometry, $r^2 = 0.9937$).

effectively the fluorescence of tryptophan located closest to the ligand binding site. By assuming 1:1 stoichiometry binding, curve fitting procedure was performed using the fluorescence spectroscopic data (Fig. 5). The resulted curve, which is in good coincidence with the titration data points allowed us to calculate the association constant of the curcumin–AGP complex ($K_a = 4 \times 10^{-4}\ M^{-1}$, see materials and methods).

To investigate whether genetic variants of AGP exhibit difference in binding of curcumin, CD displacement experiments were performed by adding *rac*-warfarin or amitriptyline to curcumin–AGP solution. The anticoagulant drug warfarin and the antidepressive agent amitriptyline are the known high affinity selective marker ligands for the F1–S and A variants of AGP, respectively.[7] It has been found that both drugs displace curcumin from its AGP binding site (Fig. 6a,b), but warfarin seemed to be more effective. At the value of 2.5 warfarin/curcumin molar ratio, the induced CEs completely disappeared and only a very weak, broad residual band can be seen located between 410 and 520 nm. Additionally, the magnitude of absorption band of curcumin decreases indicating direct competition and not allosteric effect between the molecules. Comparison of chemical structures of curcumin and the marker ligands used suggests that intermolecular hydrogen bonding and hydrophobic interactions (i.e., π–π) might play important role in the binding process of curcumin to AGP (all compounds contain hydrogen donor/acceptor functionalities and aromatic rings).

To gain deeper insight into curcumin binding mechanism of AGP, molecular models of curcumin–AGP and warfarin–AGP complexes were calculated. 3D molecular model of the F1–S variant of AGP constructed on the basis of lipocaline family sequence homology was used.[14] Results of the docking procedures of curcumin and warfarin are shown in Figure 7a,b. Based on the competition found by the CD experiments, only common protein sites were considered. Surprisingly, poor docking energy values were obtained for both molecules (especially for curcumin) docked inside the hydrophobic cavity of AGP (*site* 1). Conformation of the curcumin molecule located here is dramatically altered; by rotation around the central methylene bridge the molecule acquires 'V' shaped form (see also Fig. 8) suggesting limited space availability within the cavity. Since the two feruloyl parts are no longer conjugated in this conformation, absorption maximum of this AGP-bound curcumin should show a large blue shift. Taking into account the experimental spectra (Fig. 2) and the unfavourable docking energy, binding of curcumin inside the central cavity is unlikely.

The best docking energy result was found at the open end of the central pocket (*site* 2) where the Asn-75, Glu-69, Thr-67, Thr-76, Thr-77 and Tyr-78 residues lie close enough (within 5 Å) to form intermolecular H-bonds with the phenol and enol moieties of curcumin (Fig. 7a). Additionally, Phe-49 is in a suitable position for making hydrophobic π–π interaction with the phenol ring of curcumin. At this site, conformation of the ligand is

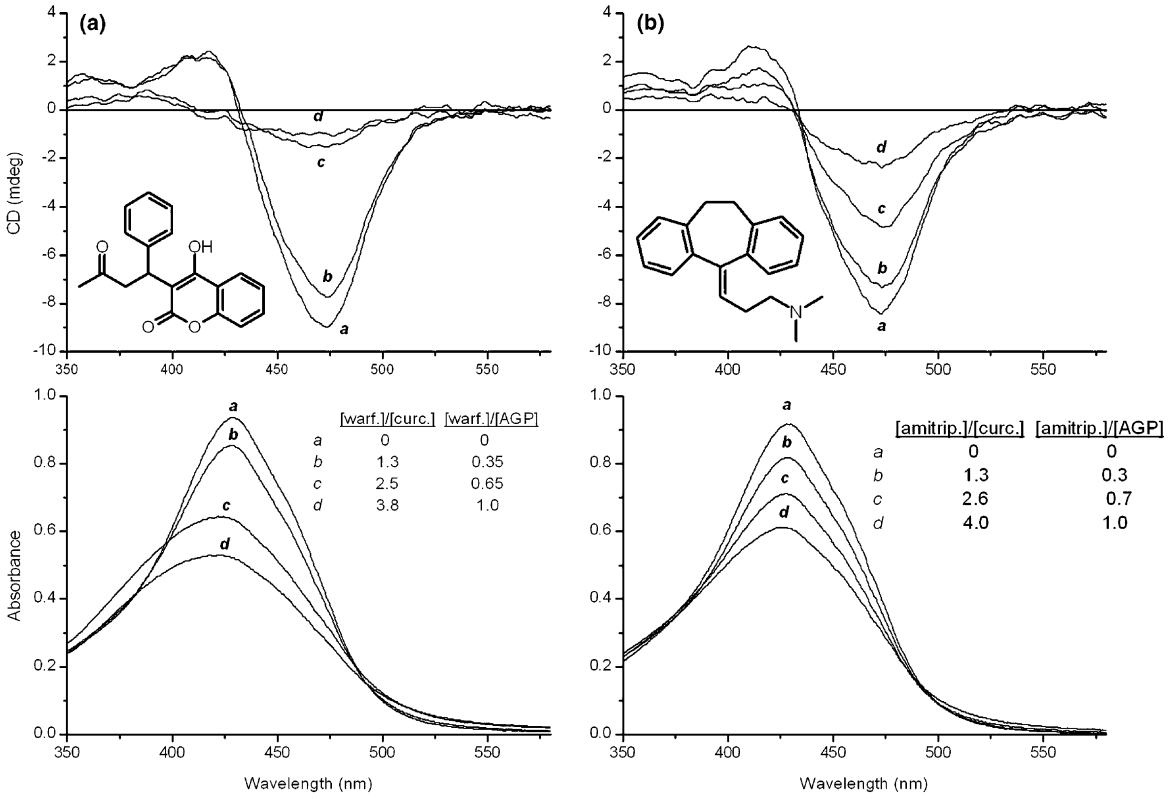

Figure 6. Induced CD and UV/vis spectra of curcumin–AGP solutions upon addition of *rac*-warfarin (a) or amitriptyline (b). Cell length 1 cm, $c_{AGP} = 1.0 \times 10^{-4}$ M, [curcumin] $= 2.5 \times 10^{-5}$ M, $T = 15$ °C.

roughly linear but closer inspection reveals that the intramolecular H-bond is broken and the feruloyl moieties are slightly twisted relative to each other (Fig. 8). Additionally, one phenyl ring is rotated and is out of the conjugation plane. This stereochemistry might account for the observed oppositely signed Cotton effects. Within the framework of the exciton coupling model, a weak intramolecular exciton interaction is supposed between the twisted, but still conjugated feruloyl chromophores (see above).

For warfarin, docking calculations explored two, energetically equivalent alternatives at *site* 2 (Fig. 7b) of which docking energies are higher only by 1.2 and 1.3 kcal/mol than that of the lowest energy result found at *site* 3 (see below). Both share a common binding space with curcumin bound at *site* 2 (cf. Fig. 7a) suggesting a possible explanation for the observed competition.

The third common binding site (*site* 3) is located on the outer surface of the protein, in a large cleft, where the curcumin molecule was found to bind in a left-handed conformation (Fig. 7a). The angle between the long axes of the twisted feruloyl parts is about −110° (Fig. 8). Its docking energy is higher only by 4 kcal/mol relative to the *site* 2 ligand. Amino acid residues involved in secondary interactions between curcumin and AGP are Glu-84, Asn-15 and Phe-114. In agreement with the experimental data, this chiral conformation of curcumin would produce long-wavelength negative and short-wavelength positive CD bands due to left-handed intramolecular exciton coupling between the feruloyl chromophores. Accordingly, this wide and flexible area of AGP may serve not only for curcumin binding but also for binding of other aromatic compounds such as acridin orange and related dye molecules.[19,20]

The lowest docking energy for warfarin was found at this site (Fig. 7b) where, similarly to *site* 2, the molecule shares a common binding region with curcumin.

4. Conclusion

Chiroptical spectroscopic and fluorescence quenching data presented here prove that the plant derived dietary agent curcumin binds to human α_1-acid glycoprotein. The resulting complex shows induced extrinsic optical activity due to the chiral conformation of curcumin bound by the asymmetric protein host. CD displacement experiments suggested that as a single molecule, curcumin is able to interact both with F1–S and A genetic variants of AGP. The relatively low association constant (4×10^4 M^{-1}) and that the curcumin–AGP complex was stable only below room temperature suggested the binding to occur at the surface of the protein. Molecular modeling calculations showed that two potential binding sites exist both being located on the outer region of AGP: the open end of the central hydrophobic cavity

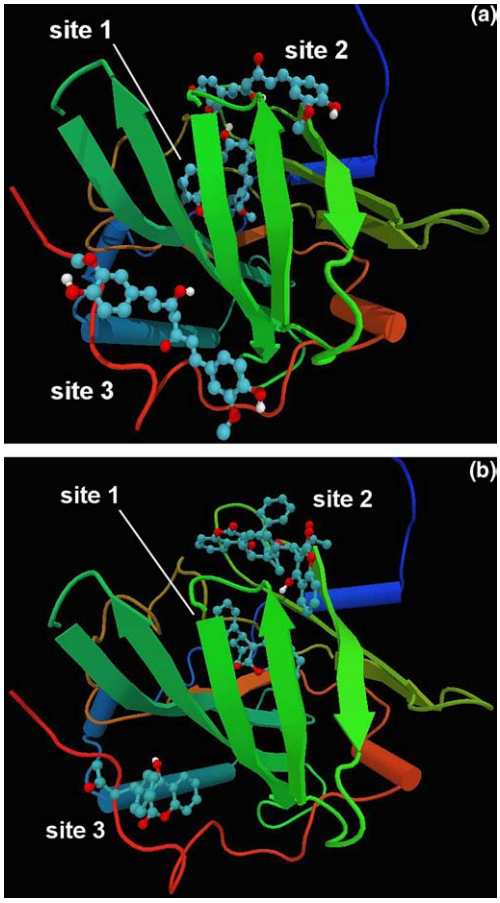

Figure 7. (a) 3D Molecular model of AGP with curcumin molecules obtained by docking procedure (C, blue; O, red; H, white); (b) 3D molecular model of AGP with warfarin molecules obtained by docking procedure (C, blue; O, red; H, white).

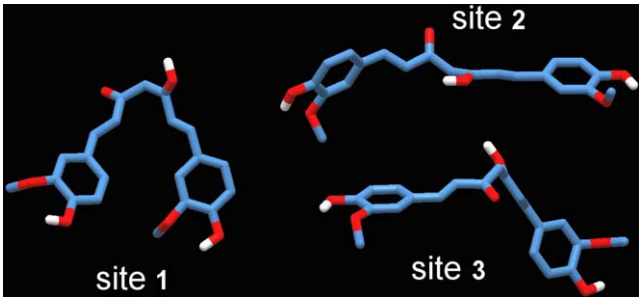

Figure 8. Conformers of curcumin found at *sites* 1–3, respectively (C, blue; O, red; H, white).

and a surface cleft. A possible significance of this superficial binding is that curcumin may alter the interaction of AGP molecule with cell membranes, viruses and biopolymers involved in the acute phase reaction.[5,21] Although HSA seems to be the most important carrier of curcumin in physiological conditions, in case of several diseases with increased AGP and decreased HSA levels[9] AGP binding may also influence pharmacological effects of curcumin. These results also highlight that curcumin derivatives and structurally related compounds such as vanilloids,[22] stilbene derivatives,[23] lignans etc., might also interact with AGP supporting the need for further studies in that field.

Acknowledgements

Financial support from the National R & D Fund (1/047 NKFP Medichem) and from the European Community (QLK2-CT-2002-90436) are gratefully acknowledged.

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