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Dedicated to Professor Eugen Segal
on the occasion of his 80th anniversary

INDUCED CHIRALITY OF GENISTEIN UPON BINDING TO ALBUMIN: CIRCULAR DICHROISM AND TDDFT STUDY

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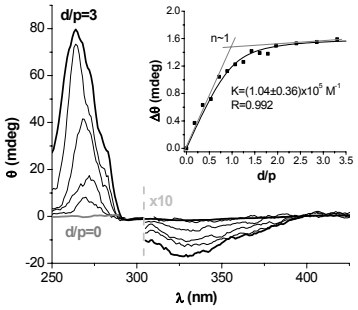
Experimental induced circular dichroism spectra of the achiral isoflavone genistein upon binding to human serum albumin were correlated with theoretical spectra obtained by time-dependent density functional theory calculations on the neutral and three anionic species of genistein in several conformations. Coupling these methods allowed us to identify the mechanism responsible for chirality induction in genistein upon binding, namely the distortion of the molecular conformation of the ligand upon inclusion into the sterically-restricted protein binding pocket. The bound genistein species and its conformation were identified and the binding parameters were estimated.



INTRODUCTION

Non-covalent, specific ligand–protein interactions may be responsible for inducing chirality to achiral ligand molecules upon their binding to asymmetric protein pockets.[1] As opposed to classical absorption spectroscopy that cannot distinguish between chromophores of the free and bound ligand, circular dichroism spectroscopy is sensitive to the altered optical properties of the bound, chiral ligand. The appearance of an induced circular dichroism (ICD) signal of the latter yields qualitative and quantitative information on the binding process, including the stereochemistry of the interaction, the localization and number of binding sites and the binding constants.

Our previous studies employed flavonols as model ligands.[2,3] Apart from their well-known biological importance,[4] compounds in the flavonoid class are particularly interesting for our study as several species are present in solution at physiological pH. By combining ICD spectroscopy with time-dependent density functional theory (TDDFT) calculations, one is able to distinguish between these species on the basis of their CD spectra and to identify the one(s) that bind to the protein. Moreover, we have shown that, by correlating to theoretical results, ICD spectroscopy provides a means of elucidating the conformation of the bound ligand. In the present paper, we apply the aforementioned methods to the study of the interaction between an isoflavone, genistein

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Fig. 1), and human serum albumin (HSA), in order to identify the ligand species that binds to the protein and its conformation, and to estimate the binding parameters.

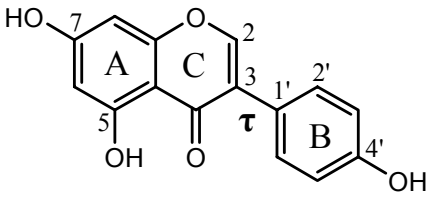

Fig. 1 – Molecular structure of genistein and numbering of ring positions.

RESULTS AND DISCUSSION

Experimental absorption and induced circular dichroism of genistein upon binding to HSA

Genistein presents three ionisable groups with pKa values of 7.2, 10 and 13,[5] corresponding to the deprotonation of the hydroxyl groups in position 7 (anionic species A7), 4' (A4') and 5 (A5), respectively. At physiological pH, the predominant species in solution are neutral (N) genistein and A7. The absorption spectra of genistein at pH 7.4 in absence and presence of HSA are given in Fig. 2. One observes the presence of two absorption bands located at 264 and 328 nm. Note that these bands correspond to both free and bound genistein. In what follows, the band of interest is at 328 nm, as it is not overlapped with protein bands and will be relevant in the ICD spectrum.

As genistein is optically inactive, it presents no CD band when free in solution. However, upon binding to HSA, an ICD signal of the former appears in its light absorbing region (Fig. 3). The two ICD bands have different intensities and opposite signs: the band at 264 nm is very intense and positive, while the band at 328 nm is weak and negative.

The stoichiometry and binding constant of the genistein–HSA complex have been obtained from plots of the absolute ICD values of the band at 328 nm *vs.* d/p (inset of Fig. 3), as described in the Experimental section. The formation of a 1:1 complex has been evidenced, with a binding constant of 1.04×10^5 M^{-1}, in very good agreement with the value 1×10^5 M^{-1} determined by Mahesha *et al.* by means of equilibrium dialysis.[6]

The explanation we propose for the observation of an ICD signal of genistein upon binding to HSA refers to the inclusion of the achiral ligand into the sterically-restrictive HSA binding pocket, which alters the conformation of the isoflavone, yielding a chiral bound ligand. We consider that the asymmetry element is the dihedral angle around the C and B fragments (τ = 2,3,1',2' in Fig. 1). In solution, due to the unrestricted rotation, several conformations are possible, the ICD signals cancelling each other due to opposite signs. In the first step of the binding process, the free rotation about the C–B torsion allows the isoflavone to adapt to the limited space available. Once inserted into the binding pocket, the rotation becomes hindered and a distorted conformation is stabilized. The spectral properties of genistein are modified accordingly, the most significant change being the appearance of ICD bands. However, the experimental data alone offer no indication on the nature and conformation of the genistein species that binds to HSA. Therefore, in order to obtain support on the proposed chirality induction mechanism and to have information on the binding species, combining the experimental results with theoretical data is required.

Time-dependent density functional theory calculations

The calculations predict a barrier to rotation of 1 kcal/mol for N genistein, confirming the free rotation in solution and thus the lack of dichroic signal. The simulated electronic CD (ECD) and absorption spectra of different species and conformations are presented in Figs. 4 and 5, and the band positions are summarized in Table 1. Fig. 5A presents the theoretical absorption spectra of the four genistein species (N, A7, A4' and A5) at the equilibrium geometries. We can discard A4' and A5 as binding species on the basis of their pKa values, as well as on the basis of the energies of their calculated equilibrium geometries, higher than A7 with 6 and 8 kcal/mol, respectively. This indicates, according to the relative Boltzmann populations, that they are practically non-existent in solution, leaving only N and A7 into discussion. One can observe that the simulated absorption spectra provide little indication on the identity of the bound species, on the basis of only the positions of the bands. The best match would be the N species, which has two bands at 262 and 331 nm, while in the spectrum of A7 the bands are shifted by 14 and 23 nm, respectively.

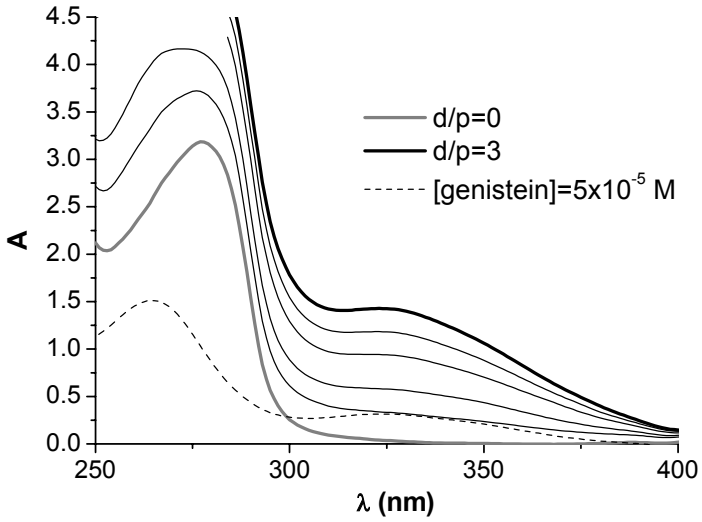

Fig. 2 – Absorption spectra of the genistein–HSA system at d/p = 0–3; [HSA] = 1×10^{-4} M.

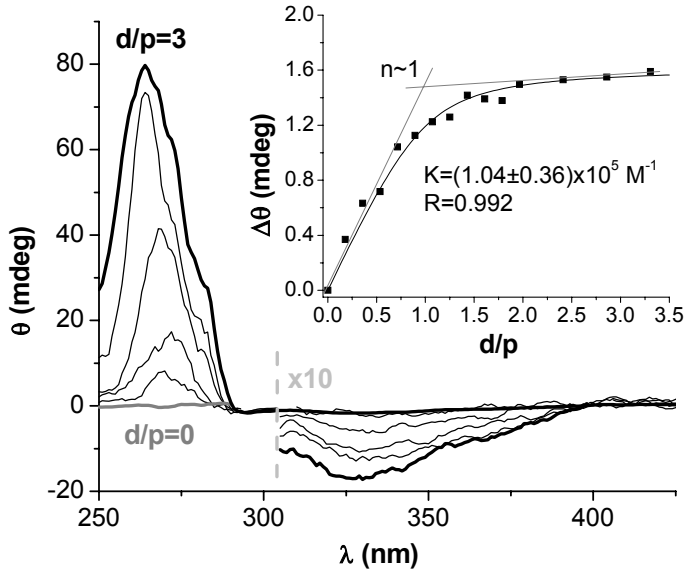

Fig. 3 – Induced circular dichroism spectra of the genistein–HSA system at d/p = 0–3; [HSA] = 1×10^{-4} M. Inset: Determination of the binding parameters of the genistein–HSA complex.

Differently, the sign and relative magnitude of the bands in the simulated ECD spectra are quite distinguishable, providing, by comparison with the experimental ICD spectrum, a means of identifying the binding species and geometry. As it can be seen in Fig. 4, the ECD spectrum strongly depends on the conformation adopted by the molecule, which influences not only the intensity and position of the band, but also its sign. This characteristic can be used to identify the binding conformation and species in a hindered medium.[2, 3] For instance, N has one positive and one negative band for τ values of –20 to –46 deg and the bands change sign for values of –60 to –90 deg. The same is valid for all anionic species.

The simulated ECD spectrum of A7 genistein presents two or three bands, depending on the conformation adopted. The conformation of –40 deg gives rise to two bands of positive (275 nm) and negative (315 nm) signs, but they are shifted comparing to the experimental values. As regards the other anionic species, they present bands above 350 nm for all calculated conformations. The best match with the experimental spectrum is for N genistein, which presents two bands at 262 nm (positive) and 331 nm (negative) for the equilibrium geometry corresponding to an angle τ of –46 deg (Fig. 5A).

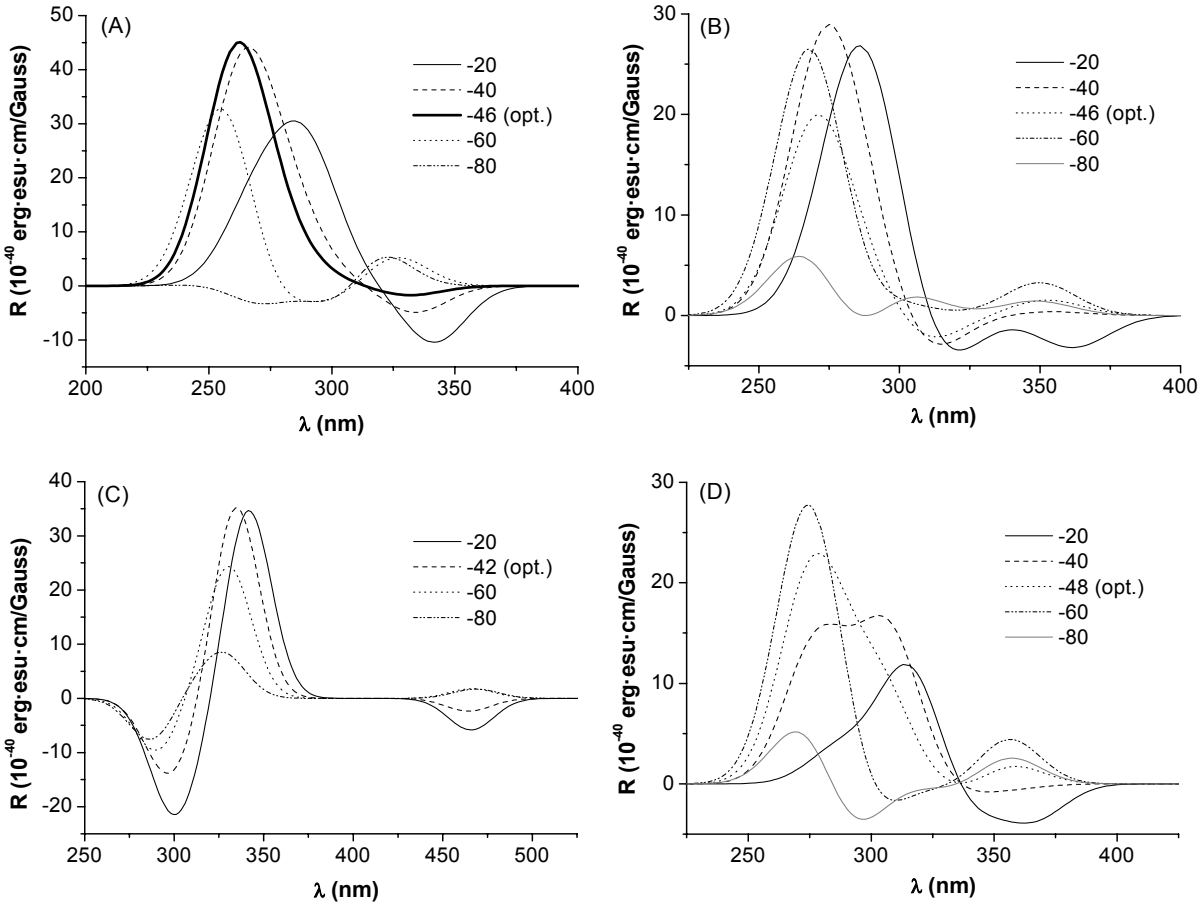

Fig. 4 – Simulated electronic circular dichroism spectra of genistein N (A), A7 (B), A4' (C) and A5 (D) in different conformations.

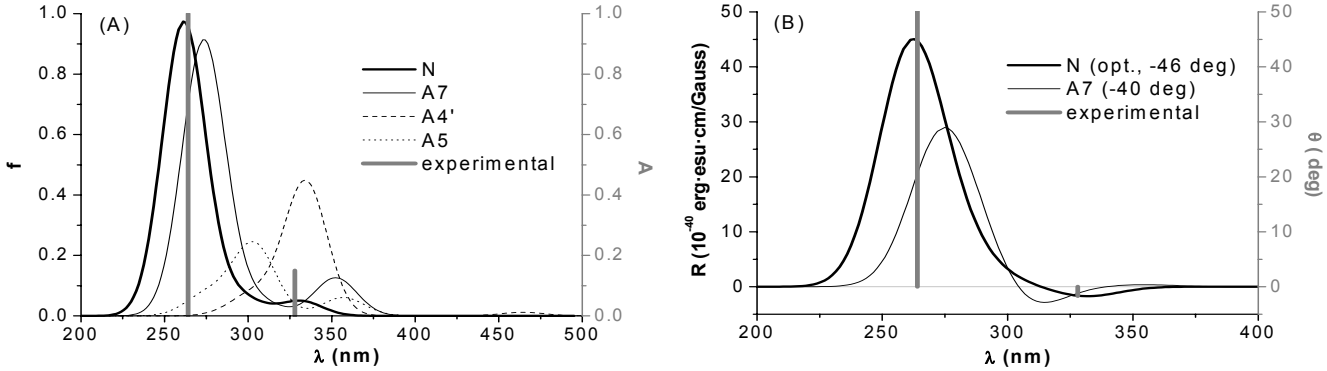

Fig. 5 – (A) Simulated absorption spectra of the four genistein species at the optimized geometry. (B) Simulated electronic circular dichroism spectra that best match the experiment. The position of the experimental bands is depicted as lines.

Table 1

Position and sign of the simulated ECD bands of the four genistein species adopting different conformations

Species	τ (deg)	λ/nm (sign)	Species	τ (deg)	λ/nm (sign)
N	–20	284 (+); 341 (–)	A7	–20	286 (+); 322 (–); 362 (–)
	–40	266 (+); 334 (–)		–40	275 (+); 315 (–)
	–46	**262 (+); 331 (–)**		–46	271 (+); 313 (–); 352 (+)
	–60	255 (+); 293 (–); 327 (–)		–60	267 (+); 350 (+)
	–80	272 (–); 294 (–); 322 (+)		–80	264 (+); 308 (+); 349 (+)
A4'	–20	300 (–); 342 (+); 466 (–)	A5	–20	282 (+); 315 (+); 361 (–)
	–42	297 (–); 334 (+); 465 (–)		–40	281 (+); 303 (+); 358 (–)
	–60	289 (–); 330 (+); 466 (–)		–48	278 (+); 306 (+); 357 (+)
	–80	287 (–); 327 (+); 468 (–)		–60	274 (+); 310 (–); 356 (+)
experimental		**264 (+); 328 (–)**		–80	269 (+); 297 (–); 356 (+)

EXPERIMENTAL

Absorption and circular dichroism measurements

To a 1×10^{-4}M HSA solution prepared in pH 7.4 phosphate buffer 0.1 M were added aliquots of a 1×10^{-3} M genistein solution in ethanol to achieve d/p (drug to protein) molar ratios in the range 0–3. We checked that no change in the HSA dichroic spectrum occurs upon addition of up to 20 % ethanol. In order to observe an induced dichroic signal, the concentrations were of the 10^{-4} M magnitude.

CD and UV-vis spectra were recorded on a Jasco J-815 CD spectrometer at 25ºC in the wavelength range 250–500 nm. The time constant, scan speed, bandwidth/resolution and sensitivity were set at 4 s, 100 nm/min, 1 nm and 100 mdeg, respectively. Each spectrum was signal-averaged three times. The ICD signal of HSA-bound genistein, θ, was obtained as the CD of the genistein–HSA complex minus the CD of genistein and HSA at the same wavelengths, and was expressed as ellipticity in millidegrees (mdeg):

$$\theta = \theta_{\text{genistein–HSA}} - (\theta_{\text{genistein}} + \theta_{\text{HSA}}) \quad (1)$$

The stoichiometry of the complex was estimated as the d/p value at the intersection of two linearly fitted domains of the plot θ *vs.* d/p.[7, 8] The binding constant was determined using eq. (2),[9] which assumes that the formation of a 1:1 genistein–HSA complex is responsible for the appearance of the ICD signal:

$$\Delta\theta = \frac{k}{2}([HSA]+[genistein]+K^{-1} - \sqrt{([HSA]+[genistein]+K^{-1})^2 - 4[HSA][genistein]}) \quad (2)$$

where Δθ is the ellipticity change due to complex formation (absolute value), [HSA] and [genistein] represent total concentrations and $k = 32982.1\Delta\varepsilon l$ (Δε is the extrinsic molar circular dichroic absorption coefficient of genistein bound to HSA in M^{-1} cm^{-1} and $l = 1$ is the path length in cm).

Theoretical calculations

Geometry optimization of the neutral and three anionic species of genistein was carried out by density functional theory (DFT) calculations, using the PBE functional and the 6-31++G(d,p) basis set in the frame of the Gaussian09 package.[10] The solvent (water) effect was introduced by the Polarizable Continuum Model.[11] Several conformations were considered by modifying the torsion angle τ (2,3,1',2' in Fig. 1) in the range 0 to –90 deg, considering the molecular symmetry. The simulated absorption and ECD spectra of these conformers were obtained by time-dependent DFT (TDDFT), PBE/6-31++G(d,p) and plotted using Gabedit 2.3.5,[12] with a full width at half maximum of 15 nm. The CD spectrum for a negative τ value is the mirror-image of that for a positive value, due to the axial symmetry of the rotating fragment.

CONCLUSIONS

The achiral isoflavone genistein binds to HSA in 1:1 stoichiometry, resulting in a chiral conformation of the bound ligand that leads to the observation of an experimental ICD spectrum for the complex. The genistein species and conformation that give rise to this signal were evidenced by comparing the ICD data with theoretical ECD spectra. Thus, neutral genistein binds to HSA in a conformation characterized by a value of the torsion angle τ equal to –46 deg. This method provides a rapid approach for characterising the stereochemistry of flavonoid binding to HSA, and can be extended to other achiral ligands. Although it neglects the intermolecular interaction between the ligand and HSA, the method gives a reasonable explanation for the observed induced chirality and readily offers valuable insight on the nature and conformation of the bound ligand.

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