Binding of a chiral drug to a protein: an investigation of the 2-(3-benzoylphenyl)propionic acid/bovine serum albumin system by circular dichroism and fluorescence

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A combined approach using global analysis of circular dichroism multiwavelength data and time resolved fluorescence was applied to investigate the interaction of $R(-)$- and $S(+)$-ketoprofen with bovine serum albumin in buffer solution at neutral pH. A characterization of the most stable drug : protein adducts of 1 : 1 and 2 : 1 stoichiometry, as individual chemical species, was obtained. The stability constants and the absolute circular dichroism spectra of the diastereomeric complexes were determined. The spectra of the 1 : 1 conjugates are opposite in sign, those of the 2 : 1 complexes are both negative, but different in shape from each other (peaks at 338 and 342 nm for $S(+)$- and $R(-)$-ketoprofen, respectively). A tryptophan residue was shown to be involved in the binding of the drug, in the primary site for the $R(-)$ and in the secondary site for the $S(+)$ enantiomer, thereby showing that chiral recognition by the protein causes the site of highest affinity being not the same for both optical antipodes.

Introduction

The nature and the strength of non-covalent interactions between molecules is at the basis of a large number of phenomena of contemporary interest in chemistry, material science and biology. For control of selectivity and maximization of ligand-substrate binding energies understanding of the factors which govern the intermolecular interactions is a major issue. In biosystems, where the complex structural organization of biomacromolecules in helices or sheets display “handedness”, ligand chirality plays a crucial role. This holds true in particular for drugs which display their action as pure optical isomers. In these systems knowledge about stereospecific interactions with biomolecules is a prerequisite for design, simplification of pharmacological profiles and elimination of the so-called “isomeric ballast”. A step to gain insight into the biochemical consequences of drug optical asymmetry can be taken through the study of their interaction with proteins, in the aim to obtain a characterization of the diastereomeric adducts, as individual chemical species in solution. In this respect, gaining detailed spectroscopic information, specific of the ligand-receptor site, is a key point. Such information cannot be achieved in the context of binding studies using conventional separation techniques. Moreover it also is hardly provided by spectroscopic techniques probing at solid-liquid interfaces, due to the complexity of the sample organization and difficult interpretation of data. Although using spectroscopic methods to determine the number of binding sites and binding constants can be problematic in general, in the present contribution we show how the combined application of circular dichroism (CD)† with global analysis and time resolved fluorescence to the system 2-(3-benzoyl-phenyl)propionic acid (ketoprofen, KP; see Chart 1) and bovine serum albumin (BSA) at KP/BSA “low” molar ratios (see below) allowed to reach a description of the chiral recognition ability of the protein and a characterization of the binding sites of highest affinity.

The partner molecules of this study were chosen because of their favourable features with respect to both binding and chemical reactivity. KP is a photosensitive drug, pharmacologically active almost exclusively in the $S(\pm)$ form. Its photoreactivity within a protein matrix, due to the presence of the benzophenone moiety, has strong relevance to photoaffinity labelling applications and to therapeutical use of the drug itself (photoallergic side-reactions are known to be mediated by

† Abbreviations: CD, circular dichroism; KP, 2-(3-benzoyl-phenyl)propionic acid (ketoprofen); NSAID, non-steroidal anti-inflammatory drug; BSA, bovine serum albumin; HSA, human serum albumin; Trp, tryptophan; Tyr, tyrosine.
protein–drug covalent photoadducts. BSA is an effective physiological carrier for a large variety of xenobiotics and a potent chiral selector, able to discriminate between enantiomers of many drugs (ref. 9 and citations therein). Previous studies of stereospecific recognition of KP enantiomers by human serum albumin (HSA, ref. 10 and citations therein) and BSA (refs. 10–12 and citations therein), have shown that the latter has the best discriminating properties.

By CD titration experiments and application of a method of global analysis of multiwavelength data, the binding constants and the absolute CD spectra of the KP : BSA diastereomeric adducts of 1 : 1 and 2 : 1 stoichiometry were determined by exploring the molar ratio range 0.4–2.2. A parallel time resolved study of the intrinsic protein fluorescence, carried out in the same experimental conditions used in the CD experiments, allowed to further characterize the binding sites of the KP enantiomers and to gain insight into the chiral recognition ability of BSA at the molecular level.

**Materials and methods**

**Materials**

S-(+)-2-(3-Benzoylphenyl)propionic acid (ketoprofen, KP), racemic KP (rac-KP), bovine serum albumin (BSA, 99%, essentially globulin and fatty acid free) were purchased from Sigma.

**Preparation of R(−)-2-(3-Benzoylphenyl)propionic acid**

The preparation of R(−)-KP was accomplished by a biocatalytic procedure consisting of three sequential steps and exploiting lipase from *Candida antarctica*, of known R stereopreference, adsorbed on acrylic resin (Novozyme 435).

**Step I (esterification).** KP (3 g, 11.8 mmol) was dissolved in toluene (300 ml) containing triethyl orthoformate (5.8 ml, 35.4 mmol) and lipase (6.5 g). The suspension was placed into a thermostated shaker (45 °C, 300 rpm) and the progress of the reaction was monitored by chiral HPLC. After 2 h the conversion reached 45% and the reaction was stopped and the enzyme filtered off. The KP ethyl ester and the unreacted KP were separated by selective partition with a sodium bicarbonate solution. The organic phase, evaporated to dryness furnished R(−)-KP ethyl ester with 60% ee (1.4 g).

**Step II (hydrolysis).** R(−)-KP ethyl ester (1.3 g, 4.6 mmol, ee 60%) was dissolved in acetonitrile (130 ml) containing water (2 ml) and *Candida antarctica* lipase (6.5 g). The suspension was incubated at 45 °C under shaking (300 rpm) and the reaction continued for 45 h up to 65% conversion. Removal of the enzyme, followed by solvent evaporation under reduced pressure, left a residue that was partitioned between tert-butylmethyl ether and sodium bicarbonate solution. The aqueous phase, after acidification with H$_2$SO$_4$ N, was extracted tert-butylmethyl ether to give R(−)-KP (730 mg, ee 75%).

**Step III (esterification).** R(−)-KP (700 mg, 2.7 mmol, ee 75%) was subjected to esterification for 80 min, using the same conditions as described in the above step I, to give the corresponding R(−)-KP ethyl ester with 92% ee. After conventional chemical hydrolysis, followed by crystallization using benzene/hexane (3 : 10), R(−)-KP was obtained (360 mg, ee >97%; [α]$_D$ = 54.4, (c = 1 CH$_2$Cl$_2$),$^3$ +54.4 (c = 2.71 CH$_2$Cl$_2$)).

KP methyl ester was obtained by stirring overnight at 80 °C a methanol solution of KP (5 × 10$^{-3}$ M) in the presence of 1% sulfuric acid. The solution was then evaporated and the product was isolated by liquid chromatographic on silica gel column using n-hexane/dichloromethane (20 : 80, v : v) as eluent. The compound was fully characterized by mass spectrometry (ESI-MS) and $^1$H-NMR.

Phosphate buffer 0.01 M at pH 7.4 was used as solvent for spectroscopic and fluorescence measurements. Water was purified by passage through a Millipore MilliQ system.

3-Ethylbenzophenone (3-EB) was obtained by photoradiation of a 10$^{-3}$ M KP solution in 0.1 M phosphate buffer pH 7.4. A volume of 330 ml of solution was exposed to light from a high pressure 150 W Xe lamp for ca. 2 h. Light components below 324 nm were cut off. Magnetic stirring and argon bubbling were maintained during the irradiation. After addition of NaOH 0.01 M and extraction with CH$_2$Cl$_2$ the main product, 3-EB, was separated by column chromatography and fully characterized by $^1$H NMR.

**Sample preparation**

A series of identical aliquots of BSA were weighted and dissolved each in identical volumes of either pure buffer or KP solutions at the required concentration. The samples were gently stirred up to complete protein dissolution and were carefully protected from ambient light during the manipulations in order to avoid photodegradation of ketoprofen, occurring in aqueous solutions at neutral pH with very high quantum yields. All the experiments described below were performed at a constant BSA content of 3 mg ml$^{-1}$ (4.5 × 10$^{-5}$ mol dm$^{-3}$). This value was chosen because it was in the linear region of the dependence of the BSA ellipticity on the protein concentration. The analysis of experiments performed at constant drug but varying BSA concentration proved to be much more difficult because of the dependence of the drug binding constants on the protein concentration (see below).

**Spectroscopy and time resolved fluorescence**

All the measurements were performed at 25 °C. Ultraviolet absorption spectra were recorded on a Perkin-Elmer L49 spectrophotometer using 0.2 cm path cells.

Circular dichroism spectra were obtained with a Jasco J-715 spectropolarimeter in cells of 1 cm pathlength. The spectra were registered in the 290–400 nm range, by using accumulation and time integration for improvement of signal to noise ratios.

Fluorescence emission spectra were obtained on a Spex Fluorolog 111A spectrofluorimeter. Right angle detection geometry was used. Experiments were carried out in cells of 1 cm pathlength. Fluorescence lifetimes were determined by means of a time-correlated single photon counting system (IBH Consultants Ltd.) in air-equilibrated solutions. The nanosecond flashlamp, filled with deuterium, was thyatron-driven at 40 kHz; the instrumental response function had a full width at half maximum of 2 ns. The absorbance of the solutions was ca. 0.1–0.3 in the usual 1 cm cell at the excitation wavelength of 295 nm. Emission was collected at right angles. For optically dense solutions a 0.5 cm path cell was used; the emitted light was collected from the first millimeter of the excited solution.

Fluorescence decay profiles were analysed by the least-squares method, assuming multiexponential decay functions and deconvolution of the instrumental response. The software package was provided by IBH Consultants Ltd. The resolution limit after deconvolution was ∼0.2 ns.

**Calculations**

The assessment of the best complexation model and the determination of the association constants of the complexes were performed by global analysis of CD spectra obtained at 10–12 different ketoprofen concentrations, using the whole 290–400 nm wavelength range for the calculations. The commercially available computer program SPECFIT/32 (Spectrum

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The CD spectra of $S\cdot(+)-$ and $R\cdot(-)-$ KP in phosphate buffer 0.01 M, pH 7.4, at 25°C, in the region 290–400 nm are reported in the inset of Fig. 1A. The $\Delta\alpha(\lambda)$ is characterized by a weak band at $\lambda$ = 340–350 nm, corresponding to the aromatic carbonyl $\pi,\pi^*$ $S_1 \leftrightarrow S_0$ transition, positive for $S\cdot(+)-$ and negative for $R\cdot(-)-$ KP, and a weak negative signal around 300 nm, corresponding to the onset of the $\pi,\pi^*$ $S_2 \leftrightarrow S_0$ band (3). The origin of such a small intrinsic CD in the lowest energy band has been attributed to an interconversion of chiral conformers promoted by the flexibility of the benzophenone moiety. The ionization of the carboxyl group of the propionic substituent, completely dissociated at this pH ($pK_a$ = 4.7, ref. 14), is not expected to influence significantly the CD in this spectral region, due to the peripheral location of the propionic acid functionality, containing the asymmetric carbon center, with respect to the benzophenyl moiety.

BSA in the same wavelength range, exhibits a negative CD, corresponding to the onset of the strong negative protein bands at 209/222 nm. The ellipticity follows a linear relationship vs. the BSA concentration up to $5 \times 10^{-5}$ mol dm$^{-3}$. Above this limit a deviation from linearity was observed and was attributed to the occurrence of molecular aggregation. This process affects the binding ability of the BSA molecules, making unfeasible the determination of the binding constants by means of titration experiments with constant KP and variable BSA concentration. Indeed concentration dependent binding constants were reported for HSA.$^{10,16}$ For this reason all the experiments described below were performed at a constant BSA concentration.

Circular dichroism of BSA-KP complexes

Fig. 1A and 2A show the effect of addition of $S\cdot(+)-$ and $R\cdot(-)-$ KP at various concentrations on the ellipticity of a 4.5 × 10$^{-3}$ mol dm$^{-3}$ BSA solution. In these experiments molar ratios KP:BSA were kept between 0.4 and 2.2 to limit the number of protein sites occupied by the drug.$^{11,12}$ The intrinsic $\theta$ signal of BSA was subtracted to better evidence the changes due to the formation of the associates. The ellipticity of KP in the 320–380 nm region is much stronger in the protein matrix than in buffer and is characterized by a well defined vibrational progression of $ca. 1200$ cm$^{-1}$. This structure is typical of $\gamma,\delta$-unsaturated asymmetric ketones in non-polar solvents, and has been attributed to a strong rigidity imposed to the benzophenyl moiety by the protein matrix and to a hydrophobic character of the environment of the ketone.$^{11}$

Each set of experiments were globally analysed using an iterative numerical procedure (see Experimental). This analysis afforded the stoichiometry of the most stable complexes and the values of the binding constants (Table 1). A complexation model with simultaneous presence of both 1 : 1 and 2 : 1 KP : BSA complexes was found to apply quite well. The agreement obtained between calculated and experimental ellipticity values can be appreciated in Fig. 1B and 2B for key wavelengths.$^{\dagger}$

A model involving the concomitant presence of 3 : 1 KP : BSA complexes was also checked. For $S\cdot(+)-$ KP the best fit to experimental data did not improve in quality, clearly indicating that there was no significant amount of such complex in the used molar ratio interval, in agreement with the presence of a group of two binding sites with a substantially higher affinity with respect to additional sites. Accordingly, in HSA a factor $ca. 100$ in the mean affinity constants for racemic KP was reported to exist between a group of two primary sites and a group of six-seven secondary sites.$^{13}$ In the case of $R\cdot(-)-$ KP an acceptable fit was achieved under this hypothesis also, but it did not correspond to actual convergence in the optimisation procedure. Relevant binding parameters were characterized by large errors and were therefore discarded. On the basis of these results it was concluded that, in the conditions of the experiment, formation of a complex where a BSA molecule is charged with three $R\cdot(-)-$ KP molecules is not needed for the reproduction of the CD signals.

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**Results**

Circular dichroism of KP enantiomers and BSA

The CD spectra of $S\cdot(+)-$ and $R\cdot(-)-$ KP in phosphate buffer 0.01 M, pH 7.4, at 25°C, in the region 290–400 nm are reported in the inset of Fig. 1A. The $\Delta\alpha(\lambda)$ is characterized by a weak band at $\lambda$ = 340–350 nm, corresponding to the aromatic carbonyl $\pi,\pi^*$ $S_1 \leftrightarrow S_0$ transition, positive for $S\cdot(+)-$ and negative for $R\cdot(-)-$ KP, and a weak negative signal around 300 nm, corresponding to the onset of the $\pi,\pi^*$ $S_2 \leftrightarrow S_0$ band (3). The origin of such a small intrinsic CD in the lowest energy band has been attributed to an interconversion of chiral conformers promoted by the flexibility of the benzophenone moiety.$^{11}$ The ionization of the carboxyl group of the propionic substituent, completely dissociated at this pH ($pK_a$ = 4.7, ref. 14), is not expected to influence significantly the CD in this spectral region, due to the peripheral location of the propionic acid functionality, containing the asymmetric carbon center, with respect to the benzophenyl moiety.

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negative sign for $S(+)-$ and $R(-)-$ KP, respectively; the 290–300 nm region is characterized by a positive sign for both isomers. In the 2 : 1 spectra the carbonyl region has negative sign with absolute minima located at 358 and 342 nm for the $S(+)-$ and $R(-)-$ KP, respectively; a positive signal at 290 nm, an isoelliptic point at 294 nm and a negative peak at 300 nm are observed with $S(+)-$ KP, whereas a positive band at 290 nm and an isoelliptic point at 300 nm are observed with $R(-)-$ KP.

Association of a racemic KP was studied by CD titration at $4.5 \times 10^{-5}$ mol dm$^{-3}$ BSA and total drug concentration varying from $2 \times 10^{-5}$ mol dm$^{-3}$ to $1.0 \times 10^{-4}$ mol dm$^{-3}$ (10 concentrations, data not shown). The data were globally analysed using a model based on the contemporary presence of the 1 : 1 complexes of each enantiomer and a 1 : 1 : 1 BSA : $R(-)-$ KP : $S(+)-$ KP mixed complex. The association constants of the 1 : 1 complexes were fixed to the values of Table 1 and the relevant molar ellipticities were those of Fig. 1C and 2C. The binding constant of the 1 : 1 : 1 complex was optimized as a parameter. An excellent agreement between calculated and experimental ellipticity values was found with $\log K_{ij1}(\text{mol}^{-2}\text{dm}^6) = 9.92 \pm 0.15$. The calculated $\Delta \varepsilon$ values for the 1 : 1 complex showed features of both the 1 : 1 and the 2 : 1 species, i.e. negative sign in the n,n* region, overall intensity close to those of the diastereomeric 2 : 1 associates, similar size for the 342 and 358 nm vibronic bands, a very weak negative band at 300–305 nm, a positive sign at 290 nm.

The interaction of KP-methyl ester with the protein was also examined by CD titration in the same experimental conditions used for KP in the carboxylate form. A racemic mixture of the ester was used. The generated signal was unstructured and characterized by a positive sign at 290–300 nm n,n* positive band in the 310–400 nm n,n* region with maximum at 335 nm, in complete agreement with previous findings. These results were taken as an indication for the involvement of the carboxylate group in the binding of KP enantiomers to BSA. The same conclusion had been reached with flavoprotein.

**Fluorescence of BSA-KP complexes**

To check for a possible involvement of aromatic aminoacid residues in the binding of KP to BSA, the protein fluorescence was studied at increasing KP concentrations, with medium and BSA concentration identical to those used in the CD experiments. With excitation at 295 nm the fluorescence of BSA ($\lambda_{\text{max}} = 344$ nm) is essentially due to the emission of tryptophan (Trp), whose $1L_{\text{em}}$ excited state is located at this energy. Steady state emission measurements cannot give information on the protein–drug interaction, because competitive light absorption by the non-fluorescent ketone introduced a trivial effect of quenching of the protein emission intensity. Therefore time resolved measurements were needed.

A $4.5 \times 10^{-5}$ mol dm$^{-3}$ BSA solution was excited at 295 nm and the fluorescence was collected at 330 and 400 nm, i.e. on the blue and red side of the emission band maximum. The native protein exhibited good biexponential emission decays, with lifetimes $\tau_1$ and relative amplitudes ($A_1, A_2; \sum A_i=1$, with $A_i$ preexponential factors) slightly different at the two detection wavelengths: $\tau_1 = 3.9$ ns (20%) and $\tau_2 = 6.7$ ns (80%) ($\chi^2 = 1.09$) at $\lambda_{\text{em}} = 330$ nm, $\tau_1 = 4.2$ ns (27%) and $\tau_2 = 7.5$ ns (73%) ($\chi^2 = 1.19$) at $\lambda_{\text{em}} = 400$ nm. This effect is well known in protein fluorescence and is attributed to heterogeneity in the Trp environments. However we notice that this effect was rather small in our conditions. Addition of $S(+)$- and $R(-)$- KP did not introduce further components in the decays and a biexponential analysis remained appropriate. Of the two lifetimes of the native protein, $\tau_2$ was slightly affected and $\tau_1$ was reduced more and more at increasing KP concentrations (Table 2). The dependence of the rate constants $k_1$ and $k_2$ on KP concentrations is reported in the Table 2. The dependence of the rate constants $k_1$ and $k_2$ on KP concentrations is reported in the Table 2.
Table 2  Lifetimes from biexponential analysis of fluorescence emission decays of BSA-KP mixtures at $\lambda_{ex} = 295$ nm and $\lambda_{em} = 330$ nm ($\phi$ were all better than 1.2). BSA $4.5 \times 10^{-3}$ mol dm$^{-3}$ in phosphate buffer 0.01 mol dm$^{-3}$ pH 7.4, 25 $^\circ$C.

<table>
<thead>
<tr>
<th>KP Conc/mol dm$^{-3}$</th>
<th>$\tau_1$/ns$^a$</th>
<th>$\tau_2$/ns$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S(+)-KP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.8</td>
<td>7.1</td>
</tr>
<tr>
<td>3.0 $\times 10^{-5}$</td>
<td>3.4</td>
<td>6.4</td>
</tr>
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<td>3.1</td>
<td>6.3</td>
</tr>
<tr>
<td>6.0 $\times 10^{-5}$</td>
<td>2.9</td>
<td>6.2</td>
</tr>
<tr>
<td>1.2 $\times 10^{-4}$</td>
<td>2.3</td>
<td>5.6</td>
</tr>
</tbody>
</table>

| R(−)-KP               |                  |                  |
| 0                     | 3.8              | 7.1              |
| 5.0 $\times 10^{-6}$  | 3.0              | 6.7              |
| 1.0 $\times 10^{-5}$  | 2.5              | 6.5              |
| 2.0 $\times 10^{-5}$  | 2.7              | 6.5              |
| 3.0 $\times 10^{-5}$  | 1.8              | 6.1              |
| 4.0 $\times 10^{-5}$  | 2.1              | 6.1              |
| 6.0 $\times 10^{-5}$  | 1.7              | 5.8              |
| 9.0 $\times 10^{-5}$  | 1.8              | 5.6              |
| 1.1 $\times 10^{-4}$  | 1.8              | 5.5              |
| 1.2 $\times 10^{-4}$  | 1.9              | 5.5              |
| 1.4 $\times 10^{-4}$  | 1.8              | 5.5              |

$^a$ Uncertainty within 7%.

Fig. 3A and 4A. As to $k_1$, a steep increase with a substantially linear course for $S(+)$-KP and a saturating behavior for $R(−)$-KP were found. As to $k_2$, the overall variations were $\leq$20%.

A deeper insight into the meaning of these data was obtained by examining the dependence of $k_1$ on the concentrations of the 1 : 1 and 2 : 1 complexes, calculated on the basis of the equilibrium constants of Table 1. For $S(+)$-KP $k_1$ did not correlate with the concentration of the 1 : 1 complex (Fig. 3B), but was linearly dependent on that of the 2 : 1 complex (slope is 7.1 $\times 10^{12}$ dm$^3$ mol$^{-1}$ s$^{-1}$, Fig. 3C). On the contrary for $R(−)$-KP $k_1$ increased linearly with the concentration of the 1 : 1 complex up to its maximum value (Fig. 4B, slope is $(9.3 \pm 1.0)$ $\times 10^{12}$ dm$^3$ mol$^{-1}$ s$^{-1}$), whereas it showed a sharp increase below 3 $\times 10^{-6}$ mol dm$^{-3}$ followed by a wide plateau up to 2 $\times 10^{-5}$ mol dm$^{-3}$, if the concentration of the 2 : 1 complex was considered (Fig. 4C). It was concluded that most of the quenching of Trp emission is produced in a specific site which is the secondary binding site for $S(+)$-KP and the primary one for $R(−)$-KP.

The slopes of the linear plots in Fig. 3C and 4B represent apparent quenching constants for the Trp singlet excited state two-three orders of magnitude larger than those expected for a collisional process ($10^{-10}$ dm$^3$ mol$^{-1}$ s$^{-1}$) and therefore are indicative of a ground state interaction of the drug with the protein.22 The small changes observed on $k_2$ are consistent with an indirect effect.

Discussion

Serum albumins are known to contain two main sites, specialized for binding of drugs of medium size, such as most of the NSAIDs. The structure of these sites was determined by X-ray crystallography in HSA25,26 and can be assumed to be similar in BSA, whose X-ray structure is not available, because the two proteins are 80% homologue as regards the primary sequence.15 These two sites, previously defined as site I and site II by Sudlow et al.,27 are located in subdomains IIA and IIIA, respectively.25,26

BSA is endowed by two Trp residues; one of them, namely Trp 134, is located in subdomain IIA, in a region not directly involved in binding of NSAIDs, whereas the other one, i.e. Trp 212, is placed in site I (subdomain IIA).15,25,26 Although multipexponential fluorescence decays in proteins containing a few tryptophans generally preclude assignment of each exponential component to a specific Trp residue,23,24 the biexponential Trp emission decay in BSA (see Table 2 and Fig. 3A and 4A) can be reasonably attributed to the substantially different microenvironments relevant to the two tryptophan residues above. This simple interpretation has been already adopted in the case of other two-tryptophan proteins.22,28 All this considered, the decay kinetics of BSA in presence of KP supports a scenario where the rate constant $k_2$, little perturbed by drug binding, is assigned to Trp 134, whereas the most affected rate constant $k_1$ is attributed to Trp 212. Alternatively, by admitting the possibility of substantial heterogeneity in protein conformations, $k_2$ could be assigned to protein conformations not significantly involved in drug binding at these molar ratios. Whatever the rationale for the $k_2$ behavior, the linear dependence of $k_1$ on the concentration of the complexes (Fig. 3C and 4B) leads to identify site I in subdomain IIA as the
site of highest affinity for $R$-(-)-KP and the secondary one for $S$-(-)-KP. It is reasonable to admit that site II in subdomain IIIA represents the main binding site for $S$-(-)-KP and the secondary one for $R$-(-)-KP. The satisfactory reproduction of the CD of the rac-KP : BSA system (data not shown, see results section), within the model of coexistence of 1 : 1 $S$-(-)-KP : BSA, 1 : 1 $R$-(-)-KP : BSA plus 1 : 1 1 $S$-(-)-KP : $R$-(-)-KP : BSA conjugates, furtherly support these conclusions. The fact that chiral recognition of KP by BSA involves location of the “first bound” molecule of each drug enantiomer in different parts of the biomolecule is directly relevant to the interpretation of displacement data in competitive binding experiments involving rac-KP.\(^2\)

The binding between KP and the protein matrix appears to be stereoselective in both sites, where specific interactions and essentially hydrophobic conditions are capable of imposing a high rigidity to the aromatic carboxyl chromophore. This is supported by the shape of the CD spectra of the diastereomeric complexes, well distinct and structured with occupation of either one or both protein sites (Fig. 1C and 2C). Chiral recognition manifested by the spectra was not reflected in the association constants for both the 1 : 1 and the 2 : 1 associates. Indeed differences in $K_{ij}$ between $S$-(-) and $R$-(-) did not exceed the experimental uncertainty (Table 1).

It was previously proposed that in this system binding of KP to a hydrophilic site of BSA would generate an unstructured positive CD band with both enantiomers.\(^1\),\(^12\) This was not confirmed by the present study. In our experimental conditions an unstructured positive CD band developed only if the KP solutions were not adequately protected from ambient light during the manipulations and was attributed to sample photodegradation. Indeed KP in phosphate buffer at pH 7.4 under-exceeds the experimental uncertainty (Table 1).

An unstructured, positive CD, analogous to that with 3-EB, was generated with the KP-ester. This indicates that the association of KP enantiomers to BSA at neutral pH is assisted by the ionic interaction between the carboxylic group of the propicinic substituent of the drug and a positively charged group of the protein. Moreover, the $\Delta_{\pi}$ values, 250–300 nm (Fig. 1C and 2C) support that aromatic aminoacids (Trp or Tyr) are involved in the binding. A stacking interaction via the $\pi$ systems has already been proposed for the binding of KP to flavoprotein.\(^2\)

We notice that the correlation between CD changes and specific interactions at each binding site is not straightforward in the higher order complexes, because the overall protein conformation could be modiﬁed upon sequential association of drug molecules to subdomains IIA and IIIA, which share a common interface.\(^2\) However, at least for the 1 : 1 BSA-KP complexes, a theoretical rationalization of the stereospecificity effects observed is possible. Indeed the optical activity induced in a chiral environment by a chiral molecule can be described by the sum of three terms, relying on one-electron, dipole–dipole (d-d) and electric–magnetic interaction (m–m) mechanisms. The first term derives from the interaction of the electric and magnetic transition dipole moments relevant to different electronic states of the chromophore and represents a small contribution in case of state mixing due to the electrostatic field of the host cavity. The second term, d–d, is particularly suited to describe the induced circular dichroism of guests with low-lying, allowed $\pi,\pi^*$ states and has been widely utilised, in the approximate form, using the polarizability of the host states.\(^2\)

The third term, m–m, can be very important for symmetry-forbidden or weakly allowed $\pi,\pi^*$ transitions, such as in the present case, and indeed has been shown to adequately reproduce the circular dichroism of carbonyls\(^2\) and peptides.\(^3\)

According to Tinoco,\(^3\) this term is expressed by:

$$R_{00}(m–\mu) = -2 \sum_{\mu_1} \sum_{\mu_2} \Im \left\{ V_{\mu_0,\mu} \frac{\mu_\mu_1 \mu_\mu_2 + \mu_\mu_1 \mu_\mu_2}{\hbar (\nu_\mu_1 – \nu_\mu_2)} \right\}$$

(1)

where $V_{\mu_0,\mu} = \mu_\mu_0 \cdot T_{ij} = \mu_\mu_0$

and

$$T_{ij} = \left\{ 1 - \frac{3R_{ij} R_{i0}}{R_{ij}} \right\} \frac{1}{R_{ij}}$$

(2)

In eqn (1) $m$ and $\mu$ stand for the magnetic transition dipole moment of the guest and electric transition dipole moment of the host, respectively, $h$ for Planck’s constant and $Im$ for imaginary part. The sums extend over all the excited states $a$ and $b$, having frequencies of $\nu_a$ and $\nu_b$, and over the bonds $i$ and $j$ of the chromophore and of the surrounding host residues, respectively. The geometrical factor $V$ of eqn (2) contains $T_{ij}$, the dipole interaction tensor, which is a direct function of the distance $R_{ij}$ between the bonds $i$ and $j$ (eqn (3)), and is responsible for the stereospecificity effects in host–guest binding.

We notice that the structure of a variety of cyclodextrin complexes, determined by this way, was accurate enough to account for the spectroscopic, photophysical and photochemical behavior of such systems.\(^3\) A reliable calculation of this term in the present case would require knowledge of the spatial coordinates of the aminoacids residues surrounding the drug chromophore in the protein pocket as well as optimisation of the geometry of the protein–drug associated species. The X-ray structure of HSA can be used to this purpose. This treatment, which was outside the purpose of the present paper mainly devoted to the experimental determination of the stereospecificity effects, is presently under consideration in our laboratory.

Conclusions

In this study, by an approach involving both spectroscopic and photophysical methods, a specific characterization of the drug : protein conjugates has been achieved. The knowledge gained on the sites of binding and on the properties of the diastereomeric complexes, can be used to gain insight into the geometry of the association. Indeed the CD spectra of the 1 : 1 conjugates, remarkably different each other and, presumably, not much influenced by conformational changes in the protein moiety (which, on the contrary, could substantially affect the spectral properties of the 2 : 1 species) can be combined with suitable molecular mechanics and rotational strength calculations and give stereospecific structural information.

Finally, it worth noting that the photodegradation quantum yield of $R$-(-)-KP had been reported to be ca. 40% higher than that of $S$-(-)-KP at molar KP : BSA ratio of 1.27.\(^2\) In the light of the information gained in the present study, the photochemistry of KP enantiomers in the BSA matrix was reinvestigated and found to be stereoselective and dependent on the stoichiometry of the association.\(3\)

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References