Induced circular dichroism as a tool to investigate the binding of drugs to carrier proteins: classic approaches and new trends

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Abstract

Induced circular dichroism (ICD) is a spectroscopic phenomenon that provides versatile and useful methods for characterizing the structural and dynamic properties of the binding of drugs to target proteins. The understanding of biorecognition processes at the molecular level is essential to discover and validate new pharmacological targets, and to design and develop new potent and selective drugs. The present article reviews the main applications of ICD to drug binding studies on serum carrier proteins, going from the classic approaches for the derivation of drug binding parameters and the identification of binding sites, to an overview of the emerging trends for the characterization of binding modes by means of quantum chemical (QC) techniques.

The advantages and limits of the ICD methods for the determination of binding parameters are critically reviewed; the capability to investigate the binding interactions of drugs and metabolites to their target proteins is also underlined, as well as the possibility of characterizing the binding sites to obtain a complete picture of the binding mechanism and dynamics. The new applications of ICD methods to identify stereoselective binding modes of drug/protein complexes are then reviewed with relevant examples. The combined application of experimental ICD spectroscopy and QC calculations is shown to identify qualitatively the bound conformations of ligands to target proteins even in the absence of a detailed structure of the binding sites, either obtained from experimental X-ray crystallography and NMR measurements or from computational models of the complex.

Highlights

- ICD is a powerful and versatile tool for binding studies on drug/protein systems.
- ICD is suited to determine the binding site and affinity of drugs to serum carriers.
- ICD and QC calculations unveil drug binding modes without previous structural data.

Keywords

Induced circular dichroism; Drug binding; Carrier proteins; Biorecognition; Quantum chemistry.

Acronyms

ACE	affinity capillary electrophoresis
BSA	bovine serum albumin
BLR	bilirubin
CD	circular dichroism
DZP	diazepam
ETA	ethacrynic acid
HPALC	high-performance affinity liquid chromatography
HSA	human serum albumin
ICD	induced circular dichroism
ITC	isothermal titration calorimetry
LCA	lithocholate
MD	molecular dynamics
MM	molecular modeling
MS	mass spectrometry
MST	microscale thermophoresis
NMR	nuclear magnetic resonance
PBZ	phenylbutazone
QC	quantum chemistry
QM	quantum mechanics
rHA	recombinant human albumin
RSA	rat serum albumin
SPR	surface plasmon resonance
TD-DFT	time-dependent density functional theory
UV	ultraviolet

1. Introduction

The characterization of molecular recognition processes is one of the primary objectives of experimental and theoretical chemistry. This is particularly true in medicinal chemistry, where intermolecular interactions lie at the heart of most of the physiological and pathological processes. The understanding of a biological process deserves the characterization of the recognition phenomena at molecular level, such as the binding of drugs to enzymes or protein receptors [1-4]. This is essential to discover and validate new pharmacological targets, and to design and develop new potent and selective drugs. Drug/target protein interactions are even more intriguing when the drugs are chiral, because their pharmacodynamics and pharmacokinetics are often significantly determined by their absolute configuration [5-7]. The emerging area of protein-protein interactions as drug targets further enlarge the possibilities for therapeutic interventions, in comparison to the conventional approach of addressing receptors and enzymes. While this potential is up to now largely untapped, some successful examples have shown that protein-protein interactions can be modulated by drug-like small molecules, natural products and peptides, thus supporting this approach as a valuable strategy in drug discovery [8-11].

The binding of drugs and metabolites to plasma proteins is fundamental in drug discovery and development. How the drug distribution can be affected by competition phenomena in the drug binding to serum proteins is indeed a widely investigated topic, because the drug displacement can be clinically relevant: this is particularly true when drugs show a high protein-bound fraction, because even small changes in the bound fraction determine a significant impact on the circulating free fraction [7]. In the present article, the main applications of induced circular dichroism (ICD) spectroscopy to the investigation of the binding of drugs and biologically active compounds to carrier proteins will be reviewed; the focus will be on the classic approaches for the derivation of drug binding parameters and the identification of binding sites, and a survey of recent reports on the characterization of binding modes by ICD combined with state-of-the-art computational techniques.

1.1 Current approaches to investigate biorecognition processes

Many techniques are used to get information on the protein binding processes [12-15], such as equilibrium dialysis [16, 17], ultrafiltration [18], high-performance affinity liquid chromatography (HPALC) [13, 19, 20], affinity capillary electrophoresis (ACE) [21-25], isothermal titration calorimetry (ITC) [26, 27], microscale thermophoresis (MST) [28], optical biosensors based on surface plasmon resonance (SPR) [29-31], fluorescence [32], circular dichroism (CD) [33-36] and nuclear magnetic resonance (NMR) [37, 38] spectroscopies, X-ray crystallography [37, 39], and mass spectrometry (MS) [40, 41].

Some of these biophysical techniques, including HPALC and SPR-based optical biosensors, are based on the immobilization of one of the binding partners to a chromatographic support or to a functionalized thin gold film, respectively. Surface-bound techniques allow quantitative binding studies and, in particular SPR, a reliable determination of on- and off-rates. The development of the immobilization procedure, however, is rather time consuming and often difficult to achieve, because the dynamic behavior of the bound partner during the binding event can be affected, and consequently the binding event may be altered. ITC provides the advantages of operating in solution, does not require labeling and is widely used to determine the affinity constant, the stoichiometry and the thermodynamic parameters. The limit of this technique is the low sensitivity, as it requires relatively high amounts of samples, not always easily available when dealing with biological samples. MST is a quite promising solution-based method, and its applicability to determine the affinity of the binding process should be very wide, thanks to the free choice of buffers for the analysis and the ability of the technique to analyze biological fluids; on the other hand, a binding partner should be fluorescently labeled. Nevertheless, label-free assays can be performed, in the case of protein binding experiments, by exploiting the intrinsic fluorescence of the protein.

Structural information on proteins and protein-ligand complexes at the atomic level of resolution can be mainly obtained by X-ray crystallography in the solid state and by NMR in solution. CD is also widely employed to get information on the protein binding process, but it is a low resolution structural technique, where only overall structural features are described. However, CD is a much less demanding technique in terms of both sample consumption and time requirements, and it is well suited to explore and elucidating chirality. Furthermore, CD allows to easily monitor the binding process in different experimental conditions and investigate the dynamic of the binding process. CD can also give information on the folding/unfolding process of the protein as a function of pH, temperature and ionic strength, or even in the presence of competitors. CD is certainly the most successful technique for determining the molecular configuration of chiral molecules [42] and for monitoring the stereoselectivity in the molecular recognition phenomena [7, 13, 33-35]. This last aspect concerns the study and the analysis of the ICD signal arising from the interaction between the ligand and the host, which allows the determination of the binding parameters for the interacting molecules and the identification of the binding sites of the host. Furthermore, detailed information on the binding mode can be obtained, because one of the main sources of ICD is the conformational change of both the interacting molecules upon binding. Thus, the stereoselectivity of the binding process can be investigated, and the conformation of the drug bound to the target protein can be determined, even when the guest molecule is not chiral. This information can be an invaluable aid in drug discovery and development, giving structural details on the ligand/target protein biorecognition process.

1.2 Induced circular dichroism (ICD)

Molecules display CD when left and right circularly polarized radiations are absorbed to a different extent. The CD of molecules arises from their rotational strengths (R_j), i.e. the scalar products of the electric ($\boldsymbol{\mu}_j$) and magnetic ($\mathbf{m}_j$) dipole moment vectors of the transitions ($0 \rightarrow j$)

induced by absorption; more specifically, quantum chemistry describes the rotational strength as the imaginary part of this scalar product by means of the Rosenfeld equation (Equation 1) [43]:

$$R_j = \mathrm{Im}\left(\boldsymbol{\mu_j} \cdot \mathbf{m_j^*}\right) = \mathrm{Im}\left(\langle \Psi_0 | \hat{\boldsymbol{\mu}} | \Psi_j \rangle \cdot \langle \Psi_j | \hat{\mathbf{m}} | \Psi_0 \rangle\right) \quad (1)$$

where Ψ_0 and Ψ_j are the wavefunctions of the ground and j-th excited states, respectively. In classical terms, the scalar product of two vectors is non-zero when **(a)** both vectors have a non-zero magnitude, and **(b)** they are not orthogonal to each other. Consequently, if its electric and magnetic dipole moments fulfill both conditions, the transition will display a rotational strength giving rise to CD. Non-chiral molecules in an isotropic medium do not display CD because the symmetry of the system causes all the transitions to never fulfill both requirements simultaneously; on the other hand, the dissymmetric arrangement of atoms is the driving force for the occurrence of non-zero rotational strengths in chiral molecules and the main reason for the importance of CD spectroscopy in stereochemical characterization.

Within this framework, ICD can be generally defined as the effect of perturbations to the symmetry of non-chiral molecules resulting in the generation of non-zero rotational strengths [44]. These perturbations may arise from a variety of sources, such as the interaction with an anisotropic medium (e.g. a chiral solvent), the restriction of conformational freedom arising from intermolecular interactions or the coupling with the transition dipole moments of the chromophores of an interacting molecule: an important requirement is that the interaction must induce a specific and preferential mutual orientation between the interacting molecules, regardless of their non-chiral or chiral nature, such that the induced rotational strengths are not superimposed to (or canceled out by) opposite contributions of other random orientations. The generation of an ICD effect is therefore a powerful indicator of the increased degree of structural order in the system: the resulting stereochemical and thermodynamic information can be fruitfully exploited to characterize the properties of supramolecular assemblies, e.g. host/guest complexes and aggregates [45, 46].

The classic example of an ideal supramolecular complex endowed with ICD involves a chiral, non-absorbing (transparent) host molecule interacting with a non-chiral, absorbing guest molecule: upon binding, the symmetry of the non-chiral guest is perturbed by the chiral environment of the host giving rise to an induced rotational strength. Moreover, the transparent nature of the host allows a direct and clear observation of the ICD arising from the guest: the latter feature is another fundamental requirement for a meaningful and correct use of ICD as an analytical tool. In fact, ICD effects may span over the whole spectrum, but relevant information about the system can only be interpreted if the individual contributions of host and guest to the overall CD spectrum are clearly recognizable, at least in a specific range of wavelengths: this is usually the case when the ICD bands of the guest are related to transitions lying at longer wavelengths than those of the guest.

A noteworthy consequence of the definition of ICD as a phenomenon involving non-chiral molecules is that changes in the chiroptical response of chiral molecules caused by intermolecular interactions should not be considered as proper ICD effects, although the two phenomena clearly share the same origin and early attempts at a theoretical treatment of ICD actually considered both non-chiral and chiral systems as potential sources of ICD effects [47]. For this reason, the employment of CD spectroscopy to monitor changes in the secondary structure of proteins caused by drug/protein and protein/protein interactions is not generally considered as an application of ICD and will not be treated in the present review. On the other hand, the observed changes in the chiroptical properties of small chiral molecules may be considered as ICD effects in some limiting cases, such as the onset of peculiar CD profiles upon binding that can be attributed to symmetric chromophores not displaying a CD response in solution: this is the case, for instance, of the benzophenone moiety of ketoprofen (KPF), which displays characteristic ICD profiles when binding to albumins of different species irrespective of the absolute stereochemistry of its propionic moiety [48-51].

2. Drug/protein binding: classic ICD applications

2.1. Characterization of binding sites

ICD methods have been widely applied to get information on the binding sites of drugs and metabolites to plasma proteins, and in particular to albumin [7, 13, 52-57] and to α-1-acid-glycoprotein [34, 58, 59]. The technique has been also successfully applied to better characterize the binding sites on human serum albumin (HSA) [60-62]. As an example, a significant contribution in the characterization of the HSA binding of carprofen, a drug that binds to sub-domain IIIA of HSA (site II, according to the terminology introduced by Sudlow [63-65]), was obtained by an ICD study. The site-to-site displacement of this drug was monitored using ibuprofen, another site II-specific drug. The low affinity binding site was shown to be site I (sub-domain IIA), and in particular at the azapropazone region of this large drug binding cavity, with the support of equilibrium dialysis displacement experiments [60, 61].

Recently, a systematic study on the characterization of a third major binding site on HSA (site III) has been reported, using biliverdin as a specific marker of the site. The importance of sub-domain IB was proved for both primary and secondary binding of various drugs and pharmaceutically active natural compounds. The variation of the ICD effect for biliverdin was investigated by addition of increasing concentrations of anticancer drugs, anticoagulants, steroid agents, and non-steroidal anti-inflammatory drugs [62]. A complex inter-domain allosteric network should then be considered for HSA, where drug binding areas of each domain can interact with each other.

Applications of ICD have been frequently reported for the investigation of the binding properties of bioactive compounds to serum carriers by means of competition experiments with ligands that are known to bind to specific binding sites on the protein, acting then as markers [7, 33-35, 66, 67]. Different co-binding situations can be observed by monitoring the change in the ICD of the drug/protein complex upon the addition of increasing concentrations of the competitor. Since the ICD signal is generated only by the binding complex, the distinction between free and bound

fractions is a relatively easy task: the simplest case is the stereoselective binding of non-chiral ligands, where the insurgence of ICD effects reflects the accommodation of the guest inside the binding site.

When no significant differences between the linear combination of the CD spectra of the binding partners and the CD spectrum of the complex can be observed, the binding event may still occur even if the complex does not show any ICD effect; in these circumstances, the binding study requires the use of selective probes for specific binding sites that show well defined ICD spectra. As an example, phenylbutazone (PBZ), diazepam (DZP) and bilirubin (BLR) have been used as markers for specific binding sites on HSA. These compounds are not chiral, thus the observed ICD spectra, obtained as the difference between the CD spectrum of the ligand/protein complex and the CD spectrum of the protein (Figure 1), reflect selectively the contribution of the bound marker [68]. In order to have selective information, the ICD signal of the bound marker should be in a spectral region where no contributions of the competitor and of the protein are present or they are negligible. Therefore, the studied drug can be used as the competitor or as the marker, depending on the structures and spectroscopic properties of the two interacting ligands [52].

As an example, the reversible binding of ethacrynic acid (ETA) to HSA was investigated by the ICD method using this non-chiral diuretic drug alternatively as the marker or as the competitor [69]. The nature of the interaction can be qualitatively evaluated by the behavior of the ICD of marker/HSA complexes upon addition of increasing concentrations of the competitor. When the ICD signal does not change the binding is independent, while a significant change indicates a competition, either direct or allosteric in nature. The ICD signals for the main HSA binding markers, i.e. PBZ, DZP and BLR, were therefore monitored in the presence of ETA as the competitor (Figure 2). A direct competition, i.e. a co-binding to the same binding site, reduces the ICD signal of the bound marker to zero by increasing the stoichiometry of the competitor/marker molar ratio: this behavior was observed for ETA with DZP, the selective marker for the binding site II of the serum carrier (Figure 2B). In the case of allosteric interactions, an increase or a decrease of

the ICD signal can be observed. An increase of the ICD signal indicates a cooperative binding due to an increased affinity of the marker, resulting in a higher concentration of the marker/protein complex: this behavior was observed for ETA with BLR, the HSA site III marker (Figure 2C).

Figure 1. CD and ICD spectra (ellipticity θ in mdeg) of the 1:2 complexes between rHA and selected binding markers in phosphate buffer 10 mM pH 7.4 (1 cm cell). **A:** PBZ, site I marker. **B:** DZP, site II marker. **C:** BLR, site III marker. Redesigned with permission from ref. [68].

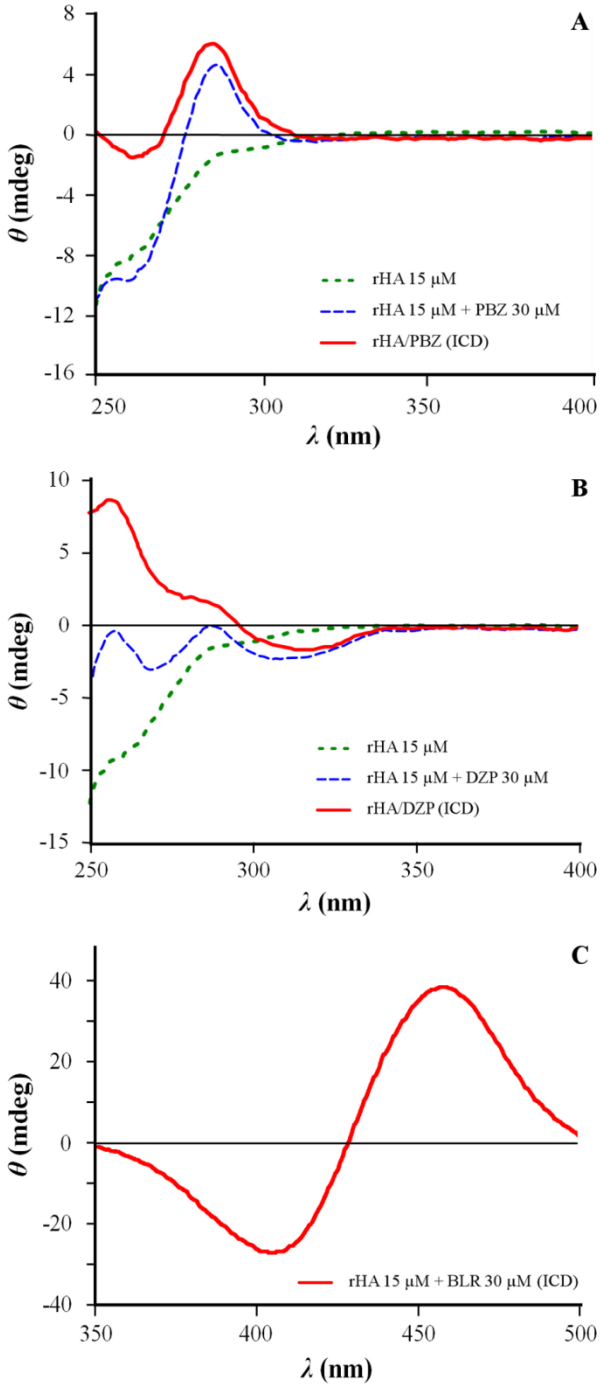
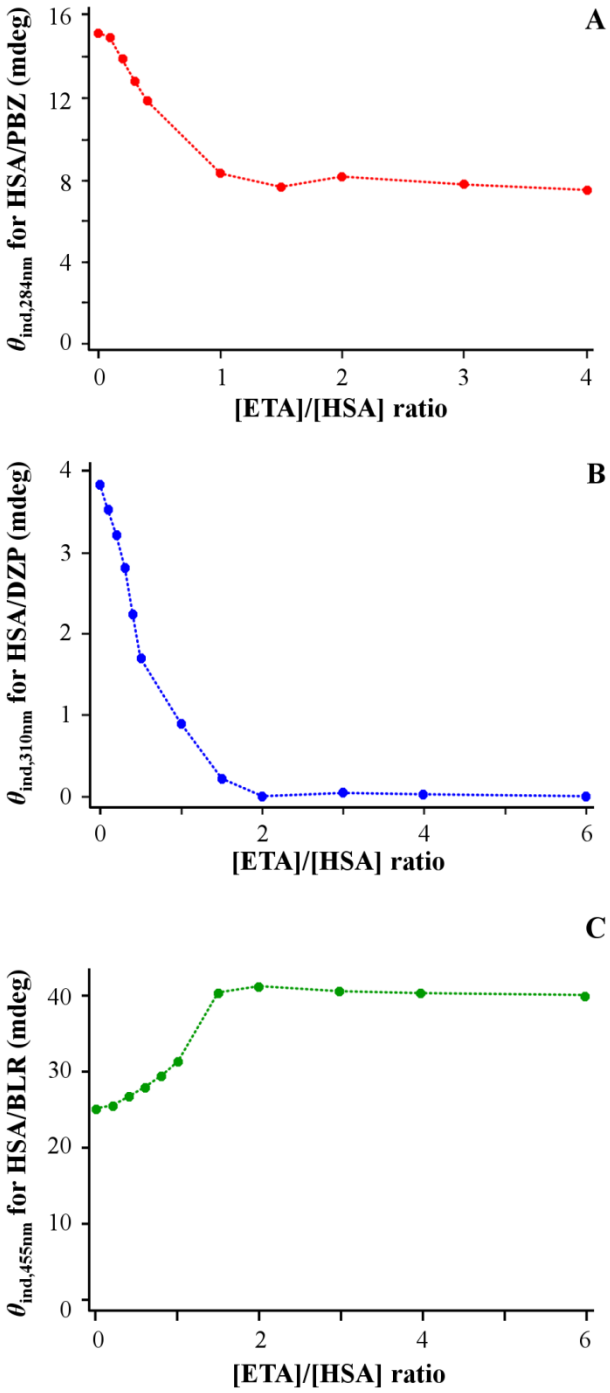


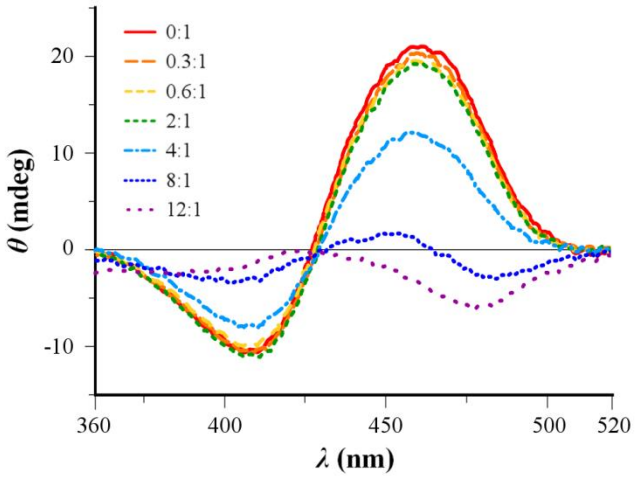
Figure 2. Change of the ICD signal (induced ellipticity θ_{ind} in mdeg) of HSA/marker 1:1 complexes in the presence of ETA, monitored at a single wavelength as a function of the ETA/HSA molar ratio ([HSA] = 30 µM, phosphate buffer 20 mM pH 7.4, 1 cm cell). **A:** binding site I, PBZ as marker (λ = 284 nm). **B:** binding site II, DZP as marker (λ = 310 nm). **C:** binding site III, BLR as marker (λ = 455 nm). Redesigned with permission from ref. [69].



On the other hand, a decrease of the ICD signal is indicative of a non-cooperative co-binding, because the marker affinity is negatively affected and the concentration of the marker/protein complex is reduced consequently: this was the behavior of ETA with PBZ, the marker for site I of HSA (Figure 2A).

The ICD method presented above should be carefully validated, because changes in the observed ICD signal may be also due to conformational changes of the marker inside the binding site. As an example, when BLR is used as marker to investigate the HSA binding of lithocolate (LCA), the ICD of the bound BLR did not change significantly upon addition of LCA acid up to a 2:1 LCA/BLR molar ratio; this result was in agreement with competition experiments carried out by equilibrium dialysis on the same system. However, a dramatic change in the ICD spectrum of the BLR/HSA complex was observed at higher concentrations of LCA, with a significant decrease of the ICD signal and a final inversion of the CD couplet with maxima at about 460 and 410 nm (Figure 3). Thus, the decrease of the BLR/HSA ICD signal in the presence of high concentrations of LCA was not determined by a change of the bound fraction of BLR; instead, the decrease was due to the change in the prevalent axial chirality of the bound conformation of BLR from *P* to *M* [70].

Figure 3. ICD spectra (ellipticity θ in mdeg) of the HSA/bilirubin 1:1 complex in the presence of LCA, measured at increasing LCA/HSA molar ratios ([HSA] = 15 µM, phosphate buffer 10 mM pH 7.4, 1 cm cell). Redesigned with permission from ref. [70].



The analysis of the ICD data collected by competition studies, therefore, allows the identification of the competition mechanism and the calculation of the affinity constants for the stereoselective binding of both the marker and the competitor to their target protein [52]. However, the change of the ICD signal observed for indirect competition phenomena may arise from a variation in the stereoselectivity of the binding event, as discussed above for the HSA binding of BLR. Since these two possibilities cannot be formally distinguished, the characterization of the binding properties should be confirmed by independent techniques, such as equilibrium dialysis, affinity chromatography or other spectroscopies. ICD analysis specifically detects interactions at stereoselective binding sites, even in the case of competition experiments. This represents a unique advantage, particularly in the case of HSA, where the binding of drugs and metabolites is usually stereoselective only for the highest affinity binding sites: in this respect, it should be emphasized that only these binding sites are likely involved in the real binding events in vivo, taking into account the relative concentrations of HSA and drugs in plasma. Other methods, such as equilibrium dialysis, fluorescence, UV spectroscopy and SPR, are more suited to study multisite binding.

2.2. Determination of binding parameters

ICD has been usefully employed also to determine the association constant of ligands binding to their target proteins. However, the applications of chiroptical spectroscopies to the determination of binding parameters are much less frequent in literature than those of other spectroscopies, in particular fluorescence. The first analysis of ICD data to calculate the affinity constant was reported for the binding of PBZ and oxyphenylbutazone to HSA [71]. Many reports have then shown that the binding parameters obtained by CD are comparable with those obtained using other methods [52, 54, 57, 72, 73]. The calculation of the affinity constant can be obtained by linear or non-linear regression analysis of the ICD titration data as obtained by increasing the concentration of the ligand, while the concentration of the protein is maintained constant [54, 71]. These methods

monitor the changing of the ICD signal as a function of the drug/protein molar ratio. A complication can arise when the concentration of the protein is too low with respect to that of the ligand, because of the possible involvement of secondary binding sites. Moreover, the stoichiometry of the binding even can also be determined by plotting the ICD signal versus the drug/protein molar ratio [54, 57].

The binding parameters have been reliably determined for several drug/HSA complexes [54, 57, 72, 73], for the trans-parinaric acid binding to β-lactoglobulin [74] and for peptide-protein or protein-protein binding complexes [75, 76]. Following another common experimental set-up for the titration of guest/host complexes, increasing concentrations of the protein can be added to a fixed concentration of the ligand until a constant value of the ICD signal is obtained, as a demonstration of binding site saturation [77]. In this case, monitoring any changes of the ICD effect as a function of the protein/ligand ratio is easy and reliable, because the total ligand concentration, the critical parameter, does not change during the experiment.

As an alternative approach, the effect of sequential dilutions on the ICD signal of the drug/protein complex at fixed stoichiometry can be analyzed and the affinity constant determined [52]. In this case, the path length of the cell is increased proportionally with the dilution of the complex, and the decrease of the ICD signal correlates with the association constant of the complex: if the binding is relatively weak, the observed change of ICD with dilution will be high. In the simplest case, i.e. a ligand (L) binding to a stereoselective site on the target protein (P) at a 1:1 stoichiometric ratio, the affinity can be expressed by the equilibrium association constant K_a and determined by means of a linear equation that combines Equation 2 with the Lambert-Beer law adapted to the ICD measurements (Equation 3) [52]:

$$L + P \rightleftarrows LP\ ; \qquad [L] = [P]\ ; \qquad p = [LP] + [P]\ ;$$

$$K_a = \frac{[LP]}{[L][P]} = \frac{[LP]}{(p-[LP])^2}\ ; \tag{2}$$

$$\Delta A_{\text{ind}} = \Delta\varepsilon_{\text{ind}} \cdot [LP] \cdot l \; ; \tag{3}$$

$$\frac{p}{\sqrt{\frac{\Delta A_{\text{ind}}}{l}}} = \frac{1}{\Delta\varepsilon_{\text{ind}}} \cdot \sqrt{\frac{\Delta A_{\text{ind}}}{l}} + \frac{1}{\sqrt{K_a \cdot \Delta\varepsilon_{\text{ind}}}} \; . \tag{4}$$

In the equations, the subscript refers to the induced nature of the ICD effect arising from the *LP* binding complex, l is the path length of the cell, and p is the initial concentration of the protein. Equation 4 allows to determine K_a and $\Delta\varepsilon_{\text{ind}}$ from the slope and the intercept of a linear fit of the experimental ICD data at an appropriate wavelength of monitoring (ΔA_{ind}). This approximation, however, is limited to the quantitative analysis of single-site binding phenomena [52].

The reliability of the method has been verified for the target protein binding of strongly bound ligands. As an example, the binding constants of PBZ, DZP, KPF and BLR were determined for the binding to albumins from different species [49]. The affinity of some specific markers for HSA binding sites were determined also for recombinant human albumin (rHA), in comparison to HSA [69], to verify that the binding properties were maintained with the recombinant protein. Further studies investigated the binding properties of modified albumins at the highest affinity binding sites, using specific markers. In particular, the affinity constants were determined for a Cys34-modified rHA [69] and for a sample of rHA modified by covalent binding of penicillin [68]. Similar models can be extended to different scenarios of drug interaction with proteins and nucleic acids, and even to the study of multi-site binding phenomena [77, 78].

3. Drug/protein binding modes: ICD and computational chemistry

The insurgence of ICD effects due to the binding of small non-chiral guests to macromolecular hosts can sometimes provide very detailed information on the structure of the guest/host complex. This situation usually occurs when the interaction of the guest with the binding site of the host displays strong binding affinity and stereoselectivity, resulting in a significant distortion of the structure for the guest. However, the analysis and exact interpretation of the structural information carried by a particular ICD effect are not at all trivial tasks, even if the individual contributions of the interacting molecules to the ICD signal of the complex can be unambiguously distinguished.

Taking advantage of the latest developments and advances in the field of computational chemistry, a peculiar approach is emerging in the last few years: the ICD spectra of host/guest complexes are compared to the theoretical CD signals calculated for different structural arrangements of the guest molecule, with the aim of characterizing the bound conformation of the guest inside the binding site of the host. The best techniques to perform such calculations are provided by quantum chemistry (QC), and in particular by time-dependent density functional theory (TD-DFT) [79], which are nowadays reaching a remarkable level of accuracy and efficiency in predicting the electronic properties of molecular systems of ever increasing size [80]. The scientific production about QC methods for the prediction of chiroptical properties is growing steadily over the last decades [81-84]; as a consequence, the use of chiroptical spectroscopies as powerful characterization tools has also experienced a revived interest [85].

In the field of pharmaceutical and biomolecular analysis, the most straightforward and popular application of the combination of experimental chiroptical spectroscopies and QC calculations certainly aims at the assessment of the absolute configuration of chiral drugs of unknown stereochemistry, due to the paramount importance of chirality in the definition of the activity of drugs. As soon as the developments in QC methods allowed to obtain more reliable and accurate agreements between calculated and experimental properties for a larger number of chemical

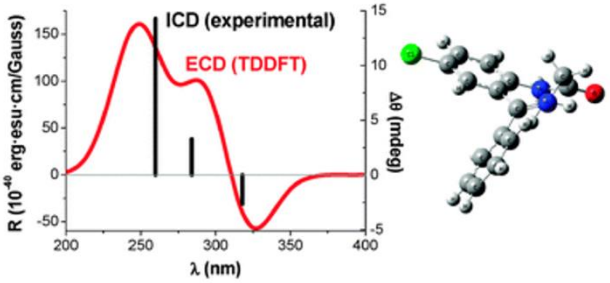
systems, the attention of some researchers started to focus on the capabilities of QC methods to reproduce more peculiar chiroptical phenomena, such as the ICD observed for drug/protein complexes [86, 87]. The different approaches that can be employed for the purpose largely depend on the nature of the drug/target adduct, i.e. on the type of mechanism at the origin of the ICD effect. Two steps, however, are always necessary:

(a) the possible structures of the drug/protein complex must be derived experimentally or modeled theoretically by computational methods. Therefore, the first requisite of the chosen approach is its ability to model the structure of the drug/target complex in an appropriate fashion.

(b) the resulting structures are used to perform QC calculations of the theoretical CD spectrum of the complex, which is then compared to the experimental ICD signal in order to verify the reliability of the prediction and characterize the geometry of the drug/protein complex.

If an experimental structure of the drug/protein complex showing ICD is already available, e.g. from X-ray crystallography or NMR measurements, its theoretical chiroptical properties can be directly calculated by QC methods using the experimental structure and then compared to the ICD signal. Since X-ray crystallography provides the highest levels of detail in the description of the binding event at the atomic scale but requires non-physiological conditions to collect data, the combination of experimental and theoretical CD spectroscopies may be used to confirm the real occurrence in solution of a particular binding mode observed in the solid state. This approach was recently applied to further confirm the origin of the well known ICD spectrum of diazepam (DZP) bound to HSA [87]: when TD-DFT calculations were performed on the bound *M*-conformer of DZP taken from the crystal structure of the HSA/DZP complex (PDB ID: 2BXF) [88], the resulting theoretical CD spectra and the experimental ICD of the complex were found to be in excellent agreement (Figure 4).

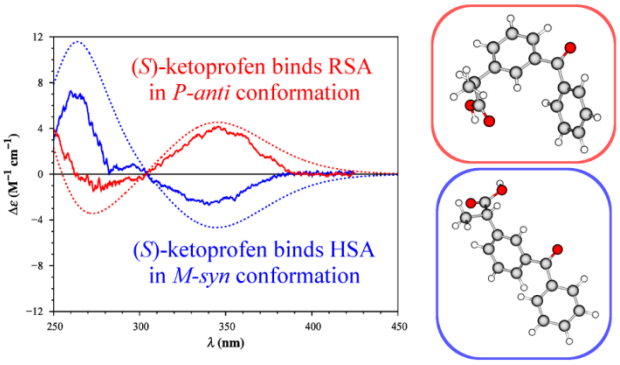
Figure 4. Comparison between the theoretical CD spectrum (calculated at the B3LYP/6-31G(d,p) level) of DZP bound to HSA, as taken from the crystal structure of the complex (PDB ID: 2BXF), and the experimental ICD signal in solution [33]. Reprinted with permission from ref. [87].



X-ray and NMR structures, however, are usually not available for drug/protein complexes, due to the strong experimental conditions required for the preparation of samples. In such circumstances, the complex needs to be modeled by means of computational methods; subsequently, the accuracy of the model needs to be verified by experimental techniques. Purely computational approaches to identify the structure of the drug/protein adducts are provided by molecular dynamics (MD) [89] and molecular docking [90], which both rely on molecular mechanics (MM) to simulate the behavior of large molecular assemblies and are widely used to investigate the binding of drugs to their biological targets. Once a structure of the drug/protein complex is identified through one of the aforementioned methods, QC calculations are performed to determine the theoretical CD spectrum of the complex and verify the agreement with the experimental ICD signal [86]. MD and molecular docking, however, require that the X-ray structure of the isolated target protein be already available or at least derived by homology modeling [91]; additionally, the binding site for the drug should be identified beforehand by means of experimental techniques in order to obtain a reasonable prediction of the real drug/target adduct. MD methods were recently applied to the interpretation of ICD effects in order to characterize the binding of nalidixic acid [92], evodiamine and rutaecarpine [93] to HSA, as well as the binding of licochalcone A [94, 95] and the enantiomers of KPF [50] to HSA and bovine serum albumin (BSA).

If the ICD signal mainly stems from structural constraints on the conformational flexibility of the drug caused by the interactions with the binding site of the protein, then the binding mode may be identified by QC methods through an alternative approach, i.e. a thorough conformational search followed by CD calculations on each possible conformer of the drug. [87] Using the QC conformational search method, the conformer whose theoretical CD spectrum shows the highest correlation with the experimental ICD spectrum of the drug/protein complex is identified as the bound conformation of the drug inside the binding site. The most important step for this QC approach is the conformational analysis: since the bound conformation of the drug is stabilized by multiple interactions with the residues of the binding site, it may or may not be present as a conformer in gas phase or solution. Therefore, the possible conformers should be systematically generated and investigated by arbitrary scans of the conformational space of the drug even though the explored conformations would not be present at equilibrium, e.g. by rotating flexible dihedral angles or forcing a twist in otherwise constrained and planar structures by small steps of fixed amplitude. In addition, all the protonation states of the drug possibly occurring in solution should be considered independently as distinct chemical entities: the equilibrium among different protonation states and conformations of the drug bound to the protein can then be considered by averaging the corresponding theoretical CD spectra in different proportions until a reasonable correlation with experimental data is reached. [87] A number of drug/carrier protein systems have been investigated through this approach, such as the 3-carboxy-coumarin/BSA complex [96] or the interactions of flavonoids to HSA [57], in particular of kaempferol [97], genistein [98] and fisetin [99]; a very detailed report on these application is given in a recent perspective review [87]. A similar approach was also employed to rationalize the species-dependent ICD effects observed for the complexes of KPF to HSA and rat serum albumin (RSA) [49, 51]: TD-DFT calculations revealed that (*S*)-KPF is bound to serum albumins with completely different arrangements of the substituted benzophenone moiety, assuming a *P-anti* conformation when bound to RSA and a *M-syn* conformation when bound to HSA (Figure 5).

Figure 5. Comparison between the theoretical CD spectra (calculated at the PBE0/def2-TZVPP′D level) of two different conformers of (*S*)-KPF (*P-anti* and *M-syn*) and the experimental ICD signals of (*S*)-KPF/RSA and (*S*)-KPF/HSA complexes in solution [49]. Reprinted with permission from ref. [51].



Irrespective of the method used to derive the structure, the last step needed to obtain a theoretical prediction of ICD is the derivation of the CD spectrum for the drug/protein complex by means of time-dependent QC calculations. At this stage, all the different levels of theory developed over the years may be used, going from the relatively inexpensive but less accurate semi-empirical methods [100] to the highly accurate but extremely expensive post-Hartree-Fock methods [101]. A popular choice for the purpose is certainly TD-DFT, because its reasonable balance between chemical accuracy and computation cost represents a great advantage when dealing with large molecular systems [80, 82]. Nevertheless, QC methods are still unable to deal with whole drug/protein complexes, so the input structures for CD calculations must be necessarily reduced in size and focused on the relevant part of the complex, i.e. the drug and the residues of the binding site surrounding it.

If the drug can be considered as the main source of the ICD effect, one possible approach is to determine the theoretical CD signal for the bound conformation of the drug, without considering the protein explicitly in the QC calculations. Such calculations may be performed in gas-phase conditions or using continuum solvation models [102] to mimic the electrostatic environment inside the binding pocket [87]. This method may result useful to understand the actual contribution of the drug to the overall ICD spectrum, and is actually the only possible approach when the bound

conformation is determined through QC conformational search. In fact, the host molecule and the binding site are not considered at all in these models: the attention is fully focused on the guest molecule. As a consequence, the interactions between drug and target protein should mainly affect the structural properties of the drug with limited coupling effects on the electronic properties of the drug in order to predict the ICD signal of the drug/protein adducts correctly using this method. Conversely, even when the bound conformation of the drug is correctly identified, no information can be obtained on the possible interactions with the protein, and the reliability of the results is consequently limited to a qualitative level. In this framework, MD and docking both share an important advantage over the QC conformational search method since they consider the whole drug/target complex, giving a more complete description of the interactions between the two chemical entities which may be eventually exploited to perform more complex CD calculations using multi-layer QM/MM or QM/QM methodologies [103-105]. On the other hand, the structures obtained with the MD and docking methods are the result of MM calculations, which are less accurate than QC calculations in the prediction of chemical structures by definition. The choice among the different approaches, therefore, only depends on the complexity of the system under investigation and on the level of chemical accuracy necessary for the theoretical models to reproduce the experimental results.

4. Conclusions

ICD spectroscopy is a useful method for determining drug binding parameters and identifying binding sites on the target protein. One of its main advantages is represented by the possibility to analyze drug/protein systems in solution, allowing the investigation of the binding process in different experimental conditions. Labelling is not required; however, the binding should be stereoselective and produce an ICD signal in a spectral region where the contribution of the protein to the overall CD spectrum of the complex is null or negligible. The analysis of the ICD signal is particularly informative in the case of competition studies, whenever a compound is tested for its binding properties towards target proteins or a better characterization of a binding site is needed to obtain a complete picture of the binding mechanism and dynamics. The main limitations of the method are related to the structural and spectroscopic features of the interacting molecules, which must fulfill certain requirements in order to generate an ICD effect suitable for analytical purposes.

However, the most important peculiarity of ICD is the possibility of exploring and elucidating chirality and stereochemistry: this feature is nowadays exploited to investigate the structure of drug/protein complexes at the microscopic level with the aid of state-of-the-art computational methods. The combined application of experimental ICD spectroscopy and QC calculations, indeed, allows the qualitative identification of the bound conformation of ligands to target proteins even without experimentally determined X-ray and NMR structures or computational models of the binding complex. In this respect, the study of biorecognition processes by ICD spectroscopy will surely benefit from the fruitful interaction between theoretical and experimental chemistry towards more accurate and reliable predictions of the spectroscopic and chiroptical properties of large chemical systems.

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