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Biomolecular studies by circular dichroism

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1. ABSTRACT

In this review, we shall outline the basic principles of circular dichroism (CD) indicating the types of structural information relevant to the study of biomolecules, such as proteins or DNA. We are mainly interested to show the utility of this technique to study protein-ligand, DNA-ligand and protein-DNA interactions.

2. INTRODUCTION

Circular dichroism (CD) spectroscopy is a valuable biophysical tool for studying biomolecules structure such as proteins or DNA, in solution (1, 2). Although it is not able to provide the detailed residue-specific information available from Nuclear Magnetic Resonance (NMR) and X-ray crystallography, CD has two major advantages: it is possible to perform measurements on small amounts of material in physiological buffers and it provides one of the best methods for monitoring any structural alterations that might result from changes in environmental conditions, such as pH, temperature and ionic strength.

In this review, the principal features of protein and nucleic acid CD spectra are described. It would be impossible to do a complete review including all the recent applications in which CD spectroscopy is involved. Therefore we will focus on several specific applications performed recently by us and others in order to show the general usefulness of CD as a biophysical technique to study Protein-ligand, DNA-ligand and Protein-DNA interactions.

2.1. Basic principles of CD

When light passes through a chromophore solution it may interact with the sample in two main ways: it may be refracted on its passage through the solution or it may be absorbed. Refraction is quantified by the refractive index, n, of the solution while absorption is quantified by the molar extinction coefficient**,** Epsilon (E). Optically active samples have distinct molar extinction coefficients for left (E_L) and right (E_R) circularly polarized light ($E_L \neq E_R$). This difference may be expressed as delta E. From Lambert- Beer Law the difference in the absorbance of left and right circularly polarized light delta A, can be given by,

(Equation 1) delta A = $A_L - A_R$ = delta E *c l*

where *c* is the concentration and *l*, the path length. When A_L and A_R are absorbed to different extents, the resulting radiation would be said to possess elliptical polarization. This difference in absorbance, *delta A*, of the two components, is a measure of CD, but it is generally reported in terms of the ellipticity (Theta) in degrees, there is a simple numerical relationship between delta A and ellipticity in degrees given by equation 4

(Equation 2) CD = $A_L - A_R$

(Equation 3) Theta =$\tan^{-1}$ (b/a)

where here b and a are the minor and major axes of the resulting ellipse.

(Equation 4) Ellipticity =Theta = = 33.0 ($A_L - A_R$)

If delta *E* or delta *A* or ellipticity (*Theta*) is plotted against wavelength, a CD spectrum may be obtained as shown in Fig.1. For comparison of results from different samples it is necessary to consider molarity,

(Equation 5) Molar ellipticity [Theta] = M [Theta]/ 100 *c l* in degree. cm^2/ decimol

here *M* designates the molecular weight; c is the concentration in degree/cm^3 and *l* is the length path in cm

In summary, CD signals are observed in the same spectral region as the absorbance bands of a particular chromophore, showing that the chromophore or its molecular environment is asymmetric. It can be measured easily by exposing a sample alternately to left-hand and right-hand circularly polarized light and detecting just the differential absorption, so the observed CD is quite small, i.e., ellipticities are typically in the range 10 mdeg, corresponding to a difference in absorbance of the order of $10^{-4.}$ This difference can be determined quite accurately with modern instrumentation. However, it is important to take careful attention to the experimental conditions in order to ensure that meaningful data are obtained (3, 4).

2.2 General aspects of CD measurements for proteins and DNA

CD spectroscopy is the method of choice for quick determination of protein, peptide and DNA secondary structure.

CD bands of peptides (5) and proteins (6) appear in two spectral regions, the far and near –UV region. The amide region or the far-UV (170-250 nm) is dominated by contributions of the peptide bonds, whereas CD bands in the near-UV region (250-300 nm) are originated from aromatic amino acids. In addition, disulphide bonds give rise to several CD bands. Peptides and proteins that lack non-amino acids chromophores (e. g., prosthetic groups) do not exhibit absorption or CD bands at wavelengths above 300 nm.

The amide band group is the most prominent chromophore of peptide and proteins to be observed by CD spectroscopy. Two electronic transitions of the amide chromophore have been characterized. The n-pi* transition is usually quite weak and occurs as a negative band around 220 nm. The energy (wavelength) of the amide n-pi* is sensitive to hydrogen bond formation. The pi-pi* transition usually is stronger, and is registered as a positive band around 192 nm and a negative band around 210 nm. As mentioned the information that can be obtain by CD spectroscopy is somewhat limited compared to NMR (7) or X-ray diffraction, however CD data are valuable as a preliminary guide to observe peptide and protein conformation and analyze their conformational transitions under a wide range of conditions (8, 9). CD of proteins in this region is normally given as a mean residue ellipticity, $[Theta]_{MRW}$, which based on the concentration of the sum of amino acids in the protein solution under investigation. If the molar protein concentration and the number of amino acids are known, the concentration of residues is simple, the product of both number. If the protein concentration is known in mg/ml, the concentration of amino acids can be calculated by assuming a mean residue weight (MRW) of 110 per amino acid residue.

(Equation 6) $[Theta]_{MRW}$= [Theta] / Cp n l x10

where *Cp* is the molar concentration of the protein, *n* the number of amino acid residues and *l* is the length path in cm.

For data in the aromatic region, CD is frequently given as delta Epsilon, mainly because in this case only a small number of aromatic amino acids or prosthetic groups contribute to the CD signal, but [*Theta*], based on the protein concentration, and $[Theta]_{MRW}$, based on the residue concentration, are also found.

The amount of protein or peptide required for CD can be gauged from the need to keep the absorbance less than the unit. Typical cell path lengths for far-UV work are in the range 0.01 to 0.05 cm and the protein concentrations are in the range 0.2 to 1 mg/ml. Depending on the design of the cell being used, the volume of the sample required can be ranged from about 1 ml to 50 microliter, so it should be as little as 10 microgram, but usually 100 to 500 microgram of sample is required. This would be greater if analysis of the near-UV and visible regions are required, these signals are weak and the protein concentration is in the range of 0.5 to 2 mg/ml (Figure 1).

For CD measurements of DNA samples, although isolated purine and pyrimidine bases are planar, intrinsically optically inactive and hence do not exhibit a CD signal, when they are incorporated into nucleosides and nucleotides, the glycosylic bond from C-1´atom of the sugar to either N-9 of purines or N-1 of pyrimidines gives rise to a chiral perturbation of the UV absorption of the base. The CD signal of a nucleic acid increases with length due to cooperativity of chiral interaction between contiguous bases.

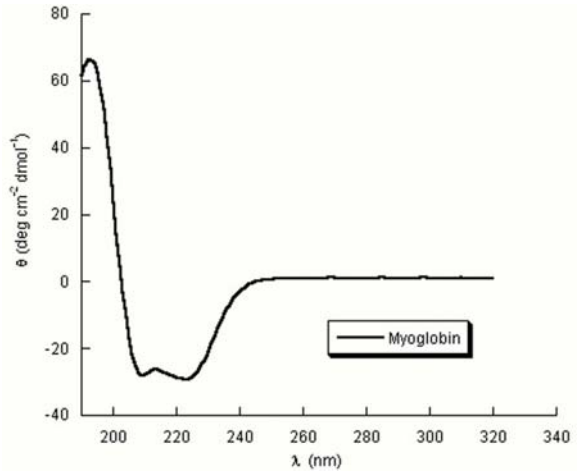

Figure 1. Example of protein CD spectra: Myoglobin (1 microMolar) in MilliQ water.

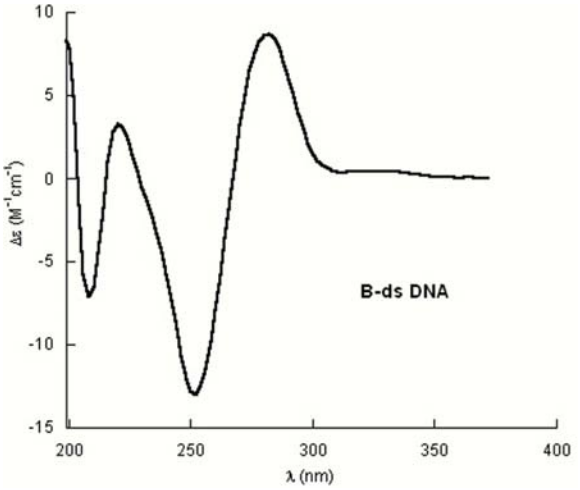

Figure 2. Example of B-ds DNA CD spectra: 5'-CGCAGTTTACGCTTTTTGCGTAAACTGCG-3´ (10 microMolar) in 12 mM phosphate buffer, 120 mM NaCl pH= 7.0.

Spectral studies of DNA have employed far UV as well as infrared light, but most analyses use ultraviolet light within 180–300 nm range, where bases of DNA absorb light (10). CD bands are often expressed in terms of ellipticity, Theta [degrees] or more convenient as a difference in the molar extinction coefficients delta Epsilon [M^{-1} cm^{-1}] (Figure 2). This technique is extremely sensitive, permitting work with DNA amounts as low as 25 microgram /milliliter.

CD experiments of these biomolecules provide not only information about their secondary structure but also it can be used to follow the interaction of ligands to proteins, peptides or nucleic acids. The CD spectrum of each component in solution is directly proportional to its concentration, and the total spectrum arises from the sum of all component spectra. If ligand binding induces extrinsic optical activity in the chromophores of the bound ligand, an induced CD signal is observed, which is directly proportional to the amount of macromolecule-ligand complex formed, and hence it can be used to construct a binding isotherm. Alternatively, ligand binding may result in a conformational change in the macromolecule, and the resultant change in its intrinsic CD signal allows quantifying the binding.

2.3. Practical aspects of CD for biomolecules

In this section it is given a brief outline of some important experimental aspects of obtaining CD data in order to obtain reliable data. Further details of these aspects can be found in a detailed review written recently by Price *et.al*. (11).

The light source for most CD instruments is a xenon arc, which gives good output over the range of wavelengths (178 to 1000 nm) used for virtually all studies on proteins and DNA. It is necessary to flush the instrument with N_2 gas in order to remove O_2 from lamp housing and the sample compartment, this a) prevents ozone formation minimizing damage to the optical system; and b) allows measurements to be made below 200 nm.

To obtain reliable CD data, it is important to pay attention to the instrument and the sample. As far as the instrument is concerned, regular maintenance and calibration with suitable chiral standard such as 1S-(+)-10-camphorsulfonic acid is essential. In order to improve the quality of the data is important to adjust some experimental parameters such as: the time constant, the scan rate, the number of scans and the bandwidth. In most cases, suitable "steady state" CD spectra in the near and far UV can be obtain using a time constant of 2 sec, a scan rate of 10 nm/min, accumulation of two to four scans and a bandwidth of 2 nm or less.

It is also important that the sample should be homogeneous and should be free of highly scattering particles by either centrifugation or passage through a suitable filter. The total absorbance of the sample should not exceed about one unit, otherwise the spectral noise will become excessive and, above a certain point, an automatic cut off may operate leading to an apparent decline of CD signal to zero. It is essential to minimise absorption due to other components in the mixtures such as buffers, supporting electrolytes, solvents etc. Phosphate, borate and low molarities Tris (20mM) have low absorbances above 190 nm in cells of path length 0.1 cm or less, these buffers can give between them a suitable coverage of pH values from 6 to about 9.5. For pH values from 4 to 6 it is usually used carboxylate group which have high absorbance bellow 200 nm. In this case it is important to work with dilute buffer solutions and to run a "blank" CD spectrum to ensure not excessive noise or other artefacts in the spectra. In conclusion, to obtain reliable CD spectra it is important to follow the above consideration and follow an established protocol (12).

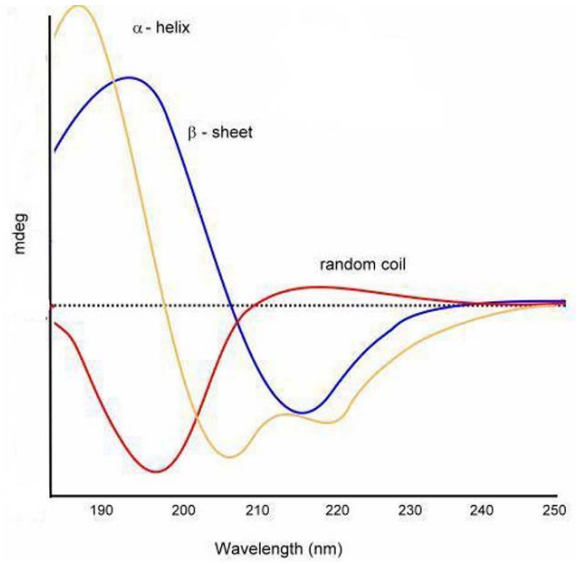

Figure 3. Different "pure" protein secondary structure by CD.

3. STRUCTURAL INFORMATION ON PEPTIDES AND PROTEINS AVAILABLE FROM CD

3.1. Protein and peptide secondary structure

Proteins and peptides share many common conformational motifs, including alpha helixes, beta pleated sheets, poly-L-proline II-like helixes and turns, which have characteristic far UV (178-250 nm) CD spectra. For example, the alpha helix motif displays large CD bands with negative ellipticity at 222 and 208 nm, and positive ellipticity at 193 nm. Short peptides usually do not form stable helixes in solution; however, it has been shown that the addition of 2, 2, 2,-trifluoroethanol (TFE) leads to an increase in the helix content of most peptides (13). Beta - sheets are less well-defined in proteins, compared to the alpha helix, and can be formed in a parallel manner. They exhibit a broad negative band near 218 nm and a large positive band near 195 nm, while disordered extended chains have a weak broad positive CD band near 217 nm and a large negative band near 200 nm (Figure 3). The poly (Pro) II (PII) conformation is increasingly recognized as an important element in peptide and protein conformation and CD is one of the most useful methods for detecting and characterizing it. For poly (Pro) peptides it is observed a strong negative band at 206 nm and a weak positive band at 225 nm.

The spectrum of a protein is basically the sum of the spectra of its conformational elements, and thus CD can be used to estimate secondary structure. One interesting approach is to deconstruct a protein into a series of synthetic peptides that are then analyzed by CD (14). In addition, the chromophores of the aromatic amino acid proteins are often in very dissymmetric environments resulting in distinctive CD spectra in the near UV (250-300 nm), which can serve as useful probes of protein tertiary structure. There are several excellent reviews (15, 16, 17) which compare and evaluate most of the currently available computer methods for analyzing CD spectra to obtain the secondary structure of proteins. Basically they are based on the exciton coupling of the 190 nm pi-pi*, while the spectrum is sensitive to the direction of the pi-pi* transition dipole moment, especially on short alpha-helixes (18).

The standard exciton-based model for predicting peptide CD spectra works well for alpha helixes and beta sheets but it fails to reproduce the PII CD spectrum because it does not account for mixing of the n-pi* and pi-pi* transitions with transitions in the deep UV, which are significant for the PII conformation. Recently it has been proposed an exciton model extended to include this mixing, using *ab initio* derived bond polarizability tensors to calculate the contributions of the high-energy transitions (19).

As mentioned CD has many more facets than just be a tool to estimate protein structure. For example, it is an excellent technique for determining the thermodynamics and kinetics of protein folding and denaturation (as shown in Fig.2), one interesting use among others is the study of membrane protein folding and conformational changes which occur during their activation and regulation (20). Moreover, it has been used to monitor changes in ellipticity as a function of temperature and concentration in order to determine the enthalpy of folding of the GCN4 transcription factor, which undergoes a two-state transition between a folded two-stranded alpha helical coiled coil and a monomeric disordered state (21). CD is also an easy quick experiment to follow the effects of mutations on protein folding and stability, and it has been used to evaluate the alpha helix content of GCN4 peptide derivatives and their dimerization process (22-24).

A recent review has shown how CD spectroscopy is a useful technique for studying protein-protein interactions in solution. It has been used CD measurements in the far UV region (178-260 nm) which arise from the amides of the protein backbone and is sensitive to the conformation of the protein. Thus CD can determinate whether there are changes in the conformation of proteins when they interact. Moreover it is possible to observe changes in the near UV (350-260 nm) and visible regions arise from aromatic and prosthetic groups. Because CD is a quantitative technique, changes in CD spectra are directly proportional to the amount of the protein-protein complexes formed, and these changes can be used to estimate binding constants. Changes in the stability of the protein complexes as a function of temperature or added denaturants (Figure 4), compared to the isolated proteins, can also be used to determinate binding constants (25).

Elastomeric proteins are widespread in the animal kingdom, and their main function is to confer elasticity and resilience to organs and tissues. From a conformational point of view, all of the elastomeric proteins that have been analyzed show a dynamic equilibrium between folded (mainly beta turns) and extended (polyproline II and beta strands) conformations that could be related to the origin of the high entropy of the relaxed state. CD spectroscopy represents the proper spectroscopic technique to be used overall because of its

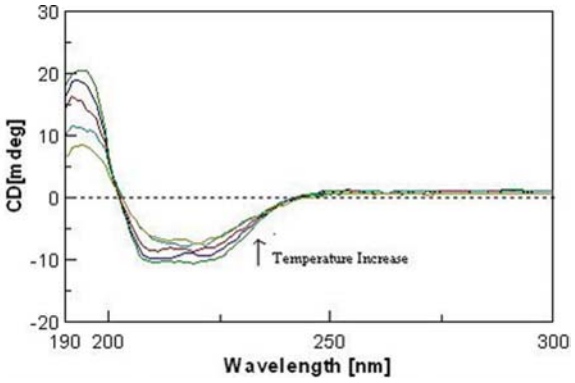

Figure 4. Example of protein thermal denaturation CD spectra: Catalase (5.9 microMolar) in MilliQ water: 25, 40, 55, 70 and 90 ºC.

particular sensitivity to the presence of PPII structure. It has been used in biomolecular studies of elastin, abductin, and lamprin (26).

The availability of recombinant prion proteins (recPrP) has been exploited as a model system to study PrP-mediated toxicity, conversion and infectivity. It has been hypothesized that the central event in the pathogenesis of prion diseases is the conversion of PrP(C) to PrP (Sc). This involves a dramatic increase in beta sheet conformation as PrP(C) is converted to PrP(Sc) and it is widely believed that this conformational change affects the undefined function of PrP(C). Although there are many methods available to monitor the changes in the structural make up of PrP mutants and oligomers formed with respect to disease relevance, circular dichroism is one of the most popular methods used as presented in a recent review (27).

Gel entrapment combined with CD spectroscopy has been used to analyze beta lactoglobulin secondary structural changes that occur at the early stages of refolding. Beta lactoglobulin is a predominantly beta sheet protein that folds by forming excess alpha helixes within milliseconds with this methodology it was possible to monitor the changes in secondary structure on a time scale of minutes or hours by far-UV circular dichroism spectroscopy. Analysis of kinetics and transient spectra allowed defining the sequence of folding events that consist of alpha helical formation, beta sheet core formation, and alpha to beta transition (28).

Time-resolved CD spectroscopy has been used directed towards the problem of rhodopsin photointermediates on the microsecond to seconds time scale. From these experiments it can be inferred that several isochromic intermediates (absorbing near 380 nm) are present on this time scale, and CD measurements promise to provide more information about these crucial intermediates preceding G-protein activation. Measurements were made in lauryl maltoside suspensions of rhodopsin since there rotational diffusion of rhodopsin was complete within 1 microsecond, allowing room temperature measurements to be made starting at the lumirhodopsin stage without complications due to linear birefringence (29).

3.2. Protein-small molecule interaction

As mentioned above CD spectroscopy has probed to be an excellent tool for following protein-ligand interactions, mainly because of its ability to sensitively detect protein conformational changes. For this reason it has been emerged as an important tool for drug discovery, enabling screening for ligand and drug binding, and detection of potential candidates for new pharmaceuticals.

Pharmacological and pharmacodynamic properties of biological active natural and synthetic compounds are crucially determinate via their binding to human proteins. Several spectroscopic techniques are available to study these mainly non-covalent interactions. CD spectroscopy, being sensitive to the chirality of ligand molecules induced by the asymmetry protein environment, has widely and successfully been applied for many decades. Chiral conformation of the ligand due to conformational adaptation to its binding site, or interaction between ligand molecules held in chiral arrangement relative to each other by the protein sites, results in one or more induced CD bands with different shape, sign and intensity. These extrinsic Cotton effects present in light absorbing region of the optically active or inactive ligand molecules give qualitative and quantitative information of the binding process. It can provide valuable data on the stereochemistry, location and nature of the binding sites (30).

CD has been used in combination with other biophysical tools, such as Potenciometry and Pendant Drop Tensiometry, in order to study the influence of polifluorinated amphiphiles, in concrete sodium perfluorooctanoate (SPFO) in its interaction with Human Serum Album (HSA) and Immunoglobulin G (IgG) proteins. These types of amphiphiles self associated in discrete objects, such vesicles and tubules and may be used in pharmaceuticals. HSA-SPFO studies performed by CD suggested a compactation of the protein due to the association with the surfactant given by an observed increase of alpha helix content (31). Finally it has been shown that a conformational transition was observed as a function of temperature, these data were analyzed to obtain the thermodynamics parameters of unfolding. These observations indicate that the presence of surfactant drastically changes the melting unfolding, acting as a structure stabilizer and delaying the unfolding process (32). On contrary IgG-SPFO studies have shown that such interaction lead to the destruction of the native structure of IgG and the formation of unfolded protein-surfactant complexes even at low surfactant concentration (33).

A practical example in the field of drug development was the development of protein kinase inhibitors for cancer's treatment. Since alpha 1-acid glycoprotein (AAG) is the principal plasma binding component of some kinase inhibitors, it has been evaluated the binding of a series of marketed and experimental kinase inhibitors to AAG by using CD spectroscopy approach

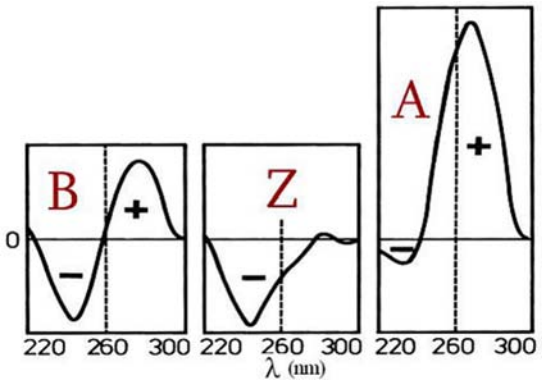

Figure 5. Schematic structures of DNA B, A, and Z observed by CD

(34). It has also been used in high-throughput monitoring of protein folding (35). In this direction, one interesting approach has been presented recently in a review, which focuses on the combination of biochromatography and CD as an effective approach for characterization of albumin binding sites and their enantioselectivity. Furthermore, applications to the study of changes in the binding properties of the protein arising by the reversible or covalent binding of drugs are discussed, and examples of physiological relevance reported. Perspectives of these studies reside in supporting the development of new drugs, which require miniaturization to facilitate the screening of classes of compounds for their binding to the target protein, and a deeper characterization of the mechanisms involved in the molecular recognition (36).

Crystal structures have opened the door to understanding the mechanism and ligand specificities of Monoamine-oxidases (MAO A and MAO B). It has been reviewed that their functional properties influence catalysis under different cellular conditions, suggesting flexibility in the MAO protein. This flexibility was confirmed by CD spectroscopy by observation of altered oxidation kinetics and a changed redox potential in the presence of flavin (37).

CD measurements have been very useful to provide some relevant information in Wilson disease. This disease is an autosomal recessive disorder of copper metabolism which results in the toxic accumulation of copper in the liver and brain, causing the hepatic and/or neurological symptoms. It has been observed by CD that zinc also binds to Wilson disease copper-binding domain [WCBD] which conformational changes completely different from those induced by copper. Finally, a chimeric protein consisting of the WCBD and truncated ZntA, a zinc-transporting ATPase lacking the N-terminal domain, has been constructed and analyzed for metal ion selectivity. These results suggest that the core determines the metal ion specificity of P-type ATPases, and the N-terminal metal-binding domain may play a regulatory role (38).

The bacterium Staphylococcus aureus is responsible for numerous hospital-acquired infections ranging from superficial wound lesions to more severe infections such as pneumonia, osteomyelitis and septicemia and, in some cases, death. The Isd (iron regulated surface determinant) proteins expressed by S. aureus and selected other bacteria are anchored to the bacterial cell wall and membrane and are involved in extract heme from Hb as an iron source. The knowledge of the overall heme-scavenging mechanism on the bacterial surface is limited. To explore this, it is necessary to use a combination of techniques to characterize both the dynamic and steady-state heme-binding properties of these proteins. UV-visible absorption and MCD (magnetic CD) spectroscopy provide diagnostic spectral data sensitive to the axial ligands, the spin state and oxidation state of the central heme-iron (39).

4. STRUCTURAL INFORMATION ON NUCLEIC ACIDS AVAILABLE FROM CD

4.1. DNA structure and stability

CD spectroscopy in the UV region provided essential information about the conformational properties of nucleic acids in solution, in spite of the fact that its use remains mostly based on comparative and empirical grounds because calculations reproduce the CD spectra only qualitatively at best. However, it is possible to characterized the different conformation of DNA which includes B-family structures, A-form, Z-form, guanine quadruplexes, cytosine quadruplexes, triplexes and other less characterized structures because each have a characteristic spectra (Figure 5).

This type of spectroscopy demonstrated its usefulness for DNA conformational studies for small and long oligonucleotides by showing, for example, that ethanol induced the B–A transition in solution and salts induced B–Z and other conformational transitions in DNA (40) (Figure 6).

Recently several chemometric methods have been proposed that have the ability to efficiently classify DNA structures from circular dichroism data. It has been performed a dataset including 50 experimental spectra corresponding to different DNA structures (random coil, duplex, hairpin, reversed and normal triplex, parallel and antiparallel G-quadruplex, and *i*-motif) and it has been analyzed by means of unsupervised hierarchical clustering analysis, principal component analysis and partial least squares discriminator analysis. The results have shown that those methods allow efficiently the classification of DNA structures from CD spectra. Moreover, these classification methods also provided the most characteristic wavelengths used in the classification procedures (41).

It has been also possible to monitor the changes in the CD signal increasing the temperature of the sample solution, monitoring DNA melting directly and making possible to observe the structural transition from folded duplex to random coil single strands (42), this approximation has been used in order to monitor the stability of a duplex DNA hairpin with two bases mismatched (43). Moreover, it has been suggested by CD experiments that guanine itself is strong enough to stabilize

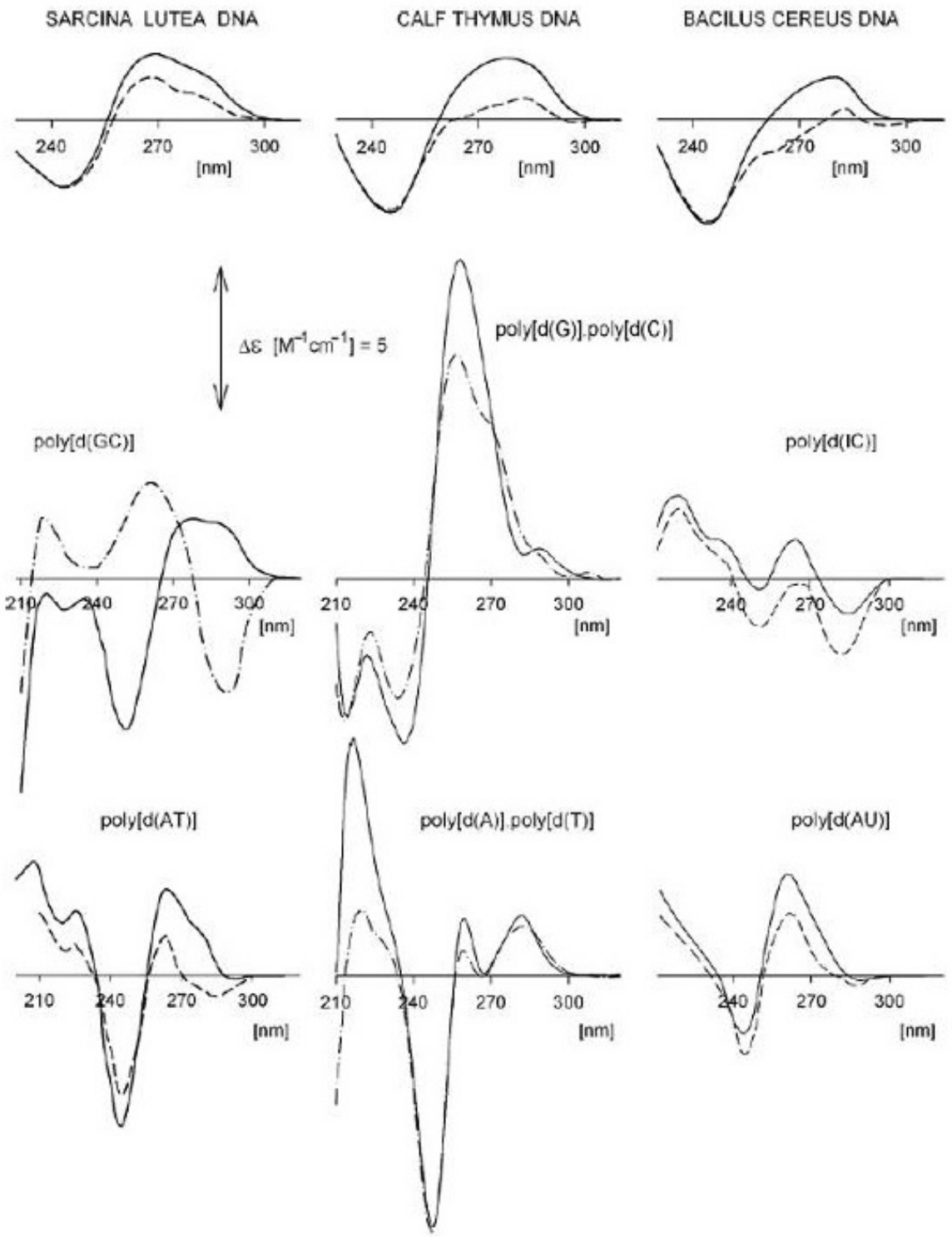

Figure 6. Sequence-dependent CD spectrum. Upper row: native DNAs from Sarcina lutea (71% G + C), calf thymus (42% G+C) and Bacillus cereus (33% G+C). Middle and bottom rows: synthetic polynucleotides. The spectra were measured in 10mM sodium acetate, pH 7 (solid lines), 5M NaCl (dashed lines) and 3.5M NaCl (dash-dotted line). The spectrum of poly [d (GC)] in 3.5M NaCl corresponds to Z-form. Salt was increased by directly adding a high concentration stock solution to cells containing DNA; the salt and DNA concentrations were corrected for the volume increase. For details of sample preparation and CD acquisition see Ref. 40.

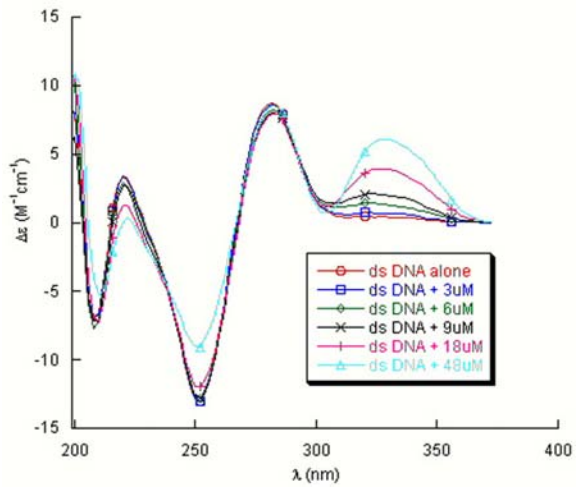

Figure 7. Titration CD experiment: dsDNA 5´-GCGAAGTTGCG-3´ and increasing concentration of Dystamicin in 12 mM phosphate buffer, 120 mM NaCl pH= 7.0. For details of sample preparation and CD acquisition see Ref. 43

various secondary structures of DNA, which is a relevant property to think about the origin and evolution of molecular replicators (44).

The double-stranded double-helical structure of DNA, elucidated by Watson and Crick over fifty years ago, is one of the scientific icons of the 20th Century (45). This structure, termed B-DNA, has the two anionic sugar phosphate backbones wrapped around each other in a right-handed double-helix, with the bases hydrogen-bonded together in pairs (A with T and G with C) in the heart of the helix. B-DNA is believed to be the most prevalent form of DNA in biological systems although other double-helical forms such as the left-handed Z-DNA and right-handed A-DNA (shorter and fatter than B-DNA) have been observed by crystallography.

Molecular recognition of B-DNA can take place in 5 distinct ways: major groove recognition; minor groove recognition; sugar-phosphate backbone binding; intercalation between the base pairs and covalent binding or metal-coordination to the bases. Meanwhile major groove recognition is mainly preferred by proteins and DNA which will be describe below (46), minor groove recognition and intercalation is preferred by synthetic agents (47). Incorporation of a transition metal into an intercalator is an attractive way of imparting positive charge to the complex. Depending on the precise complex this can be either the major or the minor groove (48, 49). Structurally similar complexes have been reported to occupy different grooves, complicating prediction in design and this will not be described here.

4.2. DNA –small molecules interaction

Considerable research effort has focused on the design of synthetic minor groove binders and such agents have found clinical application in treating cancers and protozoal diseases and as anti-viral and anti-bacterial agents (50). Minor groove binding agents include the natural molecule distamycin A, and synthetic diarylamidines (such as DAPI, berenil, and pentamidine) and bis-benzimidazoles (such as Hoechst 33258).

When this highly chromatic ligands bind, in a dissymmetric fashion to DNA they may generate large CD bands, called Cotton effect, in the region of their absorbance spectrum. The change in ellipticity as a function of substrate concentration can be used to estimate the binding constants. In the case of distamycin, it has been used CD in order to differentiate DNA binding modes: it has been found that 1:1 and 2:1 drug-duplex complexes exhibit distinctly different circular dichroic spectral characteristics and can, thus, serve as a diagnostic tools for binding mode differentiation. Observation of CD intensity profiles at 265 or 275 nm as a function of drug to DNA ratios can reveal the extent of binding cooperativity for 2:1 complex formation at a 5-base-paired binding site (51) (Figure 7).

This has been done, in order to understand the rate changes in a native ligation reaction between tripyrrole derivatives mediated by ds DNA(43). Dervan's Lab. has extensively studied the pairing rules for minor groove recognition of polyamides based on *N*-methylpyrrole (Py) and *N*-methylimidazole (Im) units (52). One interesting approach of minor groove binders with electrochemical properties has been reported recently, for this purpose it had been coupled to a system Py-Im a ferrocene core. CD titration studies revealed that one of these derivatives could bind to the target DNA with an association constant of 10^7 M^{-1} (53). The induced CD (ICD) bands of DNA-Single-Walled Carbon NanoTubes (SWCNT) at 274 and 243 nm were first used to detect trace Hg ions in solution. These DNA-SWCNTs sensors are easy to fabricate and use in detecting mercury ions at the nM level in solution (54).

CD spectroscopy has been extensively employed in the field of DNA intercalators, such as organic or inorganic octahedral (particularly ruthenium (II) and rhodium (III)) and square-planar (particularly platinum (II)) complexes which acts as potential anticancer agents and diagnostic agents (55). Recently chromatographic experiments combined with CD spectroscopy has showed that temperature has an important effect on plasmid secondary structure if adenine-rich inserts are present in a plasmid structure, it has also been suggested that base composition could also be responsible for the induction of specific interactions with histidine ligands (56).

Combined studies of CD spectroscopy, with high resolution RMN and restrained molecular dynamic simulation are another representative example of the importance of combined biophysical tools in order to elucidate the interaction of a design small molecule and its interaction with a specific DNA binding motif (57). Ent-DDI, a designed synthetic agent, is a left-handed wedge-shaped spirocyclic molecule whose aglycone portion is an enantiomer of DDI, which mimics the spirocyclic geometry of the natural product. NMR and CD data indicate that the

DNA in the DDI-bulged DNA complex undergoes a larger conformational change upon complex formation in comparison to the ent-DDI-bulged DNA, explaining the different binding affinities of the two drugs to the bulged DNA. In addition, there are different placements of the bulge bases in the helical duplex in the two complexes. One bulge base (G7) stacks inside the helix, and the other one (T8) is extrahelical in the DDI-bulged DNA complex, whereas both bulge bases in the ent-DDI-bulged DNA complex prefer extrahelical positions for drug binding. Elucidation of the detailed binding characteristics of the synthetic spirocyclic enantiomers provides a rational basis for the design of stereochemically controlled drugs for bulge binding sites.

Currently it has been found a small molecule that disrupts G-Quadruplex DNA structure and enhances gene expression (58). CD spectroscopy has been used to elucidate the effects of two high-affinity triarylpyridines on the folded conformations of c-kit 1 and c-kit 2 G-quadruplex DNA. It has been observed that one of their triarylpyridines can remodel G-quadruplex DNA in a concentration-dependent manner and also increase the level of gene expression in cells. The CD spectra of c-kit 2 suggested mainly parallel quadruplex populations as indicated by a strong positive band at 260 nm and a negative band at 240 nm. A titration of the ligand has been performed into a pre-annealed solution of G-quadruplex in buffer containing 100 mM KCl and 10 mM Tris. HCl at pH 7.4. Upon titration of up to 10 microMolar into a solution of c-kit 2, there was an increased of the band; however, upon addition of higher concentrations of the ligand, the CD signal intensity at 260 nm was found to decrease in a dose dependent fashion until a point at 100 microMolar beyond which no further reduction in the molar ellipticity was observed. The decrease in the CD signal suggested ligand-dependent disruption of the stacking between the bases of the G-quadruplex tetrads, consistent with an apparent unfolding effect. A similar effect was observed when the ligand was titrated into c-kit 1. A plot of molar ellipticity against concentration of the ligand gave a sigmoidal-shaped curve, indicative of a co-operative effect, where the binding of the ligand at one site increases the affinity for ligand binding at another site. Given that G-quadruplex DNA potentially has more than one binding site, one possible explanation was that the ligand, at higher concentrations, may bind at multiple sites and thus start to remodel the secondary structure.

4.3.. DNA –protein interaction

For most proteins, there is only a weak signal from aromatic amino acids in the region of the CD spectrum between 250 and 300 nm compared to that seen for nucleic acids. Experiments involving the addition of protein can thus be conveniently carried out in this wavelength range, as hereby described. Below 250 nm, both proteins and DNA have optical activity and any experiments here may require resolution of the spectrum into protein and DNA components (59, 60, 61).

CD spectroscopy has been extensively used in the design of DNA binding peptides. GCN4 is a well studied transcription factor (Tf) from bZip family which in the presence of its cognate DNA forms a dimer of uninterrupted alpha helixes. This Tf has a bipartite DNA-binding domain, consisting of a coiled-coil leucine zipper dimerization domain and a highly charged basic region that directly contacts DNA. Meanwhile the leucine zipper is sufficient for dimerization, forming a stable, two stranded, parallel coiled coil; the basic region is largely unstructured in the absence of DNA, but adopts a helical structure upon DNA binding (62). This particular feature has became CD spectroscopy a classic tool in order to monitor the increase in alpha helix content at 222 nm as a quick experiment to evaluated bZip mimetics. CD difference spectra were obtained by subtracting the CD spectrum of the corresponding DNA solution from the CD spectra of the protein-DNA complex (Figure 8).

It has been also possible to check that the DNA duplexes were consistent with B-form DNA, with a minimum at c.a. 247 nm, where the protein contributes little, if any, to the observed signal, assuming that the DNA was B-form in the complex and that the increased magnitude of the signal at 222 nm was due to a helix-coil transition in the basic region of bZip proteins.

By linking to bZip basic regions through an azobenzene moiety Mascarenas' group had obtained a peptide whose sequences specific DNA-binding affinity could be modulated by light (64). Another system proposed was based on a bipartite major/minor groove binding mode of a designed peptide. This system had a GCN4 basic region modified in the C-terminal in order to incorporate a linker and a distamycin derivative which is a well known minor groove binder as mentioned above. This somewhat mimics the recognition strategy used by several natural Tfs, such as homeodomains, with an alpha helix inserted into the major groove, and another recognition element (usually the N or C terminal end of the protein) inserted into the minor groove of adjacent sequences (64). Recently, it has been presented a non covalent version of the system (65). In these systems, it has observed not only the band at 222 nm which correspond to the alpha helix but also the new band observed at 330 nm corresponding to the distamycin derivative insertion in the DNA minor groove.

The Notch pathway is a short-range signaling mechanism between neighboring cells that results in changes in gene expression. Extracellular interactions between Notch receptors and ligands trigger proteolytic cleavage of the receptor Notch. Recently, it has been reviewed several studies using CD in addition with other biophysical techniques and computational methods which have scrutinized how the CSL-Notch IC-Mastermind ternary complex forms and the role of individual domains play in this process. These detailed analyses have provided a wealth of molecular insights into the assembly of a Notch pathway active transcription complex but have also left several intriguing questions (66).

CD spectroscopy also has been used in studies of a single-stranded DNA binding protein; such is the case of g5p, of the Tfs group of bacterial viruses. The CD spectrum

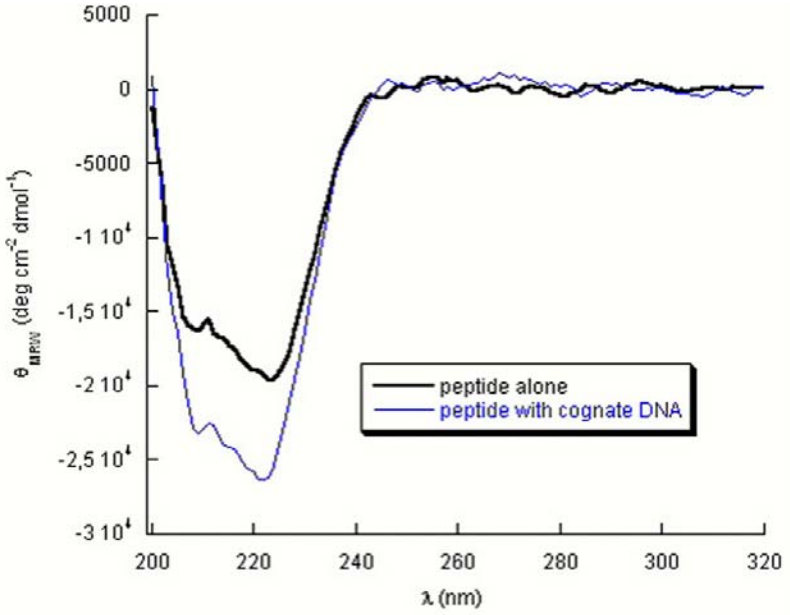

Figure 8. CD spectra of GCN4 disulphur peptide alone (5 microMolar, red) and in the presence of a cognate dsDNA (5´-GCGATGACTCATGCG-3´) (5 microMolar, blue) in phosphate buffer (10mM). For details of sample preparation and CD acquisition see Ref. 63

of the g5p is dominated by a positive tyrosine La band at 229 nm, to which the protein tyrosine contributes. The La band becomes much less positive upon binding of g5p to nucleic acids. CD spectra of mutant proteins identified a single tyrosine, Y34, which is largely responsible for this CD perturbation. At >250 nm, CD perturbations of nucleic acids can be monitored during g5p binding, and it has been identified two distinct modes of binding of the g5p at physiological ionic strength (0.2 M NaCl). SELEX selection of sequences bound preferentially by g5p yielded a G-rich sequence that is closely related to telomere sequences and has CD properties of a G tetraplex. CD spectroscopy showed that the presumed G-quadruplex form was maintained within saturated g5p·DNA complexes (67).

5. CONCLUSIONS

In the post-genomic era becomes to be important the knowledge increase of the structure Protein and DNA in solution. In this context CD spectroscopy has emerged as a very useful quick and cheap tool to study the secondary structure of these biomolecules and their interaction not only among them but also with small molecules in solution. The power of this technique is increased by its use in combination with other biophysical tools

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