Course: PG Pathshala-Biophysics

Paper 10: TECHNIQUES USED IN MOLECULAR BIOPHYSICS II (Based on Spectroscopy)

Module: 21. Basic principle of CD spectroscopy and instrumentation

Content Writer : Dr. Imtaiyaz Hassan Jamia Millia Islamia, New Delhi

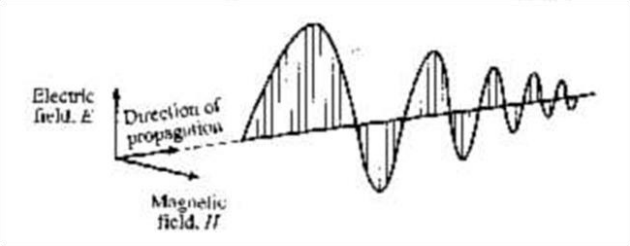
Introduction: circular dichroism (CD) is a special type of absorption spectroscopy which measures the differences in the absorption of left- and right-handed circularly polarized light arise because of structural asymmetry. The CD speptropolarimeter is used to monitor the CD signal (differential absorption of left and right handed circularly-polarized light) of molecules is with respect to the wavelengths and can give both positive and negative signals. CD spectroscopy can only be implicated for chiral molecules despite of difference in their size, with a special attention to the large biological molecules. Primarily CD is used to analyze secondary structural content of proteins like α-helix and β-sheets or changes in conformation of macromolecules upon changing the their environment such as temperature or pH. A zero CD intensity is observed in the absence of any regular structure in protein. CD is an effective tool to monitor structural changes occurred in protein or DNA due to interaction with other molecules. We can derive structural, kinetic and thermodynamic parameters of macromolecule in general, and of proteins specifically. CD data is generally represented in a unit called mean residue ellipticity (MRE), which is a measure of structural content in a protein/DNA, expressed in terms of degrees-cm^2/dmol.

Objective: In this module we discuss following things in detail,

1. Circularly polarized light
2. Physical principles of CD
3. Optical Rotatory Dispersion
4. The CD instrument: the spectropolarimeter
5. Information available from CD studies of proteins
6. Experimental conditions and general maintenance
7. Summary

1. Circularly polarized light

Light is an electromagnetic radiation which accomplishes an isotropic distribution of the electric and magnetic field vectors. If the electric and magnetic field vectors are oscillating at right angle to each other then such light is termed as linearly polarized and its direction of the polarization is specified by the electrical field vector.



Linear Polarized Light

However, if two linearly polarized light beams are superimposed in such a way that have a phase shift of λ/2 and their electric and magnetic field vectors are perpendicular to each other, a circularly polarized light is produced. Hence, resultant amplitude vector of the electric field will produce a rotation on a spiral perpendicular to the direction of the propagating light wave (see Figure 1).

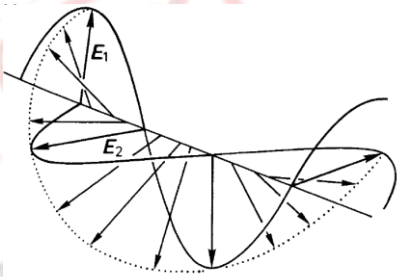


Figure 1: Generation of of circularly polarized light. "By superposition of two linearly polarized light beams with a phase difference of a quarter wavelength. The displacement of 90 ° (or λ/4) is generated by a 1/4 wave plate that is sliced parallel to the optical axis".

However, the circularly polarized light having two different components as left- and right-handed springs which rotates anticlockwise and clockwise, respectively. The frequencies of left- and right-handed springs are related to the frequency of the light. Once, both frequencies are combined together after passing through an optically isotropic medium a plane polarized light is produced. On the other hand, eliptically polarized light is produced by superimposing two circularly polarized waves of different amplitude.

2. Optical Rotatory Dispersion

Both left and right circularly polarized beams possesses different angular velocities when it pass through a sample and thus produce ellipse which is known as optical rotation. The measurement of the optical rotation in dependence of the used wavelength is the optical rotatory dispersion (ORD) as shown in the Figure 2.

When two circular polarized light of an equal and opposite amplitude and phase are superimposed, the linear polarized light is produced.

When left- and right hand polarized lights are differentially absorbed, their component leads to ellipticity (CD) and optical rotation.

Figure 2: Showing relationship between linearly, circularly, and elliptically polarized light.

The left and right circularly polarized light beams are traveling at different speed if substances present in the medium posses an optical activity, are absorbed at different extent. Furthermore, if both left and right handed polarized light having different refractive indices and their components are combined, the plane-polarized radiation will be rotated by an angle α which is expressed as,

$$\alpha = \frac{n_l - n_r}{\lambda}$$

"where n_1, n_r are the indices of the refraction for left-handed and right-handed polarized light and α is in radians per unit length (from λ)

$$\text{rotation}\left(\text{rad cm}^{-1}\right) = \phi = \frac{\pi}{\lambda}\left(n_L - n_R\right)$$

where *n* is refractive index, λ is wavelength of light and Φ angle of rotation

Usually reported as a specific rotation [α], measured at a particular T, concentration and λ (normally 589; the Na D line),

$$\text{Molar rotation } [\Phi] = [\alpha] \times MW \times 10^{-2}$$

$$[\alpha] = \frac{\alpha}{c'd'}$$

$$[\alpha] = \frac{10^2 \alpha}{lc}$$

l = pathlength in decimeters $\qquad c = \frac{g}{100\,mL}$

Concentration of an optically active substance, 'c', expressed in g cm^{-1} and d' = thickness of the sample in decimeters,

$$[M] = M[\alpha] \times 10^{-2} = \frac{M\alpha \times 10^{-2}}{c'd'}$$

M = molecular weight of the optically active component
the 10^{-2} factor is subject to convention and is not always included in [M]"

Comparison of CD with ORD

The ORD curve is produced when plot molar rotation [α] or [M] (y axis) with respect to the wavelength λ (at x axis). The ORD curve is positive or negative when clockwise or counterclockwise rotations are plotted (Figure 3). ORD is based solely on the index of refraction. On the other hand, CD is presented as the ratio of the semiminor and semimajor axes of the ellipse. CD plots are generally Gaussian in shape while ORD is S-shaped. ORD spectra are dispersive whereas CD spectra are absorptive. Optical rotation and CD can be derived mathematically from each other if all spectral information is available. ORD and CD spectra provide similar information, however, each technique can be used for different applications and have their own limitations.

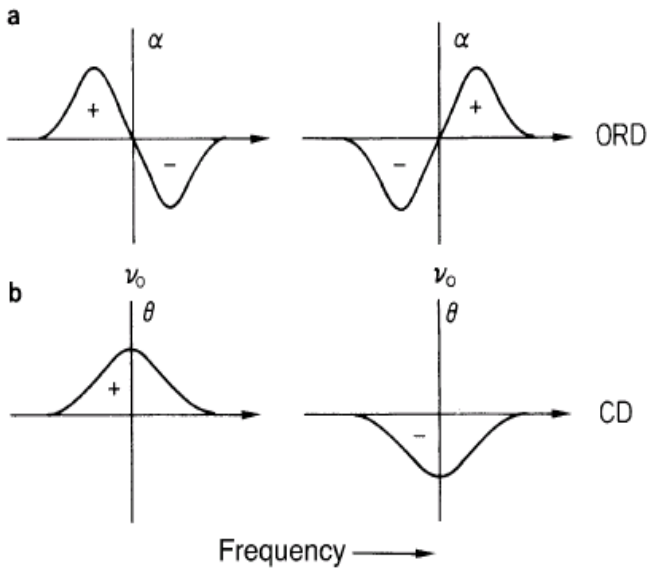


Figure 3: Showing ORD spectra are dispersive (called a *Cotton effect* for a single band) whereas CD spectra are absorptive. The two phenomena are related by the so-called König-Kramers transforms.

3. Physical principles of CD

In principle if a linear polarized light is passed through a medium with chiral molecule (optically active substance), both speed and absorption coefficients (εL and εR) of the two circularly polarized components are different. Such difference in the absorption coefficient is measured an equipment known as circular dichroism spectrophotometer or a circular dichrograph. Since both left and right circularly polarized light absorbs differentially, an elliptically polarized light emerges from the sample. Thus, in practice the ellipticity is determined from the difference of the absorption coefficients by CD spectroscopy which is expressed in terms of,

$$\theta_\lambda = const\ (\varepsilon L - \varepsilon R)\ .\ c.\ d$$

"where *d* is the path length and *c* the concentration of the sample.

Const is given by

$$\text{Const} = \frac{180}{4\pi} \ln(10) \approx 33$$

The molar ellipticity is then given by,

$$[\theta]\lambda = \frac{Mr.\Theta\lambda}{10.d.c}$$

*M*r is the molar mass in g·mol^{-1}. If the molar extinction coefficients of the left and right circularly polarized light are known then the molar ellipticity can be expressed as

$$[\theta]\lambda = 3300\ \varepsilon$$

The dependence of the ellipticity on the wavelength of the incident light produces the CD spectrum which has an absorption band. The positive and negative CD is observed as a function of the wavelength, depending which of the two circularly polarized components is absorbed stronger".

Optical activity or chirality

Chirality is a property of individual molecule. A chiral molecule, which is optically active, absorbs L-CPL and R-CPL differently which happens to be the basis of CD. A chiral molecule has two enantiomeric forms that are almost identical but are actually different molecules. When 4 different atoms as functional groups singly bonded to a given carbon atom in an organic molecule, the carbon atom is said to be asymmetric or chiral centre or chiral atom (Figure 4). In chiral molecule, 4 different functional groups can be arranged in two ways that represent non-super imposable mirror image of each other. Such a carbon atom is asymmetric and it can exist in two isomeric forms called enantiomers and is called a chiral atom or chiral centre. Enantiomers are also called optical or stereoisomer ((Figure 4). They are chemically identical in their reactions, but they differ in vary characteristic physical property, the ability to rotate the plane polarized light.

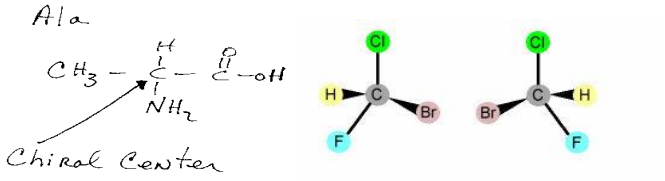


Figure 4: A chiral carbon with four different atoms attached.

Optical activity is solely depends on the chirality of molecules and thus only chiral substances (compounds that have an asymmetric carbon) are characterized by the CD. Asymmetry in the chromophore in protein (amide groups, aromatic groups and disulfide bridges) is induced by the chirality and chemical environment. Chirality is therefore resulted from the overall structure of a macromolecule. Hence, chirality is only the basis for the investigation of protein conformation by CD spectroscopy.

Chromophore:

A chromophore is a part of molecule that shows absorbance. Chromaphores in protein is generally divided into three groups namely peptide bond, amino acid and prosthetic groups.

Peptide bond: Peptide backbone and secondary structures like α-helix and β-sheet show absorbance in far-UV region from 170-250 nm. A weak intensity n→p* transition is observed at 210-220 which is also the lowest energy transition in the peptide chromophore. α-helical form of protein also shows the n→p* transition as a small shoulder near 220 nm. Another intense band at 190 nm is responsible for the peptide bond absorption in the p→p* transition.

Amino acid side chains: In proteins tryptophan, tyrosine, phenylalanine and disulphide bonds show absorbance in near ultraviolet region. However, the contributions to the CD spectra in the far UV is usually negligible. The disulfide group is an intrinsically asymmetric chromophore and showing a broad CD absorption around 250 nm.

Prosthetic group: Many proteins have prosthetic group which show absorbance. For example cytochrome c show absorbance in the Soret region with maximum absorbance around 409 nm.

4. The CD instrument: the spectropolarimeter

CD instruments (known as spectropolarimeter) is used to measure the difference in absorbance between the LEFT and RIGHT circularly polarised components (ΔA=AL−AR) which is generally expressed in terms of the ellipticity (θ) in degrees. In actual scenario, $\theta = \tan^{-1}(b/a)$ where b and a are the minor and major axes of the resulting ellipse.

The components of CD machine are shown in the Figure 5. "In CD spectropolarimeter a plane polarized radiation is produced by passage of light from the source (LS) through 2 prisms (P1 and P2) and passes through a series of mirrors (M0 to M5) and slits (S1 to S3). The ordinary ray (O) is focused by a lens (L), and passed through a filter (F) to the modulator (CDM). The circularly polarized components are then passed through the shutter (SH) to the sample compartment, before detection by the photomultiplier (PM)".

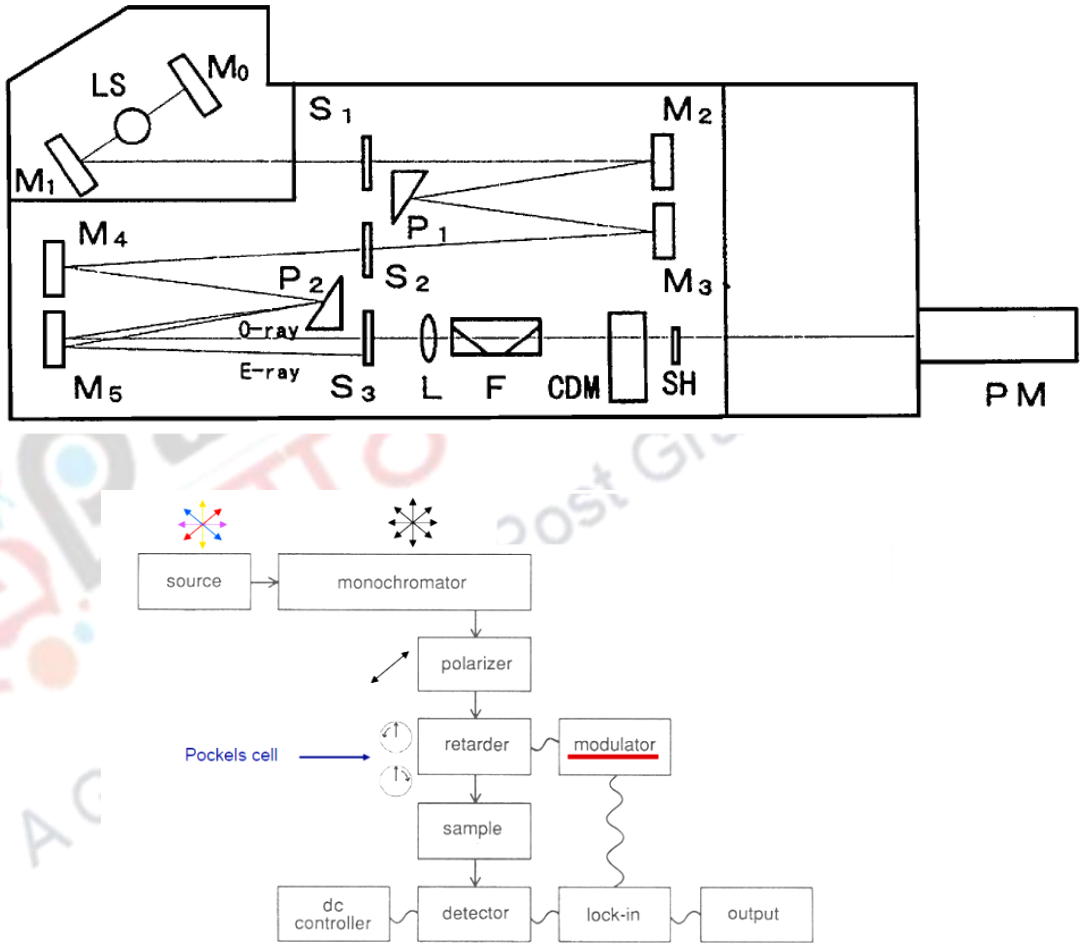


Figure 5: Showing block diagram of a spectropolarimeter (Jasco J-815).

Radiation is split into the two circularly polarized components by passing through a modulator which is made up of piezoelectric quartz crystal, subjected to an alternating (50 kHz) electric field. The modulator will transmit each of the two components in turn. If one of the components

is absorbed by the sample to a greater extent than the other, the resultant radiation would now be elliptically polarized, observed in the form of CD signal.

5. Information available from CD studies of proteins

CD signal is produced due to the interaction of circularly polarized light with chiral molecules. Most of the biologically important molecules such as proteins, DNA and RNA are chiral in nature. CD is greatly influenced by the three-dimensional structure, a CD signature. Furthermore, CD is also used to compare two macromolecules (or the same molecule under different conditions) for different purposes such as, to ascertain that a purified protein is correctly folded, to determine the structural differences due to mutation, etc. Far UV CD measurements entails a quantitative estimates of secondary structures which may be compared with other techniques. A typical CD spectra of protein with different secondary structural information is shown in the Figure 6.

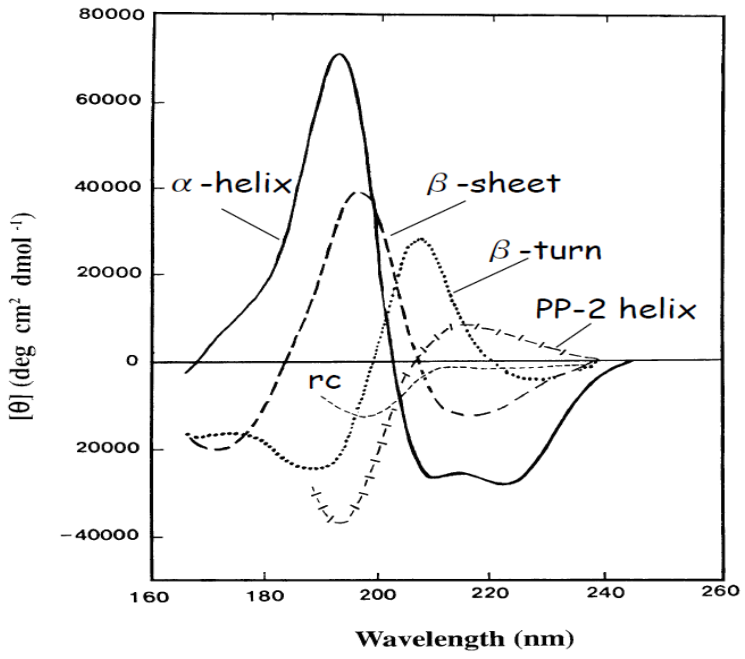


Figure 6: Showing a typical CD spectra. CD in the far UV region (180-240 nm) - where the peptide bond absorbs light- reports on the overall content of secondary structure. An all helix polypeptide has an ellipticity of -38000 deg cm^2 $mol^{-1}res^{-1}$ at 222 nm An all random polypeptide has an ellipticity of -1200 deg cm^2 $mol^{-1}res^{-1}$ at

222 nm. In general, the CD signal at 215 nm indicates the sheet content and the signal at 208 nm and 222 nm are used to calculate the helical content.

CD is extensively used to see the dynamic changes in the DNA structures. In particular, structural changes induced by temperature, pH, ligands or co factor binding, or denaturants can easily be estimated by the CD measurements (Figure 7). CD is also used to measure stability of a protein with varying parameters such as high concentration, extremes of pH, elevated temperatures and long term storage times.

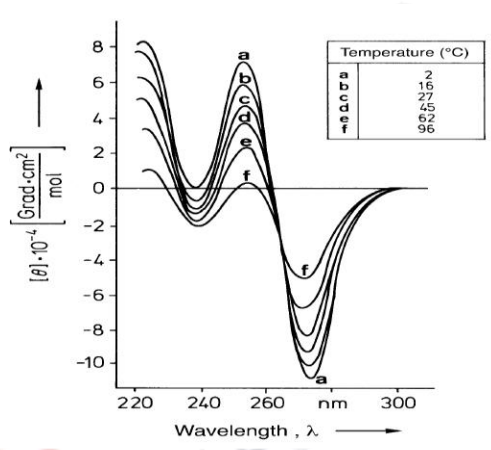


Figure 7: Showing the effect of temperature on adenosine 5 nicotate in 2M NaCl. At low temperature bases and stacked while at high temperature bases are dissociated.

6. Experimental conditions and general maintenance

CD is being widely used to estimating structural information of DNA or proteins in the form of transparent solution. Therefore, understanding of appropriate experimental design, key aspects of instrument calibration and sample characterization are essentially important. Following guidelines will be useful to get a reliable CD data.

CUVETTES: CD spectra are collected by a transparent quartz cuvettes) with a path length 0.01 to 1 cm which should be carefully handled, used and stored. Generally, quartz cells should be washed with concentrated nitric acid followed by distilled water, then ethanol, followed by drying with a vacuum pump.

PREPARATION OF BUFFERS: Buffers used for CD spectroscopy are transparent, filtered, degassed and must not contain any optically active materials which possesses a maximum of total absorbance below one. Phosphate, Tris and borate are commonly used buffers for the far UV CD studies. The spectrum of the CD buffer must be subtracted from the spectrum of the sample during data analysis.

PREPARATION OF PROTEINS SOLUTION: Samples for CD spectroscopy must be at least 95% pure. For secondary structure measurements protein solution must be 95% pure and its concentration range from 0.005 to 5 mg/ml depending on the path length. However, lowest concentration and additives are preferred. Dialysis or gel filtration is generally employed to sample before going to CD measurements to remove any insoluble aggregates which causes light scattering and absorption flattening.

CALIBRATION OF CD INSTRUMENT: A number of methods are available for calibrating the wavelength of the CD instrument. "These are the use of rare earth element filters (e.g., holmium oxide which has peaks at 279.4 nm, 361.0 nm and 453.7 nm), benzene vapour (241.7 nm, 253.0 nm and 260.1 nm) and neodymium glass (586.0 nm). CSA (1S-(+)-10-camphorsulphonic acid) is widely used as a standard for magnitude calibration of CD instruments, because it has CD peaks in the near and far UV (290.5 nm and 192.5 nm). Pantolactone which has a strong band at 219 nm may also be used for calibrating CD instruments".

EXPERIMENTAL CONDITIONS: Defining experimental condition is critical for CD measurements. It includes "Settings parameters such as bandwidth, time constant, scan rate and number of scans are essentially important to improve the data quality. Increasing the bandwidth will allow more light to fall on the sample. Increasing number of scans generally enhanced the signal/ noise (S/N) ratio which is proportional to the square root of the number of scans".

Summary

CD is an extensive equipment used to investigate the secondary structure and thermodynamic stability of DNA and proteins. CD signal arose because of difference in absorption of left- and right-handed circularly polarized light at different wavelength (λ). CD signal is only produced by

interaction of circularly polarized light with a chromophore in a chiral environment. CD spectrum of a protein contain the information about three components of secondary structure: α-helical, β-sheet, and random coil. Value of CD is generally expressed in terms of mean residue ellipticity, θ (degree·cm2/dmol) which indicates the difference in molar extinction coefficients ($\Delta\varepsilon = \varepsilon L - \varepsilon R$). The molar ellipticity $[\theta]$ is can also be presented as $[\theta] = 3298\ \Delta\varepsilon$. A proper handling is necessary during CD measurements. Additives, buffers and stabilizing compounds should be optimized properly before measuring the CD spectra.

End of Module

Thank you

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