

Circular-Dichroism in The Study of Protein Structure

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ABSTRACT

The protein folding, form (secondary and tertiary structural features) can be studied by using Circular Dichroism (CD) spectroscopy which gives us an insight into the function of the protein. Secondary structural features are analyzed in far ultra violet (UV) region (240-180 nm) to get the structural features like α -helix and β -sheet while tertiary structural features are analyzed in near UV region (350-260 nm) which reflects the presence of aromatic amino acid side chains. Circular dichroism is mostly used for the initial investigation of protein samples. CD spectroscopy is a fast, economic, reliable and non-destructive method for the study of conformation and stability of a protein. It is a comparative study, where results obtained are compared with an innovator sample (reference/ standard sample) and useful in obtaining the information of protein structure, the ratio of various forms and the rate at which structural changes occurring could be attained. In this article we have briefed the CD approach in the study of protein structure.

KEYWORDS: Circular Dichroism, Chiral, Protein structure, Spectroscopy.

Introduction

Optical Activity: Certain organic compound or its solution when placed in the path of a plane polarized light they rotate the light through certain angle (Fig 1). This property of the compound to rotate plane polarized light is called optical activity. For a compound to be optically active, it must be chiral in nature (Asymmetric character). Optical activity is not seen in *symmetrical* molecules.

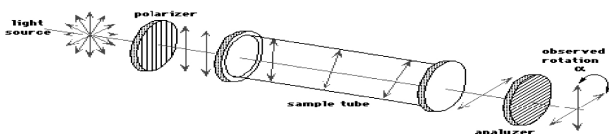


Fig. 1: Rotation of the polarized light

The isomer that rotates the plane of polarization to the left is called levo isomer (-) and to the right is called dextro isomer (+).

As shown in the fig 2 passing unpolarized light through a linear polarizer will convert it to a linearly polarized light. Passing linearly polarized light through a quarter-wave plate with its axes at 45° to its polarization axis will convert it to circularly polarized light.

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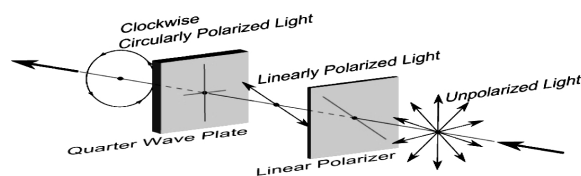


Fig2: Conversion of unpolarized light to circularly polarized light

A plane polarized light is a mixture of left circularly polarized light (LCPL) and right circularly polarized light (RCPL) and they are in equal magnitude and are in phase with each other.

Linear polarization or **plane polarization** of electro-magnetic radiation is a confinement of the electric field vector or magnetic field vector to a given plane along the direction of propagation.

The orientation of a linearly polarized electromagnetic wave is defined by the direction of the electric field vector. If the electric field vector is vertical (alternately up and down as the wave travels) the radiation is said to be vertically polarized as shown in the fig 3 and If the electric field vector is horizontal the radiation is said to be horizontally polarized.

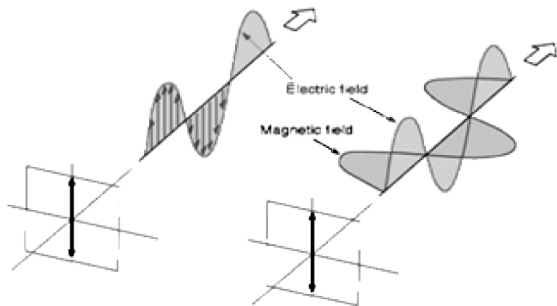


Fig 3: Vertically polarized light

Circular dichroism (fig 4) is the result of absorption of left-handed circularly polarized light (L-CPL) and right-handed circularly polarized light (R-CPL) to different extents so that the emergent beam is an elliptically polarized light and occurs when the molecule is asymmetric^[1]. Circular dichroism is measured as a function of wavelength hence it is termed as circular dichroism spectroscopy.

i.e., $CD = \Delta A(\lambda)$
 $(\Delta A \text{ is difference in absorption} = A(\lambda)_{LCPL} - A(\lambda)_{RCPL}$
 $\lambda \text{ is the wave length})$

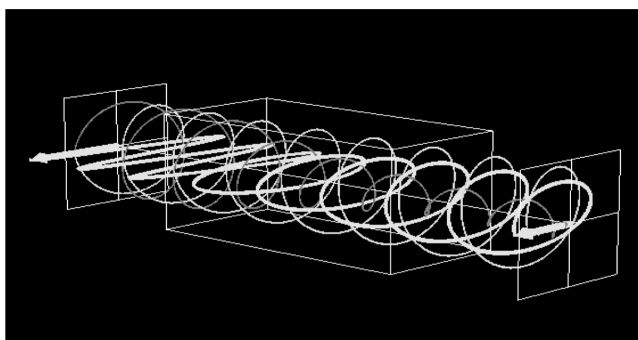


Fig 4: Circular Dichroism

- CD occurs when a molecule contains one or more chiral chromophores (light-absorbing groups) which lead to the absorption of one circularly polarized light to greater extent than the other and hence the CD signal over the corresponding wavelengths will be non-zero^[2].
- Dichroism is used to denote direction-dependent light absorption. A circular dichroism signal can be positive or negative i.e. if L-CPL is absorbed to a greater extent than R-CPL the CD signal is positive and if R-CPL is absorbed to a greater extent than L-CPL the CD signal is negative.

A CD signal is obtained when the sample under analysis is chiral or asymmetric as one of these reasons: where,

- It possess a chiral structure (which is intrinsic)
- It has a covalent link to a chiral centre in the molecule, or

- It is present in an environment which is asymmetric by virtue of the molecule which causes disposition of the electric and magnetic dipoles.

The CD of molecules is measured over a range of wavelengths i.e. the measurements are carried-out in the UV-visible region of the electro-magnetic spectrum.

CD and Proteins

Circular dichroism is mainly used to study the secondary structure of proteins (**Fig 5**) along with the conformation of peptides and nucleic acid as it requires little sample in the concentration of 100µg/ml or even less than that^[3].

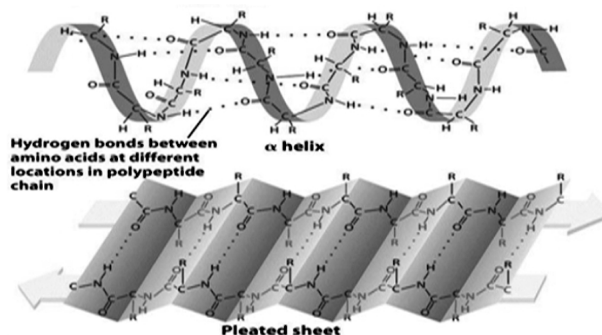


Fig 5: Secondary structure of protein

Molar Ellipticity: The CD spectrum is often reported in degrees of ellipticity (θ), which is a measure of the ellipticity (**Fig 6**) of the polarization and is given by:

$$\tan \theta = \frac{E_R - E_L}{E_R + E_L}$$

where E is the magnitude of the electric field vector.

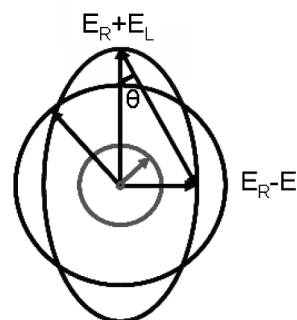


Fig 6: Degrees of ellipticity

Elliptically polarized light (purple) is the superposition of LCP (red) and RCP (blue) light. θ is the angle between the magnitude of the electric field vector at its maximum and its minimum.

Elliptical polarized light (violet) is composed of **unequal** contributions of left (red) and right (blue) circular polarized light.

i.e., $\theta = 3298.2 \Delta \epsilon$.

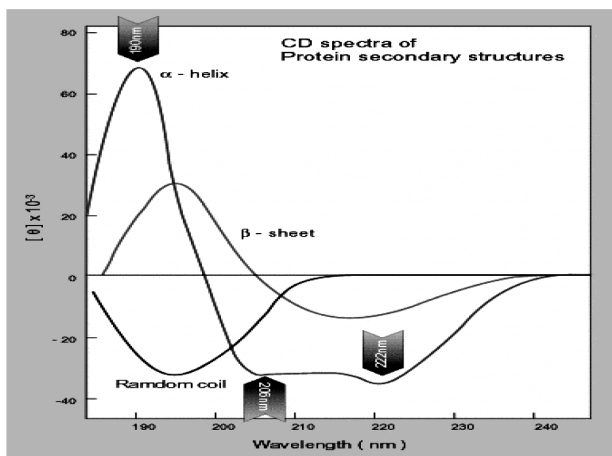


Fig. 7: CD spectra of Protein secondary structure

Fig. 7 represents an all α -helix, an all β -sheet and a random coil protein (parent characteristic feature for secondary structure of proteins). With reference to the origin '0' if the signals/peaks obtained in the CD spectrophotometer are above the origin then they are positive(+) peaks & if they are below the origin then they are negative (-) peaks[4].

A secondary structure signal is obtained for a biomolecule or protein in circular dichroism because the peptide bond is asymmetric[5]. The amide group of the peptide bond in far UV range 260-180nm undergo two transitions of low energy i.e. π^* and 0^* exhibiting signals around 210-230nm and 180-200nm.

The transition π^* is responsible for the CD signal at 222nm (a negative peak) and the transition 0^* is responsible for signal at 208nm (a negative peak) and one more at about 190nm (a positive peak). These three signals are the characteristics of the α -helix spectrum.

The transition 0^* is also responsible for signal at 198nm (a positive peak) which is a characteristic feature of β -sheet spectrum.

Hence in the far UV region (260-180nm), which corresponds to peptide bond absorption, the CD spectrum can be analysed to give the content of regular secondary structural features such as alpha-helix and beta-sheet.

A negative band in 190-200 nm and weak positive CD band at 220-230 nm is indicative of substantial disordered structure representing random coil.

The CD spectrum in the near UV region (350-260 nm) reflects the environments of the aromatic amino acid side chains and thus gives information about the tertiary structure of the protein (Fig 8). The presence of significant signal near-UV is a good indication that the protein is folded into a well-defined structure.

In the near UV region (350-260 nm), CD signal is obtained if and only if a protein sample contain

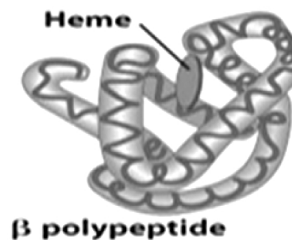


Fig. 8: Tertiary structure of protein

aromatic amino acid/s (Fig 9) viz., Tryptophan, Tyrosine and or Phenylalanine. CD signal is obtained by 0^* transition. Disulphide bonds also contribute to CD signal at 260nm which is related to π^* transition but this signal is wider as compared to aromatic signals.

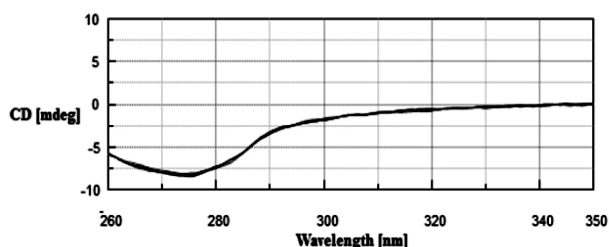


Fig.9: CD signal of tertiary structure of proteins (aromatic amino acids)

Each of the aromatic amino acid has its own characteristic wavelength profile. Tryptophan gives a CD signal between 300-290 nm, tyrosine shows signal between 285-275 nm and phenylalanine shows signal between 270-255 nm. These signals arise due to the vibrational transitions occurs due to excited states of different vibrational levels. In some cases aromatic side chains leads to CD signals in far UV region below 240 nm and leads to complications in the analysis of samples.

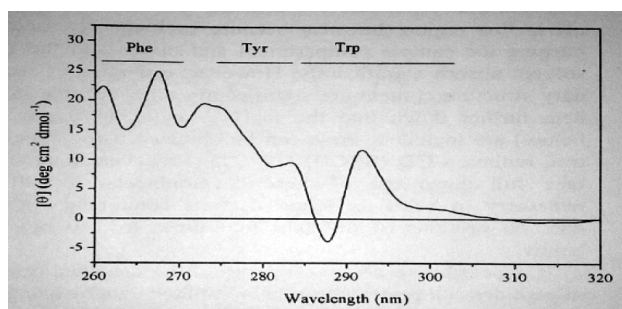


Fig.10: The near UV CD spectrum for type II dehydroquinase from *Streptomyces coelicolor*.

The wavelength ranges corresponding to signals from phenylalanine, tyrosine and tryptophan side chains are indicated, but it should be emphasised that there can be considerable overlap (Fig 10) between the tyrosine and tryptophan signals[6, 7].

Instrumentation

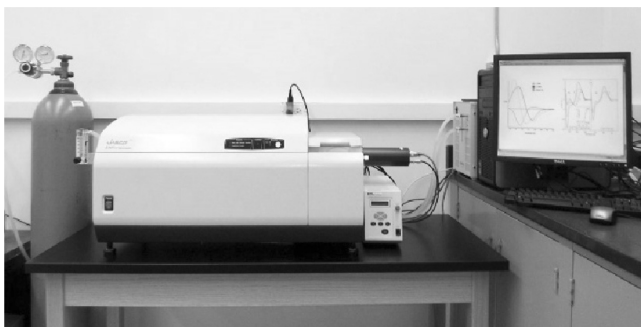


Fig. 11: The CD spectrophotometer

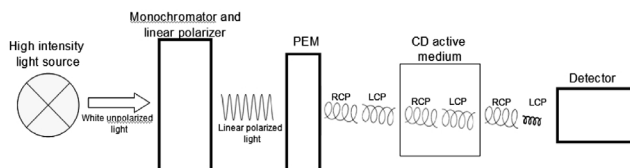


Fig. 12: Illustration of a CD spectrophotometer

Circular dichroism can be measured using a spectrophotometer (Fig 11& 12) which works by Modulation method. It is commonly employed for CD where plane polarized light is passed through a *modulator* to which alternating electric field is applied, as a result of this the plane polarized light is split into right and left components. The modulator consists of a thin plate of fused silica coupled with a piezoelectric quartz crystal. The transmitted radiation from the modulator is detected by the photomultiplier[6,8].

Dynode voltage module indicates the absorption of light (in volts, V) and measures the response of the photomultiplier during analysis, indicating the quantity of photons that are scattered or not absorbed by the sample. The limit for reliable reading is [00V but upto 700V are accepted but one should cautious to reduce the noise generated by absorption. Voltage more than 700V damage the instrument hence the limit should not be exceeded.

Experimental set up:

- If the objective is to determine the secondary structure of a protein, the spectrum must be recorded from 260-180nm or lower.
- Below 200nm oxygen, buffers & salts absorb strongly and the signal to noise ratio falls abruptly. This is offset by averaging multiple scans.
- As a consequence: the shorter the path-length of the cuvette the better the CD signal, so it is usually best to run at <100µg/ml in a cuvette of path-length 1mm.
- If spectra are required in the range above 250nm, 10mm path-length cell is used.

Practicalities

- CD is based on measuring a very small difference between two large signals, hence must be carried out carefully.
- Quartz cells path lengths between 0.1cm and 1 cm is to be used.
- For accurate secondary structure estimation one must know concentration of sample.

A homogeneous sample of protein should be used and it should be free of scattering particles. For far UV CD analysis path-length of the cell should be typically 1mm and sample concentration should be in the range of 0.1-1 mg/ml. Volume of the sample required is 50µl to 200µl and the total volume of the cell/cuvette is 400µl.

For near UV CD analysis path-length of the cell should be typically 10mm and sample concentration should be in the range of 0.1-1 mg/ml. Volume of the sample required is 0.5-1ml and the total volume of the cell/cuvette is 2ml.

Useful information about CD can be obtained at room temperature or lower than that where the conformational states of the protein can be known[9,10].

Typical Conditions for CD

- Protein Concentration: 0.25 mg/ml or less.
- Cell Path Length: 1 mm
- Volume: up to 400 µl
- Need very little sample: 0.1 mg

To reach the best signal to noise ratio, protein concentration is an important parameter to be considered. For a path length of 1mm concentration of 0.01% and for path-length of 10mm concentration of 0.1% can be approximately used.

Preparation of samples:

- Samples for CD spectroscopy must be at least 95% pure by the criteria of HPLC or gel-electrophoresis.
- For secondary structure measurements, sample concentration range from 1-100µg/ml depending on the pathlength of the cell.

Pure water(double-distilled) or 10mM phosphate buffer can be used to dilute the protein sample. Chemical reagents used to prepare buffer must be of standard quality without any impurities as they may scatter light. Hence filtering the buffers is advisable.

Initializing the CD spectrophotometer

- CD machines have very powerful lamps (xenon arc) that promote the ionization of oxygen to ozone. Ozone is toxic and also will quickly destroy the mirrors in the optics of the machine. Hence flush the chamber

with nitrogen gas. Constant flow of nitrogen needed is usually 3-25L/min for measurements in 260-180nm.

- Xenon arc lamp is used as the source of radiation which has to be switched on at least 60min to warm up before the analysis is carried out.
- D(+)-camphor sulphonic acid(10mM) is used for calibration of the instrument.
- Cells used must be of quartz and should be handled with gloves. They can be cleaned with distilled water, ethanol or acetone. To remove any proteins sticking to the cells Chromic acid or 50% nitric acid can be used followed by plenty of pure distilled water.

Various settings can be adjusted in the instrument to improve the results of analysis[11,12, 13, 14].

- Bandwidth
- Time constant/Scan rate
- Number of scans
- Sensitivity

To obtain reliable CD data, regular maintenance and calibration with suitable chiral standard (1S-(+)-10-camphorsulphonic acid) is utmost essential. In order to avoid distortion of the spectrum the product of the time constant and the scan speed should be less than 0.5 nm which is to be considered as a basic need.

Bandwidth:It is the measure of the precision by which a required wavelength of light is selected by the monochromator. More light will fall on the sample when band width is increased but will leads to decrease in the ability to resolve spectral bands. For CD studies the bandwidth should be equal to or less than 1 nm even lower to 0.1 nm are used in the near UV spectrum particularly to resolve fine structures of proteins.

Time constant/scan rate:It is a measure of the time over which the CD data are averaged and will depend on the precise mode of operation of the instrument.

Number of scans:Signal to noise ratio is important to obtain a good resolved CD signal. In order to improve the signal/noise (S/N) ratio, number of scans should be increased. S/N ratio is directly proportional to the number of scans.

Sensitivity: Different sensitivities- low, standard and high sensitivity can be adjusted to achieve smoothness of the CD spectra.

The stability of the sample and the instrument under the conditions employed and the balance between scan speed, time constant, sensitivity and number of scans is essential to improve the quality of the data.

CD studies at different temperatures:

Study of proteins at different temperature is very essential to know their stability and the folding and unfolding of the secondary structure in order to analyse samples in question.Hence Peltier Type Cell device (PTC) is introduced along with CD which controls the temperature inside the cell with the help of an external circulating bath which maintains varied temperature in the cell. The cell temperature can be maintained from -10⁰C to +110⁰C allowing a wide range for temperature stability study.

Advantages

- CD technique is non-destructive, requires little sample and one can use cells of different path length in the same instrument for the study of a wide range of samples.
- It is possible to recover the sample under analysis and conduct further experiments on the same sample.
- CD studies can be carried out in various experimental conditions including temperature,pH,agitation, oxidation and shelf life.
- CD is used to study the folding and unfolding of proteins, in protein engineering field to assess structural characteristics of mutant proteins and conformational changes.
- A better spectrum can be obtained for a given sample in both far and near UV CD measurement with ease and rapidity (20-30 min).
- CD is used for the analysis of biopharmaceuticals for their correctly folded active conformation.

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