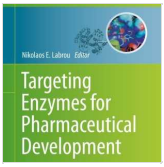

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Enzyme–Ligand Interaction Monitored by Synchrotron Radiation Circular Dichroism

Book Chapter

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ABSTRACT

CD spectroscopy is the essential tool to quickly ascertain in the far-UV region the global conformational changes, the secondary structure content, and protein folding and in the near-UV region the local tertiary structure changes probed by the local environment of the aromatic side chains, prosthetic groups (hemes, flavones, carotenoids), the dihedral angle of disulfide bonds, and the ligand chromophore moieties, the latter occurring as a result of protein–ligand binding interaction. Qualitative and quantitative investigations into ligand-binding interactions in both the far- and near-UV regions using CD spectroscopy provide unique and direct information whether induced conformational changes upon ligand binding occur and of what nature that are unattainable with other techniques such as fluorescence, ITC, SPR, and AUC.

This chapter provides an overview of how to perform circular dichroism (CD) experiments, detailing methods, hints and tips for successful CD measurements. Descriptions of different experimental designs are discussed using CD to investigate ligand-binding interactions. This includes standard qualitative CD measurements conducted in both single-measurement mode and high-throughput 96-well plate mode, CD titrations, and UV protein denaturation assays with and without ligand.

The highly collimated micro-beam available at B23 beamline for synchrotron radiation circular dichroism (SRCD) at Diamond Light Source (DLS) offers many advantages to benchtop instruments. The synchrotron light source is ten times brighter than a standard xenon arc light source of benchtop instruments. The small diameter of the synchrotron beam can be up to 160 times smaller than that of benchtop light beams; this has enabled the use of small aperture cuvette cells and flat capillary tubes reducing substantially the amount of volume sample to be investigated. Methods, hints and tips, and golden rules tomeasure good quality, artifact-free SRCD and CD data will be described in this chapter in particular for the study of protein–ligand interactions and protein photostability.

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GUIDELINES

1 Introduction

Circular dichroism (CD) is a powerful technique which enables the monitoring of local and global changes in the structure and conformation of proteins. CD spectroscopy enables the selective monitoring of specific chromophores of the protein including peptide backbone in the far-UV region (180–250 nm), aromatic side chains of amino acid residues and dihedral angles of disulfide bonds in the near-UV region (250–350 nm), and prosthetic groups (hemes, flavones, carotenoids) in the visible region (400–800 nm). Bespoke benchtop CD instruments commonly use a xenon light source. Synchrotron radiation circular dichroism (SRCD) beamlines utilize the light produced at synchrotrons as the light source that is brighter than standard xenon light sources [1], and with much higher photon flux in the vacuum-UV region (130–200 nm). The highly collimated microbeam generated with the SRCD beamline B23 at Diamond Light Source (DLS) had enabled to reduce the amount of sample required for standard CD measurements by using lower volume cuvette cells with 1–2 mm diameter aperture [1, 2] and the high-throughput CD (HTCD) using 96 plates [3]. The higher photon flux of B23, however, can affect the photostability of the investigated protein by inducing structural denaturation [1]. Although this effect can be minimized or eliminated by reducing the slit width of the double-grating monochromator of B23 or rotating the cuvette cells around the axis parallel to the incident beamlight [4, 5], the UV protein denaturation has been used as an assay to determine the photostability and also qualitatively to determine ligand-binding interactions for UV-transparent ligands like fatty acids and carbohydrates [4, 5]. The rate of UV denaturation is significantly affected, usually decreased, if the ligand is interacting with the protein, otherwise, the rate with and without ligand is unaffected [4, 5]. In addition to qualitative investigations into ligand-binding interactions, recent advances saw the development of small-volume titration techniques that are most important when investigating the ligand-binding interactions of limited materials and has been well demonstrated with membrane proteins [6–15].

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A large number of SRCD spectra can be collected during a single experiment, particularly when using the HTCD with 96 plates or UV denaturation assays at B23, and for Users who are allocated a specific number of 8-h shifts, it is important to be able to analyze on-the-fly to ensure that the experimental conditions are suitable for data collection. For this reason, software has been developed at B23 which allows for the analysis of CD data in .ols, .csv, and .txt format [16], making it compatible with data collected on other CD instruments off-site.

CD instruments measure the difference in absorbance of left- and right-circularly polarized light of chiral molecules as a function of wavelength expressed in nanometers (nm) otherwise known as CD spectroscopy [17]. Absorbance is therefore an important experimental parameter which must be considered when planning an experiment, and such considerations are made by application of the Beer-Lambert law $A = \varepsilon.c.l$, where A is absorbance, ε is specific molar extinction coefficient which is specific for the chromophore studied, c is concentration, and l is pathlength. In most cases an optimum A (also known as optical density OD) of 0.8 is recommended, with a maximum of 1.5. For proteins, the amide bond is the main chromophore that absorbs in the far-UV region (180–250 nm) [18], while the aromatic side chains of tryptophan, tyrosine, and phenylalanine amino acid residues and disulfide bonds are the chromophores that absorbs in the near-UV region. Prosthetic groups such as hemes, NAD and FAD cofactors, and carotenoid pigments are the chromophores that extend the absorption of light in the visible region (400–800 nm). The folding of proteins is promptly determined by SRCD/CD spectroscopy in the far-UV region for which the content of secondary structure can be estimated using a variety of algorithms [18–21] that are also available is several suite of programs [16, 22–24]. The CD/SRCD in the near-UV region is sensitive to the local environment of the aromatic side chains of Trp, Tyr, and Phe, and dihedral angle of disulfide bonds of cystine residues and has been used successfully to probe qualitatively and quantitatively ligand-binding interactions [25].

2 Materials

2.1 Fused Silica Cuvettes

Common cuvettes employed for measurements of SRCD/CD spectra in the far-UV region are cylindrical or rectangular cells with a 0.02 cm pathlength made of fused silica. Low-volume titrations are conducted with a cell with a small-diameter channel which leads down to a small-volume reservoir. The reservoir window is made of black fused silica to mask accordingly the area of the incident light passing through the sample.
For measurements conducted in the near-UV region using larger pathlengths, e.g., 1 cm, these cells have black walls and small aperture (2 mm × 2 mm) windows. This restricted surface for light entry permits the use of small sample volumes (for a 1 cm pathlength, **70 µl** of solution is required for these specialized cuvettes compared to a standard cuvette requiring a sample volume > **1 mL**).

2.2 Buffer Systems Specificity

2.2.1 Buffering System of Choice

For measurements in the far-UV region, sulfonate- (HEPES, MES, MOPS, PIPES) [17] and carboxylate-rich (glycine, acetate, citrate) [26] buffering systems are not recommended as these buffers tend to absorb at 200 nm due to the n→π∗ electronic transitions of the S=O and C=O bond, respectively, and alternative buffers should be used instead. Regularly, phosphate buffer (concentrations of < **[M]25 Milimolar (mM)**) is suggested to be the most appropriate buffer system for CD measurements, and low concentrations of Tris or Tris-acetate-EDTA (TAE) (< **[M]25 Milimolar (mM)**) are also acceptable, provided that pH adjustment is not done with HCl as this would introduce or increase the existing content of chloride anions (Cl^-), which absorb in the far-UV region. pH adjustment of buffers should be achieved using more UV-transparent phosphoric acid, nitric acid, acetic acid, citric acid, and borate [17].

2.2.2 Salts

As discussed previously, the presence of high concentrations of Cl^- is undesirable for studies in the far-UV region below 200 nm. If monovalent salts are required for maintenance of the ionic stabilization in the system to be studied, UV-transparent fluoride anions (F^-) should be substituted for Cl^- [17, 25]. However, it is important to ascertain that the fluoride anions is not affecting the protein folding compared to that with chloride anions, which can be conducted by comparing the CD spectra in the overlapping transparent region that usually can be achieve

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down to 210–205 nm. Sulfate salts can also be used as an alternative [17, 25]. For a 0.02 cm and longer pathlengths, less salt concentration is better for CD measurements; otherwise the 200 nm or higher wavelength cutoff will prevent accurate secondary structure estimations. For pathlengths of 0.02 cm and longer, no more than [M]**50 Milimolar (mM) NaCl** should be used, which could be increased to [M]**100 Milimolar (mM)** – [M]**150 Milimolar (mM) NaCl** with 0.01 cm. Higher concentrations of NaCl that need to be used (e.g., > [M]**500 Milimolar (mM)**) will require even narrower cell pathlengths that can be achieved only with demountable cells of 0.0050 or 0.0020 cm as it will be otherwise impossible to clean standard cuvette cells of these pathlengths. Of course, it will require higher protein concentration following the Beer's law. For example, as a rule of thumb, an average protein concentration of [M]**0.4 mg/mL** (for proteins highly helical the concentration could be as high as [M]**0.5 mg/mL** , while for disordered or highly beta-sheet proteins, the concentration would be [M]**0.3 mg/mL** as the UV absorption is higher) will enable a good signal-to-noise quality CD spectrum using a cuvette cell of 0.02 cm pathlength. Therefore, halving the pathlength to 0.01 cm will require double the protein concentration to obtain the same CD spectrum. With B23, the smallest pathlength available for standard measurements is 5 µm which requires a protein sample volume of **2 µl** at a concentration of [M]**16 mg/mL** .

2.2.3 Buffer Additives

Addition of buffer additives should also be carefully assessed based on their UV absorption properties. For protein samples which have been purified using high concentrations of imidazole, such as from affinity purification columns, buffer exchange of the protein to remove all imidazole is required as it absorbs too strongly in the ~210 nm region due to its large extinction coefficient [17]. The addition of histidine in the protein formulation should be therefore avoided for the same reason. Similarly, urea and guanidine denaturant agents should be avoided unless the protein denaturation is the aim of the experiment. High concentrations of glycerol (< [M]**10 % (v/v)**) should also be avoided to enable the CD to be measured below 200 nm [22]. The four carboxylate groups of ethylenediaminetetraacetic acid (EDTA) absorb strongly below 200 nm and therefore should not be used in concentrations above [M]**1 Milimolar (mM)** for measurements in the far-UV region using a 0.02 cm pathlength [17]. For higher EDTA concentration, a smaller pathlength should be used but a compromise has to be reached as the protein concentration should be increased as well that will decrease the EDTA-protein molar ratio.
If a reducing agent is required, beta-mercaptoethanol (BME) is preferred to dithiothreitol (DTT). DTT strongly absorbs across the UV region—DTT_{red} (the active for which maintains the reduced state of proteins) below 260 nm across the far-UV region and DTT_{ox} (the result of the eventual oxidization of DTT over time from the environment) below 340 nm and across the near-UV region [27, 28]. Tris (2-carboxymethyl) phosphine-HCl (TCEP-HCl) is also compatible with CD but is highly acidic; therefore, the suitability of a potentially acidic environment for the protein should be considered (no more than [M]**1 Milimolar (mM)**) [29, 30]. DMSO should also be avoided for measurements in the far-UV region as it absorbs strongly in the UV region (λ_{max} 220 nm, cutoff is <260 nm) and therefore is unsuitable for far-UV measurements [31, 32]. If an organic solvent is required, most other types, e.g., hexane and trifluoroethanol, are transparent down to 185 nm; however, it is important to note that these solvents may induce structural changes to the protein being studied [22].

2.3 Protein Samples

Protein samples should be soluble, homogenous, and pure; therefore, purification techniques including size-exclusion chromatography (SEC) should be used in addition to standard immobilized metal affinity chromatography (IMAC) to ensure highly pure samples which can be checked using gel electrophoresis and mass spectroscopy [22]. Solid particles can lead to light scattering and distortion of measured CD spectra [17]; therefore, effort should be made to remove them from samples before data collection. Centrifugation of the sample and only using the supernatant for measurements or filtering samples through a 0.2 µm filter before loading into the cuvettes for data collection can be adopted to ensure samples are solid free [17]. Protein concentration determined spectroscopically absorbance should follow the Beer-Lambert law measuring either the absorbance at 280 nm for proteins containing aromatic amino acids or at 205 nm for nonaromatic amino acid containing peptides/proteins [18]. This should be performed with the final protein product (after purification and removal of solid particles) prior to any CD measurement.

2.4 CDApps Software

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CDApps software has been developed to analyze SRCD and CD data (.ols, .csv, .txt format). Developed at Diamond B23 beamline, the software is available to use on-site for analysis, and can be copied on a USB stick along with video tutorial files for analysis and training off-site. Information regarding beamline software including manuals can be found on the beamlines software page http://www.diamond.ac.uk/Instruments/Soft-Condensed-Matter/B23/manual/Beamline-software.html.

The software has progressed from its original conception as detailed in [16] which focused on the planning and data processing of standard, thermal melt, UV denaturation and titration experiments to have a more intuitive interface as well as the processing capability for high-throughput CD (HTCD) data sets from a 96-well plate [3] and CD imaging applications [33].

3 Methods

Circular dichroism experiments conducted at B23, Diamond Light Source, can all be planned and processed using CDApps [16].
For all experiments, background measurements (background buffer equivalents) should be collected which can be subtracted from all experimental data files. The most recent upgrades for the data collection software include the option to collect UV absorption data which can be used as an internal control for each sample measured.
Optimum absorbance readings are ~0.8, for titrations the range recommended is from 0.4 to a maximum of 1.5–1.6 [34]. Within these limits, the chromophore concentration will be sufficient for detection and without being too high to saturate the detector distorting the spectral profile. A recommendation before starting a series of measurements is to know the absorbance in terms of intensity magnitude and wavelength maxima for all components of the solutions such as solvent (buffer), solute (protein/peptide), additives, ligands, and any other chemical agent present therein (*see* Subheading 2.2.1 and **Notes 1–4**). CDApps can help to calculate the absorption of mixtures from the information input into the software (pathlength to be used, extinction coefficient at specific wavelengths, and molecular weights of materials) for the individual components during planning of the experiments [16].
Before commencing measurements, an air baseline should be recorded for the instrument once the experimental parameters (wavelength range, wavelength increment, integration time) have been set up. This will allow for the data collection software to calculate the absorbance simultaneously during CD signal collection.

4 Notes

1. Conduct measurements in the “most UV-transparent” buffer possible, achieved using buffering systems at their lowest working concentration for the system of interest. Phosphate buffer is the most appropriate buffer system of choice.
2. Minimize or eradicate the concentration of chloride anions present. Substitute with fluoride salts where possible, and adjust the pH of buffers using non-chloride acids including nitric and phosphoric acids. This is especially important when measuring low-concentration protein solutions (<1 mg/mL) in the far-UV region.
3. Use only very pure products for measurements (>95% pure), and chemicals which are at least analytical grade purity.
4. Ensure no bubbles are present in the solutions before measurements. Degas buffers beforehand, and when cuvettes are filled, ensure no bubbles are present by tapping gently.
5. Pathlengths of cuvettes used for measurements can be changed depending on the concentration of the protein solution being measured in accordance to the Beer-Lambert law [35].
6. For samples with weak signals, it is recommended to check the absorbance of the sample. If it is within the ideal limits, then an alternative is to increase the number of scans collected. The underlying reason for increasing the number of scans is because $signal \simeq \frac{1}{n}$. Therefore, the average spectrum from more scans (4, 9, or 16) or increasing the integration time will improve the signal:noise ratio.
7. When cleaning cuvettes, especially when using protein solutions and after thermal melt or long-term UV denaturation experiments, be sure to use concentrated nitric acid to clean thoroughly wet cells from wash of 96% ethanol. Also thoroughly rinse and dry the cells before reuse to remove residual acid which can change the pH affecting the protein folding.
8. Remove all debris and solid particulates by centrifugation beforehand and using only the supernatant, or

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filtering the solution through a 0.2 µm filter.

9. Be sure that the HV of the PMT detector does not exceed the recommended voltage from the CD instrument manufacturer (for B23 is about 600 V, which is common with other benchtop instruments using the PMT detector) as this is a sign of oversaturation of the detector. If this occurs, remove the portion of spectra for which this occurs before analysis or decrease the sample concentration, or reduce the cell pathlength accordingly.
10. Observe the absorbance and discount measurements which are above 1.5. Reduce the concentration to reduce the absorbance, or alternatively decrease the cell pathlength. Also consider the absorbance contribution from the ligand as this should be factored in for ligand-binding studies, especially titration experiments.
11. In cases where the ligand absorbs at 280 nm, a lower starting protein concentration can be used (0.4 mg/mL) to allow for the absorbance contribution of the ligand throughout the titration.
12. When conducting titration experiments in increasing volume, ensure the total added ligand volume does not exceed 20% of the original volume (CDApps will warn when a 15% dilution has been reached). This ensures any changes observed are solely as a result of ligand binding rather than any potential “concentration effects” influencing the proteins conformation or local tertiary structure. To aid this, use ligand stocks at the highest possible concentration (usually in the region of 10–20 times the final concentration of ligand required for the titration).
13. When conducting ligand-binding studies ensure thorough and gentle mixing in the solution before measurement, avoiding the formation of bubbles (see Note 4). Check for a homogenous appearance. Look for signs of insufficient mixing, e.g., streaking of solutions, and rectify before measurement. This is incredibly important when conducting titration experiment, and care should be taken to thoroughly mix after the addition of each aliquot.
14. Determine the incubation time required for the system to stabilize after the addition of ligand by determining the time required for consecutively measured spectra to overlap and no longer change. The determined time can be used as standard for subsequent measurements.
15. Care should be taken to monitor for precipitation during the titration, a sign of unsuitable conditions for the protein (e.g., though changes to the pH of the solution upon addition of the ligand) and the possibility of reduced protein concentration which should be accounted for during the analysis for accuracy especially for measurements in the far-UV region and SSE. Solid particulates should be removed to prevent light scattering.
16. The extent of UV denaturation is specific to a protein. Use experience of the proteins behavior and stability to judge the number of scans to collect.
17. When using CDApps for analyses of data sets more complicated than standard 1 to *n* measurements (UV denaturation, Thermal Melt, 96 HT format), only one dataset can be analyzed at a time.
18. When analyzing data for the 96-HT format, be sure to complete the experimental details and parameters for all of the scans in the file.
19. Ensure the number of scans in the workbook match the number of scans in the uploaded data files.
20. After selection of the HTCD analysis options, experimental parameters (e.g., concentration and units, Δε, molecular weight, and pathlength) will have to be re-entered. A dialog box will ask if you want the value to be applied to all of the scans in the file, click OK if this is the case, otherwise they can be manually inserted for each individual scan under the “Experiment details” section in CDApps.

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SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

ABSTRACT

CD spectroscopy is the essential tool to quickly ascertain in the far-UV region the global conformational changes, the secondary structure content, and protein folding and in the near-UV region the local tertiary structure changes probed by the local environment of the aromatic side chains, prosthetic groups (hemes, flavones, carotenoids), the dihedral angle of disulfide bonds, and the ligand chromophore moieties, the latter occurring as a result of protein–ligand binding interaction. Qualitative and quantitative investigations into ligand-binding interactions in both the far- and near-UV regions using CD spectroscopy provide unique and direct information whether induced conformational changes upon ligand binding occur and of what nature that are unattainable with other techniques such as fluorescence, ITC, SPR, and AUC.

This chapter provides an overview of how to perform circular dichroism (CD) experiments, detailing methods, hints and tips for successful CD measurements. Descriptions of different experimental designs are discussed using CD to investigate ligand-binding interactions. This includes standard qualitative CD measurements conducted in both single-measurement mode and high-throughput 96-well plate mode, CD titrations, and UV protein denaturation assays with and without ligand.

The highly collimated micro-beam available at B23 beamline for synchrotron radiation circular dichroism (SRCD) at

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Diamond Light Source (DLS) offers many advantages to benchtop instruments. The synchrotron light source is ten times brighter than a standard xenon arc light source of benchtop instruments. The small diameter of the synchrotron beam can be up to 160 times smaller than that of benchtop light beams; this has enabled the use of small aperture cuvette cells and flat capillary tubes reducing substantially the amount of volume sample to be investigated. Methods, hints and tips, and golden rules tomeasure good quality, artifact-free SRCD and CD data will be described in this chapter in particular for the study of protein–ligand interactions and protein photostability.

ATTACHMENTS

Hussain2020_Protocol_EnzymeLigandInteractionMonitor.pdf

FILES

Standard CD Measurements
Version 1
by satyavati Kharde

UV Denaturation of Proteins Monitored by Circular Dichroism in the Far-UV Region (180–260 nm)
Version 1
by satyavati Kharde

Low-Volume Titrations for Ligand Binding Monitored by Circular Dichroism
Version 1
by satyavati Kharde

Processing of Circular Dichroism Data Collected at B23
Version 1
by satyavati Kharde

Citation: Rohanah Hussain, Charlotte S. Hughes, Giuliano Siligardi (09/02/2021). EnzymeÃçÂÂLigand Interaction Monitored by Synchrotron Radiation Circular Dichroism. https://dx.doi.org/10.17504/protocols.io.bnxfmfjn