

An Introduction to Circular Dichroism

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Useful references:

Basic References:

- Spectrophotometry and Spectrofluorimetry, Gore, M. (ed.), 2000. Oxford University Press, Oxford, UK. Chapter 4: Introduction to Circular Dichroism by Rogers and Ismail. Pg 99-139.
- Johnson, W. C. Proteins (1990) **7**, 205

Secondary Structure Calculations:

- Greenfield and Fasman. Biochemistry (1969) **8**, 4108
- Johnson, W. C. Proteins (1999) **35**, 307

Melting Curves:

- Yadav and Ahmad. (2000) Analytical Biochemistry **283**, 207
- Gehrmann, Douglas, Bányai, Tordai, Patthy and Llinás. JBC (2004) 279 46921

Protein Concentration:

- Pace, Vajdos, Fee, Grimsley and Gray. Protein Science (1995) **4** 2411

Basics of CD

Circular Dichroism (CD) is defined as the difference in absorption of left and right circularly polarized light.

$$CD(\lambda) = A_{\ell}(\lambda) - A_{r}(\lambda)$$

In principle, CD can be measured for any frequency of electromagnetic radiation. In practice most CD involves UV or visible light. This guide concerns recording far-UV (260nm – 180nm) CD spectra of proteins, suitable for estimating the secondary structure.

The far-UV CD spectrum detects the chirality of the amide chromophore. This chromophore typically has an extinction coefficient of $\sim 10^5$. Typical CD signals will be on the order of $\sim 10^1$, five orders of magnitude smaller. Hence it is important to keep in mind that you are measuring a tiny difference between two large numbers (the absorbance of left and right circularly polarized light vs. wavelength). It should not be surprising that a CD spectrum takes much longer to record than a simple UV. Allow yourself ~ 2 hours to record a spectrum of a protein.

Recording a spectrum

What do I need?

Obviously you will need a protein sample. This sample needs to be in the correct concentration range. Since you are making an absorbance measurement, ultimately there is a detector counting photons. If the absorbance of the sample + buffer + cuvette is too high, not enough light will reach the detector and hence a meaningful spectrum will not be recorded. This is referred to as “saturation”. Likewise, if the absorbance of the sample + buffer + cuvette is too low, then the differential absorbance of left and right circularly polarized light will be below the detection threshold.

Fortunately JASCO makes it easy to gauge saturation. It is possible to observe high tension (HT) voltage. HT voltage is roughly proportional to absorbance. If the HT voltage goes above ~600 then the detector is saturated. The amplitude of the spectrum will oscillate wildly. If you think the protein concentration is too high, you should dilute and try again. Keep in mind that the cuvette, buffers and salt all have an absorbance. Good's buffers are often bad below 200nm (TES is an exception). Cl⁻ ions are bad below 200nm. Also you can reduce the path length to reduce the optical density of the sample + buffer + cuvette (see below). In my case (a protein domain of ~65-80 amino acids) a concentration of ~10 μ M with water as the solvent worked well.

You also need a quartz cuvette. For my measurements I used a Starna 1mm quartz cuvette with the 9mm spacer. This minimizes the sample used (<200 μ L). Moreover the absorbance of the sample was low enough to record down to 182 nm routinely.

Next you need N_2 gas. CD machines require a high energy lamp to generate UV frequencies. These lamps convert ambient oxygen to ozone which corrodes the optics of the instrument. To avoid this it is **imperative** that all users purge with N_2 . There should be a chart by the CD in the CMA telling people what flow rate to use. As a matter of routine, I would purge the instrument for 15 minutes before turning on the lamp, during the entire measurement and 15 minutes after turning off the lamp. Since it damages the instrument to run without N_2 purge, it is critical to make sure that there is enough N_2 for the entire measurement before starting. If memory serves me correctly one tank provides \sim 8 hours of N_2 .

Finally (this is counterintuitive) you will need a blank. The blank takes into account that the machine baseline is NOT flat. The cuvette may even have its own CD spectrum. And there is a slight drift in the baseline from month-to-month or week-to-week. Hence I would always record a spectrum of my buffer (pure water) in the same cuvette, with the same

acquisition parameters as my protein sample. I would then subtract the buffer spectrum from the protein spectrum to account for the baselines.

Parameters to set

There are only a few parameters, which I will go over briefly. Most are self-explanatory.

- Wavelength range – for far-UV I'd try 260nm to 182nm. If you cannot measure down to 182nm you can truncate your data in the JASCO program or export the data as a text file and truncate in EXCEL.
- Scan speed – I would normally scan at ~10 nm/min.
- Number of scans – I would normally accumulate 10 scans.
- Integration time – integration time scales with signal to noise. It is advisable to maximize the integration time. Keep in mind though that the upper limit is determined by the scan speed and bandwidth. If I remember correctly the software will complain if the scan speed*integration > 200. I think I would normally use 16s. You can check my data to confirm this. My data should be in the Users directory under Llinas/Justin. Spectra recorded during 2000 and 2001 would be a good place to start.

- Bandwidth – I normally wouldn't play with this. Check my files and use what I used.
- Data Interval – determines how often data is collected. 1nm should suffice.
- Sensitivity – if memory serves me there is a parameter called “sensitivity.” In my opinion this is a badly misnamed parameter. It determines the range to be displayed in the output window during acquisition. This does not effect the measurement in any way.

CD units

For some reason the JASCO software displays the spectrum in units of ellipticity $[\theta]$ vs. wavelength. Ellipticity is the ratio of the minor to major axes of the ellipse traced out by the electric field vector that emerges when linearly polarized light is incident on the sample. Fortunately it is easy to convert this to Molar Absorbance units.

$$[\theta] = 32980 \cdot C \cdot l \cdot (\epsilon_{\ell} - \epsilon_r)$$

Where C is the concentration in Molar, l is the pathlength in cm, ϵ_{ℓ} is the molar extinction coefficient for left circularly polarized light and ϵ_r is the molar extinction coefficient for right circularly polarized light. See the JASCO manual for details on this calculation.

Note well, because this is important. The concentration is in “of residue” units. This is very critical if you plan on calculating the secondary structure. What I mean is that if the concentration is 10 μ M of a 60 residue protein, then the “of residue” concentration is 600 μ M (60 * 10). See the JASCO manual for details.

Calculations of Secondary Structures Percentages

The major assumption used to calculate the secondary structure percentage is that the CD spectrum is a linear combination of the CD spectrum of “pure α -helix”, “pure β -sheet”, etc. The weighting factors of this linear combination are equal to the fraction of that secondary structure element. The program I am most familiar with is CDSSTR from the lab of Curtis Johnson at Oregon State. CDSSTR calculates a basis set of secondary structure elements from a set of spectra from well-characterized proteins. This gives more consistent and stable results than using a “typical” spectrum for each secondary structure motif. Practical aspects of this program are well discussed in the chapter by Rogers and Ismail. The one comment I would add is that the calculation is quite sensitive to errors in concentration. The paper by Pace, *et al.* gives a good method to estimate the concentration.

I would routinely measure a UV spectrum for every protein sample that I would measure a CD spectrum. This is also helpful to see saturation.

Melting Curves

Melting curve set up is similar to setting up a spectrum. The major difference is that you are scanning over a range of temperatures, rather than wavelength. Usually you observe the signal at 222nm. Since you are observing at a wavelength $> 200\text{nm}$ you are not constrained to use a 1mm cuvette. You could increase the signal-to-noise ratio by using a 1cm cuvette, although you use more sample. Also the absorbance of salts and buffers are less of an issue at 222nm. I would recommend keeping an eye on the HT voltage to insure that the detector is not saturated. Also you should keep in mind that there is a slight lag between the temperature of the cell and the temperature of the protein in the cuvette. If you scan temperature too rapidly you will see transient effects. I believe I scanned at about 1 degree Celsius per minute. You can check the agreement between the temperature in the software and the temperature of the cell displayed on the temperature control unit.

Analysis of melting curves range from simple to very complex. The JASCO software will take a 1st derivative to help you locate the inflection

point which is approximately equal to the melting temperature of the protein. Detailed van't Hoff analysis can be used to extract the enthalpy and entropy of melting. See the papers by Yadav and Ahmad, and Gehrmann, Douglas *et al.* for details.