



Abstract: The study of protein stability *in vitro* is of enormous importance for a variety of reasons. In the development of protein-based products such as biotherapeutics, knowledge of the stability is essential in avoiding unwanted consequences such as aggregation or immunotoxicity.

Circular Dichroism spectroscopy (CD) is one of the most widely used methods of establishing stability because of the wealth of information that it produces, a consequence of its versatility and ease of use. This article will describe why this is the case and provide a flavour of what CD can offer as a technique in the field of protein stability and why CD should be a go-to technique when addressing questions in this area.

CHIRASCAN SERIES APPLICATION NOTE

Protein Stability Measurements using Quantitative Circular Dichroism (*q*CD) Spectroscopy

INTRODUCTION

CD spectroscopy is a widely used tool for evaluating stability. Protein stability is generally assessed by subjecting a protein sample to an external stress and observing how resistant the protein's higher order structure is to that stress. The most frequently used stress in such measurements is temperature.

THERMAL DENATURATION

As the temperature of a protein sample is raised, a point will be reached where the three dimensional protein structure will begin to unfold and with further heating will undergo a transition to an unfolded state. The midpoint of this transition is referred to as the melting temperature (T_m) and is a frequently used indicator of protein stability. More complex proteins with multiple domains and subunits can have several transitions, which are often distinguishable.

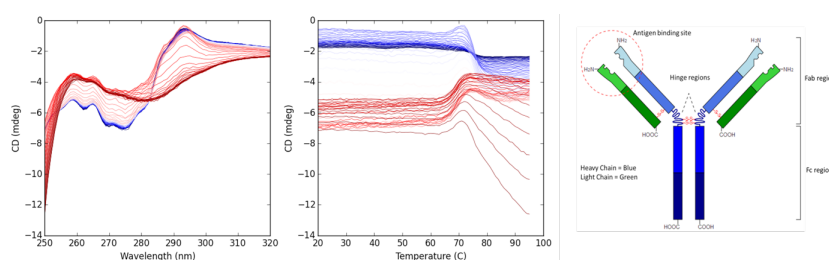


Figure 1. Raw thermal denaturation circular dichroism data of a monoclonal antibody, such as those used as biotherapeutics, recorded in the near-UV range of the spectrum. The data can be analysed globally using Applied Photophysics' proprietary Global 3 software to give melting temperatures and enthalpy changes.

Author

James Law, PhD

KEYWORDS

- | | |
|------------------------|-------------------------|
| ▶ Stability | ▶ Chemical Denaturation |
| ▶ Circular Dichroism | ▶ Thermodynamics |
| ▶ Thermal Denaturation | ▶ Fluorescence |

Measurement of a T_m is usually carried out either through spectroscopic techniques such as circular dichroism and fluorescence, or through the use of differential scanning calorimetry (DSC). Along with a T_m , fitting of the data can yield estimates for the enthalpy changes accompanying the transitions.

In a CD thermal denaturation experiment, the measured signal arises directly from the secondary and tertiary structures of the protein. Different structural elements give rise to particular CD features and it is the disruption of these higher order structures that is observed during the thermal ramp. CD therefore provides structural information in addition to the thermodynamic parameters, meaning there is confidence that the observed signal changes correspond directly to changes in higher-order structure. This level of structural detail cannot be accessed by most other spectroscopic and calorimetric techniques.

Benefits of a thermal denaturation experiment by Circular Dichroism

Fast: a typical T-ramp takes less than 60 minutes to obtain full spectral data across a wide range of temperatures.

Accurate: an in-sample thermocouple measures sample temperature at each data point. T_m s acquired on a Chirascan show excellent reproducibility.

Simple: there is no need to prepare a reference sample to exactly match the protein's buffer conditions as is required for DSC. Only the protein is required. The heating is software controlled from the instrument's user interface.

Economical: only a small amount of sample required, typically 50 μ g in the far-UV.

Robust: global analysis uses all the data to extract T_m s and van't Hoff enthalpies ($\Delta H_{\text{van't Hoff}}$).

Orthogonal: CD, absorbance, fluorescence and light scattering are measured in a single experiment.

Automation: lights-out operation possible using semi-automated (6 samples) and fully automated (many samples) variants.

Other techniques have their own benefits. For example, differential scanning calorimetry (DSC) directly measures the heat energy being absorbed by the sample during unfolding. This allows two different enthalpy changes to be calculated, a van't Hoff enthalpy and a calorimetric enthalpy. Comparing these two values can establish information about the unfolding model of the protein. This can of course be useful but note that accurate protein concentrations must be known in order to obtain meaningful results [1]. In practice, as many complimentary methods should be used as is feasible to increase confidence in results. For this reason, recent approaches to characterising protein stability have mathematically combined data from different techniques into a single figure [2].

In light of the above discussion, the scope of a thermal denaturation CD

experiment can be extended with a CCD fluorescence spectrometer accessory (exclusive to Applied Photophysics). This accessory enables the simultaneous acquisition of an entire orthogonal dataset of an alternative physical property (i.e. fluorescence) in a single experiment. For example, we can measure CD thermal profile in the far-UV which provides information relating to the secondary structure of the protein. At the same time we can measure the environmental changes occurring at the fluorescent side chains, allowing a different, albeit related, T_m to be calculated that reflects tertiary structural changes.

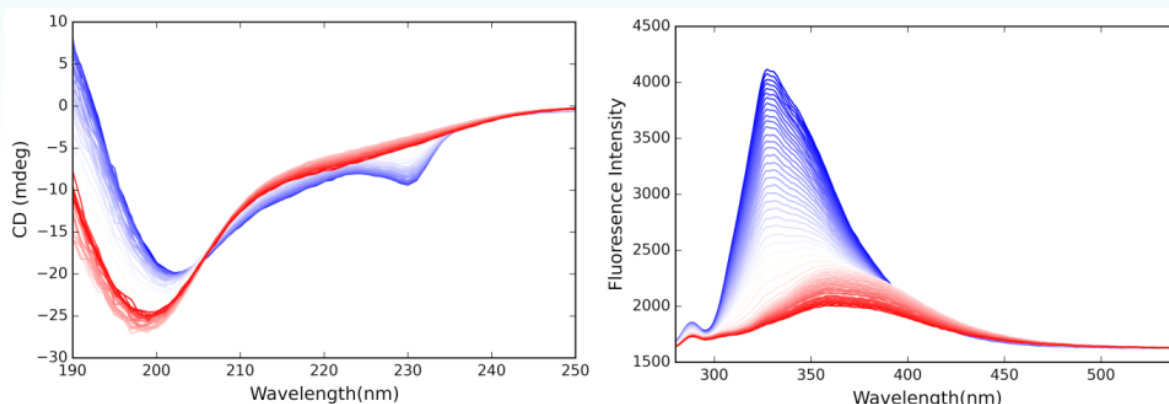


Figure 2. Left: temperature-dependent far-UV CD spectra of α -chymotrypsin. Right: temperature-dependent fluorescence emission spectra recorded simultaneously (excitation 285nm, 6 seconds per spectrum).

A parallel fluorescence dataset can therefore provide further information about the system being studied in a way that maximises sample efficiency and saves time.

CD spectroscopy is exceptionally useful in stability studies outside the realm of thermal denaturation in a way that differential scanning calorimetry is not. For example, chemical denaturation is a parallel method to thermal denaturation and again provides thermodynamic information relating to protein higher-order structure stability.

CHEMICAL DENATURATION

Chemical denaturation is particularly useful in cases where aggregation is observed during a thermal melt experiment and where thermal unfolding is irreversible. The protein is exposed to increasing concentrations of a denaturant (e.g. urea or guanidinium hydrochloride) and the stability of the protein's higher-order structure is measured as a function of denaturant concentration. There are known empirical relationships that can equate these observations to thermodynamic parameters allowing a calculation of the Gibbs free energy of unfolding [3]. Applied Photophysics provides several solutions to facilitate chemical denaturation experiments, notably a titration accessory (Figure 4) that provides a means of carefully controlling the amount of denaturant added to an optical cell in an automated fashion. Plate-based titration, using the Chirascan-auto q CD, offers a tremendous advantage over a manual system and is the subject of a separate note.[4][5]

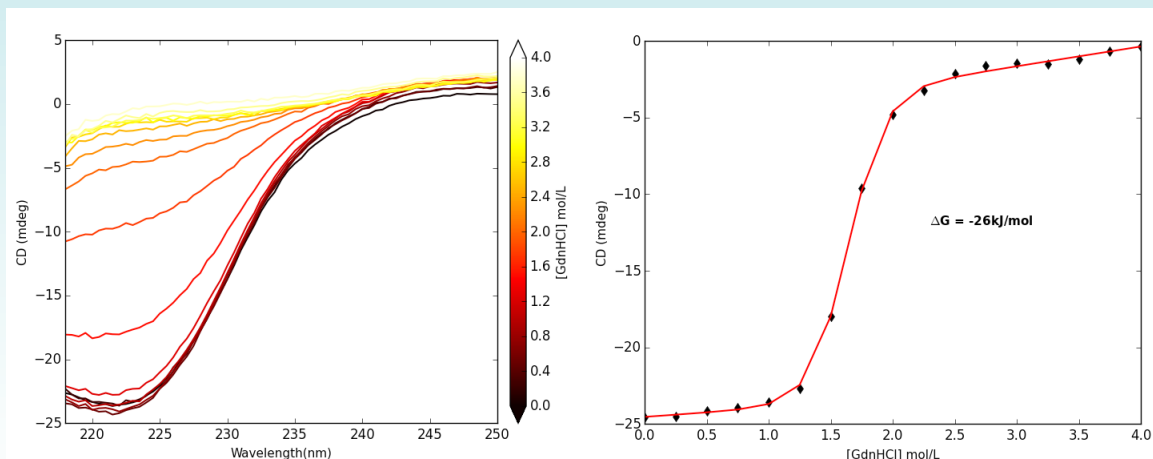


Figure 3. Left: CD spectra of cytochrome c as the concentration of guanidinium hydrochloride is increased from 0 to 4M. Right: CD at 220nm plotted against guanidinium hydrochloride concentration for the raw data (black diamonds) and data fitted to an equation to obtain ΔG of unfolding (red line).

KINETIC STABILITY

CD spectroscopy can also be used to measure how stable a protein sample is over a period of time. If a change to a sample has occurred over the course of weeks and months, we can continually assess whether the protein structure has remained intact over this period without the need to perform a forced denaturation each time. Statistical comparative methods exist for testing for small but significant changes in higher order structure from CD spectra. Applied Photophysics' proprietary *q*BiC software provides a means of quantifying change in CD spectra, establishing whether or not the change is statistically significant.

ADDITIONAL METHODS

The fact that the CD spectrum of a protein is representative of its higher order structure means that we can subject the protein sample to any number of stresses and observe if a measurable change in higher order structure has occurred. These include freeze-thawing, agitation and pH adjustment, and treatment with any external molecules (assuming light throughput through the sample remains appreciable). Again in this case we can use *q*BiC software to determine whether observed spectral changes are statistically significant or not.

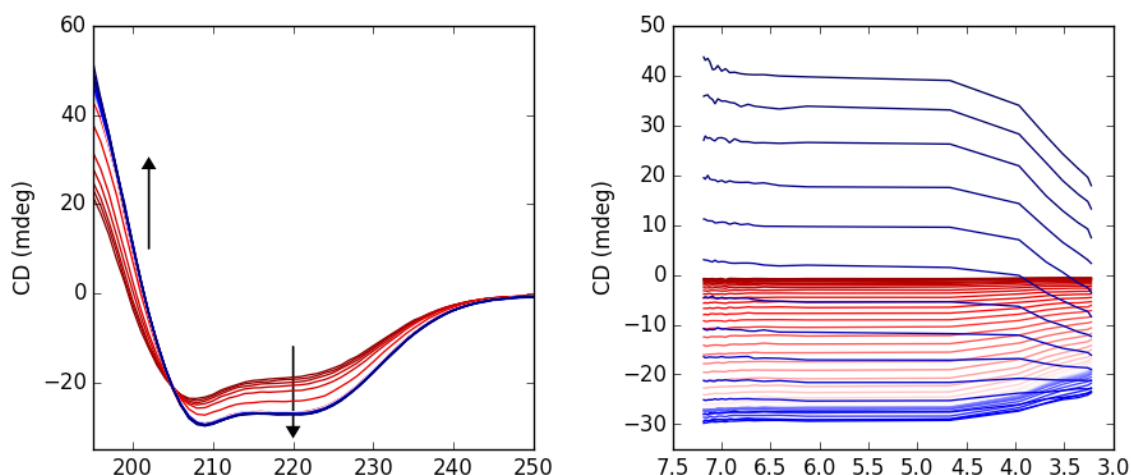


Figure 4. Left: changes to the CD spectrum of bovine serum albumin (0.2mg/mL pH7 phosphate buffer) as the sample is titrated with Na₂HPO₄. Right: change in CD at each wavelength as the pH is increased, it can be seen that significant change to the spectrum begins at approximately pH 5.



Figure 5: Titration unit and pH probe for Chirascan, both fully integrated into the control software.

REFERENCES

- [1] C. M. Johnson, "Differential scanning calorimetry as a tool for protein folding and stability," *Arch. Biochem. Biophys.*, vol. 531, no. 1–2, pp. 100–9, Mar. 2013.
- [2] M. A. Alsenaidy, N. K. Jain, J. H. Kim, C. R. Middaugh, and D. B. Volkin, "Protein comparability assessments and potential applicability of high throughput biophysical methods and data visualization tools to compare physical stability profiles," *Front. Pharmacol.*, vol. 5, p. 39, Jan. 2014.
- [3] T. O. Street, N. Courtemanche, and D. Barrick, "Protein folding and stability using denaturants," *Methods Cell Biol.*, vol. 84, no. 07, pp. 295–325, Jan. 2008.
- [4] S. Fiedler, L. Cole, and S. Keller, "Automated circular dichroism spectroscopy for medium-throughput analysis of protein conformation," *Anal. Chem.*, vol. 85, no. 3, pp. 1868–72, Feb. 2013.
- [5] L. C. Sebastian Fielder, Sandro Keller, "Protein Stability by Chemical Denaturation," *Appl. Photophysics*, pp. 1–12.

4207Q295 v1.0

Applied Photophysics Limited
 21, Mole Business Park, Leatherhead,
 Surrey, KT22 7BA, UK
 Tel: +44 1372 386 537
 Fax: +44 1372 386 477
 Email: info@photophysics.com

Applied Photophysics, Inc.
 100 Cummings Center, Suite 440-C
 Beverly, MA, 01915, USA
 Tel: +1 978 473 747
 Email: demo@photophysics.com