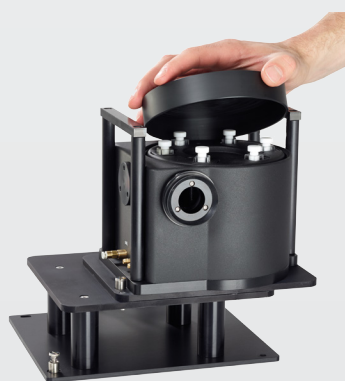




Abstract: This application note demonstrates the combined use of Circular Dichroism (CD) and intrinsic fluorescence to map the stability of a protein in different pH conditions. A single experiment was performed in which a Chirascan-plus™ equipped with a 6-Cell Peltier Cell Holder and a Chirascan CCD Emission Fluorometer measured the thermal stability of α -chymotrypsin in six pH conditions. High quality, temperature dependent CD and fluorescence spectra were obtained using only 50 μ g of protein for each condition. The addition of the fluorescence measurements to the equivalent CD experiment added less than 10 seconds per spectrum.



CHIRASCAN SERIES APPLICATION NOTE

pH Stability Screening with the Chirascan-plus CD Spectrometer and CCD Fluorescence Accessory

INTRODUCTION

For a protein to be functional, it needs to be folded into its three dimensional native state. The extent to which a protein maintains this structure is directly related to its kinetic and thermodynamic stability. Knowledge of a protein's stability is desired in many application areas for example in the case of developing protein drug formulations, where it is essential to have the protein in a stable environment.

Circular Dichroism (CD) is an exceptionally sensitive method for determining protein stability and is routinely used for this purpose. The CD signal of a protein is characteristic of its higher order structure, and changes in the CD spectrum can be attributed to structural changes in the protein. One way in which CD spectroscopy is used to evaluate protein stability is to subject the molecule to thermal stress and measure the native structure's resistance to it.

An alternative spectroscopic signal used to measure protein thermal stability is intrinsic fluorescence. Here the change in fluorescence from naturally occurring fluorescent side chain residues like tryptophan can be measured as the temperature is increased. This reveals how the environment of the residue changes during heating. CD and intrinsic fluorescence reflect different physical properties and measurement of both simultaneously provides a more complete representation of the structural and conformational changes accompanying denaturation.

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KEYWORDS

▶ Protein Stability	▶ CCD Fluorescence Accessory
▶ Circular Dichroism	▶ 6-Cell Peltier Cell Holder
▶ Fluorescence	▶ pH Screening

EXPERIMENTAL

We set out to map the thermal stability of α -chymotrypsin in six different pH conditions using CD spectroscopy and protein intrinsic fluorescence. To do this, α -chymotrypsin (Sigma Aldrich) was dissolved in a series of 15 mM citrate-phosphate buffers (pH 3 to pH 8) at a concentration of 0.1 mg/mL. Six 2 mm pathlength cells with clear side windows for fluorescence were each loaded with 0.5 mL of protein solution and placed in the Chirascan-plus™ equipped with a 6-Cell Peltier Cell Holder and a CCD accessory. Samples were heated continuously at 0.12°C per minute from 25°C to 75°C while absorbance and CD spectra were acquired simultaneously from 245 nm to 195 nm in 1 nm steps with a sampling time of 1 second per point. After each wavelength scan, the samples were excited at 285 nm for 6 seconds (8 nm bandwidth) whilst the fluorescence emission spectrum was recorded. Fitting of CD data was carried out in Applied Photophysics' Global3 software. Melting temperatures were calculated from the fluorescence data by fitting the wavelength of maximum emission as a function of temperature to a sigmoidal model. The total duration of the experiment was less than 7 hours and resulted in 300 individual CD, absorbance and fluorescence spectra (i.e 900 spectra in total).

RESULTS

The CD and fluorescence data acquired in the thermal ramp experiment are shown below in Figure 1.

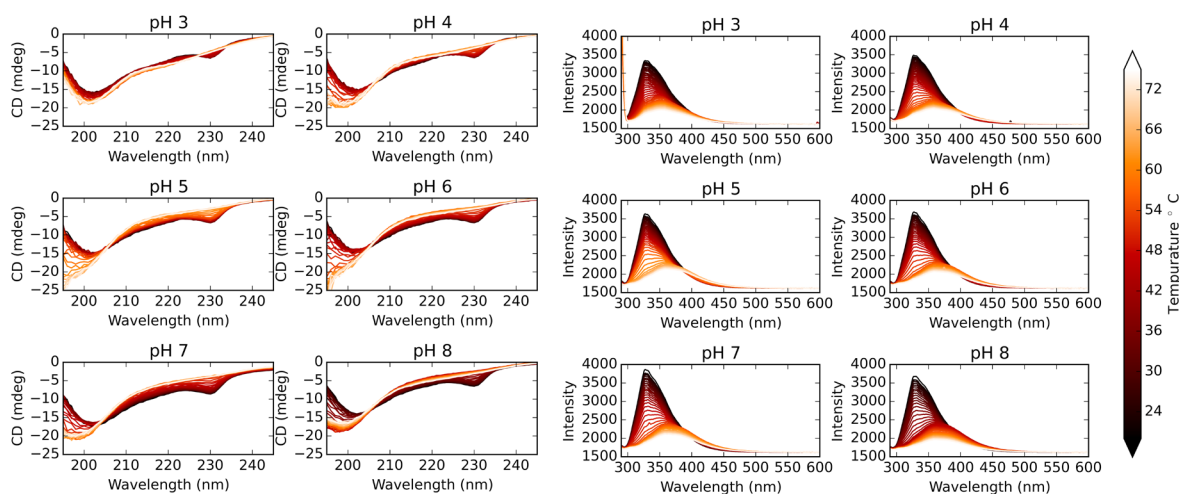


Figure 1. Left – CD data for the 6 pH conditions. Right- Fluorescence spectra of the 6 pH conditions.

The temperature dependent CD spectra were fitted to a two-state, one transition model in Applied Photophysics' Global3 analysis software. This fitting process calculates the midpoint of the transition (the melting temperature, T_m) and the van't Hoff enthalpy change. The fluorescence data were also fitted to a one transition model by fitting the change in maximum emission wavelength with temperature. The results are shown in Table 1. The melting temperatures calculated with each technique agree to well within 1°C suggesting that a change in environment of the fluorescent residues corresponds with the deformation of the protein's secondary structural elements.

Cell Position	pH	T _m °C (Intrinsic Fluorescence)	T _m °C (Circular Dichroism)	van't Hoff enthalpy, kJ/mol (Circular Dichroism)
1	3	48.3 + 0.1	48.9 + 0.2	346.7 + 15.5
2	4	53.1 + 0.1	53.2 + 0.1	405.4 + 8.9
3	5	51.5 + 0.1	51.5 + 0.2	474.1 + 15.3
4	6	49.0 + 0.2	49.4 + 0.1	535.8 + 14.1
5	7	45.6 + 0.1	45.3 + 0.2	458.6 + 8.7
6	8	41.9 + 0.1	41.3 + 0.1	495.9 + 8.7

Table 1. Thermodynamic parameters. Errors are estimates of the standard error of the fitted parameters returned by the fitting algorithm.

All the CD and fluorescence data acquired from the single experiment were used to construct an Empirical Phase Diagram (EPD) which displays a useful pictorial representation of a protein's stability.[1] The EPD is shown in Figure 2. The colour changes represent structural changes of the protein in the conditions explored, allowing the transitional phases of the protein to be visualised. In Figure 2, the stable phases are depicted by a fuchsia pink colour which is predominant at lower temperatures. The loss of this colour at elevated temperatures represents the loss of the protein's higher order structure as the conditions become unfavourable. It is clear that pHs 4 and 5 represent the most thermally stable buffer conditions for this protein.

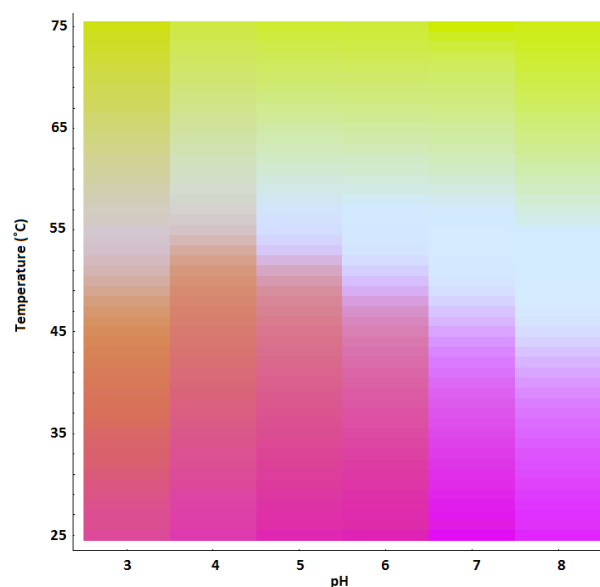


Figure 2. Empirical Phase Diagram constructed from the data in Figure 1

CONCLUSION

The ability to measure both protein circular dichroism and intrinsic fluorescence emission simultaneously provides orthogonal probes for loss of structure during unfolding. Combining this with the use of a 6-Cell Peltier Cell Holder maximises productivity when screening buffer conditions, as up to 6 conditions can be measured in a single experiment. The results from such an experiment can be compiled into an empirical phase diagram which provides a clear pictorial representation of the stability information. In addition to fluorescence, the CCD accessory may also measure light scattering if the onset of aggregation occurs during stress to the protein (aggregation was not seen in the example).

REFERENCES

[1] N. R. Maddux, S. B. Joshi, D. B. Volkin, J. P. Ralston, and C. R. Middaugh, "Multidimensional methods for the formulation of biopharmaceuticals and vaccines," *J. Pharm. Sci.*, vol. 100, no. 10, pp. 4171-4197, 2011.

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