Biophysical Journal, Volume *111*

Supplemental Information

Circular Dichroism Spectroscopy of Collagen Fibrillogenesis: A New Use for an Old Technique

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Supporting Methods

Transmission Electron Microscopy

Type-I collagen samples (Telo-EPC) were prepared and imaged as described previously (1). In brief, collagen suspensions were prepared as described in the Rheology section of the Methods in the main text and incubated for 30 minutes at 37 °C in microfuge tubes. A 10 μL sample of the supernatant of the gel was plated on a plastic Petri dish, and an extra-thick carbon mesh copper grid was placed on the droplet for 5 minutes. The supernatant was removed using filter paper, and then the grid was stained with 1% phosphotungstic acid for 5 minutes. Samples were dried overnight and imaged using a JEM-100CX TEM microscope.

Rheology

Type-I collagen samples (Telo-EPC) for rheological testing were prepared and loaded on the rheometer as described in the main text. The temperature was immediately raised to 37 °C, and the sample was continually oscillated to 0.5% strain at 1 rad/s for 10 minutes while measuring the resultant torque to obtain the temperature-dependent sample storage and loss moduli (G′ and G″, respectively).

Gelatin samples were assayed for hydrogel formation. Samples were prepared at 25 mg/mL in PBS, and were fully solubilized by heating to 37 °C. Rheology measurements were performed differently for gelatin compared to type-I collagen, which forms a hydrogel at low temperatures (~4 °C) rather than physiological temperature (37 °C). To perform these measurements, samples were plated at 25 °C, then the temperature was increased to 37 °C to ensure the gelatin was fully fluid, decreased to 4 °C at a rate of 2 °C/minute, and held at 4 °C for 15 minutes to assay for hydrogel formation. Measurements of three separately prepared samples were taken and averaged.

Light Scattering

Light scattering measurements were performed on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) with a 3mW He-Ne laser at $\lambda = 633$ nm, collecting backscattered light at $\theta = 173°$. A built-in Peltier element controlled sample temperature during measurements to within ±0.1 °C. Scattering intensities and autocorrelation functions were determined from the average of either three or five correlation functions, with a typical acquisition time of 60 s per correlation function. Type-I (Telo-EPC) and type-III collagen solutions were prepared in PBS buffer (final concentration of 0.1 mg/mL). Samples were loaded into low-volume quartz batch cuvettes (ZEN2112) and equilibrated to 37 °C. Measurements of three separately prepared samples of each type were taken every minute for 30 minutes and averaged.

ICA Software

Source code for Independent Component Analysis is provided in the supporting file `itpp_ICA.zip`. It includes the it++ library (http://itpp.sourceforge.net) and supporting code for implementing ICA on CD data provided in the AVIV CD spectrometer output file format. Instructions for installation, required supporting libraries and usage are provided in `READ_ME.txt`. Code developed specifically for this project and it++ including modifications to the library are distributed under the pre-existing GNU General Public License (http://www.gnu.org/copyleft/gpl.html).

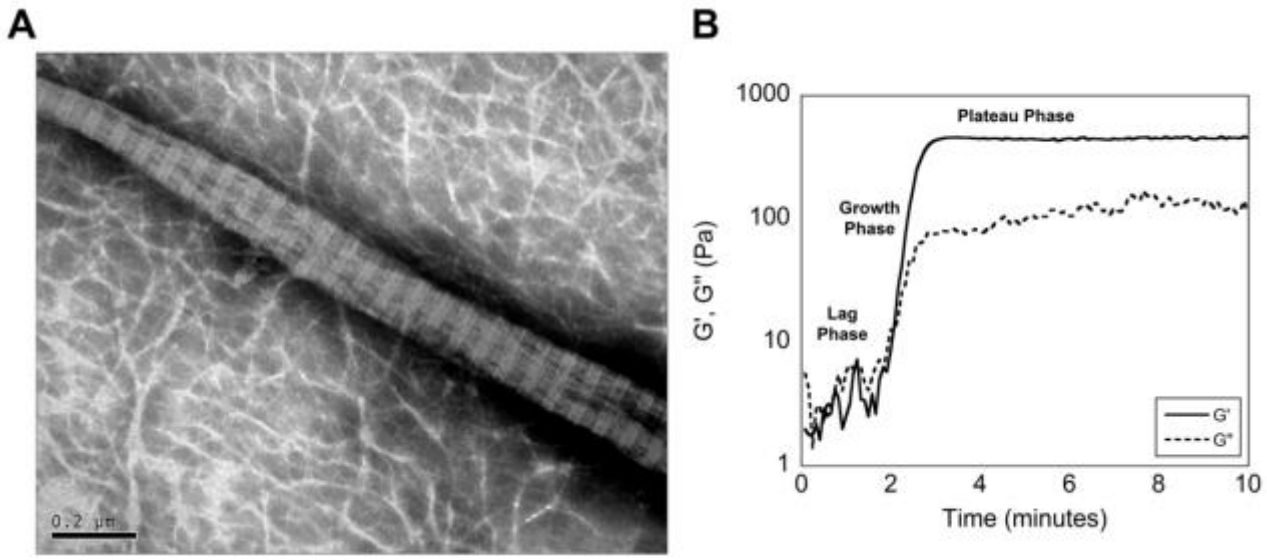

Figure S1. A) Ordered D-banding in collagen fibrils was visualized by Transmission Electron Microscopy. B) Rheology allows for characterization of the mechanical properties of type-I collagen hydrogels; the process of collagen fibrillogenesis manifests as a lag phase, where the storage modulus and loss modulus (G′ and G′′, respectively) are negligible, a growth phase of fibril formation, exhibited as an increase in both G′ and G′′, and a plateau phase indicative of fibril stabilization, where G′ and G′′ are constant.

Experiment Number	Samples	Wavelength (nm)	Temperature (°C)	Averaging Time (s)	Wavelength Increment (nm)	Temperature Step (°C)	Equilibration Time (min)	Notes
1	Telo-EPC, Telo-ABM, Atelo-ABM, RT, Type-III, Gelatin	260-195	4	10	1	N/A	2	• All samples at 0.1 mg/mL except Telo-EPC at 0.1, 0.05, 0.025, and 0.01 mg/mL
		222	4-60	10	N/A	0.33	2	• All samples at 0.1 mg/mL except Telo-EPC at 0.1, 0.05, 0.025, and 0.01 mg/mL • Performed from 4 °C to 60 °C and returned to 4 °C
2	Telo-EPC	260-200	4-60	3	2	0.33	6	• N/A
3	Telo-EPC with and without type-I collagenase	240-198	4	3	2	N/A	2	• Telo-EPC prepared at 0.05 mg/mL in PBS • Sample volumes of 250 µL
		240-198	37	3	2	N/A	2	• Samples from above given 1 hour for self-assembly
		240-200	37	3	2	N/A	2	• 50 µL of either 0.04 mM $CaCl_2$ (Fisher) in PBS (Fisher) or 3 mg/mL type-I collagenase (C1030, Sigma) in 0.04 mM $CaCl_2$ in PBS was added to each cuvette prior to measurement

Table S1. Parameters of CD Spectroscopy experiments for each set of collagen samples with additional notes to clarify procedures.

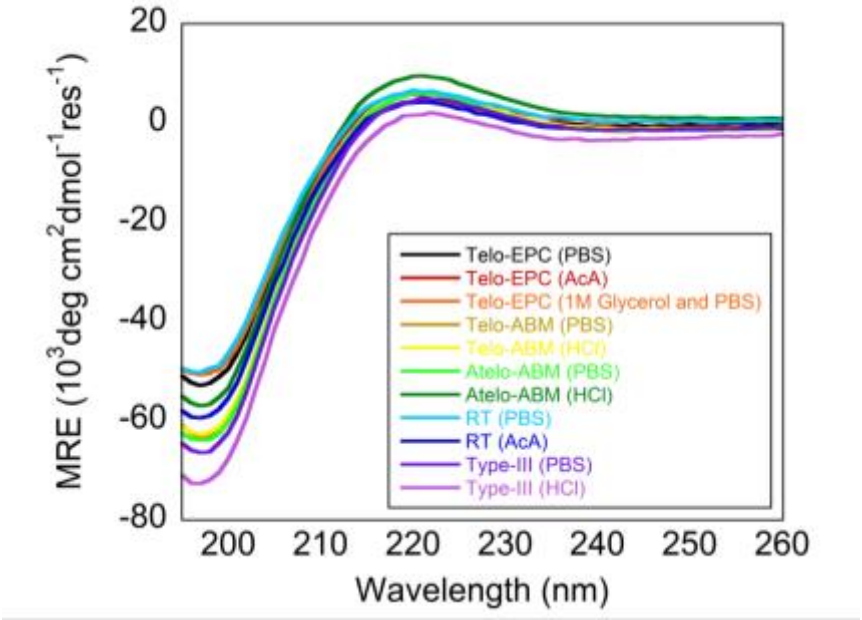

Figure S2. Mean residue ellipticity (MRE) of collagen samples was measured from 195 – 260 nm at 4 ºC. All collagens, regardless of buffer, demonstrated triple-helical content via CD measurements as a positive peak at 222 nm and a negative peak near 195 nm.

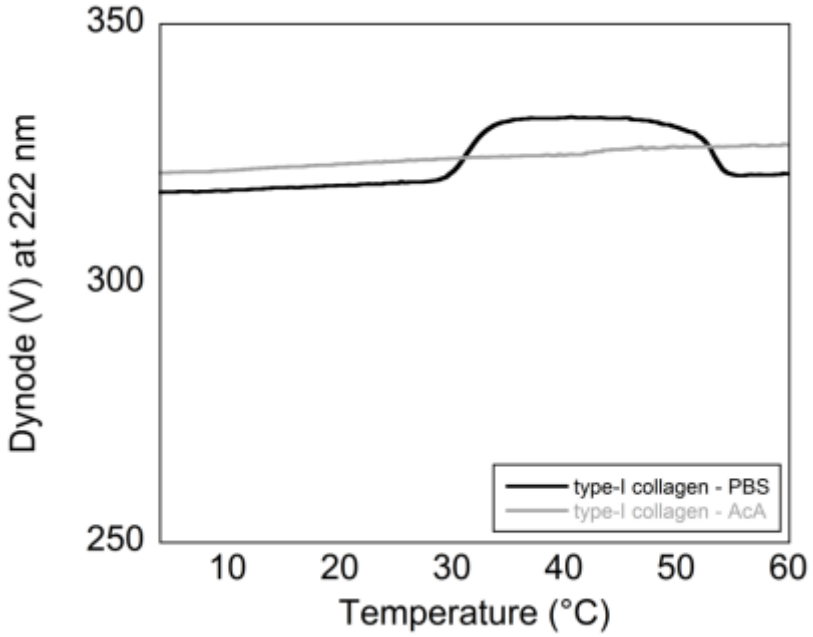

Figure S3. Representative example of the change in dynode voltage at 222 nm throughout a temperature melt experiment (Fig. 1 *A*) with type-I collagen (Telo-EPC) in acidic (AcA) and physiological (PBS) buffers. The dynode voltage did not exceed 600 V, which indicated that the change in ellipticity was due to structural changes in the protein sample and not the result of light scattering.

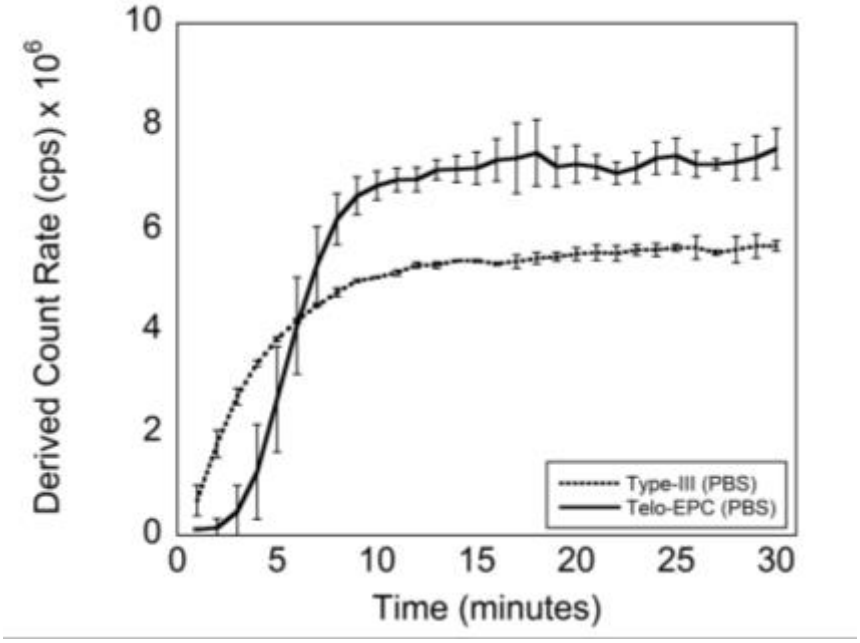

Figure S4. The derived count rate of type-I (Telo-EPC) and type-III collagen samples was monitored at 37 ºC as samples formed fibrils. Error bars represent the average measurement for three samples. Telo-EPC and type-III samples were both capable of self-assembly, demonstrated as an increase in derived count rate during heating presumably due to fibril formation. Type-I and type-III collagen are very difficult to isolate from one another, therefore, it is not possible to determine if the increase in scattering as seen in these results is due to fibrillogenesis of solely type-III or type-I and type-III fibrils.

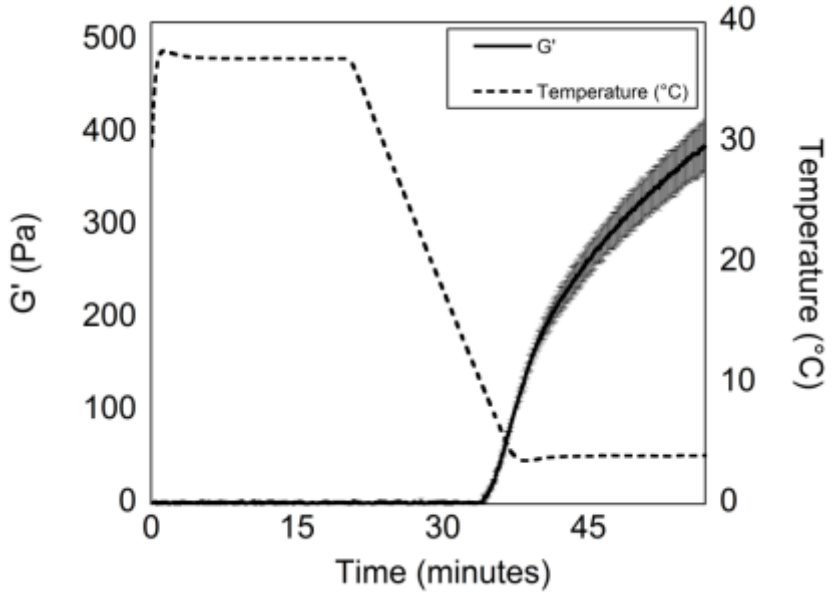

Figure S5. The increase in storage modulus at temperatures below 10 °C indicates that gelatin is capable of forming a hydrogel at relatively high concentrations and low temperatures. Error bars represent the average measurement of three samples.

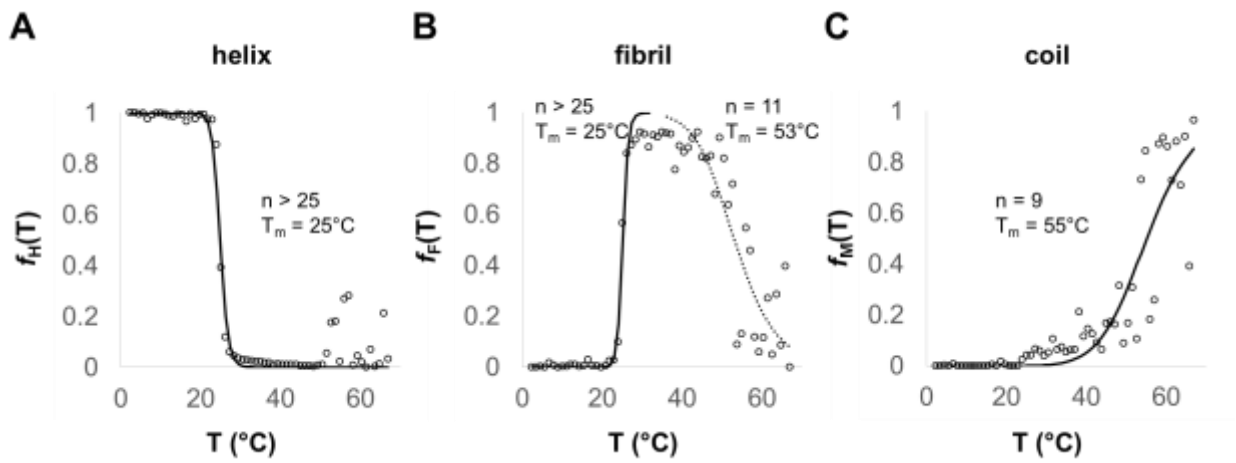

Figure S6. $f_H(T)$, $f_F(T)$ and $f_M(T)$ corresponding to fractional helix (A), fibril (B), and coil (C) species from deconvolution of the Telo-EPC melt (Fig. 5 and Fig. 6). Fractional populations were fit to a Hill-plot function: $f = T^n / (T^n + T_m^n)$, with n as the steepness of the transition at temperature T_m. Helix and coil (A and C) fractional populations were each fit to one transition. Fibril (B) fractional populations were fit to two transitions corresponding to the emergence (solid line) and loss (dotted line) of this species.

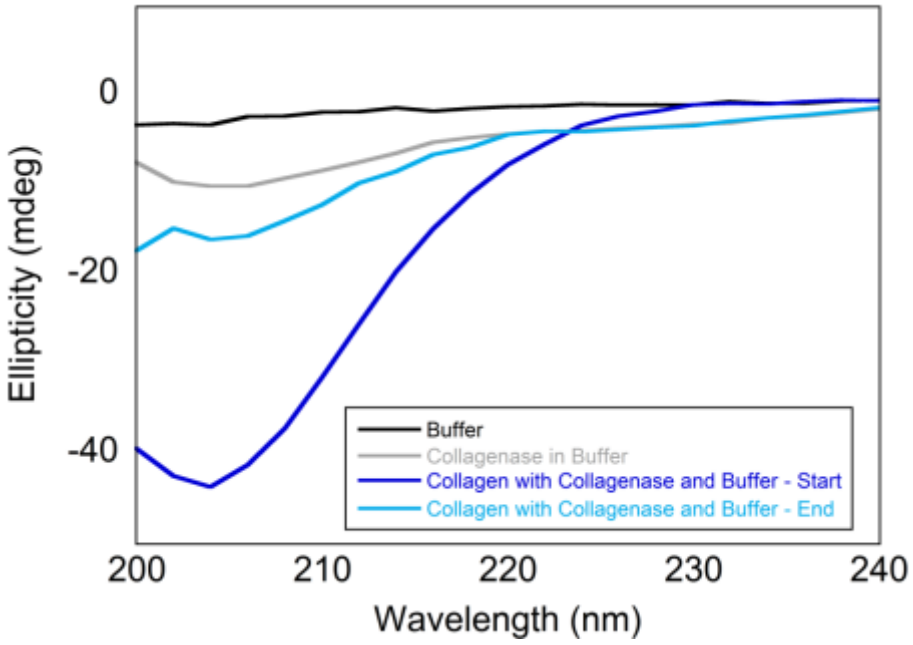

Figure S7. Raw wavelength spectra of buffer, type-I collagenase in buffer, and the starting and ending spectra of type-I collagen with collagenase from 200 – 240 nm. There are no buffer/enzyme subtraction or MRE correction in this figure. The collagenase signal was slightly more negative in the lower wavelength regions compared to the buffer signal. When collagen and collagenase were in the cuvette at the beginning of the measurement, the measurement was primarily our fibrillar collagen with the negative peak at ~204 nm. When the collagen was digested we saw a similar spectra compared to the original collagenase signal, presumably with some fragments of type-I collagen remaining.

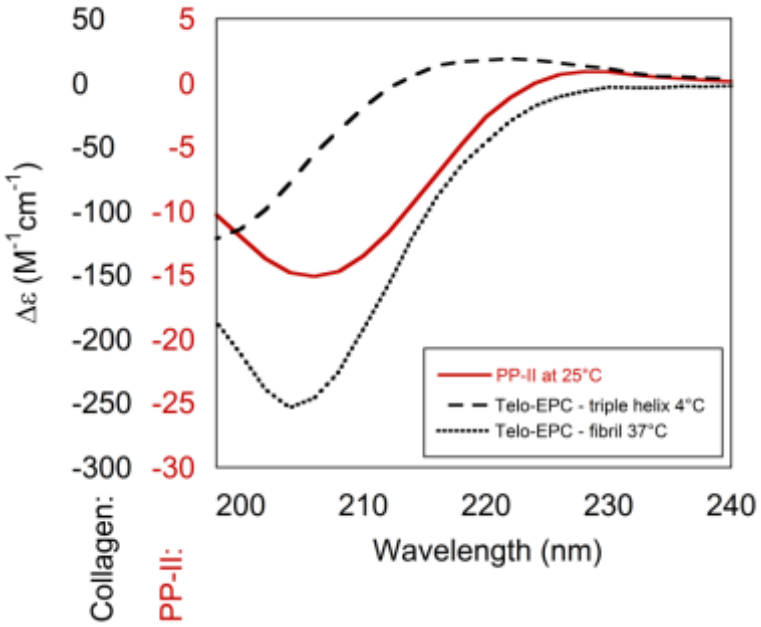

Figure S8. Wavelength spectra of Telo-EPC in triple-helical (4 °C) and fibril states (37 °C) (from Fig. 5 *A*) were compared to the poly-proline II (PP-II) signal obtained by Lopes et al (2). To ensure the units were the same, MRE of our collagen signals was converted to Δε (3-5). Note that the collagen signals are an order of magnitude greater than the PP-II signal. Signal information for PP-II was downloaded from the Protein Circular Dichroism Data Bank (6). The PP-II from Lopes et al (2) was purchased from Sigma-Aldrich, and had a molecular weight of 1,000 Da – 10,000 Da in comparison to the collagen triple-helix, which is ~350,000 Da. The Telo-EPC triple-helix signal and PP-II signal are clearly distinct, particularly when comparing the positive peak at 222 nm and the lack of a negative peak at 204 nm. The CD signature of Telo-EPC fibril signal and PP-II are similar in spectral shape, but the amplitude of the peaks differ by over an order of magnitude.

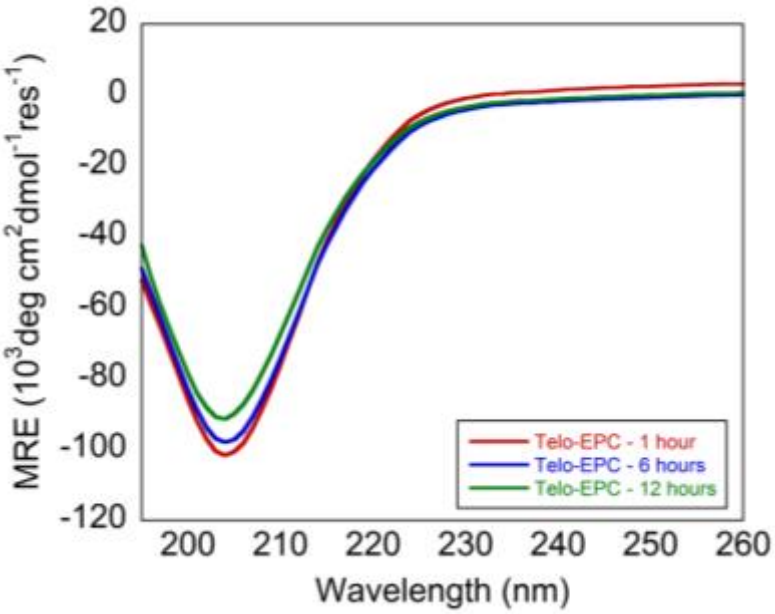

Figure S9. Wavelength spectra of Telo-EPC were measured every hour for 12 hours as outlined in Table S1, Experiment 1, but at 37 ℃. The negative peak at 210 nm was stable over the course of 12 hours.

Supporting References

1. Drzewiecki, K. E., A. S. Parmar, I. D. Gaudet, J. R. Branch, D. H. Pike, V. Nanda, and D. I. Shreiber. 2014. Methacrylation induces rapid, temperature-dependent, reversible self-assembly of type-I collagen. Langmuir : the ACS journal of surfaces and colloids 30:11204-11211.

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6. Wallace, B., and R. Janes. 2016. Protein Circular Dichroism Data Bank Department of Crystallography, Institute of Structural and Molecular Biology, Birkbeck College, University of London, School of Biological and Chemical Sciences, Queen Mary, University of London, UK.