

Supplemental Experimental Procedures:

Expression and Purification of Mutant Proteins: Expression of mutant proteins were performed according to the manufacturer's protocol (Novagen). Following cell lysis (1), the supernatant was treated with methanol (40% final concentration) at 4° C for 2 1/2 hours. Alternatively, mutant proteins expressed in inclusion bodies were dissolved in 8M urea at room temperature for 2 h. In either case, the resulting suspension was centrifuged at 3000g for 30 minutes. The supernatant was dialyzed against 50 mM Tris-HCl pH 8.4. The dialysate was treated with ammonium sulfate 45-75% saturation. The resulting precipitate was dissolved in 50 mM Tris-HCl pH 8.4 and applied to a Sephadex G-100 column (2.5 x 100 cm) equilibrated with 50 mM Tris-HCl, 100 mM NaCl, pH 8.4. The fraction containing the mutant protein was dialyzed against 50 mM Tris-HCl, pH 8.4 and applied to a DEAE Sephadex A-25 column. Bound protein was eluted with a 0-0.8 M NaCl gradient. Eluted fractions containing mutant proteins were centrifugally concentrated (Amicon, Centricon-10). Purity of mutant proteins was verified by SDS tricine gel electrophoresis. The protein concentration was determined by the biuret method.

Steady State Fluorescence Spectroscopy: Estimation of quantum yield of Trp in tear lipocalin mutants was performed using NATA as a fluorescence standard; calculations were performed assuming the quantum yield of NATA in aqueous solution to be 0.13 (2)

$$\phi = \phi_R \frac{I}{I_R} \times \frac{OD_R}{OD}$$

ϕ , I , OD - quantum yield, the integrated fluorescence intensity, optical density of Trp mutants, respectively. The subscript R refers to the reference fluorophore (NATA).

To calculate the integrated fluorescence intensity I , it was necessary to construct the blue side of the Trp emission spectra. The spectrum of an elementary component on

wavelength (nm) scale can be described by a biparametric (maximum amplitude and position) log-normal function (3). Log-normal function (3) is fitted to Trp emission spectra and the obtained parameter was used to construct the blue side of spectra:

$$I(\lambda) = I_m \exp \left[- \frac{\ln 2}{\ln^2 p} \ln^2 \left(\frac{a - 1/\lambda}{a - 1/\lambda_m} \right) \right]$$

where $I_m = I(\lambda_m)$ is the maximal fluorescence intensity; λ_m is the wavelength of the band maximum; $p = (1/\lambda_m - 1/\lambda_-)/(1/\lambda_+ - 1/\lambda_m)$ is the band asymmetry parameter; $a = 1/\lambda_m + (1/\lambda_+ - 1/\lambda_-)p/(p^2 - 1)$ is the function-limiting point; and $\lambda_+ = 10^7 / (0.831 \cdot 10^7 / \lambda_m + 7,070)$ and $\lambda_- = 10^7 / (1.177 \cdot 10^7 / \lambda_m - 7,780)$ are the wavelength positions of half-maximal amplitudes. In some cases for better fitting, the second component was added.

Fluorescent lifetime Measurements: Measurements were made with a Coherent Laser system (Verdi V-8 pump laser (532 nm), dye ring laser 899-01 with Rhodamine 6G (590 nm), frequency doubler MBD200 (295 nm)). The excitation wavelength was set at 295 nm. Emission (bandwidth 6 nm) was observed at fluorescence λ_{\max} . The light was modulated by a Pockels cell modulator. The available frequency range was 0.1-310 MHz. P-terphenyl in ethanol was used as a reference standard ($\tau = 1.05$ ns). Data analyses were performed with nonlinear least-squares programs from SPEX or from the Center for Fluorescence Spectroscopy (M. L. Johnson), University of Maryland at Baltimore, School of Medicine, Baltimore, MD. The goodness of fit was assessed by the χ^2 criterion (χ^2 ranged from 0.9 to 2.7).

Distance Measurements by RET: Efficiency of radiationless energy transfer, E , can be calculated from

$$E = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_D \rangle}$$

where $\langle \tau_{DA} \rangle$ and $\langle \tau_D \rangle$ are the fluorescence lifetime of the donor in the presence and absence of acceptor, respectively. The cysteine substituted mutant proteins were used to calculate the donor lifetimes in the absence (unlabeled) and presence (labeled) of the acceptor. Under the assumption that adding the dansyl group does not interfere with the conformation of the tryptophan residue, an attempt was made to fit the data by global analysis methods.

According to Förster's theory (4), the efficiency of radiationless energy transfer, E , between a donor, D, and an acceptor, A, is given by

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

where R is the distance between the donor and the acceptor. The Förster distance R_0 (in angstroms) at which 50% energy transfer occurs is obtained from

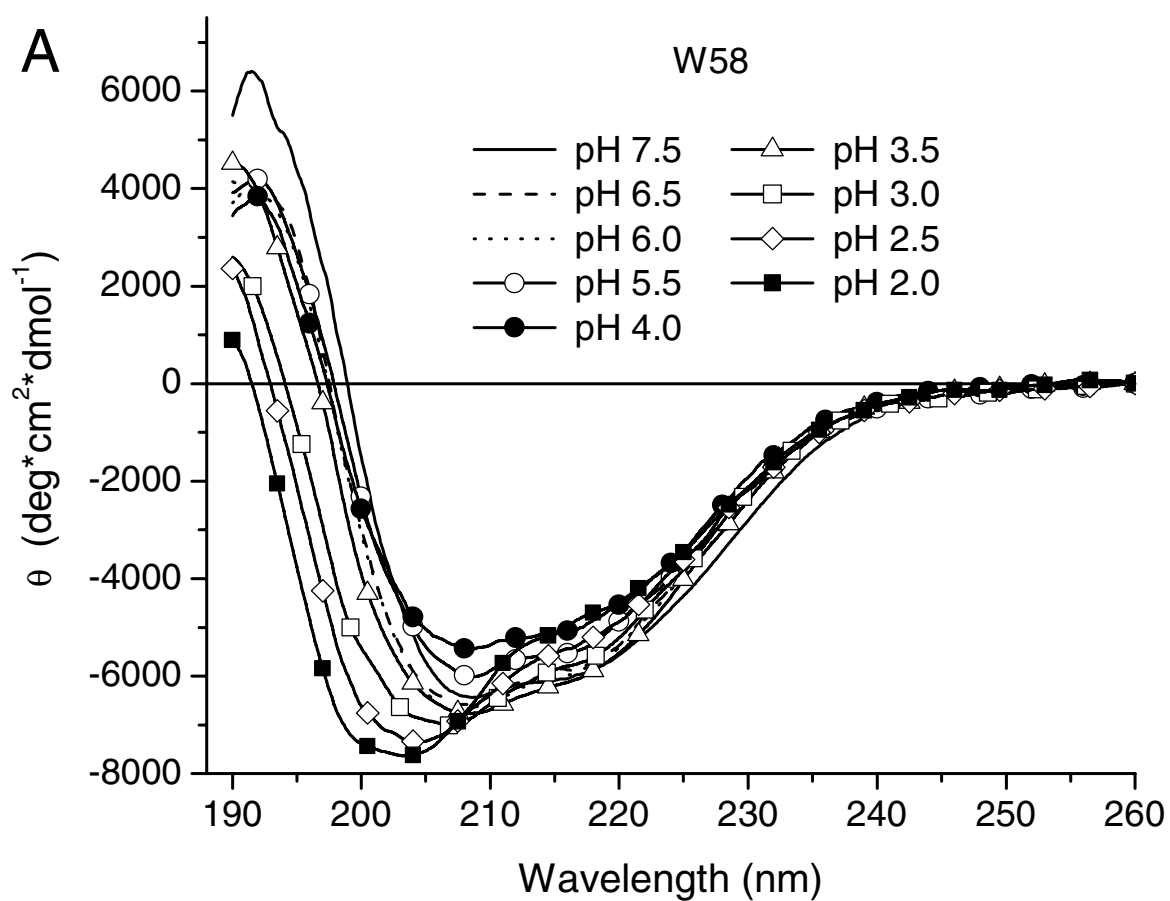
$$R_0^6 = 8.79 \times 10^{-5} [\kappa^2 n^{-4} \phi_D J(\lambda)]$$

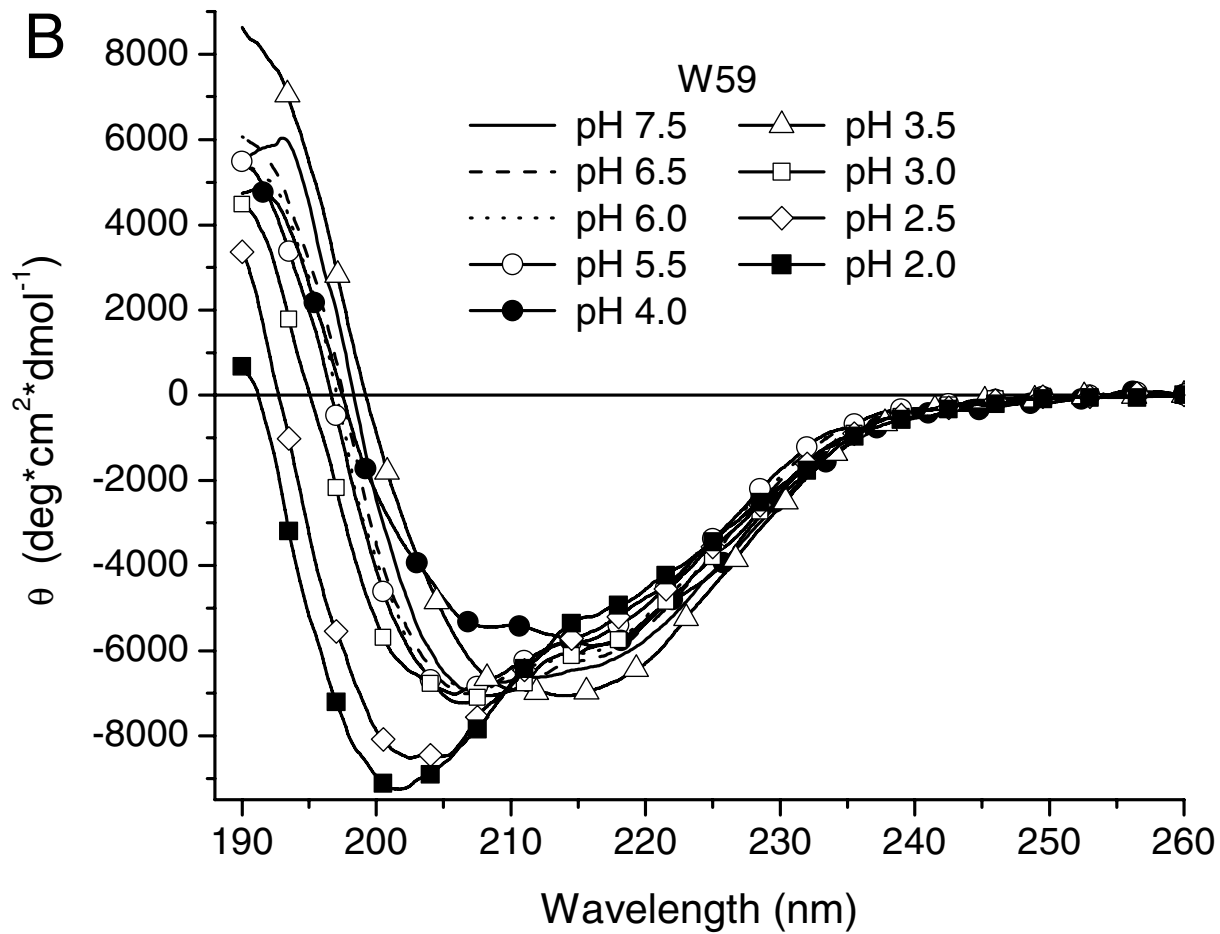
The refractive index, n , of the medium is taken to be 1.5 (5). ϕ_D is the quantum yield of the donor in the absence of the acceptor. Because distance measurements were applied to loop region, the orientation factor, κ^2 , was assumed to be 2/3 which is appropriate for dynamic random averaging of the donor and acceptor. Spectral overlap integral $J(\lambda)$ (in $M^{-1} \text{ cm}^{-1} \text{ nm}^4$) between the emission spectrum of the donor $F_D(\lambda)$ and the absorption spectrum of the acceptor $\varepsilon_A(\lambda)$ is defined as

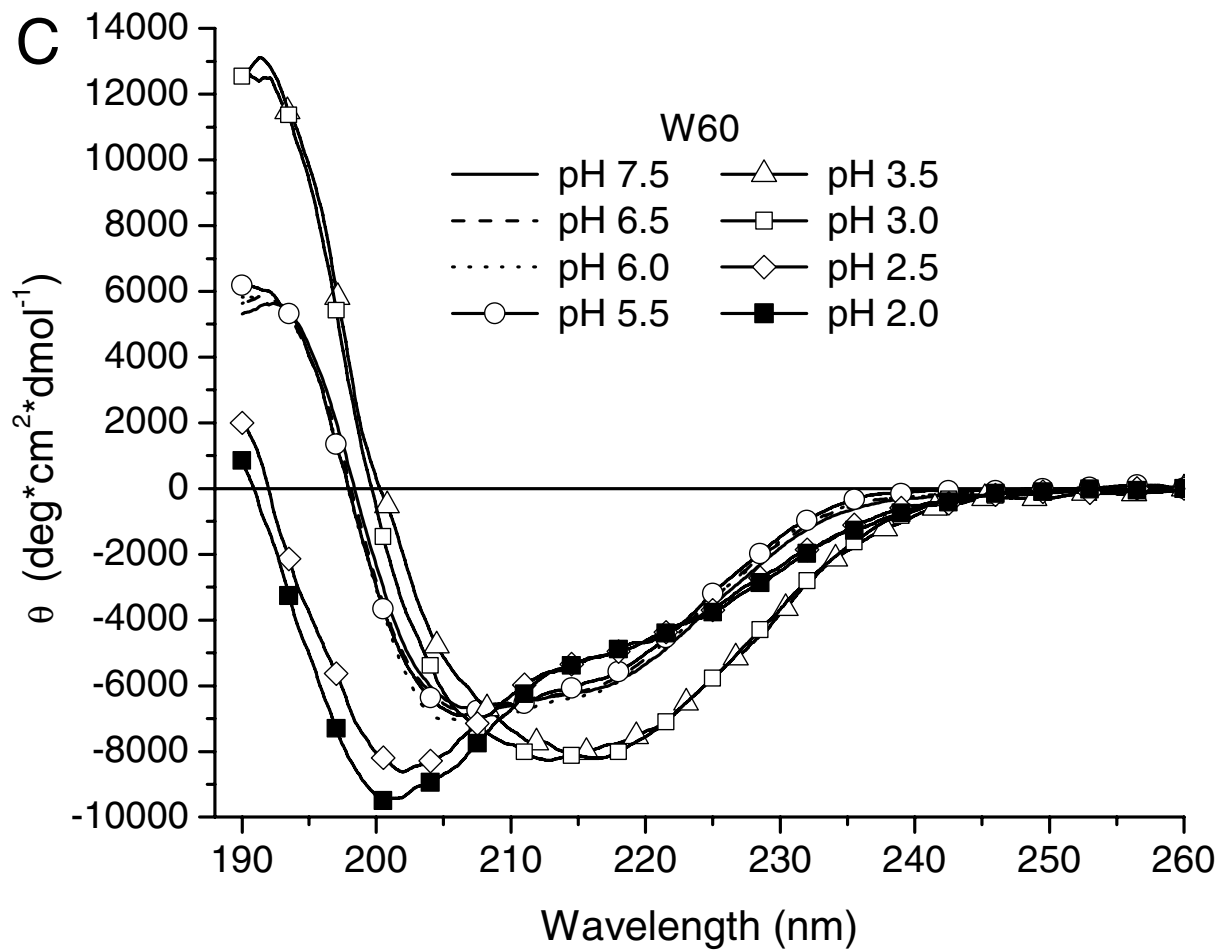
$$J(\lambda) = \frac{\int F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda}{\int F_D(\lambda) d\lambda}$$

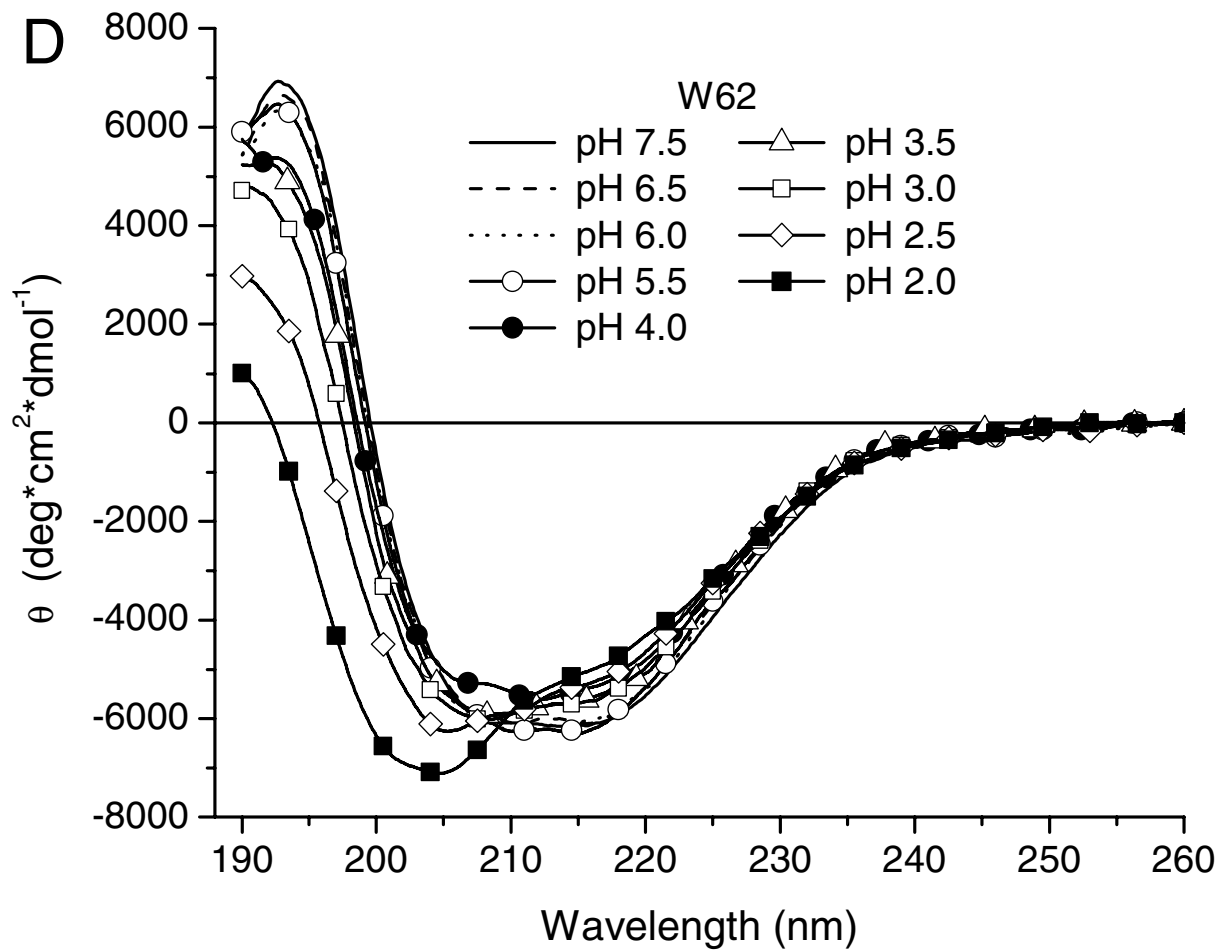
These experiments were performed at pH 6.8 instead of 7.3 because the dansyl labeling efficiency of cysteine improved at pH 6.8 and the fluorescent lifetimes were essentially unchanged.

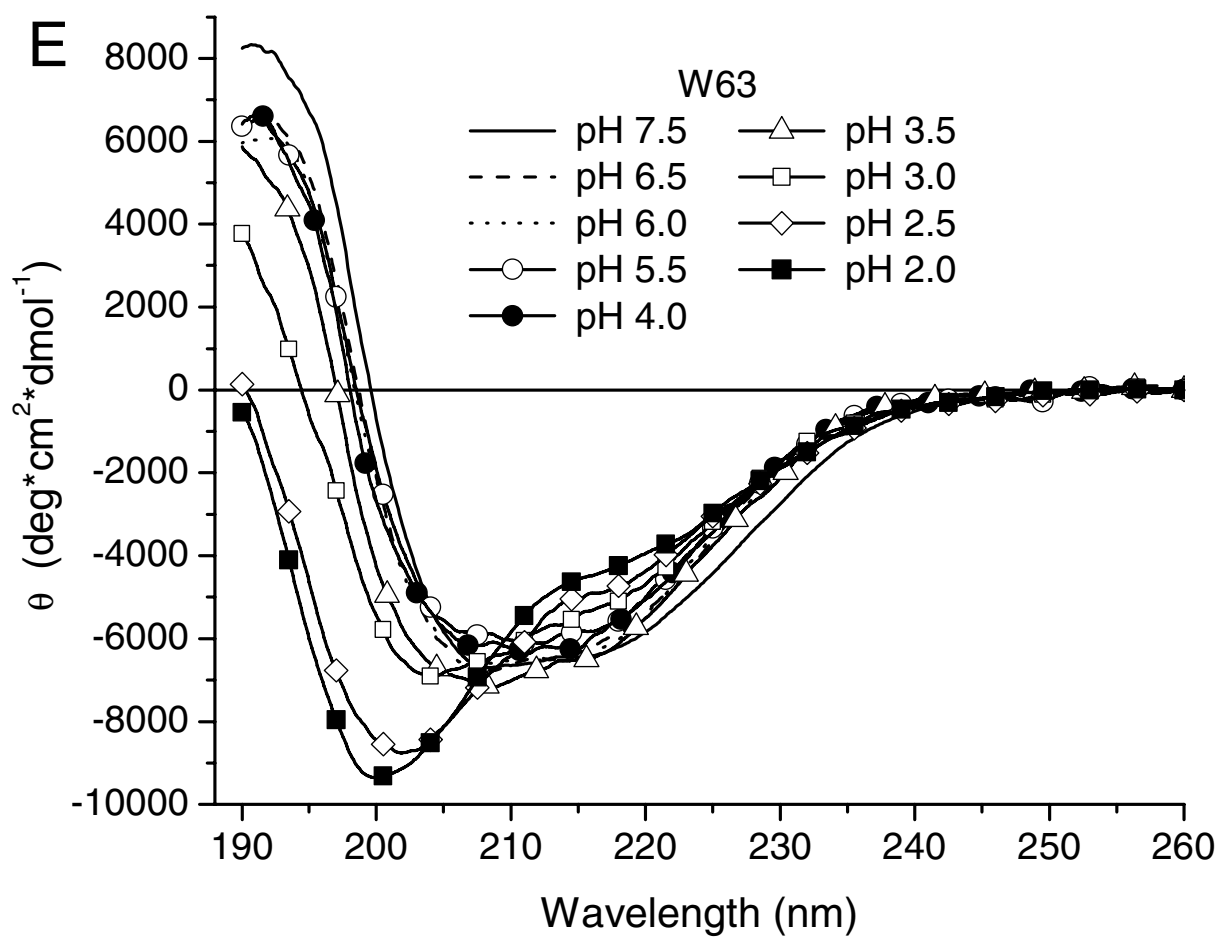
Supplementary Figures

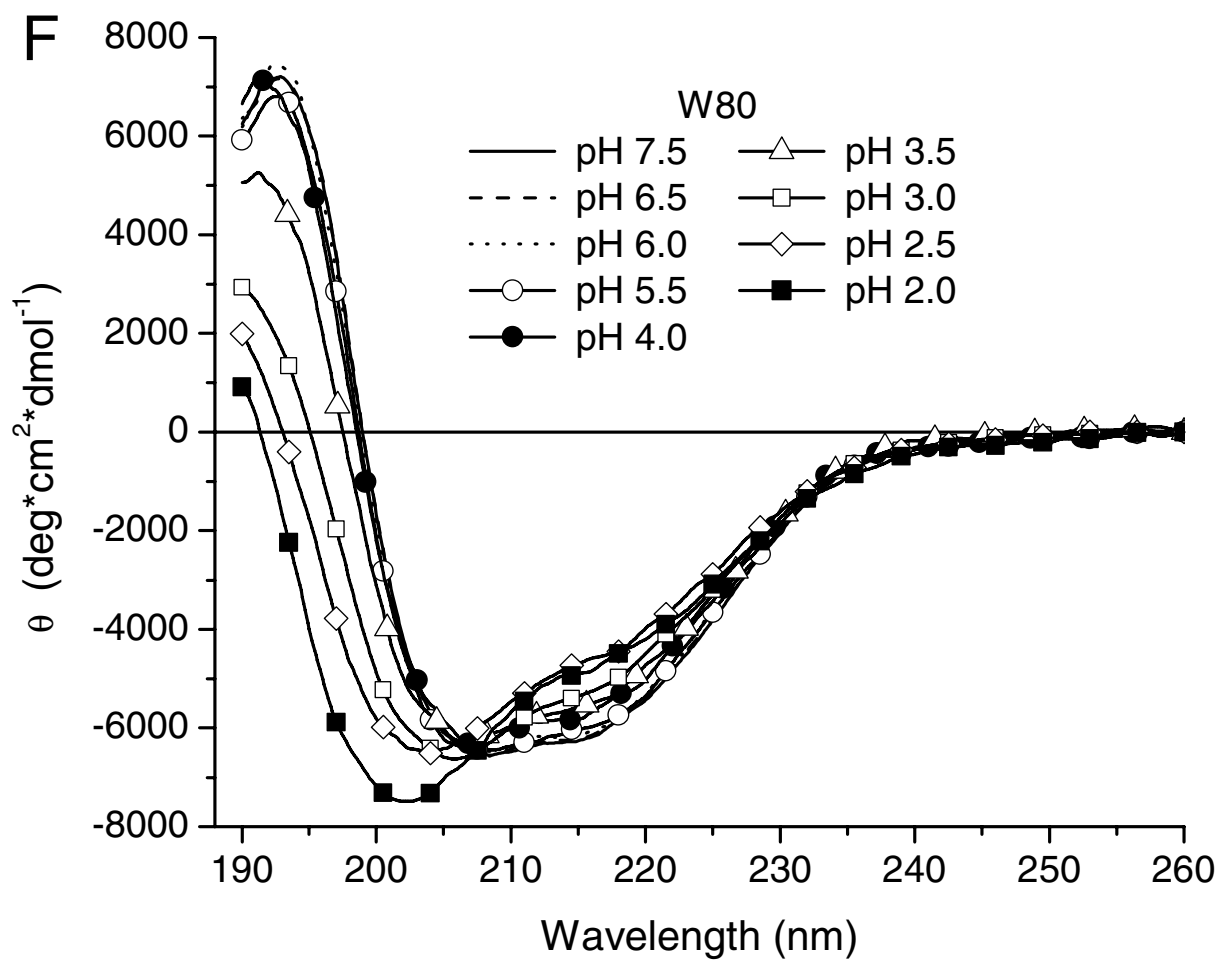


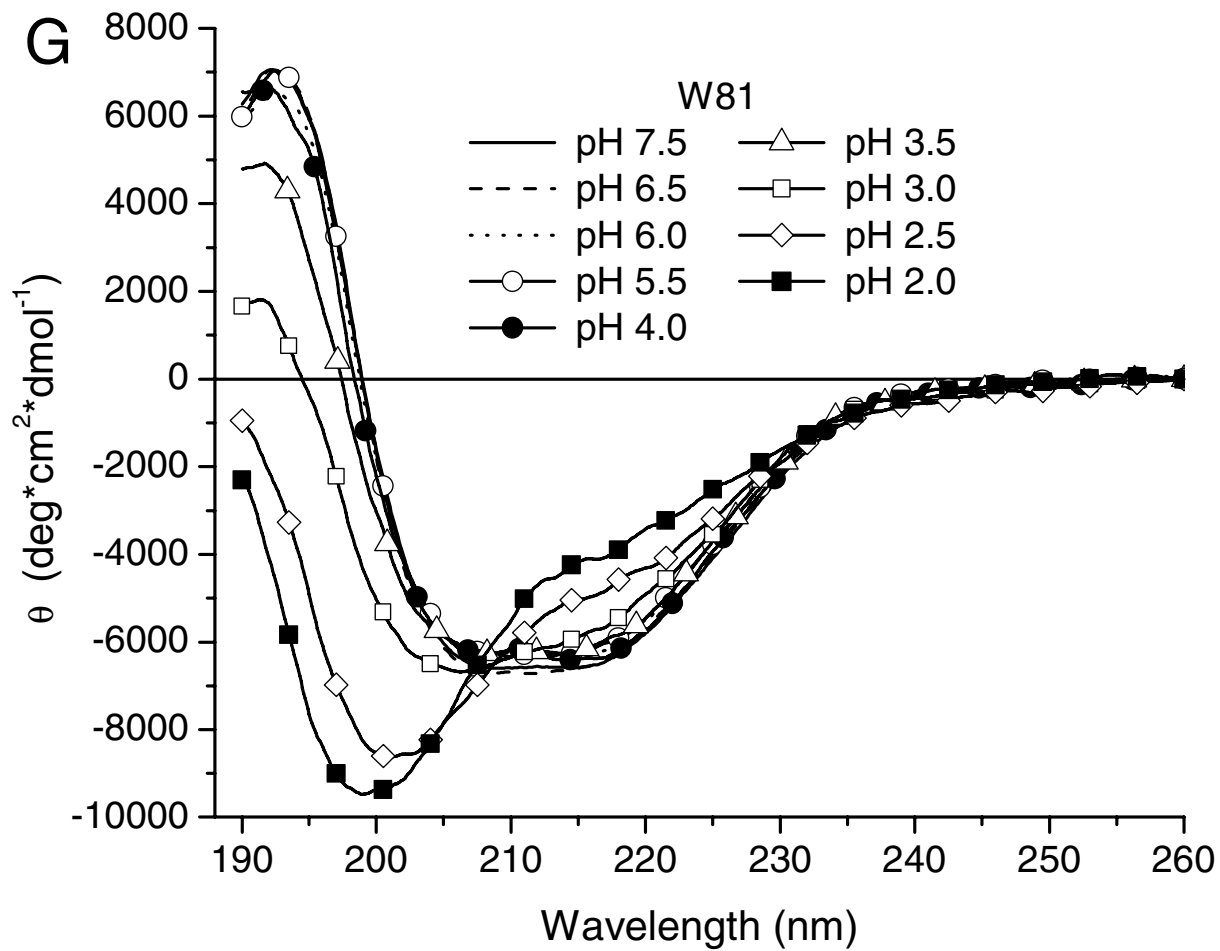


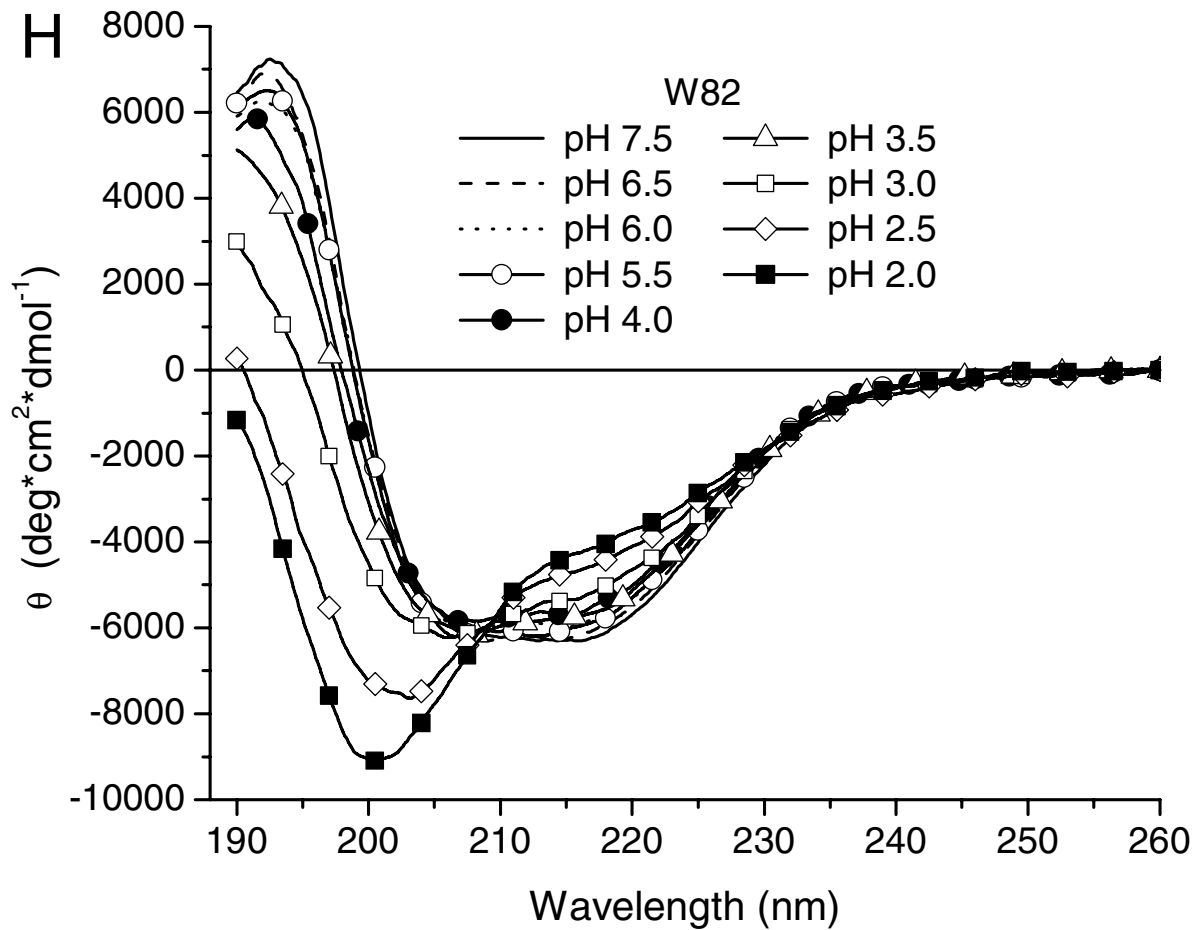


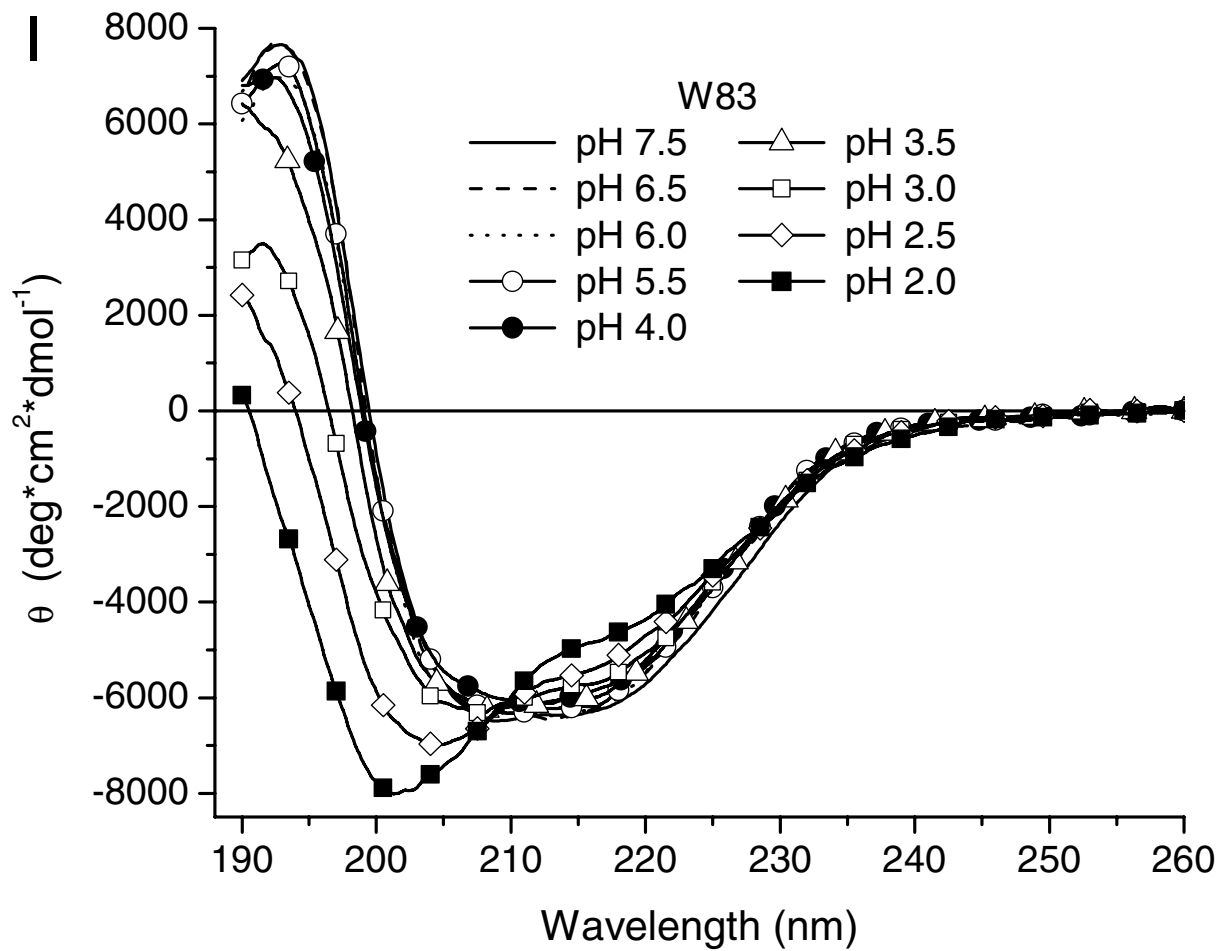


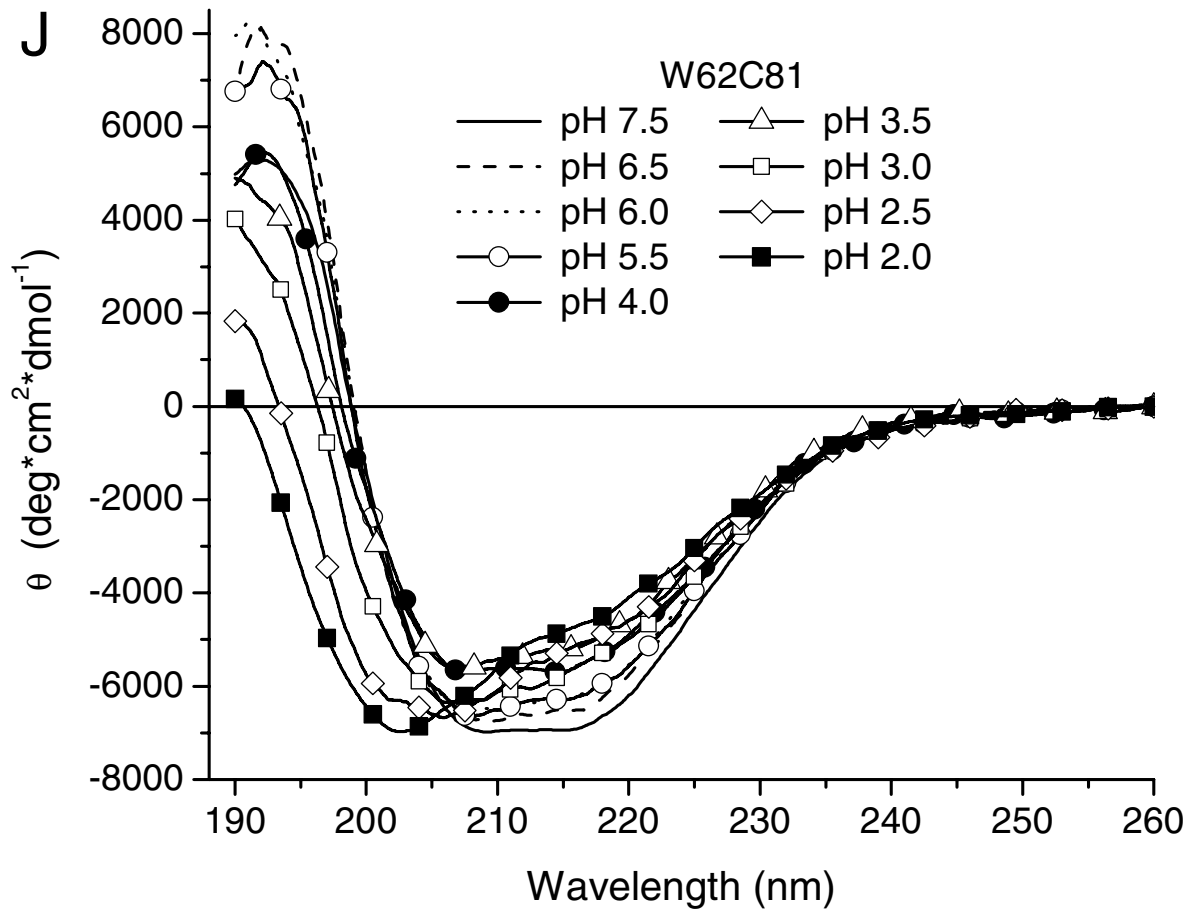


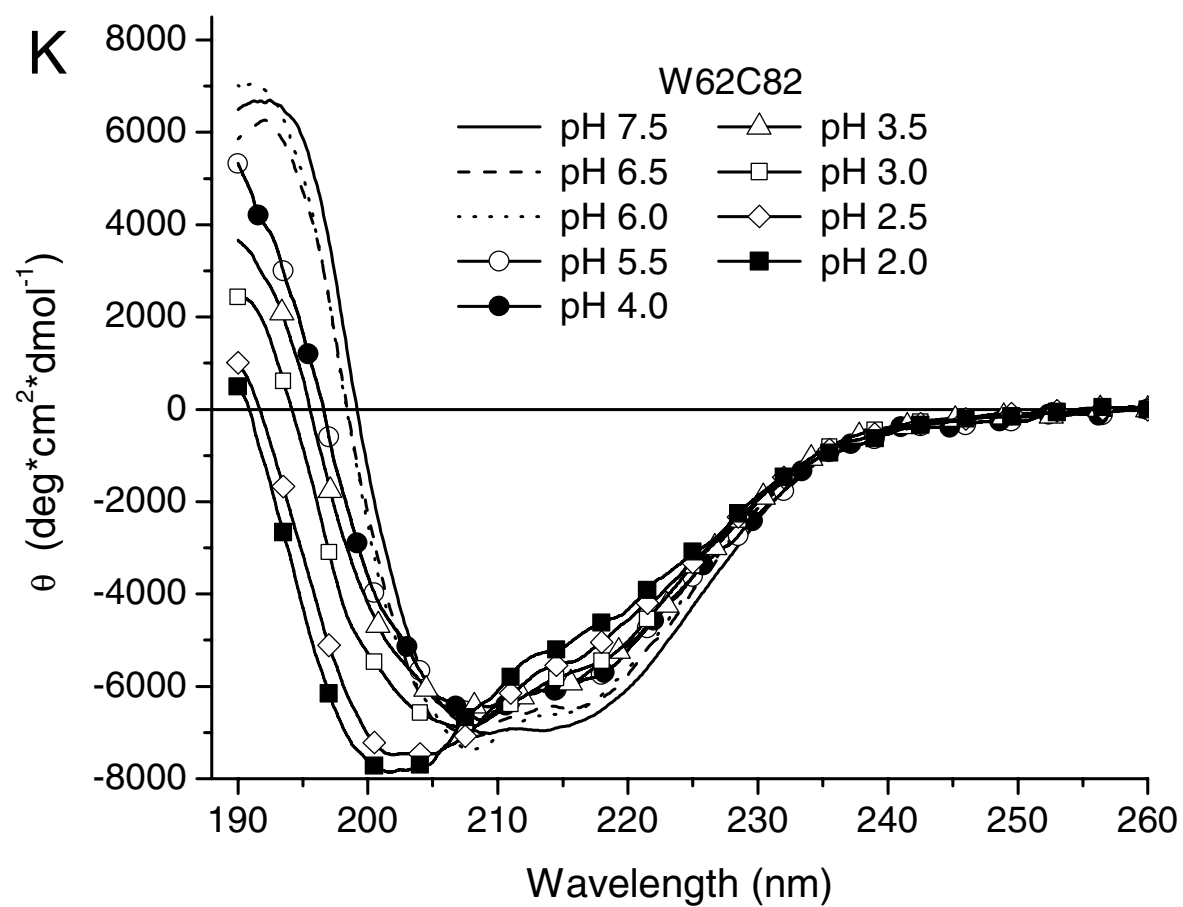


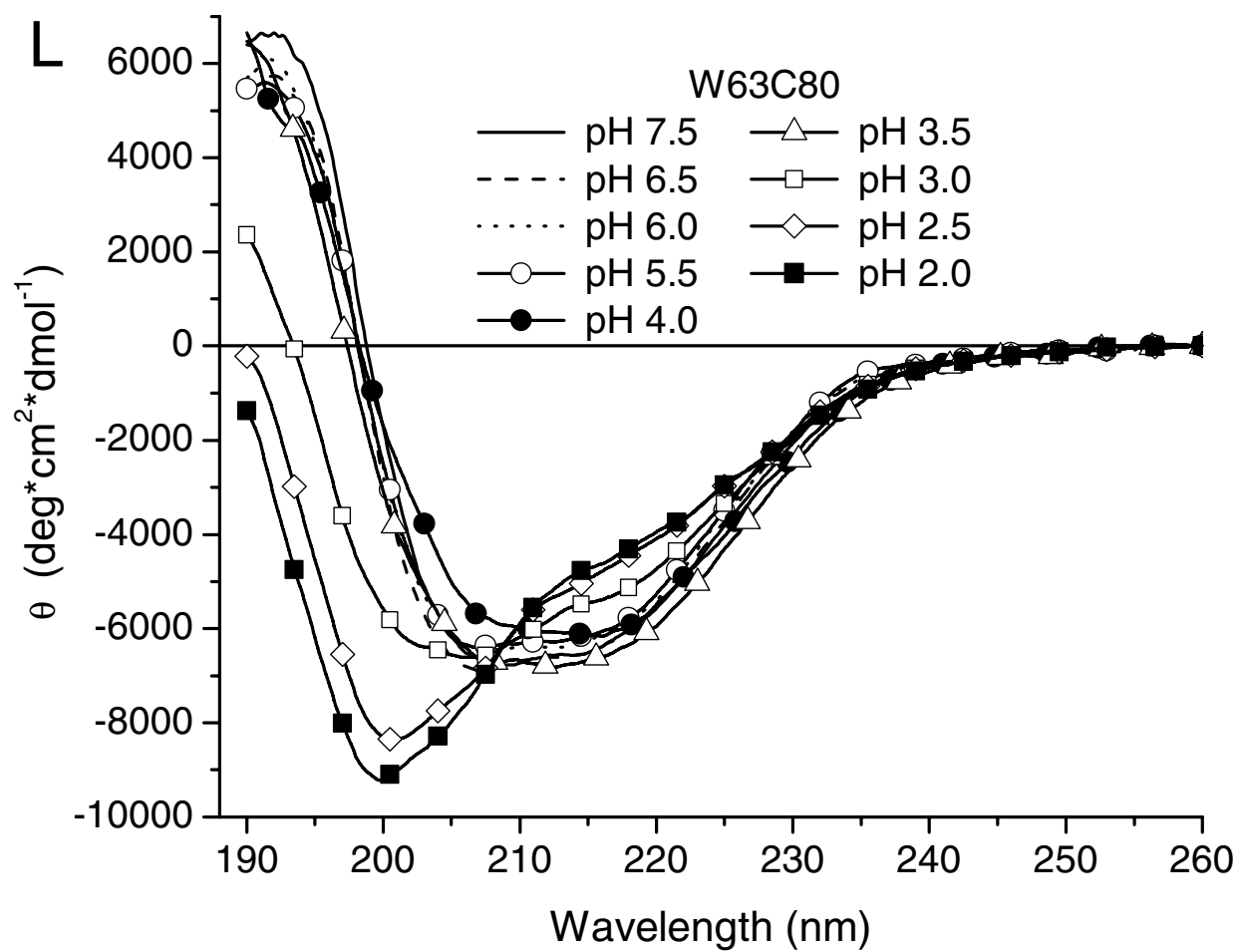


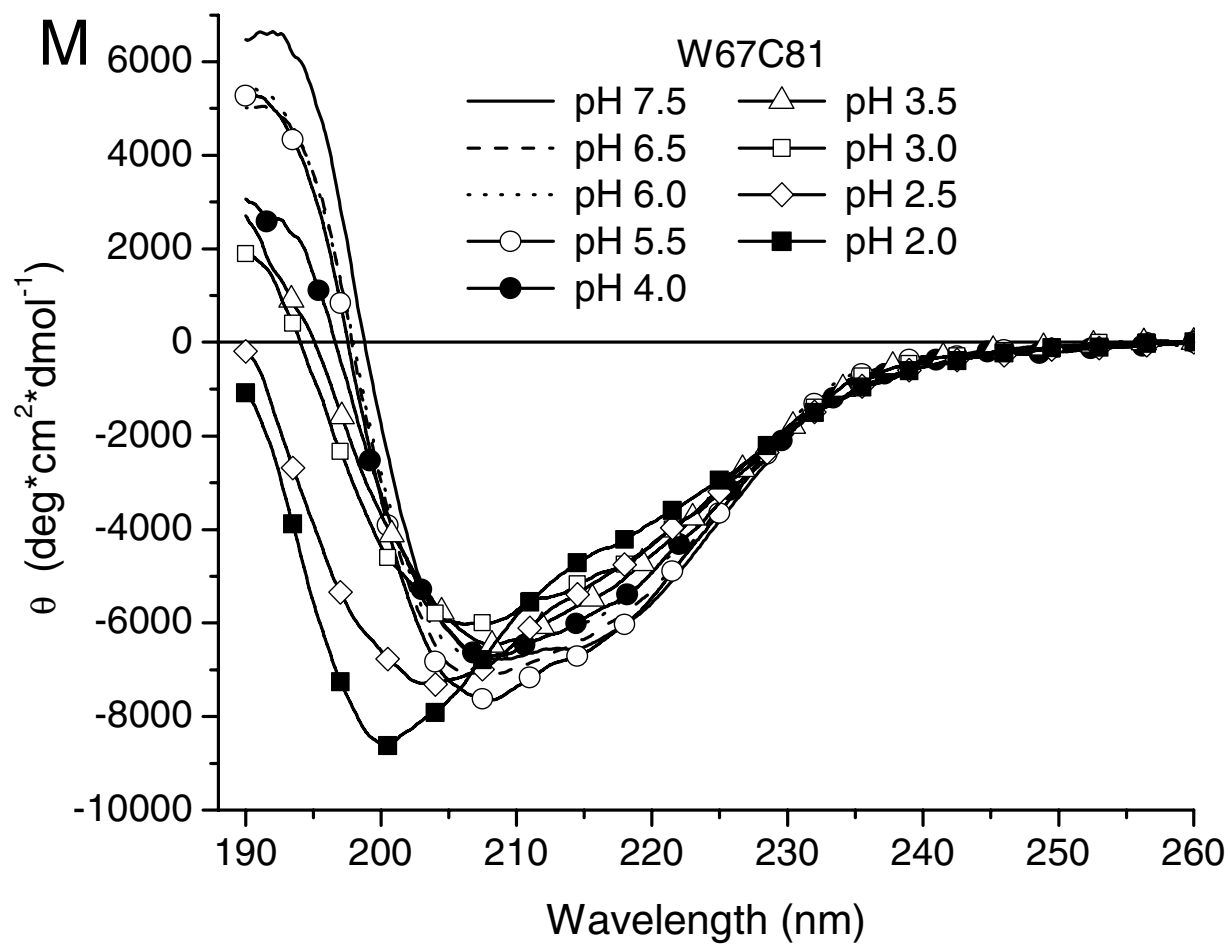


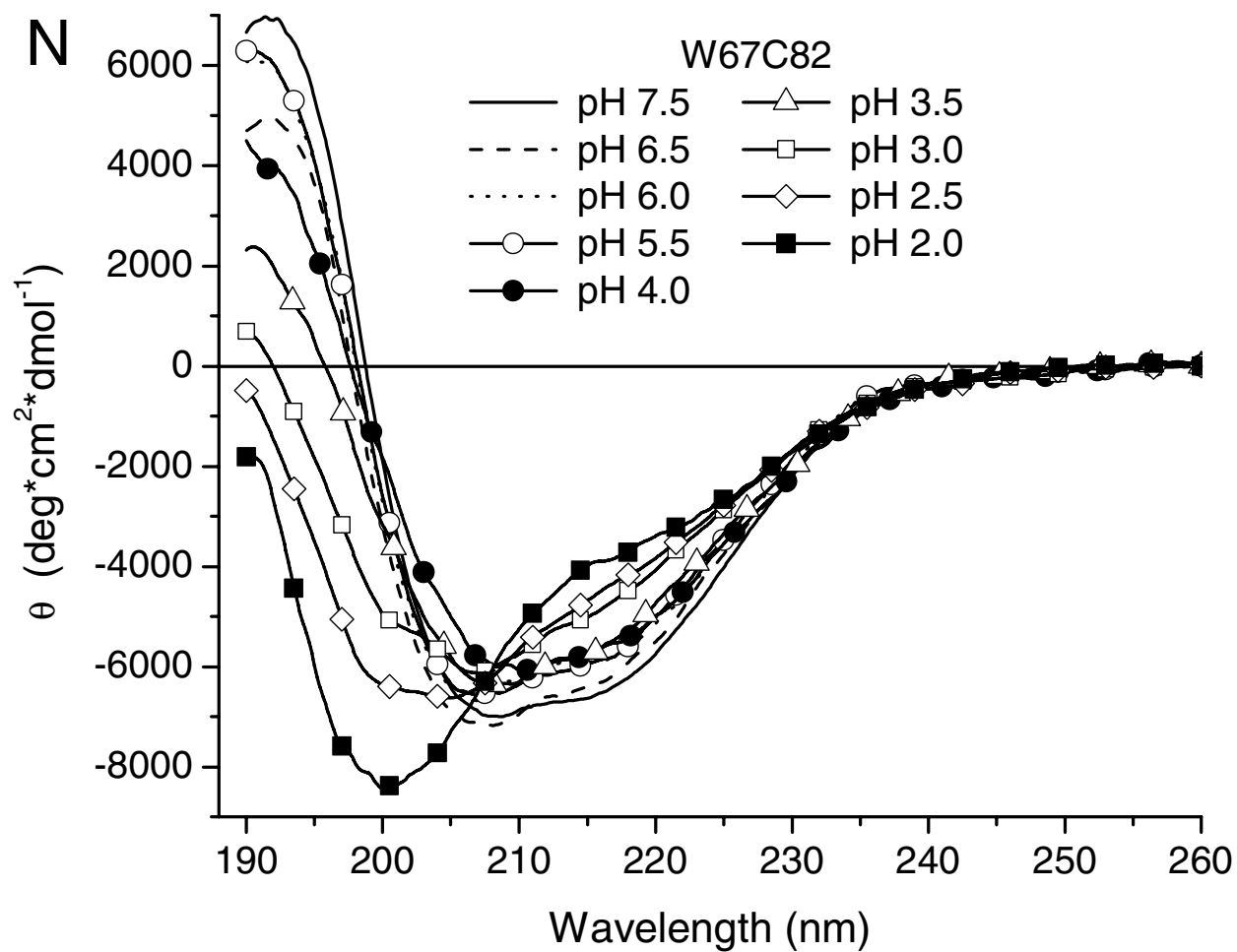




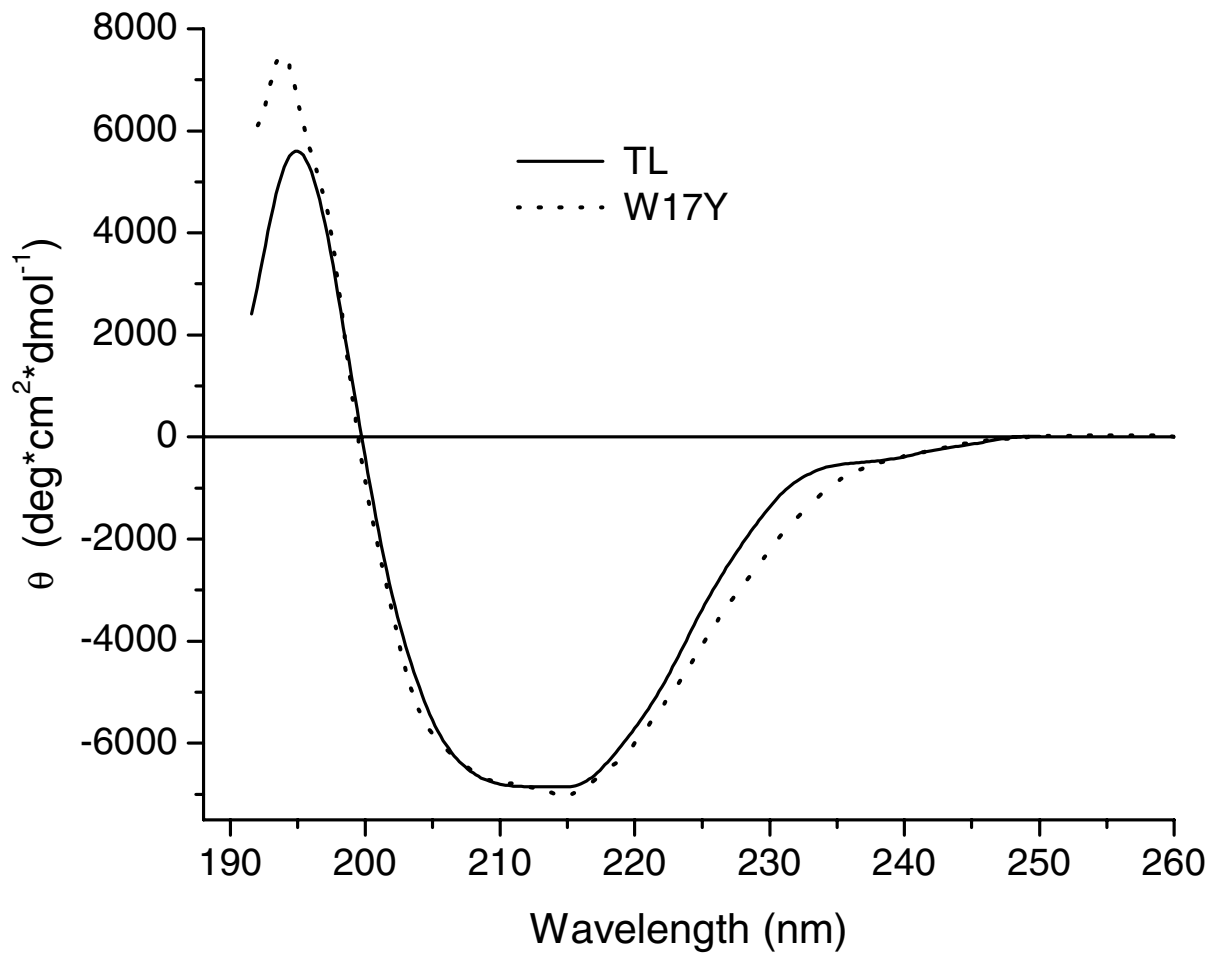




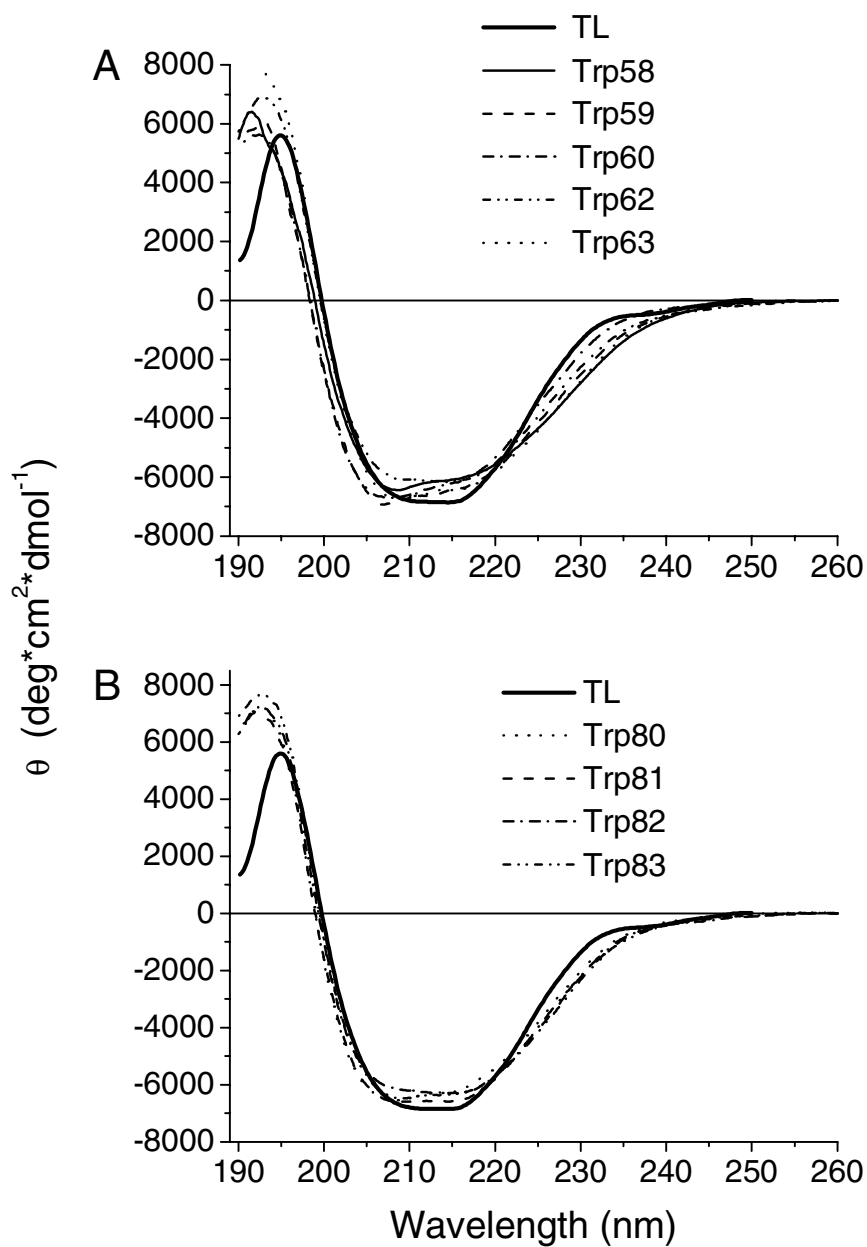




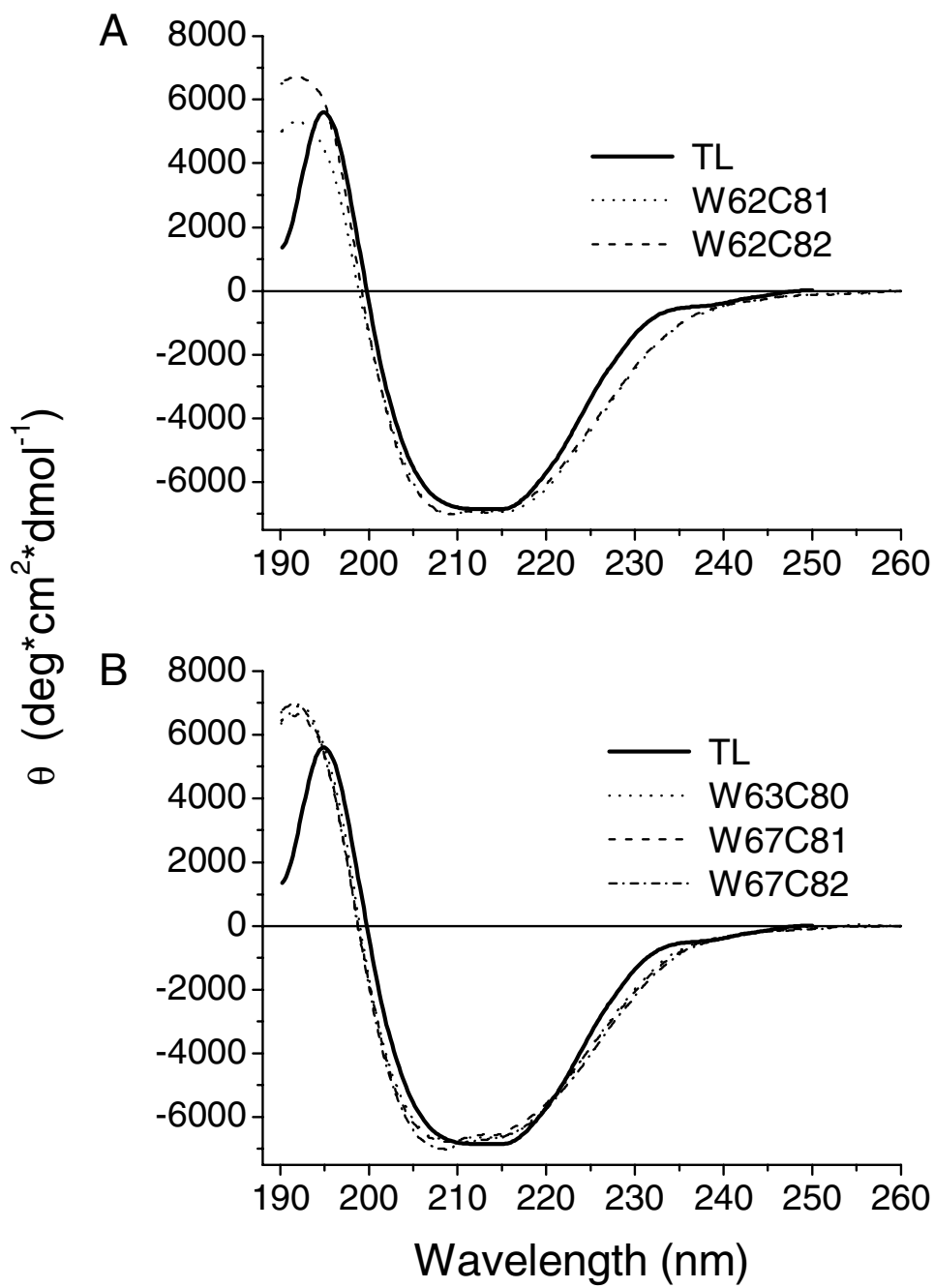
Supplementary Figure 1A-N. Far UV CD spectra of Trp and Trp/Cys mutants at pH 7.5-2.0. To show that the structure of the various Trp and Trp/Cys mutants are similar to TL over the entire pH range far-UV CD spectra were performed.



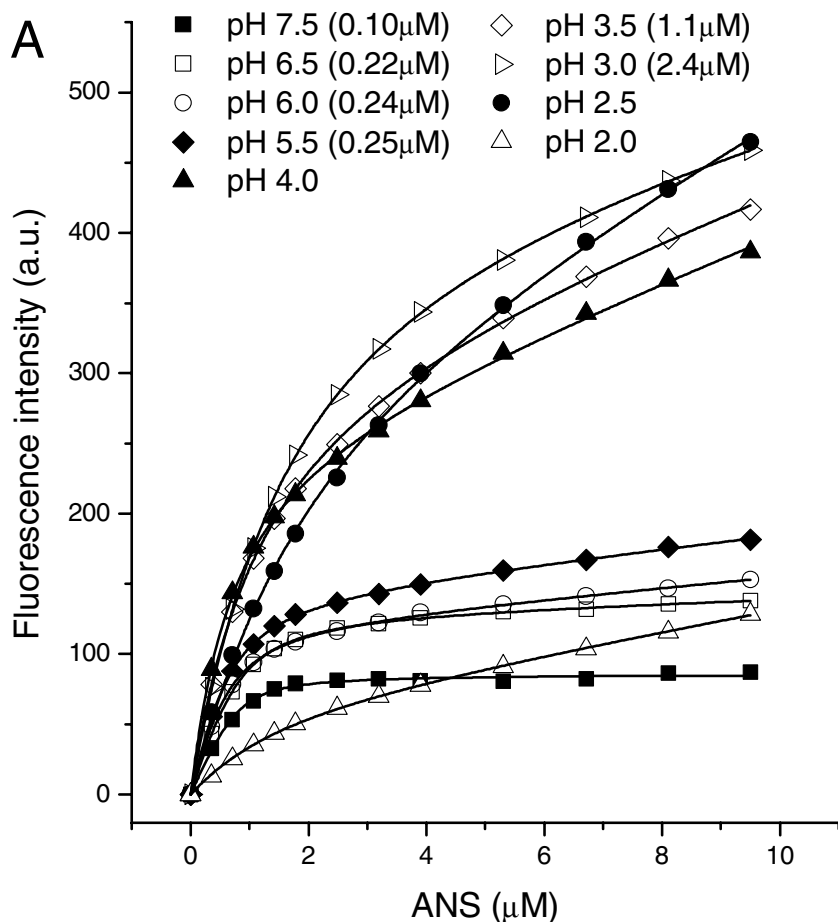
Supplementary Figure 2. Far UV-CD spectra of W17Y and TL at pH 7.5. W17Y is used as a template for all tryptophan mutations. Minor spectral differences at 220-235 nm are attributable to the loss of positive contribution to the far-UV CD from tryptophan 17 in the mutant protein



Supplementary Figure 3. Far UV-CD spectra of proteins with single tryptophan mutants at pH 7.5. Spectra of the proteins with single tryptophan mutations made in the interstrand loops CD and EF are shown in Figures 3a and 3b, respectively. β -sheet structure is evident in each of the mutants. In fact the spectra of EF strand mutants are nearly superimposable; the differences from TL are uniform among the mutants (Figure 3b).



Supplementary Figure 4: Far UV CD spectra of single Trp and single Cys mutants of TL at pH 7.5: (A) W62C81 and W62C82; (B) W63C80, W67C81, and W67C82. TL is shown for comparison.

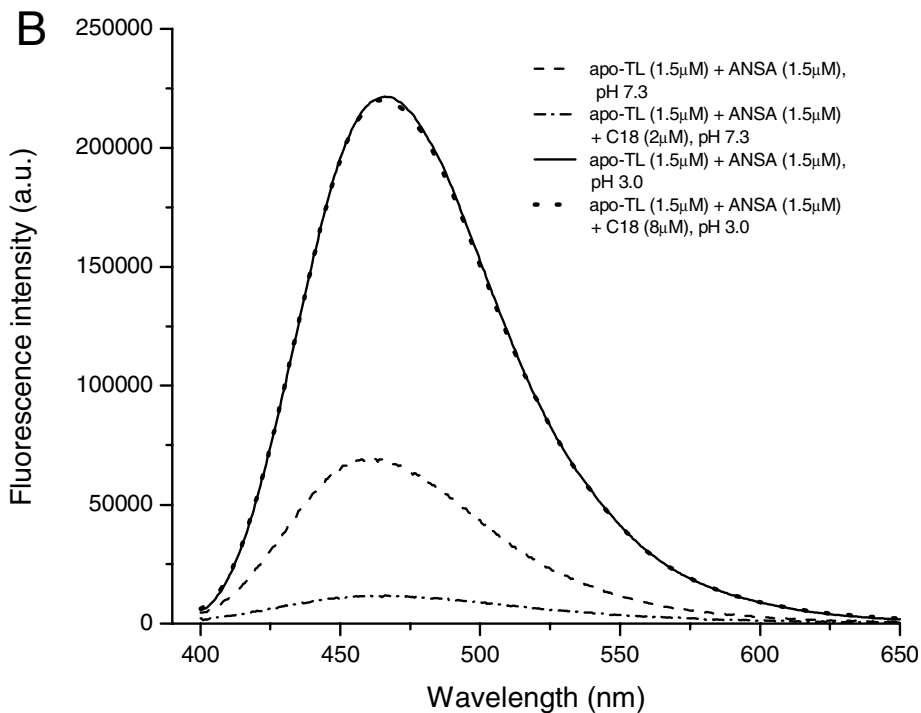


Supplementary Figure 5: (A) ANSA binding to apo-TL (1.2 μM) at various pH values monitored by ANSA fluorescence. K_d's, shown in parentheses, indicate greater affinity above pH 5.5 despite less fluorescent intensity.

ANSA binding to apo-TL shows 2 modes (Figure 5a). The nature of ANSA binding to apo-TL at low pH (3.0-4.0) is different from that observed at pH 5.5-7.5. Inspection of the plots reveals two distinct modes of binding. The mode of binding at pH 5.5-7.5 is characterized by saturation of binding at high ANSA concentration with a minor contribution, perhaps from non-specific binding, seen mainly at pH 5.5-6.5. The overall fluorescent intensity in pH range 5.5-7.5 is much lower than that observed at pH 3.0-4.0. At pH 3.0-4.0, saturation is not reached despite an 8 molar excess of ANSA compared to

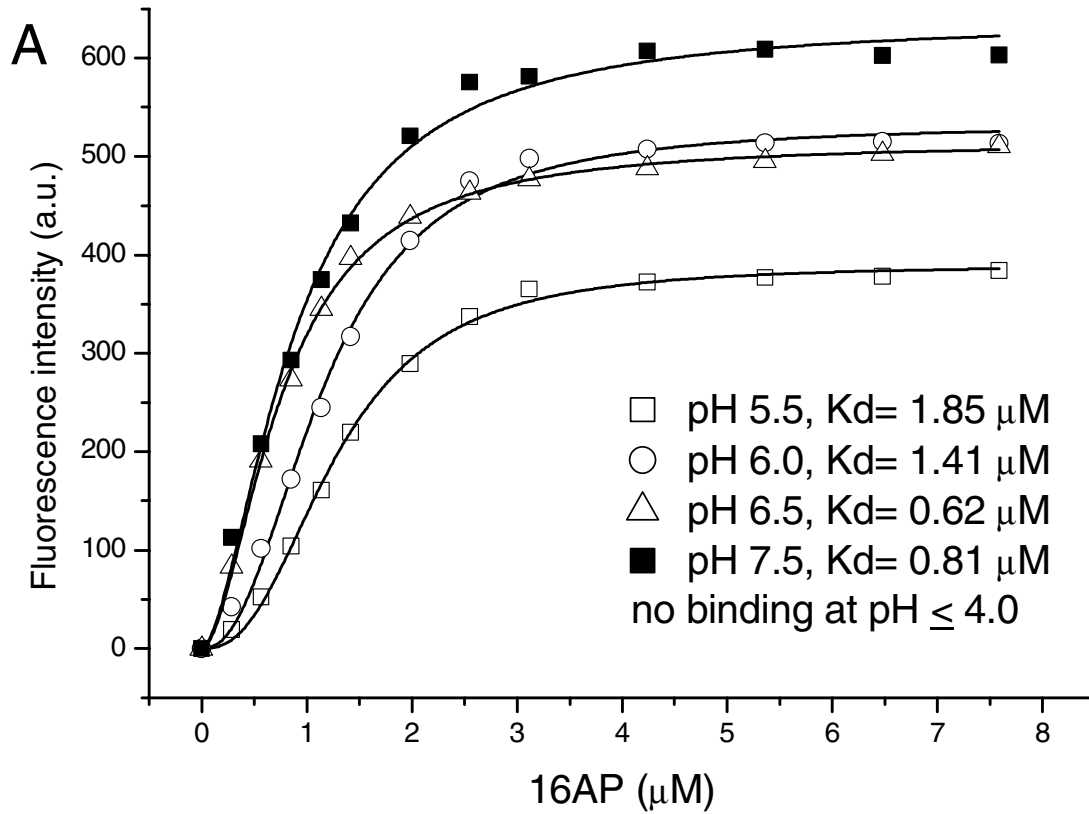
apo-TL. The K_d at pH 3.0-4.0 is an order of magnitude higher than at pH 5.5-7.5.

Therefore, the binding affinity at each pH range is different. The additional hydrophobic sites that emerge at acidic pH have less affinity for ANSA than what is observed at pH 5.5-7.5.



Supplementary Figure 5: (B) Fluorescence emission spectra. Competition of ANSA binding site of apo-TL by stearic acid at pH 7.3 and 3.0. The existence of distinct binding sites in TL for each mode was confirmed from the fluorescence spectral characteristics of bound ANSA and displacement of ANSA with stearic acid (Figure 5b). Stearic acid is known to bind exclusively in the cavity of TL (6, 7). At pH 7.3, the addition of stearic acid in equimolar concentrations to the apo-TL-ANSA complex resulted in nearly complete loss of fluorescent intensity, confirming that at neutral pH, ANSA is almost exclusively bound within the calyx of TL. The emission maximum at pH 7.3 is blue

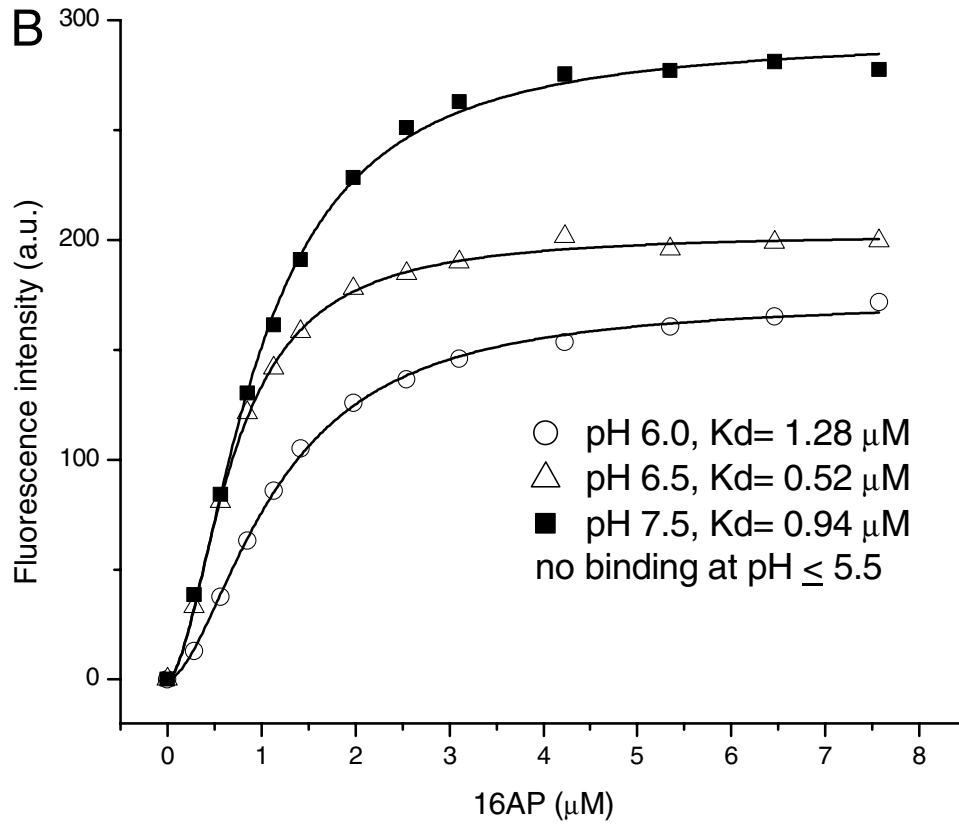
shifted ($\lambda_{\text{max}} = 461.3 \text{ nm}$) compared to that at pH 3.0 ($\lambda_{\text{max}} = 465.6 \text{ nm}$) indicating that the intracavitary site is more hydrophobic than the external binding site.



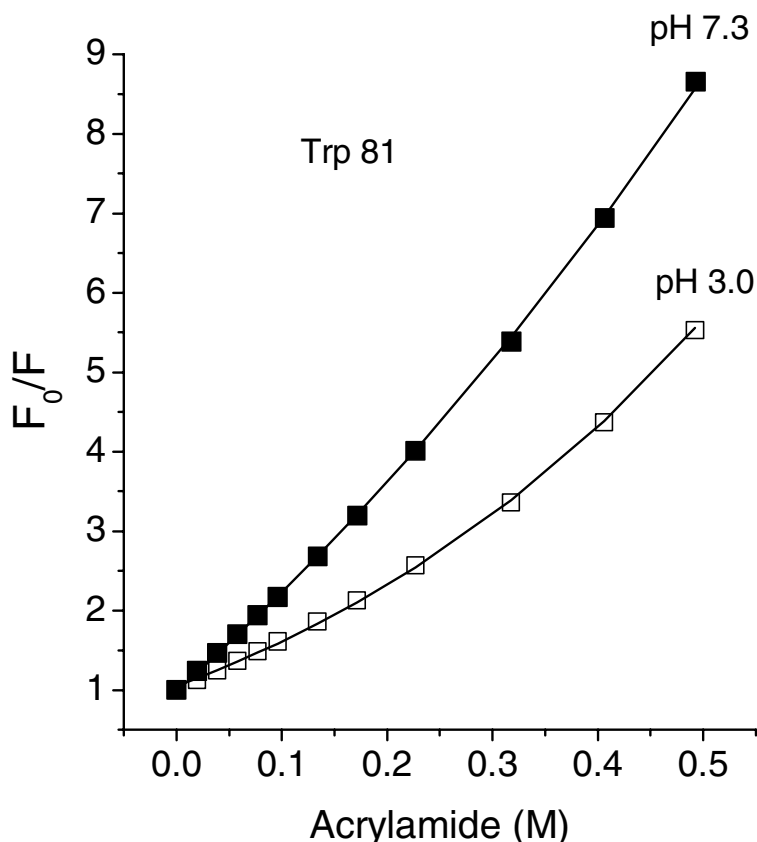
Supplementary Figure 6A: 16AP binding to apo-TL and apo-W59 (1.2 μM) mutant protein at various pH values monitored by fluorescence figures 6a and 6b, respectively. The titration curves of 16AP with all other loop single Trp mutants are quite similar (data not shown). Fluorescence titration curves are sigmoidal at low pH indicative of cooperative binding. But at pH 7.5 binding curves could be fitted satisfactorily with a formula for non-cooperative binding. At the pH values tested the degree of cooperativity ranged from 1.4-2.5 gradually increasing at lower pH. TL is presumably a dimer in pH 7.5 but has a tendency to form tetramers after delipidation (8). One possibility is that

cooperativity of binding at low pH reflects changes in the oligomeric state of the protein.

Binding of 16 AP with apo-TL and apo-W59 at varying pH.



Supplementary Figure 6 (B) 16AP binding to apo-W59 (1.2 μM) at varying pH.



Supplementary Figure 7: Acrylamide quenching of Trp fluorescence for mutant protein W81 pH 7.3 and 3.0. A typical Stern-Volmer plot that is representative of quenching experiments with tryptophan mutants I shown in Figure 7.

Supplementary References

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