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Selective Excitation of the $^1L_\alpha$ State of Tryptophan in Collagen-like Peptides Can Reveal the Formation of a Heterotrimer

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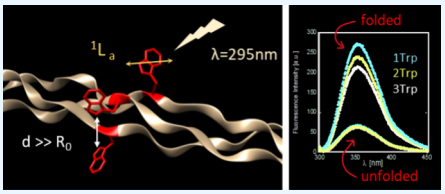
ABSTRACT: Fluorescence emission from tryptophan residues has been often used to probe the protein structure due to its transition dipole moment sensitivity to the local environment. We report the fluorescence study of a collagen-like peptide heterotrimer modified with the tryptophan in the X position $(X\text{-}Y\text{-}Gly)_n$ that shows the diminished fluorescence in a homotrimer versus a heterotrimer when the $^1L_\alpha$ state is selectively excited. This behavior is only observed in folded peptides, below the helix-to-coil transition temperature, and can be explained by long-range interactions between the tryptophans located on different strands within the triple helix, not by the change in the local environment. Our results suggest that tryptophan homotransfer is possible at distances much longer than the R_0 (0.5−0.7 nm) previously estimated. These observations imply that the energy transfer between the $^1L_\alpha$ states of proximal tryptophans can be facilitated by constraining their rotation by the helix and, thus, can be employed as a reporter of heterotrimer formation in biosensors.



INTRODUCTION

The triple helix is a characteristic secondary structure of the family of fibrillar collagens that can be found in skin, tendons, cartilage, and bones.[1] The most abundant is collagen type I, which forms a heterotrimer in the folded conformation. The heterotrimer is an assembly where three strands form a helix, and at least one of the strands has a different amino acid sequence from the other two. The triple helix of collagen type I is formed by the assembly of two α1 chains and one α2 chain. Small quantities of a collagen type I homotrimer, three α1 chains, found in adult skin and embryonic tissues, have been associated with multiple diseases like cancer, fibrosis, osteogenesis imperfecta, periodontal disease, and osteoporosis.[2] The homotrimeric form of collagen type I has an impaired ability to form fibrils and has increased resistance to proteases.[2]

In addition to naturally occurring collagen, the triple helix motif is also observed in the collagen-like peptides (CLP) that recently have been extensively studied as collagen models for structural investigations, disease models, functional materials, and sensing elements.[3−8] The triple helix is based on three peptide chains, polyproline type II turns (PPII-like), that are supercoiled and have a characteristic G-X-Y repeating sequence, where glycine residues form the core of the helix and X and Y positions are most commonly occupied by proline and hydroxyproline, respectively. In general, the X or Y position can be occupied by any residue, and both positions are characterized by easy access of the solvent to the side chains of the residues.[9]

The helix-to-coil transition (unfolding, loss of the helical secondary structure in favor of a random coil secondary structure) is irreversible in natural collagens, but it is reversible in CLPs and can be programmed by the choice of sequence and the number of residues in the sequence.[4] In addition, the three peptide chains can be either identical, forming a homotrimer after folding, or different, forming a heterotrimer either an ABB or ABC type, depending on the chain combination.[10] The reversible folding and formation of the heterotrimer enabled the development of fluorescence-based sensors for detection of collagen-related diseases.[11−13]

Fluorescence-based sensors and assays are one of the most versatile reporters in detection and identification of biomarkers, mutations, and structural changes of protein and peptides, which are linked to diseases.[14] Xiao's group showed that modifying a CLP peptide with a FAM (5-carboxyfluorescein) fluorescence probe at the N-terminus enables detection of specific single-point mutations based on fluorescence proximity quenching, which is avoided when a fraction of CLP trimers is in the heterotrimer assembly.[11] The same group has also shown that TPE (1-(4-carboxyphenyl)-1,2,2-triphenylethene) conjugated to peptides and combined with graphene oxide sheets can be used to detect charged CLP via aggregation-induced emission.[12] Wennemers' group recently studied CLP modified to chemoselectively target endogenous aldehydes generated by LOX (lysyl oxidases), which are associated with impaired tissue function in fibrotic and malignant diseases of connective tissues.[13] To image the

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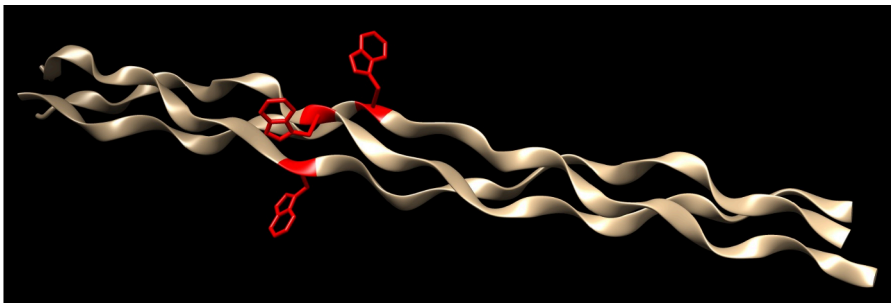
Scheme 1. Structure of the Folded Triple Helical Homotrimer Peptide with Tryptophan in the W13 Position (WOG)[a]



[a]In a heterotrimer, either two (1W) or one (2W) peptide strand is replaced with strands containing phenylalanine in the same position (F13).

location of cross-linked fibers in vitro and in vivo, the single CLP strand is modified with 3-carboxymethyl-6,8-difluoro-7-hydroxy-4-methylcoumarin (modified Pacific Blue dye), a probe that upon binding and reaction with LOX becomes fluorescent. Correct sensor operation depends on the possibility of forming a helix as a heterotrimer with fibrotic collagen in tissues.[13]

Tryptophan is one of the most frequently used fluorescence probes in the study of proteins due to its common presence in native proteins and ease of point mutation to install it within the native structure.[15] Its spectroscopic characteristics and sensitivity to the local environment make tryptophan an excellent intrinsic probe. This sensitivity originates in the large charge separation (changes in the transition dipole, the electric dipole moment associated with the transition between the two states) in the excited state and the presence of two overlapping singlet excited states: ^1L_a and ^1L_b.[16] Due to the different alignment (90°) between the two transition dipole moments, their interactions with the local environment are different. The fluorescence emission is possible from either of the states and is determined by the local environment.[16] Consequently, the presence of the two states can also complicate the interpretation of the data.[17,18]

Most published studies of the collagen triple helix with tryptophan as an intrinsic probe were limited to studies of excessive cross-linking often observed with aging and diabetes.[19] One exception is the study by Simon-Lukasik et al. who demonstrated that tryptophan-modified CLP can be used in detailed homotrimer studies of the structure and dynamics of CLP peptides using time-resolved fluorescence and fluorescence polarization.[20] These authors showed that tryptophan in folded CLP homotrimers exhibits a very long half-life for both positions X and Y and that position X allows for much larger unrestricted motion of the side chain; thus, it is better suited for sensor development.

Here, we present steady-state fluorescence studies of CLP modified with tryptophan in the X position (W13) and a complementary peptide modified with phenylalanine in the X position (F13) and folded into a homo- or heterotrimer (Scheme 1). When the ^1L_a state is selectively excited, differences in the intensity of fluorescence reflect the number of tryptophan residues in the heterotrimer and thus can be used as a reporter of heterotrimer formation. This observation was unexpected because the distance between the adjacent tryptophan residues is large in comparison to the typical distances of tryptophan energy transfer.

MATERIALS AND METHODS

Peptides. All peptides in this study were synthesized, purified, and characterized (HPLC, MS) at the Tufts Core Facility. In all peptides, except for FL8, the N-terminus was blocked by acylation and the C-terminus by amidation to prevent unwanted interactions. The FL8 peptide had an FITC tag conjugated to the N-terminus via a β-alanine-glycine-glycine (Aβ-GG) linker.

All peptides were annealed at 60 °C for 10 min, slowly cooled, and kept at 4 °C for 24 h to allow for trimer assembly. To prepare heterotrimers, the individual peptides were dissolved in a stock solution, combined in the desired ratio, and then annealed.

Fluorescence. All fluorescence spectra were acquired by using a Shimadzu RF-5301 spectrofluorometer with a xenon lamp as an excitation source. Spectra were recorded with 1 nm incremental increases, a slit width of 5, and slow mode acquisition. For temperature control, a water jacket cell holder was designed and made at the CSULB CNSM machine shop. Stock solutions of 0.5 mM peptides were prepared in a 10% DPBS buffer (pH 7.4, Dulbecco's), with or without urea (Fisher BioReagents), and placed in a 1 cm quartz cuvette. The final concentration of WOG peptides used in all experiments was 150 μM. Tryptophan, used as a fluorescence standard, was purchased from Sigma Chemical Co. and used without further purification. All the spectra were recorded in triplicate, where the calculated RSD for all fluorescence intensity measurements was between 0.3 and 0.7%.

Absorption. Absorption spectra were acquired with either a Shimadzu UV-1900i double-beam spectrophotometer or a VWR UV-1600PC single-beam spectrophotometer in a 1 cm quartz cuvette. Solutions were prepared as described for fluorescence studies.

Circular Dichroism. All CD experiments were performed using a JASCO J-810 spectropolarimeter equipped with a Peltier temperature control system containing a quartz cell. The annealed peptide solutions (20 μM) were transferred into a 2 mm path-length quartz cuvette, and a 50 nm/min scan rate was used to acquire the spectra. Folding and thermal stability studies were performed by monitoring the decrease in molar ellipticity at 225 nm in a temperature range of 15−60 °C at a ramp rate of 0.1 °C/min. The first derivative of the thermal unfolding curves was calculated using JASCO Spectra Manager II software to determine the peptide helix-to-coil transition temperature (T_m). Three repeated scans per sample were acquired.

Quantum Yield. Peptide solutions were prepared in the range of 2.5×10^{-5} to 1×10^{-4} M, and the same solution was

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used to measure absorption and fluorescence spectra in triplicates. Fluorescence spectra for relative QY measurements were integrated at energies between 290 and 500 nm. Tryptophan was used as the reference, with QY_W = 0.13 at 25 °C. QY values for the peptide (QY_{pep}) were calculated according to the equation:

$$QY_{pep} = QY_W \frac{Grad_{pep}}{Grad_W}\left(\frac{n_{pep}}{n_W}\right)^2$$

where "Grad" indicates the slope of the integrated intensity vs maximum absorbance plot and n is the refractive index of the solution (the same in peptide and tryptophan solutions).[21]

RESULTS

The application of self-assembly of fluorescent CLP to detect either point mutation in heterotrimer assembly or fibrosis in a collagen-rich tissue relied on conjugation of an external fluorescent probe to the CLP.[11,13] This type of fluorescent sensing is based on either proximity quenching (FAM) or enzyme activation (modified Pacific Blue) that is affected by folding into a trimer. We have demonstrated this behavior with peptides previously used in our lab (FL8, Table 1).[22]

Table 1. Peptide Sequences and Helix-to-Coil Transition Temperature (T_m)

name	peptide sequence	trimer	T_m (exp) (°C) (±0.5 °C)
WOG	$(POG)_4WOG(POG)_4$	homo	47.7
FOG	$(POG)_4FOG(POG)_4$	homo	50.3
1W	$(POG)_4WOG(POG)_4$: $(POG)_4FOG(POG)_4$	hetero, ratio = [1:2]	50.7
2W	$(POG)_4WOG(POG)_4$: $(POG)_4FOG(POG)_4$	hetero, ratio = [2:1]	50.1
FL8	FITC−A_βGG−$(POG)_8$	homo	63.7

When in a folded conformation (T < 63.7 °C), the fluorescence from FITC conjugated to the N-terminus is significantly quenched in comparison to the unfolded peptide (80 °C) (Figure 1, gray). For comparison, the effect of temperature on fluorescence emission of FITC is also shown (white).[23] If the trimer system is designed to fold into a heterotrimer and the peptide assembly has only one strand modified with FITC, then FITC fluorescence is not attenuated due to proximity quenching.[11]

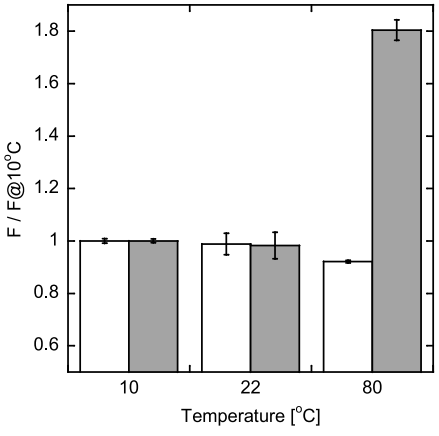

Figure 1. Normalized fluorescence intensity of FITC (white) and FL8 (gray). Normalization was performed with respect to fluorescence intensity at 10 °C. The error bars represent the standard deviation calculated from 3 independent measurements.

Often in the study of protein behavior, the intrinsic probe is preferred over an external fluorescence probe, and tryptophan is frequently used for this purpose. We used the two peptides, WOG and FOG (Table 1), that include either tryptophan (W) or phenylalanine (F) in the X position (W13 or F13). We chose these sequences based on the transition (helix-to-coil) temperature, T_m, predicted by the Brodsky model to have very similar T_m; thus, none of the peptides would be preferentially assembled as a homotrimer.[4] To promote assembly of the heterotrimer, the peptides WOG and FOG were combined in the desired ratio, annealed, and slowly cooled. Using circular dichroism (CD) spectroscopy, we measured T_m for homotrimers WOG and FOG and heterotrimers 1W (WOG•FOG•FOG) and 2W (WOG•WOG•FOG), see Figure 2. The CD spectra show characteristic features for CLP: $\pi-\pi^*$ amid transition at 197 nm and positive $n-\pi^*$ transition at 224 nm (Figure S1).[24] When the intensity of the 224 nm peak is monitored with an increase of temperature, the first derivative of the unfolding curve indicates the T_m of the peptide (Figure 2).

Peaks of the unfolding curves indicate that all the peptides have the same T_m = 50 °C (±0.5), with the small deviation of WOG, which has 47.7 °C (Table 2); thus, the predominant trimer in each solution is guided by the molar ratio of WOG and FOG peptides.

In the folded WOG homotrimer, the three W13 are positioned on the outside of the helix and, according to Simon-Lukasik et al., afforded fast side-chain motions with significant conformational freedom and exposure to the solvent (water).[20] Indeed, our measured maximum fluorescence intensity in folded and unfolded WOG peptides is the same (λ = 351 nm), and this wavelength indicates that the tryptophan environment is hydrophilic in both conformations. However, the fluorescence intensity of the unfolded peptide was significantly lower than that of the folded conformation. To fully unfold the peptide, we decided not to use thermal denaturation (due to the strong dependence of tryptophan fluorescence on collisional quenching) but rather to use urea as a denaturing agent.[25] Figure 3 shows our measured of fluorescence intensity of the WOG peptide with respect to the urea concentration. The fluorescence intensity decreases with an increase in urea concentration until an 8 M solution is reached, after which fluorescence intensity stays constant. Based on these findings, in all experiments that tested unfolded peptides, we used 8 M urea.

The intensity and wavelength of maximum fluorescence emission of tryptophan vary widely in protein systems.[15] In CLP, the position of W13 is well-established, and there is no surprise that the wavelength of maximum emission is unchanged upon unfolding since the environment of tryptophan in folded and unfolded peptides is very similar: fully exposed to water. We also observed, however, a significant change in the intensity of fluorescence emission between the folded and unfolded peptides. Thus, the relative quantum yield in the WOG peptide has been measured with comparison to tryptophan using the standard QY = 0.13 (Table 2 and Figure S2).[16,25] The QY of the folded WOG peptide is significantly higher (0.24) than the QY of tryptophan at the same temperature, as well as in the solution of urea (0.15). An increase in the temperature of the WOG solution causes a

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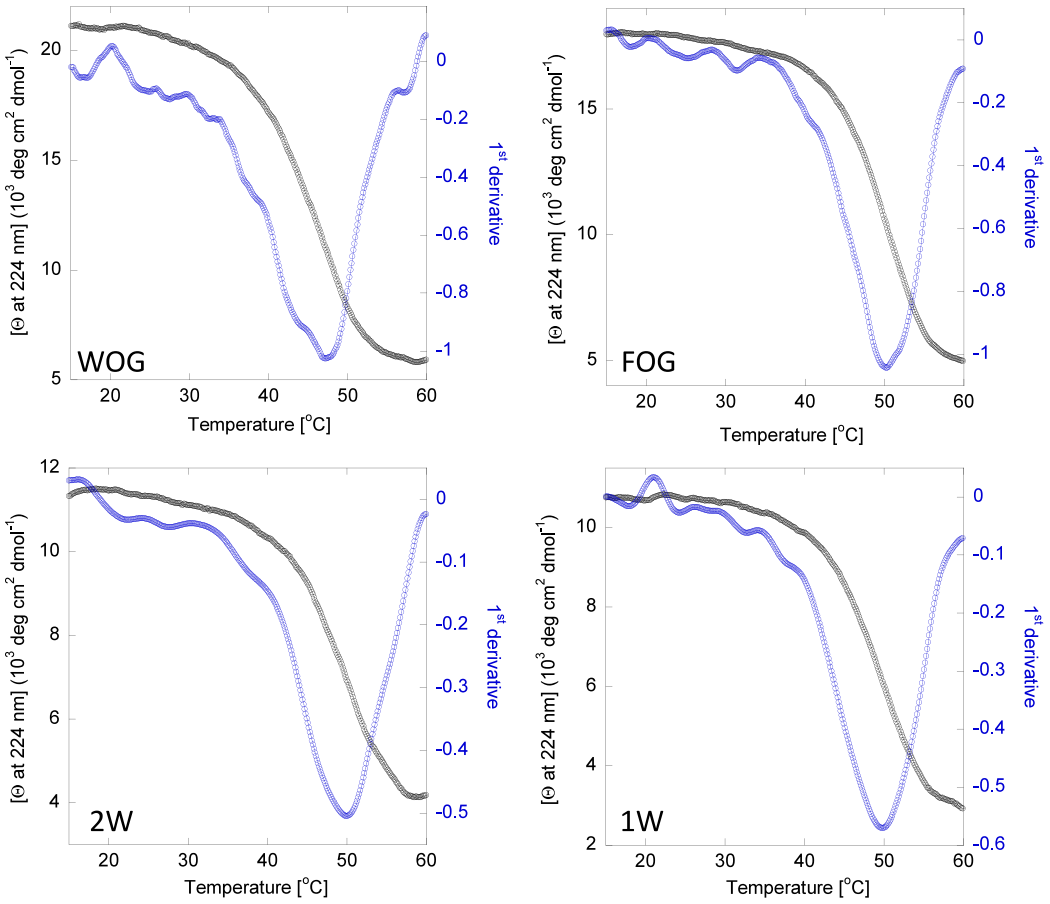

Figure 2. Molar ellipticity at 224 nm (black) of WOG, FOG, 2W, and 1W and the first derivative (blue). Thermal unfolding shows that the peak (224 nm) is eliminated in a cooperative transition that indicates a helix-to-coil transition.

Table 2. Relative Quantum Yield (QY) Measurements with Conditions[a]

peptide	conditions	slope	QY
WOG	25 °C	16,021 (R = 0.99)	0.24
WOG	25 °C, 8 M urea	10,279 (R = 0.98)	0.15
WOG	60 °C	1286 (R = 0.96)	0.02
tryptophan	25 °C	8657 (R = 0.98)	0.13#

[a]The reference probe is tryptophan at 25°C (0.13), indicated by #. The one-way ANOVA indicated that the slope$_{(WOG)}$ is significantly different from the slope$_{(tryptophan)}$; $p \ll 0.001$.

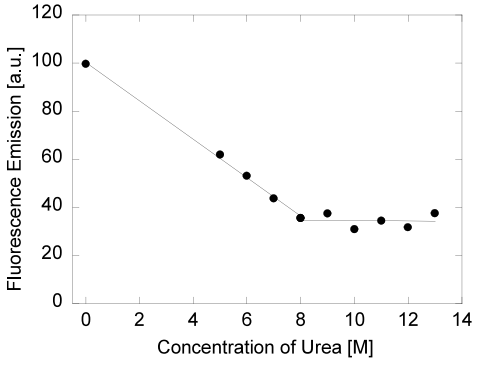

Figure 3. Maximum fluorescence intensity of the WOG peptide at λ_{EM} = 351 nm (λ_{EX} = 280 nm) in urea solutions. The error bars represent the standard deviation calculated from 3 experiments and are smaller than the points on the graph.

dramatic decrease in QY (0.02) as a result of peptide unfolding and an increase in collisional quenching. To determine if the increase in QY in the folded peptide is related to absorption or emission transition dipole moment, we compared the absorption spectra of WOG with and without urea (Figure 4). The same absorbance in both cases indicates that the absorption dipole moment is the same in folded (black) and unfolded (urea, white) peptides; thus, the increase in QY

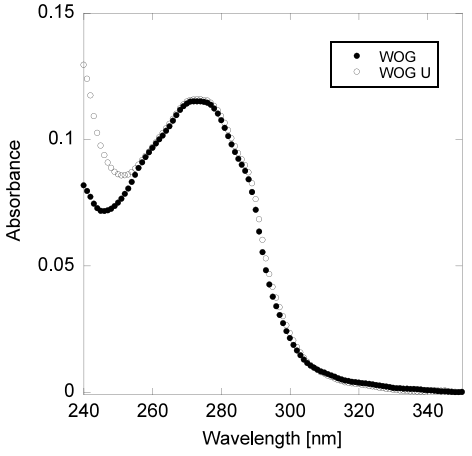

Figure 4. Representative absorption spectra of the WOG peptide without (black) and with (white) urea. The peptide concentration was 30 μM in a 10% phosphate buffer.

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observed in the folded WOG peptide must be related to the changes in the emission transition dipole moment. Therefore, any changes in fluorescence intensity must be related to the excited state of tryptophan in the WOG peptide.

Traditionally, protein with a tryptophan residue in the sequence as a fluorescence reporter is excited close to the maximum absorption wavelength at 280 nm.[15] In the WOG peptide, the excitation at 280 nm results in an emission spectrum with two peaks: small at 307 and large at 351 nm. To establish the most suitable excitation wavelength, the fluorescence emission was measured at 307 (black) and 351 nm (white) with respect to the excitation wavelength (Figure 5). The two selected excitation wavelengths resulted in either

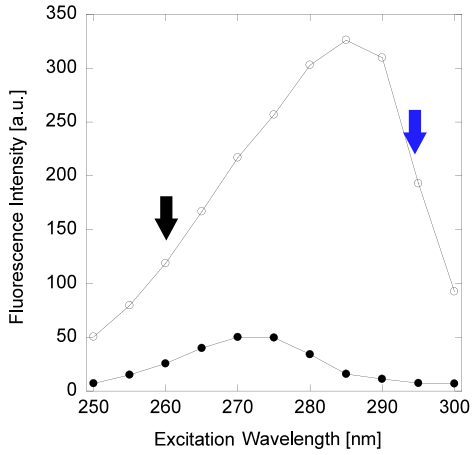

Figure 5. Fluorescence excitation spectra of the WOG peptide measured at λ_{EM} = 307 nm (black) and λ_{EM} = 351 nm (white) fluorescence emission wavelengths. The arrows point at two excitation wavelengths used in this work, λ_{EX} = 260 nm and λ_{EX} = 295 nm (see the text). Lines are added to guide the eyes.

double (260 nm excitation, black arrow) or single (295 nm excitation, blue arrow) emission peaks in WOG peptides. In Figure 6a, the normalized fluorescence intensity shows that application of 295 nm excitation (blue) eliminates the emission at 307 nm in the WOG peptide in a folded conformation. The same experiment performed in the 8 M urea solution containing WOG (Figure 6b) in the unfolded conformation results in similar behavior. Interestingly, when excitation is performed with 260 nm (black), the ratio of maximum fluorescence intensity of the 351/307 peak is significantly different in folded and unfolded states. In Figure 7, we compare the fluorescence intensity of WOG (black) in folded (a) and unfolded (b) states with the excitation wavelength at 260 nm. The peak at 351 nm drastically increases upon folding into a homotrimer, while the peak at 307 nm does not change. In addition to the WOG peptide, we have investigated the FOG (red) peptide homotrimer and heterotrimers 2W (green) and 1W (blue), Figure 7.

The FOG homotrimer incorporates phenylalanine in the F13 position, which is the same location as tryptophan in the WOG peptide. We observed a broad emission peak from phenylalanine between 285 and 310 nm when 260 nm excitation is used and a lack of emission with 295 nm excitation, which is expected for a phenylalanine residue (see normalized absorption spectra in Figure S3).[16] The heterotrimers 2W and 1W are formed by varying the ratio of WOG and FOG peptides, but the concentration of the WOG peptide is kept constant at 150 μM. In the solution containing urea (Figure 7b,d) and unfolded peptides, both excitation wavelengths result in the same fluorescence intensity at 351 nm for WOG, 2W, and 1W peptides. We view this as a control experiment to ensure that the differences in intensity observed upon folding are not related to differences in the peptide concentration. The emission peak at 307 nm (Figure 7a,b), present when excited with 260 nm, is a combination of fluorescence from tryptophan and phenylalanine. While the concentration of WOG is constant, the concentration of FOG must be adjusted proportionally to achieve the molar ratios of 2:1 and 1:2 in 2W and 1W, respectively. The intensity of the 307 nm peak follows changes in the concentration of the FOG peptide. Due to the overlap between the two emission peaks and required differences in the concentration of the FOG peptide, small differences in the intensity of the 351 nm peak in the folded state (Figure 7a) are difficult to interpret.

When 295 nm is used for excitation, there is a significant difference (Figure 7c) in the fluorescence intensity between homotrimer WOG and heterotrimers 2W and 1W. What is surprising is that the most intense peak is observed for the heterotrimer with a single WOG peptide, and the least intense is observed for the WOG homotrimer. The net concentration of WOG is identical in all solutions, which is confirmed by

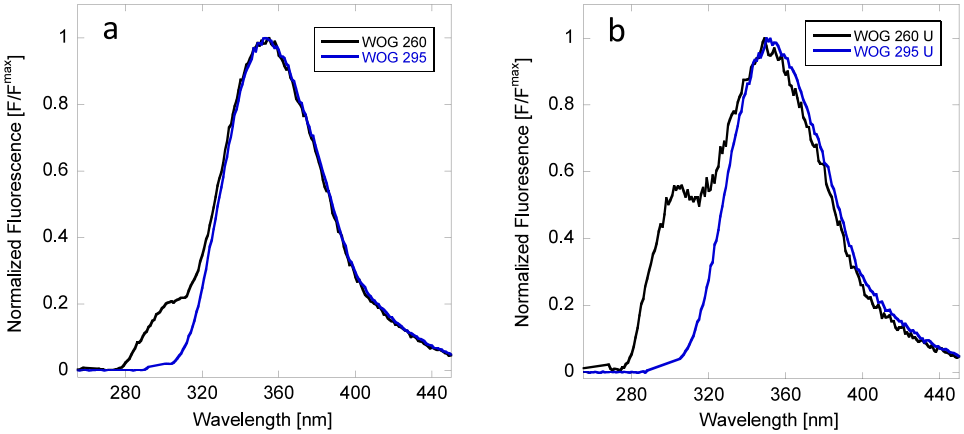

Figure 6. Normalized (F/F_{max}) fluorescence emission spectra of the WOG peptide in folded (a) and unfolded, 8 M urea, (b) states. The excitation was performed at λ_{EX} = 260 nm (black) or 295 nm (blue). The concentration of WOG was 150 μM in a 10% phosphate buffer.

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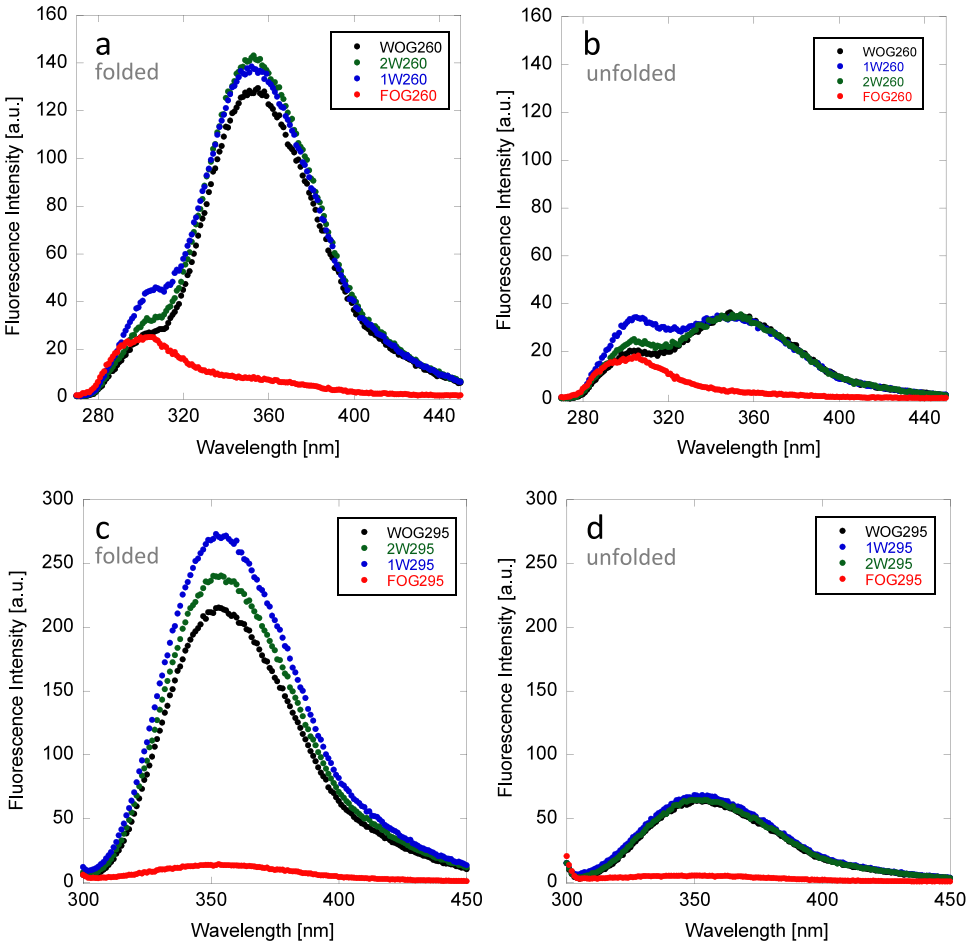

Figure 7. Fluorescence emission spectra of WOG (black), 2W (green), 1W (blue), and FOG (red) peptides excited with λ_{EX} = 260 nm (a,b) or λ_{EX} = 295 nm (c,d) in folded (a,c) or unfolded, 8 M urea, (b,d) states. The final concentration of all peptide solutions was 150 μM in a 10% phosphate buffer.

measuring the fluorescence of the solution after unfolding (Figure 7d).

The heterotrimer solutions 1W and 2W may contain small fractions of the homotrimers or the other heterotrimer. While the exact fraction of unwanted trimers cannot be calculated, we considered the effect of folding on maximum fluorescence intensity. In Figure 7c, one can assume that the maximum fluorescence intensity of WOG is associated with a 100% homotrimer. If one considers the heterotrimer 1W, it has the highest maximum fluorescence intensity of all the trimers; thus, if there are any other trimers present, they would lower that intensity. Therefore, the "cleaner" the solution, the larger the effect on the fluorescence intensity due to folding.

DISCUSSION

Homotrimer. In protein folding, one of the most used intrinsic probes used to study this process is tryptophan. Its fluorescence emission is sensitive to the local environment; thus, changes in fluorescence report the changes to the protein structure.[15] By incorporating residue W13 in CLP WOG, we observed a large increase of fluorescence after folding at 351 nm and no change in the emission wavelength. The lack of shift in the emission wavelength is consistent with the crystal structure of the CLP, where the side chain of the residues in the X position is directed to the outside of the helix.[9] Thus, in both folded and unfolded conformations, there is free access of the solvent (water) to the W13 residue; thus, no shift is observed.

The large increase in fluorescence intensity upon folding is most likely related to restricting rotation of W after the formation of the triple helical backbone, even though the X position affords significant rotational flexibility of W13 (18 low-energy conformers have been identified).[20] Restricting the rotation by forming a rigid-rod trimer will result in a significant increase of emission exemplified by the change in quantum yield between folded (QY = 0.24) and unfolded (QY = 0.15) states.[26]

Initially, to excite the WOG peptide, we used the excitation close to maximum absorption (Figure 4) at 280 nm and observed two fluorescence emission peaks (in both the folded and unfolded states): small at 307 nm and large at 351 nm. The intensity of the 307 nm peak was not dependent on folding, unlike the 351 nm peak. We decided to excite each of the transitions independently. We were not able to identify an excitation wavelength to isolate the 307 nm peak, but we were able to isolate the 351 nm peak by excitation at 295 nm (Figure 5, blue arrow). All possible excitation wavelengths to observe the emission at 307 nm are fully encompassed by excitation wavelengths that result in emission at 351 nm. To see both peaks at the smallest ratio, we used 260 nm excitation. Because the intensity of the 307 nm peak is not sensitive to WOG folding, while the intensity of the 351 nm peak is very

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sensitive (Figure 6), we concluded that the emission must originate from two different states.

The fluorescence spectrum of W in an aqueous environment and in proteins when exposed to a polar environment is characterized by single, redshifted broad emission with a maximum emission wavelength of 350−365 nm.[15] In nonpolar environments, the peak is blueshifted and develops a second smaller peak of 290−305 nm.[27] The change in emission spectra originates from the presence of two overlapping transitions to the ^1L_a and ^1L_b state (Figure S4).[28] While in the polar solvent, the lowest excited state from which the emission is observed is ^1L_a. By contrast, in a nonpolar environment, the states crossover, and the emission is observed from the ^1L_b state.[29] Several absorption anisotropy studies of tryptophan and its derivatives were able to decouple each state's contribution to total anisotropy, and the sharp peak (most redshifted) is associated with the transition to the ^1L_b state; thus, the second emission peak at 290−305 nm observed in nonpolar environments is identified as emission from the ^1L_b state.[30−32] Eftink et al. showed that the ^1L_a state fully encompasses the ^1L_b state, but the excitation at 295 nm preferentially excites only the ^1L_a state.[17] From these reasons, we believe that the emission peak that we observe at 307 nm is associated with the ^1L_b state. This hypothesis is also supported by the difference in intensities of the two peaks because the dipole moment associated with the ^1L_a state is larger than the ^1L_b state.[16]

Victorovich et al. compared the fluorescence emission of tryptophan for several proteins and observed two resolvable emission peaks: 330 and 360 nm in most systems and 308 and 330 nm in nonpolar solvents (hexane).[27] The authors explain their observation in terms of stabilized or destabilized ground and excited ^1L_a states; however, if we apply their proposed transition diagram to explain the emission that we observed, at 307 and 351 nm, we will have to assume that in the WOG peptide, we have emission from two states, the stabilized and destabilized excited ^1L_a state, to two ground states, stabilized and destabilized; this combination of states is not observed by Victorovich et al.[27] Moreover, the transition observed at 307 nm must represent the stabilized, by the interaction of the indole ring with the hydrophobic environment (van der Waals interactions), ground state, which is unlikely in our system.

Lastly, the energy difference between two emission peaks observed in our system is 4100 cm^{-1}, which is a very large separation if one assumes that the emissions at 307 and 351 nm represent emissions from two vibrational states of the ^1L_a excited state. In Victorovich et al.'s work, the maximum observed energy difference was 2795 cm^{-1}.[27] It is more likely that the peaks at 307 and 351 nm present in WOG peptides excited at 260 nm represent emission from two electronic singlet states, ^1L_a and ^1L_b, respectively.

Heterotrimer. Observation of reversible folding and unfolding of CLP has increased the understanding of collagen assemblies and its diseases and has launched several applications in which folding/unfolding plays an integral part of sensing processes. In both biological and sensing applications, the assembly process inevitably involves the formation of heterotrimers, where the sequences of two (ABB type) or three (ABC type) peptides are different. Thus, interactions between the residues within the CLP helix are attracting a great deal of attention.

Excitation of a heterotrimer with 260 nm results in the two fluorescence emission peaks at 351 (WOG) and 307 nm (WOG and FOG). In the folded and unfolded conformations, differences in intensity of the 307 nm peak seem to reflect differences in the concentration of the FOG peptide (Figure 7a,b). While the intensity of the 351 nm peak shows small differences, there is no clearly established dependence on the type of heterotrimer. When 295 nm excitation is used to excite the fluorescence in the folded heterotrimer (Figure 7c), there is a significant difference in intensity, with the lowest intensity observed in the trimer with three tryptophans and the highest for the heterotrimer with a single tryptophan, all in the W13 position. The presence of phenylalanine in the F13 position in heterotrimers ensures that the hydrophobic environment in the 13 position is the same in WOG, 2W, and 1W peptides and that the access of water to the W-site is the same as well. In addition, the assembly of the trimers into the different register trimers should also be identical due to the same character of the sequence, i.e., the same length, position of W or F, and T_m.[4,33] Moreover, the proximity of the peptide backbone, often implicated in the quenching of W emission in proteins and peptides, is also the same in all trimers.[34,35] Nevertheless, there are distinctive differences in the fluorescence intensity of the folded WOG, 2W, and 1W trimers. Since the excitation is performed at 295 nm, the only state that is excited is ^1L_a; thus, the differences in emission intensity of the 351 nm peak must be related to interactions between the tryptophans within the trimers. The increased number of tryptophans in the trimers lowers the intensity of the 351 nm peak: $I_{3W} < I_{2W} < I_{1W}$; therefore, the energy transfer between proximal tryptophans (homotransfer) must be possible since it results in fluorescence quenching.

Simon-Lukasik et al. observed fast depolarization in the time-resolved anisotropy but only in species with a long lifetime (8.4 ns), which is a predominant (95%) lifetime of the excited state in the WOG peptide.[20] The authors explained the depolarization as resulting from rapid side-chain rotational motion but considered the possibility of homotransfer between adjacent tryptophans. The distance between adjacent tryptophans in the WOG peptide (from the crystal structure, 1.1−1.7 nm) is two to three times the R_0, the Forster energy transfer distance, (0.5−0.7 nm) estimated for tryptophan homotransfer; thus, they concluded that homotransfer is an unlikely, but not impossible, event.[36] In the CLP heterotrimer system, the side-chain rotational motion of tryptophan is the same in each trimer as their position and environment are the same, yet the intensity of the fluorescence is different. If one assumes that the fluorescence intensity of folded WOG is a baseline, then the increase of intensity observed in heterotrimers 2W and 1W can be interpreted either as removal of a quencher (energy homotransfer between adjacent tryptophans) or addition of the donor (in this case, phenylalanine) to provide additional energy transfer to tryptophan. If the latter was true, the increase in fluorescence intensity would be much greater with 260 nm excitation, when the phenylalanine is fully excited, which is not the case. In addition, the energy transfer between phenylalanine and tryptophan is difficult due to a very small spectral overlap, but it has been observed by Golbik et al. in the collagen type IV heterotrimer.[37] The transfer efficiency was only 13%, and the calculated distance between the probes was 2.605 nm.[37] The FRET was observed only if the applied excitation was 260 nm. When the excitation was performed at 295 nm, phenylalanine did not participate in energy transfer. Thus, in our case, the observed differences must originate in the energy homotransfer between adjacent tryptophans, even

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though the distance between them is large, as Golbik et al. showed that these distances are accessible within the triple helix.[37]

It is also worth noting that the FOG peptide shows, especially in a folded conformation, a very broad, low intensity peak at 349 nm, which is not expected for phenylalanine.[16] It presents itself as a shoulder with 260 nm excitation and a peak with 295 nm excitation. Because the fluorescence intensity is additive, we considered that the differences in heterotrimer fluorescence can simply originate from the addition of WOG and FOG fluorescence, but the analysis presented in Table 3

Table 3. Analysis of the Fluorescence Intensity in WOG, FOG, 2W, and 1W Peptide Trimers

	ratio		fluorescence intensity [a.u.]			
folded peptide	WOG	FOG	I_{total} (exp)	I@351 WOG	I@351 FOG	W→W ET (calc)
WOG	2	0	210	210	0	33.4
FOG	0	2	12.8	0	12.8	0
1W	2	4	269	243.4	25.6	0
2W	2	1	240	233.6	6.4	9.8

shows that the intensity of the FOG peak at 351 nm is too low to explain these differences. The origins of the 349 nm peak in the FOG trimer are not clear. Higher-order assemblies that are based on phenylalanine have been shown to exhibit a fluorescence peak that is redshifted, as in our system, and are characterized as a J-aggregate formed due to $\pi-\pi$ stacking and hydrophobic interactions.[38] However, the fluorescence emission from J-aggregates is characterized by a narrow bandwidth, which we do not observe, and sterically, it would be possible only as an intramolecular assembly, which we did not observe. Thus, we cannot claim that the 349 nm peak observed in the FOG trimer is due to formation of J-aggregates.

CONCLUSIONS

We observed energy homotransfer between the adjacent tryptophans in CLP trimers at lengths much longer than reported R_0 when excitation involved only the ^1L_a state, which allows a distinction between homo- and heterotrimers. Additionally, these findings show that energy transfer in folded protein systems may greatly exceed the calculated distances for energy transfer. These observations imply that energy transfer between the $^1L_\alpha$ states of proximal tryptophans can be facilitated by constraining their rotation by the helix; thus, it can be employed as a reporter of heterotrimer formation in biosensors. The signal generation is based on the energy homotransfer between proximal tryptophans; thus, the presence of phenylalanine, rare in collagen, is not necessary to detect the formation of heterotrimers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c03600.

Supplementary figures including circular dichroism spectra, QY determination, absorption spectra, and electronic state diagram in tryptophan (PDF)

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Author Contributions

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ABBREVIATIONS

CLP, collagen-like peptide; P, proline; O, hydroxyproline; G, glycine; W, tryptophan; F, phenylalanine; A_β, beta-alanine; FITC, fluorescein; CD, circular dichroism; QY, quantum yield; R_0, Förster distance of the pair of a donor and an acceptor

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