Intramolecular Quenching of Tryptophan Fluorescence by the Peptide Bond in Cyclic Hexapeptides

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Abstract: Intramolecular quenching of tryptophan fluorescence by protein functional groups was studied in a series of rigid cyclic hexapeptides containing a single tryptophan. The solution structure of the canonical peptide c[β-PyTWF] (Py, phosphotyrosine) was determined in aqueous solution by 1D- and 2D- 1H NMR techniques. The peptide backbone has a single predominant conformation. The tryptophan side chain has three χ1 rotamers: a major χ1 = −60° rotamer with a population of 0.67, and two minor rotamers of equal population. The peptides have three fluorescence lifetimes of about 3.8, 1.8, and 0.3 ns with relative amplitudes that agree with the χ1 rotamer populations determined by NMR. The major 3.8-ns lifetime component is assigned to the χ1 = −60° rotamer. The multiple fluorescence lifetimes are attributed to differences among rotamers in the rate of excited-state electron transfer to peptide bonds. Electron-transfer rates were calculated for the six preferred side chain rotamers using Marcus theory. A simple model with reasonable assumptions gives excellent agreement between observed and calculated lifetimes for the 3.8- and 1.8-ns lifetimes and assigns the 1.8-ns lifetime component to the χ1 = 180° rotamer. Substitution of phenylalanine by lysine on either side of tryptophan has no effect on fluorescence quantum yield or lifetime, indicating that intramolecular excited-state proton transfer catalyzed by the ε-ammonium does not occur in these peptides.

Tryptophan fluorescence has been extensively studied to test peptide and protein conformation changes, due to the sensitivity of both emission wavelength and intensity to the local environment of the indole chromophore. The wavelength of maximum emission ranges from about 310–350 nm, depending on the electrostatic environment. The fluorescence quantum yield ranges from near zero to 0.35 with lifetimes up to 10 ns. The large variations in intensity and fluorescence lifetime τ are due to nonradiative pathways that compete with emission for deactivation of the excited state.

\[
\tau^{-1} = k_r + k_{nr}
\]

where \( k_r \) and \( k_{nr} \) are the radiative and nonradiative rates. In addition to the intersystem crossing rate \( k_{si} \), the nonradiative rate of indole contains contributions from three environmentally sensitive processes, solvent quenching \( k_{si} \), excited-state proton transfer \( k_{pt} \), and excited-state electron transfer \( k_{et} \), which depend on the proximity of water, proton donors, and electron acceptors.

\[
k_{nr} = k_{sc} + k_{si} + k_{pt} + k_{et}
\]

Six protein functional groups representing eight amino acid side chains plus the peptide bond quench 3-methylindole fluorescence in intramolecular quenching experiments. Most peptides and proteins containing a single tryptophan have multieponential fluorescence decays. Although excited-state processes are still invoked to account for the complexity, the multiple fluorescence lifetimes are generally attributed to ground-state heterogeneity. The rotamer model proposes that the multiple lifetimes represent multiple ground-state rotamers of the tryptophan.

tophan side chain. 2,14,15 There are six low-energy canonical conformations dictated by the torsional angles about the C3N=C4C5 (γ1 = ±60°, 180°) and C5C6-C7 (γ2 = ±90°) bonds. If proximity of the indole ring to water or quenching functional groups differs among rotamers, those rotamers will have different fluorescence lifetimes. If interconversion among rotamers is slower than the fluorescence time scale, the fluorescence decay will be multiexponential with relative amplitudes proportional to the rotamer populations. For small peptides in solution or bound to membrane vesicles, several groups have proposed that the two or three fluorescence decay components represent χ1 rotamers. 16–19 The three decay amplitudes of [Trp3]oxytocin were correlated with χ1 rotamer populations determined by 1H NMR. 20 In addition to rotamers of the tryptophan side chain, microconformational states of peptides and proteins with different local environments of the indole ring constitute a source of ground-state heterogeneity. Finally, relaxation of the protein matrix surrounding the indole ring during the lifetime of the excited state would also produce a complex decay.

This contribution tests the rotamer model in a cyclic hexapeptide containing a single tryptophan and five other amino acids whose side chains do not quench 3-methylindole fluorescence. The cyclic peptide is closely related to a somatostatin analogue previously shown to have a rigid backbone in organic solvent. 21 The solution structure of the cyclic peptide in aqueous solution is determined by 1D- and 2D-1H NMR. The tryptophan fluorescence is characterized by steady-state and time-resolved techniques, and the dependence on solvent isotope and temperature is determined. Fluorescence decay amplitudes are correlated with the NMR-determined χ1 rotamer populations of the tryptophan side chain. Excited-state electron-transfer rates for intramolecular quenching of the six χ1, χ2 rotamers by the peptide bonds are calculated from Marcus theory using distances estimated by molecular modeling and parameters estimated from the electron-transfer rate of N-acetyltryptophanamide (NATA). The fluorescence lifetimes of the six rotamers are calculated using these electron-transfer rates together with experimental values for the intersystem-crossing and water-quenching rates previously determined for NATA. 7 The observed fluorescence decay is interpreted in terms of the χ1 rotamer populations and the calculated χ1, χ2 rotamer lifetimes.

Experimental Section

Materials. Oxime resin was either purchased from Novabiochem or synthesized. 22 N-α-N-Boc-α-proline, -l-phenylalanine, -l-tryptophan, -l-threonine, and -l-leucine, N-α-N-Boc-Nα-benzoylcarbonyl-l-lysine, 2-[ (H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)], α-hydroxybenzotriazol-1-yloxytrichloroethane (HOBt) were purchased from Novabiochem. N-α-N-Boc-α-phosphoserine diisopropyl ethylamine was purchased from Advanced Chemtech. O-(2-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was purchased from Perceptive Biosystems. All other reagents and solvents were highest grade available.

Synthesis. Peptides were synthesized manually using oxime resin at 25 °C. 23 The oxime resin offers two advantages for the synthesis of small cyclic peptides containing tryptophan: simultaneous cyclization and cleavage from the resin under mild conditions and efficient isolation from multimeric side products. 24 The disadvantage of using Boc resin is that the repetitive trifluoroacetic acid (TFA) deprotection may result in modification of peptide side chains. The extent of cyclization using the oxime resin is highly sequence-dependent. 25 Therefore, the amino acid that was coupled to the resin was permitted in the sequence c[DPpYTFWF] except for d-Pro and pTyr (phosphotyrosine, pY). The synthesis starting with Thr gave the best overall yield of purified peptide. Thr (3 mmol) was coupled to 1 g of resin (substitution level 0.3–0.5 mmol/g) using 0.35 mM diisopropylcarbodiimide in 6 mL of CH2Cl2 for 24 h. The solvent was removed, and unreacted sites on the oxime resin were capped by addition of 6 mL of dry dimethylformamide (DMF) containing 0.2 M acetic or trimethylacetic anhydride and 0.3 M diisopropylethylamine (DIEA). The coupled amino acid was deprotected with 25% TFA in 4–6 mL of CH2Cl2 for 15–20 min. Amino acid coupling reactions were carried out by shaking at room temperature for 45 min in 6 mL of dry DMF containing 0.25 M HBTU, 0.1 M HOBt, and 0.5 M DIEA, except for d-Pro where HATU replaced HBTU. The extent of coupling was monitored by a ninhydrin color test on a small aliquot of resin after each step. 26 After the last deprotection, the peptide was cyclized and cleaved from the resin by reaction for 48 h with 0.5 M acetic acid and 1.5 M triethylamine in 6–8 mL of dry DMF. 27 For c[DPpYTFWF] and c[DPpYTFFL], the organic phase was removed by filtration and added dropwise to cold diethyl ether, and the white precipitate was removed by centrifugation. Alternatively, the DMF was removed by vacuum distillation, which gave a viscous orange residue. For c[DPFTK(z)WF] and c[DPFTFK(z)], the organic layer was added dropwise to cold water, and the white precipitate was removed by centrifugation. The resulting pellet or residue was dissolved in a small volume of 1:1 CH2Cl2/H2O and purified by RP-HPLC on a Vydac C-18 semi-prep column using a gradient of CH3CN/0.1% TFA: 20–55% for 60 min for c[DPpYTFWF] and 40–100% for 40 min for the other peptides.

Deprotection of the ε-amino group of lysine was done by catalytic hydrogenation. The peptide was dissolved in a minimal volume (5–10 mL) of glacial acetic acid and placed in a hydrogenation flask. A catalytic amount of 10% Pd on carbon was added, and the suspension was purged with argon three times and then charged to 50 psi with hydrogen and shaken overnight. After the hydrogenation was complete, the Pd catalyst was filtered off, and the flask was washed five times with small aliquots of ethyl acetate. The ethyl acetate/acidic acid solvent was removed under vacuum to yield the crude, yellow deprotected peptide. The peptides were purified by HPLC using a gradient of 20–55% CH2CN/0.1% TFA for 60 min. Peptide molecular weights were verified by MALDI-MS. Yields of purified peptide were 8–17% relative to the substitution level of the oxime resin. The low yields are probably due to TFA-mediated side chain reactions or cleavage of the linear sequence during the cyclization step.

3H Nuclear Magnetic Resonance. All NMR experiments were performed using a Varian Inova 600 MHz spectrometer. For experiments in H2O, 2 mg of peptide was dissolved in 600 μL of 10 mM phosphate buffer, 1 mM NaN3, 10% D2O and pH was adjusted to 3.5 with dilute HCl. Chemical shifts were referenced to 3-(trimethylsilyl)-propan-2,2,3,3-d4 (TSP). For experiments in 100% D2O, 1 mg of...
peptide was dissolved in 270 μL of D2O (Cambridge Isotope) in a Shigemi NMR tube. Chemical shifts δ of amide protons were measured in 5° increments from 5 to 55 °C. Temperature coefficients −dδ/dT were determined from the slope of a plot of chemical shift vs temperature by least-squares.

Spin-type assignments for c[α-PpYTFWF] were made from TOCSY spectra27,28 that used a mixing time of 70 ms, spectral width of 7000.4 Hz, and 2048 data points in 256 t1 increments of 24 transients. Water suppression was performed by low-power irradiation with a presaturation delay pulse of 1 s. NOESY spectra29 used mixing times up to 300 ms and WATERGATE water suppression sequence.30 NOESY spectra were acquired with 1024 data points in 256 t1 increments of 32 transients. Interproton distances from NOESY experiments were calculated using the isolated two-spin approximation theory.31 A reference distance of 2.82 Å from the indole NH to H7 of the indole ring was taken from the crystal structure.32 PE-COSY spectra33 were acquired with a spectral width of 6600 Hz and 4096 data points in 440 t1 increments of 80 transients.

NMR data were processed using the Felix program (Molecular Simulations, Inc.) on an INDY-IP22 work station (Silicon Graphics, Inc.). Convolution difference water deconvolution was used to remove the residual water signal in the spectra. Data were routinely zero-filled to 2K data points in both dimensions, with the exception of the PE-COSY, which was filled to 8K in D1 dimension and 2K in D2 dimension. In all spectra, a Gaussian and a 90°-shifted sinebell squared apodization function were used in the D1 and D2 dimension, respectively. The data were then phase corrected and baseline corrected.

Structure calculations were performed using the NMR-Refine module of InsightII (Molecular Simulations, Inc.) in revision. A set of 30 structures was calculated in DGII from 59 NOE-derived distance restraints comprising 23 intraresidue, 23 sequential, and 13 nonadjacent restraints, two of InsightII (Molecular Simulations, Inc.). A set of 30 structures was obtained. The dihedral angle between the planes of the NMRchitect ring was taken from the crystal structure.32 PE-COSY spectra were acquired with a spectral width of 6600 Hz and 4096 data points in 440 t1 increments of 80 transients. The residual water signal in the spectra. Data were routinely zero-filled to 2K data points in both dimensions, with the exception of the PE-COSY, which was filled to 8K in D1 dimension and 2K in D2 dimension. In all spectra, a Gaussian and a 90°-shifted sinebell squared apodization function were used in the D1 and D2 dimension, respectively. The data were then phase corrected and baseline corrected.

Structure calculations were performed using the NMR-Refine module of InsightII (Molecular Simulations, Inc.) in revision. A set of 30 structures was generated in DGII from 59 NOE-derived distance restraints comprising 23 intraresidue, 23 sequential, and 13 nonadjacent restraints, two hydrogen bond restraints, 13 J dihedral angle restraints, and six chiral restraints.34 The NOE restraints were grouped as strong, medium, and weak, which corresponded to 1.8–2.7, 1.8–3.3, and 1.8–5.0 Å distance ranges. The two hydrogen bond restraints were incorporated on the basis of the reduced temperature coefficients for the amide protons of Thr1 and Phe6. They were defined as O⋯N distances of 2.6–3.2 Å between residues 3 and 6.35 These restraints did not bias the structure calculations as judged by repeating the calculations without hydrogen bond restraints. The DGII calculation was done using 10,000 steps of simulated annealing and 500 steps of conjugate gradient optimization to an RMS gradient of 0.001. Structures were further optimized to an RMS gradient of 0.001. Structures were further minimized in Discover using the CVFF force field and a dielectric constant of 78.0 for water.36 A 500-step steepest descent minimization without cross and Morse potentials was performed until the maximum residuals. Decay curves were simulated with 1.8×104 counts in the Beechem global analysis program.39

where αi(λ) is the amplitude at wavelength λ and τr is the lifetime of component i. Lifetimes but not amplitudes were linked in global analyses. Individual decay curves were also deconvolved by the maximum entropy method (MEM).40

where αi(λ,τ) is the fluorescence lifetime distribution at wavelength λ. Quality of fit was judged by the value of reduced χ2, the weighted residuals, and the shape of the autocorrelation function of the weighted residuals. Decay curves were simulated with 1.8×104 counts in the peak using experimental instrumental response functions of 10 and 24 ps/channel in the Beechem program. The radiative rate kᵣ is calculated from

$$kᵣ = \Phi/\bar{τ}$$

where \( \bar{τ} \) is the average lifetime

$$\bar{τ} = \sum_i \frac{α_i(λ) τ_i}{\sum_i α_i(λ)}$$

**Results and Discussion**

**Backbone Conformation of c[α-PpYTFWF]**. The backbone state structure of a closely related peptide, c[α-PFTK(z)WF], is the average lifetime

$$\bar{τ} = \sum_i \frac{α_i(λ) τ_i}{\sum_i α_i(λ)}$$


in DMSO was reported by Kessler et al.\(^\text{(21)}\) The salient features of the Kessler peptide are (1) a rigid peptide with a single predominant backbone conformation and a trans conformation of the Phe\(^\text{-D-Pro}\) peptide bond from the H,C COSY spectrum, (2) a \(\beta\)I turn about Phe\(^\text{-Trp}\) and a \(\beta\)II' turn about D-Pro\(^\text{-pTyr}\). Vicinal \(^3\)J\(\text{N-H}_\alpha\) coupling constants from 1D-spectra of c[D-PpYTFWF] in H\(_2\)O are similar to the coupling constants for c[D-PFTK(z)WF] in DMSO (Table 1). The upfield shifted D-Pro\(^\text{H}_\alpha\) resonance at 4 ppm is additional evidence for a \(\beta\)-turn about D-Pro\(^\text{-pTyr}\).\(^\text{(2,42)}\) The small temperature coefficient of the Phe\(^\text{N-H}\) chemical shift suggests one intramolecular hydrogen bond at Thr\(^\text{-CO} \cdots\text{NH-Phe}\) as found in the Kessler peptide (Table 1). The temperature coefficient of Thr\(^\text{N-H}\) in the intermediate range is consistent with the other hydrogen bond suggested by Kessler.\(^\text{(21)}\)

Two facts point to a single backbone conformation for c[D-PpYTFWF] in aqueous solution. First, there is no detectable cis–trans isomerization of the Phe\(^\text{N-D-Pro}\) peptide bond in the NMR data. Second, four of five \(^3\)J\(\text{N-H}_\alpha\) coupling constants in Table 1 fall outside the 6–8 Hz range attributed to conformational averaging. The overall structure of c[D-PpYTFWF] was generated from distance geometry calculations using restraints derived from NOE and J coupling data. There were no NOE distance restraint violations greater than 0.5 Å in the distance geometry calculations. The average structure from the family of 17 structures is shown in Figure 2. The average overall backbone RMSD is 0.43 ± 0.05 Å; the all-atom RMSD is 1.90 ± 0.06 Å.

**Side Chain Conformation.** The rotamer populations for \(\chi_1 = \pm 60^\circ\), \(180^\circ\) conformations of amino acid side chains in c[D-PpYTFWF] were calculated from \(^3\)J\(\text{H}_{\text{N}}-\text{H}_{\text{A}}\) coupling constants using the Pachler equations.\(^\text{(2,43)}\) Coupling constants for Phe\(^\text{II}\) and Trp\(^\text{III}\) were determined from the 1D-spectrum; coupling constants for Ptyr\(^\text{II}\) were determined from the PE-COSY spectrum. Table 1 compares the \(\chi_1\) rotamer populations \(\text{pl} = \text{III}\) for c[D-PpYTFWF] in D\(_2\)O and c[D-PFTK(z)WF] in DMSO. The populations for amino acids at the same position in the sequence are similar in the two peptides. Trp\(^\text{III}\) adopts a major \(\chi_1\) side chain conformation with two equally populated minor conformations.

| \(\text{Phe}^\text{III}\) | \(\text{Trp}^\text{II}\) | \(\text{Thr}^\text{I}\) | \(\text{Phe}^\text{I}\) | \(\text{Phe}^\text{II}\) | \(\text{Thr}^\text{II}\)| \(\text{Phe}^\text{II}\)| | \(\text{Phe}^\text{III}\) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 90 | 180 | 90 | 180 | 90 | 180 | 90 | 180 |

\(^{\text{II}}\text{Phe}\) and \(^{\text{II}}\text{Trp}\) were determined from the 1D-spectrum; coupling constants for Pt yr\(^\text{II}\) were determined from the PE-COSY spectrum. Table 1 compares the \(\chi_1\) rotamer populations \(\text{pl} = \text{III}\) for c[D-PpYTFWF] in D\(_2\)O and c[D-PFTK(z)WF] in DMSO. The populations for amino acids at the same position in the sequence are similar in the two peptides. Trp\(^\text{III}\) adopts a major \(\chi_1\) side chain conformation with two equally populated minor conformations.

\(^{\text{II}}\text{Phe}\) and \(^{\text{II}}\text{Trp}\) were determined from the 1D-spectrum; coupling constants for Pt yr\(^\text{II}\) were determined from the PE-COSY spectrum. Table 1 compares the \(\chi_1\) rotamer populations \(\text{pl} = \text{III}\) for c[D-PpYTFWF] in D\(_2\)O and c[D-PFTK(z)WF] in DMSO. The populations for amino acids at the same position in the sequence are similar in the two peptides. Trp\(^\text{III}\) adopts a major \(\chi_1\) side chain conformation with two equally populated minor conformations.
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Protein Eng.
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two Trp H
rotamer. NOE intensities alone can be interpreted at best

Figure 2. Mean structure of c[\text{-}PpYTFWF] from distance geometry and
energy minimization calculations.

The NOESY spectrum showed a single set of cross-peaks
between Hα and Hβ protons of Trp\(^{5}\), consistent with fast
averaging of the side chain conformation on the NMR time
scale. A semiquantitative analysis of local NOE intensities
facilitated stereospecific assignments of the Trp\(^{5}\) Hβ protons
and identification of the major \(\chi_1\) rotamer as \(-60^\circ\), under
the basic assumption that a major \(\chi_1\) rotamer dominates the
cross-peak intensities.\(^{44}\) The NOE intensity is about 1.5-fold larger
for the Hα-Hβ cross-peak than for the Hα-Hδ cross-peak of
Trp\(^{5}\). There is also 1.4-fold stronger NOE intensity between
Trp\(^{5}\) amide NH-Hβ\(r\) than NH-Hδ\(r\). These two NOE intensity
ratios indicate a predominant conformation with \(\chi_1 = -60^\circ\).

The presence of multiple interconverting \(\chi_1\) rotamers makes it
impossible to determine \(\chi_2\) rotamer populations for each \(\chi_1\)
rotamer. NOE intensities alone can be interpreted at best
semiquantiatively. Nevertheless, NOE intensities between the
two Trp Hβ protons and the indole ring H2 and H4, which
depend solely on \(\chi_2\) conformation, occur with comparable
intensities in all data, suggesting little \(\chi_2\) preference for Trp\(^{5}\)
in this peptide. The presence of both H2 and H4 cross-peaks with
H\(\alpha\), which indicate primarily \(\chi_1\), \(\chi_2 = 60^\circ, -90^\circ\), 180\(^\circ\), 90\(^\circ\) and
\(\chi_1, \chi_2 = -60^\circ, 90^\circ\), 180\(^\circ\), -90\(^\circ\), respectively, excludes
correlation between \(\chi_1\) and \(\chi_2\). The data suggest the presence of up to six Trp\(^{5}\) side chain rotamers in c[\text{-}PpYTFWF].

Fluorescence Spectroscopy. The fluorescence emission
maxima \(\lambda_{max}\) and quantum yields \(\Phi\) of c[\text{-}PpYTFWF], c[\text{-}PFTKWF], and c[\text{-}PFTFWK] in H\(_2\)O and D\(_2\)O are given in
Table 2. The broad, featureless emission spectra of the peptides
are essentially identical to the spectrum of NATA in aqueous
solution. The quantum yields of the peptides and NATA are
equally identical to the spectrum of NATA in aqueous

Table 2. Fluorescence Data\(^{a}\)

<table>
<thead>
<tr>
<th>solvent</th>
<th>(\lambda_{max}) (nm)</th>
<th>(\Phi_{350}^b)</th>
<th>(\Phi_{280}^c)</th>
<th>(k^d_r) (10(^{-15}) s(^{-1}))</th>
<th>(k_n) (10(^{-15}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)O</td>
<td>348 0.17 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>2.9 ± 0.1</td>
<td>5.7 ± 0.7</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>D(_2)O</td>
<td>350 0.20 ± 0.02</td>
<td>0.21 ± 0.03</td>
<td>3.21 ± 0.06</td>
<td>6.5 ± 0.9</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>c[\text{-}PpYTFWF]</td>
<td>350 0.16 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>3.06 ± 0.09</td>
<td>5.5 ± 0.7</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>c[\text{-}PFTKWF]</td>
<td>350 0.20 ± 0.02</td>
<td>0.21 ± 0.02</td>
<td>3.30 ± 0.06</td>
<td>6.4 ± 0.6</td>
<td>23.9 ± 0.8</td>
</tr>
<tr>
<td>c[\text{-}PFTFWK]</td>
<td>349 0.17 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>2.9 ± 0.2</td>
<td>6.1 ± 0.5</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>D(_2)O</td>
<td>351 0.19 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>3.2 ± 0.1</td>
<td>6.9 ± 0.4</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>NATA(^c)</td>
<td>350 0.15 ± 0.02</td>
<td>2.6 ± 0.4</td>
<td>6 ± 1</td>
<td>33 ± 6</td>
<td></td>
</tr>
<tr>
<td>D(_2)O</td>
<td>350 0.18 ± 0.03</td>
<td>3.3 ± 0.5</td>
<td>5 ± 1</td>
<td>25 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) 25°C. \(^{b}\) Mean values with standard deviations from 5 to 20 experiments. \(^{c}\) Values for cyclic peptides calculated from lifetime data in Table 3. \(^{d}\) Values for cyclic peptides calculated using quantum yields measured at 295 nm excitation wavelength. \(^{e}\) Data from ref 66.

were deconvolved in global analyses with the lifetimes linked.
The decay curves for the peptides gave good fits to three
exponential functions with \(\chi_2\) values of 1.4–1.6 and lifetimes
of about 3.8, 1.8, and 0.3 ns in H\(_2\)O (Table 3). Triple exponential
fits were an improvement over double exponential fits, which
had \(\chi_2\) values of 1.9 and nonrandom weighted residuals and
autocorrelation functions. MEM analysis, in which the decay
model is not predetermined, supports a triple exponential decay.
The relative amplitudes \(\alpha_1\) appear independent of emission
wavelength: about 0.7 for the 3.8-ns component, 0.16 for the
1.8-ns component, and 0.14 for the 0.3-ns component. These
amplitudes correlate very well with the \(\chi_1\) rotamer populations
determined from \(^3J_{\text{H}_\alpha-\text{H}_\beta}\) coupling constants (Table 1), which
associates the 3.8-ns lifetime component with the \(\chi_1 = -60^\circ\)
rotamer.

Radiative and nonradiative rates were calculated from quan-
tum yield and lifetime data using eqs 1, 5, and 6. Intramolecular
static quenching in dipeptides reduces the apparent value of
radiative rate, which is smaller in H\(_2\)O than D\(_2\)O due to water quenching, which is an isotopically
sensitive temperature-dependent quenching process.\(^{5}\) The finding
of the same quantum yields, lifetimes, and deuterium isotope


5184–5195.
The temperature dependence of the fluorescence lifetime of NATA in water has two Arrhenius factors,

\[ r^{-1} = k_o + A_1 \exp(-E_{i}^*/kT) + A_2 \exp(-E_{j}^*/kT) \]  

(7)

where \( k_o = k_1 + k_{isc} \) is the temperature-independent rate, \( A_1 \) is the frequency factor, \( E_{i}^* \) is the activation energy, \( R \) is the gas constant, and \( T \) is temperature in K. The two Arrhenius terms represent solvent quenching \( k_{i} = A_1 \exp(-E_{i}^*/RT) \) and excited-state electron transfer from the indole ring to the amides \( k_{ii} = A_2 \exp(-E_{j}^*/RT) \). Fluorescence decays of c[d-PpYTFWF] were measured in H\(_2\)O and D\(_2\)O from 5 to 55 \(^\circ\)C and deconvoluted in single curve analyses. The long lifetime \( r_1 \) decreased monotonically over the temperature range studied, from 5.14 ns at 7.2 \(^\circ\)C to 1.97 ns at 55 \(^\circ\)C in H\(_2\)O. The temperature dependence of the intermediate and short lifetimes was obscured by correlation among the decay parameters of these minor components in the three exponential fits. Plots of \( \ln(r_1 - k_o - k_{ij}) \) versus \( 1/T \) for the long lifetime data were made, using \( k_i \) values from Table 2 and \( k_{isc} \) and \( k_{ij} \) values for NATA. Arrhenius parameters obtained from the intercept and slope of the lines were: \( A_2 = 1.9 \times 10^{11} \) s\(^{-1}\) and \( E_{i}^* = 4.4 \) kcal/mol in H\(_2\)O and \( A_2 = 5.3 \times 10^{11} \) s\(^{-1}\) and \( E_{j}^* = 4.9 \) kcal/mol in D\(_2\)O. Electron-transfer rates calculated at 25 \(^\circ\)C are 1.1 \times 10^8 \) s\(^{-1}\) in H\(_2\)O and 1.4 \times 10^8 \) s\(^{-1}\) in D\(_2\)O. The \( k_{ij} \) values for the long lifetime component of c[d-PpYTFWF] are slightly lower than the values for NATA (2.0 \times 10^8 \) s\(^{-1}\) in H\(_2\)O and 2.2 \times 10^8 \) s\(^{-1}\) in D\(_2\)O).  

**Excited-State Electron Transfer.** Two models for producing different local environments of Trp 5 can explain the multitudes of ground-state species arising from the six side chain rotamer populations of the tryptophan side chain as proposed by the rotamer model,14,15 (2) Different lifetimes result from multiple conformations of the peptide backbone. The latter is unlikely because of the strong NMR evidence for a rigid backbone. Moreover, the \( \chi_1 \) side chain rotamer populations of Trp\(^5\) determined from NMR correlate very well with the relative amplitudes of the fluorescence decay. By assuming that the radiative rates of the rotamers are the same, the different lifetimes would be due to differences in the total nonradiative rate. On the basis of intermolecular quenching experiments,8 we expect only three dynamic quenching mechanisms in c[d-PpYTFWF]: intsystem crossing, water quenching, and peptide bond quenching by excited-state electron transfer. We assume that the intsystem crossing and water quenching rates are the same for all six Trp\(^5\) rotamers and equal or similar to the rates in NATA. In this scenario, the three Trp\(^5\) lifetimes would result from differences in peptide bond quenching among rotamers. The total electron-transfer rate \( k_{ij} \) of rotamer \( j \) is the sum of the individual electron-transfer rates from Trp\(^5\) to each of the six peptide bonds in c[d-PpYTFWF]  

\[ k_{ij} = \sum_i k_{ij} \]  

(8)

where \( k_{ij} \) is the quenching rate of rotamer \( j \) by peptide bond \( i \).

In a system with weak coupling between donor and acceptor, the electron-transfer rate decreases exponentially with distance \( r \).

The electronic coupling of donor and acceptor in proteins may be through-space, hydrogen-bonding, or covalent.47 Electron transfer occurs from the highest occupied molecular orbital (HOMO) of the donor to the lowest unoccupied molecular orbital (LUMO) of the acceptor. For tryptophan-containing peptides, excited indole is the electron donor. In aqueous solution, emission and therefore also electron transfer occur from the \( ^1\text{Le} \) state. The carbonyl carbon of the peptide bond is probably the electron acceptor. The covalent bond distances are the same for all rotamers. A through-bond electron transfer mechanism could not produce lifetime heterogeneity unless the through-bond electron-transfer rate were highly dependent on the orientation of the indole ring and the peptide bond. Moreover, the through-space distances between indole and various carbonyl carbons are a few Å shorter than the through-bond distances. Hence, we only consider through-space electron-transfer mechanisms. An orientation dependence of \( k_{ij} \) has been shown for heme-heme intramolecular electron transfer in cytochromes, even when donor—acceptor distances were comparable in different proteins.49 The effect of orbital overlap has been treated by several workers.50–52 The electron-transfer rate may be expressed as a function of distance and orientation by

\[ k_{ij}(i, j) = B \left( f(\theta_{ij}) \exp(-\beta r_{ij}) \right) \]  

(9)

where \( B \) is a preexponential factor independent of both \( r \) and \( \theta(\theta_{ij}) \) is an orientation function, and \( \beta = 1.7 \) Å\(^{-1}\) is the distance decay factor commonly used for through-space electron transfer in proteins.53 The donor HOMO of the \( ^1\text{Le} \) state of indole is fairly well described by the LUMO of the ground state.45,54 The acceptor LUMO is the \( \pi^* \) antibonding orbital of the carbonyl C=O bond. We calculate the overlap of these orbitals very crudely with the simple orientation function \( f(\theta_{ij}) = \cos^2 \theta_{ij} \) for overlap of \( S-P \) orbitals.55 \( \theta_{ij} \) is the dihedral angle between the planes containing the indole ring and peptide bond \( i \) in rotamer \( j \). In the spherical element approximation, the \( S \) orbital represents the LUMO of the indole ring, and the \( P \) orbital represents the \( \pi^* \) antibonding orbital of the carbonyl.

To calculate electron-transfer rates of the six possible Trp\(^5\) rotamers in c[d-PpYTFWF] from eqs 8 and 9, we need a value for \( B \). NATA and c[d-PpYTFWF] have the same donor—acceptor pair; therefore, we assume that the electron-transfer parameters \( B \) and \( \beta \) in eq 9 are about the same for both molecules. NATA mimics a tryptophan-containing peptide with peptide bonds \( i-1 \) and \( i \) on either side of the indole ring. The fluorescence decay is a single exponential with a 2.9-ns lifetime at 25 \(^\circ\)C. Presumably, the single-exponential represents a multitude of ground-state species arising from the six side chain rotamers with free rotation about each peptide bond. The \( k_{ij} \) value for NATA in H\(_2\)O is almost certainly a composite value

from these multiple species, so that \( B \) is likewise a composite value. We estimate \( B \) using the electron-transfer rate of NATA from

\[ k_{\text{et}}(\text{NATA}) = \left( \frac{B}{6} \right) \sum_j \left( \langle f(\theta_{i-1,j}) \rangle \exp(-\beta r_{i-1,j}) + \langle f(\theta_j) \rangle \exp(-\beta r_{ij}) \right) \]  

(10)

The summation over \( j = 1 \rightarrow 6 \) assumes equal populations of the six canonical rotamers of the indole side chain. To simulate free rotation about the \( \phi, \psi \) bonds in each rotamer, 12 values of \( \langle f(\theta_{i-1,j}) \rangle \exp(-\beta r_{i-1,j}) \) and \( \langle f(\theta_j) \rangle \exp(-\beta r_{ij}) \) for peptide bonds \( i-1 \) and \( i \) were computed and averaged. Using \( k_{\text{et}}(\text{NATA}) = 2.0 \times 10^8 \text{ s}^{-1} \) at 25 °C, eq 10 gives \( B = 1.1 \times 10^{13} \text{ s}^{-1} \). Assuming no orientation dependence with \( f(\theta_j) = 1 \), eq 10 gives \( B = 3.3 \times 10^{11} \text{ s}^{-1} \).

Individual electron-transfer rates \( k_{\text{et}}(i,j) \) from rotamer \( j \) of Trp\(^5\) to peptide bond \( i \) in c[\( \alpha\)-PpYTFWF] were calculated from eq 9, and the total electron-transfer rate \( k_{\text{et}}(j) \) for each rotamer was then calculated from eq 8 (Table SI). In general, for distances \( >6 \text{ Å} \) or orientations of the indole ring and the peptide bond \( >70^\circ \), the \( k_{\text{et}}(i,j) \) values are negligible (\( <10^7 \text{ s}^{-1} \)). In the six rotamers, the peptide bonds on either side of the indole ring contribute \( >84\% \) of the total \( k_{\text{et}}(j) \). The \( k_{\text{et}}(j) \) values vary by a factor of 7 among rotamers with peptide bond quenching being fastest in the 60°, 90° rotamer and slowest in the 60°, 90° rotamer. Neglecting orientation, the \( k_{\text{et}}(j) \) values vary by less than a factor of 4.

Table 4 gives the fluorescence lifetimes for the six rotamers. Lifetimes were calculated from eqs 1 and 2 with \( k_{\text{pl}} = 0 \), using \( k_{\text{et}}(j) \) for the peptide from Table 2 and \( k_{\text{inc}} + k_{\text{es}} = 8.4 \times 10^7 \text{ s}^{-1} \) for NATA at 25 °C. For \( \beta = 1.7 \text{ Å}^{-1} \), the lifetimes range from 1 to 4 ns. For each \( \chi_1 \) rotamer, the calculated lifetimes of the \( \chi_2 \) = ±90° rotamers differ by less than a factor of 2. Such closely spaced lifetimes are difficult to resolve in a multieponential decay. With this in mind, we rationalize the observed and calculated lifetimes of c[\( \alpha\)-PpYTFWF] according to the rotamer model as follows.

(I) Assume that the rotamers do not interconvert on the fluorescence time scale, that the observed fluorescence decay amplitudes \( \alpha \) equal the NMR-determined \( \chi_1 \) rotamer populations, and that each \( \chi_1 \) rotamer has a major \( \chi_2 \) rotamer. This assigns the 4.0-ns lifetime calculated for the 60°, 90° rotamer to the observed 3.8-ns component, either the 1.7- or 2.0-ns lifetime calculated for the \( \chi_1 = 180° \) rotamer to the observed 1.8-ns component, and the 1.0-ns lifetime calculated for the 60°, 90° rotamer to the observed 0.3-ns component. Case I agrees with the \( \chi_2 \) rotamer populations of tryptophan zwitterion,\(^{56} \) although not with the apparently uncorrelated \( \chi_1, \chi_2 \) rotamer populations of the cyclic peptide. To test whether Case I is consistent with the time-resolved fluorescence data, we simulated triple exponential fluorescence decays using calculated lifetimes and amplitudes equal to the respective \( \chi_1 \) rotamer populations of Trp\(^5\) in Table 1. Eight simulations using the 4.0, 1.7, and 1 ns values recovered lifetimes (amplitudes) of 3.9–3.9 (0.71 ± 0.07), 1.5–1.9 (0.16 ± 0.02), and 0.14–0.55 ns (0.13 ± 0.06) with \( \langle \bar{\tau} \rangle = 3.0 ± 0.2 \text{ ns} \). Nine of 10 simulations using the 4.0, 2.0, and 1 ns values recovered lifetimes (amplitudes) of 3.4–5.7 (0.69 ± 0.06), 1.5–2.1 (0.19 ± 0.05), and 0.15–0.35 ns (0.11 ± 0.03) with \( \langle \bar{\tau} \rangle = 3.0 ± 0.5 \text{ ns} \). Only one simulation recovered the 1-ns value for the short lifetime with \( \bar{\tau} = 3.06 \text{ ns} \).

(II) Assume that the \( \chi_1 \) rotamers do not interconvert on the fluorescence time scale, that the observed fluorescence decay amplitudes equal the NMR-determined \( \chi_1 \) rotamer populations, but that the observed lifetimes represent some form of average lifetime of the two \( \chi_2 \) rotamers. For example, if the \( \chi_2 \) rotamers interconvert rapidly, then the lifetimes would converge to a single value\(^{14} \)

\[ \frac{1}{\tau} = (k_{90} + K k_{90})/(1 + K) \]  

(11)

where \( k_{90} \) and \( k_{90} \) are the decay rates and the equilibrium constant \( K = p_{90}/p_{90} \) is the population ratio of \( \chi_2 \) rotamers. A value of \( K = 0.11 \) was calculated from eq 11 using the observed 3.8-ns lifetime of the \( \chi_1 = 60° \) rotamer and the calculated decay rates of the two \( \chi_2 \) rotamers, which corresponds to 90° population for \( \chi_2 = 90° \) as assumed in Case I.

Table 4 also shows the effect of \( \beta \) on the calculated lifetimes of the six rotamers, \( \beta \) values of 1.4, 1.7, and 2.6 Å\(^{-1} \) have been used for through-space electron transfer in proteins.\(^{57–59} \) The 60°, 90° rotamer has the longest lifetime, and the 60°, 90° rotamer has the shortest lifetime throughout this range. As expected, the lifetime difference between these rotamers is

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Table 4. Calculated Fluorescence Lifetimes for Rotamer \( j \) of Trp\(^5\) in c[\( \alpha\)-PpYTFWF]a

<table>
<thead>
<tr>
<th>( \beta (\text{Å}^{-1}) )</th>
<th>60°, 90°</th>
<th>60°, 90°</th>
<th>60°, 90°</th>
<th>60°, 90°</th>
<th>60°, 90°</th>
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<tbody>
<tr>
<td>1.4</td>
<td>0.38 (0.08)</td>
<td>2.8 (3.9)</td>
<td>1.9 (2.7)</td>
<td>1.7 (2.7)</td>
<td>1.4 (2.9)</td>
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<tr>
<td>1.5</td>
<td>0.55 (0.13)</td>
<td>3.1 (4.0)</td>
<td>2.1 (2.6)</td>
<td>1.8 (2.6)</td>
<td>1.5 (2.8)</td>
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<tr>
<td>1.6</td>
<td>0.80 (0.21)</td>
<td>3.4 (4.0)</td>
<td>2.3 (2.5)</td>
<td>1.9 (2.6)</td>
<td>1.6 (2.7)</td>
</tr>
<tr>
<td>1.7</td>
<td>1.10 (0.33)</td>
<td>4.0 (4.0)</td>
<td>2.6 (2.5)</td>
<td>2.0 (2.5)</td>
<td>1.7 (2.7)</td>
</tr>
<tr>
<td>1.8</td>
<td>2.00 (0.60)</td>
<td>3.7 (3.9)</td>
<td>2.4 (2.3)</td>
<td>1.8 (2.2)</td>
<td>1.6 (2.4)</td>
</tr>
<tr>
<td>1.9</td>
<td>3.6 (0.90)</td>
<td>3.6 (4.1)</td>
<td>2.3 (2.3)</td>
<td>1.6 (2.3)</td>
<td>1.4 (2.5)</td>
</tr>
<tr>
<td>2.0</td>
<td>5.8 (1.5)</td>
<td>3.7 (4.1)</td>
<td>2.3 (2.2)</td>
<td>1.5 (2.0)</td>
<td>1.3 (2.3)</td>
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<tr>
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<td>2.3 (2.1)</td>
<td>1.5 (2.0)</td>
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<tr>
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<tr>
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<td>1.2 (1.8)</td>
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<td>2.6</td>
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<td>1.9 (1.9)</td>
<td>1.0 (1.7)</td>
<td>0.93 (1.8)</td>
</tr>
</tbody>
</table>

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a Numbers in parentheses calculated assuming no orientation dependence.
creases with $\beta$ for both orientation-dependent and orientation-independent mechanisms. Orientation has the most effect on the lifetime of the 60°, −90° rotamer and the least effect on the two $\chi_1 = -60^\circ$ rotamers. Cases I and II predict the 3.8- and 1.8-ns lifetimes quantitatively, but not the 0.3-ns lifetime. The predictions are robust to $\beta$ values between 1.7 and 2.1 Å.

**Conclusions**

This paper provides definitive evidence for the rotamer model of tryptophan photophysics for peptides in aqueous solution. c[δ-PpYTFWF] has a rigid backbone, making side chain rotamers the only source of conformational heterogeneity. The three fluorescence decay amplitudes correlate well with the three $\chi_1$ rotamer populations of Trp$^3$ determined by $^1$H NMR. The three fluorescence lifetimes can be explained by differences among $\chi_1$, $\chi_2$ rotamers in the rate of intramolecular excited-state electron transfer from the indole ring to two to four peptide bonds in c[δ-PpYTFWF]. Except for the shortest lifetime, remarkably good quantitative agreement is obtained with calculated lifetimes using a simple model and reasonable assumptions. Two relatively innocuous approximations were made to calculate fluorescence lifetimes, namely (1) The radiative, intersystem-crossing, and water-quenching rates are the same in all rotamers. (2) The intersystem-crossing and water-quenching rates are the same in NATA and c[δ-PpYTFWF]. Intersystem crossing plus water quenching $k_{isc} + k_q$ contribute only about 30% of the average total nonradiative rate (Table 2) or 10−40% in individual rotamers (Table SI). Likewise, $k_e + k_{isc} + k_q$ contributes at most about 50% of the total decay rate. Small differences in these contributions would be overshadowed by the larger electron-transfer rates.

The electron-transfer calculations attempt to establish a quantitative relationship between fluorescence lifetime and molecular structure. Given the many assumptions, is the agreement between calculated and experimental lifetimes entirely fortuitous? We assumed a through-space electron transfer mechanism with orientation dependence. Electronic propagation in peptides probably involves a combination of through-space and through-bond mechanisms, like the pathway model used for protein electron transfer. Moreover, we made a number of simplifying assumptions to estimate electron-transfer rates, the most significant being: (1) Orientation dependence of electron transfer from the excited indole ring to the carbonyl carbon was approximated by overlap of $S\rightarrow P$ orbitals. The assumption of a spherical molecular orbital for $^1I_a$ neglects the nodal structure of the ground-state LUMO. $^5$ (2) Distances and relative orientations were measured for canonical conformations of $\chi_1$, $\chi_2$ rotamers. (3) The Marcus parameter $B$ was estimated from the electron-transfer rate of NATA and $\beta$ was borrowed from ground-state electron-transfer theory. Finally, we assumed that the middle of the indole ring is the electron donor. The donor atom closest to the acceptor, indole C3 in our case, is normally used in protein electron-transfer calculations. We also did calculations with indole C3 as electron donor. Not surprisingly, there was less variation in electron-transfer rates among rotamers, factors of four with orientation and only two without orientation. Much more work with well-defined model systems is needed to place the excited-state electron-transfer calculations on firm ground.

Excited-state relaxation processes have been invoked to explain the complex fluorescence decays of proteins.9−11 Dipolar solvent relaxation on the fluorescence time scale is amply documented for indole derivatives in viscous solvents.96−62 We may exclude solvent relaxation as a source of lifetime heterogeneity in the cyclic peptides on the following two grounds: (1) The fluorescence decays of the cyclic peptides appear independent of emission wavelength. At the 10-nm resolution of our time-resolved emission spectral data, $\lambda_{max} = 350$ nm for both 3.8- and 1.8-ns components and 340 nm for the 0.3-ns component. The short lifetime component constitutes <2% of the total intensity, so its spectrum may include a contribution from the water Raman at 331 nm. (2) Solvent relaxation of tryptophan zwitterion has a 1.2-ps relaxation time in aqueous solution at room temperature.$^{63}$ It is unlikely that the relaxation time would be much slower for a fully solvent exposed tryptophan in a small peptide. On the other hand, rotamer interconversion during the lifetime of the excited state is a possibility. The good agreement between NMR-determined ground-state rotamer populations and fluorescence decay amplitudes suggests that $\chi_1$ rotamer interconversion is slower than the fluorescence time scale, albeit faster than the NMR time scale. In molecular dynamics simulations using an all atom representation, $\chi_2$ rotamer interconversion is faster than $\chi_1$ rotamer interconversion.$^{64}$ The short lifetime component may be indicative of excited-state conformer interconversion.65 If so, the lifetime heterogeneity would still be due to at least three ground-state rotamers with different lifetimes, but the amplitudes and decay times would no longer simply represent the rotamer populations and lifetimes.

Although the peptide bond is a weak intermolecular quencher of 3-methylindole fluorescence, it is an efficient intramolecular quencher in NATA and the cyclic peptides. This strongly suggests that peptide bond quenching is an important quenching mechanism in proteins. The quenching rate will depend on the number, distance, and possibly orientation of peptide bonds within about 6 Å of the indole ring. Sillen et al. proposed that electron-transfer quenching by the peptide bond may be the main determinant of the lifetime of tryptophan in proteins in the absence of strong quenchers.$^{13}$ They used molecular dynamics to determine tryptophan rotamer populations and Marcus theory to predict the three fluorescence lifetimes of two single tryptophan proteins. The details of their lifetime calculations differ from those presented here. In c[δ-PpYTFWF], the peptide bond is assumed to be the only intramolecular quenching group. Although phenylalanine is not an intermolecular quencher of 3-methylindole fluorescence,$^8$ the phenyl ring has been proposed to be a highly efficient intramolecular quencher of buried tryptophans in proteins.$^{12}$ The presumed quenching mechanism involves an atypical NH$\rightarrow\pi$ hydrogen bond, which requires a particular orientation of the indole nitrogen relative to the phenyl ring. There are three other aromatic residues in c[δ-PpYTFWF] besides tryptophan: pTyr, Phe, and Phe$.^6$ However, these
residues are probably not intramolecular quenching groups in the cyclic peptides. First, substitution of either phenylalanine by lysine in c[D-PFTKWF] and c[D-PFTFWK] has no effect on fluorescence quantum yield and lifetimes. Second, molecular modeling shows that the particular conformation for NH···π hydrogen bonding is equally possible in all tryptophan \( \chi_1 \) rotamers. Last, NH···π hydrogen bonding is not likely to occur in aqueous solution.

Acknowledgment. This work was supported by NIH Grant GM42101.

Supporting Information Available: Table of electron transfer rates for rotamer \( j \) of Trp\(^5\) of c[D-PpYTFWF] (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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