Probing Water Environment of Trp59 in Ribonuclease T1: Insight of the Structure–Water Network Relationship

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Supporting Information

ABSTRACT: In this study, we used the tryptophan analogue, (2,7-aza)Trp, which exhibits water catalyzed proton transfer isomerization among N(1)-H, N(7)-H, and N(2)-H isomers, to probe the water environment of tryptophan-59 (Trp59) near the connecting loop region of ribonuclease T1 (RNase T1) by replacing the tryptophan with (2,7-aza)Trp. The resulting (2,7-aza)Trp59 triple emission bands and their associated relaxation dynamics, together with relevant data of 7-azatryptophan and molecular dynamics (MD) simulation, lead us to propose two Trp59 containing conformers in RNase T1, namely, the loop-close and loop-open forms. Water is rich in the loop-open form around the proximity of (2,7-aza)Trp59, which catalyzes (2,7-aza)Trp59 proton transfer in the excited state, giving both N(1)-H and N(7)-H isomer emissions. The existence of N(2)-H isomer in the loop-open form, supported by the MD simulation, is mainly due to the specific hydrogen bonding between N(2)-H proton and water molecule that bridges N(2)-H and the amide oxygen of Pro60, forming a strong network. The loop-close form is relatively tight in space, which squeezes water molecules out of the interface of α-helix and β2 strand, joined by the connecting loop region; accordingly, the water-scarce environment leads to the sole existence of the N(1)-H isomer emission. MD simulation also points out that the Trp-water pairs appear to preferentially participate in a hydrogen bond network incorporating polar amino acid moieties on the protein surface and bulk waters, providing the structural dynamic features of the connecting loop region in RNase T1.

INTRODUCTION

The role of water molecules in proteins, which is believed to be crucial in such functions as biorecognition1,2 and enzymatic reaction,3-6 remains unexplored territory. Scientists have made tremendous efforts to gain understanding of the water molecules in proteins via indirect measurements, such as by simulating molecular dynamics and probing the polarity of the local environment.7,8 However, the lack of a more direct method of sensing water molecules or, more specifically, “biowater” has long been recognized as a weakness in evaluating their possible functionality in protein. To overcome this hurdle, we recently have unveiled a novel tryptophan analogue 2,7-diazatryptophan ((2,7-aza)Trp, see Scheme 1), which, upon replacing tryptophan, successfully recognizes the presence of water in proteins. (2,7-aza)Trp has the advantageous property of water catalyzed proton transfer,9 giving an N(1)-H 345 nm emission band and a prominent green N(7)-H isomer 500 nm emission. The ratimetric changes of these multiple emissions offer an unprecedented opportunity to sense the water microsolvation of proteins. Exploiting a structurally undetermined protein “human thromboxane A2 synthase (hTXAS)” by site-specifically replacing Trp residues with this water sensitive bioprobe ((2,7-aza)Trp), we then demonstrated that it was plausible to sense the water environment in protein without disrupting its native structure.9

In this contribution, we moved a step forward to probe a single Trp containing protein ribonuclease T1 (RNase T1). RNase T1 from Aspergillus oryzae is a small extracellular enzyme composed of 104 amino acid residues. It cleaves single-
stranded RNA by catalyzing the hydrolysis of phosphodiester bonds specifically at the 3'-side of guanosine nucleotides. X-ray studies of single crystal RNase T1 revealed that the secondary structure of RNase T1 includes a long α-helix and two β-sheets connected by extended loop regions (see Figure 1). RNase T1 contains a single tryptophan residue (Trp59), the water environment of which has been receiving considerable attention. The Trp59 is located on the β2 strand at the interface between the α-helix and β1–3 strands joined by the connecting loop region. Mutation of Trp59 to a tyrosine has been shown to decrease the thermal transition temperature by more than 4 °C as well as to enhance enzymatic catalysis, indicating that the microenvironment of Trp59 may play an important role in folding as well as in modulating the geometry of the RNase T1 active site. RNase T1 has been used extensively as a model for the study of protein folding because it can be reversibly unfolded and refolded by heating and high concentration of denaturants, such as urea and guanidine hydrochloride (GuHCl), without forming noticeable amounts of aggregates.

In past research, the Trp fluorescence of RNase T1 has revealed a peak wavelength at 322 nm, which resembles Trp in nonpolar media. However, the study of the charge transfer reaction rate constant (k_rxn) of singlet oxygen with the Trp59 residue in RNase T1 implied a relatively polar microenvironment around Trp59. It is known that both water and protein contribute, in various ratios, to the Trp emission shift. Water exposure per se is not sufficient for a red shift. The charged groups lie close to Trp and, as a result, dominate the Trp spectral shift, while water may even create a blue shift in such environments. For RNase T1, Trp59 is close to the amide backbone and thus exhibits different temperature and viscosity dependencies from those of exposed Trp residues in other proteins. These results once led to the conclusion that using tryptophan emission alone to monitor the microenvironment around Trp59 in RNase T1 was inadequate. More importantly, based on X-ray crystallography, it has also been postulated that Trp59 of RNase T1 is involved in the hydrogen bond (H-bond) network with structurally conserved water molecules. Nevertheless, the X-ray resolved structure more or less describes a single static state, making the dynamics/
thermodynamics information at the molecular level insufficient.28 To shed light on the water and, hence, its possible associated hydrogen bonding network around Trp59 of RNase T1, in this study, we combined the fluorescence spectroscopy of wild type, 7-azatryptophan ((7-aza)Trp59—) and 2,7-diazatryptophan ((2,7-aza)Trp59—) substituted RNase T1 to probe the water environment in the proximity of Trp59. We then interpreted the resulting fluorescence bands and their corresponding relaxation dynamics in terms of the existence of two major conformational loops in RNase T1, in which Trp59 is subject to different environments and, hence, different water interactions.Supplementary support was also rendered by simulation of molecular dynamics. The result provides new insight into the microenvironment relationship of structure and the water network in RNase T1.

**METHODS**

**Synthesis.** The detailed synthetic procedures and compound characterizations are elaborated in the Supporting Information.

**Expression and Purification of Recombinant RNase T1.** The recombinant RNase T1 from *Aspergillus oryzae* with in-frame *Omp A2* secretion signal peptide39 and 6xHis tag at the N-terminus was constructed in a pCW-based vector for overexpression driven by *Ptac* promoter. The construct was validated by DNA sequencing prior to protein expression. It was then transformed into a Trp auxotroph *Escherichia coli* (E. coli) strain (ATCC 23231) for protein expression. Transformed *E. coli* were cultured in 1 L of M9 minimal medium supplemented with 2 mM MgSO₄, 0.4% glucose, 0.1 M CaCl₂, 100 μg/mL ampicillin, and 1 mM l-Trp. Growth was facilitated with 200 rpm of shaking at 37 °C until OD₆₀₀ reached 0.8, after which the cells were collected by centrifugation and resuspended in 1 L M9 medium supplemented as above, but without l-Trp. The culture was then incubated at 30 °C (30 min, 200 rpm), after which 2 mM (7-aza)Trp/(2,7-aza)Trp and lactose (40 g) were added. The culture was then incubated further at 30 °C (18–20 h, 180 rpm) before harvest by centrifugation. To release recombinant RNase T1 from periplasmic space, collected cells were subjected to osmotic shock following a modified protocol of Koshl and Botstein.30 The pellet of a 1 L culture was resuspended in 40 mL of ice-cold permeabilizer buffer (50 mM Tris-HCl, pH 7.5 containing 15% sucrose) and kept on ice for 30 min. After centrifugation, the cells were resuspended in ice-cold permeabilizer buffer, incubated for another 30 min on ice, and centrifuged again. The supernatants from the two washing steps were combined with culture supernatant and subjected to purification of recombinant RNase T1 by Ni-NTA column (Qiagen).

**MD Simulations of Water Solvated Wild-Type RNase T1 and (2,7-aza)Trp Mutant.** The RNase T1 crystal structure coordinates (PDB entry 9RNT)30 were taken as the initial configuration for system construction for MD simulations. Simulations for the protein were conducted using the Cerius² suite of program in version 4.831 and Amber 11.0 packages.32,33 The Amber ff99SB34 protein force field was adopted and combined with a set of GAFF35 parameters for the description of the mutant (2,7-aza)Trp59. The water hydration system of RNase T1 includes a two-stage process, in which the positions of water oxygen in crystallography are first used to construct primary hydration waters and then further explicit water hydration is sampled in consideration of the protein water accessible/buried surfaces.36–38 A combination approach of canonical Monte Carlo simulation and annealing MD simulation is performed in order to obtain (assess) a thermodynamically reasonable hydration level.39 The basic strategy involves the generation of the positions, orientations, and number of water molecules in the target protein’s amino acid residue grid, followed by final refinement by minimization using a set of scripts for the program. We used the TIP4P water model because dynamical properties such as the diffusion constants are in good agreement with experimental results.40 Our system contained 4441 explicit TIP4P water molecules with the initial system density set to 0.75 g/mL, followed by tuning the length of the system box, until the system converged (i.e., <1% in temperature fluctuations) to a stabilized density of about 1.002 g/mL with the MD box size decreased to 58.17 × 55.86 × 49.00 Å. In each step, the size of the box was then reduced slightly from its original size (i.e., <1% of the last reduced volume). The system was initially minimized with a decreasing harmonic restraint force and constraint conducted in three steps. First, only the water molecules added during protein hydration were allowed to move. Next, the movement was extended to all residues of the RNase T1 (or the (2,7-aza)Trp mutant) and crystal water molecules, except for the core structure of site-specific water around Trp (or (2,7-aza)Trp). Finally, all atoms were allowed to move freely. In each step, energy was minimized by a combination of the steepest descent method for 100000 steps and the conjugated gradient method for another 100000 steps. After minimization, the systems ran the molecular dynamics in NVT ensemble. The list of nonbonding pairs was produced using a 10.0 Å cutoff. Long-range electrostatic interactions were handled using the smoothed particle mesh Ewald algorithm41 with a real space cutoff length of 8 Å. The cutoff length for the Leonard-Jones potential was set at 15 Å. All bond lengths were constrained using the SHAKE algorithm.42 We performed a set of explicit water MD simulations for wild-type as well as for (2,7-aza)Trp...
replacement RNase T1 with periodic boundary conditions using a truncated triclinic box with lengths of 58.17 × 55.86 × 49.00 Å and 4441 solvated water molecules. The Langevin thermostat was used to maintain the system temperature. Each system was gradually heated to the target temperature of 300 K over 1 ns equilibrium and another 4 ns trajectory collection for further analyses (more detail in the SI).

## RESULTS AND DISCUSSION

Photophysics of Trp Analogues Substituted-RNase T1. To examine the conformation and microsolvation of the relevant Trp position, RNase T1 was synthesized in Trp- auxotrophic E. coli cells and proved to be capable of cotranslational incorporation by site-specifically replacing Trp residues with (2,7-aza)Trp. As for single (2,7-aza)Trp substitution at Trp59, multiple emissions were resolved and the spectra could be well fitted by three components with peak wavelengths at 345, 380, and 495 nm (Figure 2), which are respectively assigned to the emissions from N(1)-H, N(2)-H, and N(7)-H proton-transfer isomers (see Scheme 1). Several significant remarks can be made from this steady state result. First of all, the appearance of the N(7)-H green emission band unambiguously proves the existence of water molecules surrounding the (2,7-aza)Trp59. Upon electronic excitation of the N(1)-H species, catalyzed by the water molecules, the surrounding the (2,7-aza)Trp59. Upon electronic excitation wavelength: 310 nm (the concentration is 43 mM sodium phosphate). Also shown are the deconvoluted spectra based on three emission bands (dotted line, see text for detail). The excitation wavelength: 310 nm (the concentration is 43 μM with 1 cm path lengths cell).

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existence of N(2)-H isomer in (2,7-aza)Trp59-RNase T1 is unexpected and quite worthy of study, for it may imply the existence of a specific hydrogen bonding interaction for (2,7-aza)Trp in (2,7-aza)Trp59-RNase T1, stabilizing the N(2)-H isomer (vide infra).

To gain deeper insight into the spectroscopy-structure correlation, the dynamics of relaxation of the corresponding multiple emission bands in (2,7-aza)Trp59-RNase T1 were measured, and pertinent data are listed in Table 1. First, upon monitoring of the very long wavelength of 520 nm, which is attributed to the tautomer N(7)-H emission, the relaxation dynamics could be well fitted by a single exponential rise (with negative pre-exponential factor, see Table 1) and decay component with time constants of 0.19 and 0.69 ns, respectively. The 380 nm emission band is well ascribed to the excited N(1)-H species, which undergoes water catalyzed proton transfer reaction. Accordingly, the remaining 1.35 ns component is of great interest.

![Figure 2. Emission spectra of (2,7-aza)Trp (blue solid line) and (2,7-aza)Trp-RNase T1 (red solid line) in neutral water (buffered by sodium phosphate). Also shown are the deconvoluted spectra based on three emission bands (dotted line, see text for detail). The excitation wavelength: 310 nm (the concentration is 43 μM with 1 cm path lengths cell).](image)

### Table 1. Photophysical Properties of Various Azaindole and Azatryptophan Analogues in Neutral Water and RNase T1

<table>
<thead>
<tr>
<th>Analogues</th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt; (nm)</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt; (nm)</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (nm)</th>
<th>τ&lt;sub&gt;b&lt;/sub&gt; (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-azaindole</td>
<td>288</td>
<td>386</td>
<td></td>
<td>0.91</td>
</tr>
<tr>
<td>(7-aza)Trp</td>
<td>289</td>
<td>400</td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td>2,7-diazaindole</td>
<td>295</td>
<td>335</td>
<td>320</td>
<td>0.22</td>
</tr>
<tr>
<td>(2,7-aza)Trp</td>
<td>370</td>
<td>400</td>
<td></td>
<td>10.10</td>
</tr>
<tr>
<td>(7-aza)Trp</td>
<td>495</td>
<td>550</td>
<td></td>
<td>0.21 [rise], 1.30</td>
</tr>
<tr>
<td>RNase T1</td>
<td>280</td>
<td>322</td>
<td>330</td>
<td>1.05 [23%], 3.46 [77%]</td>
</tr>
<tr>
<td>(7-aza)Trp59-RNase T1</td>
<td>300</td>
<td>372</td>
<td>380</td>
<td>1.37 [70%], 7.35 [30%]</td>
</tr>
<tr>
<td>(2,7-aza)Trp59-RNase T1</td>
<td>300</td>
<td>345</td>
<td>330</td>
<td>0.17 [73%], 1.35 [27%]</td>
</tr>
<tr>
<td>(2,7-aza)Trp59-RNase T1 + 6 M GuHCl</td>
<td>380</td>
<td>380</td>
<td>3.10</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>495</td>
<td>520</td>
<td>0.21 [rise], 0.69</td>
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</tr>
<tr>
<td>380</td>
<td>380</td>
<td>3.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>495</td>
<td>520</td>
<td>0.19 [rise], 0.69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The wavelength at which the measurement of relaxation dynamics was monitored. <sup>b</sup>Values are ±0.03 ns in uncertainty. Values in parentheses indicate percentages, and those in brackets denote the rise component.
Prior to further assignment of the above results, we then performed two more relevant experiments. First, for 2,7-diazaindole in polar, aprotic solvent such as acetonitrile, in which only N(1)-H species exists and ESPT is prohibited due to the lack of water, only the 345 nm N(1)-H emission was resolved, with a lifetime of 1.55 ns. Second, upon methylating the N(1)-H proton in 2,7-diazaindole, forming the N(1)–CH₃ derivative to stop ESPT, the emission peak wavelength was observed at 350 nm with a lifetime fitted to be 1.35 ns. It was thus reasonable to assign the 1.35 ns emission band around 345 nm to the normal N(1)-H emission of (2,7-aza)Trp in (2,7-aza)Trp-RNase T1, in which the N(1)-H species does not undergo ESPT.

The above spectroscopy and dynamics results led us to propose the existence of two types of (2,7-aza)Trp59 (or Trp59) environment in (2,7-aza)Trp59-RNase T1 (or RNase T1). This viewpoint may not be new, since the wild type RNase T1 has been reported to exhibit two distinctly different Trp emission lifetimes of 1.40 and 3.88 ns. Accordingly, two Trp59 environments in RNase T1 have been proposed. However, Trp is insensitive to polarity changes; especially, its excited-state relaxation is greatly quenched by amide bonds. Therefore, further insight into the Trp environments in wild type RNase T1 is pending. What is new in this study is that according to the results of (2,7-aza)Trp59-RNase T1, one Trp59 environment should be richer in water than the other such that water catalyzed proton transfer takes place in the former case, but not in the latter.

It is noteworthy that whether the Trp59 site is water-rich or scant may not be directly related to the polarity environment surrounding it. To further examine the polarity environment, Trp59 in RNase T1 was replaced by (7-aza)Trp (see Scheme 1a). (7-aza)Trp has been widely used as a polarity probe in proteins. The parent molecule of (7-aza)Trp, that is, 7-azaindole, undergoes an excited-state charge transfer from pyrrolic to pyridyl moiety, and hence its emission is sensitive to the solvent polarity, which can be red-shifted from 325 nm in cyclohexane to 395 nm in water. In this study, as shown in Table 1, the ~375 nm fluorescence of (7-aza)Trp59-RNase T1 also revealed fast and slow single exponential decay components with lifetimes of 1.37 and 7.33 ns, respectively (see Figure 2). Due to the large difference in lifetime, it was feasible for us to perform the temporal spectral evolution of these two components by deconvoluting the respective time trace, followed by the integration of the corresponding intensity as a function of emission wavelength. The results, shown in Figure 3, revealed negligible differences between short and long decay emissions, all being maximized at ~375 nm. The 375 nm emission clearly revealed the relatively polar environment for (7-aza)Trp59 in RNase T1.

Based on the fluorescence pH titration experiment, Petrich and co-workers concluded that 7-azaindole also undergoes water catalyzed N(1)-H → N(7)-H proton transfer in the electronically excited state. In neutral water, according to the decay of N(1)-H emission, the rate of water catalyzed ESPT takes place with a time constant of ~1 ns. This is in sharp contrast to 7-azaindole in dimethyl sulfoxide, wherein the N(1)-H emission exhibits a lifetime as long as 9.3 ns. However, the key difference between 7-azaindole and 2,7-diazaindole, and likewise between (7-aza)Trp and (2,7-aza)Trp, lies in the prompt protonation of the N(7)-H tautomer for 7-azaindole ((7-aza)Trp) in the excited state, followed by a rapid nonradiative deactivation. The fast (~<1 ns) protonation in neutral water is due to the high basicity of the N(1) site for the excited N(7)-H tautomer. Therefore, despite the water-catalyzed ESPT, the resulting N(7)-H tautomer emission is not observable in both 7-azaindole and (7-aza)Trp. In sharp contrast, for 2,7-diazaindole (or (2,7-aza)Trp), the electron withdrawing property of the N(2) nitrogen leads to the drastic decrease of N(1) basicity, such that an excited-state protonation at the N(1) site does not occur in the N(7)-H tautomer, giving a prominent green N(7)-H emission in neutral...
water, as elaborated above. Accordingly, in agreement with that deduced from (2,7-aza)Trp59-RNase T1, the 1.37 and 7.33 ns emission components in (7-aza)Trp59-RNase T1 are best ascribed to two origins, that is, two different (7-aza)Trp environments in (7-aza)Trp59-RNase T1, in which water-catalyzed ESPT takes place in (7-aza)Trp9 on one of the (7-aza)Trp59-RNase T1 conformers, giving a short-lived (1.37 ns) N(1)-H emission, while ESPT is prohibited in the other conformer due to the water-scant environment of (7-aza)Trp59, resulting in a long-lived (7.33 ns) N(1)-H emission.

For all wild type RNase T1, (7-aza)Trp59-RNase T1, and (2,7-aza)Trp59-RNase T1, two distinctly different N(1)-H emission dynamics were observed with the lack of a sequential type of kinetic relationship. This led us to conclude that the two RNase T1 conformers are not interconvertable during the excited-state lifespan. Instead, the two emission components originate from the equilibrium established between two conformers in the ground state. We then made a further attempt to analyze the population ratio for these two conformers by monitoring the respective N(1)-H emission. Theoretically, the transition moment is mainly governed by the electronic configuration, which should not be altered by the surrounding environment. Assuming the same fluorescence transition moment, the initial population ratio of the N(1)-H at \( t = 0 \), that is, the fitted pre-exponential factor, for short versus long components in (7-aza)Trp59-RNase T1 and (7-aza)Trp59-RNase T1 was calculated to be \(~73:27\) and \( 70:30 \), respectively (see Table 1), which, within the experimental and fitting error, are mutually consistent, reaffirming the existence of two major conformers for RNase T1.

Finally, though not an issue for (7-aza)Trp59 and Trp59 due to the lack of N(2) nitrogen, the existence of the N(2)-H isomer in (2,7-aza)Trp59 is worthy to pursue because it may pinpoint a specific type of interaction that provides supplementary substructural information. It has been concluded that the population of N(2)-H isomer in (2,7-aza)Trp is more or less 2% in neutral water and is negligible in proteins such as (2,7-aza)Trp substituted hTXAS9 and ascorbate peroxidase (APX)44 (vide supra). As shown in Scheme 1, the N(2)-H cannot be produced from the ESPT of N(1)-H during the excited-state lifespan. This is supported by the system response (60 ps) limited rise time of the 380 nm N(2)-H emission. Therefore, the 380 nm emission originating from the excitation of the ground-state N(2)-H in (2,7-aza)Trp59-RNase T1 is unambiguous, indicating a specific type of interaction involving, most likely, the H-bond formation with the N(2)-H proton to account for its stabilization. Indirect and qualitative support for this viewpoint may be provided by the 2.1 ns lifetime of the 380 nm N(2)-H emission in (2,7-aza)Trp59-RNase T1, which is much shorter than that of ~10 ns for (2,7-aza)Trp in water.9 Also, we noticed that the intensity ratio among the triple emission bands in (2,7-aza)Trp59-RNase T1 was different from that of (2,7-aza)Trp in neutral water. Upon carefully examining the X-ray crystal structure of RNase T1, we found that Trp59 was close to Ala19 and Pro60 of the backbone and surrounded by traces of water molecules (see Figure 1). We thus suspect that the existence of this exceptional N(2)-H isomer in (2,7-aza)Trp59-RNase T1 may be correlated to a multiple H-bonding network. This viewpoint, together with the two distinct Trp environments, concluded from the above result, was further supported by the structural analyses of RNase T1 ((2,7-aza)Trp59-RNase T1) elaborated below.

**Molecular Dynamics (MD) Simulation on RNase T1**

Structure and Trp-Water Network. We then carefully examined the reported crystal structure data of RNase T1 (PDB: 9RNT).26 Figure 1 illustrates the possible water environment in the vicinity of Trp59, which reveals a water-network chain extending from wat-2 (close to Trp59) to outside bulk water via wat-3 and wat-5. RNase T1 has been studied extensively using MD simulations.52–55 Most studies have adopted the implicit solvent model and involved trajectories of much less than 1 ns, except for a few simulations of length longer than several ns.53–55 There still remains plenty of room in the interpretation of water dynamics coupled with protein motion and, hence, its enzymatic functionality. In this study, we performed a set of explicit water hydration simulations in an aim to probe the role of water in the protein structure and its motion for wild type as well as (2,7-aza)Trp substituted RNase T1. Here we present the analyses of the MD trajectory over 4 ns after initial equilibration for 1 ns. We found structural fluctuations occurring on time scales up to several ns. During the trajectory analyses, significant loop motions, as illustrated in Figure 4a, naturally occurred, implying the

![Figure 4](image-url)
Figure S5. Molecular dynamic simulations illustrate the water-coupled protein motion and thus switching between loop-close and loop-open forms of RNase T1. Also shown are the Trp59 site with hydration layer and outer bulk water. Only water molecules in the layer within 8 Å from the indole ring are shown for clarity.

chain-loop motion from a trans to a cis configuration that has been considered to facilitate a kinetic intermediate transition to the unfolding state. Accordingly, the related residue configurations were evaluated and are depicted in Figures 4a and S7. From Figure 4b, we also noted that the Trp rotamer underwent significant changes. Figure 4c depicts the distance evolution regarding the relative residues Pro60, Glu58, Ala19, Pro30, and Arg77 lining the Trp site and, hence, demonstrates the structural changes resulting from the interactions among the Trp site, the α-helix, and the loop backbone residues. It can be seen that the ring of Pro60 interacts strongly with the Trp59 rings. In addition, Glu58 is tethered to Arg77 via H-bonds. These residues all exhibited significant correlation motions in the simulation, and such motions could stabilize the interactions between Pro60 and Trp59. The results thus suggest that the intermolecular interactions among Trp59, Pro60, Glu58, and Arg77 residues determine the core structure of the connecting loop motion region (highlighted in Figure 4a). It is also worthy to note that the change in distance between Ala19 and Trp59 is associated with the helix motion, which undergoes significant displacement between loop-open and loop-close forms. The α-helix is closer to the Trp site in the loop-close form. The loop residues then fluctuate amid the transition such that the helix is more disordered and the Trp site is opened with higher water accessibility. Evidently, the fluctuation of loop residues is accompanied by the changes of Trp59 (or (2,7-aza)Trp59)-water network intermediate structures.

To gain more detailed insight, we then illustrated the MD snapshots of hydrated RNase T1 in loop-close and loop-open forms. Figure S5 depicts the Trp-water, Trp-protein, and protein-water interactions during the structural transition in a complementary way. Detailed analyses showed a dynamic exchange of hydrating water molecules with bulk water, which occurs at all times. We expect that most of the energetically favorable structure relaxation occurs between Trp59-water pairs in the loop region of RNase T1. There seems to be a synergy between the helix motion and displacement of Trp59 in regulation of the water accessibility, causing different Trp59/water contacts in a thermal equilibrium manner. Trp-water pairs appear to preferentially participate in a H-bond network incorporating amino acid moieties on the protein surface and bulk waters.

Figure 6a shows detailed analysis of time-dependent Trp59-water pair relaxation and distance evolution between indole-C(2)-H and local surrounding water molecules. Their exchange with bulk water molecules is also analyzed. The results indicate that even at the boundaries of the Trp site, water molecules fluctuate substantially, some making long excursions (4 ns-long over 150 Å) between hydration shell and bulk waters, whereas others have a longer residency time. The initially resident inner water molecules may also be replaced by outside bulk water. In order to probe the water network around the Trp site, the distance analysis was enlarged by 2–12 Å (see Figure 6a, lower part). Clearly, the trajectory begins at the Trp site filled with water; but soon, within the first 0.2 ns, water leaves the loop region and is exchanged with bulk water. In the course of the following 0.5 ns, there appear to be two types of structures, loop-close and loop-open forms, where the water micro-environment around the Trp site is different. One loop is accessible most probably by a single H-bond string of the water network (see snapshot at 1 ns, loop-close form in Figure 6b); the other is considered to be a water-filled state with double H-bond strings of the water network outreaching to the bulk water reservoirs (see snapshot at 2 ns, loop-open form in Figure 6b). Rarely, a more complicated string of water molecules was also observed (not shown here).

The plot of water accessibility of the Trp site as a function of the evolved time (Figure 6) demonstrates a repeated switching between loop-open and loop-close states. Our result also suggests that the loop of the Trp site is able to accommodate 3–5 water molecules across the cleft and then connect with the bulk water layer (see Figures 5 and S7). There is an apparent correlation regarding the presence of water in the Trp site and the protein transition motion between loop-open and loop-close forms. In addition, there exists an H-bond network in which water molecules specifically form hydrogen bonds with the carbonyl groups of both Trp59 and Pro60. Features of its...
location suggest that water may have a very long residency time at this location and that its H-bond formation with N−H group of (2,7-aza)Trp59 stabilizes the N(2)-H isomer, as revealed by Figure 6c (also, see trajectory analysis in SI, Figure S8).

Overall, by means of explicit water MD simulations, the Trp-enclose loop switching was revealed in open and close forms as a result of water coupled protein motion. The close form has a single string H-bond water network and hence is relatively water-scant, prohibiting the formation of (2,7-aza)Trp N(2)-H isomer. Conversely, the open form is filled with a double H-bond string water network and hence is water-rich, which promotes the formation of N(2)-H via additional N(2)-H···H2O H-bond stabilization. Water catalyzed ESPT takes places for the N(1)-H isomer in the loop-open forms, resulting in a prominent green N(7)-H tautomer emission. The MD simulated changes of the specific H-bond network are thus consistent with the multiple emissions of (2,7-aza)Trp59-RNase T1 observed experimentally.

Examining Trp59 (2,7-aza)Trp59) Specific H-Bonding Formation. As proposed above, the H-bond pairs exist between water and Trp59, as well as the carbonyl groups of Pro60 amide backbone in the β2 strand, which is postulated to be sensitive to the chemical/thermal driven dynamics on the protein environment. We thus carried out an experiment by unfolding RNase T1 using GuHCl. It has been well established that GuHCl molecules bind to peptidic bonds and accelerate the denaturing process by masking the hydrogen bonds within protein. As a result, the emission spectra of (2,7-aza)Trp59-RNase T1, shown in Figure 7a, exhibited a significant blue shift of the major peak from 372 to 350 nm upon unfolding with GuHCl. We then quantitatively deconvoluted the emission to N(1)-H (∼345 nm), N(2)-H (∼380 nm), and N(7)-H (∼495 nm), shown in Figure 7b. All pertinent fitting parameters are listed in Table S1. Obviously, upon the addition of 3 M GuHCl, the relative N(2)-H emission intensity decreased from 60 to 32%, and was further reduced to 27% upon the GuHCl concentration being increased to 6M. Note that the GuHCl dynamic quenching of N(2)-H emission was negligible, as evidenced by the increasing lifetime of the 380 nm emission (∼3 ns) after the addition of GuHCl. Therefore, the result can be well rationalized by the rupture of the Trp59 H-bond pairs near the connecting loop region destabilizing the N(2)-H isomer, and hence, we observed vestiges of spectral fingerprints in Figure 7b. The results thus
Figure 7. (a) Emission spectra of (2,7-aza)Trp59-RNase T1 (red solid line), denatured with 3 M GuHCl (blue dash line) and 6 M GuHCl (green dot line). (b) Identification of the triple emission bands by the deconvolution of photoluminescence spectra using triple emission bands (see text for detail). $\lambda_{em}$ = 310 nm.

We carried out fluorescence spectroscopy of wild type (containing a single tryptophan, Trp59), 7-azatryptophan ((7-aza)Trp59), and 2,7-diazatryptophan ((2,7-aza)Trp59) substituted RNase T1 to probe the water environment of Trp59 near the connecting loop region. The resulting emission bands and their associated relaxation dynamics, together with the molecular dynamics simulation, led us to several conclusions.

1. Equilibrium exists between two Trp59 containing conformational loops in RNase T1, namely, the water-rich loop-open and water-scarce loop-close forms, where the electronically excited Trp59 is subject to two different environments of relaxation. The interconversion between these two forms must be long ($\gg$ ns), as evidenced by two distinctive Trp, (7-aza)Trp, and (2,7-aza)Trp (N(1)-H) relaxation dynamics in wild type, (7-aza)Trp-RNase T1, and (2,7-aza)Trp-RNase T1 system.

2. In the loop-open form, water catalyzed (2,7-aza)Trp59 N(1)-H → N(7)-H excited-state proton transfer takes place, giving rise to a prominent 495 nm green emission band. The existence of N(2)-H isomer with distinct 380 nm emission is due to the specific hydrogen bonding between N(2)-H proton and water molecule that bridges N(2)-H and backbone Pro60, forming a multiple H-bond network.

3. The loop-close form is relatively tight in space, which squeezes water molecules out of the interface of the $\alpha$-helix and $\beta$-strand, joined by the connecting loop region; this water-scarce environment leads to the existence of solely N(1)-H isomer emission.

4. MD simulation indicated a hydrogen bond network incorporating polar amino acid moieties on the protein surface and bulk waters, providing the transition feature of the water accessibility to the connecting loop region in regulation of the RNase T1 dynamics.

This paper thus unveils the above-mentioned new insights into the structure-water network relationship in RNase T1 and demonstrates the powerful combination of Trp, (7-aza)Trp, and (2,7-aza)Trp to probe the water environment of a specifically targeted Trp in proteins.

ASSOCIATED CONTENT

Supporting Information

1. Synthesis and characterization. 2. The relative emission intensity of (2,7-aza)Trp. 3. Determination of RNase T1 activity. 4. The temperature-dependent emission spectra of (7-aza)Trp-RNase T1. 5. Photophysical measurement. 6. MD simulations of water solvated (2,7-aza)Trp-RNase T1. 7. Trajectory analysis of (2,7-aza)Trp-water network of (2,7-aza)Trp-RNase T1 system. 8. Water accessible and buried surfaces of RNase T1 protein. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES


(25) Cerezuelo, Vers 3.5; Molecular Simulations Inc.: 9685 Scranton Road, San Diego, CA, U.S.A.


(31) Cerezuelo, Vers 3.5; Molecular Simulations Inc.: 9685 Scranton Road, San Diego, CA, U.S.A.


(34) Cerezuelo, Vers 3.5; Molecular Simulations Inc.: 9685 Scranton Road, San Diego, CA, U.S.A.


(37) Cerezuelo, Vers 3.5; Molecular Simulations Inc.: 9685 Scranton Road, San Diego, CA, U.S.A.


(40) Cerezuelo, Vers 3.5; Molecular Simulations Inc.: 9685 Scranton Road, San Diego, CA, U.S.A.


(45) Chen, L. X.; Longworth, J. W.; Fleming, G. R. Picosecond Time-
Resolved Fluorescence of Ribonuclease T1. A pH and Substrate
W. Protein Dynamics. A Time-Resolved Fluorescence, Energetic and
Molecular Dynamics Study of Ribonuclease T1. *Biophys. Chem.* 1987,
26, 247–261.
(47) Wong, C. Y.; Eftink, M. R. Biosynthetic Incorporation of
Tryptophan Analouges into Staphylococcal Nuclease: Effect of 5-
Hydroxytryptophan and 7-Azagtryptophan on Structure and Stability.
*Protein Sci.* 1997, 6, 689–697.
(48) Negreire, M.; Gai, F.; Bellefeuille, S. M.; Petrich, J. W.
(49) Chen, Y.; Rich, R. L.; Gai, F.; Petrich, J. W. Fluorescent Species
of 7-Azaindole and 7-Azagtryptophan in Water. *J. Phys. Chem.* 1993,
97, 1770–1780.
(50) Chen, Y.; Gai, F.; Petrich, J. W. Single-Exponential Fluorescence
Decay of the Nonnatural Amino Acid 7-Azagtryptophan and the
(51) Chen, Y.; Gai, F.; Petrich, J. W. Solvation of 7-Azaindole in
Alcohols and Water: Evidence for Concerted, Excited-State, Double-
10166.
(52) Moors, S. L.; Jonckheer, A.; De Maeyer, M.; Engelborghs, Y.;
Ceulemans, A. Tryptophan Conformations Associated with Partial
Surfaces of β-Hairpin and α-Helical Peptides Generated by Replica
Exchange Molecular Dynamics with the AGBNP Implicit Solvent
(54) Axelsten, P. H.; Prendergast, F. G. Molecular Dynamics of
Tryptophan in Ribonuclease-T1. II. Correlations with Fluorescence.
Molecular Dynamics Simulations of Ribonuclease T1: Analysis of the
Effect of Solvent on the Structure, Fluctuations, and Active Site of the
(56) Kiefhaber, T.; Schmid, F. X. Kinetic Coupling Between Protein
Folding and Prolyl Isomerization. II. Folding of Ribonuclease A and
(57) Beauchamp, D. L.; Khajehpour, M. Probing the Effect of
Water–Water Interactions on Enzyme Activity With Salt Gradients: A
Case-Study Using Ribonuclease T1. *J. Phys. Chem. B* 2010, 114,
16918–16928.
(58) Roseman, M.; Jencks, W. P. Interactions of Urea and Other
(59) Mayo, S. L.; Baldwin, R. L. Guanidinium Chloride Induction of
Partial Unfolding in Amide Proton Exchange in RNase A. *Science
(60) Pace, C. N. Determination and Analysis of Urea and Guanidine
Hydrochloride Denaturation Curves. *Methods Enzymol.* 1986, 131,
266–280.