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Fluorescence Properties of a Tryptophan Residue in an Aromatic Core of the Protein Subunit of Ribonuclease P from *Escherichia coli*

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Escherichia coli ribonuclease P (RNase P), a ribonucleoprotein complex which primarily functions in tRNA biosynthesis, is composed of a catalytic RNA subunit, M1 RNA, and a protein cofactor, C5 protein. The fluorescence emission spectrum of the single tryptophan residue-containing C5 protein exhibits maxima at 318 nm and 332 nm. Based on a comparison of the emission spectra of wild-type C5 protein and some of its mutant derivatives, we have determined that the 318 nm maximum could be the result of a complex formed in the excited state as a result of hydrophobic interactions between Trp109, Phe18 and Phe73. The analogous tryptophan fluorescence emission spectra of wild-type C5 protein and the barstar mutant W38F/W44F, taken together with the detailed structural information available for barstar, provide a possible explanation for the unusual emission spectrum of C5 protein.

Keywords: Trp fluorescence; RNase P protein subunit; hydrophobic interactions

Ribonuclease P (RNase P) is essential for the maturation of tRNAs and is composed of M1 RNA (377 nucleotides) and C5 protein (119 amino acid residues) in *Escherichia coli* (Altman *et al*., l995; Pace & Brown, 1995). While M1 RNA is the catalytic subunit of RNase P from *E. coli*, the presence of C5 protein enhances the rate of the reaction catalyzed by M1 RNA alone *in vitro* and is required for enzymatic function *in vivo*. The role in RNase P catalysis of various conserved amino acid residues in C5 protein has been discussed in the accompanying paper (Gopalan *et al*., 1997). To complement the functional analyses of the various mutants, we employed fluorescence spectroscopy to examine the role of certain aromatic residues in the structure of C5 protein. We have used mutant derivatives of C5 protein to analyze how the microenvironment around the lone tryptophan residue (Trp109) influences the fluorescence emission spectrum of the protein.

A tryptophan and six phenylalanine residues are the aromatic amino acid residues in C5 protein (Hansen *et al*., 1985). The emission spectrum of wild-type C5 protein, obtained upon excitation at 280 nm, is not a smooth Lorentzian curve as is expected for a protein containing a single, uncomplexed tryptophan residue (Figure 1A). There is a plateau between 315 nm and 335 nm, possibly due to overlapping spectra. Derivative spectroscopy, a method for analyzing spectroscopic information, offers a simple tool for the resolution of overlapping peaks (Demchenko, 1986a; Padros *et al*., 1984). The calculation of derivative spectra ($dE/d\lambda$) involves computing the difference between the emission intensity at a given wavelength (λ) and the emission intensity when the wavelength is shifted by a defined amount ($\lambda + \Delta\lambda$). Performing this simple mathematical operation multiple times yields higher-order derivative spectra (for example, the second derivative, $\delta^2E/\delta\lambda^2$). The minima in the second-order derivative spectrum correspond to peaks in the original spectrum. Based on the position of the minima in the second-order derivative of the emission spectrum of C5 protein, we can infer maxima at 318 and 332 nm in the emission spectrum. Since both maxima are clearly blue-

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Abbreviations used: RNase P, ribonuclease P; $ptRNA^{Tyr}$, precursor of tyrosine tRNA.

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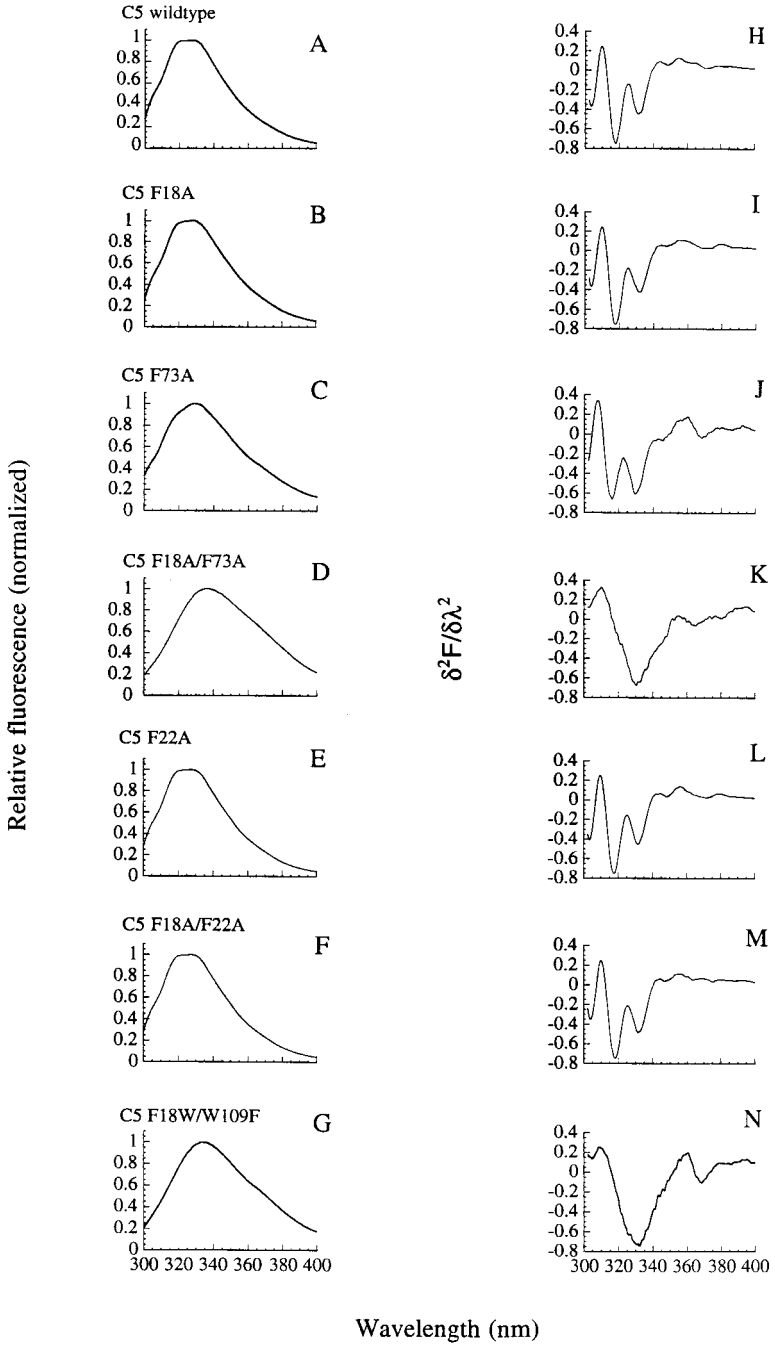

Figure 1. Peak normalized emission spectra of wild-type C5 protein and its various mutant derivatives (A through G). The emission spectra were obtained by excitation of the protein samples at 280 nm. The second derivatives of the various emission spectra are depicted in H through N. The intrinsic protein fluorescence was recorded on a F-4500 Hitachi fluorimeter. A thermostatted cuvette was used to ensure that all the measurements were performed at 25°C. The bandpass for both excitation and emission was 5 nm, the scan speed was 240 nm per minute and the response time was automatically adapted by the device. Details of the site-directed mutagenesis of C5 protein, overexpression and purification of mutant derivatives in BL21 (DE3) cells are provided in the accompanying paper (Gopalan *et al.*, 1997). After purification of the various mutant derivatives of C5 protein on a CM Sephadex column, the protein samples were stored at −70°C. Prior to CD or fluorescence spectroscopic measurements, the various protein samples were treated with 10 mM DTT for one hour at room temperature. This step is essential in order to prevent aggregation of the protein during dialysis. The samples were then dialyzed to remove the urea in a step-wise fashion and the storage buffer was exchanged for 10 mM potassium phosphate, 400 mM potassium chloride, 1 mM DTT (pH 7.5).

shifted compared to the emission maximum at 354 nm for denatured C5 protein (data not shown), we conclude that Trpl09 is in an apolar environment. When the tryptophan emission of a protein occurs at shorter wavelengths relative to the emission of tryptophan in water, the blue shift is accounted for by the shielding of the tryptophan residue from water by the hydrophobic protein interior (Lakowicz, 1983).

The fluorescence emission spectrum of C5 protein, which consists of overlapping peaks, can be the result of various microstates of Trp109 in the same structural position, but the interactions of which with functional groups in the local environment vary in the excited state (Demchenko, l986). To understand further the emission spectrum of C5 protein, we examined the microenvironment (i.e. nearby amino acid residues) around Trp109. In the accompanying study (Gopalan *et al.*, 1997), we gained additional insight regarding the Trp109 microenvironment from biochemical and genetic studies.

Unlike mutant derivatives of C5 protein in which conserved aromatic amino acid residues are altered individually, mutants in which pairs of conserved aromatic residues are mutated simultaneously lead to a drastic decrease in activity of RNase P (Gopalan *et al.*, 1997). For example, with

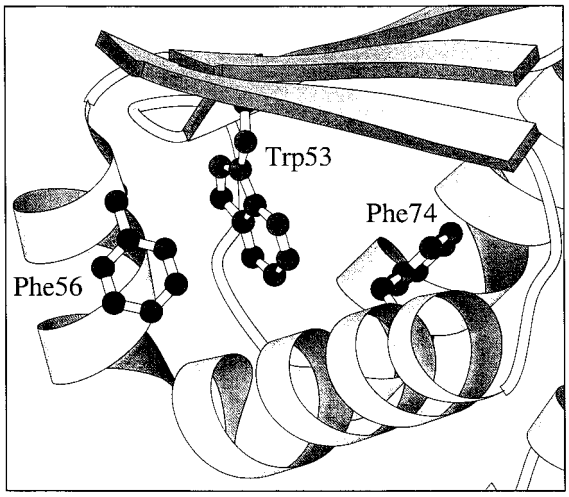

Figure 2. The hydrophobic core consisting of Trp53, Phe56 and Phe74 in barstar. The distances between the C^{γ} atoms in these residues are: Phe56-Trp53, 4.92 Å; Phe74-Trp53, 8.49 Å; Phe56-Phe74, 11.08 Å. The angles between the planes containing the chromophores are: Phe56-Trp53, 73.2°; Phe74-Trp53, 76.3°; Phe56-Phe74, 144.2°.

the precursor for tyrosine tRNA ($ptRNA^{Tyr}$) as the substrate, C5 F18A, C5 W109A and C5 F18A/W109A exhibit 34%, 69% and <1%, respectively, of the initial velocity observed with the wild-type C5 protein. After examining the effects of various mutations on C5 protein function, we hypothesized the presence of a hydrophobic core (or cores) in C5 protein that involves at least Phe18, Phe22, Phe73 and Trp109 (Gopalan *et al.*, 1997), and that certain phenylalanine residues in C5 protein might influence the fluorescence emission properties of Trpl09. This notion led us to compare the emission spectra of wild-type C5 protein and mutant derivatives (in which the phenylalanine residues were mutated) with each other and, also, with that of the protein barstar.

Barstar is a small protein inhibitor of the extracellular RNase barnase in *Bacillus amyloliquefaciens* (Hartley, 1989). The fluorescence emission spectrum of the barstar mutant W38F/W44F, which lacks Trp38 and Trp44 but still possesses a lone tryptophan residue at position 53, is similar to that of C5 protein and provides a basis for understanding our results with C5 protein. Even though the barstar mutant has only one tryptophan residue, the second-order derivative spectrum clearly resolves two peaks with maxima at 318 nm and 330 nm (data not shown; G. Schreiber & A. R. Fersht, unpublished observation). In barstar, Trp53 is flanked by Phe56 and Phe74 and is part of a hydrophobic core (Lubienski *et al.*, 1994). Phe56 and Phe74 are at distances of 4.92 Å and 8.49 Å from Trp53, respectively (Figure 2). Time-resolved fluorescence anisotropy measurements using wild-type barstar and its mutant derivative W38F/W44F suggest that the environment of Trp53 (in barstar) is rigid and devoid of any flexibility (Swaminathan *et al.*, 1996).

The analogous tryptophan fluorescence emission spectra of wild-type C5 protein and the barstar mutant W38F/W44F, taken together with the detailed structural information available for barstar, provide a possible explanation for the 318 nm and the 332 nm sub-bands in the emission spectrum of C5 protein. The 318 nm sub-band in the fluorescence emission spectrum of C5 protein could be due to a complex formed in the excited state as a result of hydrophobic interactions between the single tryptophan residue (Trp109) and one or more neighboring phenylalanine residues. The 332 nm sub-band would then correspond to the emission of Trp uncomplexed in the excited state. Similarly, in the case of barstar, the high energy band at 318 nm is likely to be due to a hydrophobic interaction between Trp53, Phe56 and Phe74 (Lubienski *et al.*, 1994).

To examine our hypothesis that the emission spectrum of C5 protein includes a component due to a hydrophobic interaction between Trp109 and one or two phenylalanine residues, we obtained the emission spectra of the following mutants: C5 F18A, C5 F22A, C5 F73A, C5 F18A/F22A, C5 F18A/F73A and C5 F18W/W109F. The fluorescence emission spectra and the respective second-order derivative spectra of the wild-type C5 protein and these mutants are depicted in Figure lA through G and H through N, respectively. The mutants C5 F18A, C5 F22A and C5 F18A/F22A display emission spectra similar to the wild-type C5 protein (Figure 1). The second-order derivative of the emission spectrum of C5 F73A reveals a weaker contribution of the 318 nm sub-band relative to that observed with the wild-type C5 protein. The emission spectra of the two mutants C5 F18A/F73A and C5 F18W/W109F are smooth Lorentzian curves, as expected for proteins with a single, uncomplexed tryptophan residue. The emission maximum for both mutants is 330 nm, indicating that the Trp is in an apolar environment and that the mutants possess some tertiary structure. The 318 nm minimum, present in the wild-type C5 protein spectrum, is clearly absent in the second-order derivative spectra of C5 F18A/F73A and C5 F18W/W109 mutants.

We have used the change in fluorescence as a function of urea concentration to demonstrate that the two peaks in the fluorescence emission spectrum of C5 protein are the result of various microstates of Trp109 in the same structural position. The intrinsic fluorescence of C5 protein decreases upon denaturation with urea. Therefore, the folding and unfolding of C5 protein can be monitored by fluorescence spectroscopy. Figure 3 reveals the decrease in intensity of the emission at both 318 nm and 332 nm as a function of increasing urea concentration. It is evident that the sharp transitions between the initial folded and the final unfolded states are superimposable regardless of whether we monitor this transition at 318 nm or

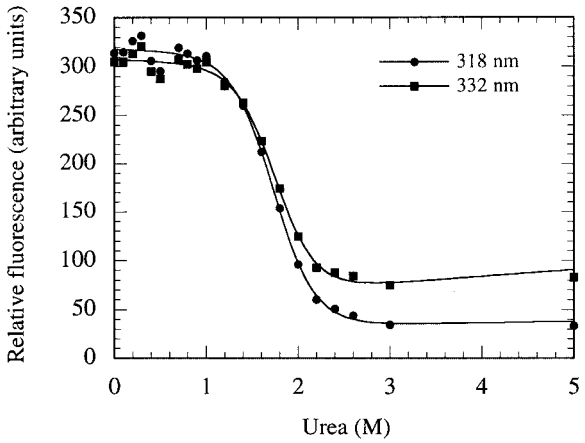

Figure 3. Urea-induced denaturation of C5 protein. The change in intensity of the fluorescence emission at 318 nm and 332 nm, upon excitation of the protein sample at 280 nm, with increasing concentration of urea is indicated. The fluorimeter parameters are as indicated in the legend to Figure 1. Stock solutions of different concentrations of urea were prepared and stored as 0.8 ml aliquots at −20°C. For each data point indicated in the Figure, 0.1 ml of ~30 μM C5 protein in 90 mM potassium phosphate, 3.6 M potassium chloride, 9 mM DTT (pH 7.5) was added to 0.8 ml of a defined concentration of urea. The unfolding was allowed to proceed for five hours at room temperature. The samples were centrifuged briefly (14,000 *g* for one minute) prior to obtaining fluorescence measurements.

332 nm. The midpoint of unfolding (i.e. where the native and unfolded states are equally populated) is calculated to be 1.73 (±0.03) M urea (based on data for 318 nm emission) or 1.75 (±0.04) M urea (based on data for 332 nm emission).

The difference in free energy between the folded and unfolded states of any protein is defined as the conformational stability (ΔG) and can be determined using a urea denaturation curve (Pace, 1986). The data from the transition region in the denaturation curve fit the equation $\Delta G = \Delta G^{H_2O} - m[D]$, where ΔG^{H_2O} is the value of ΔG in the absence of denaturant, m is a measure of the dependence of ΔG on the denaturant concentration and $[D]$ is the denaturant concentration. The calculations were performed essentially as described elsewhere (Santoro & Bolen, 1988; Clark & Fersht, 1993). Using the urea denaturation curve (based on data for 318 nm emission) in Figure 3, we have calculated $\Delta G^{H_2O} = 4.5$ kcal mol^{-1} and $m = 2.6$ (±0.3) kcal mol^{-1} M^{-1} for the wild-type C5 protein. Based on data for 332 nm emission, we have calculated $\Delta G^{H_2O} = 4.7$ kcal mol^{-1} and $m = 2.7$ (±0.3) kcal mol^{-1} M^{-1} for the wild-type C5 protein. Since the m value reflects the degree of exposure of the interior of the protein upon urea-induced denaturation, a high m value of 2.6 kcal mol^{-1} M^{-1} is consistent with the presence of a hydrophobic core in C5 protein.

Table 1. The half-intensity width values for the emission spectra of wild-type C5 protein and its various mutant derivatives

Protein	Half-intensity width (nm)
Wild-type C5	47.2
C5 F18A	48.3
C5 F73A	55.0
C5 F18A/F73A	63.9
C5 F22A	47.2
C5 F18A/F22A	49.5
C5 F18W/W109F	60.0

The emission spectrum of the wild-type C5 protein is the sum of two peaks (Figure 1). Therefore, it is not possible to classify the environment of Trp109 on the basis of the half-intensity width value of the emission spectrum (47.2 nm) according to the classification system of Burstein (1977). Nevertheless, it is noteworthy that the half-intensity width values of 55, 63.9 and 60 nm, observed for the emission spectra of C5 F73A, C5 F18A/F73A and C5 F18W/W109F, respectively, are larger than that observed with the wild-type C5 protein (Table 1). These three mutants exhibit emission spectra in which the 318 nm sub-band is either reduced or completely absent (Figure 1). The substitution of large, aromatic residues by Ala is likely to create a small cavity which enables solvent molecules to surround Trpl09. These solvent-mediated dipole-dipole interactions can account for the larger half-intensity width values observed with the mutants C5 F73A, C5 F18A/F73A and C5 F18W/W109F compared to the wild-type C5 protein.

Using mutant derivatives of C5 protein in which various conserved phenylalanine residues were mutated either alone or as a pair, we have demonstrated that the 318 nm sub-band is weaker in the F73A mutant and is not significantly altered in the F18A mutant (relative to the wild-type C5 protein). However, the 318 nm sub-band is completely eliminated in the double mutant C5 F18A/F73A. It is likely, therefore, that Phe18 and Phe73 are both needed for the excited state complex involving Trp109, Phe18 and Phe73. The transition moment responsible for the emission band at 318 nm of Trp109 must be absolutely dependent on the unique electronic and conformational states of the phenylalanine residues that flank this residue. The spatial orientation of Phe18 and Phe73 relative to Trp109 might enable the formation of a high-energy excited state complex. This observation is reinforced by our finding that the mutant C5 F18W/W109F fails to exhibit the 318 nm sub-band. Therefore, in this mutant, we conclude that Trp109 is not flanked by phenylalanine residues in the same spatial orientation as the wild-type C5 protein.

The changes in fluorescence properties of the mutant derivatives of C5 protein, compared to the wild-type, cannot be due to gross alterations in protein structure. Although mutants C5 F18A/F73A and C5 F18W/W109F display emission spectra unlike that of wild-type C5 protein in that there

is only one peak at 330 nm, it is evident that we are examining the properties of Trp109 in the folded state of these mutant derivatives. The peak at 330 nm is clearly blue-shifted compared to the emission maximum at 354 nm observed with denatured C5 protein and is indicative of Trp in an apolar environment (although the water of hydration may be different compared to the wild-type C5 protein). Moreover, all the mutant derivatives used in this study displayed significant secondary structure and are not disordered, as determined by CD spectroscopy (data not shown). The mutants C5 F73A and C5 F18W/W109F function at close to wild-type levels both *in vivo* and *in vitro* (Gopalan *et al*., 1997) and must, therefore, be folded into a tertiary structure essential for RNase P catalysis.

Often, aromatic residues in proteins form a network of three or more interacting aromatic side-chains and these interactions stabilize the tertiary structure of the protein (Burley & Petsko, 1985; Singh & Thornton, 1985). Since aromatic-aromatic interactions can serve as nucleation sites in protein folding, further information regarding the arrangement of aromatic side-chains of C5 protein will be particularly valuable in understanding the tertiary structure of this protein.

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