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Fluorescence Spectrum of Barnase: Contributions of Three Tryptophan Residues and a Histidine-Related pH Dependence

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Received December 6, 1990; Revised Manuscript Received March 21, 1991

ABSTRACT: Fluorescence spectra of wild-type barnase and mutants in which tryptophan and histidine residues have been substituted have been analyzed to give the individual contributions of the three tryptophan residues. The spectrum is dominated by the contribution of Trp-35. The fluorescence intensity varies with pH according to an ionization of a pK_a of 7.75. This pK_a is close to that previously determined by NMR titration of the C2–H resonances of His-18 as a function of pH (Sali et al., 1989). This histidine residue is close to Trp-94. The pH dependence of the spectrum is abolished when either His-18 or Trp-94 is mutated, and so appears to be caused by the His-18/Trp-94 interaction. The spectral response of this interaction can serve as a probe of the folding pathway and of electrostatic effects within the protein. Changes in the fluorescence spectra on substitution of Trp-94 and His-18 suggest that there is net energy transfer from Trp-71 to Trp-94.

The extracellular ribonuclease barnase (Hartley & Barker, 1972) produced by the prokaryote *Bacillus amyloliquefaciens* is being used extensively as a model protein for biophysical studies on protein folding and stability (Kellis et al., 1988, 1989; Matouschek et al., 1989, 1990; Serrano & Fersht, 1989; Serrano et al., 1990; Bycroft et al., 1990; Horovitz et al., 1990). It is a small (110 residues, 12 382 Da), monomeric, single-domain enzyme, formed by a 5-stranded, twisted antiparallel β-sheet and 2 α-helices (residues 6–18 and 26–34), the first of which packs against the β-sheet (Mauguen et al., 1982).

The major probe that is being used to chart the folding and unfolding pathways of barnase is its fluorescence emission spectrum (Kellis et al., 1989). It is important, therefore, to understand the factors that contribute to this spectrum in the native protein. Tryptophan residues make the major contribution to the fluorescence emission spectra of proteins when present. The fluorescence properties of these residues are particularly sensitive to the environment of their side chains. There are three tryptophan residues in barnase, at positions 35, 71, and 94 (Figure 1). Trp-35 is near the C-terminal end of the second α-helix and relatively far away (22–25 Å) from the other two tryptophans. Trp-71 is in a hydrophobic region at the beginning of the second strand of the β-sheet. Trp-94 is at the beginning of the fourth strand of the β-sheet and only 10 Å apart from Trp-71. The fractions of solvent-exposed surface of the side chains of the three tryptophans are 0.075 (Trp-35), 0.029 (Trp-71), and 0.219 (Trp-94). There are numerous close contacts between Trp-94 and a histidine residue (His-18) that lies at the C-terminal end of the first α-helix.

We have mutated each of the three tryptophans into significantly less fluorescent residues in order to assess the contribution of individual tryptophans to the fluorescence emission spectrum of barnase. Three mutant proteins were constructed, in each of which a single tryptophan residue was replaced by another aromatic amino acid. To study the specific contribution of Trp-94 to the total fluorescence spectrum and the influence of His-18, two more mutant proteins were made; one in which His-18 was replaced by glycine and one in which Trp-94 was replaced by leucine.

We report here that the major contributor to fluorescence intensity in the protein is Trp-35 and that there is probably energy transfer between the two other tryptophan residues. We also report that there is a pH dependence of fluorescence intensity caused by a tryptophan–histidine interaction, and discuss the use of this effect to study electrostatic interactions within the protein.

Materials and Methods

Materials

Radiochemicals were from Amersham International plc., Buckinghamshire, U.K. The buffers used in the fluorescence experiments, Tris [tris(hydroxymethyl)aminomethane] and Bis-Tris ([bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane]), and Torula yeast RNA were obtained from Sigma, St. Louis, MO. SP-Trisacryl-M was purchased from IBF biotechnics, Villeneuve La Garenne, France. Dialysis tubing was purchased from Spectrum Medical Industries Inc., Los Angeles, CA. All other reagents were of analytical grade and were purchased either from Sigma or from Amersham.

Escherichia coli BL21(DE3) pLysS cells were a gift from Dr. F. W. Studier. The plasmid pTZ18U and the helper phage M13KO7 were obtained from Pharmacia, Uppsala, Sweden. The wild-type barnase gene was cloned into the plasmid pUC9 by Paddon and Hartley (1987) and donated to us. A 1.4-kb *Eco*RI–*Hin*dIII restriction fragment of this recombinant plasmid containing the structural gene for barnase fused to the promoter and signal sequence of the *E. coli* alkaline

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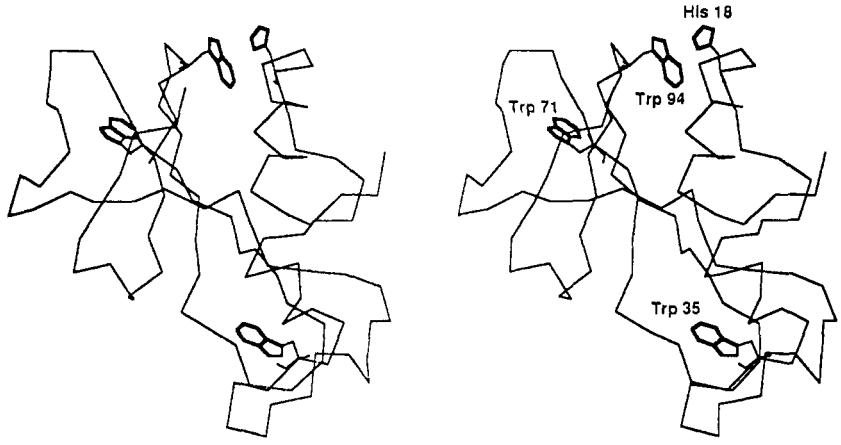

FIGURE 1: Stereoview of the structure of barnase showing the relative positions of the three tryptophan residues, and His-18.

phosphatase gene was subcloned into the plasmid pTZ18U (Serrano et al., 1990).

Methods

Mutagenesis. Single-stranded DNA was obtained from the modified plasmid pTZ18U, harbored in *E. coli* TG2 after infection with the helper phage M13K07. Site-directed mutagenesis was carried out by using the method of Sayers et al., (1988) and the kit supplied by Amersham (using one-fifth of the amounts of reagents recommended in the kit). The following oligonucleotides were used to direct the mutations:

Trp-35 →
Phe: 5′-TTGATGCCACG*A*AGCCGAGGG-3′

Trp-71 →
Tyr: 5′-CGCTTCACGA*T*ATGTTCGTCC-3′

Trp-94 →
Phe: 5′-TTTGTAAATCAGG*A*AGTCGCTTGAG-3′

Trp-94 →
Leu: 5′-GTAAATCAGCA*AGTCGCTTGA-3′

His-18 →
Gly: 5′-CAGGTAGCTTAC*C*ATATGTCTGAA-3′

(an asterisk follows mismatched bases).

Trp-71 was mutated to tyrosine and not phenylalanine because several homologous proteins have tyrosine at that position (Hill et al., 1983). Mutants were identified by sequencing their ssDNA according to the chain termination method (Sanger et al., 1977) using the Sequenase (Tabor & Richardson, 1987) kit supplied by United States Biochemical Corp., Cleveland, OH.

Expression and Purification of Barnase. The mutant ssDNA was used to transfect BL21(DE3)(F⁻,*ompT*,*rBmB*−) (Grodberg & Dunn, 1988; Studier & Moffatt, 1986). To confirm identification of mutants, plasmids obtained from individual colonies of transfected BL21(DE3) cells were used to transfect *E. coli* TG2 cells. These were in turn infected with the helper phage, and ssDNA was prepared and sequenced. Purification of the proteins was performed as described previously (Serrano et al., 1990).

Protein Concentration. Extinction coefficients of the native proteins were determined by the method improved by Gill and von Hippel (1989). The extinction coefficients of the denatured proteins were calculated from the number of tryptophan and tyrosine residues in the protein using the tabulated molar extinction coefficients. The extinction coefficients of the native enzymes were calculated from the UV absorbance of solutions of identical concentration of native and denatured enzymes. Protein concentrations were determined from the UV absorbance at 280 nm and the appropriate extinction coefficients.

Enzymatic Activity. RNA hydrolysis by wild-type and mutant enzymes was assayed at 25 °C by monitoring the decrease in absorbance at 298.5 nm on addition of the enzyme to a 2 mg/mL torula yeast RNA solution in 0.1 M ionic strength Tris-HCl, pH 8.5 (Rushizky et al., 1963).

Fluorescence Spectra and pK_a Determination. Stock buffer solutions (Tris or Bis-Tris of ionic strength I = 100 mM) at different values of pH were prepared by adding varying volumes of 1 M Tris or Bis-Tris in distilled water to 200 μL of 5 N HCl and completing the volume to 10 mL with distilled water. The solutions were then frozen. Before an experiment was performed, each buffer solution was diluted 1:10 to a final ionic strength of 10 mM. Aliquots of 25 μL of the stock protein solutions in distilled water ($A_{280} \sim 1.5$) were added to 875 μL of diluted Tris or Bis-Tris buffers in a cuvette thermostated at 25 °C. The solution was mixed by single-inversion in order to minimize adsorption of the enzyme to the cuvette. Spectra or emission fluorescence intensity values at a chosen wavelength were recorded on a Perkin-Elmer LS-50 luminescence spectrometer immediately after the temperature had reached 25 ± 0.1 °C. The excitation wavelength was 295 nm, and the bandwidth for both excitation and emission was 10 nm. Spectra were corrected by subtracting the spectrum of 875 μL of the buffer mixed with 25 μL of distilled water. The pH of the mixed solutions was measured at the end of the experiment with a radiometer Copenhagen PM64 pH meter, calibrated against standard buffers supplied by Radiometer Copenhagen. The sigmoidal titration curves of fluorescence intensity of the wild-type protein and the Trp-71 → Tyr and Trp-35 → Phe mutants were fitted to the equation of a single ionization equilibrium.

RESULTS

Expression and Yield of Mutants. Mutant proteins were expressed with yields of between 3.4 and 9.0 mg/L. These yields are similar to those previously reported in our laboratory with our expression system. Although the mutant proteins involved replacement of bulky residues with smaller ones, there was no difficulty in their expression. All mutants have an activity between 50 and 100% of that of wild-type enzyme.

Extinction Coefficients. The molar extinction coefficients (M^{-1} cm^{-1}) at 280 nm of the wild-type and mutant proteins in water were found to be the following: wild type, 27 411; Trp-35 → Phe, 21 547; Trp-71 → Tyr, 23 339; Trp-94 → Phe, 22 481; Trp-94 → Leu, 22 277. The molar extinction coefficient for His-18 → Gly was assumed to be the same as that of the wild-type protein. The extinction coefficient (in units of mg^{-1} cm^{-1}) for the wild-type protein is thus 2.21. This value

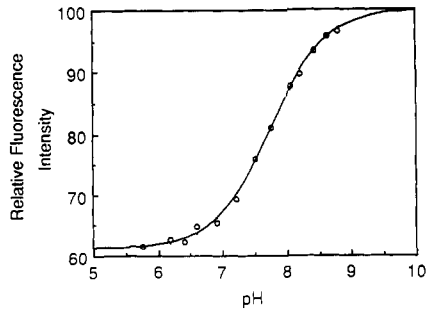

FIGURE 2: pH titration of His-18 in wild-type barnase. The intensity of fluorescence emission of solutions of different pH in either Tris or Bis-Tris buffers (ionic strength = 10 mM) containing 1 μM barnase was recorded at 340 nm. The solid line represents the best fit to a single ionization equilibrium by nonlinear regression.

Table I: pK_a of His-18 in Barnase and Mutants, As Measured by the Change in Fluorescence Emission Intensity at the Indicated Wavelength[a]

protein	emission wavelength (nm)	pK_a
wild type	340	7.75 ± 0.04
Trp-35 → Phe	336	7.75 ± 0.04
	346	7.77 ± 0.03
Trp-71 → Tyr	340	7.74 ± 0.04

[a] Experiments performed at 25 °C at an ionic strength of 0.01 M.

Table II: Wavelength Emission[a] Maxima and Relative Intensities[b] at These Wavelengths for Wild-Type and Mutant Proteins

protein	λ_{max} (pH 5.5) (nm)	relative intensity	λ_{max} (pH 9.4) (nm)	relative intensity
wild type	336.0	100.0	340.5	158.5
His-18 → Gly	347.5	262.8	347.5	247.7
Trp-35 → Phe	336.0	30.2	346.0	89.1
Trp-71 → Tyr	336.5	82.7	340.5	123.9
Trp-94 → Phe	331.5	199.8	331.5	182.6
Trp-94 → Leu	331.5	191.8	331.5	179.9

[a] Experiments performed at 25 °C at an ionic strength of 0.01 M. [b] Relative to wild type at pH 5.5 = 100%.

is about 5% higher than the value previously quoted of 2.09 mg^{-1} cm^{-1} (Lees & Hartley, 1966).

pH Dependence of Fluorescence Intensity. The titration curve of fluorescence intensity of the wild-type protein as a function of pH is shown in Figure 2. The titration curve is sigmoidal and fits a theoretical curve for the ionization of a single residue with a pK_a of 7.75 ± 0.02. The same type of curve and same pK_a values were found for the mutant proteins Trp-35 → Phe and Trp-71 → Tyr. The emission intensities for each protein were monitored at the λ_{max} of fluorescence intensity at high pH. The ratio of intensities at high to low pH is 1.63 for the wild-type protein, 2.96 for the mutant Trp-35 → Phe, and 1.52 for the mutant Trp-71 → Tyr. The emission intensities of the mutants Trp-94 → Phe, Trp-94 → Leu, and His-18 → Gly are constant (±3.5%) in the pH range examined.

pK_a values of His-18 as determined from fluorescence titration curves are shown in Table I. Values for all three proteins, wild type and the mutans Trp-71 → Tyr and Trp-35 → Phe, are very close to 7.75 ± 0.02. The same value of pK_a is obtained when the fluorescence intensity as a function of pH for Trp-35 → Phe is monitored at two different wavelengths.

Wavelength emission maxima of the fluorescence of the mutant proteins are shown in Table II. No change in the wavelength of fluorescence intensity maxima between low and high pH is noted in mutants which lack either Trp-94 (Trp-94 → Phe and Trp-94 → Leu) or His-18 (His-18 → Gly). The

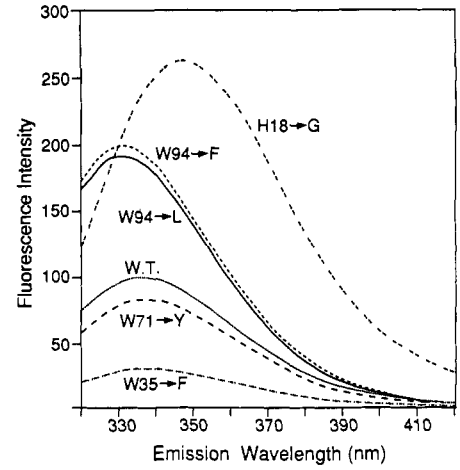

FIGURE 3: Fluorescence emission spectra of the different mutants in Bis-Tris buffer (ionic strength = 10 mM) at pH 5.5. All spectra were recorded at the same protein concentration of 4 μM. The maximal value of the intensity of emission of the wild-type protein has been arbitrarily taken at 100.

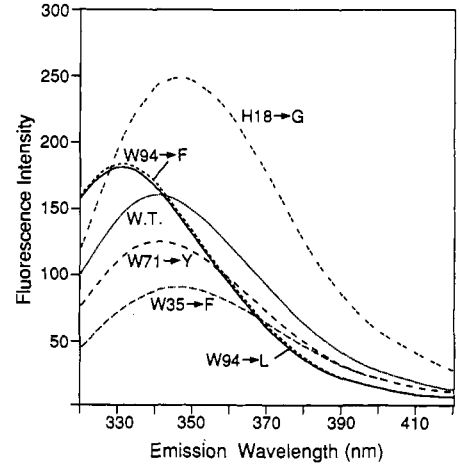

FIGURE 4: Fluorescence emission spectra of the different mutants in Tris buffer (ionic strength = 10 mM) at pH 9.4. All spectra were recorded at the same protein concentration of 4 μM. Emission intensities are relative to the maximal value of intensity of emission of the wild-type protein at pH 5.5 (see legend to Figure 3).

replacement of Trp-94 by either leucine or phenylalanine causes a blue shift (4.5 nm at low pH and 9.0 nm at high pH) of the fluorescence emission spectrum relative to the wild-type protein, while a red shift of 11.5 nm at low pH and 7.0 nm at high pH is observed on mutation of His-18 to glycine. The wavelength maxima of the fluorescence emission of the mutant Trp-71 → Tyr are the same as those of the wild-type protein both at low and at high pH. When Trp-35 is mutated to phenylalanine, there is a red shift of 5.5 nm at high pH relative to the wild-type protein, while the maximum at low pH remains the same.

Fluorescence emission spectra of the different proteins are shown in Figure 3 for pH 5.5 and in Figure 4 for pH 9.4. In the mutant His-18 → Gly, the maximal fluorescence intensity increases relative to the wild-type protein by about 150% at low pH, and about 55% at high pH. The intensity of fluorescence is also increased relative to the wild-type protein when Trp-94 is removed (mutants Trp-94 → Phe and Trp-94 → Leu). This increase is on the order of 100% at low pH, and about 15% at high pH. When Trp-35 is replaced by phenylalanine, there is a decrease of more than 70% of the fluorescence intensity at low pH, and about 45% at high pH. The replacement of Trp-71 by tyrosine has little effect on the

intensity of the fluorescence spectrum.

Discussion

pH Dependence of Fluorescence Intensity: Influence of a Histidine Residue. A sigmoidal curve with an inflection point at pH 7.75 is obtained when the emission fluorescence intensity of wild-type protein at a fixed wavelength is plotted as a function of pH (Figure 2). The emission spectrum is due mainly to tryptophan groups, since the protein was excited at 295 nm. The pH dependence of fluorescence intensity suggests, therefore, the presence of an ionizable group with a pK_a of 7.75 that affects a tryptophan residue in barnase. Candidates for such a group are histidine residues. There are two histidine residues in barnase, at positions 18 and 102. One of them, His-18, makes close contacts with a tryptophan residue (Trp-94, Mauguen et al., 1982). We have constructed mutants in which the interaction between Trp-94 and His-18 is eliminated by mutating either Trp-94 or His-18. A flat plot is obtained when the intensity of the fluorescence emission of the mutant proteins, His-18 → Gly, Trp-94 → Phe, and Trp-94 → Leu, that lack either His-18 or Trp-94 is titrated from pH 5.6 to pH 8.7 (data not shown). No change is observed in the wavelength of fluorescence intensity maxima between low and high pH in these mutants (Table II). Their fluorescence emission spectra are, therefore, essentially pH independent in this range. Mutant proteins in which the interaction between His-18 and Trp-94 is maintained, but one of the two other tryptophans is substituted, i.e., Trp-35 → Phe and Trp-71 → Tyr, show a sigmoidal pattern of titration which fits a single ionization curve with the same pK_a as wild type (Table I). When the fluorescence emission of the mutant Trp-35 → Phe is monitored at two different wavelengths, the same pK_a value is obtained. This is expected for a process involving the ionization of a single group. It is evident from these results that the pH dependence of fluorescence in barnase is associated with the simultaneous presence of both Trp-94 and His-18, and not with the other two tryptophans. The decrease in fluorescence at low pH would result from excessive quenching of the fluorescence of Trp-94 by a protonated histidine residue in position 18. The phenomenon of quenching of tryptophan fluorescence by protonated histidine residues has been observed first by Shinitzky and Goldman (1967). Since the fluorescence intensity of a mutant which lacks histidine (His-18 → Gly; see Figures 3 and 4) is greater than that of the wild-type protein, we suggest that both the unprotonated and the protonated forms of histidine can quench the fluorescence of the tryptophan, and that the quenching by the protonated form is greater.

The pK_a of His-18 obtained from the fluorescence titration curves (7.74–7.77) in H_2O is about 0.2 pK_a unit lower than the value obtained by NMR from a plot of the C2–H resonances of His-18 as a function of pD in D_2O (Sali et al., 1989). The higher value in D_2O is caused by an H/D isotope effect. The pK_a values measured by fluorescence are close to the value of 7.71 which was determined from the pH dependence of the stability of the protein in water, under conditions where the stability of the protein varies with the ionization of His-18 (Sali et al., 1989). The very similar values of pK_a that are obtained for the different mutant proteins indicate that no major structural changes have occurred at the Trp-94/His-18 site on mutation at positions 35 and 71. Neither does any gross change occur at the active site of the protein since all mutants retain the enzymatic activity.

Fluorescence Emission Spectra: Individual Tryptophan Contributions and Possible Energy Transfer between Trp-71 and Trp-94. The fluorescence emission spectra of the different proteins at low (5.5) and high pH (9.4) are shown in Figures 3 and 4 at identical concentrations. These values of pH were chosen so that His-18, when present, is at least 95% protonated or 95% unprotonated for all proteins examined (unpublished results).

Contribution of Trp-35. When Trp-35 is mutated, there is a decrease of more than 70% of the fluorescence intensity at low pH, and about 45% at high pH. This residue is, therefore, the major contributor of fluorescence emission in the wild-type protein, both at low and at high pH. An alternative explanation to the decrease in fluorescence on mutation of Trp-35 could be a change in the environment of the two other tryptophans on mutation at position 35. This is unlikely, since both tryptophans are far from position 35 and the pK_a of His-18, which is highly dependent on its interaction with Trp-94 (unpublished results), is unchanged (Table I). Mutation of Trp-35 results in a red shift relative to the wild-type protein at high pH (Table II). This can be explained by assuming that, in the absence of Trp-35, the emission spectrum of this mutant protein at high pH is dominated by the contribution of Trp-94 (see below).

Contribution of Trp-71. When Trp-71 is mutated to tyrosine, there is only a small decrease in fluorescence intensity, of about 20% at both low and high pH. Trp-71, therefore, contributes the least to the fluorescence intensity of the wild-type protein. This is also suggested by the observation that the wavelength maxima of fluorescence emission of the mutant Trp-71 → Tyr at low and at high pH (Table II) are the same as those of the wild-type protein. Again, on mutation at position 71, there is probably no change in the environment of tryptophan-94, since no change was seen in the pK_a of His-18. Trp-71 is the most buried residue of the three tryptophans, and therefore one would expect its contribution to the emission spectrum to be greater. The reason for the small contribution of Trp-71 to the emission spectrum in the wild-type protein is probably energy transfer to Trp-94 (see below).

Contribution of Trp-94. The intensity of fluorescence relative to the wild-type protein is increased when Trp-94 is removed (mutants Trp-94 → Phe and Trp-94 → Leu), by about 100% at low pH and about 15% at high pH. This indicates either that Trp-94 in the wild-type protein provides a negative contribution to the total fluorescence intensity or, alternatively, that there is a change in the environment of the other two tryptophans on mutation at position 94. This is unlikely, since both a relatively conservative mutation at position 94, Trp-94 → Phe, and a less conservative mutation, Trp-94 → Leu, show essentially the same fluorescence emission spectra. Further, the two mutant proteins remain active. A more plausible explanation for the negative contribution to fluorescence of Trp-94 is that, in the wild-type protein there is energy transfer from one of the two other tryptophans to this residue, but that its fluorescence emission is not enhanced concomitantly because of a quenching effect exerted by His-18. The emission spectra of the buried residues Trp-71 and Trp-35 (as indicated by the blue shift of the mutants Trp-94 → Phe and Trp-94 → Leu) are blue-shifted relative to Trp-94 (see Table II). The blue-shifted emission spectra of Trp-35 and Trp-71 provide an effective overlap (required for energy transfer) with the absorption spectrum of Trp-94 in the UV region. The emission fluorescence of this transferred energy is effectively quenched by the presence of His-18. This becomes evident when His-18 is mutated to glycine. This mutant exhibits a 150% increase of fluorescence intensity at low pH (55% at high pH) relative to the wild-type protein at the same pH values. Removal of the histidine residue is expected to

increase the exposure to solvent of Trp-94 and therefore decrease the fluorescence intensity. The observed effect is opposite, i.e., enhancement of fluorescence intensity. This can be attributed to the removal of the quenching effect of His-18. Since the distance between Trp-71 and Trp-94 (10 Å) is much smaller than the distance of either residue to Trp-35 (22–25 Å), net energy transfer probably occurs from Trp-71 to Trp-94. This also explains the small contribution of Trp-71 to the fluorescence spectrum of the wild-type protein. Finally, the higher exposure to solvent of Trp-94 in the mutant His-18 → Gly and its increased contribution to the fluorescence spectrum relative to the other tryptophan residues explain why this mutant exhibits the most red-shifted spectrum of all.

The fluorescence changes found in this study provide useful probes for future studies. The pK_a of His-18 can, for example, be determined with high precision, and can be used to quantify electrostatic interactions between His-18 and other charged residues in wild-type and mutant proteins. The kinetics of folding for mutants of tryptophans may give clues on the cooperativity of folding.

Conclusions

The following picture of the fluorescence emission spectrum of barnase emerges from our studies: Trp-35 located near the C-terminal end of the second α-helix and far from the two other tryptophan residues is the major contributor to the spectrum. The fluorescence of Trp-94 which lies at the fourth strand of the β-sheet is quenched by the presence of a nearby histidine (His-18). Trp-71 is located at the beginning of the second strand of the β-sheet 10 Å away from Trp-94 and is probably involved in energy transfer to this residue. The quenching of fluorescence by histidine-18 can be utilized as a probe of the folding pathway of barnase and to quantify accurately electrostatic interactions within the protein.

Registry No. Trp, 73-22-3; His, 71-00-1; barnase, 37300-74-6.

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