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Biosynthetic incorporation of tryptophan analogues into staphylococcal nuclease: Effect of 5-hydroxytryptophan and 7-azatryptophan on structure and stability

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Abstract

5-Hydroxytryptophan (5HW) and 7-azatryptophan (7AW) are analogues of tryptophan that potentially can be incorporated biosynthetically into proteins and used as spectroscopic probes for studying protein–DNA and protein–protein complexes. The utility of these probes will depend on the extent to which they can be incorporated and the demonstration that they cause minimal perturbation of a protein's structure and stability. To investigate these factors in a model protein, we have incorporated 5HW and 7AW biosynthetically into staphylococcal nuclease A, using a trp auxotroph *Escherichia coli* expression system containing the temperature-sensitive lambda *c*I repressor. Both tryptophan analogues are incorporated into the protein with good efficiency. From analysis of absorption spectra, we estimate ~95% incorporation of 5HW into position 140 of nuclease, and we estimate ~98% incorporation of 7AW. CD spectra of the nuclease variants are similar to that of the tryptophan-containing protein, indicating that the degree of secondary structure is not changed by the tryptophan analogues. Steady-state fluorescence data show emission maxima of 338 nm for 5HW-containing nuclease and 355 nm for 7AW-containing nuclease. Time-resolved fluorescence intensity and anisotropy measurements indicate that the incorporated 5HW residue, like tryptophan at position 140, has a dominant rotational correlation time that is approximately the value expected for global rotation of the protein. Guanidine-hydrochloride-induced unfolding studies show the unfolding transition to be two-state for 5HW-containing protein, with a free energy change for unfolding that is equal to that of the tryptophan-containing protein. In contrast, the guanidine-hydrochloride-induced unfolding of 7AW-containing nuclease appears to show a non-two-state transition, with the apparent stability of the protein being less than that of the tryptophan form.

Keywords: 7-azatryptophan; CD studies of staphylococcal nuclease A; 5-hydroxytryptophan; nuclease A (staphylococcal); thermodynamics of unfolding; time-resolved fluorescence studies of staphylococcal nuclease A; tryptophan analogues

There has been much interest in recent years in the strategy of incorporating unnatural amino acids into proteins, for example, as a means of specifically perturbing the chemical nature of a particular side chain to test for its contribution to the function of a protein. To cite a few examples, Schultz and coworkers have used a cell-free expression system (chemically attaching the desired unnatural amino acid to suppressor tRNA and then placing the amber codon at the desired position in the mRNA) to incorporate a number of unnatural amino acids, mostly aliphatic amino acid analogues, at various positions in T4 lysozyme (Noren et al., 1989; Ellman et al., 1992; Mendel et al., 1992) and staphylococcal nuclease (Judice et al., 1993; Thorson et al., 1995). The small amount of analogue-containing protein produced was then subjected to thermal unfolding studies, which revealed modest changes in the apparent transition temperature. Using an alternate enzymatic coupling method, Jackson et al. (1994) have studied the effect of an unnatural amino acid, 4-fluorohistidine, on the catalytic activity of ribonuclease A.

Groups led by Szabo and Ross have focused on the incorporation of analogues of the amino acid tryptophan and have used whole-cell biosynthetic procedures, taking advantage of the fact that tryptophanyl tRNA synthetase will catalyze tRNA acylation with a few analogues of this aromatic amino acid. Szabo and coworkers have incorporated 5-hydroxytryptophan (5HW) and 7-azatryptophan (7AW) into oncomodulin (its Y57W mutant), rat parvalbumin (its F102W mutant), and *Bacillus subtilis* tryptophanyl tRNA synthetase (Hogue et al., 1992, 1995; Hogue, 1994). Similarly, Ross and coworkers have incorporated 5HW biosynthetically into bacteriophage λ *c*I repressor and insulin (Ross et al., 1992; Laue et al., 1993; Laws et al., 1995). Also, Heyduk and

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Callaci (1994) have incorporated 5HW into the sigma subunit of RNA polymerase, and Soumillion et al. (1995) have incorporated 7AW into phage lambda lysozyme. An advantage of the whole-cell biosynthetic methods (over cell-free expression systems, chemical, or enzymatic synthesis methods) is that larger amounts of protein potentially can be produced.

The main reason for the interest in incorporating 5HW and 7AW into proteins is that the absorbance spectrum of these analogues is red-shifted with respect to that of tryptophan (see Results). Consequently, it should be possible to selectively excite either 5HW or 7AW, in proteins, in the presence of tryptophans of other proteins or DNA bases. In fact, Laue et al. (1993) have exploited the red-shifted absorbance of 5HW to directly monitor (in an ultracentrifuge) the binding of 5HW-lambda repressor to DNA. The term "spectrally enhanced" proteins was coined for such 5HW- or 7AW-containing proteins. Both 5HW and 7AW have red-shifted absorbances (more prominent for the former), but they differ in their fluorescence quantum yields and Stokes shifts. The amino acid 5HW (or 5-hydroxyindole) has a quantum yield similar to that of tryptophan and has a small and solvent-insensitive Stokes shift (i.e., its emission maximum is about 339 nm in water). In contrast, 7AW has a low quantum yield and a large Stokes shift in water. The yield and Stokes shift of 7AW depend on the protic nature of the solvent and is related to the existence of different tautomeric species in solution (Avouris et al., 1976; Négrerie et al., 1991; Chapman & Marconcelli, 1992; Chen et al., 1993). Although weakly fluorescent in water, the 7-azaindole ring shows much stronger fluorescence in aprotic solvents such as acetonitrile, suggesting that 7AW might be very sensitive to its microenvironment in a protein. Figure 1 shows the chemical structure of 5HW and 7AW.

Because there appear to be such good prospects for the advantageous use of such spectrally enhanced proteins, it is important to determine the extent to which the tryptophan analogues perturb the structure and stability of a protein. In the present study, we have undertaken to do this and we have selected as the protein for this study staphylococcal nuclease. This protein, and its many mutants, have been the subject of numerous structure/stability/folding studies, and thus there is a wealth of information about this protein (Shortle et al., 1988; Carra et al., 1994; Shortle, 1995). Importantly, it has a single tryptophan residue, Trp 140, whose fluorescence properties have been characterized extensively (Brochon et al., 1974; Eftink et al., 1991, 1996), and nuclease has an excellent expression system and protein purification procedure (Shortle & Meeker, 1989). Here we present results of our attempt to modify the expression system for the incorporation of the tryptophan analogues, 5HW and 7AW, and we present a characterization of the enzymatic activity, optical spectroscopy, and time-resolved fluorescence of the resulting proteins to test whether any obvious perturbation of the structure of these proteins occurs. Finally, we present a study of the guanidine-HCl-induced unfolding of the proteins, in order to compare the stability of the modified proteins with the stability of the tryptophan-containing wild type.

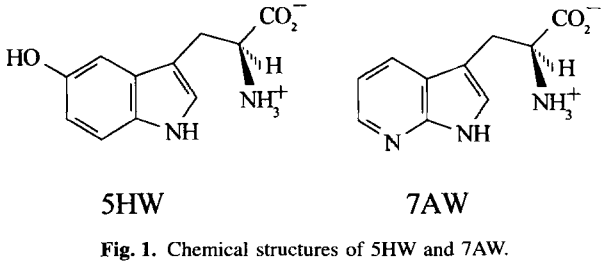

Fig. 1. Chemical structures of 5HW and 7AW.

Results

Protein yield, purity, and enzymatic activity

When the expression system described in Materials and methods is supplemented with either tryptophan, 5HW, or 7AW, we isolated 32–72 mg, 40–65 mg, and 26–66 mg, respectively, as typical yields of purified nuclease (per liter of medium). For comparison, the basal level of nuclease production was less than 5 mg/L without a tryptophan supplement. The isolated proteins were subjected to denaturing PAGE; in each case, a single electrophoretic band was seen.

The enzymatic activity of each protein was determined using DNA as substrate and monitoring the change in absorbance at 260 nm when the polynucleotide is hydrolyzed. The activity of 5HW- and 7AW-containing nuclease was 92% and 80%, respectively, of the value for tryptophan-containing nuclease.

Optical spectroscopy-free amino acids

The absorption and fluorescence emission spectra for tryptophan, 5HW, and 7AW (the free amino acids in buffer) are shown in Figure 2A and B. This figure clearly shows the red-shift for the absorbance of 5HW and 7AW. We find the following fluorescence

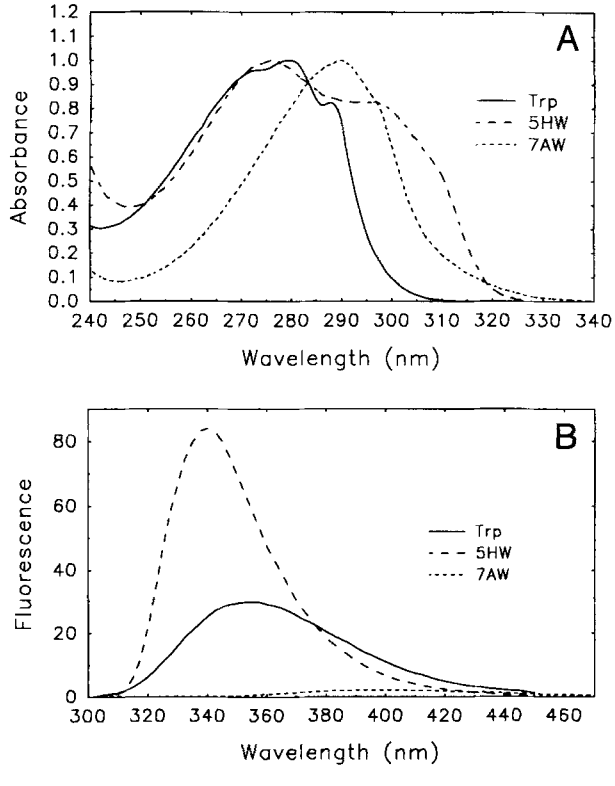

Fig. 2. A: Absorption spectrum of tryptophan, 5HW, and 7AW. The spectra are normalized to have the same maximum absorbance. **B:** Fluorescence spectrum of tryptophan, 5HW, and 7AW. (Aqueous buffer, pH 7.3, room temperature; excitation at 295 nm, 5-nm slits.)

quantum yields, Φ, and emission maxima, λ_{max}, for the chromophores at pH 7, 20 °C, and excitation at 280 nm: tryptophan, Φ = 0.14 (used as standard), λ_{max} = 353 nm; 5HW, Φ = 0.275, λ_{max} = 339 nm; and 7AW, Φ = 0.017, λ_{max} = 403 nm. Note the low fluorescence intensity and red-shifted emission of 7AW. (We find that the fluorescence quantum yield of 5HW and 7AW depend on excitation wavelength, as was reported previously by Gai et al. (1994) for 5-methoxyindole and 7-azaindole. For example, with excitation at 295 nm, we find Φ = 0.097 for 5HW and Φ = 0.007 for 7AW, assuming a wavelength-independent value for tryptophan.)

The low-temperature fluorescence (excitation) anisotropy spectra for the three compounds is shown in Figure 3. Tryptophan shows the familiar pattern (due to the existence of overlapping ^1L_a and ^1L_b electronic transitions), with the anisotropy reaching a limiting anisotropy plateau of about 0.3–0.32 at 300 nm (where there is selective excitation into the ^1L_a oscillator) (Valeur & Weber, 1977; Eftink et al., 1990). The other two chromophores show a lower anisotropy at all wavelengths. 5HW reaches a plateau of approximately 0.25 at its red excitation edge, where it is believed that there is selective excitation into its ^1L_b oscillator (Lami, 1976), and has a value of ~0.2 at 300–305 nm. As reported previously by Rich et al. (1993), 7AW has an extensive red edge plateau (at 310–330 nm), but its limiting anisotropy is only about 0.16. (Of particular importance is the observation that the anisotropy of 7AW is only about 0.05–0.1 in the excitation range of 300–305 nm, where the anisotropy decay studies for the proteins (see below) were performed. Because we must use 300–305 nm as an excitation wavelength with our laser in time-resolved fluorescence anisotropy measurements, this low anisotropy for 7AW limits such studies with proteins containing this chromophore.)

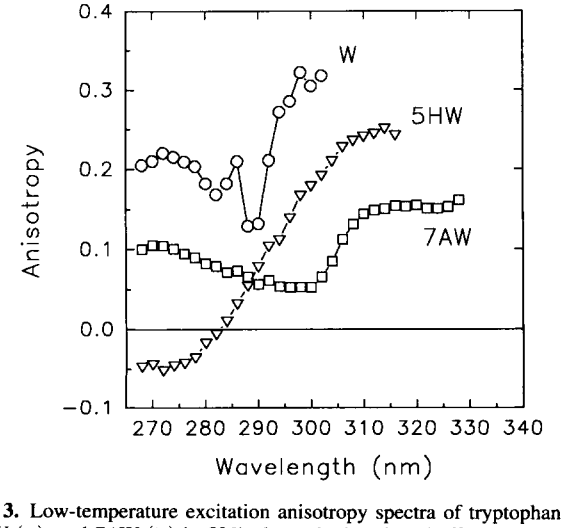

Fig. 3. Low-temperature excitation anisotropy spectra of tryptophan (○), 5HW (▽), and 7AW (□) in 50% glycerol–phosphate buffer, 77 K.

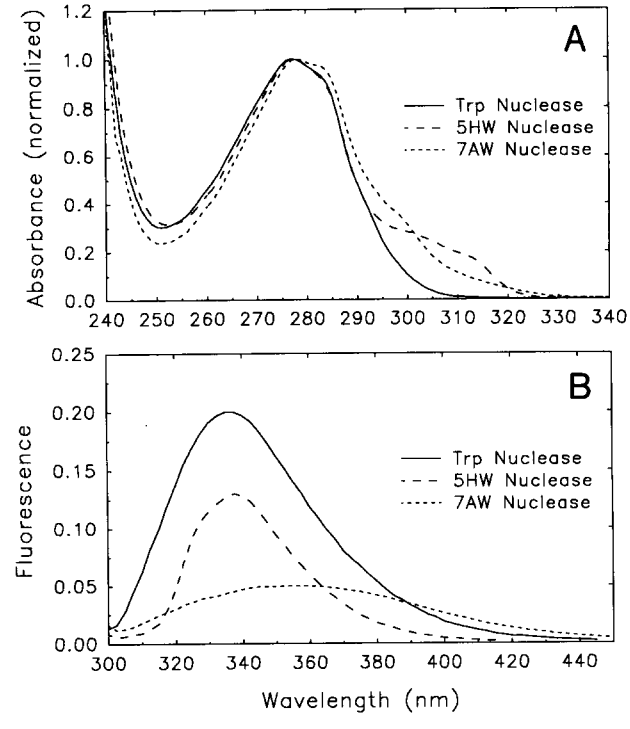

Fig. 4. A: Absorption spectrum of tryptophan-, 5HW-, and 7AW-containing nuclease. **B:** Fluorescence emission spectrum of tryptophan-, 5HW-, and 7AW-containing nuclease. (Aqueous buffer, pH 7.3, 20 °C; excitation at 295 nm, 5-nm slits.)

Optical spectroscopy proteins

The absorption and fluorescence spectra of tryptophan-, 5HW-, and 7AW-containing nuclease are shown in Figure 4A and B (native protein conditions). The contribution from 5HW is obvious in the absorption spectrum, which extends to about 320 nm; the contribution from 7AW is also pronounced. In an attempt to determine the extent of 5HW and 7AW incorporation into the proteins, the absorption spectrum of the nuclease was also recorded in 5 M guanidine-HCl solution and these spectra were fitted (via nonlinear least-squares) by summing the component spectra (also in 5 M guanidine-HCl) of seven tyrosine residues, three phenylalanines, plus variable amounts of tryptophan or the tryptophan analogue. Shown in Figure 5 are these spectra and fits (solid lines). The fitting parameters (see the legend) indicate that 5HW nuclease contains ~95% of the analogue and that 7AW nuclease contains ~98% of the analogue.

The steady-state fluorescence spectrum (Fig. 4B) of 5HW-containing nuclease has a maximum at about the same wavelength as that of the free amino acid. Compared to the tryptophan-containing protein, the 5HW-containing variant and has a similar emission maximum and a lower quantum yield (with excitation at 295 nm), but the shape of the emission spectrum of the latter is much narrower. The fluorescence of 7AW-containing protein has a low quantum yield and has a broader emission spectrum that is centered at a longer wavelength of 355 nm. Whereas the yield is lower and the maximum is red-shifted for 7AW-nuclease, in comparison with the tryptophan-containing protein, the fluorescence of the incorporated 7AW is much stronger and bluer than that of free 7AW in neutral buffer. (Compare short-dashed curves in Figures 4B and 2B. The quantum yield for the incorporated 7AW is larger than that of free 7AW in buffer whether one compares to the Φ of the latter with 280 nm or 295 nm excitation.)

The near-UV CD spectra of the proteins are shown in Figure 6A. The different tryptophan analogues give rise to different spectral patterns. It is difficult to interpret these patterns, but the existence of spectra in the 280–310-nm region must be attributed primarily to absorbance by the tryptophans (Omenn et al., 1969), and the existence of non-zero ellipticity indicates that there is a persistent

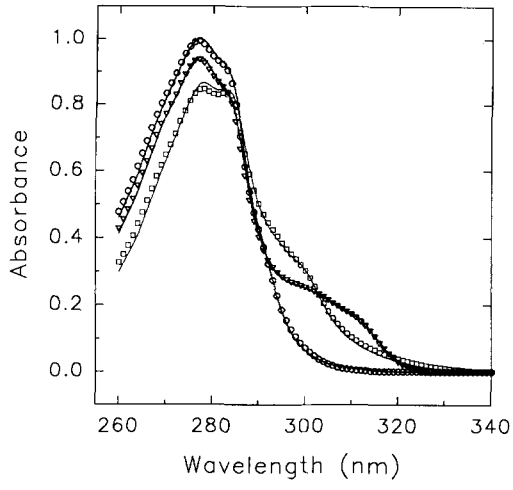

Fig. 5. Fitting of absorption spectra of tryptophan- (○), 5HW- (▽), and 7AW-containing (□) nuclease in 5 M guanidine-HCl, to estimate percent incorporation of the tryptophan analogue. Amino acid component (basis) spectra were obtained in 5 M guanidine-HCl. Analyses included seven tyrosines and three phenylalanines as fixed components. The number of tryptophans, n_{trp}, and tryptophan analogue, $n_{analogue}$, and the protein concentration were varied in the fit. For tryptophan-containing protein, the solid line fit is with $n_{trp} = 0.96 \pm 0.01$ and $n_{5HW} = 0.04$ (an equivalent fit is obtained with $n_{trp} = 1.01 \pm 0.01$ and $n_{7AW} = -0.01$). For 5HW nuclease, the fit is with $n_{trp} = 0.05$ and $n_{5HW} = 0.95 \pm 0.01$. For 7AW nuclease, the fit is with $n_{trp} = 0.02$ and $n_{7AW} = 0.98 \pm 0.01$.

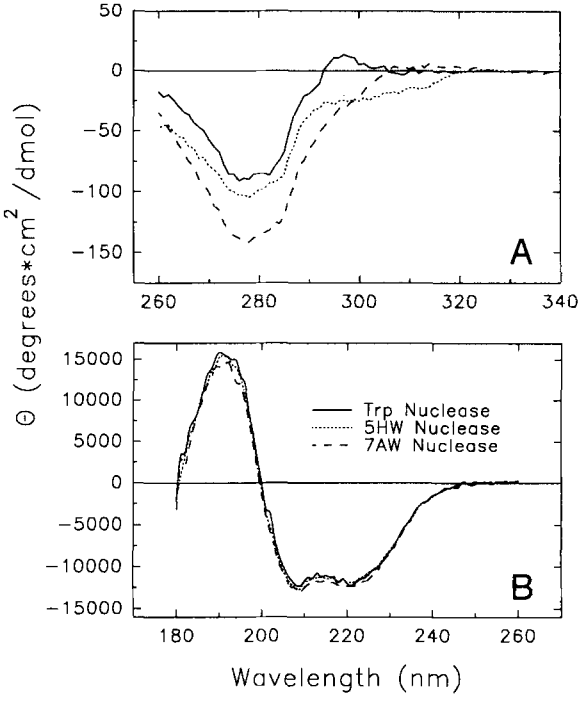

Fig. 6. A: Near-UV CD spectrum of tryptophan-, 5HW-, and 7AW-containing nuclease. **B:** Far-UV CD spectrum of the three proteins. (Aqueous buffer, pH 7.3, 20 °C, 0.6-nm bandwidth; 10-mm cell for near-UV; 0.2-mm cell for far-UV; protein concentrations of ~30 μM.)

three-dimensional structure around the tryptophan analogues. The aromatic ellipticity of 5HW and 7AW are both larger in magnitude than that of tryptophan in these proteins at 275–280 nm. Also, for 5HW- and 7AW-containing protein, the ellipticity does not reach the baseline until longer excitation wavelength, as would be expected due to the extended red absorbance of these chromophores.

The far-UV CD spectrum of the three proteins (Fig. 6B) shows a similar pattern. Analysis of the CD spectra using the program SELCON (Sreerama & Woody, 1993) yields the following estimates of secondary structure elements: for tryptophan-containing nuclease, 41.6% α-helix, 15.9% β-sheet, and 24.2% random; for 5HW-containing nuclease, 40.4% α-helix, 14.6% β-sheet, and 26% random; and for 7AW-containing nuclease, 40.8% α-helix, 16.6% β-sheet, and 23.7% random.

Time-resolved fluorescence studies

Frequency domain fluorescence lifetime data for the three proteins are shown in Figure 7A (excitation at 300 nm for tryptophan-containing protein and at 305 nm for 5HW- and 7AW-containing protein). The data were analyzed in terms of a bi-exponential decay law and the results of this analysis are shown in Table 1. 7AW-containing nuclease has the shortest mean lifetime ($= \Sigma \alpha_i \tau_i$) of 2.6 ns; the intensity decay data for 5HW-containing nuclease fall between those for the other two forms of the protein.

Differential polarized phase and modulation data (anisotropy decay data) are shown in Figure 7B for tryptophan and 5HW-containing nuclease. The anisotropy of 7AW-containing nuclease was so small (see Fig. 3 for the anisotropy of immobilized 7AW) when exciting at 305 nm that we did not obtain usable data for this protein. For the tryptophan and 5HW variants of the protein, the

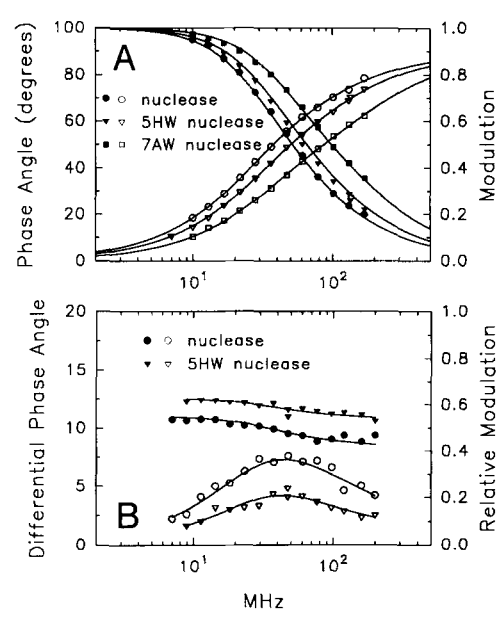

Fig. 7. Time-resolved fluorescence data for tryptophan (○,●), 5HW (▽,▼), and 7AW (□,■) containing nuclease. **A:** Frequency domain fluorescence lifetime data. Solid lines are fits to a bi-exponential decay law, with decay times and amplitudes given in Table 1. **B:** Differential polarized phase angle and relative modulation (anisotropy decay) data. Solid lines are fits to a bi-exponential rotational model, with rotational correlation times and amplitudes given in Table 1. Conditions: phosphate buffer, pH 7.3, 20 °C, excitation at ~300 nm for tryptophan and ~305 nm for 5HW and 7AW; emission observed through a 335-nm cut-on filter.

Table 1. *Fluorescence properties of nuclease variants*[a]

Property	Tryptophan	5HW	7AW
Quantum yield (295 nm excitation)	0.205	0.139	0.050
Emission λ_{max} (nm)	336	338	355
Lifetimes			
τ_1 (ns)	5.62 ± 0.04	4.74 ± 0.07	4.35 ± 0.07
τ_2 (ns)	1.81 ± 0.04	1.41 ± 0.05	1.83 ± 0.02
α_1	0.81	0.65	0.32
$\langle\tau\rangle$ (ns)	4.90	3.55	2.62
χ^2	1.29	5.15	1.24
Anisotropy parameters			
ϕ_1 (ns)	13.1 ± 1.0	12.6 ± 1.1	ND
ϕ_2 (ns)	1.4 ± 0.5	0.26 ± 0.40	ND
$g_1 r_o$	0.291 ± 0.004	0.219 ± 0.004	ND
$g_2 r_o$	0.029	0.011 ± 0.013	ND
χ^2	1.78	1.10	

[a]All parameters are for 20 °C, pH 7. Phase/modulation fluorescence lifetime data were fitted with standard deviations of $\sigma_p = 0.2$ degrees and $\sigma_m = 0.01$ for the phase angle and modulation, respectively. The phase and modulation data were fitted to a multi-exponential decay law as described in Eftink and Ghiron (1989). A third lifetime component of $\tau_3 = 0.005$ ns was included to account for scattered light; this third component had an amplitude of less than 5% in all occasions. The mean fluorescence lifetime, $\langle\tau\rangle$ is defined as $\sum \alpha_i \tau_i$. Anisotropy decay data were fitted with a bi-exponential decay law (nonassociated) with standard deviations of $\sigma_p = 0.4$ degrees and $\sigma_m = 0.01$ for the differential polarized phase angle and relative modulation, respectively, as described in Eftink et al. (1991). For the tryptophan-containing protein, the sum of the $g_i r_o$ values was fixed at 0.32, a limiting value for a completely immobilized indole ring. For the other proteins, the $\sum g_i r_o$ was not fixed at a particular value because the excitation wavelength of 300 nm may not be at the limiting anisotropy for the chromophore.

anisotropy decays were fitted by a bi-exponential decay law, with the resulting parameters given in Table 1. In both cases, a long rotational correlation time of ~13 ns is determined. A shorter correlation time is also found for both proteins.

Guanidine-HCl-induced unfolding data

As shown in Figure 8A and B, the unfolding of tryptophan- and 5HW-containing nuclease are well described as a two-state tran-

Table 2. *Thermodynamics parameters for the guanidine-HCl induced unfolding of nuclease variants*[a]

Protein	$\Delta G^\circ_{o,1,un}$ (kcal/mol)	m_1 (kcal/mol/M)	$\Delta G^\circ_{o,2,un}$ (kcal/mol)	m_2 (kcal/mol/M)	χ^2
Trp-containing	5.01	5.93	—	—	2.24
	(4.70–5.32)	(5.59–6.27)			
5HW-containing	5.08	5.68	—	—	2.42
	(4.73–5.42)	(5.32–6.03)			
7AW-containing					
Two-state	2.18	4.50	—	—	7.02
	(1.95–2.42)	(4.19–4.81)			
Three-state[b]	3.05	6.61	3.64	4.91	1.10
	(2.72–3.38)	(5.91–7.30)	(3.14–4.16)	(4.36–5.49)	

[a]Unfolding studies were performed at 20 °C at pH 7.3, 0.02 sodium phosphate buffer. Global fit of CD (at two wavelengths) and fluorescence data to Equations 1–4 using NONLIN, with weighting of the data sets via the standard deviations of each data point. Values in parentheses are 67% confidence intervals. We note that fits (either individual data sets or the global set) for Trp nuclease and 5HW nuclease showed an improvement in χ^2 of less than a factor of two in going from a two-state to a three-state transition, so only the former is reported. For 7AW nuclease, the fits of either individual data sets or the global set always showed an improvement in χ^2 of greater than a factor of five on going from a two-state to a three-state model.

[b]An alternate type of three-state fit, having only slightly higher χ^2, was obtained for a fit that assumes the existence of a mixture of noninterconverting native states, that is, for the case in which there is a mixture of 7AW nuclease and some Trp nuclease (as an impurity due to incorporation of residual tryptophan), with each protein undergoing a two-state unfolding transition. We constrained this fit to have the $\Delta G^\circ_{o,un}$ and m values for Trp nuclease be the same as those determined independently for the latter protein, and we assumed that the native and unfolded signals (and slopes) were the same for both 7AW nuclease and Trp nuclease. We used this model to fit only the CD data (at 222 and 228 nm), because it is not reasonable to expect the fluorescence signals of the two fluorophores to be similar (and because tryptophan is not excited at 310 nm). A fit of this "mixture" model gives $\Delta G^\circ_{o,un} = 2.79$ kcal/mol and $m = 5.85$ kcal/mol/M for 7AW nuclease, with 80.6% of the sample being the 7AW protein.

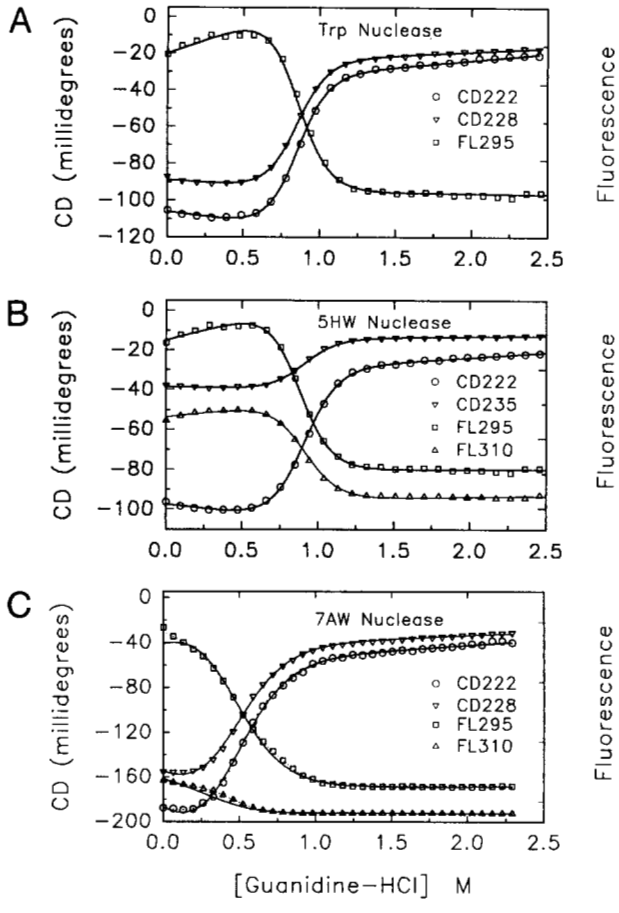

Fig. 8. A: Guanidine-HCl-induced unfolding of wild-type nuclease A; the data (CD measurements at two wavelengths, 222 and 228 nm, and fluorescence intensity measurements with 295-nm excitation, 340-nm emission) were fitted globally with a two-state model. **B:** Guanidine-HCl induced unfolding of 5HW-containing nuclease, fitted globally (CD data at two wavelengths, 222 and 235 nm; fluorescence data at both 295-nm and 310-nm excitation, 340-nm emission) with a two-state model. **C:** Guanidine-HCl-induced unfolding of 7AW-containing nuclease, fitted globally (CD data at two wavelengths, 228 and 228 nm; fluorescence data at 295 nm and 310 nm, emission at 360 nm) with a two-state (solid lines) and three-state (dotted lines) model for unfolding.

sition, as indicated by the excellent global fit of CD and fluorescence data sets to Equations 1–4 (see parameters listed in Table 2). The data in Figure 8 were obtained on the same samples using a multidimensional CD-fluorometer (Ramsay et al., 1995). The $\Delta G^{\circ}_{o,un}$ and m values for these two proteins are almost the same ($\Delta G^{\circ}_{o,un}$ = 5–5.1 kcal/mol and m = 5.7–5.9 kcal/mol/M), indicating that 5HW does not perturb the stability of nuclease. With 5HW nuclease, it is noteworthy that the data collected with excitation at 310 nm shows excellent agreement with the CD data and with fluorescence data collected with 295 nm excitation. This is additional proof that the 5HW group is incorporated in the protein.

Data in Figure 8C for 7AW-containing nuclease, however, show a broader transition and are only fitted modestly well by a two-state model, with $\Delta G^{\circ}_{o,un}$ = 2.2 kcal/mol and m = 4.5 kcal/m/M. A global analysis with the three-state model improves the fit significantly, as indicated by the χ^2 in Table 2. In view of the consideration that the apparent deviation from two-state may be due to a small amount of tryptophan-containing protein impurity, we have fitted the data (CD only) to a "mixture" model (see legend of Table 2), which allows for the presence of about 19% tryptophan-containing impurity in the 7AW nuclease sample. We cannot discount this fit, although our above analysis of the absorbance spectrum of 7AW should have easily revealed 19% impurity, and although the 310-nm fluorescence data in Figure 8C can only have detected transition(s) involving 7AW, we cannot discount the presence of a minor amount of tryptophan-containing impurity.

Discussion

The expression system (see Materials and methods) gives excellent yields of tryptophan analogue-containing nuclease. The results presented here are with 5HW and 7AW. We have also used this expression system to incorporate several other tryptophan analogues (Wong, 1996). The efficiency of this system is very good for 5HW, where analysis of the absorption spectrum indicates that ≥95% of the protein contains 5HW at position 140. With 7AW, analysis of the absorption spectrum also indicates a very high percentage (≥98%) incorporation of this analogue. Other laboratories, using other expression systems, have achieved >90% incorporation of 5HW and 7AW into proteins, but have usually found that there is more "leakage" (i.e., incorporation of residual or cannibalized tryptophan instead of the analogue) with 7AW; achieving 50–70% incorporation of 7AW into a protein is usually considered to be good. Thus, the present expression system is very favorable for incorporation of tryptophan analogues. We note that Soumillion et al. (1995) recently also have obtained very efficient biosynthetic incorporation (~98%) of 7AW into lambda phage lysozyme.

The spectral enhancement of these nucleases is illustrated by the absorption spectra (Fig. 4A) and emission spectra (Fig. 4B) of the 5HW- and 7AW-containing proteins. In each case, excitation at ~310 nm will be able to selectively excite the tryptophan analogue, even in the presence of tryptophan residues (e.g., in other proteins). The 5HW analogue has a fluorescence quantum yield that is similar to that for tryptophan; the quantum yield is lower for 7AW in the protein (but the yield of the latter is much higher than that of free 7AW in buffer). The emission maximum of 355 nm for 7AW in nuclease is at a much shorter wavelength than that of 403 nm for 7AW in water, which also suggests that this residue is not fully solvent exposed in the protein. Schlessinger (1968) found the incorporation of 7AW into alkaline phosphatase resulted in a blue-shift in the emission maximum for 7AW to 370 nm in the protein, similar to its blue-shift in ethanol. More recently, Hogue (1994) found a fluorescence maximum of 350 nm and quantum yield of 0.15 for 7AW incorporated biosynthetically into the single tryptophan site of *B. subtilis* tryptophanyl tRNA synthetase. The intensity decay of the 7AW in the latter protein was found to be multi-exponential, with a long component of 10 ns. Thus, 7AW can have wide-ranging fluorescence properties, which appear to reflex the solvent exposure of this chromophore. The values of λ_{max} = 355 nm, Φ = 0.07, and $\langle\tau\rangle$ = 2.6 ns (with a 3.6-ns long component) that we find for 7AW at position 140 of nuclease suggest that the residue is buried, but not quite as much as that in tryptophanyl tRNA synthetase.

Because 7AW may have such a broad range (sub-nanosecond to 10 ns) of lifetime components, this suggests that occasionally it may provide an advantage in anisotropy decay studies by matching the intensity decay time with the rotational correlation time. However, the low-temperature anisotropy of 7AW is lower than that of tryptophan or 5HW over the entire excitation range, as shown in

Figure 3. With our argon ion laser, which enables 305 nm excitation for anisotropy decay measurements, the limiting anisotropy of 7AW will be less than 0.1 at this wavelength, thus making anisotropy decay measurements difficult with this probe (i.e., the maximum differential phase angle in Figure 7B would be only about 2°). Excitation of 7AW at a longer wavelength (e.g., 315 nm, at which $r_o \approx 0.155$) should still enable useful anisotropy decay data to be collected. Alternatively, the low anisotropy of 5HW ($r_o = 0$ at about 283 nm) and 7AW ($r_o = 0.05$ at 295–300 nm) may be useful in studies of complexes between tryptophan-containing and 5HW- (or 7AW-) containing proteins/peptides, because the anisotropy of the former will dominate at the above wavelengths, but the anisotropy of the latter will dominate at wavelengths above 300 nm (of course, the relative intensities of the emitting centers at the respective wavelengths will also determine the contribution to the anisotropy signal).

The incorporation of these tryptophan analogues into nuclease appears to have little or no effect on the protein's structure. Full enzymatic activity is retained with each analogue. A similar secondary structure is found. The observation of the same long rotational correlation time, ϕ_1, for the tryptophan- and 5HW-containing proteins suggests that the overall globular shape is similar with each analogue. Most importantly, the stability (i.e., $\Delta G^\circ_{o,un}$ and guanidine-HCl m value) of nuclease is essentially unchanged upon incorporation of 5HW, indicating the absence of perturbation by this analogue. With 7AW, there appears to be a reduction in the stability of the protein. If analyzed in terms of a two-state model, the $\Delta G^\circ_{o,un}$ for 7AW nuclease is less than half of the value for the normal protein and the m value is reduced greatly. The multiple data sets for the guanidine-HCl-induced unfolding of 7AW nuclease can be better fitted by a three-state model, which suggest loss of cooperativity of the unfolding transition, or by a model that includes the presence of a small amount (<20%) of tryptophan-containing protein as an impurity. For the latter type of fit, we arrive at the same overall conclusion, that 7AW nuclease is less stable ($\Delta G^\circ_{o,un} = 2.8$ kcal/mol) than the tryptophan-containing protein (but the recovered m value is similar for the two proteins). Although our analysis of the absorbance spectra indicates that the 7AW nuclease sample contains no more than a few percent tryptophan impurity, we cannot say with certainty that the apparent deviation from a two-state model (Fig. 8C) is not due to some tryptophan impurity protein.

Because 5-hydroxyindole and 7-azaindole have the additional polar hydroxyl and ring nitrogen, respectively, not found in indole, and because tryptophan residues are usually buried in apolar regions of proteins, the general expectation is that these analogues should destabilize a protein. Clearly, this is not seen for 5HW nuclease, and the effect of 7AW incorporation appears to be more complicated than a simple reduction in $\Delta G^\circ_{o,un}$. Besides introducing potential hydrogen bond donating and accepting groups, another potential perturbation introduced by these analogues is an additional proton dissociating group (i.e., the hydroxyl group of 5HW) and proton associating group (i.e., the pyridino nitrogen of 7AW). In the recent study by Soumillion et al. (1995), incorporation of four 7AW residues into lambda phage lysozyme was found to destabilize the protein modestly at neutral pH and to shift the acid-induced unfolding curve to higher pH, most likely due to the fact that the 7AW groups introduce additional sites for protonation of the unfolded state (i.e., pK_a of 4.29 for 7AW).

Upon inspecting the X-ray structure of nuclease, we find that position 5 of the indole ring of Trp 140 is relatively solvent accessible, whereas position 7 is buried. If the analogue-containing proteins have similar structure around this residue, then the observed destabilization by 7AW incorporation is consistent with the burial of the pyridino nitrogen at position 7. It is interesting to note that, besides being solvent accessible, the 5-hydroxy group is expected to be within hydrogen bonding distance of the side-chain amino groups of both Lys 110 and Lys 133. Not only may this lead to a stabilizing interaction, but it leads to the prediction that the pK_a of the 5-hydroxy group in 5HW nuclease will be perturbed.

The overall result of the present study is that both 5HW and 7AW can be incorporated with >95% efficiency into nuclease and that the spectral and activity results are consistent with a very minimal perturbation of the three-dimensional structure of this protein. Whereas 5HW also is found not to perturb the thermodynamic stability of nuclease, 7AW causes some loss of the stability (and possibly a loss of cooperativity in the unfolding process) of the protein. It would be premature to generalize these results to other proteins, but they suggest that the biosynthetic incorporation of tryptophan analogues into a protein can be tolerated, with some destabilization of the native state, particularly by 7AW.

Materials and methods

Materials

L-Tryptophan, L-5-hydroxytryptophan, D,L-7-azatryptophan, L-amino acids (used as minimal medium supplements), NaEDTA, Tris, Tris-HCl, Fast Flow S-Sepharose (Pharmacia), urea (molecular biology grade), and guanidine hydrochloride (molecular biology grade) were obtained from Sigma Chemical Co. The tryptophan was recrystallized from water:ethanol before use. Bacto-tryptone (Difco), bacto-yeast extract (Difco), bacto-agar (Difco), MOPS, and agarose were obtained from Fisher Biotech. Biotin and Tricine were obtained from Amresco.

Escherichia coli strain AR120/pWT, which overproduces wild-type nuclease A, was a gift from Dr. W. Stites, University of Arkansas.

Expression system and protein isolation

The plasmid from AR120 was isolated and transformed into UM1, an *E. coli* strain auxotrophic for tryptophan and harboring a lambda lysogen coding form cI857, the temperature sensitive lambda repressor. UM1 was constructed by P1 transduction (Schleif & Wensink, 1981) as follows. P1 phage was grown on TAP106 (*leu thi hsdR rpsL supE lacΔU169 galKam*(λ*kil* cI857Δ*bio* A N::Kan, a gift from Dr. Donald Court, NCI-Frederick Caner Research and Development Center) (Patterson et al., 1993) and was used to infect the trp auxotroph CY15077 (W3110 *tna*A2 Δ*trp*EA2, a gift from Dr. Charles Yanofsky, Stanford University). Transductants were selected for kanamycin resistance and then checked for the presence of both a tryptophan and a biotin requirement. Cells were rendered competent by the $CaCl_2$ method (Silhavy et al., 1984). The plasmid for nuclease was transformed into UM1.

Cells from a −70 °C culture were inoculated into 5 mL of Neidhardt's defined medium (Neidhardt et al., 1974) with supplements of the other 19 amino acids, thiamine-HCl and biotin (Wong, 1996) and were incubated at 30 °C. When in exponential growth phase, 1 mL of this culture was transferred into 1 L of medium in four 1-L flasks, and was shaken at 250 rpm with aeration until the culture reached an $OD_{600} \approx 1$. The cells were collected by cen-

trifugation (2,000 × *g*, 10 min, 10 °C) and were resuspended in medium, prewarmed to 40 °C, containing the tryptophan analogue and freshly added ampicillin. The culture was split into eight 1-L flasks and incubated with shaking (250 rpm) for 3 h at 40 °C. Cells were harvested and resuspended in 50 mL of ice cold buffer, containing 6 M urea, 25 mM Tris-HCl, and 5 mM NaEDTA, pH 8.1.

The protein was then isolated from the cells following the procedure of Shortle and Meeker (1989). Protein purity was confirmed by SDS-PAGE (Laemelli, 1970) and silver staining (Wray et al., 1991). Protein concentrations were determined by the SDS-Lowry assay (Markwell et al., 1978). The molar concentration of each protein was determined from absorbance measurements using the appropriate molar extinction coefficients. The following molar extinction coefficients (at 280 nm) were used: $\epsilon = 1.59 \times 10^4$ M^{-1} cm^{-1} for tryptophan-containing nuclease; 1.54×10^4 M^{-1} cm^{-1} for 5HW nuclease; and 1.65×10^4 M^{-1} cm^{-1} for 7AW nuclease. These values were calculated from the relationship and chromophore extinction coefficients given by Pace et al. (1995), along with molar extinction coefficients (280 nm) of 4.83×10^3 and 5.97×10^3 M^{-1} cm^{-1} for 5HW and 7AW in neutral aqueous solution. The molar extinction coefficients of the proteins were also determined directly based on the SDS-Lowry assay; values agree within 5% of those calculated above.

Enzyme assay

The activity of the nuclease variants was determined using the method of Cuatrecasas et al. (1967), which measures the increase in absorbance at 260 nm due to the hydrolysis of DNA.

Optical spectroscopy

Absorbance spectra were obtained with a Shimadzu UV160A. Molar extinction coefficients were determined experimentally by dissolving a weighed amount of protein into neutral buffer and measuring the absorbance. The buffer used for this and most of the studies was 20 mM NaH_2PO_4, pH 7.3. The absorption spectrum of the 5HW- and 7AW-containing proteins were analyzed by summing the expected contributions of the tryptophan analogue and phenylalanine and tyrosine residues. This was done by first obtaining basis spectra for the amino acids in 5M guanidine-HCl solution, pH 7, 0.02 M sodium phosphate (ϵ_{tyr} = 1,338 cm^{-1} M^{-1} at λ_{max} = 276 nm; ϵ_{trp} = 5,638 cm^{-1} M^{-1} at λ_{max} = 280 nm; ϵ_{5HW} = 4,898 cm^{-1} M^{-1} at λ_{max} = 277 nm and = 3,825 cm^{-1} M^{-1} at the 300 nm shoulder; and ϵ_{7AW} = 5,816 cm^{-1} M^{-1} at λ_{max} = 291 nm). The absorbance spectra of the proteins were then recorded in the same 5 M guanidine-HCl solution. The latter spectra were fitted, between 260–340 nm, with the basis spectra using the nonlinear least-squares program in SigmaPlot 5.0 (Jandel Scientific Software). In the fitting procedure, the number of tyrosine and phenylalanine residues was fixed at seven and three, respectively, the number of tryptophan and analogue residues was a floated fitting parameter (with the condition that the sum of n_{trp} + $n_{analogue}$ = 1.0), as was the total concentration of protein.

CD spectra were obtained with an AVIV 62DS spectrophotopolarimeter, equipped with a thermoelectric cell holder. Near-UV CD spectra (260–360 nm, 0.6 nm bandwidth) were acquired at 20 °C using an integration time of 6 s and using a 10-mm pathlength cell. Far-UV CD spectra (200–260 nm, 0.6 nm bandwidth) were obtained using an integration time of 3 s and using a 0.2-mm pathlength cell. The protein concentration for these measurements was ~30 μM. The mean residue ellipticity was calculated using a mean residue mass of 119 (149 amino acids for nuclease) and a molecular mass of 17.8 kDa for nuclease.

Steady-state fluorescence spectra were recorded with a Perkin-Elmer MPF44A spectrophotofluorometer, which is equipped with a thermo-jacketed cell holder. Spectra were measured at 20 °C. Fluorescence quantum yields were determined by cutting and weighing paper. Tryptophan, for which the quantum yield is 0.14, was used as a reference for quantum yield determinations.

Time-resolved fluorescence studies

These measurements were made using an ISS frequency domain instrument, together with an Innova (Coherent) argon ion laser, which has lines at 300–305 nm. Using an interference filter centered at 310 nm, the excitation from this laser has an effective wavelength of 305 nm, which was used for the 5HW- and 7AW-containing variants. Alternatively, with an interference filter centered at 290 nm, the effective wavelength of this excitation system is 300 nm, which was used for the tryptophan-containing protein. Intensity decay data were collected at 20 °C and were analyzed as described elsewhere (Eftink & Ghiron, 1987). Likewise, anisotropy decay data were collected with this frequency domain instrument and were analyzed as described elsewhere (Eftink et al., 1991).

Guanidine-HCl induced unfolding studies

Equilibrium unfolding studies were performed with our modified AVIV CD instrument, which also monitors steady-state fluorescence of the sample. Using a computer controlled syringe pump and an acquisition program described elsewhere (Ramsay et al., 1995), we have collected unfolding data at 20 °C by monitoring the CD signal at two wavelengths (e.g., 222 nm and 228 nm) and the steady-state fluorescence signal (excitation at 295 nm). In the case of 5HW- and 7AW-containing variants, an excitation wavelength of 310 nm was also used for the fluorescence measurements. At this wavelength, the respective tryptophan analogue is selectively excited. For each protein, the fluorescence emission was collected at 340 nm or 360 nm through a 10-nm interference filter. The standard deviation for each data type (actually, for each data point) was also determined.

Such unfolding data (two CD and one or two fluorescence signals versus guanidine-HCl concentration) were then globally fitted with a two-state unfolding model. For a two-state transition, N ⇌ U, where N is the native state and U is the unfolded state, the following equations relate the spectroscopic signal, S, to the thermodynamics of the transition.

$$K_{un} = [U]/[N] \tag{1}$$

$$X_N = 1/Q;\ X_U = K_{un}/Q;\ \text{where } Q = 1 + K_{un} \tag{2}$$

$$S = \Sigma X_i \cdot (S_{i,o} + [\mathbf{d}] \cdot \delta S_i/\delta[\mathbf{d}]), \tag{3}$$

where K_{un} is the unfolding equilibrium constant, X_i are mole fractions, Q is the partition function, $S_{i,o}$ is the signal (either CD or fluorescence) in the absence of denaturant, and $\delta S_i/\delta[\mathbf{d}]$ is the dependence of each intrinsic signal on denaturant concentration (i.e., $\delta S_i/\delta[\mathbf{d}]$ is a baseline slope for each pure state).

The standard free energy change for unfolding, $\Delta G^\circ_{un} = -RT \cdot \ln K_{un}$, is related to denaturant concentration, [**d**], by

$$\Delta G^\circ_{un} = \Delta G^\circ_{o,un} - m \cdot [\mathbf{d}], \tag{4}$$

where $\Delta G^{\circ}_{o,un}$ is the standard free energy change for unfolding of the protein in the absence of denaturant and m is the dependence of ΔG°_{un} on [**d**] (Pace, 1986). The program NONLIN was used for the nonlinear least-squares analyses (Johnson & Fraiser, 1995) and the standard deviations of each type of data were used in the global analyses (Ramsay & Eftink, 1994). Similarly, fits of a three-state model were performed as described elsewhere (Ramsay et al., 1995).

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