Incorporation of Tryptophan Analogues into Staphylococcal Nuclease: Stability toward Thermal and Guanidine-HCl Induced Unfolding†

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ABSTRACT: The tryptophan analogues, 5-hydroxytryptophan, 7-azatryptophan, 4-fluorotryptophan, 5-fluorotryptophan, and 6-fluorotryptophan, have been biosynthetically incorporated into Staphylococcal nuclease, its V66W mutant, and the Δ137−149 fragment of the latter mutant. The guanidine−HCl induced unfolding and thermal unfolding of these proteins were studied to characterize the effect of incorporation of these tryptophan analogues on the thermodynamic stability of the proteins. The three proteins have tryptophan residues at positions 140 (in wild type) and 66 (in the Δ137−149 fragment of V66W) and at both positions (in V66W). The unfolding data show that 5-hydroxytryptophan does not perturb the stability of wild-type nuclease, but it destabilizes the fragment and causes the V66W mutant to unfold in a more cooperative manner. 7-Azatryptophan is found to destabilize all three proteins. 4-Fluorotryptophan is slightly stabilizing of the three proteins, but the other two fluorotryptophans do not alter the stability of the proteins.

Mutagenesis studies have enabled numerous studies of the effect of amino acid substitutions on protein structure and stability. The protein investigated here, Staphylococcal nuclease, has been one of the most thoroughly characterized proteins via the combination of mutagenesis and stability studies. Mutagenesis methods are normally limited to substitution at any position of one of the 20 natural amino acids, although some synthetic and cell-free strategies have been developed to extend the available amino acids (*1*−*3*). For example, Schultz has used a cell-free biosynthetic procedure that involves placing the amber codon at a particular position in a mRNA and carrying out biosynthesis in the presence of chemically aminoacylated (with the desired analogue) suppressor tRNA. With this procedure they have incorporated several nonnatural amino acids into specific cites in T4 lysozyme (*3, 4*) and Staphylococcal nuclease (*5*).

The cell-free suppressor tRNA procedure is very general but is difficult and would be very costly to scale up. In some cases, the in vivo protein synthesis machinery will tolerate certain analogues of amino acids and can thus be tricked to produce large quantities of protein containing an analogue. One such case is the amino acid tryptophan. There has been a number of recent investigations in which analogues of tryptophan, such as 5-hydroxytryptophan (5HW), 7-azatryptophan (7AW), or one of the fluorotryptophans, have been biosynthetically incorporated into proteins (*6*−*14*). The primary goal of such studies is usually to take advantage of the spectroscopic properties of the tryptophan analogue as a specific probe of the proteins' structure and function (*15, 16*). Less attention has been paid, so far, to the effect of incorporated tryptophan analogues on the stability of a protein's native state. This is the goal of the work reported in this article.

We have previously reported the incorporation of 5HW and 7AW into Staphylococcal nuclease (*17*), and in the preceding paper, we have extended this study to the incorporation of these and other tryptophan analogues into wild-type nuclease, a V66W mutant, and the Δ137−149 fragment, V66W′ (*18*). Nuclease was selected because nuclease serves as something of a standard for studies of protein stability (*19*−*23*) and because the set of proteins includes one with a single tryptophan at position 140 (wild type), one with a single tryptophan at position 66 (V66W′), and one with both tryptophan residues (*24, 25*). We demonstrated in our previous studies (*18*) that a high degree of incorporation of the analogues has been achieved and that the proteins containing the analogues (which can be called alloproteins) seem to have a structure similar to that of the tryptophan-containing wild type.

In the present communication we present studies of the thermal and guanidine−HCl induced unfolding of these alloproteins in order to quantitatively characterize the effect of the tryptophan analogues on the stability of the protein. The intent is to determine whether any of the analogues cause a significant perturbation of the thermodynamics of the protein. By studying a set of proteins with the analogues at two different positions, we hope to see whether any perturbing effect depends on the location of the amino acid analogue in the protein. The V66W mutant has previously been reported to show a non-two-state unfolding transition, with the population of an equilibrium folding intermediate (*24, 25*). The Δ137−149 fragments of nuclease have been shown in several studies to have marginally stable structures, with the degree of retained secondary and tertiary structure being very sensitive to amino acid substitutions (*30, 31*). Tryp-

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[1] Abbreviations: 4FW, 4-fluorotryptophan; 5FW, 5-fluorotryptophan; 6FW, 6-fluorotryptophan; 5HW, 5-hydroxytryptophan; 7AW, 7-azatryptophan; CD, circular dichroism; V66W, mutant of nuclease with V66 replaced by W66; V66W′, the Δ137−149 fragment of V66W, which lacks the last 13 amino acids.

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tophan at position 66 (forming V66W′) is one of the substitutions that stabilizes the fragment, which appears to retain the residual β-barrel structure of the N-terminal subdomain of the nuclease. Thus, the proteins V66W and V66W′ provide good tests of the effect of analogue substitution on the thermodynamics of protein unfolding.

MATERIALS AND METHODS

The materials used were described in the previous article. Guanidine-HCl (ultrapure) was obtained from U.S. Biochemical Co. (Cleveland, OH).

Methods. The octanol/water partition coefficient of Trp and the various analogues was determined by preparing a ~1 mM solution of the compounds in 0.02 M sodium phosphate buffer, pH 7.05. Three milliliters of these solutions was added to 3 mL of octanol, and the two-phase system was vortexed a couple of minutes and allowed to settle. After centrifuging to separate the phases, the absorbance of the aqueous phase was measured, using an appropriate octanol-equilibrated buffer blank. The absorbance of the Trp analogue in the octanol phase was calculated by the difference from the absorbance of a control solution of the analogue, and the partition coefficient was calculated as $A_{octanol}/A_{buffer}$.

Thermal unfolding of the proteins was monitored using our automated multidimensional circular dichroism/fluorometer, as described elsewhere (*26*). Typically we monitor CD signals at two or three wavelengths and fluorescence signals at one or two excitation wavelengths as the temperature of the sample is ramped at a constant rate. The temperature inside the stirred cuvette is recorded along with the spectroscopic signals. The data are analyzed with a nonlinear least-squares method, as described below. Guanidine−HCl induced unfolding studies were made with the same instrument as described by Ramsay and co-workers (*27*).

Thermodynamic Model. Data for the guanidine−HCl induced unfolding of the proteins were fitted by a two-state unfolding model, as described by the following equations.

$$\mathrm{N} \rightleftarrows \mathrm{U}; \quad K = [\mathrm{U}]/[\mathrm{N}] \tag{1}$$

$$Q = 1 + K; \quad X_\mathrm{N} = 1/Q; \quad X_\mathrm{U} = K/Q \tag{2}$$

$$\langle\alpha\rangle = X_\mathrm{N}(\alpha_{\mathrm{N,o}} + [\mathbf{d}]\delta\alpha_\mathrm{N}/\delta[\mathbf{d}]) + X_\mathrm{U}(\alpha_{\mathrm{U,o}} + [\mathbf{d}]\delta\alpha_\mathrm{U}/\delta[\mathbf{d}]) \tag{3}$$

where X_N and X_U are the mole fractions of the native, N, and unfolded, U, states, $\langle\alpha\rangle$ is the calculated signal (corresponding to the experimental CD or fluorescence signal) as a function of denaturant concentration, [**d**], $\alpha_{\mathrm{N,o}}$ and $\alpha_{\mathrm{U,o}}$ are the signals for the N and U states in the absence of denaturant, and $\delta\alpha_\mathrm{N}/\delta[\mathbf{d}]$ and $\delta\alpha_\mathrm{U}/\delta[\mathbf{d}]$ are the slopes (assumed to be linear) of the pre-transition and post-transition baseline regions in a plot of $\langle\alpha\rangle$ vs [**d**]. The equilibrium constant, K, is related to the standard free energy change for the unfolding transition, ΔG°_{un}, by the following relationship

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

where $\Delta G^\circ_{o,un}$ is the free-energy change for the N $\rightleftarrows$ U transition in the absence of denaturant (a measure of the stability of the protein at the reference condition), and $m = -\delta\Delta G^\circ_{un}/\delta[\mathbf{d}]$ is a parameter that describes the sensitivity of the unfolding transition to denaturant concentration. This linear dependence of ΔG°_{un} on [**d**] has been experimentally supported in several cases. Although there are other models for relating ΔG°_{un} to [**d**], the above equation is the simplest and will be accepted for this study.

Equations 2−4 were fitted to raw CD and fluorescence data, as a function of denaturant concentration, to obtain the fitting parameters $\Delta G^\circ_{o,un}$, m, $\alpha_{\mathrm{N,o}}$, $\alpha_{\mathrm{U,o}}$, $\delta\alpha_\mathrm{N}/\delta[\mathbf{d}]$, and $\delta\alpha_\mathrm{U}/\delta[\mathbf{d}]$. The fitting was performed using the program NONLIN (*27*, *28*).

Thermal unfolding data were also described by the two-state model with the following relationship for ΔG°_{un} as a function of temperature

$$\Delta G^\circ_{un}(T) = \Delta H^\circ_{un}(1 - T/T_{un}) + \Delta C_p[T - T_{un} - T\ln(T/T_{un})] \tag{5}$$

where ΔH°_{un} and is the enthalpy change for the unfolding transition at the transition temperature, T_{un}, and ΔC_p is the heat capacity change for unfolding.

Equations 2, 3, and 5 were fitted to CD and fluorescence data, as a function of temperature, to obtain the fitting parameters, ΔH°_{un}, T_{un}, and ΔC_p, as well as $\alpha_{\mathrm{N,o}}$, $\alpha_{\mathrm{U,o}}$, $\delta\alpha_\mathrm{N}/\delta\mathrm{T}$, and $\delta\alpha_\mathrm{U}/\delta\mathrm{T}$ (where the $[\mathbf{d}]\delta\alpha_i/\delta[\mathbf{d}]$ terms in eq 4 are replaced by $T\delta\alpha_i/\delta T$).

In some cases a three-state model, N $\rightleftarrows$ I $\rightleftarrows$ U, was fitted to denaturant induced or thermally induced unfolding transitions, as described by Ramsay and co-workers (*27*). The purpose in doing so is not so much to determine the thermodynamics of each step, but to compare the overall quality of the fit with that of the two-state model in order to see if there has been a loss of cooperativity in the unfolding of one of the proteins, as compared to wild-type nuclease. In performing these fits we allowed the relative spectroscopic signal, α_I, of the intermediate species to be a fitting parameter, but to minimize the number of floating parameters, we made the assumption that the slope of the intermediate's signal, that is, $\delta\alpha_\mathrm{I}/\delta[\mathbf{d}]$, is the average of the baseline slopes for the native and unfolded states.

RESULTS AND DISCUSSION

Octanol/Water Partitioning of Analogues. Before presenting the results for the unfolding studies with the proteins, we first present information about the hydrophobic character of the individual tryptophan analogues. Xu and co-workers (*29*) have previously reported the octanol/water partition coefficients of Trp and the fluorotryptophans. We have repeated these measurements using pH 7.05 phosphate buffer, rather than water. We find $\Delta\Delta G_{transfer}$ ($= RT \ln (P/P_o)$, where P is the partition coefficient of the analogue, and $P_o = 0.055$ is the value for Trp) values of 0.37, 0.59, and 0.71 kcal/mol for 4FW, 5FW, and 6FW, in comparison to Trp. That is, the fluorotryptophan analogues have higher octanol/water partition coefficients and thus are more hydrophobic than Trp by a slight degree. These $\Delta\Delta G_{transfer}$ values are similar to those of Xu and co-workers, with the exception that we find an inversion in the order of 5FW and 6FW. Though they only have a slightly larger atomic radius than hydrogen atoms, the fluorine atoms of these analogues apparently cause

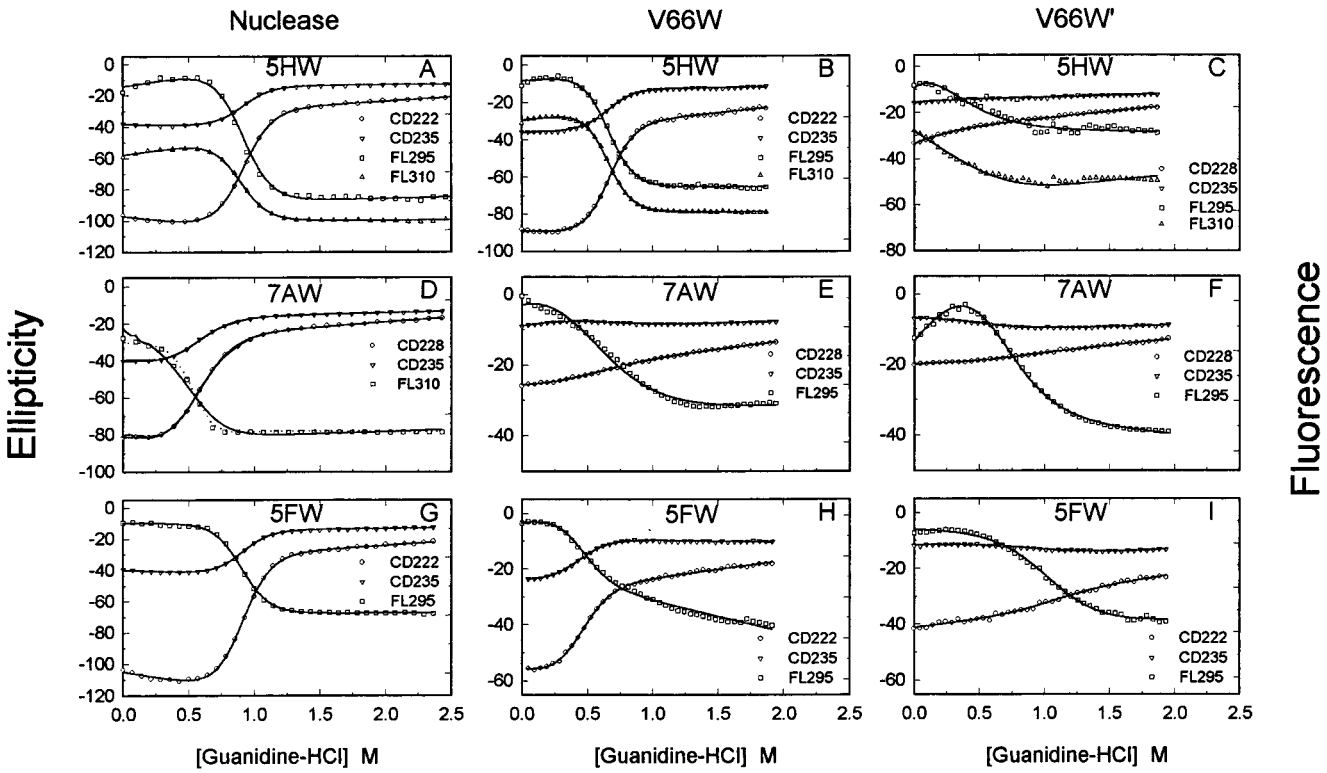

FIGURE 1: (top row) Guanidine−HCl induced unfolding of 5HW variants of WT nuclease (A), V66W (B), and V66W′ (C) at pH 7.3, 20 mM phosphate buffer, 25 °C. Raw CD data at two wavelengths and steady-state fluorescence data (excitation at both 295 and 310 nm, emission at 340 nm) were collected on the same sample using a multidimensional CD/fluorometer, and the data were fitted with a two-state model by global analysis. The solid line is the theoretical fit. (middle row) Guanidine−HCl induced unfolding of 7AW variants of WT nuclease (D), V66W (E), and V66W′ (F). Fluorescence data were collected with excitation at 295 and 310 nm, emission through a 360 nm interference filter. (bottom row) Guanidine−HCl induced unfolding of 5FW variants of WT nuclease (G), V66W (H), and V66W′ (I). Fluorescence emission at 340 nm.

a modest change in the hydrophobic character of the indole rings, which may have an effect on protein stability of alloproteins containing these analogues. In contrast, the octanol/water partition coefficients of 5HW and 7AW are both at least a factor of 10 smaller than that of Trp (the actual partition coefficients for 5HW and 7AW are too small for accurate measurement), indicating that the latter two analogues have $\Delta\Delta G_{transfer}$ values of less than −1.4 kcal/mol and are less hydrophobic than Trp. This result is expected since the 5-hydroxyl group of 5HW and the 7-imino group of 7AW will have hydrogen bonding tendencies.

Guanidine−HCl Induced Unfolding. Figure 1 shows examples of data for the unfolding of selected alloproteins (5HW in WT, V66W, and V66W′ in panels A−C; 7AW in WT, V66W, and V66W′ in panels D−F; 4FW in WT, V66W, and V66W′ in panels G−I). Note that in each case there is a drop in the fluorescence of the tryptophan (or analogue) of each of the proteins upon unfolding. Also, there is usually a decrease in the magnitude of the negative ellipticity in the range 222−235 nm upon unfolding, except for those cases where the protein appears to be largely unfolded in the absence of denaturant. The data were fitted by a two-state model to obtain $\Delta G°_{o,un}$ and m for the unfolding transitions. The resulting parameters are given in Table 1.

The data for WT nucleases were fitted well, in all cases except for the 7AW-containing protein, by a two-state model. The resulting values of $\Delta G°_{o,un} \approx 5-5.5$ kcal/mol and m ≈ 5.5−6 kcal/(mol M) are similar to those for the Trp-WT nuclease, again with the exception of the 7AW protein, for which the $\Delta G°_{o,un}$ and m values are lower.

Table 1: Parameters Describing the Guanidine−HCl Induced Unfolding of WT Nuclease and V66W′ Alloproteins[a]

protein/ analogue	$\Delta G°_{o,un}$ (kcal/mol)	m (kcal/ (mol M))	$[\mathbf{d}]_{1/2}$	χ^2
WT nuclease				
Trp	5.01	5.93	0.84	2.24
5HW	5.08	5.68	0.89	2.42
7AW[b]	2.18	4.50	0.49	7.02
4FW	5.53	5.84	0.95	2.31
5FW	5.22	5.80	0.90	2.47
6FW	5.50	5.98	0.92	1.96
V66W′				
Trp	2.37	2.76	0.86	0.94
5HW[c]	0.99	1.01	0.98	1.55
7AW[c]	1.43	2.76	0.52	1.54
4FW	2.40	2.62	0.92	0.79
5FW	1.83	2.14	0.86	1.11
6FW	2.04	2.29	0.89	0.79

[a] Buffer conditions: 20 mM phosphate, pH 7.3, 25 °C. Parameters are for a two-state global fit of two CD data sets and one fluorescence data set. [b] The three-state model was also fitted to the data for 7AW-nuclease, yielding an improved χ^2 of 1.10 and $\Delta G°_1 = 3.05$ kcal/mol, $m_1 = 6.61$ kcal/(mol M), $\Delta G°_2 = 3.64$ kcal/mol, and $m_2 = 4.91$ kcal/(mol M). [c] The low $\Delta G°$ and/or m values, and the small amplitude of the changes in both CD and fluorescence signals with increasing [Guanidine−HCl], indicate that the transitions (if any) are very gradual and make questionable the listed thermodynamic parameters for these two fragments.

The data for each of the V66W′ fragments can be fitted by a two-state model, although the transitions are more gradual than that for the WT protein. For example, for Trp-

Table 2: Parameters Describing the Guanidine−HCl Induced Unfolding of V66W: Two-State and Three-State Fits

protein/ analogue	$\Delta G°_1$ (kcal/ mol)	m_1 (kcal/ (mol M))	$[\mathbf{d}]_{1/2}$ (M)	$\Delta G°_2$ (kcal/ mol)	m_2 (kcal/ (mol M))	$[\mathbf{d}]_{1/2}$ (M)	χ^2
V66W							
Trp	1.30	3.87	0.34				15.8
	2.26	5.32	0.42	2.75	2.98	0.92	2.03
5HW	4.01	6.16	0.65				3.03
	4.00	6.16	0.65	4.24	1.24	3.40	3.01
7AW	2.23	2.90	0.77				3.01
	0.35	3.36	0.10	1.70	2.05	0.83	1.74
4FW	2.72	5.23	0.52				3.76
	2.47	4.71	0.52	5.74	5.01	1.15	1.84
5FW	1.86	4.97	0.37				6.30
	2.18	4.87	0.45	3.15	3.19	0.99	1.10
6FW	2.00	4.55	0.44				5.94
	2.33	4.91	0.47	2.01	2.43	0.83	2.05

V66W′ the $\Delta G°_{o,un}$ and m values are approximately half those for the WT protein. Also, since the fragments each have reduced α-helix content, the CD signals show a much smaller change in amplitude as guanidine−HCl is added. For each of the V66W′ alloproteins, the fluorescence of tryptophan (or analogue) at position 66 drops upon unfolding, although the percentage drop is not quite as large at that for position 140 in the full-length protein. Transitions for fragments containing 4FW, 5FW, and 6FW have about the same $\Delta G°_{o,un}$ and m values as those for the Trp-containing fragment. The fragments containing 7AW and 5HW, especially the latter, show an even broader unfolding transition, with $\Delta G°_{o,un}$ and m values lower than those for the Trp-V66W′. These results indicate that the fluorotryptophans, substituted at position 66, do not significantly perturb the fragment, as compared to tryptophan at this position. 7AW and 5HW in position 66, on the other hand, appear to significantly destabilize the fragment; as mentioned in the legend of Table 1, the values listed for $\Delta G°_{o,un}$ and m for the 7AW and 5HW fragments are considered to have great uncertainty.

The data for the V66W mutant alloproteins also show broad transitions in some cases. It has previously been shown that Trp-V66W is better described by a three-state model than by a two-state model. Nevertheless, since the goal here is to compare the apparent stability of various alloproteins, we present parameters for two-state fits in Table 2. The most obvious difference between the various V66W alloproteins is that the 5HW variant has a much greater stability and a sharper transition than the tryptophan form (i.e., $\Delta G°_{o,un} \approx 4$ kcal/mol and $m = 6$ kcal/(mol M) for 5HW-V66W, compared to $\Delta G°_{o,un} \approx 2$ kcal/mol and $m = 5$ kcal/(mol M) for Trp-V66W). Also, the transition for 5HW-V66W is very well described as a two-state transition, whereas Trp-V66W shows a slight deviation from two-state. 4FW-V66W also seems to be at least 1 kcal/mol more stable (and seems to have a higher m and to be more clearly two-state) than is Trp-V66W. 5FW- and 6FW-V66W have transitions similar to that for Trp-V66W.

7AW-V66W shows a very broad transition and shows very small changes in its CD signal as guanidine−HCl is added. The latter result is consistent with the finding in the previous article that 7AW-V66W has a less negative ellipticity in the far-UV region, which suggests that this alloprotein is partially unfolded in the absence of denaturant and may retain structure only the β-barrel region. The drop in fluorescence that is seen, as guanidine−HCl is added to 7AW-V66W, appears to reflect the gradual unfolding of the residual structure. If this is the case, it is difficult to compare the apparent thermodynamics, for a two-state fit, for the unfolding of 7AW-V66W with that of the other proteins.

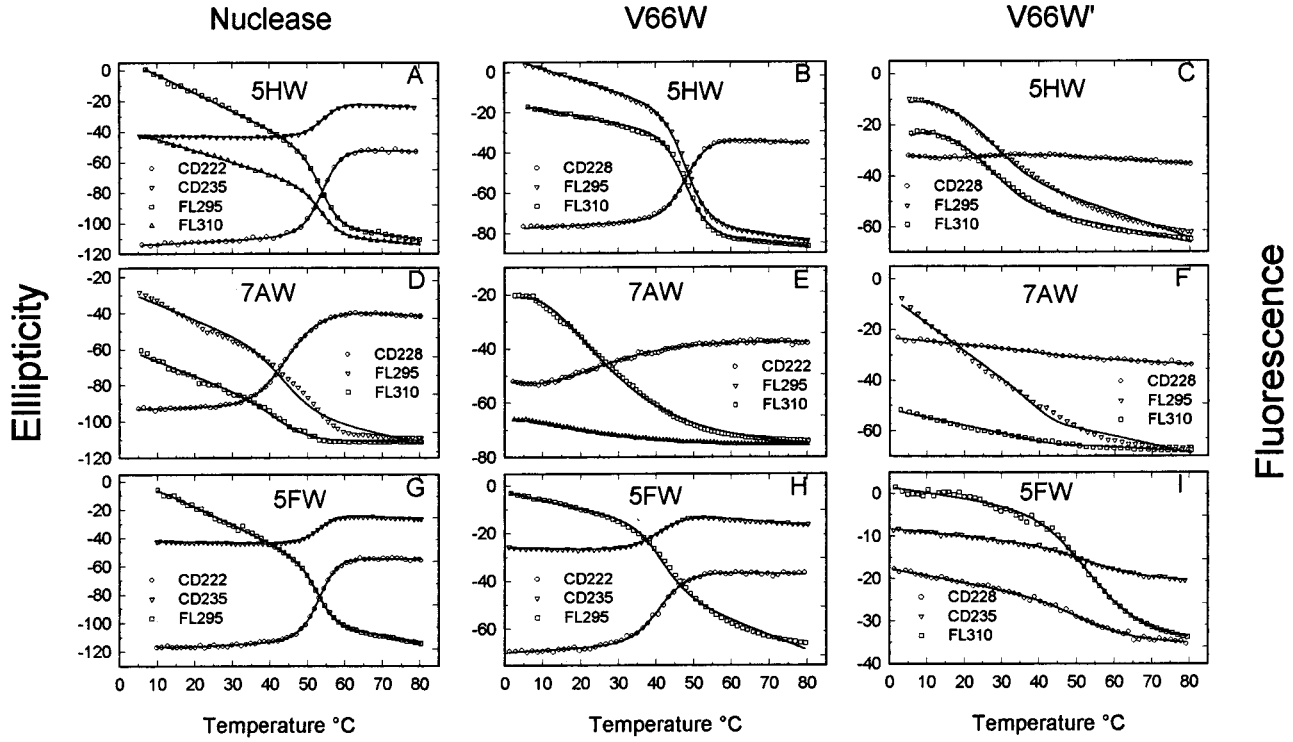

FIGURE 2: (top row) Thermal unfolding of 5HW variants of WT nuclease (A), V66W (B), and V66W′ (C). Conditions as in Figure 1. (middle row) Thermal unfolding of 7AW variants of WT nuclease (D), V66W (E), and V66W′ (F). (bottom row) Thermal unfolding of 5FW variants of WT nuclease (G), V66W (H), and V66W′ (I).

In an attempt to make a more reasonable interpretation of the unfolding data for the V66W alloproteins, we have performed a three-state fit of these proteins. In Table 2 we give a comparison of the two-state and three-state fits for the V66W alloproteins. For some, but not all, of these V66W alloproteins there is a significant improvement in the goodness-of-fit (the global chi squared, χ^2). For example, for Trp-V66W the χ^2 is reduced by a factor of ~8 on going to a three-state model. Similar, though less dramatic, improvements in χ^2 are seen with 5FW-V66W and 6FW-V66W forms. Consistent with the above comments about the sharpness of the transition for 5HW-V66W, this alloprotein shows no improvement in going to a three-state model. With 7AW-V66W the three-state fit is only a modest improvement. This analysis shows that the second unfolding transition for Trp-V66W (as well as for 5FW-V66W and 6FW-V66W) has $\Delta G°_{o,un}$, m, and $[\mathbf{d}]_{1/2}$ values that are similar to those for the two-state fit for 7AW-V66W (i.e., $\Delta G°_{o,un} \approx 2.5$ kcal/mol, $m \approx 3$ kcal/(mol M), and $[\mathbf{d}]_{1/2} \approx 0.8-0.9$ M). These values are also reasonably similar to those for the unfolding of Trp−V66W′. Although these comparisons do not prove that 7AW-V66W retains a β-barrel structure, the unfolding data and far-UV CD spectra are consistent with this interpretation.

Thermal Unfolding. Shown in Figure 2 are examples of data for the thermal unfolding of the alloproteins (5HW in the three proteins in panels A−C; 7AW in the three proteins in panels D−F; and 5FW in the three proteins in panels G−I). The data were fitted with a two-state model to obtain the thermodynamic parameters given in Tables 3 and 4. Far-UV CD spectra taken at 20 °C at the beginning and end of a heating (to 80 °C)/cooling cycle showed at least 90% recovery in the CD minima at near 220 nm, indicating reversibility in the thermal unfolding of each of the proteins.

For WT nuclease, we fitted the data with a model either in which the ΔC_p is fixed at 0, or in which the ΔC_p is allowed to float. A modest improvement in the goodness-of-fit parameter, χ_2, is found with each WT alloprotein when a nonzero ΔC_p is considered. The T_{un} and $\Delta H°_{un}$ were all about 52−54 °C and ~80 kcal/mol, respectively, for these proteins, with the exception of 7AW-containing WT, which has significantly lower values for these parameters.

The thermal unfolding data for V66W′ show broader transitions, which, as in the guanidine−HCl studies, have only a small variation in CD signal but a more convincing variation in fluorescence signal with temperature. A two-state model was fitted to the data for each protein with the assumption that $\Delta C_p = 0$ (see Table 3). Inclusion of a nonzero ΔC_p resulted in essentially no improvement in χ^2 for most cases, and therefore, we do not consider the ΔC_p term for V66W′ alloproteins. Compared to the Trp-V66W′ ($\Delta H°_{un} = 25$ kcal/mol, $T_{un} = 49$ °C), the fluorotryptophan variants have very similar thermodynamic parameters, whereas the 5HW and 7AW variants are less stable. This result is consistent with the guanidine−HCl studies and indicates that 5HW and 7AW, in position 66, are destabilizing to the residual structure of the Δ137−149 fragment.

The thermal unfolding data for V66W were fitted with a two-state model, for comparison purposes (see Table 4). Due to the broadness of the transitions, it was not possible to include a ΔC_p in the analyses. (We also fitted the data for the V66W alloproteins to a three-state model, as discussed below. With the exception of 5HW-V66W, there is an improvement in the χ^2 of more than a factor of 2 with the three-state model.) 5HW-V66W has higher T_{un} and $\Delta H°_{un}$ values (two-state) than the Trp-V66W. This is consistent with the results of guanidine−HCl unfolding and indicates that this protein is more stable than Trp-V66W. This result is somewhat perplexing, since the results with WT nuclease and V66W′ show that 5HW at position 140 does not perturb or stabilize WT nuclease and that 5HW at position 66 destabilizes the fragment. Note that 5HW-V66W is the only V66W alloprotein for which there is no significant improvement in χ^2 on going to a three-state fit. The above results can be interpreted in terms of the two-domain model for nuclease presented by Carra and co-workers (*21*, *22*), where the N $\rightleftarrows$ I $\rightleftarrows$ U unfolding process can be related to the unfolding of the α-helix domain (primarily the long C-terminal α-helix) and the β-domain (N-terminal β-barrel hydrophobic core region). Assuming that there is coupling between the unfolding of these domains and that the first transition reflects the unfolding of the less stable α-domain, the observation that 5HW-V66W appears to unfold as a two-state system can be explained in terms of the free-energy level of I (intermediate species having the α-domain unfolded and the β-domain folded) always being near that of U. In

Table 3: Thermodynamic Parameters for the Thermal Unfolding of WT Nuclease and V66W′

protein/ analogue	$\Delta H°_{un}$ (kcal/mol)	T_{un} (°C)	ΔC_p (kcal/(mol K))	χ^2
WT Nuclease				
Trp	79.65	53.15	⟨0⟩	1.35
	(76.91−82.40)	(53.05−53.26)		
	80.53	53.12	2.60	0.78
	(78.11−83.03)	(53.01−53.22)	(2.43−2.80)	
5HW	79.84	53.99	⟨0⟩	1.91
	(75.52−84.30)	(53.82−54.06)		
	80.61	53.88	2.52	1.20
	(76.65−83.92)	(53.70−54.06)	(2.16−2.80)	
7AW	41.93	45.20	⟨0⟩	2.11
	(39.28−44.48)	(44.83−45.58)		
4FW	78.16	54.29	⟨0⟩	2.06
	(74.88−81.55)	(54.16−54.33)		
	80.20	54.22	2.44	1.34
	(77.10−83.39)	(54.08−54.35)	(2.28−2.61)	
5FW	77.37	52.97	⟨0⟩	1.68
	(74.06−80.57)	(52.83−53.11)		
	77.97	52.87	2.68	1.39
	(74.86−80.58)	(52.72−53.02)	(2.32−2.98)	
6FW	79.88	53.60	⟨0⟩	2.54
	(76.78−82.89)	(53.45−53.74)		
	79.29	53.51	2.78	1.72
	(76.69−81.64)	(53.39−53.63)	(2.53−3.01)	
V66W′				
Trp	25.05	49.14	⟨0⟩	1.14
	(23.09−27.01)	(48.21−50.08)		
5HW[a]	19.71	23.32	⟨0⟩	2.66
	(18.07−21.35)	(21.35−25.30)		
7AW[a]	13.10	44.82	⟨0⟩	1.82
	(11.55−14.54)	(43.47−46.20)		
4FW	26.60	52.01	⟨0⟩	1.20
	(24.71−28.55)	(50.81−53.21)		
5FW	26.99	52.74	⟨0⟩	1.15
	(25.13−28.85)	(51.98−53.50)		
6FW	24.76	51.73	⟨0⟩	1.26
	(22.84−26.74)	(50.52−52.99)		

[a] The low $\Delta H°_{un}$ and/or T_{un}, along with the small amplitude of the changes in CD and fluorescence signal, indicate a very gradual transition (if any) and make questionable the reported thermodynamic parameters for these fragments.

Table 4: Thermodynamic Parameters for the Thermal Unfolding of V66W: Fits of the Two-State and the Three-State Models[a]

protein/analogue	$\Delta H°_{un,1}$ (kcal/mol)	$T_{un,1}$ (°C)	$\Delta H°_{un,2}$ (kcal/mol)	$T_{un,2}$ (°C)	χ^2
V66W					
Trp	41.94	43.32			6.44
	(38.96−45.02)	(43.07−43.62)			
	39.52	44.96	21.07	47.78	1.12
	(36.58−41.98)	(44.23−45.78)	(18.09−24.36)	(44.50−50.82)	
5HW	71.46	48.46			2.10
	(67.97−74.85)	(48.27−48.65)			
	21.47	35.86	67.53	47.99	1.28
	(17.86−25.20)	(32.19−39.45)	(65.70−69.31)	(47.82−48.17)	
7AW	13.72[b]	15.60[b]			4.63
	(12.75−14.73)	(13.63−17.24)			
	23.25	21.37	35.85	50.48	1.18
	(19.97−26.63)	(20.57−22.19)	(26.22−45.96)	(48.28−52.54)	
4FW	52.10	44.06			2.81
	(48.22−56.11)	(43.66−44.44)			
	47.74	44.10	58.92	57.70	1.34
	(44.93−50.11)	(43.69−44.58)	(40.85−78.10)	(55.91−59.20)	
5FW	38.58	42.80			9.63
	(34.40−42.49)	(42.05−43.57)			
	40.36	42.28	29.40	52.59	1.090
	(38.86−41.92)	(41.85−42.68)	(25.26−33.83)	(51.00−54.24)	
6FW	41.47	43.57			5.08
	(38.47−44.57)	(43.11−44.03)			
	41.43	44.10	25.16	53.28	0.93
	(39.90−42.97)	(43.76−44.46)	(22.06−28.45)	(51.45−55.17)	

[a] For each protein, the first line is a two-state fit and the second line is a three-state fit. Fits are for heat capacity changes of 0. The only V66W alloprotein for which inclusion of a nonzero ΔC_p term improves the χ^2 (for a two-state fit) by as much as a factor of 2 is 5HW-V66W, for which we obtain $\Delta C_p = 2.47$ kcal/(mol K). [b] The low $\Delta H°_{un}$ and T_{un} indicate a very gradual transition and make questionable the listed thermodynamic parameters for the two-state fit for 7AW-V66W.

other words, the presence of 5HW residues either stabilizes the α-domain or strengthens its coupling with the β-domain, so that the I ⇄ U transition occurs very easily following the difficult N ⇄ I transition.

Whereas 5HW is sometimes stabilizing and sometimes destabilizing, the effect of 7AW is consistently seen to destabilize WT nuclease, V66W′, and V66W. For V66W, the presence of 7AW at positions 66 and 140 in V66W causes a significant broadening of the thermal transition. When fit to a three-state process, the first transition for 7AW-V66W is found to occur at near room temperature, much lower than that found for three-state fits for any of the other proteins. Among the fluorotryptophan alloproteins, 4FW-V66W is slightly more stable than Trp-V66W toward thermal unfolding, as indicated by higher $\Delta H°_{un}$ and T_{un} values (for a two-state fit). A similar conclusion (slight stabilizing effect of 4FW) was observed for the guanidine−HCl unfolding of V66W. The 5FW and 6FW forms of V66W have $\Delta H°_{un}$ and T_{un} values that are similar to those of Trp-V66W, indicating that these analogues are nonperturbing. The three-state model improves the fits for all V66W alloproteins (except the 5HW variant). The three-state fits for the fluorotryptophan alloproteins are similar to the fits for Trp-V66W. The three-state fits of 7AW-V66W indicate that the first transition is partially complete at room temperature (i.e., T_{un1} for the first transition is ∼20 °C). It is thought that the first transition is associated with the unfolding of the C-terminal α-helix of the protein. If substitution of 7AW at position 140 causes a melting of the C-terminal α-helix below room temperature, this would explain the loss of far-UV CD ellipticity in the 220 nm region for 7AW-V66W, as reported in the preceding article.

CONCLUSIONS

The following conclusions can be drawn regarding the substitution of tryptophan analogues into positions 66 and 140 of WT nuclease, V66W′, and V66W.

(1) 5HW has a mixed effect. It does not perturb the stability of WT nuclease (position 140), but it destabilizes the V66W′ fragment (when at position 66). When 5HW is present in both position 66 and position 140 in 5HW-V66W, there is an increase in the apparent degree of cooperativity (more two-state-like) in the thermal unfolding of the protein, which can be explained as an increase in the strength of coupling between the two subdomains of the protein.

(2) 7AW destabilizes WT nuclease, V66W′, and V66W, indicating that this tryptophan analogue perturbs the globular structures when substituted at either position 66 or position 140. Although only two positions have been studied, this result suggests that 7AW may have a general destabilizing effect on protein structure. Since the 7-azaindole ring has a polar imino nitrogen at position 7 of the aromatic ring, one would expect that this nitrogen would prefer to be hydrogen bonded and that such a hydrogen bond would have to be sacrificed to bury the 7-azaindole ring into an apolar region of a protein. The natural environment of an indole side chain of a tryptophan residue in a protein would not be expected to accommodate a hydrogen bond acceptor at position 7.

(3) The three fluorotryptophans have a small effect on the stability of the proteins; 4-fluorotryptophan seems to have a small stabilizing effect. These results are reasonable since (a) fluorine has a covalent radius of 1.35 Å, which is only slightly larger than the covalent radius of 1.2 Å for a hydrogen atom, (b) the electronegative fluorine atoms will act as weak hydrogen bond acceptors and will increase the charge separation in the indole ring, and (c) the fluorine

atoms make the tryptophan analogue slightly more apolar, as measured by their larger partition coefficient into octanol. The balance of these effects seems to lead to an insignificant or slight stabilizing effect on the native state of the proteins studied here.

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