J. Am. Chem. Soc. 1993, 115, 4457–4460

Introduction of a Trp Residue into α_4 as a Probe of Dynamics

Tracy M. Handel,[†] Scott A. Williams,[‡] Dora Menyhard,[†] and William F. DeGrado[*,†,§]

Contribution from the DuPont Merck Pharmaceuticals Company, P.O. Box 80328, Wilmington, Delaware 19880-0328, Regional Laser and Biotechnology Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and The Johnson Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received November 16, 1992

Abstract: To investigate the dynamic properties of α_4, a designed four-helix bundle protein, a Leu residue sequestered in the hydrophobic core of the bundle was changed to Trp. If the interior of the protein was very loosely packed, this replacement should be accommodated quite readily. On the other hand, if the protein was reasonably well-packed (as in the native state or an intermediate late on the folding pathway), the substitution should perturb the packing and destabilize the protein. In fact, the mutation of Leu to Trp was strongly destabilizing, decreasing the free energy of folding by approximately 3–4 kcal/mol. Time-resolved fluorescence measurements suggest that the Trp side chain might be held in two or more distinct orientations in the interior of the protein although it is not held in so many orientations as to average the near-UV signal in the CD spectrum. Finally, fluorescence anisotropy decay showed that the Trp indole side chain was immobilized on the nanosecond time scale, indicating there is a larger barrier for rotation of side chains in the interior of α_4 as compared to Trp-containing peptides in more fluid media such as homogeneous liquids, SDS micelles, or bilayers.

De novo protein design[1] is a recently developed approach that has considerable promise for the study of protein structure and function. This approach is critically testing our understanding of protein structure and also is laying the groundwork for the design of proteins and biomimetic polymers whose properties have not been anticipated in nature. Although several de novo designed proteins have recently been described,[2] the ultimate goal of designing a protein that folds into a well-defined and experimentally determined tertiary structure has yet to be demonstrated. One of the early problems associated with the proteins designed to date has been that they appear to adopt structures that are more dynamic than native proteins. In this respect, the designed structures appear to resemble "molten globules",[3] a term broadly used to describe non-native states of proteins that are nonetheless compact and contain native-like secondary structure. Recently, molten globules have been extensively studied because they are believed to resemble protein folding intermediates. Relative to the native state, molten globules are slightly larger and have much broader thermal unfolding transitions, and their interior-facing residues adopt a large number of equilibrating rotomeric states.

One de novo designed protein, α_4,[4,5] has been particularly well-characterized and appears to possess hybrid characteristics of a protein in the native and molten globule states.[6,7] This protein is intended to fold into a structure with four identical helices interconnected by three loops (Figure 1), and a considerable body of evidence[4–8] suggests that it adopts a fold or a family of related folds that approximates this conformation. Similar to native proteins, α_4 is compact and globular and has a high degree of secondary structure and an extremely high thermodynamic stability.[4–7] However, the change in heat capacity (ΔC_p) for unfolding α_4 is intermediate between that expected for the native and molten globule states, and the protein binds ANS (1-anilino-8-naphthalenesulfonate),[6] presumably reflecting a mobile hydrophobic interior. Also, the appearance of the ^{1}H-NMR spectra suggests that the backbone is relatively well-structured while the side chains are free to adopt multiple low-energy conformations.[6,8] Taken together, these data suggest that the protein folds into a structure similar to the design with four antiparallel helices ordered around a hydrophobic core, but the exact registration of the helices and the topology of the bundle may be dynamically averaging, and therefore imprecisely defined.

In order to further characterize the structure of α_4, we have adopted an approach that we hope will have general utility for the study of the structural plasticity and dynamic properties of non-native states such as molten globules or folding intermediates. We substituted a large, bulky Trp residue in place of a smaller Leu residue in the buried interior of α_4, and the structural and thermodynamic consequences of this mutation were then determined. We reasoned that if the interior of α_4 is very loosely packed, such as in the interior of a micelle, then a Trp should be accommodated quite readily. Although the indole side chain of Trp is not uniformly apolar (its indole N–H can serve as an effective proton donor), it should nevertheless be expected to exist stably on the interior of a protein if its apolar core is highly mobile and partially hydrated.[9] Thus, the replacement might

[†] DuPont Merck Pharmaceuticals Company.
[‡] Regional Laser and Biotechnology Laboratory, University of Pennsylvania.
[§] Department of Biochemistry and Biophysics, University of Pennsylvania.

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SEQUENCE of Ac-α_4 and L_6W-α_4

Helix1: Gly-Glu-Leu-Glu-Glu-Trp-Leu-Lys
Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly
Helix2: Gly-Glu-Leu-Glu-Glu-Leu-Leu-Lys
Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly
Loop: Pro-Arg-Arg

Ac-α4: Ac-Helix2-Loop-Helix2-Loop-Helix2-Loop-Helix2-CONH2
L6W-α4: Ac-Helix1-Loop-Helix2-Loop-Helix2-Loop-Helix2-CONH2

Figure 1. Sequences of Ac-α_4 and L_6W-α_4. The peptides were synthesized by solid-phase methods on a Milligen 9050 peptide synthesizer using Fmoc-protected amino acids and purified by reverse-phase HPLC described in the experimental section.

even enhance the thermodynamic stability of a highly molten protein through the hydrophobic effect. On the other hand, if the protein is reasonably well-packed (such as in the native state or an intermediate late in the folding pathway), the introduction of such a large side chain would perturb the packing and destabilize the protein.[10] Baldwin and co-workers[11] have used a similar strategy of introducing large, bulky residues into helix/helix packings, to probe the interactions stabilizing the molten globule state of apomyoglobin. In this work, we build on this basic approach by selecting Trp as the residue to be introduced. This allowed us to evaluate not only the thermodynamic consequences of the mutation but also the dynamic effects of the substitution through time-resolved fluorescence measurements.

Results

Synthesis of Ac-α_4 and L6W-α_4. In previous work, we prepared α_4 and mutants of this protein by expressing a foreign gene in bacteria. Because we are also interested in introducing a variety of noncommonly occurring amino acids at multiple locations within α_4, we investigated whether this 73-residue protein could be synthesized and purified to homogeneity, as has been accomplished by Mutter, Rivier, and co-workers for a de novo designed protein of similar size.[12] Using similar techniques it has been possible to prepare Ac-α_4 (Figure 1) and an analogue of this protein, L6W-α_4, in which Leu 6 is substituted by Trp. Ac-α_4 differs from the original α_4 in that it lacks the N-terminal Met occurring in the cloned material and also has N-terminal acetyl and C-terminal carboxamide blocking groups.

Solution Characteristics of Ac-α_4 and L6W-α_4. The bulk structural properties of these two proteins appear to be similar in many regards. For instance, they elute from a Sephadex G-50 size-exclusion column[4] at identical positions, indicating that the proteins have similar hydrodynamic radii (the calculated and experimentally determined MW for L6W-α_4 are 8649 and 10 000 kDa, respectively). Also, the far-UV CD spectrum of L6W-α_4 is nearly identical to that of α_4 ($[\theta_{222}]$ = −19 000 vs −18 000 deg cm^2 dmol^{-1} for Ac-α_4 and L6W-α_4, respectively). Thus, the introduction of a Trp residue has not significantly perturbed the gross physical properties of the protein.

Examination of the steady-state fluorescence spectrum of L6W-α_4 provides information concerning the environment of the Trp in the protein.[13] Its fluorescence emission maximum is 329 nm, which can be compared to approximately 350 nm for Trp in water vs 319 nm for the single, solvent-inaccessible Trp in ribonuclease T_1.[14] Thus, it is likely that the Trp in L6W-α_4 is largely buried but has some solvent accessibility, at least in some of its conformational states.

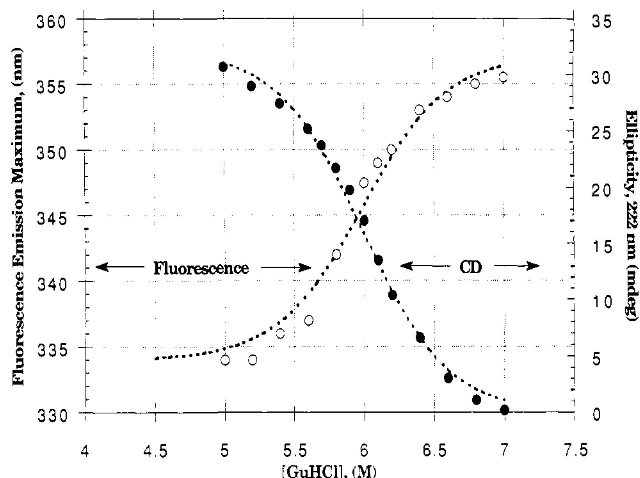

Figure 2. CD signal at 222 nm and fluorescence emission wavelength of L_6W-α_4 as functions of GuHCl. The lines through the data points are theoretical curves calculated from the equation and the parameters described in Table I.

Table I. Summary of the Thermodynamic Parameters Obtained from Guanidine Denaturation of Ac-α_4 and L_6W-α_4 (See Also Figure 2) as Monitored by the Ellipticity at 222 nm on an AVIV 62DS CD Spectrophotometer[a]

protein	midpoint [GuHCl], M	ΔG_{H2O}, kcal mol^{-1}	$RT\Delta\beta°$, kcal mol^{-1} M^{-1}
Ac-α_4	6.3	−15.4	−2.60
L6W-α_4	6.0	−11.8	−1.97

[a] The data were fit to the equation $\Delta G_{obs} = \Delta G_{H2O} - RT\Delta\beta°$[GuHCl], where ΔG_{obs} is $-RT \ln (f/(1-f))$ (f is the fraction of folded protein), ΔG_{H2O} is the free energy of folding in the absence of denaturant, and $-RT\Delta\beta°$ is the change in the molar cosolvation free energy (which is a measure of the cooperativity of the transition).

Thermodynamic Stability of Ac-α_4 and L6W-α_4. Figure 2 illustrates the guanidine (GuHCl) induced unfolding curve for L6W-α_4 monitored by either Trp fluorescence or by far-UV CD. As is the case for the great majority of small, single-domain proteins, both techniques show a single coincident transition.[15] This finding indicates that the protein unfolds via an essentially two-stage process in which intermediates are not significantly populated at equilibrium.[15] Although the two proteins unfold at similar concentrations of GuHCl, the unfolding of Ac-α_4 is more cooperative than that of L6W-α_4 (Table I). In the absence of GuHCl, L6W-α_4 is also considerably less stable than the parent protein, Ac-α_4; extrapolation of the stability data to zero GuHCl by the method of Santoro and Bolen[16] indicates that L6W-α_4 is less stable than Ac-α_4 by 3.6 kcal/mol.

Time-Resolved Fluorescence Measurements. Time-resolved fluorescence measurements of Trp-containing proteins provide information concerning the number of Trp rotamers and their rates of interconversion on the nanosecond time scale. Measurement of the isotropic fluorescence decay of L6W-α_4 gave three components of approximately equal intensity, providing possible evidence for multiple conformers of the Trp side chain (Figure 3).[17] However, it should be appreciated that Trp residues in native proteins often show multiple exponentials, and the origin of this behavior is controversial.[17b]

The mobility of the Trp side chain can be assessed by measuring the polarization decay for the protein. Figure 4 shows that the data are well-described by a single exponential with a 7-ns

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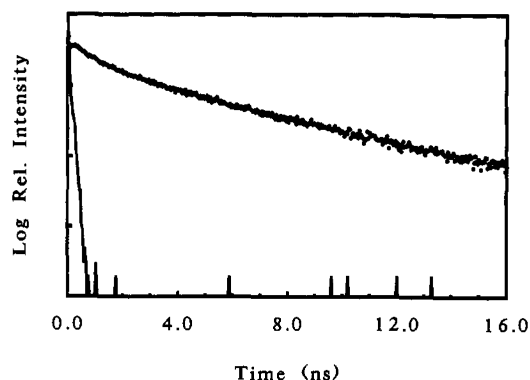

Figure 3. Time-dependent isotropic fluorescence decay of L_6W-α_4. The isotropic fluorescence decay was measured using a time-correlated single-photon counting system with a 25-ps fwhm instrument response function. The instrument response (–) and fluorescence decay (●) are plotted on a logarithmic scale. Curves were generated using the RLBL LIFETIME program (G. Holton, Department of Chemistry, University of Pennsylvania) with typical $\chi^2 < 1.1$ for these measurements.

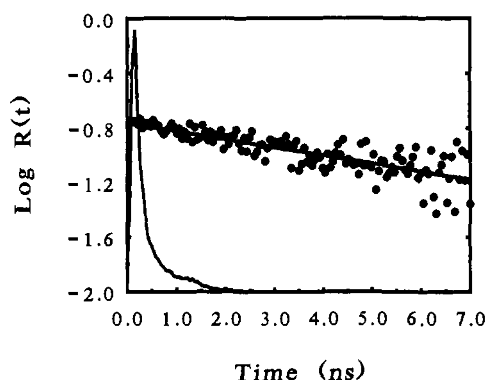

Figure 4. Fluorescence anisotropy decay of L_6W-α_4. A representative 7-ns segment of the anisotropy decay (●) is presented on a logarithmic scale. As with the isotropic fluorescence decay, the data were obtained by the photon counting technique and measured over three time domains of 1, 7, and 25 ns full scale. A $\chi^2 < 1.1$ was obtained when fitting the data to a single rotational decay model. The addition of a second decay did not result in a statistically significant improvement in the fit to the data.

correlation time, which we attribute to overall tumbling of the bundle. The absence of correlation times faster than overall bundle rotation, which would correspond to segmental motions of the Trp, indicates that the Trp is effectively immobilized within the protein core. Thus, if this residue undergoes conformational averaging, it must occur on a time scale significantly slower than approximately 10 ns.

The value of 7 ns for the overall tumbling time of the bundle is somewhat higher than observed for complexes of ANS with the α_1B tetramer or the α_2 dimer,[6] indicating that either the transition dipole moments, intrinsic to each probe, are sampling separate semielliptical radii or there is an expansion of the spherical hydrodynamic radius induced by introduction of a Trp residue; or equally plausible, there is limited surface exposure of hydrophobic side chains, resulting in weak protein–protein aggregation. We know, from several unpublished polarized time-resolved experimental results, that molten globule-like derivatives of α_4 show a small degree of aggregation at micromolar to millimolar concentrations. Such aggregation is evidently too weak to be detected by size-exclusion chromatography.

Near-UV CD. The CD of proteins in the aromatic region has been previously used to assess whether they assume native conformations. In general, native proteins show CD spectra in the near UV that arise from the asymmetric environments of the aromatic amino acids. The spectrum of L6W-α_4 is similar in shape and close in magnitude to that of another four-helix bundle

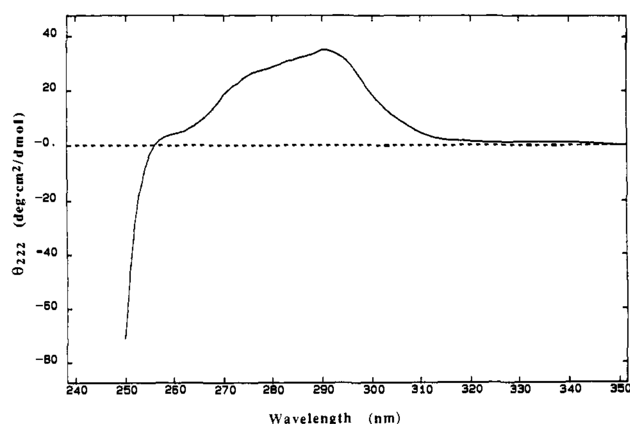

Figure 5. Near-UV CD spectra of L_6W-α_4. The ellipticity is given as mean residue ellipticity (i.e., the molar ellipticity for the single Trp divided by the number of residues in the protein).

protein, native interleukin-2 (Figure 5).[18] This observation contrasts with most molten globules, which show limited circular dichroism in the near UV, presumably because the aromatic side chains adopt a large number of different orientations that lead to averaging of the signal.[3]

Discussion and Conclusions

The findings of this investigation support our earlier conclusion that α_4 has a structure intermediate between the native and molten globule states. Introduction of a large, bulky, apolar Trp residue on the interior of the protein destabilizes the protein, indicating that the interior is reasonably well-packed and unlike the interior of a micelle. It is difficult to interpret the decrease in cooperativity for L6W-α_4 as compared to Ac-α_4. One possible interpretation would be that the protein no longer folds in an all-or-nothing manner.[15] However, this appears to be unlikely on the basis of the observation that the transition is identical irrespective of whether the helical content or the solvent exposure of the Trp was monitored (by far-UV CD and fluorescence, respectively).[15] A more likely possibility is that the solvent exposure of the interior core of L6W-α_4 is somewhat greater than that for Ac-α_4. This explanation would also be consistent with the observed decrease in the stability of the L6W-α_4.

A surprising finding of this work is that the stability determined for Ac-α_4 was less than the value previously determined for a bacterially expressed version of the protein, α_4, which contains Met as the first amino acid and also lacks the N-terminal acetyl and C-terminal carboxamide groups of Ac-α_4. The bacterially expressed protein was reported to have a stability of –22 kcal/mol, while the corresponding value for Ac-α_4 is –15.4 kcal/mol. Although the value for Ac-α_4 is still extremely favorable and out of the range typically observed for native proteins, the difference in these two values is significantly greater than our experimental error. We have recently found that the observed stability of bacterially expressed α_4 depends on the method used in its purification and that when it is purified by reversed-phase HPLC (as for the chemically synthesized protein), its stability is similar to that of Ac-α_4. We have also found that α_4 binds to apolar molecules,[6] suggesting that the difference in observed stability might have arisen from the presence of bound, hydrophobic molecules in the original preparation; in fact NMR spectra confirmed the presence of aromatic impurities. Such hydrophobic molecules might not be expected to be removed by the previous purification method which included ion-exchange chromatography and size-exclusion chromatography.

The spectroscopic studies provide a description of the dynamic properties of the Trp side chain within the protein. The multiple lifetimes for the indole side chain provide some evidence that it

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might be held in two or more distinct orientations on the interior of the protein. However, the anisotropic decay shows a single exponential (corresponding to the overall tumbling of the protein), which indicates that the conformers of the Trp do not significantly interconvert on the 1–10-ns time scale. Thus, there is a large barrier for rotation of the Trp side chains in the interior of Ac-α_4 as compared to Trp-containing peptides in more fluid media such as homogeneous liquids, SDS micelles, or bilayers.[19] Finally, the similarity of the aromatic CD spectrum to a native four-helix bundle would indicate that the Trp side chain is not held in so many conformers as to randomize its signal.

In summary, these studies provide a picture of a protein in which the packing has been substantially disturbed by the introduction of a Trp side chain. The bulky indole side chain in all probability adopts more than one conformation, but these conformers exchange slowly on the 1–10-ns time scale. These findings are consistent with our earlier conclusion that α_4 is a protein with properties intermediate between those of a molten globule and a native protein. The large decrease in stability upon addition of a bulky side chain and the immobilization of the Trp residue are expected for a protein in a native conformation. On the other hand, data amassed in previous papers clearly show that the protein is not fully native. Thus, the apolar clustering of hydrophobic Leu residues is sufficient to cause the condensation of the protein core and to direct the folding trajectory almost to a native state. To fully specify a native state, however, requires the introduction of more specific packing interactions,[20] hydrogen bonds, or metal-binding sites.[6,7]

Experimental Section

Materials. The peptides were synthesized on a Milligen 9050 synthesizer by solid-phase methods using Fmoc (9-fluorenylmethoxycarbonyl) protected amino acids. Fmoc L-amino acids and Fmoc L-amino acid pentafluorophenyl (OPfp) esters were purchased from Milligen. Synthetic cycles included 45-min coupling reactions followed by 10-min capping steps using solutions of 0.5 M pyridine and 0.5 M acetic anhydride in DMF (Gly, Glu, Leu, Lys). Arg, Trp, and Pro residues were not capped but were double-coupled for an additional 45 min. The peptides were cleaved for 2 hours in trifluoroacetic acid (TFA)/anisole/thioanisole/ethanedithiol (9:0.2:0.5:0.3) and subsequently precipitated with cold ether. The peptides were doubly purified by reverse-phase HPLC on a preparative Vydac C4 column using gradient elution, first with TFA buffers H_2O/TFA (99.9:0.1)–CH_3CN/H_2O/TFA (90:9.9:0.1) and subsequently with triethylammonium phosphate (TEAP) (pH 2.5) buffers H_2O/TEA/H_3PO_4 (96.7:2:1.3)–CH_3CN/H_2O/TEA/H_3PO_4 (60:38.7:0.8:0.5).[21] Prior to lyophilization, peptides were desalted on a Pharmacia Fast Desalting FPLC column eluted with 10% acetic acid. The purified materials were >95% pure on the basis of analytical HPLC and electrospray mass spectrometry.

Physical Measurements. Stabilities were determined by monitoring the CD signal (222 nm) and fluorescence emission wavelength of L_6W-α_4 as functions of GuHCl. Guanidine denaturation curves were fit[16] to the equation $\Delta_{Gobs} = \Delta_{GH2O} - RT\Delta\beta°[\mathrm{GuHCl}]$, where ΔG_{obs} is $-RT \ln (f/(1-f))$ (f is the fraction of folded protein), ΔG_{H2O} is the free energy of folding in the absence of denaturant, and $-RT\Delta\beta°$ is the change in the molar cosolvation free energy (which is a measure of the cooperativity of the transition).

Near-UV CD spectra of L_6W-α_4 were recorded on an AVIV 62DS CD spectrophotometer in a 0.5-cm cell at a concentration of 0.53 mM in 10 mM Hepes (*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) pH 7.5.

Isotropic fluorescence decay measurements were made using a time-correlated single-photon counting instrument with a 25-ps fwhm response time. Anisotropy decay was measured over three time regimes of 1, 7, and 25 ns. The χ^2-square of the fitted parameters was less than 1.1 for these measurements using the RLBL LIFETIME software package (Holton, G. Ph.D. Thesis, University of Pennsylvania, Philadelphia, PA).

Acknowledgment. We thank Spencer Anthony-Cahill for providing the results on Ac-α_4. We also thank Robin Hochstrasser for encouragement and support.

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