Hydrogen Bonded Cluster Can Specify the Native State of a Protein

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Received June 10, 1999

De novo protein design[1−5] provides an attractive approach for examining the balance of forces stabilizing the native, folded states of proteins. Considerable evidence indicates that hydrophobic interactions provide a very strong driving force that stabilizes the structures of proteins.[6−8] Other forces including hydrogen bonding, electrostatics, van der Waals interactions, and aromatic stacking are often less favorable, but important for conformational specificity.[9] Such interactions can provide an important role in specifying a unique conformation relative to a dynamically averaging ensemble of folded states,[10−13] often called the molten globule.[14,15] We have developed a series of dimeric four-helix bundle proteins as a model system for probing the relative role of these interactions in protein folding.[16−20] These peptides resemble coiled coils, which have been extensively studied as models for protein folding,[2,21−25] in that they are folded as dimers but unfolded as monomers. However, unlike coiled coils, the helices in these dimeric four-helix bundles have lengths similar to those frequently observed in natural proteins,[26] and they assemble into globular proteins rather than highly elongated rodlike structures. The initial members of this series of dimeric 4-helix bundles adopted dynamic, molten globule-like conformations.[16−18] However, subsequent refinements to the design led to α_2D, which adopted a unique well-defined structure in solution, shown in Figure 1.

The sequence of α_2D differs from its molten globule-like precursor, α_2C, at three positions, residues 7, 26, and 30. In α_2D, residue 7 is a Glu side chain, which is largely exposed to solvent and adopts multiple rotamers in solution. By contrast His26 and His30 are involved in a partially solvent-exposed hydrogen-bonded interaction with symmetry-related side chains at the helix 2-helix 2′ interface of α_2D (Figure 1). Similar hydrogen bonding between His residues is frequently observed in natural proteins.[27] The interacting His residues appear to be in the neutral, nonionized state in folded α_2D. The thermal stability of α_2D is pH dependent, and titrates with a pK_a near 6 as would be expected for one or more histidine side chains (Supporting Information). To further address the role of these side chains in stabilizing a unique tertiary structure, we prepared two variants of α_2D, H26F, and H30K, in which these His side chains were individually reverted to the original residue in α_2C. His 26 occurs at a partially buried position and was replaced by Phe as in α_2C. Additionally, to examine the role of aromaticity versus hydrogen bonding, residue 26 was also changed to Asn. These changes are nearly isosteric, and were easily accommodated in computer models of the structure. His30 occupies a more solvent-exposed position, and was changed to Lys, as in α_2C. This residue was also changed to Asp, which can unambiguously serve as a hydrogen bond acceptor. Examination of the properties of these peptides indicates that a hydrogen-bonded cluster observed in α_2D is indeed important to its unique, native structure.

Variable-temperature, far-UV CD spectroscopy clearly showed the variants of α_2D adopted helical structures, which underwent cooperative, reversible folding/unfolding transitions (Supporting Information). Changes to the solvent-exposed His30 had a relatively minor effect on the aggregation state or the stability of the dimer. H30D was more stable than α_2D by approximately 0.4 kcal/mol while H30K was destabilized to a similar extent (Table 1). Remarkably, however, these changes at His30 gave rise to large changes in the dynamic properties of the peptide (Figure 2). The proton NMR spectrum of H30K are broad, indicating that the protein is populating multiple conformations in rapid-to-intermediate exchange on the millisecond time scale. By contrast, H30D, which can accept a hydrogen bond from His26′, shows a very well dispersed NMR spectrum, which is similar to that of α_2D and typical of a uniquely folded protein.

Very different results were observed for changes to the buried His26 at which substitution markedly affected both the stability as well as the aggregation state. Replacing His26 with a nonpolar Phe gave rise to a large increase in T_m; conversely, substituting His26 with the more polar side chain Asn markedly decreased T_m. Sedimentation equilibrium ultracentrifugation showed that H26F formed trimers while H26N formed dimers. Interestingly, both of these substitutions gave proteins that appeared nativelike, although their spectra were somewhat less well dispersed than α_2D. For example, H26N shows somewhat poorer dispersion in the methyl region (near 1.0 ppm). This change in chemical shift dispersion is largely a result of the loss of the aromatic groups of His26 and His 26′ that sit directly over the methyl groups of I33 at 0.24 and 0.37 ppm. Other small changes in chemical shift due to dynamics may not be ruled out from these data alone. The spectra of H26F are also relatively sharp and well dispersed, indicative of a uniquely folded structure. Therefore, the change of His26 to Phe has effected a transition from a nativelike dimer to a stable, nativelike trimeric conformation.

Previous synthetic studies have suggested that aromatic and hydrogen-bonded interactions are essential for defining structural

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(1) Beasley, J. R.; Hecht, M. H. *J. Biol. Chem.* **1997**, *272*, 2031−4.
(2) Bryson, J. W.; Betz, S. F.; Lu, H. S.; Suich, D. J.; Zhou, H. X.; O'Neil, K. T.; DeGrado, W. F. *Science* **1995**, *270*, 935−41.
(3) Richardson, J.; Richardson, D. C.; Tweedy, N. B.; Gernert, K. M.; Quinn, T. P.; Hecht, M. H.; Erickson, B. W.; Yan, Y.; McClain, D. M. E.; Surles, M. C. *Biophys. J.* **1992**, *63*, 1186−1209.
(4) Baltzer, L. *Current Opin. Struct. Biol.* **1998**, *8*, 466−470.
(5) Balaram, P. *J. Pept. Res.* In press.
(6) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1−64.
(7) Kellis, J. T., Jr.; Nyberg, K.; Sali, D.; Fersht, A. R. *Nature* **1988**, *333*, 784−6.
(8) Matthews, B. W. *Adv Protein Chem.* **1995**, *46*, 249−78.
(9) Honig, B.; Yang, A.-S. *Adv. Protein Chem.* **1995**, *46*, 27−58.
(10) Lumb, K. J.; Kim, P. S. *Science* **1996**, *271*, 1137−1138.
(11) Lumb, K. J.; Kim, P. S. *Science* **1995**, *268*, 436−439.
(12) Betz, S.; Fairman, R.; O'Neil, K.; Lear, J.; DeGrado, W. *Proc. R. Soc.* **1995**, *348*, 81−88.
(13) Broo, K. S.; Nilsson, H.; Nilsson, J.; Flodberg, A.; Baltzer, L. *J. Am. Chem. Soc.* **1998**, *120*, 4063−4068.
(14) Kuwajima, K. *Proteins* **1989**, *6*, 87−103.
(15) Ptitsyn, O. B.; Pain, R. H.; Semisotnov, G. V.; Zerovnik, E.; Razgulyaer, O. L. *FEBS Lett* **1990**, *262*, 20−24.
(16) Handel, T. M.; Williams, S. A.; DeGrado, W. F. *Science* **1993**, *261*, 879−885.
(17) Ho, S. P.; DeGrado, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 6751−6758.
(18) Raleigh, D. P.; DeGrado, W. F. *J. Am. Chem. Soc.* **1992**, *114*, 10079−10081.
(19) Raleigh, D. P.; Betz, S. F.; DeGrado, W. F. *J. Am. Chem. Soc.* **1995**, *117*, 7558−7559.
(20) Hill, R. B.; DeGrado, W. F. *J. Am. Chem. Soc.* **1998**, *120*, 1138−1145.
(21) Cohen, C.; Parry, D. A. D. *Proteins* **1990**, *7*, 1−15.
(22) Gibney, B. R.; Rabanal, F.; Skalicky, J. J.; Wand, A. J.; Dutton, P. L. *J. Am. Chem. Soc.* **1997**, *119*, 2323−2324.
(23) Gonzalez, L., Jr.; Woolfson, D. N.; Alber, T. *Nature Struct. Biol.* **1996**, *3*, 1011−8.
(24) Harbury, P. B.; Zhang, T.; Kim, P. S.; Alber, T. *Science* **1993**, *262*, 1401−1407.
(25) Hodges, R. S. *Biochem. Cell Biol.* **1996**, *74*, 133−54.
(26) Richardson, J. *Adv. Protein Chem.* **1981**, *34*, 168−340.
(27) Singh, J.; Thornton, J. M. *Atlas of Protein Side-Chain Interactions*; Oxford University Press: Oxford, 1992; Vol. 1.

10.1021/ja9919332 CCC: $19.00
Published on Web 01/15/2000

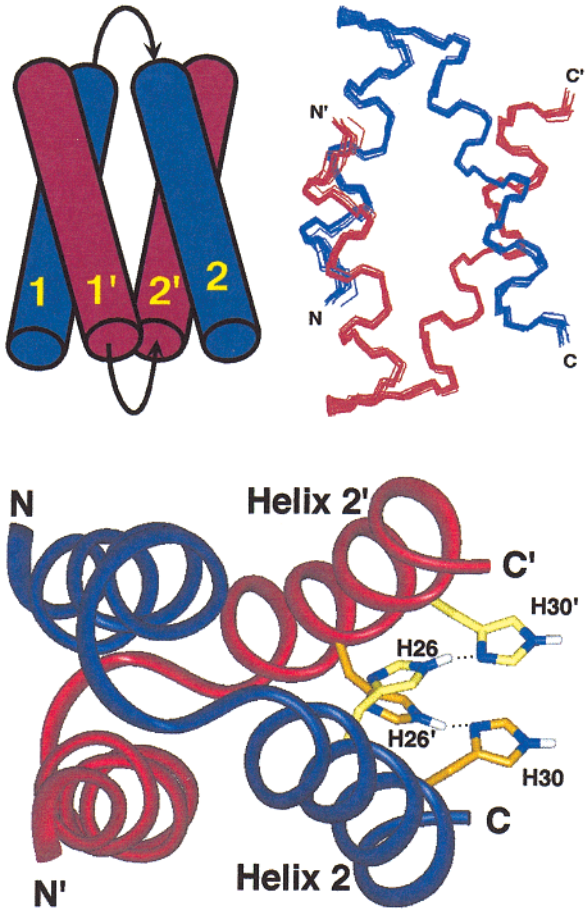

Figure 1. The structure of α_2D, Ac-GEVEEL**E**KKFKELWK-GPRRG-EIEEL**H**KKF**H**ELIKG-NH_2 (residues in bold indicate changes made to α_2C in the design of α_2D). The top, left panel shows the topology of the dimeric four-helix bundle that adopts the bisecting U motif. Superposition of the backbone (top, right panel) from the 15 lowest energy structures of α_2D calculated from NMR-derived data[20] (PDB code 1QP6). His cluster found in the anti-parallel interface between Helix 2 and Helix 2′ (bottom panel). The ϵ-NH of His26 of Helix 1 hydrogen bonds to the δ-nitrogen of His30 of Helix 2′; a reciprocal interaction occurs between the symmetry-related histidines.

Table 1. Thermodynamic Parameters for the Unfolding[a]

peptide	$\Delta G°$ (25 °C)[b]	ΔH_m (1 M)[b]	T_m (1 M)[b]	$\Delta\Delta G°$ (spec.)
α_2D	7.0	70	96.9	
H30K	6.6	68	96.0	0.4
H30D	7.3	71	87.8	−0.3
H26F	10.8	43	103.3	−3.8
H26N	5.7	61	81.7	1.3

[a] See Supporting Information for conditions. [b] $\Delta G°$, ΔH_m, and T_m are respectively the free energy (25 °C), enthalpy (at T_m), and thermal midpoint of the unfolding transitions (at 1 M standard state); units are kcal mol^{-1}.

uniqueness in coiled coils, designed peptides, and protein domains.[24,28−32] The current data illustrate how a hydrogen-bonded cluster contributes to the unique structure of α_2D. The buried residue His26 (and the symmetry-related His26′) appears to play a negative role[3,33] in defining a unique dimeric fold in α_2D, because changing this residue to Phe results in a highly stable trimeric

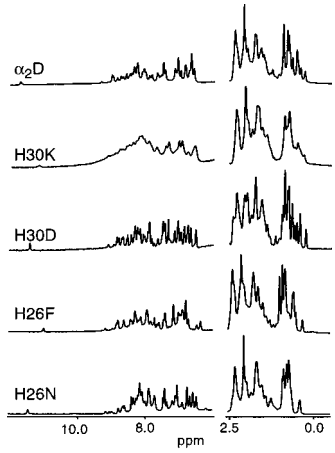

Figure 2. 1D ^{1}H-NMR spectroscopy (500.13 MHz) of 2 mM α_2D, H30K, H30D, H26F, and H26N at the temperature of maximum thermal stability (50 mM d_{11}-TRIS, pH 7.3).

state. Thus, His26 must destabilize the trimer relative to the dimer via specific hydrogen-bonded interactions, which may be formed exclusively in the dimer. H26N appears to form similar hydrogen-bonded interactions, although its stability is attenuated by the need to bury the Asn side chain in the protein core (the free energy of dehydration of an Asn side chain is more unfavorable than that of a neutral His[34]). Also, the smaller volume of Asn relative to His may result in the formation of unfavorable voids.

The more solvent-exposed His30 makes a more positive contribution toward the conformational specificity of α_2D. This residue is a hydrogen bond acceptor that is retained in H30D, but disrupted in H30K. The small decrease in stability in H30K suggests that this interfacial hydrogen bond provides a modest contribution to the thermodynamic stability of the dimer. More importantly, the dynamic behavior of H30K suggests that this hydrogen bond is necessary for specifying a uniquely folded state. These findings clearly illustrate how solvent-exposed side chains can provide thermodynamic stability while simultaneously "locking in" a distinct structure.

These results also illustrate the plasticity of protein structure, and may have evolutionary implications. α_2D represents a primordial protein at an early stage of evolution; it is a marginally stable native protein that has not been highly optimized for folding. Thus, a single residue change can trigger an oligomerization switch in H26F or the induction of dynamically averaging conformations in H30K. It is interesting to speculate that structural plasticity in similar self-associating systems may have played an important role in the early stages of the evolution of modern proteins.

Acknowledgment. The authors thank Jim Lear for software to analyze thermal denaturation and sedimentation equilibrium data. Walter Englander kindly provided NMR spectrometer time on a Bruker AMX-500. We are grateful to Jim Lear, Joel Schneider, Gregg Dieckmann, Chris Summa, and Giovanna Ghirlanda for useful discussions. This work was supported by NIH postdoctoral NRSA (to R.B.H.) 1 F32 GM18491-01 and GM54616 (to WFD), as well as the MRSEC program of the NSF.

Supporting Information Available: Data showing the pH dependence of the near-UV CD signal for α_2D and sedimentation equilibrium analysis for H26F and H30K and the concentration dependence of the thermal denaturation for α_2D, H30K, H30D, H26F, and H26N along with the fits of the Gibbs−Helmholtz equation to these data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA9919332

(28) Olofsson, S.; Baltzer, L. *Fold Des.* **1996**, *1*, 347−56.
(29) Dolphin, G. T.; Baltzer, L. *Fold Des.* **1997**, *2*, 319−30.
(30) Lumb, K. J.; Kim, P. S. *Biochemistry* **1995**, *34*, 8642−8648.
(31) Dolphin, G. T.; Brive, L.; Johansson, G.; Baltzer, L. *J. Am. Chem. Soc.* **1996**, *118*, 11297−11298.
(32) Spector, S.; Kuhlman, B.; Fairman, R.; Wong, E.; Boice, J.; Raleigh, D. *J. Mol. Biol.* **1998**, *276*, 479−489.
(33) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochemistry* **1999**, *68*, 779−819.
(34) Schaefer, M.; van Vlijmen, H.; Karplus, M. *Adv. Protein Chem.* **1998**, *51*, 1−57.