A Protein Designed by Binary Patterning of Polar and Nonpolar Amino Acids Displays Native-like Properties

Sushmita Roy,[†] Gayathri Ratnaswamy,[†] Judith A. Boice,[‡,§] Robert Fairman,[‡] George McLendon,[†] and Michael H. Hecht*,[†]

Contribution from the Department of Chemistry, Princeton University, Princeton, New Jersey 08544-1009, and Division of Macromolecular Structure, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, New Jersey 08543-4000

Received January 8, 1997[⊗]

Abstract: Large collections of *de novo* α-helical proteins can be constructed by using combinatorial methods based on a "binary code" for protein design, in which the sequence locations of polar and nonpolar amino acids are specified explicitly, but the precise identities of these residues are varied extensively. We demonstrate that a 75-residue protein chosen from such a binary code collection displays several properties similar to those of native proteins: (i) Both the chemically induced and thermally induced denaturations are cooperative; (ii) addition of the hydrophobic dye 1-analinonaphthalene-8-sulfonate (ANS) yields only minimal fluorescence; (iii) the NMR spectrum shows significant chemical shift dispersion in both the amide and methyl regions; and (iv) amide protons are protected from exchange to an extent observed in some natural proteins. These results demonstrate that binary patterning of polar and nonpolar amino acids can serve as the basis for initial steps toward the design of novel proteins with native-like properties.

Introduction

Strategies for devising *de novo* protein sequences range from screening libraries of random sequences[1−3] to rationally designing specific tertiary interactions.[4−26] Both approaches have yielded collapsed molecules with extensive secondary structure. However, the attainment of truly native-like features has proved more difficult.[27,28] While recent studies using iterative strategies of rational design, characterization, and redesign have progressed toward producing novel proteins harboring some native-like features,[16−28] the ability to produce large collections of native-like proteins remains a central challenge in molecular design.

We previously reported a combinatorial strategy for the design of large libraries of novel proteins.[29] Our strategy employs binary patterning of polar and nonpolar amino acids to generate sequences in which the locations of polar and nonpolar residues are specified explicitly, but the precise identities of the side chains are not constrained and can be varied extensively. We constructed a large combinatorial collection of synthetic genes whose protein products were designed to fold into 4-helix bundles. Sequence diversity was facilitated by encoding polar residues (Glu, Gln, Asp, Asn, Lys, or His) with the degenerate codon NAN and nonpolar residues (Phe, Leu, Ile, Met, or Val) with the degenerate codon NTN. In the resulting collection of proteins, each member has a different amino acid sequence. However, all sequences in the collection share the identical binary pattern of polar and nonpolar residues. Characterization

* Address correspondence to this author. Phone: 609-258-2901. Fax: 609-258-6746. Email: hecht@berthaw.princeton.edu.
† Princeton University.
‡ Bristol-Myers Squibb Pharmaceutical Research Institute.
§ Current address: Department of Biochemistry, Merck Research Laboratories, Rahway, NJ 07065.
⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

(1) Mandecki, W. A. *Protein Eng.* **1990**, *3*, 221−226.
(2) Davidson, A. R.; Sauer, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2146−2150.
(3) Davidson, A. R.; Lumb, K. J.; Sauer, R. T. *Nature Struct. Biol.* **1995,** *2*, 856−864.
(4) Hecht, M. H.; Richardson, J. S.; Richardson, D. C.; Ogden, R. C. *Science* **1990**, *249*, 884−891.
(5) Regan, L.; DeGrado, W. F. *Science* **1988**, *241*, 976−978.
(6) Fedorov, A. N.; Dolgikh, D. A.; Chemeris, V. V.; Chernov, B. K.; Finkelstein, A. V.; Schulga, A. A.; Alakhov, Y. B.; Kirpichnikov, M. P.; Ptitsyn, O. B. *J. Mol. Biol.* **1992**, *225*, 927−931.
(7) Ghadiri, M. R.; Soares, C.; Choi, C. *J. Am. Chem. Soc.* **1992**, *114*, 4000−4001.
(8) Zhou, N. E.; Kay, C. M.; Hodges, R. S. *J. Biol. Chem.* **1992**, *267*, 2664−2670.
(9) Dado, G. P.; Gellman, S. H. *J. Am. Chem. Soc.* **1993**, *115*, 12609−12610.
(10) Johnsson, K.; Allemann, R. K.; Widmer, H.; Benner, S. A. *Nature* **1993**, *365*, 530−532.
(11) Pessi, A.; Bianchi, E.; Crameri, A., Venturini, S.; Tramontano, A.; Sollazzo, M. *Nature* **1993**, *362*, 367−369.
(12) Schafmeister, C. E.; Miercke, L. J . W.; Stroud, R. M. *Science* **1993**, *262*, 734−738.
(13) Iwamoto, T.; Grove, A.; Montal, M. O.; Montal, M.; Tomich, J. M. *Int. J. Pept. Protein Res.* **1994**, *43*, 597−607.
(14) 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*, 935−941.
(15) Houbrechts, A.; Moreau, B.; Abagyan, R.; Mainfroid, V.; Preaux, G.; Lamproye, A.; Poncin, A.; Goormaghtigh, E.; Ruysschaert, J.; Martial, J. A.; Goraj, K. *Protein Eng.* **1995**, *8*, 249−259.
(16) Munson, M.; O'Brien, R.; Sturtevant, J. M.; Regan, L. *Protein Sci.* **1994**, *3*, 2015−2022.
(17) Desjarlais, J.; Handel, T. M. *Protein Sci.* **1995**, *4*, 2006−2018.
(18) Mutter, M.; Tuchscherer, G. G.; Miller, C.; Altman, K.; Carey, R. I.; Wyss, D. F.; Labhardt, A. M.; Rivier, J. E. *J. Am. Chem. Soc.* **1992**, *114*, 1463−1478.
(19) Fezoui, Y.; Weaver, D. L.; Osterhout, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3675−3679.
(20) Quinn, T. P.; Tweedy, N. B.; Williams, R. W.; Richardson, J. S.; Richardson, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8747−8751.
(21) Yan, Y.; Erickson, B. W. *Protein Sci.* **1994**, *3*, 1069−1073.
(22) Nautiyal, S.; Woolfson, D. N.; King, D. S.; Alber, T. *Biochemistry* **1995**, *34*, 11645−11651.
(23) Raleigh, D. P.; Betz, S. F.; DeGrado, W. F. *J. Am. Chem. Soc.* **1995**, *117*, 7558−7559.
(24) O'Shea, E. K.; Lumb, K. J.; Kim, P. S . *Curr. Biol.* **1993**, *3*, 658−667.
(25) Kuroda, Y.; Nakai, T.; Ohkubo, T. *J. Mol. Biol.* **1994**, *236*, 862−868.
(26) Struthers, M. D.; Cheng, R. P.; Imperiali, B. *Science* **1996**, *271*, 342−345.
(27) Betz, S. F.; Raleigh, D. P.; DeGrado, W. F. *Curr. Opin. Struct. Biol.* **1993**, *3*, 601−610.
(28) Betz, S. F.; Bryson, J. W.; DeGrado, W. F. *Curr. Opin. Struct. Biol.* **1995**, *5*, 457−463.
(29) Kamtekar, S.; Schiffer, J. M.; Xiong, H.; Babik, J. M.; Hecht, M. H *Science* **1993**, *262*, 1680−1685.

S0002-7863(97)00071-1 CCC: $14.00

of a representative group of these "binary code" proteins demonstrated that most of the novel sequences escape proteolysis *in vivo* and fold into collapsed α-helical structures (ref 29 and S. Roy *et al.* unpublished).

The ability of the binary patterning strategy to generate vast collections of *de novo* sequences led us to investigate whether any of the resulting proteins possess native-like features. While natural proteins fold into structures with well-packed hydrophobic cores,[30] the combinatorial basis of the binary code strategy precludes rational design of specific packing interactions. Can the binary code nonetheless generate novel proteins with native-like properties?

Initial screening[31] of proteins from our original collection[29] suggested that protein M-60 might possess native-like features. This suggestion is supported by the recent finding that M-60 readily forms crystals (S. Roy, F. Hughson, and M. H. Hecht, unpublished). In the current work, we demonstrate by several different criteria that protein M-60 exhibits several native-like properties in solution.

Results

Cooperative Denaturation. The urea denaturation profile shown in Figure 1A demonstrates that protein M-60 unfolds cooperatively and is relatively stable, having a denaturation midpoint of ∼7 M urea. The free energy of unfolding (ΔG) was calculated to be approximately 4 kcal/mol.

While cooperative sigmoidal chemical denaturation (e.g. by urea or guanidine hydrochloride) has been observed for a number of different *de novo* proteins, cooperative thermal denaturation has been more difficult to obtain.[27,28] The thermal denaturation of M-60 is shown in Figure 1B. In contrast to most earlier designed proteins, M-60 exhibits a sigmoidal thermal transition with a clear upper baseline. The transition is relatively broad, as is often observed for small natural proteins.[32] Both the chemical and thermal denaturations of protein M-60 are reversible.

ANS Fluorescence. The hydrophobic dye, 1-analinonaphthalene-8-sulfonate (ANS), is sometimes used as a rapid probe for the presence of accessible nonpolar patches. When ANS is surrounded by nonpolar moieties, significant fluorescence is observed, whereas ANS free in aqueous solution does not fluoresce. In molten globule folding intermediates nonpolar side chains are accessible to ANS, and the resulting fluorescence is sometimes used as a diagnostic for the molten globule state.[33] Natural proteins (e.g. apomyoglobin) that possess hydrophobic binding pockets bind ANS even in their native state.[34] However, most native proteins sequester nonpolar residues away from solvent and therefore do not bind ANS. Figure 2 compares the fluorescence of M-60 in the presence of ANS to several controls: apomyoglobin yields a substantial signal; ethanol yields a moderate signal, and lysozyme does not fluoresce. At the same concentration as apomyoglobin or lysozyme, protein M-60 yields a signal that is barely above baseline, and is ∼50-fold lower than that of apomyoglobin.

Chemical Shift Dispersion. Natural proteins typically give rise to NMR spectra with well-dispersed chemical shifts.[35] However, the first generation of designed proteins typically formed "molten" structures, which gave rise to broad NMR peaks and poor chemical shift dispersion. The ^{1}H NMR spectrum of protein M-60 is shown in Figure 3. The peaks in both the N−H region (6.5 to 9.5 ppm) and the methyl region (upfield of 3 ppm) show chemical shift dispersion comparable to that of naturally-occurring α-helical proteins. Particularly noteworthy are the amide proton shifted at 9.4 ppm, and the ring-current shifted methyl peaks at 0.5, 0.3, and −0.4 ppm. Such shifts indicate that the corresponding protons are in uniquely folded environments. Moreover, despite the presence of M-60 dimers at the concentration required for NMR (see below), the amide peaks are relatively sharp.

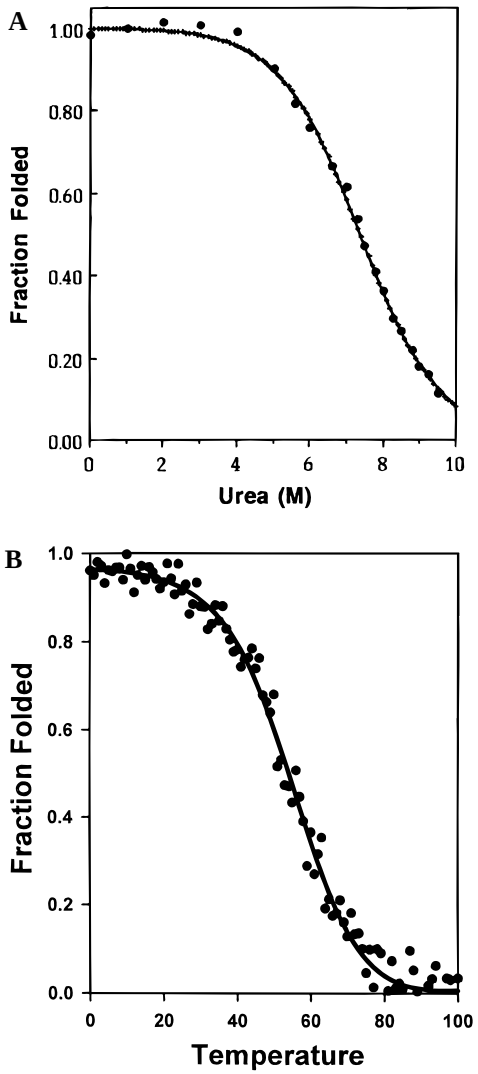

Figure 1. (A) Urea-induced denaturation of protein M-60. Protein concentration was 26 μM in a buffer containing 50 mM sodium phosphate and 200 mM NaCl, pH 7.0. (B) Thermal denaturation of protein M-60. Protein concentration was 2.6 μM in the same buffer. The midpoint is 55 °C, and the van't Hoff enthalpy (ΔH) is 25 kcal/mol. At higher protein concentrations, the T_m is increased slightly.

Protection from Amide Proton Exchange. In the structures of native proteins a population of amide protons are typically protected from exchange with solvent. In contrast, in a "molten" molecule, which interconverts between several different structures, amide protons exchange rapidly.[36,37] As shown in Figure 4, many of the amide protons in M-60 are substantially protected

(30) Richards, F. M.; Lim, W. A. *Q. Rev. Biophys.* **1994**, *26*, 423−498.
(31) Roy, S.; Helmer, K. J .; Hecht, M. H. *Folding Des.* **1997**, *2*, 89−92.
(32) Alexander, P.; Fahnestock, S.; Lee, T.; Orban, J.; Bryan, P. *Biochemistry* **1992**, *31*, 3597−3603.
(33) Semisootnov, G. V.; Rodionova, N. A.; Razgulyaev, O. I.; Uversky, V. N.; Gripas, A. F.; Gilmanshin, R. I. *Biopolymers* **1991**, *31*, 119−128.
(34) Stryer, L. *J. Mol Biol.* **1965**, *13*, 482−495.
(35) Wuthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.
(36) Hughson, F. M.; Wright, P. E.; Baldwin, R. L. *Science* **1990**, *249*, 1544−1548.
(37) Jeng, M. F.; Englander, S. W.; Glove, G. A.; Wang, J. A.; Roder, H. *Biochemistry* **1990**, *29*, 10433−10437.

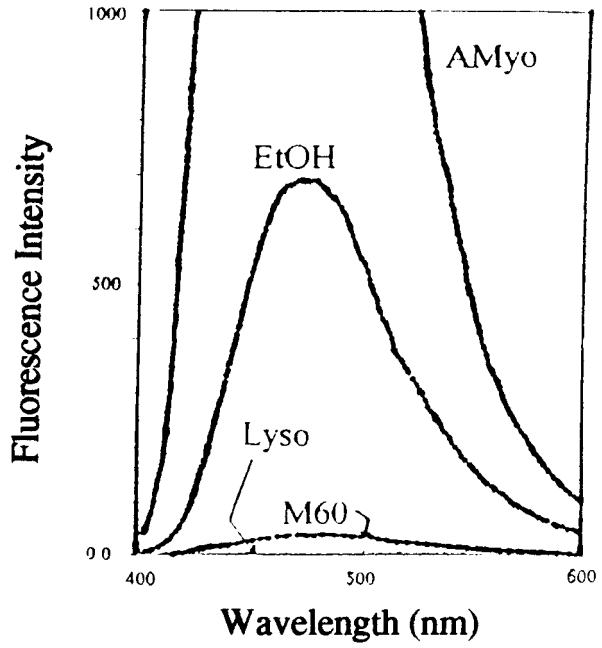

Figure 2. Fluorescence emission spectra of 1 μM ANS in the presence of apomyoglobin (AMyo), 99% ethanol (EtOH), Hen Lysozyme (Lyso), or protein M-60. Protein concentrations were 50 μM in a buffer containing 10 mM sodium phosphate (pH 7.0). Fluorescence of ANS in the presence of lysozyme is indistinguishable from the baseline for ANS alone.

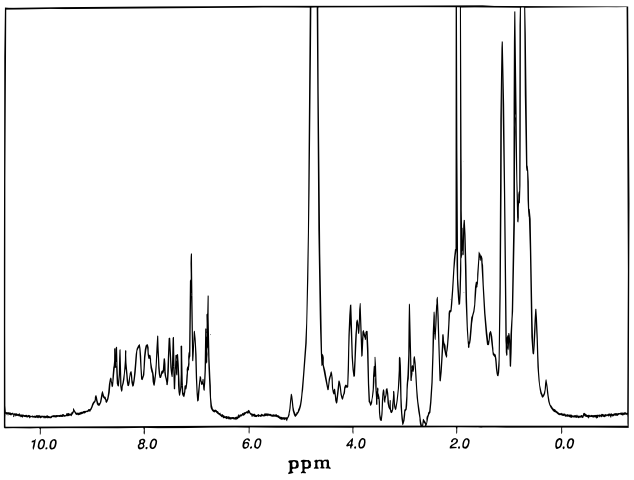

Figure 3. ^{1}H NMR spectrum of protein M-60 at 25 °C. Protein concentration was ~0.4 mM.

from exchange. Some are protected from exchange for many days, and some peaks can be observed even after 3 weeks. The rate constant of amide exchange for three of the slower exchanging peaks was calculated to be on the order of 10^{-2}/h at 25 °C (for the most well-resolved amide peak, at 9 ppm, $t_{1/2}$ is ~140 h). Since the $t_{1/2}$ for an unprotected amide proton is 0.28 min at 25 °C, pH 4.6 (see ref 38), our data indicate that the protection factor for this peak is approximately 30 000. Thus, M-60 has several protons that exchange on a moderate to slow time scale, with protection factors approximately 100-fold higher than those observed for molten globules.[36,37] These protection factors are comparable to those observed in the interior of several naturally occurring proteins.[39−41]

The protection factors obtained for the slower exchanging protons in M-60 indicate a global stability of ~5 kcal/mol. This agrees reasonably well with the value extrapolated from urea denaturation (Figure 1A). This correspondence suggests that M-60 has a core that is protected to the extent expected from the global stability of the protein.

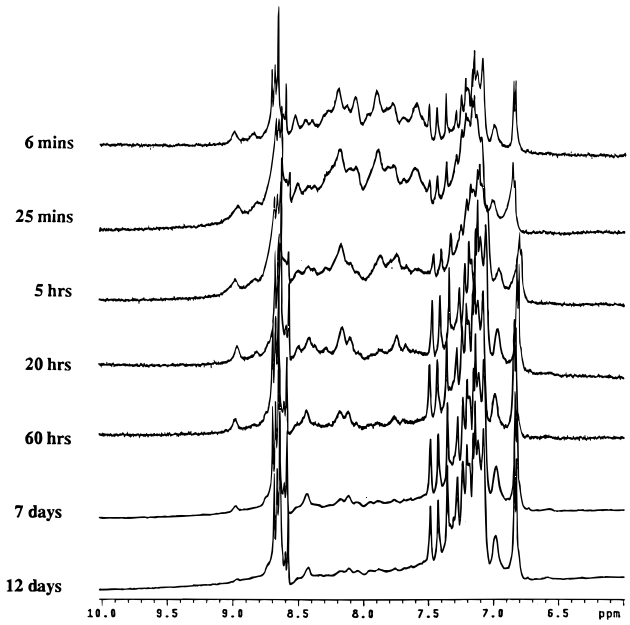

Figure 4. Time course of amide proton exchange following a 20-fold dilution of protein M-60 from a buffer containing 50 mM CD_3COOD/NaOH, 200 mM NaCl, pH 4.6 in H_2O, into a buffer containing 50 mM CD_3COOD/NaOD, 200 mM NaCl, pD 4.2 in D_2O.

Oligomeric State of Protein M-60. The results described above demonstrate several properties of M-60 that resemble those of native proteins. What is the oligomeric state of the protein responsible for these properties?

Gel filtration chromatography of protein M-60 gave rise to two peaks,[42] a monomer and a dimer (not shown). The monomer elutes at exactly the position (relative to standards) expected for a compact globular structure. Analysis of the oligomeric state by sedimentation equilibrium ultracentrifugation indicated that the dissociation constant (K_D) for the dimer is approximately 5 μM (Figure 5 and Table 1). (At high concentrations M-60 weakly associates as tetramers; $K_D = 4$ mM).

Dimerization is inhibited by high concentrations of salt (not shown), suggesting that dimerization is due to electrostatic attractions, not hydrophobic interactions. Thus, the dimerization is probably mediated by surface (i.e. polar) residues, and presumably affects neither the packing of the hydrophobic core nor the overall topology.

The native-like properties of M-60 do not depend on protein dimerization. This assertion is based on the following: (i) The α-helical content of M-60 is independent of concentration. (ii) In urea and guanidinium hydrochloride denaturation experiments no differences in the denaturation midpoint were observed between 2 and 50 μM (data not shown). (iii) The high resistance of M-60 to urea denaturation does not require dimerization: Urea-induced denaturation occurs beyond 5 M urea (Figure 1A), and gel filtration experiments demonstrate that at 5 M urea protein M-60 is monomeric (not shown). Thus the high resistance to urea must reflect the inherent stability of the

(38) Englander, S. W.; Downer, N. W.; Teitelbaum, H. *Annu. Rev. Biochem.* **1972**, *41*, 903−924.
(39) Montelione, G. T.; Wuthrich, K.; Scheraga, H. A. *Biochemistry* **1988**, *27*, 2235−2243.
(40) Marmorino, J. L.; Auld, D. S., Betz, S. F., Doyle, D. F., Young, G. B.; Pielak, G. J. *Protein Sci.* **1993**, *2*, 1966−1974.
(41) Lyons, T. A.; Ratnaswamy, G.; Pochapsky, T. C. *Protein Sci.* **1996**, *5*, 627−639.
(42) Behavior in gel filtration experiments is independant of pH over a range from 4.6 to 7.0.

Table 1. Analysis of Sedimentation Equilibrium Data[l]

		SRV[a]									
[M60],[b] μM	M_r[c]	SS[d]	4[e]	3[f]	2[g]	1[h]	1 ↔ 2[i]	1 ↔ 3[i]	1 ↔ 4[i]	1 ↔ 2 ↔ 3[j]	1 ↔ 2 ↔ 4[k]
50	14800 ± 1000	3.8	22.3	13.9	4.9	9.7	3.7	3.6	3.7		
500	19735 ± 400	4.6	53.1	25.4	13.6	60.9		3.4	9.7	2.4	2.6

[a] Square root of variance $\times 10^3$. [b] Concentration of protein loaded. [c] The apparent molecular mass as determined by a single species analysis. [d] SRV assuming a single species with the molecular mass indicated in column 2. [e] SRV assuming a tetramer of subunits each with a monomer mass of 8562. [f] SRV assuming a trimer of subunits each with a monomer mass of 8562. [g] SRV assuming a dimer of subunits each with a monomer mass of 8562. [h] SRV assuming a monomer with a mass of 8562. [i] SRV for monomer ↔ *n*-mer equilibria of increasing order up to $n = 4$. [j] SRV for monomer ↔ dimer ↔ trimer. [k] SRV for monomer ↔ dimer ↔ tetramer. [l] Data were collected at 25 °C, scanning at 240 nm for 50 μM data and 275 nm for 500 μM data. We used the HID program from the Analytical Ultracentrifugation Facility at the University of Connecticut.

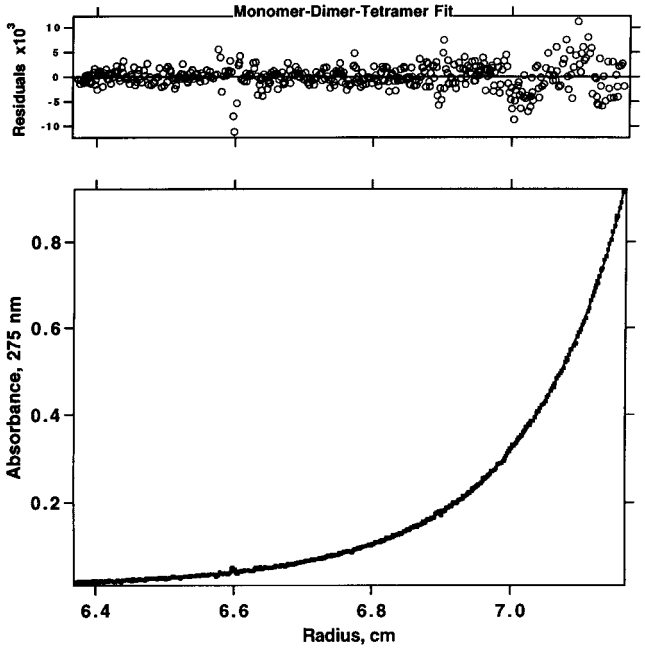

Figure 5. Sedimentation equilibrium analysis of protein M-60. The figure shows results for a protein concentration of 500 μM. The monomer−dimer−tetramer fit is shown, as are the residuals for this fit.

monomer. (iv) Dimerization does not underlie the cooperativity of the thermal denaturation, as decreased protein concentration does not diminish the cooperativity of the thermal melt (not shown). (v) Finally, in the hydrogen exchange experiment, the observed protection factors cannot be attributed solely to dimerization; the relative weakness of the dimerization ensures that a steady-state population of monomer (>5%) is available for exchange. Thus the differential protection of the dimer relative to the monomer is at most 20-fold. Indeed, Raleigh *et al.*[23] pointed out that the stronger dimerization observed in their α2 peptide system is too weak to survive the times required for protection experiments.

Discussion

Native-like States. For any polypeptide chain, one can imagine a continuum of possible states ranging from a truly random coil to a perfectly rigid structure in which all atoms are uniquely fixed in place. For any given sequence, neither extreme is significantly populated in physiological buffers at ambient temperatures. Denatured proteins, "far from adopting featureless random coils ... frequently exhibit significant amounts of residual structure."[43] At the other extreme, even fully folded proteins are not perfectly rigid, and typically possess significant flexibility.[44]

Between the denatured state and the folded state, a continuum of intermediate states is possible. Although most intermediates are short-lived under physiological conditions, some can be stabilized under special conditions. Characterization of these stabilized intermediates has revealed a continuum of states ranging from collapsed globules that are devoid of persistent structure to native-like intermediates that exhibit many features similar to those of fully folded proteins.[45] Indeed, some intermediate states are non-native by some criteria, but very much native-like by other criteria. For example, Alexandrescu *et al.* describe a subdomain of staphylococcal nuclease which "shows hydrogen exchange protection factors in the range reported for molten globules", but at the same time is sufficiently well-ordered that its native-like 3-dimensional structure could be determined by NMR.[46]

We cannot ascertain where exactly M-60 falls on this continuum of states. However, the initial characterization reported here demonstrates that by several criteria this *de novo* protein is substantially native-like: Its chemically and thermally induced denaturations are cooperative; addition of ANS yields only minimal fluorescence; the NMR spectrum shows significant chemical shift dispersion; and amide protons are protected from exchange.

In other respects, however, protein M-60 is not absolutely native-like. Its denaturation enthalpy ($\Delta H = \sim 25$ kcal/mol) is smaller than typically observed for natural proteins of comparable size (75 residues). Likewise the *m* value (~534 cal/(mol·M)) associated with the urea-induced denaturation is approximately half as large as that observed for natural proteins of similar size.[47] Thus, although the chemically and thermally induced denaturations of protein M-60 are cooperative, they are not as sharp as might be expected for fully native-like structures. Indeed, we have recently found that among our collection of *de novo* α-helical proteins, several sequences give rise to considerably sharper thermal melts than M-60 (S. Roy *et al.*, unpublished).

The initial collection of proteins reported by Kamtekar *et al.*[29] contains only 29 sequences. Preliminary screening[31] of this collection led us to study protein M-60 in greater detail. It must be stressed that this initial collection of 29 proteins represents an *extremely* small fraction of the total number of sequences (5×10^{41}) that can be encoded by the binary pattern used for this structural motif (24 buried positions and 32 surface positions, chosen from 5 nonpolar and 6 polar residues, respectively, can yield $5^{24} \times 6^{32} = 5 \times 10^{41}$ possible sequences[29]). Nonetheless, the results described here and elsewhere[31] demonstrate that from this relatively small subset of sequences, proteins can be isolated that share several properties with natural proteins. These results suggest that within the far larger set of binary code sequences

(43) Shortle, D. *Curr. Opin. Struct. Biol.* **1993,** *3,* 66−74.
(44) Creighton, T. E. Proteins: Structures and Molecular Principles 2nd ed.; (1993), W. H. Freeman and Company: New York, 1993.
(45) Nishii, I.; Kataoka, M.; Goto, Y. *J. Mol. Biol.* **1995**, *250*, 223−238.
(46) Alexandrescu, A. T.; Dames, S. A.; Wiltscheck, R. *Protein Sci.* **1996**, *5*, 1942−1946.
(47) Myers, J. K.; Pace, C. N.; Scholtz, J. M. *Protein Sci.* **1995**, *4*, 2138−2148.

that adapt this fold, there are likely to be many proteins that display properties at the native-like end of the continuum of states.

The Sequence Requirements for Native-like Properties. Theoretical studies by Dill and co-workers suggest that judicious use of polar residues at key positions can destabilize alternative hydrophobic cores and thereby play a key role in the design of unique structures.[48] Experimental support for this suggestion comes from the work of Raleigh *et al*., who showed that incorporation of polar residues at the interface between buried and exposed α-helical surfaces enhances the native-like properties of their α2 peptide.[23] However, α2 must dimerize to form a 4-helix bundle, and the resulting dimer was not sufficiently long-lived to allow Raleigh *et al*. to demonstrate amide proton protection.[23]

On the basis of their success redesigning the interfacial residues of α2, Raleigh *et al*. expressed interest[23] in whether any of the binary code proteins would exhibit native-like features without the addition of polar residues at interfacial positions. The results presented here demonstrate that proteins with native-like properties can indeed be isolated from our original collection of binary code proteins. We do not yet know how frequently such properties will occur in our own or similar collections. However, initial screens suggest that >10% of our binary code proteins give rise to NMR spectra with well-dispersed peaks[31] consistent with native-like packing. Statistical arguments suggest that among the 5×10^{41} sequences that can be encoded by our binary patterning,[29] many are likely to possess native-like properties.

Experimental Section

Protein Purification. The 75-residue sequence of protein M-60 is identical with that of protein 60 described previously[29] except for one modification, which inserts a Tyr between Met_1 and Gly_2 of the original sequence.[49] This modification was constructed to prevent cleavage of the initiator methionine *in vivo*, and also to provide a chromaphore with an absorbance at 275 nm. The full amino acid sequence of M-60 is MYGEVENILKQLKELVEGPDSGNLKDLINQLKQLIEGPSGGEL-DHFLKQLKELLHGPRSGQVKQIVHHIQHLFQR. Protein was purified by modification of a freeze−thaw, acid precipitation, and cation-exchange chromatography protocol (refs 29 and 50 and S. Roy, unpublished results), to a point where only a single band was observed by silver stained SDS-PAGE. Electrospray mass spectrometry indicated a mass of 8562, thus confirming that the N-terminal methionine is retained. Protein concentration was determined by tyrosine absorbance.[51]

Protein Denaturation Studies. The urea-induced denaturation of M-60 was monitored by recording the ellipticity at 222 nm as a function of urea concentration. A cuvette with a path length of 1 mm was used in an Aviv 62 DS spectropolarimeter. Data were recorded at 20 °C, in 50 mM sodium phosphate and 200 mM NaCl, pH 7.0. The free energy of folding, ΔG, was calculated from these data as described previously.[52]

Thermal denaturation of protein M-60 was monitored by recording the ellipticity at 222.0 nm every 1 °C from 0 to 100 °C with 0.5 min equilibration time for each data point. The path length was 10 mm. The fraction folded was calculated by using the formula $(u - e)/(u - l)$, where e is the mean residue ellipticity and u and l are the upper and lower baseline extrapolations from the curve fit.

ANS Fluorescence. Protein concentrations were 50 μM in a buffer containing 10 mM sodium phosphate, pH 7.0. All solutions contained ANS at a concentration of 1 μM. Spectra were measured on a Perkin Elmer Luminescence LS50 spectrometer at 25 °C in a 1-cm cuvette, and with an excitation wavelength of 370 nm.

NMR and Amide Proton Exchange. The ^{1}H NMR spectrum of protein M-60 (Figure 3) was measured at 25 °C at a protein concentration of ∼0.4 mM in a buffer containing 50 mM CD_3COOD/NaOH and 200 mM NaCl, 10% D_2O, pH 4.6. We recorded 128 transients on a Varian Inova 500-MHz spectrometer with presaturation of the water peak and processed on an SGI IndigoII workstation using Felix 950 (Biosym/MSI).

The time course of amide proton exchange was observed by ^{1}H NMR following a 20-fold dilution of M-60 (in 50 mM CD_3COOD/NaOH in H_2O, 200 mM NaCl, pH 4.6 at 25 °C) into D_2O (50 mM CD_3COOD/NaOD in D_2O, 200 mM NaCl, pD 4.2). Spectra were recorded at indicated times after dilution and processed on a Varian Inova 500-MHz spectrometer. To calculate the rate of exchange, peak height was normalized using 3 non-exchangeable proton signals c1, c2, and c3 at 7.5, 7.4, and 7.0 ppm, respectively, and the average rate constant was calculated. The first-order rate constant for the proton at 9 ppm is $\sim 5 \times 10^{-3}$/h and $t_{1/2}$ is ∼140 h. The intrinsic $t_{1/2}$ at pH 4.6, 25 °C, is 0.28 min.[38] This yields a protection factor of ∼30 000.

Sedimentation Equilibrium Ultracentrifugation. Sedimentation equilibrium was performed on a Beckman Model XLA analytical ultracentrifuge[53] with an AN-60-Ti rotor, and fits were obtained by using the program NONLIN.[54] Experiments were carried out with use of charcoal filled Epon cells with quartz windows. Six-channel, 1.2-mm cells were used for 50 μM samples and two channel, 3 mm path length cells were used for the 500 μM samples. Continuous radial scanning at 240 and 275 nm was used for the 50 and 500 μM samples, respectively. The 3-mm cells were scanned at 20 000 rpm, and the 12 mm path length cells were scanned at multiple speeds of 15 000, 20 000, 25 000, and 30 000 rpm. The cells were scanned every 0.001 cm, and 10 scans were averaged. The density of the solvent was determined gravimetrically and yielded a value of 1.012 g/mL. The partial specific volume of 0.75 mL/g was calculated from the weight average of the partial specific volumes of the individual amino acids.[55] At buffer conditions identical with the NMR exchange experiment (50 mM CH_3-COOH/NaOH and 200 mM NaCl, pH 4.6, at 25 °C), the dimer dissociation constant is 5 μM (range 1 to 28 μM) and the tetramer dissociation constant is ∼4 mM (range 1 to 11 mM).

Acknowledgment. We thank H. Xiong for constructing the M version of protein 60, A. Brunet, J. Beasley, F. Hughson, T. Das, J. Swan, and H. Xiong for helpful suggestions, David Maltby for performing the mass spectrometry at the UCSF Facility, and Preston Hensley for the sedimentation equilibrium data analysis software.

JA9700717

(48) Dill, K. A.; Bromberg, S.; Yue, K.; Fiebig, K. M.; Yee, D. P.; Thomas, P. D.; Chan, H. S. *Protein Sci.* **1995**, *4*, 561−602.

(49) Xiong, H. Ph.D. Dissertation; Princeton University, 1995.

(50) Johnson, B. H.; Hecht, M. H. *Biotechnology* **1994**, *12***,** 1357−1360.

(51) Gill, S. C.; von Hippel, P. H. *Anal. Biochem.* **1989,** *182*, 319−326.

(52) Fairman, R.; Chao, H. G.; Mueller, L.; Lavoie, T. B.; Shen, L.; Novotny, J.; Matsueda, G. *Protein Sci.* **1995,** *4*, 1457−1469.

(53) Giebler, R. In *The Optima XL-A: A New Analytical Ultracentrifuge with a Novel Precision Absorption Optical System, Analytical Ultracentrifugation in Biochemistry Polymer Science;* Harding, S. E., Rowe, A. G., Hortons, J. C., Eds.; Royal Society of Chemistry: Cambridge, 1992; pp 16−25.

(54) Johnson, R. M. L.; Correia, J. J.; Yphantis, D. A.; Halverson, H. R. *Biophys. J.* **1981,** *36*, 575−588.

(55) Cohn, E. J.; Edsall, J. T. *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions;* Reinhold Publishing Corporation: New York, 1943; pp 370−377.