Coiled-Coil Formation Governed by Unnatural Hydrophobic Core Side Chains

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Received March 29, 2001
Revised Manuscript Received July 8, 2001

The study of protein−protein interfaces has garnered much recent attention.[1] As a ubiquitous and tractable means of governing protein association, the α-helical coiled-coil has been the focus of numerous investigations.[2] Although considerable data have been gathered on the structure−function relationships of natural core residues, the limited number of side chain candidates restricts the design of novel assemblies.[3] Here we report introduction of core diversity in the form of an unnatural hydrophobic side chain, and demonstrate its use in the formation of a specific heterotrimer. These results point toward the development of completely unnatural interfaces that will greatly expand the scope of available applications.

Coiled-coils are noncovalent aggregates formed by the supercoiling of helical strands (typically two or three). Their primary sequence contains a heptad repeat (*abcdefg*) with hydrophobic side chains in *a* and *d* positions whose burial in a tightly packed core is the primary basis for association. In their role as mediators of protein complexation they perform manifold natural functions ranging from structural support (α-keratin, actin, etc.) to DNA binding (bZIP transcription factors), receptor oligomerization (mannose binding protein), and membrane fusion (HIV, influenza).[4] Synthetic analogues that exploit their capacity for specific association have been used to improve protein purification, direct the assembly of active protein complexes, design affinity sensors, and develop novel materials.[5] Such broad applicability emphasizes the need for maximal diversity and design flexibility.

The present system describes heterotrimerization, achieved through steric matching (Figure 1). A small side chain positioned at one core position packs against the same residue on opposing strands of a parallel homotrimer. The resulting pocket should be destabilizing, and introduction of a complementary peptide, substituted with a larger side chain, should favor heteromeric structures due to improved packing interactions. To test this principle we focused on the dimerization domain of GCN4, where the impact of natural side chain variation has been thoroughly investigated.[6] We were particularly mindful of work by Alber and co-workers, who demonstrated that small molecule ligands could stabilize a trimer containing core alanines.[7]

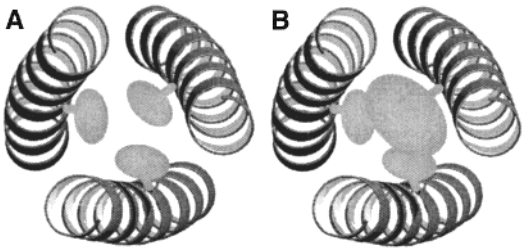

Figure 1. Heterotrimerization through steric matching. The Ala_{16} homotrimer (A) contains small methyl side chains at a central *a* residue, creating a pocket in the hydrophobic core. In the 2:1 complex of Ala_{16}:Chx_{16} (B) the larger cyclohexylalanine side chain partially fills the void, stabilizing the heterotrimer.

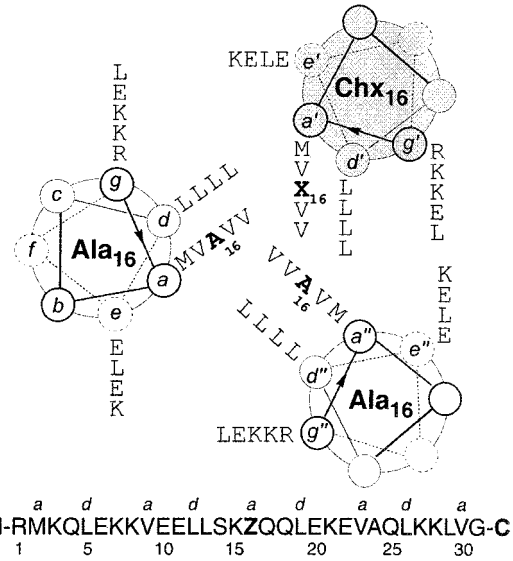

Figure 2. Helical wheel projection of the 2:1 Ala_{16}:Chx_{16} complex (viewed down the helical axis from N to C terminus). The sequence of both peptides (which differ only in the nature of Z) is also given. Altered core side chains are emboldened. Solvent exposed residues were omitted for clarity. X = cyclohexylalanine.

The peptides used here, similarly derived from GCN4, contain either alanine (**Ala_{16}**) or cyclohexylalanine (**Chx_{16}**) in place of a central asparagine residue (Figure 2).[8] Since parallel coiled-coil trimers contain segregated layers of *a* and *d* residues, side chains introduced in the same *a* position should be juxtaposed in the aggregate.[9] Accordingly, it was hypothesized that a 2:1 mixture of **Ala_{16}:Chx_{16}** should exhibit signatures characteristic of the heteromeric complex, rather than a superposition of weighted average component signals.[10]

(1) Guo, Z.; Zhou, D.; Schultz, P. G. *Science* **2000**, *288*, 2042−2045. DeLano, W. L.; Ultsch, M. H.; De Vos, A. M.; Wells, J. A. *Science* **2000**, *287*, 1279−1283.

(2) (a) Micklatcher, C.; Chmielewski, J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 724−729. (b) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779−819. (c) Kohn, W. D.; Kay, C. M.; Hodges, R. S. *J. Mol. Biol.* **1998**, *283*, 993−1012.

(3) (a) Tang, Y.; Ghirlanda, G.; Petka, W. A.; Nakajima, T.; DeGrado W. F.; Tirrell, D. A. *Angew. Chem., Int. Ed.* **2001**, *40*, 1494−1496. (b) Bilgicer, B.; Fichera, A.; Kumar, K. *J. Am. Chem. Soc.* **2001**, *123*, 4394−4399. (c) Tang, Y.; Ghirlanda, G.; Vaidehi, N.; Kua, J.; Mainz, D. T.; Goddard, W. A., III; DeGrado, W. F.; Tirrell, D. A. *Biochemistry* **2001**, *40*, 2790−2796. (d) Hill, R. B.; Hong, J.-K.; DeGrado, W. F. *J. Am. Chem. Soc.* **2000**, *122*, 746−747. (e) Harbury, P. B.; Plecs, J. J.; Tidor, B.; Alber, T.; Kim, P. S. *Science* **1998**, *282*, 1462−1467. (f) Oakley, M. G.; Kim, P. S. *Biochemistry* **1998**, *37*, 12603−12610. (g) Harbury, P. B.; Zhang, T.; Kim, P. S.; Alber, T. *Science* **1993**, *262*, 1401−1407. (h) Gonzalez, L., Jr.; Woolfson, D. N.; Alber, T. *Nat. Struct. Biol.* **1996**, *3*, 1011−1018.

(4) (a) Root, M. J.; Kay, M. S.; Kim, P. S. *Science* **2001**, *291*, 884−888. (b) Newman, J. R. S.; Wolf, E.; Kim, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13203−13208. (c) Lupas, A. *Trends Biochem. Sci.* **1996**, *21*, 375−382. (d) Cohen, C.; Parry, D. A. D. *Science* **1994**, *263*, 488−489.

(5) (a) Kern, P.; Hussey, R. E.; Spoerl, R.; Reinherz, E. L.; Chang, H.-C. *J. Biol. Chem.* **1999**, *274*, 27237−27243. (b) Petka, W. A.; Hardin, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389−392. (c) Sharma, V. A.; Logan, J.; King, D. S.; White, R.; Alber, T. *Curr. Biol.* **1998**, *8*, 823−830. (d) Chao, H.; Bautista, D. L.; Litowski, J.; Irvin, R. T.; Hodges, R. S. *J. Chromatogr. B.* **1998**, *715*, 307−329. (e) Kim, Y.-I.; Hu, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7510−7514. (f) Chang, H.-C.; Bao, Z.-Z.; Yao, Y.; Tse, A. G. D.; Goyarts, E. D.; Madsen, M.; Kawasaki, E.; Brauer, P.; Sacchettini, J. C.; Nathenson, S. G.; Reinherz, E. L. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11408−11412.

(6) O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber, T. *Science* **1991**, *254*, 539−544.

(7) Gonzalez, L., Jr.; Plecs, J. J.; Alber, T. *Nat. Struct. Biol.* **1996**, *3*, 510−515.

(8) Peptides were synthesized by standard solid-phase methods, as in: Schnöelzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180−193.

(9) Lumb, K. J.; Kim, P. S. *Biochemistry* **1995**, *34*, 8642−8648.

10.1021/ja015912v CCC: $20.00
Published on Web 10/12/2001

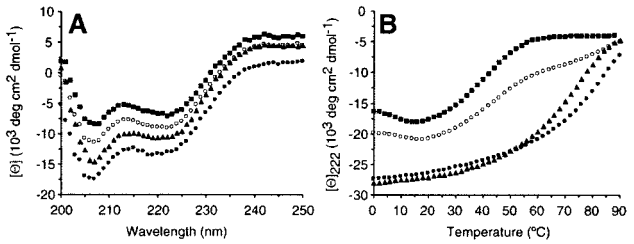

Figure 3. Circular dichroism (CD) data for solutions of Ala_{16} (■), Chx_{16} (●), and Ala_{16}:Chx_{16} 2:1 (▲), along with calculated weighted average signals for 2:1 samples (○). (A) CD spectra. Samples are 1 μM total peptide concentration in PBS buffer (10 mM phosphate pH 7.5, 150 mM NaCl).[13] (B) Thermal melt. Samples are 10 μM total peptide concentration in PBS buffer (10 mM phosphate, pH 7.5, 150 mM NaCl) containing 1.5 M guanidinium hydrochloride.[12]

Table 1. Apparent T_m Values Derived from CD Data[a]

sample	T_m (°C)	sample	T_m (°C)
Ala_{16}	47	Nap_{16}	75
Chx_{16}	87	Ala_{16}:Nap_{16} 2:1	71
Ala_{16}:Chx_{16} 2:1	77		

[a] Buffer conditions as outlined in Figure 3B.

Samples of **Ala_{16}**, **Chx_{16}**, and 2:1 **Ala_{16}**:**Chx_{16}** all exhibit circular dichroism (CD) behavior characteristic of helical peptides (Figure 3A). Pure **Chx_{16}** is significantly more helical than **Ala_{16}** and thus also than the predicted average signal of a solution in which it is the minor component. Despite this, the 2:1 mixture has a helical content approaching that of **Chx_{16}**, and considerably in excess of the weighted average value.

Thermal unfolding experiments reveal cooperative behavior in each sample (Figure 3B). Again, the 2:1 mixture produces a trace similar to **Chx_{16}**, and radically different from the weighted average. Its melting transition is sharper than the component ones, particularly **Chx_{16}**. Observed T_m values (Table 1) display the same trend.[11] Pure **Ala_{16}** melts markedly lower than either of the other two samples.[12]

Analytical ultracentrifugation experiments were performed to investigate aggregation number. Due to the intrinsic stability of these complexes, experiments were run in buffer containing 3 M guanidinium hydrochloride to obtain meaningful data about relative aggregate stabilities (Table 2).[14] While **Ala_{16}** gives a weight between monomer and dimer, 2:1 **Ala_{16}**:**Chx_{16}** exists as a trimer. Further work is required to elucidate the solution preferences of pure **Chx_{16}**, but behavior of the 2:1 mixture is consistent with specific interactions. [15]

More direct evidence for specific heterotrimer formation was obtained from an affinity tagging experiment. An analogue of **Chx_{16}** was prepared with an N-terminal $(His)_6$GlyGly sequence (**Chx_{His}**), which binds tightly to Ni-nitrilotriacetic acid (Ni-NTA) agarose beads.[16] A 3:1 solution of **Ala_{16}**:**Chx_{His}** was exposed to Ni-NTA beads. HPLC analysis of bound material revealed a molar ratio of 0.62:0.38 **Ala_{16}**:**Chx_{His}**. Since **Ala_{16}** has no tag sequence, retention of approximately two of the three equivalents available provides additional support for a specific complex.

Table 2. Molecular Weights from Analytical Ultracentrifugation[a]

sample	MW_{obs}[b]	MW_{calc} dimer[c]	MW_{calc} trimer[c]
Ala_{16}	6436	7650	11475
Chx_{16}	12790	7814	11721
Ala_{16}:Chx_{16} 2:1	11215		11557[d]
Nap_{16}	11443	7902	11853
Ala_{16}:Nap_{16} 2:1	9302		11601[d]

[a] Samples were run in PBS buffer containing 3 M GdnHCl (see text). [b] Average values obtained at two different speeds and three different concentrations for each sample (see Supporting Information for details). [c] Except where noted molecular weights are calculated for homodimers and homotrimers. [d] Calculated weight of 2:1 heterotrimer.

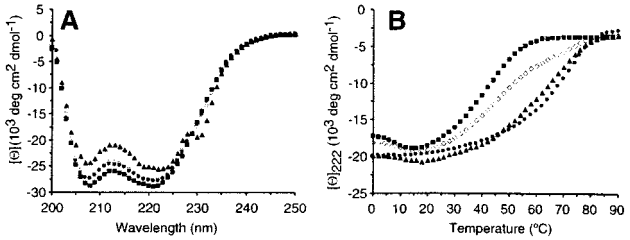

Figure 4. Circular dichroism (CD) data for solutions of Ala_{16} (■), Nap_{16} (●), and Ala_{16}:Nap_{16} 2:1 (▲), along with calculated weighted average signals for 2:1 samples (○). (A) CD spectra. Samples are 10 μM total peptide concentration in PBS buffer (10 mM phosphate, pH 7.5, 150 mM NaCl). (B) CD signal at 222 nm as a function of temperature. Samples are 10 μM total peptide concentration in PBS buffer (10 mM phosphate, pH 7.5, 150 mM NaCl) containing 1.5 M guanidinium hydrochloride.[12]

To further verify specificity for cyclohexylalanine, a control peptide was prepared with naphthylalanine at the central *a* position (**Nap_{16}**). The bulky naphthalene should be too large for even the reduced volume layer created by alanines on opposing strands. CD studies reveal that pure **Nap_{16}** indeed exhibits reduced helicity compared to either **Ala_{16}** or **Chx_{16}**, while the 2:1 **Ala_{16}**:**Nap_{16}** trace overlays almost perfectly with the calculated weighted average (Figure 4A). Similarly, the 2:1 sample displays a broad melting transition whose curve shape mimics that of the weighted average (Figure 4B). Analytical ultracentrifugation of a 2:1 **Ala_{16}**:**Nap_{16}** sample gave an observed molecular weight (9302) well below that calculated for the trimer (Table 2).[17] Tagged **Nap_{16}** (**Nap_{His}**) was dramatically less efficient than **Chx_{His}** at retaining **Ala_{16}** on Ni-NTA agarose beads.[18]

The ability of unnatural hydrophobic core side chains to direct specific aggregation represents a new recognition motif that promises a large number of programmable aggregates with biologically orthogonal interfaces. The work described above represents a first step in that direction.

Acknowledgment. We thank M. Greenberg and K. Lumb for helpful discussions. This work was supported by Colorado State University and the Petroleum Research Fund (35729-G4), administered by the American Chemical Society. The ultracentrifugation facility at CSU is supported by NIH (RR11847).

Supporting Information Available: Mass spectra of all peptides and detailed sedimentation equilibrium data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Hendsch, Z. S.; Nohaile, M. J.; Sauer, R. T.; Tidor, B. *J. Am. Chem. Soc.* **2001**, *123*, 1264−1265.

(11) T_m values were obtained according to the procedure outlined in: Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry of Macromolecules*, Part 3; W. H. Freeman: New York, 1980; p 1132

(12) Guanidinium hydrochloride (1.5 M) had to be added to observe T_m values below 90 °C for either **Chx_{16}** or the 2:1 mixture.

(13) The 1 μM data provide more visible differences between all signals. The source of unconventional curve shapes and mean residue elipticity values is unclear, and may reflect complicated behavior. Spectra at 10 μM exhibit more conventional features (including approximately equal minima at 208/222 nm).

(14) Initial experiments in aqueous buffer with no guanidinium hydrochloride at peptide concentrations from 15 to 55 μM demonstrated that **Ala_{16}**, **Chx_{16}**, and the 2: 1 mixture are all largely trimeric.

(15) The pure **Chx_{16}** gives a MW_{obs} significantly in excess of the calculated trimer, perhaps indicative of higher-order aggregation that may also be reflected in the broader melting transition discussed above.

(16) Brown, M. B.; Sauer, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1983−1988. **Ala_{16}** was not bound in the absence of **Chx_{His}**, but was retained from a 2:1:2 **Ala_{16}**:**Chx_{His}**:**Chx_{16}** mixture. See Supporting Information for details.

(17) The pure **Nap_{16}** sample, like that of **Chx_{16}**, displays considerable self-aggregation. Although the reasons for this are not obvious, the heterotrimer is clearly disfavored.

(18) **Ala_{16}**:**Nap_{His}** ratio retained from a 3:1 mixture was less than 1:3.5.