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Peptide Tic-Tac-Toe: Heterotrimeric Coiled-Coil Specificity from Steric Matching of Multiple Hydrophobic Side Chains

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Abstract: Specific coiled-coil heterotrimers result from steric matching of hydrophobic core side chains. A 2:1 heterotrimer is formed by peptides containing alanine or cyclohexylalanine, respectively, at a central core residue. Detailed thermodynamic analysis reveals that the designed complex is considerably more stable than the corresponding alanine homotrimer (ΔT_m = 25 °C, $\Delta\Delta G_{unf}$ = 4.5 kcal/mol), while control complexes with naphthylalanine or cyclopropylalanine peptides are much less stable. However, the cyclohexylalanine homotrimer is of comparable stability to the 2:1 complex, prompting an investigation of multiply substituted peptides. A specific 1:1:1 heterotrimer is formed from three independent peptide strands, each bearing one large (cyclohexylalanine) and two small (alanine) side chains at the same three core positions but in different order. The combined impact of three substitutions improves specificity to the point where each pure peptide and all pairwise equimolar mixtures form significantly less stable complexes (ΔT_m = 22−24 °C). The capacity for specific complex formation governed by multiple unnatural core side chains should facilitate design of numerous new peptide assemblies.

The development of biopolymer-inspired molecules that adopt defined structures is of considerable interest. In addition to backbone-modified species such as β-peptide and other foldamers,[1] attachment of unnatural side chains to peptide scaffolds should provide a wide variety of new complexes.[2] The structural simplicity and synthetic accessibility of α-helical coiled coils make them particularly attractive frameworks for this purpose.

The supercoiled helical strands of a coiled-coil complex contain hydrophobic side chains in the first and fourth (*a*, *d*) positions of a primary sequence heptad repeat.[3] In parallel systems, alternating layers of *a* and *d* side chains form a hydrophobic core largely responsible for complex stability. New core residue designs can build upon extensive studies of substitutions among natural core side chains.[4] The broad functional diversity of natural and synthetic sequences ensures the applicability of new recognition mechanisms.[5]

Many natural coiled-coil complexes ensure specificity of orientation, register, and aggregate number through burial of a single polar residue (frequently asparagine).[6] This strategy promotes the one complex that pairs asparagine side chains with each other rather than hydrophobic alternatives, in exchange for the stability costs associated with burial of a polar side chain. In contrast, we recently demonstrated the use of unnatural residues to confer heterotrimer specificity through steric matching of hydrophobic core side chains. Specifically, 2:1 mixtures of GCN4-derived peptides containing alanine (Ala_{16}) or cyclohexylalanine (Chx_{16}), respectively, at the central core a position form a stable heterotrimer (Figure 1).[7] Competing homotrimers contain a sterically mismatched core layer of either three alanines (Ala_{16}) or three cyclohexylalanines (Chx_{16}), resulting

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(1) (a) Winkler, J. D.; Piatnitski, E. L.; Mehlmann, J.; Kasparec, J.; Axelsen, P. H. *Angew. Chem., Int. Ed.* **2001**, *40*, 743−745. (b) Tanatani, A.; Mio, M. J.; Moore, J. S. *J. Am. Chem. Soc.* **2001**, *123*, 1792−1793. (c) Lee, H.-S.; Syud, F. A.; Wang, X.; Gellman, S. H. *J. Am. Chem. Soc.* **2001**, *123*, 7721−7722. (d) Gunther, R.; Hofmann, H.-J. *J. Am. Chem. Soc.* **2001**, *123*, 247−255. (e) Daura, X.; Gademann, K.; Schaefer, H.; Jaun, B.; Seebach, D.; van Gunsteren, W. F. *J. Am. Chem. Soc.* **2001**, *123*, 2393−2404. (f) Cubberley, M. S.; Iverson, B. L. *J. Am. Chem. Soc.* **2001**, *123*, 7560−7563. (g) Corbin, P. S.; Zimmerman, S. C.; Thiessen, P. A.; Hawryluk, N. A.; Murray, T. J. *J. Am. Chem. Soc.* **2001**, *123*, 10475−10488. (h) Cheng, R. P.; Gellman, S. H.; DeGrado, W. F. *Chem. Rev.* **2001**, *101*, 3219−3232. (i) Porter, E. A.; Wang, X.; Lee, H.-S.; Weisblum, B.; Gellman, S. H. *Nature* **2000**, *404*, 565. (j) Gellman, S. H. *Acc. Chem. Res.* **1998**, *31*, 173−180. (k) Seebach, D.; Overhand, M.; Kuehnle, F. N. M.; Martinoni, B. *Helv. Chim. Acta* **1996**, *79*, 913−941. (l) Appella, D. H.; Christianson, L. A.; Karle, I. L.; Powell, D. R.; Gellman, S. H. *J. Am. Chem. Soc.* **1996**, *118*, 13071−13072.

(2) (a) Bilgicer, B.; Fichera, A.; Kumar, K. *J. Am. Chem. Soc.* **2001**, *123*, 4393−4399. (b) 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. (c) Niemz, A.; Tirrell, D. A. *J. Am. Chem. Soc.* **2001**, *123*, 7407−7413.

(3) (a) Burkhard, P.; Strelkov, S. V.; Stetefeld, J. *Trends Cell Biol.* **2001**, *11*, 82−88. (b) Lupas, A. *Trends Biochem. Sci.* **1996**, *21*, 375−382. (c) Cohen, C.; Parry, D. A. D. *Science* **1994**, *263*, 488−489.

(4) (a) Harbury, P. B.; Kim, P. S.; Alber, T. *Nature* **1994**, *371*, 80−83. (b) Harbury, P. B.; Zhang, T.; Kim, P. S.; Alber, T. *Science* **1993**, *262*, 1401−1407. (c) O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber, T. *Science* **1991**, *254*, 539−544. (d) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779−819.

(5) (a) Kern, P.; Hussey, R. E.; Spoerl, R.; Reinherz, E. L.; Chang, H.-C. *J. Biol. Chem.* **1999**, *274*, 27237−27243. (b) Kim, Y.-I.; Hu, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7510−7514. (c) Chao, H.; Bautista, D. L.; Litowski, J.; Irvin, R. T.; Hodges, R. S. *J. Chromatogr., B* **1998**, *715*, 307−329. (d) Petka, W. A.; Hardin, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389−392. (e) Wang, C.; Stewart, R. J.; Kopecek, J. *Nature* **1999**, *397*, 417−420.

(6) Zeng, X.; Herndon, A. M.; Hu, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3673−3678. Gonzalez, L., Jr.; Woolfson, D. N.; Alber, T. *Nat. Struct. Biol.* **1996**, *3*, 1011−1018. Lumb, K. J.; Kim, P. S. *Biochemistry* **1995**, *34*, 8642−8648. Oakley, M. G.; Kim, P. S. *Biochemistry* **1998**, *37*, 12603−12610.

(7) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* **2001**, *123*, 11081−11082.

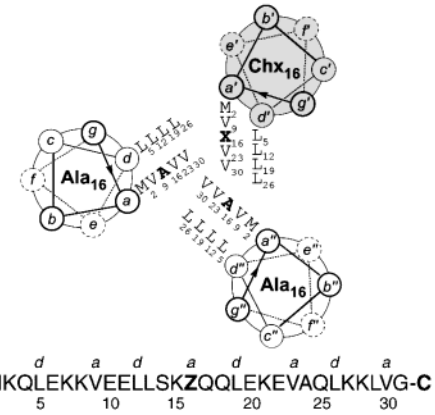

Figure 1. Helical wheel projection of the 2:1 Ala_{16}/Chx_{16} complex (viewed down the helical axis from the N to C terminus). The primary sequence shared by Ala_{16} (Z = alanine), Chx_{16} (Z = cyclohexylalanine), Nap_{16} (Z = naphthylalanine), and Cyp_{16} (Z = cyclopropylalanine) is also given. Altered core side chains are shown in bold. Solvent-exposed residues are omitted for clarity. X = cyclohexylalanine.

in a presumably destabilizing steric void or repulsion. Control experiments in which a naphthylalanine peptide (Nap_{16}) failed to form similar heterotrimers supported the specific suitability of cyclohexylalanine as a binding partner for the alanine peptide.

Despite formation of the intended complex, parallel studies on each isolated peptide demonstrated that only the alanine-bearing one was significantly less stable than the heterotrimer. In fact, both the cyclohexylalanine and naphthylalanine peptides formed complexes even more stable than the designed one. Specificity for the 2:1 complex appeared to arise mainly from poor interactions in the alanine peptide homotrimer. Thus, we sought to construct a second-generation system in which recognition specificity was enhanced sufficiently that the designed complex was more stable than its component strands. In this work we report successful design of a 1:1:1 heterotrimer that fulfills this criterion. Detailed thermodynamic analysis of the original system (along with an additional control peptide) confirmed both the unique role of cyclohexylalanine and the dramatic destabilization associated with all alanine core layers. To improve specificity, triply substituted peptides were investigated. Although peptides bearing three alanines or three cyclohexylalanines did not form the desired complexes, an alternative approach in which each strand contributes the cyclohexylalanine side chain to a different core layer was successful.

Results and Discussion

Cyclopropylalanine Control. The demonstrated lack of specific interaction between Ala_{16} and Nap_{16} was ascribed to a steric mismatch, due presumably to the overly bulky naphthalene group. To further verify the specific suitability of cyclohexylalanine, the alternative mismatch, in which the partner side chain is too small to complement the alanine pocket, was explored in the form of a cyclopropylalanine-containing peptide (Cyp_{16}, Figure 1). The results parallel those observed with Nap_{16}, and confirm that the 2:1 Ala_{16}/Cyp_{16} complex is unstable. Both wavelength and thermal denaturation circular dichroism (CD) spectra of the 2:1 mixture overlay well with the weighted average of the pure component signals, suggesting a lack of interaction. The apparent solution molecular weight obtained from sedimentation equilibrium experiments (8515) is well short of that calculated for the heterotrimer (11473).[8]

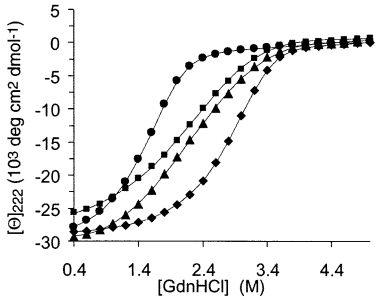

Figure 2. Guanidine hydrochloride denaturation profiles of Ala_{16} (circles), 2:1 Ala_{16}/Nap_{16} (squares), 2:1 Ala_{16}/Cyp_{16} (triangles), and 2:1 Ala_{16}/Chx_{16} (tilted squares). Lines are fits to the data (see the text). All solutions are 10 μM total peptide, in PBS buffer at 25 °C.

Table 1. Unfolding Free Energies

sample	ΔG_{unf} (kcal/mol)	sample	ΔG_{unf} (kcal/mol)
Ala_{16}	18.1 ± 0.17	Chx_{16}	24.7 ± 0.08
2:1 Ala_{16}/Chx_{16}	22.6 ± 0.04	Nap_{16}	23.5 ± 0.13
2:1 Ala_{16}/Nap_{16}	16.6 ± 0.33	Cyp_{16}	22.7 ± 0.31
2:1 Ala_{16}/Cyp_{16}	15.0 ± 0.31		

Thermodynamic Characterization. Relative stabilities of both matched and mismatched complexes were established by chemical denaturation with guanidine hydrochloride. Unfolding free energies at 25 °C were calculated by least-squares fitting to a monomer−trimer model that assumes both folded and unfolded baselines are linear functions of denaturant concentration (Figure 2).[8,9] The 2:1 Ala_{16}/Chx_{16} mixture is 4.5 kcal/mol more stable than pure Ala_{16}, while the corresponding 2:1 Ala_{16}/Nap_{16} and 2:1 Ala_{16}/Cyp_{16} complexes are 1.5 and 3.1 kcal/mol less stable, respectively (Table 1). Pure Chx_{16}, Nap_{16}, and Cyp_{16} solutions are more resistant to denaturation than any of the mixed complexes. These trends are well correlated with those observed previously via thermal denaturation and sedimentation equilibrium experiments.[7]

More detailed thermodynamic information was obtained by global analysis of thermal denaturation data from Ala_{16} solutions containing variable concentrations of guanidine hydrochloride (Figure 3).[8,10] A similar analysis of the matched 2:1 Ala_{16}/Chx_{16} mixture was performed on data from both thermal denaturations with variable guanidine concentrations and guanidine denaturations at variable temperature (Figure 4). In each case, reasonable estimates were obtained for the unfolding enthalpy at the melting temperature (ΔH_m), the melting temperature itself (T_m), and the change in heat capacity (ΔC_p) upon unfolding (Table 2). The value of ΔC_p is of particular interest as a probe of efficient core packing. Although literature values for well-packed coiled-coil structures are somewhat variable, those observed for both Ala_{16} (10.9 cal/(mol K) per residue) and 2:1 Ala_{16}/Chx_{16} (11.4 cal/(mol K) per residue) are within the expected regime.[11]

Multiply Substituted Systems. Although 2:1 Ala_{16}/Chx_{16} heterotrimer formation validates the principle of steric matching

(8) See the Supporting Information for further details.
(9) (a) Santoro, M. M.; Bolen, D. W. *Biochemistry* **1988**, *27*, 8063−8068. (b) Becktel, W. J.; Schellman, J. A. *Biopolymers* **1987**, *26*, 1859−1877. (c) Monomer−trimer equilibrium modeled as in the following: Jelesarov, I.; Lu, M. *J. Mol. Biol.* **2001**, *307*, 637−656. Duerr, E.; Jelesarov, I. *Biochemistry* **2000**, *39*, 4472−4482.
(10) Kuhlman, B.; Raleigh, D. P. *Protein Sci.* **1998**, *7*, 2405−2412.
(11) Boice, J. A.; Dieckmann, G. R.; DeGrado, W. F.; Fairman, R. *Biochemistry* **1996**, *35*, 14480−14485.

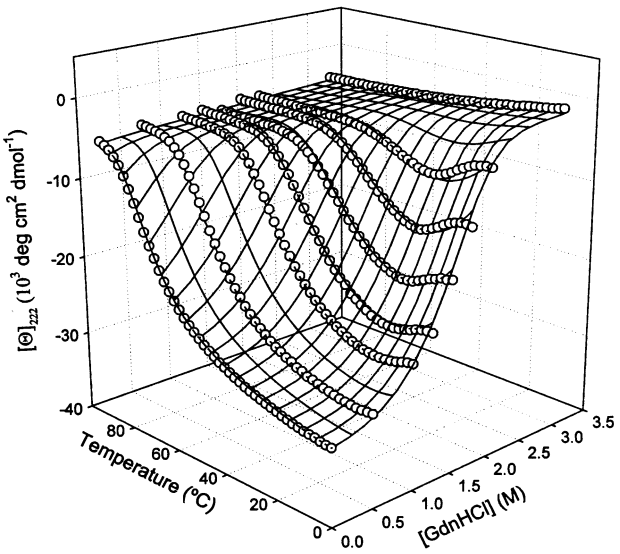

Figure 3. Global thermodynamic analysis of Ala_{16}. The solid line mesh depicts simultaneous fits to the data (open circles) from eight thermal denaturations. The peptide is 10 μM in PBS buffer with varying concentrations of added guanidine hydrochloride.

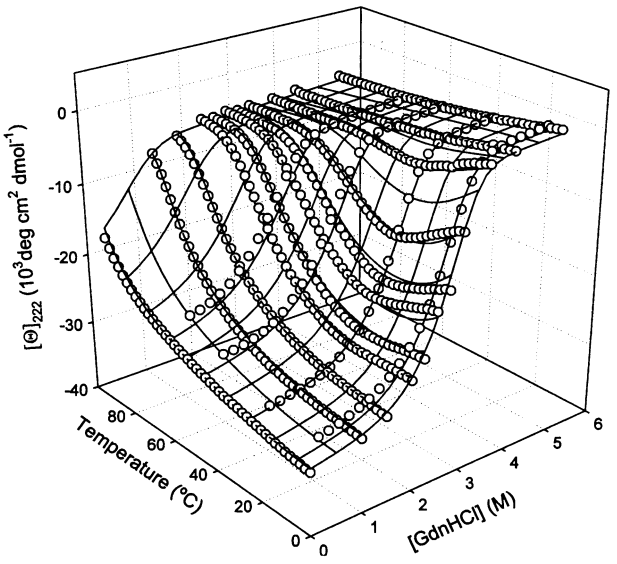

Figure 4. Global thermodynamic analysis of 2:1 Ala_{16}/Chx_{16}. The solid line mesh depicts simultaneous fits to the data (open circles) from eleven thermal and four chemical denaturations. The total peptide concentration is 10 μM in PBS buffer with varying concentrations of added guanidine hydrochloride.

Table 2. Thermodynamic Parameters

sample	ΔH_m (kcal/mol)	T_m (°C)	ΔC_p (kcal/(mol K))
Ala_{16}	61.6	71	1.06
2:1Ala_{16}/Chx_{16}	83.5	96	1.01

as a means to specificity, the designed system remains less stable than the Chx_{16} homotrimer. To further discourage alternate assemblies, trisubstituted systems were investigated. However, replacement of three consecutive core *a* residues with alanine ($Tris_{Ala}$) or cyclohexylalanine ($Tris_{Chx}$) did not achieve the desired effect. Both wavelength and thermal denaturation CD spectra of a 2:1 $Tris_{Ala}/Tris_{Chx}$ mixture are well predicted by averaging the component signals, and only $Tris_{Ala}$ suffered the anticipated reduction in homotrimer stability.[8] Coupled with the observed stability of Chx_{16} outlined above, these data suggest that in these systems core layers with steric voids are more damaging than those with steric repulsions.

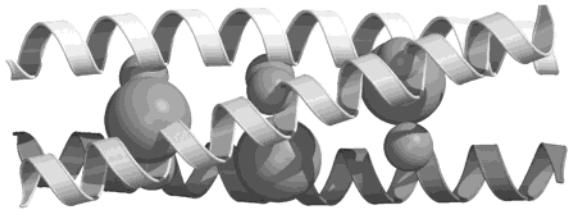

Figure 5. Specificity through multiple interactions. Large core *a* side chains pack against small ones at the same positions of other strands.

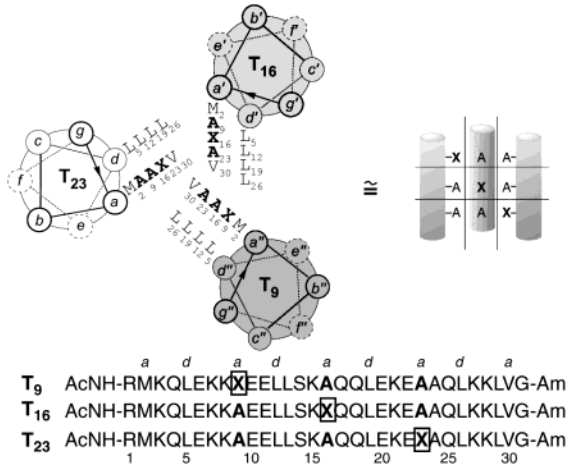

Figure 6. Helical wheel projection of the 1:1:1 $T_9/T_{16}/T_{23}$ complex (viewed down the helical axis from the N to C terminus), showing only core residues. The sequence of each peptide is also given. The tic-tac-toe arrangement of cyclohexylalanine residues (X**)** is depicted schematically at the right. Ac = acetyl and Am = amide.

To capitalize on the apparent sensitivity to all-alanine core layers, a new set of peptides was prepared in which three sequential *a* residues are replaced, two with alanine and one with cyclohexylalanine. An equimolar mixture of peptides with cyclohexyl side chains at each possible position should form a parallel 1:1:1 heterotrimer, with each contributing the cyclohexyl group for one core layer (Figure 5). The resulting three-dimensional arrangement somewhat resembles the outcome of a tic-tac-toe game, with three X groups along the diagonal (Figure 6). Other possible assemblies suffer from at least one destabilizing all-alanine core layer.

To test the ability of cooperative steric matching to impart complex specificity, separate peptides were prepared, in which the central three *a* positions are occupied by XAA (T_9), AXA (T_{16}), or AAX (T_{23}) residues. The remaining sequence is based on the first-generation system, derived in turn from GCN4 and related peptides (Figure 2).[7,12]

CD spectra of each individual peptide and the equimolar mixture argue in favor of the 1:1:1 heterotrimer as the most stable complex. All samples exhibit characteristic helical signatures, with minimum molar elipticities at 208 and 222 nm (Figure 7A). The degree of sample helicity, determined by the

(12) (a) Gonzalez, L., Jr.; Plecs, J. J.; Alber, T. *Nat. Struct. Biol.* **1996**, *3*, 510−515. (b) For a similar approach using natural side chains see: Kiyokawa, T.; Kanaori, K.; Tajima, K.; Tanaka, T. *Biopolymers* **2001**, *55*, 407−414. Kashiwada, A.; Hiroaki, H.; Kohda, D.; Nango, M.; Tanaka, T. *J. Am. Chem. Soc.* **2000**, *122*, 212−215.

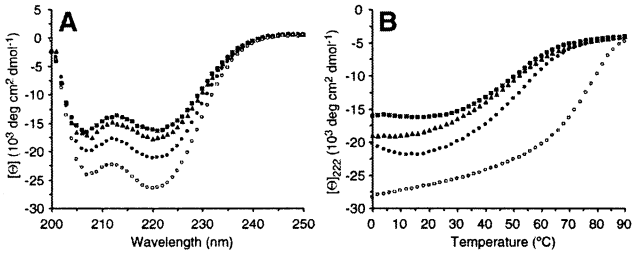

Figure 7. Wavelength scan (A) and thermal denaturation (B) CD data for solutions of T_9 (squares), T_{16} (triangles), T_{23} (closed circles), and an equimolar mixture (open circles). All samples are 10 μM total peptide in PBS buffer (10 mM phosphate (pH 7.4), 150 mM NaCl).

Table 3. Apparent T_m Values from CD Data

sample	T_m (°C)	sample	T_m (°C)
T_9	61	1:1 T_9/T_{16}	63
T_{16}	59	1:1 T_9/T_{23}	71
T_{23}	59	1:1 T_{16}/T_{23}	63
1:1:1 mixture	83		

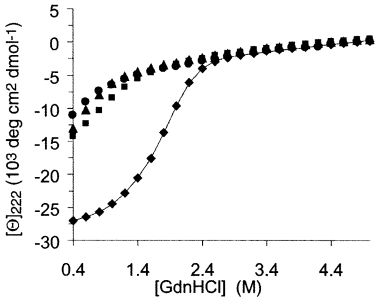

Figure 8. Guanidine hydrochloride denaturation profiles of T_9 (circles), T_{16} (squares), T_{23} (triangles), and an equimolar mixture (tilted squares). The line is a fit to the data (see the text). All solutions are 10 μM total peptide, in PBS buffer at 25 °C.

absolute value of the molar elipticity at 222 nm, is considerably larger for the 1:1:1 mixture than for any individual peptide ($[\theta]_{222} = -26200$ deg cm^2 dmol^{-1} for the 1:1:1 mixture versus those of individual peptides between -16200 and -21000).

Thermal and guanidine denaturation experiments further support enhanced stability in the designed complex. Aqueous buffer solutions of pure component strands exhibit significantly lower melting temperatures than their equimolar combination ($\Delta T_m > 20$ °C, Table 3, Figure 7B).[9] The free energy of unfolding for the 1:1:1 sample, obtained by guanidine denaturation, is 19.60 $\pm$ 0.21 kcal/mol (Figure 8). In contrast to the designed system, each individual peptide failed to exhibit a cooperative unfolding transition during guanidine titration. Thus, although no quantitative information on unfolding thermodynamics is available, homoaggregates of all three component species are considerably less stable.

To correlate observed helicity profiles with trimer formation, apparent molecular weights (MW_{app}) in PBS buffer were determined by analytical ultracentrifugation. An equimolar mixture of all three peptides gives an average value ($MW_{app} =$ 10245) close to that calculated for the heterotrimer (11556), and significantly larger than that for a dimer (7704). Each of the component peptides gives a noticeably lower average, although still in excess of the dimer value ($MW_{app} =$ 9104, 9969, and 9729, for T_9, T_{16}, and T_{23}; all individual peptides have identical masses).

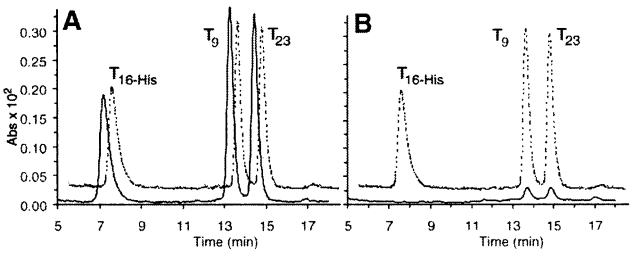

Figure 9. Ni-NTA affinity tag experiments (see the text): (A) HPLC trace of material retained from 1:1:1 (dotted line) and 1:2:2 (solid line) mixtures of $T_{16-His}/T_9/T_{23}$; (B) trace from the 1:1:1 mixture (dotted line) plotted with that from an initial solution lacking the tagged peptide (solid line). Injection volumes and extinction coefficients are identical.

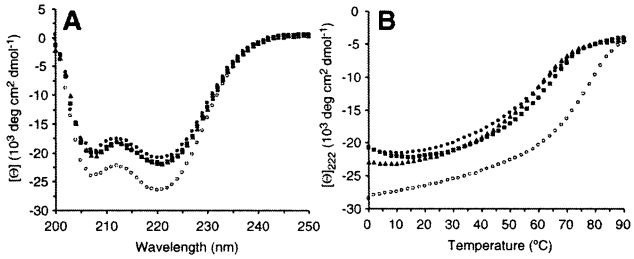

Figure 10. Wavelength scan (A) and thermal denaturation (B) CD data for solutions of 1:1 T_9/T_{16} (squares), 1:1 T_9/T_{23} (triangles), 1:1 T_{16}/T_{23} (closed circles), and 1:1:1 $T_9/T_{16}/T_{23}$ (open circles). Buffer conditions are as in the caption for Figure 7.

Complex stoichiometry was assayed using a T_{16} derivative bearing an N-terminal $(His)_6$GlyGly affinity tag (T_{16-His}), which binds to nickel nitrilotriacetic acid (Ni-NTA) groups.[7,13] Buffered peptide solutions are mixed with a slurry of Ni-NTA-functionalized agarose beads, followed by centrifugation, supernatant removal, and washing with pure buffer. Peptides bound on the beads are then eluted by treatment with imidazole buffer. Only tagged peptides and their specific binding partners are retained; thus, HPLC analysis of the material in the elution fraction reveals relative peptide concentrations. Equimolar ratios of T_{16-His} and complementary untagged peptides (T_9, T_{23}) were retained from either 1:1:1 or 1:2:2 initial mixtures (Figure 9A), indicating 1 equiv of each peptide in the stable complex. In the absence of T_{16-His}, neither untagged peptide is significantly retained (Figure 9B).

As a final verification that all three peptides are required to achieve a stable complex, CD spectra and thermal denaturation curves were recorded for each of the three pairwise equimolar mixtures (Figure 10). Though somewhat more helical than the isolated peptides, each sample remains well short of the 1:1:1 mixture ($[\theta]_{222}$ between -20700 and -21600 deg cm^2 dmol^{-1}). Similarly, no one pair exhibits a melting temperature within 10 deg of the designed system (Table 3). These results also support the significance of steric matching in controlling specificity. Each pairwise mixture can form equal concentrations of two possible 2:1 complexes, both of which have one matched and two mismatched core layers, with one of the mismatches being all alanine. In contrast, the pure component solutions examined above can form only completely mismatched complexes. Thus, observance of intermediate stability in the pairwise mixtures is consistent with the design principles.

(13) Brown, B. M.; Sauer, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1983−1988.

Conclusions

The data presented above demonstrate that steric matching of unnatural hydrophobic core side chains is an effective tool for controlled assembly of coiled-coil heterotrimers. Unstable all-alanine core layers are specifically complemented by cyclohexylalanine peptides, while sterically mismatched naphthylalanine or cyclopropylalanine species result only in additional destabilization. The ability to combine multiple interactions to improve specificity is particularly intriguing, given that isolated hydrophobic contacts may be unsuitable for that purpose. The capacity for directed aggregation demonstrated here represents a significant step in the programmed association of biopolymer-based complexes, and should permit the design of more complex assemblies.

Experimental Section

Peptide Synthesis. Amino acids (including cyclohexylalanine) were obtained from NovaBiochem (San Diego). Peptides were prepared according to the in situ neutralization protocol developed by Kent, except for Cyp_{16}, which was prepared by standard FMOC solid-phase methods.[14] Each peptide was purified by reversed-phase HPLC (C-18 column, (solvent A) 1% CH_3CN in H_2O, 0.1% (v/v) CF_3CO_2H, (solvent B) 10% H_2O in CH_3CN, 0.07% (v/v) CF_3CO_2H), and the identity of purified samples was confirmed by electrospray mass spectrometry (Finnegan LCQ-Duo). All peptides are C-terminally amidated and N-terminally acetylated; each contains an acetamidobenzoate group on the side chain nitrogen of Lys_7 as a spectroscopic label ($\epsilon_{270} = 18069$).

CD Spectroscopy. All experiments were performed on an Aviv model 202 circular dichroism spectrometer, equipped with a Microlab 500 series automated titration assembly. Sample concentrations were measured by UV absorbance of the acetamidobenzoate label at 270 nm. Wavelength data are the average of three scans from 250 to 200 nm in 1 nm steps. Thermal denaturation experiments at 222 nm were run from 0 to 90 °C in 2 deg steps, at a 2 deg/min rate of increase with 1 min of equilibration and data averaging at each temperature. T_m values were obtained from the minima of the first derivatives of θ vs $1/T$ plots.[15] Guanidinium titrations were performed using the automated titration assembly. The signal at 222 nm was recorded for solutions of constant peptide concentration with guanidine hydrochloride concentrations varied from 0 to 5 M in 0.2 M increments. Data were collected for 1 min at each step, with 10 min equilibration times (solutions were stirred during equilibration but not data collection).

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman XL-I analytical ultracentrifuge equipped with an An60-Ti rotor. Data were collected using 12 mm path length six-sector centerpieces at 270 nm. Samples were dialyzed against the reference buffer at 4 °C overnight. Data were collected at 38000 and 48000 rpm at concentrations spanning 17−55 μM. Samples were judged equilibrated (in all cases equilibration was complete in 12 h) when three consecutive scans taken 1 h apart were indistinguishable. Solvent densities and partial molar volumes were calculated in the manner prescribed by Laue.[16] Data were analyzed using Origin and fit to ideal single-species and appropriate monomer−oligomer models.

Ni-NTA Affinity Tag Experiments. A 0.5 mL sample of a 50% slurry of Ni-NTA agarose (Qiagen) in an Eppendorf tube was centrifuged for 30 s, followed by removal of the superantant. Peptide solution was added, and the tube was repeatedly inverted for 5 min. The sample was centrifuged (30 s), and the supernatant (flow-through fraction) was removed. The procedure was then repeated with 1 mL of buffer (wash fraction) and 1 mL of buffer containing 250 mM imidazole (elution fraction), except that the wash fraction was not agitated for 5 min. Solutions were analyzed by RP-HPLC. All solutions were 10 μM in total peptide, except for the 2:1:2 $T_9/T_{16-His}/T_{23}$ mixture, which was 16.6 μM, and the 1:1 T_9/T_{23}, which was 6.6 μM.

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Supporting Information Available: Mass spectra of all peptides, detailed sedimentation equilibrium, Ni-NTA affinity tag, and guanidine denaturation data, and detailed derivations of thermodynamic models (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Schnöelzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180−193.
(15) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry of Macromolecules, Pt. 3: The Behavior of Biological Macromolecules*; W. H. Freeman: New York, 1980; p 1132.
(16) Laue, T. M.; Shah, B. D.; Ridgeway, T. M.; Pelletier, S. L. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science*; Harding, S. E., Rowe, A. J., Horton, J. C., Eds.; The Royal Society of Chemistry: Cambridge, U.K., 1992; pp 90−125.