Specific Control of Peptide Assembly with Combined Hydrophilic and Hydrophobic Interfaces

Nathan A. Schnarr and Alan J. Kennan*

Contribution from the Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

Received June 27, 2002; E-mail: kennan@lamar.colostate.edu

Abstract: Designed coiled-coil heterotrimers are described whose assembly is governed by both hydrophobic and hydrophilic forces. Sterically matched hydrophobic core side-chain packing of alanine and cyclohexylalanine has been shown to promote formation of a 1:1:1 heterotrimer. Manipulation of hydrophilic glutamic acid (Glu)/lysine (Lys) pairs at each of three helical contact interfaces provides a secondary recognition mechanism. Peptides with matched cores and hydrophilic contacts form stable heterotrimers (ΔG_{unf} at 25 °C = 17.93 kcal/mol; MW_{app} = 11362 vs 11563 calcd for trimer), as do those with a single Lys/Lys (but not Glu/Glu) interface. The additional specificity engendered by simultaneous operation of two interfaces was used to design a system in which six different peptides are mixed to form three specific and independent heterotrimers in the same solution.

The self-assembly of complex systems from simple building blocks is an extremely powerful tool for molecular design. Biopolymers are particularly useful components, in light of their intrinsic self-complementarity.[1] Of natural systems, the α-helical coiled-coil is especially promising, requiring only short, contiguous blocks of amino acids whose self-affinity is well understood.[2] Thus manipulation and diversification of coiled-coil assembly strategies paves the way for rapid construction of molecular complexity.

Coiled-coils result from superhelical twisting of two or more constituent helices. Their assembly is facilitated by a primary sequence heptad repeat (*abcdefg*), which supports both hydrophobic (*a*, *d* residues) and hydrophilic (*e*, *g*) interfaces.[3] Core packing of the hydrophobic residues largely controls complex stability and aggregation number,[2] while electrostatic matching of hydrophilic side chains imparts sequence selectivity.[4] Specificity in natural and designed systems is also achieved through buried core hydrophilic residues, which pack preferentially against each other rather than their hydrophobic counterparts.[5] Although useful for homomeric systems, this mechanism does

not provide for strand differentiation in heteromeric structures. In contrast, we have recently described an alternative approach to core specificity through steric matching of hydrophobic side chains, which permits formation of a 1:1:1 heterotrimer.[6,7] The component peptides, derived from GCN4, are substituted with alanine or cyclohexylalanine in three different orders at consecutive *a* residues. Since parallel coiled-coils contain separate *a*/*d* core layers, the altered positions pack against each other in the complex. The sequences are designed such that interaction matches one cyclohexylalanine and two alanine side chains,

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* To whom correspondence should be addressed.

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while undesired arrangements suffer from multiple steric core mismatches. The use of multiple substitutions is critical for controlling specificity, since each strand presents a different core profile despite use of the same residues. The same method is less practical for buried polar interactions, since each separate substitution results in considerable erosion of overall stability.
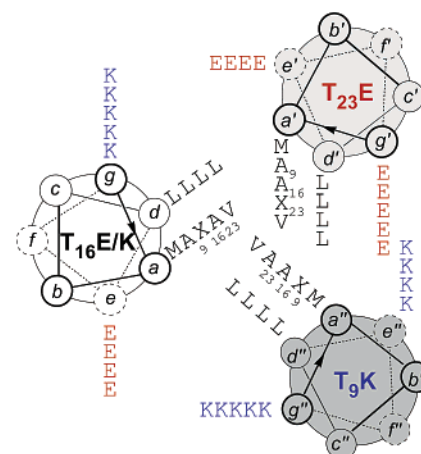
Although this approach to designing a single trimer was highly successful, the true potential of self-assembly will be tapped only by a more sophisticated system. In turn, the design of more intricate structures demands the simultaneous operation of multiple orthogonal recognition modes. As a first step in this direction, we were interested in exploring the use of two independent specificity mechanisms within the same peptide. Given the considerable body of literature on controlling coiled-coil specificity with e/g electrostatic interactions,[4] we decided to target a system that employs both the core recognition strategy we have developed and electrostatic matching of glutamic acid (Glu)/lysine (Lys) e/g residues.[8]

In this work we describe the successful execution of this design. Relative stabilities have been measured for core-matched complexes with one, two, or all three electrostatically matched e/g interfaces. Control experiments with mismatched core arrangements demonstrate that both interfaces are significant in controlling complex stability. The attendant increase in design precision is exploited in the assembly of three specific trimers from a mixture of six different peptides, a process accessible only through these methods.

Results and Discussion

The peptides used are derived from those in our previous work (Figure 1).[6] Each parent peptide contains either XAA (T_9), AXA (T_{16}), or AAX (T_{23}) at the central three a positions (A = alanine, X = cyclohexylalanine). The corresponding sequences bearing either Glu (T_nE) or Lys (T_nK) at all e/g positions were prepared, as well hybrids (T_nE/K) with Glu and Lys in all e and g positions, respectively. Different equimolar combinations of these peptides give rise to complexes featuring either zero, one, or three sterically matched 2:1 Ala:Chx core layers, along with zero, one, two, or three electrostatically matched Glu/Lys e/g interfaces.

To determine the general impact of electrostatics on these peptides, pure solutions of each new sequence were examined by circular dichroism (CD) spectroscopy. Based on literature demonstrations of like-charge repulsion at e/g interfaces, along with our work on mismatched core sequences, we expected these homotrimers to be poor candidates for stable complex formation. In fact, the Lys-substituted basic peptides (T_nK) exhibit a somewhat helical wavelength profile, but with low absolute intensity, while thermal denaturation demonstrated a noncooperative unfolding transition (Figure 2). Similar results were observed for the Glu-substituted acidic peptides (T_nE), which gave even weaker signals (Figure 3). The wavelength profiles are significantly distorted from helical norms, while the thermal unfolding curves afford slightly shallower and earlier transitions than for the Lys peptides. To verify that charge repulsion remains destabilizing with matched core sequences, a requirement for orthogonal control of complex specificity, equimolar

(8) The simultaneous application of hydrophobic and hydrophilic motifs has been extensively probed with natural amino acids. See ref 2c and references therein.

Figure 1. Peptides employed. Each sequence derives from one of three parents (T_9, T_{16}, T_{23}) by replacement of all e/g residues with Glu (T_nE), Lys (T_nK), or both (T_nE/K), as indicated. Helical wheel projection of the totally matched trimer ($T_9K:T_{16}E/K:T_{23}E$) is also given to illustrate the interfaces involved. Solvent-exposed residues omitted for clarity. **X** = cyclohexylalanine. Each peptide is N-terminally acetylated (Ac) and C-terminally amidated (Am). The positions of core modification in the parent peptides are underlined.
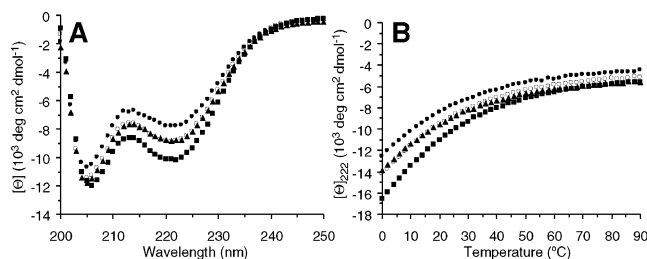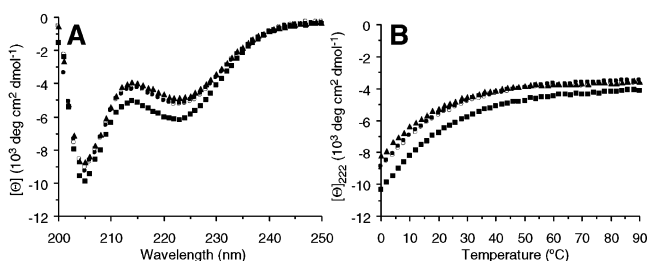

Figure 2. Wavelength scan (A) and thermal denaturation (B) CD data for solutions of T_9K (circles), $T_{16}K$ (triangles), $T_{23}K$ (squares), and an equimolar mixture (open circles). All samples are 10 μM total peptide in PBS buffer (10 mM phosphate pH 7.4, 150 mM NaCl).

mixtures of all like-charged peptides were examined (i.e., 1:1:1 $T_9K:T_{16}K:T_{23}K$ and $T_9E:T_{16}E:T_{23}E$). In each case, CD spectra are consistent with those calculated by averaging component signals, arguing against interaction to form new stable complexes.

Having demonstrated the viability of electrostatic control in these systems, we next investigated complexes with variable numbers of matches. In all cases the hydrophobic cores were completely matched, to isolate electrostatic effects. Since parallel coiled-coil trimers contain three e/g interfaces, we examined assemblies with zero, one, two, or three matched Glu/Lys contacts. As expected, the equimolar $T_9K:T_{16}E/K:T_{23}E$ mixture, in which all e/g interfaces pair Glu against Lys, is highly helical and thermally stable by CD ($[\theta]_{222} = -28\,457$ deg cm^2 dmol^{-1},
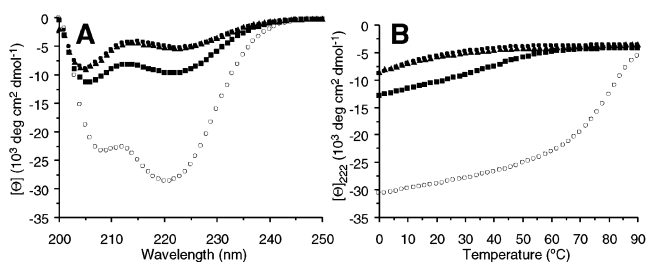
Figure 3. Wavelength scan (A) and thermal denaturation (B) CD data for solutions of T_9E (circles), $T_{16}E$ (triangles), $T_{23}E$ (squares), and an equimolar mixture (open circles). Conditions as in Figure 2.
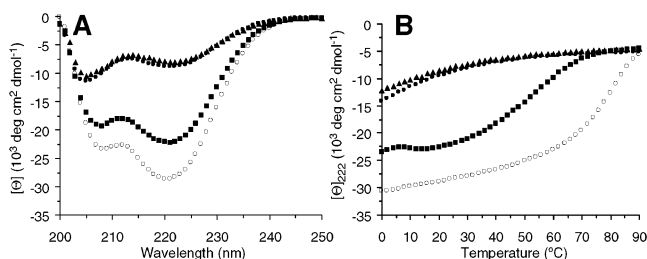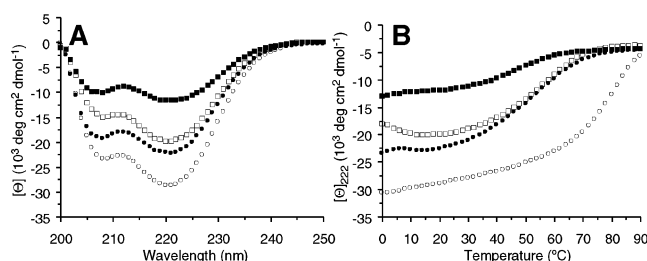

Figure 4. Wavelength scan (A) and thermal denaturation (B) CD data for equimolar solutions of $T_9K:T_{16}K:T_{23}K$ (circles), $T_9K:T_{16}E/K:T_{23}K$ (triangles), $T_9K:T_{16}E:T_{23}K$ (squares), and $T_9K:T_{16}E/K:T_{23}E$ (open circles). Conditions as in Figure 2.

Figure 5. Wavelength scan (A) and thermal denaturation (B) CD data for equimolar solutions of $T_9E:T_{16}E:T_{23}E$ (circles), $T_9E:T_{16}E/K:T_{23}E$ (triangles), $T_9E:T_{16}K:T_{23}E$ (squares), and $T_9K:T_{16}E/K:T_{23}E$ (open circles). Conditions as in Figure 2.

Tm = 83 °C, Figure 4). Sequential replacement of each Glu/Lys interface with a Lys/Lys one produced an interesting result. Although the mixture with two Lys/Lys repulsive interactions ($T_9K:T_{16}E/K:T_{23}K$) is virtually indistinguishable from the totally mismatched system examined above, the complex with one Lys/Lys juxtaposition ($T_9K:T_{16}E:T_{23}K$) is reasonably stable ($[\theta]_{222} = -22\,098$ deg cm^2 dmol^{-1}, Tm = 61 °C). In contrast, when matched interfaces are replaced by Glu/Glu interactions, even a single mismatch is almost completely destabilizing ($T_9E:T_{16}K:T_{23}E$, Figure 5). This differential instability, observed previously in disulfide bonded homodimers,[4i] provides another useful mechanism for controlling specificity.

To further assess relative strengths of the hydrophobic and hydrophilic recognition mechanisms, we examined complexes with only partially mismatched cores. The heterotrimer formed from an equimolar mixture of $T_9K:T_{16}E/K:T_{16}E$ contains 2:1, 1:2, and 3:0 ratios of alanine to cyclohexylalanine at the three core layers. It thus represents an intermediate state between the fully matched and mismatched core systems, with completely matched e/g interfaces. Its CD profile ($[\theta]_{222} = -19\,593$ deg cm^2 dmol^{-1}, Tm = 63 °C) is comparable to that observed for the $T_9K:T_{16}E:T_{23}K$ system, which contains a matched core and one repulsive Lys/ Lys e/g interface (Figure 6). The system containing two intermediate mismatches ($T_9K:T_{16}E:T_{16}K$), with

Figure 6. Wavelength scan (A) and thermal denaturation (B) CD data for equimolar solutions of $T_9K:T_{16}E:T_{16}K$ (squares), $T_9E:T_{16}E/K:T_{16}K$ (open squares), $T_9K:T_{16}E:T_{23}K$ (circles), and $T_9K:T_{16}E/K:T_{23}E$ (open circles). Conditions as in Figure 2.

Table 1. Molecular Weights from Sedimentation Equilibrium[a]

| sample	MW$_{app}$	MW$_{calcd}$dimer[b]	MW$_{calcd}$trimer
$T_9K:T_{16}E/K:T_{23}E$	11362	7709	11563
$T_9K:T_{16}E:T_{23}K$	10949	7706	11559
$T_9E:T_{16}K:T_{23}E$	8390	7712	11568

[a] Conditions as in Figure 2. [b] Average of three possible heterodimers.

only one 2:1 Ala:Chx layer and one Lys/Lys interface, retains little if any stability. Its CD spectra closely resemble those of systems with completely mismatched cores containing either matched or single Lys/Lys e/g surfaces (see Supporting Information). The capacity of each criterion, hydrophobic or hydrophilic, to control assembly preferences seems well balanced by the other.

Before proceeding with the simultaneous implementation of these specificity controls, we sought to further characterize representative complexes. Since dimers, trimers, and higher oligomers of these peptides are predicted to be comparably helical, CD is not a good measure of specificity in aggregation number. Independent verification was gathered from sedimentation experiments in the analytical ultracentrifuge. Observed molecular weights in solution were obtained for systems containing matched cores and either all Glu/Lys ($T_9K:T_{16}E/K:T_{23}E$), one Lys/Lys ($T_9K:T_{16}E:T_{23}K$), or one Glu/Glu interface ($T_9E:T_{16}K:T_{23}E$). The completely matched system affords a value close to that calculated for the heterotrimer, and a single Lys/Lys interface lowers the observed weight only slightly (Table 1). In contrast, the Glu/Glu interaction, shown to be destabilizing by CD results in an observed weight closer to that of a dimer. Quantification of complex stability was obtained from guanidine hydrochloride denaturation experiments at 25 °C (Figure 7). The completely matched assembly exhibits a cooperative unfolding pattern, while the single Lys/Lys complex displays an intermediate transition, and the single Glu/Glu complex is essentially uncooperative. Data from the matched system were fit according to a monomer–trimer model that assumes both folded and unfolded baselines are linear functions of denaturant concentration.[9] The observed unfolding free energy of 17.93 ± 0.40 kcal/mol is comparable to that of the parent $T_9:T_{16}:T_{23}$ system (19.60 ± 0.21 kcal/mol). These experiments support the viability of CD screens for reasonable complexes.

Having established the means for independent control of complex specificity by hydrophobic or hydrophilic interfaces,

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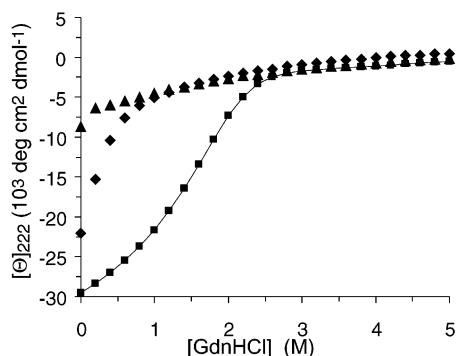
Figure 7. Guanidine hydrochloride denaturation profiles (25 °C) of **T₉K: T₁₆E/ K:T₂₃E** (squares), **T₉K:T₁₆E:T₂₃K** (diamonds), and **T₉E:T₁₆K:T₂₃E** (triangles). All solutions 10 μM total peptide.


Figure 8. Competition between all Glu/Lys and one Lys/Lys interfaces. Equimolar mixture of **T₉K**, **T₁₆E**, **T₂₃E/K**, and **T₂₃K** can form two different heterotrimers. If **T₂₃E/K** is used, the complex has fully matched electrostatic *e/g* interfaces (all Glu/ Lys). If **T₂₃K** is included instead, the assembly has one repulsive Lys/Lys interface.

we focused on its application to more complicated assembly problems. Since viable complexes result from either fully matched (all Glu/Lys) or singly mismatched (one Lys/Lys) *e/g* interfaces, we sought to directly assay their relative stabilities. An equimolar mixture of **T₉K**, **T₁₆E**, **T₂₃E/K**, and **T₂₃K** can result in formation of either heterotrimer type, depending on which **T₂₃** derivative is incorporated (Figure 8).

To determine relative complex stabilities, an affinity tag strategy we have previously employed proved useful.[6,10] A (His)₆GlyGly sequence that binds Ni-nitrilotriacetic acid (Ni-NTA) functionalized agarose beads was appended to the N-terminus of **T₁₆E** (to give **T₁₆EHis**). In the experiment, buffered peptide solutions are mixed with a slurry of Ni-NTA beads, followed by centrifugation, supernatant removal, and washing with pure buffer. Upon subsequent elution with imidazole buffer, only tagged peptides and their specific binding partners are obtained, thus HPLC analysis of the elution fraction reveals relative peptide concentrations. In the present case, analysis of the supernatant solution is also instructive, as it should contain the rejected **T₂₃** component. As expected, observed peak ratios are consistent with a significant preference for the fully matched complex (Figure 9). Specifically, the supernatant solution is enriched in **T₂₃K**, while the elution fraction is dominated by **T₂₃E/K**, the peptide required for the fully matched heterotrimer.

The preference for fully matched systems was also verified in a more complex mixture, equimolar in all nine of the new sequences (**TₙE**, **TₙK**, **TₙE/K**). The analysis is also more complicated, since each peptide can participate in either of two unique fully matched complexes or one of two other assemblies bearing a single Lys/Lys interface. Again the mixture was deconvoluted by the Ni-NTA method, this time by considering

(10) Method as in: Brown, B. M.; Sauer, R. T. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 1983–1988.
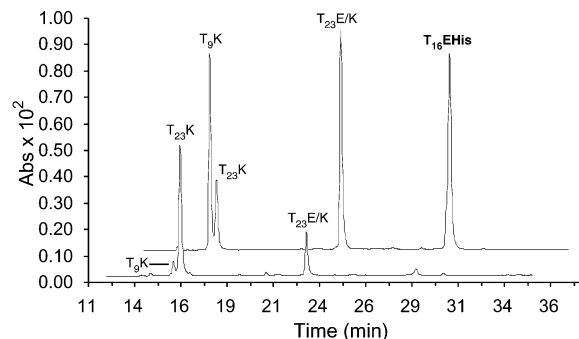
Figure 9. Ni-NTA affinity tag analysis of an equimolar **T₉K**, **T₁₆EHis**, **T₂₃E/K**, **T₂₃K** mixture. Supernatant solution (front trace) is significantly enriched in **T₂₃K**, while elution fraction (back trace) contains largely the components of the fully matched heterotrimer (**T₉K**, **T₁₆EHis**, **T₂₃E/K**). See Figure 8 for schematic diagram.
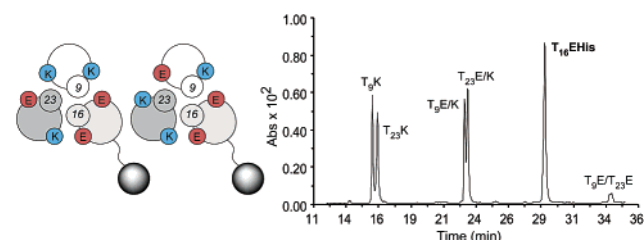

Figure 10. Ni-NTA affinity tag analysis of complex mixture. Equimolar ratios of all nine electrostatic interface peptides (**TₙE**, **TₙK**, **TₙE/K**) can afford six different fully matched complexes, with each peptide participating in two. The possibilities for **T₁₆E** are represented at left (black sphere indicates connection to Ni-NTA beads). On the right is the elution fraction from a mixture containing **T₁₆EHis**, demonstrating retention of precisely the required binding partners.

the results of three parallel experiments. A different acidic peptide (**TₙE**) was tagged in each case.

The elution fraction from one such experiment, with **T₁₆EHis** as the tagged peptide, again reveals a preference for fully matched systems (Figure 10). As expected, approximately equimolar ratios of the components from both possible matched complexes are observed. In addition, the roughly equal ratio of basic (**T₉K**, **T₂₃K**) to hybrid (**T₉E/K**, **T₂₃E/K**) peptides is consistent with the formation of these matched assemblies to the near exclusion of single Lys/Lys ones, as the latter necessarily require a 2-fold excess of basic peptides. Similar results were obtained from experiments with **T₉EHis** and **T₂₃EHis** (see supporting information).

Although these experiments demonstrated the capacity for favoring a given ensemble of structures, we were eager to identify a strategy that would allow more specific assembly control. By turning to the other viable set of interactions, complexes with one Lys/Lys interface, we have been able to promote the specific formation of three independent heterotrimer complexes from an input of only six different peptides. From a 2:2:2:1:1:1 mixture of **T₉K:T₁₆K:T₂₃K:T₉E:T₁₆E:T₂₃E,** only three specific heterotrimers can be formed while maintaining the recognition requirements developed above (Figure 11). Each complex contains two basic and one acidic peptide, with the core sequence of the acidic peptide varied from one trimer to the next. Such a rapid build up of complexity is possible only through the interaction of two distinct recognition mechanisms. The lack of such specificity associated with the fully matched systems described above also underlines the power of more diverse possible combinations.
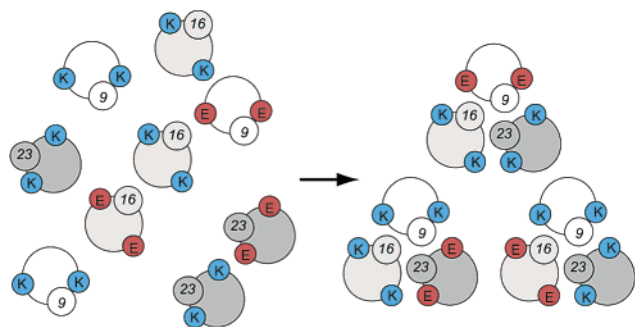
Figure 11. More specific assembly system. Mixing 2 equiv of each basic peptide (T_nK) with 1 equiv of each acidic peptide (T_nE) results in formation of three specific heterotrimers starting from six different peptides. Numbers indicate location of cyclohexylalanine in the sequence.
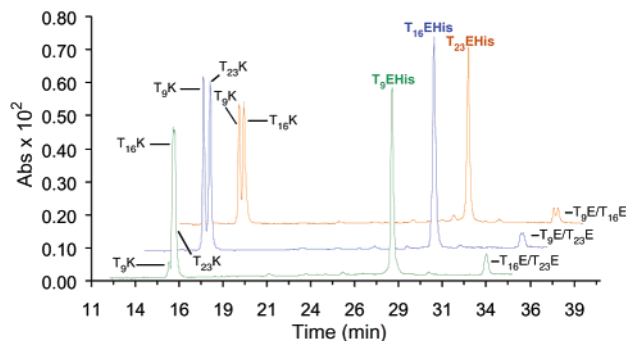
Figure 12. Ni-NTA affinity tag analysis of more complicated assembly system (see text). Traces correspond to elution fractions of experiments using T_9EHis (front), $T_{16}EHis$ (middle), and $T_{23}EHis$ (back) as tagged peptides.

Although work on each isolated system made formation of three specific heterotrimers the only likely outcome, we sought more direct evidence of successful and specific formation of the designed complexes. To verify the presence of each specific heterotrimer, elution fractions from three parallel Ni-NTA experiments with different tagged peptides (T_nE) were compared (Figure 12). In each case, the significant HPLC peaks are due only to the tagged peptide and its two specific binding partners. Taken together, these data provide strong evidence for the designed assembly.

Conclusions

The data presented above demonstrate that the combined design principles of hydrophobic core steric matching and hydrophilic interface electrostatic matching can be used to promote complicated self-assembled peptide systems. Assemblies containing matched cores but two or three mismatched hydrophilic interfaces are unstable, as are those containing a single Glu/Glu e/g mismatch. In contrast, a single Lys/Lys mismatch is tolerated sufficiently to permit stable complex formation, as is a single core alanine layer in systems with matched electrostatics. Though both of these complexes are less stable than the fully matched case, they provide additional flexibility in complex design. The use of simultaneous hydrophobic/hydrophilic design in the construction of sophisticated assemblies was illustrated by formation of three specific heterotrimers from a mixture of six peptides. This capacity for control of multiple assembly events augers well for the future design of specific and intricate peptide structures.

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.[11] Each peptide was purified by reverse-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 ($\epsilon_{270} = 18\,069$).

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 two-degree steps, at a 2 deg/min rate of increase with 1-min equilibration and data averaging at each temperature. T_m values were obtained from minima of first derivatives of θ vs $1/T$ plots.[12] 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 by 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 38 000 and 48 000 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.[13] Data were analyzed by using Origin and fit to ideal single-species 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 superanant. 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.

Supporting Information Available: Detailed molar elipticity, 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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