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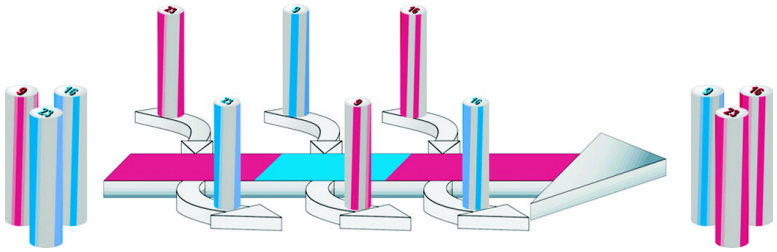

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Sequential and Specific Exchange of Multiple Coiled-Coil Components

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Abstract: The capacity for sequential and specific exchange of single peptides from coiled-coil heterotrimers is investigated. Dual hydrophobic−hydrophilic interface systems permit iterative cycles of pH-triggered strand exchange that can specifically replace one, two, or even all three initial trimer components. The resultant new complexes are either resistant to or capable of further exchange. Control experiments demonstrate that background exchange among different complexes is negligible. When triggered, however, selective displacement of the same peptide from only one of two distinct heterotrimers is feasible. Previously documented peptidic cross-linking strategies remain operative in these more intricate environments.

Introduction

The design of intricate self-assembled systems requires fine control of component interactions. Sensitivity to easily manipulated macroscopic variables (e.g., temperature, pH) is a powerful route to such control. The influence of many such factors on biopolymer folding has been well explored, and the resulting established knowledge base holds promise for their use in subtle structural tuning. Several simplifying features of α-helices in general, and of their assembly via coiled-coil formation in particular, make them an especially appealing biopolymer subclass.[1]

Coiled coils, consisting of two or more entwined helical strands, are ubiquitous modulators of protein−protein association.[2] The signature heptad repeat sequence (*abcdefg*) is now well established, as are the contributions of hydrophobic core (*a*/*d*) and electrostatic interface (*e*/*g*) residues to overall stability and specificity.[3] In general, "knobs into holes" packing of hydrophobic *a*/*d* side chains (commonly isoleucine, leucine, valine) provides the driving force for complex formation, with electrostatic matching of *e*/*g* side chains (commonly glutamic acid, lysine) forming a secondary recognition interface. In many systems a single polar core substitution (asparagine, glutamine) also contributes to specificity, albeit at the cost of stability.[4]

We have described an alternative means to control specificity with core residues, in which steric matching of bulky cyclohexylalanine and small alanine side chains governs the formation of 1:1:1 coiled-coil heterotrimers. Stable complexes permit juxtaposition of two alanines and one cyclohexylalanine at each modified core layer.[5,6]

We have further demonstrated that this specificity strategy can be coupled with known principles of *e*/*g* electrostatic matching to install two orthogonal recognition controls.[5c] Optimal complexes are formed by matching hydrophobic core residues as above, while aligning electrostatic interfaces to contain Glu/Lys pairing at each of three *e*/*g* surface contacts. The only alternative complexes stable at neutral pH contain a single Lys/Lys *e*/*g* mismatch. The need to satisfy two independent interfaces permits construction of well-defined complexes from even complicated component mixtures.

(1) (a) Woolfson, D. N. *Curr. Opin. Struct. Biol.* **2001**, *11*, 464−471. (b) Oakley, M. G.; Hollenbeck, J. J. *Curr. Op. Struct. Biol.* **2001**, *11*, 450−457. (c) Micklatcher, C.; Chmielewski, J. *Cur. Opin. Chem. Biol.* **1999**, *3*, 724−729. (d) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779−819 and references therein.

(2) (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.

(3) (a) De Crescenzo, G.; Litowski, J. R.; Hodges, R. S.; O'Connor-McCourt, M. D. *Biochemistry* **2003**, *42*, 1754−1763. (b) Arndt, K. M.; Pelletier, J. N.; Muller, K. M.; Pluckthun, A.; Alber, T. *Structure* **2002**, *10*, 1235−1248. (c) Meier, M.; Lustig, A.; Aebi, U.; Burkhard, P. *J. Struct. Biol.* **2002**, *137*, 65−72. (d) Litowski, J. R.; Hodges, R. S. *J. Biol. Chem.* **2002**, *277*, 37272−37279. (e) McClain, D. L.; Gurnon, D. G.; Oakley, M. G. *J. Mol. Biol.* **2002**, *324*, 257−270. (f) Burkhard, P.; Ivaninskii, S.; Lustig, A. *J. Mol. Biol.* **2002**, *318*, 901−910. (g) Phelan, P.; Gorfe, A. A.; Jelesarov, I.; Marti, D. N.; Warwicker, J.; Bosshard, H. R. *Biochemistry* **2002**, *41*, 2998−3008. (h) Chana, M.; Tripet, B. P.; Mant, C. T.; Hodges, R. S. *J. Struct. Biol.* **2002**, *137*, 206−219. (i) McClain, D. L.; Binfet, J. P.; Oakley, M. G. *J. Mol. Biol.* **2001**, *313*, 371−383. (j) Kohn, W. D.; Kay, C. M.; Hodges, R. S. *J. Mol. Biol.* **1998**, *283*, 993−1012. (k) Harbury, P. B.; Kim, P. S.; Alber, T. *Nature* **1994**, *371*, 80−83. (l) Harbury, P. B.; Zhang, T.; Kim, P. S.; Alber, T. *Science* **1993**, *262*, 1401−1407. (m) O'Shea, E. K.; Lumb, K. J.; Kim, P. S. *Curr. Biol.* **1993**, *3*, 658−667.

(4) (a) Kim, B.-M.; Oakley, M. G. *J. Am. Chem. Soc.* **2002**, *124*, 8237−8244. (b) Knappenberger, J. A.; Smith, J. E.; Thorpe, S. H.; Zitzewitz, J. A.; Matthews, C. R. *J. Mol. Biol.* **2002**, *321*, 1−6. (c) Akey, D. L.; Malashkevich, V. N.; Kim, P. S. *Biochemistry* **2001**, *40*, 6352−6360. (d) McClain, D. L.; Woods, H. L.; Oakley, M. G. *J. Am. Chem. Soc.* **2001**, *123*, 3151−3152. (e) Zhu, H.; Celinski, S. A.; Scholtz, J. M.; Hu, J. C. *J. Mol. Biol.* **2000**, *300*, 1377−1387. (f) Gonzalez, L., Jr.; Woolfson, D. N.; Alber, T. *Nat. Struct. Biol.* **1996**, *3*, 1011−1018. (g) Lumb, K. J.; Kim, P. S. *Biochemistry* **1995**, *34*, 8642−8648.

(5) (a) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* **2001**, *123*, 11 081−11 082. (b) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* **2002**, *124*, 9779−9783. (c) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* **2003**, *125*, 667−671. (d) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* **2003**, *125*, 6364−6365.

(6) Other approaches to heterotrimer design: (a) Kiyokawa, T.; Kanaori, K.; Tajima, K.; Tanaka, T. *Biopolymers* **2001**, *55*, 407−414. (b) Kashiwada, A.; Hiroaki, H.; Kohda, D.; Nango, M.; Tanaka, T. *J. Am. Chem. Soc.* **2000**, *122*, 212−215. (c) Nautiyal, S.; Alber, T. *Protein Sci.* **1999**, *8*, 84−90. (d) Lombardi, A.; Bryson, J. W.; DeGrado, W. F. *Biopolymers* **1997**, *40*, 495−504. (e) Gonzalez, L., Jr.; Plecs, J. J.; Alber, T. *Nat. Struct. Biol.* **1996**, *3*, 510−515. (f) Nautiyal, S.; Woolfson, D. N.; King, D. S.; Alber, T. *Biochemistry* **1995**, *34*, 11 645−11 651.

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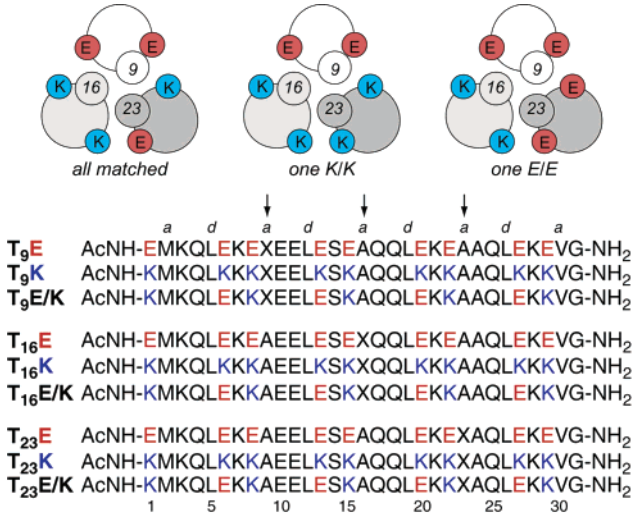

Figure 1. Peptides employed. Each sequence derives from one of three parents (T_9, T_{16}, T_{23}) by replacement of all *e*/*g* residues with Glu (T_nE), Lys (T_nK), or both (T_nE/K), as indicated. Schematic representations of totally matched (T_9E:$T_{16}K$:$T_{23}E/K$), one Lys/Lys (T_9E:$T_{16}K$:$T_{23}K$), and one Glu/Glu (T_9E:$T_{16}K$:$T_{23}E$) trimers are also given. Lettered spheres depict amino acid composition at all *e*/*g* positions, numbers indicate position of cyclohexylalanine (X) in the sequence. Each peptide is N-terminally acetylated (Ac) and C-terminally amidated. The positions of core modification are indicated by arrows. Naming of parent sequences is discussed in ref 5c.

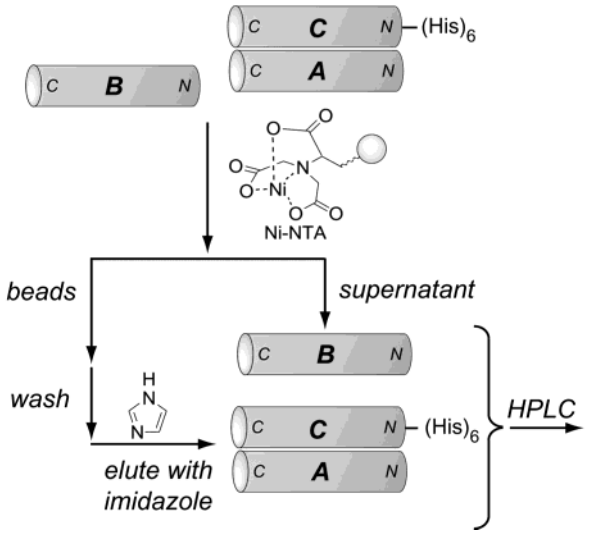

Figure 2. Ni-NTA affinity tag analysis scheme. Initially, peptide **A** is specifically bound to **C**, which bears an N-terminal Gly-Gly-$(His)_6$ affinity tag. Upon exposure to Ni-NTA agarose beads, **C** is bound through the His tag, and **A** is bound through its interaction with **C**. Only **B,** which does not interact with the beads or the tagged peptide, remains unbound. After supernatant removal and washing with blank buffer, bound material is eluted by treatment with imidazole or low pH (2.5). HPLC analysis reveals the identity of unbound (supernatant fraction) and bound (elution fraction) material. Peptides **A** and **B** are intended to represent all binding and nonbinding peptides, respectively. Thus any higher order aggregates are analyzed in the same manner.

Most recently, we have exploited the pH sensitivity of electrostatic interactions to facilitate selective exchange of a single heterotrimer strand.[5d] At low pH, a single Glu/Glu *e*/*g* interface is preferred to a Lys/Lys one. Thus a complex with one Lys/ Lys interaction, when subjected to low pH and an appropriate new peptide, will convert to a complex with one Glu/Glu interaction by strand exchange. Since the requirement for core alignment remains, only a single specific peptide is displaced in the process.

This capacity for selective replacement of a given heterotrimer strand is a unique feature of our dual-interface systems and bodes well for future focused control of assembly. Before undertaking complicated applications, we sought to validate this approach under more challenging circumstances. In particular, we aimed to probe the capacity for multiple exchange events and targeted strand replacement in heterogeneous assembly populations. Here we describe the successful application of pH-driven strand exchange in several more demanding challenges. Sequential and specific replacement of two or all three heterotrimer strands is accomplished, with two variations on the latter, including one that results in a net overall inversion of electrostatic patterning. We also demonstrate the ability to replace one specific strand in one of two different complexes, leaving untouched the same peptide in the other complex.

Results and Discussion

The dual-interface complexes employed are comprised of peptides used in our earlier work (Figure 1).[5c] The central three *a* residues in each sequence (positions 9, 16, 23) contain two alanines (A) and one cyclohexylalanine (X) in one of three possible arrangements: XAA (T_9), AXA (T_{16}), AAX (T_{23}). Derivatives of each core arrangement are equipped with glutamic acid (Glu, E), lysine (Lys, K), or a mixture of both (E/K) at all *e*/*g* positions, forming the electrostatic interfaces. Thus T_9K contains cyclohexylalanine at position 9, alanine at positions 16 and 23, and lysine in all *e*/*g* locations.

To facilitate analysis of strand exchange, two additional peptides ($T_{16}K_{His}$, $T_{23}E_{His}$) were employed.[5c] Each is related to the parent sequence by attachment of an N-terminal Gly-Gly-$(His)_6$ affinity tag. As described previously, this sequence binds specifically to Ni-nitrilotriacetic acid (Ni-NTA) functionalized agarose beads.[5,7] This interaction is exploited according to the following general scheme (Figure 2). Upon exposure of a mixture to Ni-NTA beads, tagged peptides and their specific binding partners adhere to the solid support. The supernatant, containing unbound material, is removed, and after washing with buffer to eliminate residual nonspecific interactions, the bound material is eluted by treatment with imidazole buffer or low pH. HPLC analysis of the supernatant and elution fractions reveals the identity of unbound and bound material, respectively.

Initial efforts at sophisticated strand exchanges targeted two sequential displacements, in which the product complex from a single exchange is subjected to a second pH adjustment in order to displace a different component of the original complex (Figure 3). After a one-step exchange from T_9E:$T_{16}K_{His}$:$T_{23}K$ to T_9E:$T_{16}K_{His}$:$T_{23}E$, a T_9K solution is added to the product complex instead of elution buffer, and the pH is adjusted to 9.3. This results in displacement of T_9E and restoration of a one Lys/Lys complex. The second supernatant trace reveals that T_9E has been selectively displaced in step 2, and the elution fraction reflects formation of the intended complex (T_9K:$T_{16}K_{His}$:$T_{23}E$). As in the one-step experiment, exclusive displacement of T_9E, rather than $T_{23}E$, emphasizes the power of dual interfaces. On simple electrostatic grounds, in the absence of the need for hydrophobic core alignment, either peptide would

(7) Method modeled after: Brown, B. M.; Sauer, R. T. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 1983−1988. We are very grateful to a previous referee (of ref 5a) for initially suggesting this approach.

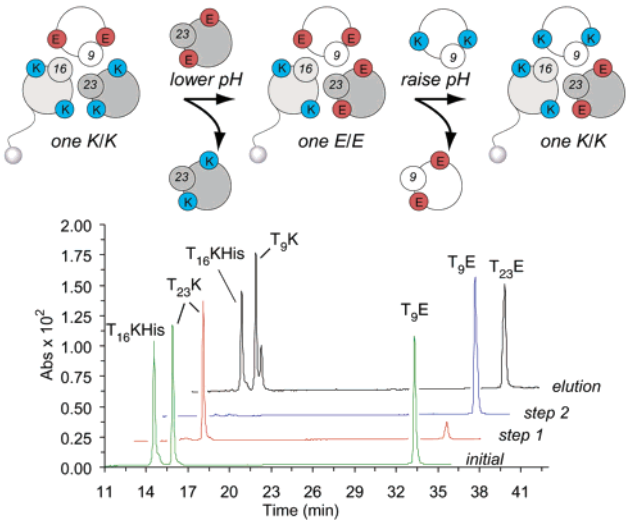

Figure 3. Two-step exchange. (above) Experiment schematic. After formation of T_9E:$T_{16}K_{His}$:$T_{23}E$, addition of T_9K at pH 9.3 affords a new T_9K:$T_{16}K_{His}$:$T_{23}E$ complex by specific displacement of T_9E. (below) Affinity analysis. Supernatants after steps 1 and 2 contain essentially only displaced peptides. Components of final complex are observed in the elution fraction.

be a logical candidate for displacement. Conversely, the exchange process is essentially shut down under conditions that screen electrostatic interactions (e.g., 2 M NaCl; see the supporting information for details).

To further extend the boundaries of sequential replacement, we next focused on a three-step process, in which all of the original heterotrimer peptides are replaced. After performing the two-step process above, two independent third steps were investigated, resulting in the formation of either all matched or one Glu/Glu complexes (Figure 4). The all matched complex prevents subsequent exchange, as it is relatively insensitive to further pH variance. The alternative route, which continues the oscillation between stable Lys/Lys and Glu/Glu contacts, can in principle support additional exchange. The complementary approaches to either stable or dynamic substitution products emphasize the flexibility of these methods. The latter one also results in complete inversion of each original electrostatic interface, providing access to the full continuum of relative charge arrangements.

The feasibility of both three-step processes was confirmed by the usual affinity tag methods (Figure 4). After conversion to the T_9K:$T_{16}K_{His}$:$T_{23}E$ trimer as above, the bound material was treated with either $T_{16}E/K$ or $T_{16}E$ at pH 5.5, affording all matched (T_9K:$T_{16}E/K$:$T_{23}E$) or single Glu/Glu (T_9K:$T_{16}E$:$T_{23}E$) complexes, respectively. Supernatant traces after steps 1 and 2 parallel those in previous experiments. Since the third exchange step actually displaces the tagged peptide, the newly formed complex is now found in the supernatant of step 3. In both cases, the principle components of these traces are as expected. Elution fractions reveal largely the presence of isolated $T_{16}K_{His}$, along with residual amounts of several peptides.

The results above demonstrate that strand exchange in isolated dual-interface heterotrimeric coiled coils can be readily controlled. General utility of these strategies also demands that they operate in the context of heterogeneous assembly populations. Before addressing this issue directly, we sought to determine whether strand exchange in the absence of stimuli would occur among distinct and stable complexes. Such background changes in heterotrimer composition would, of course, be detrimental in systems where triggered exchange was desired.

To test for strand exchange we preformed the T_9E:$T_{16}K_{His}$:$T_{23}K$ heterotrimer (one Lys/Lys *e*/*g* interface) and treated it with the complex formed from T_9K:$T_{16}E/K$:$T_{23}E$ (all matched interfaces) at pH 9. Interchange of the T_{16} derivatives between these complexes generates two new heterotrimers but maintains the overall number of matched/mismatched interfaces (Figure 6). The experiment was performed in three slightly different ways, to avoid artifacts from the analysis method. In addition to simply adding the second complex, a version was performed in which the all matched system contains a single His tag sequence ($T_{23}E_{His}$ instead of $T_{23}E$). If binding to the agarose beads is for some reason critical to exchange, it should be observed in this experiment. Finally, the His tag bearing complex was preincubated with an independent set of Ni-NTA beads, followed by mixing of both bead populations.

The results from all of these experiments demonstrate that independent, preformed, stable complexes do not exchange in the absence of pH triggering, over the time course of these experiments (Figure 6).[8] The supernatant from the first experiment contains only the peptides from the second complex (which are expected as that complex has no affinity tag). The other

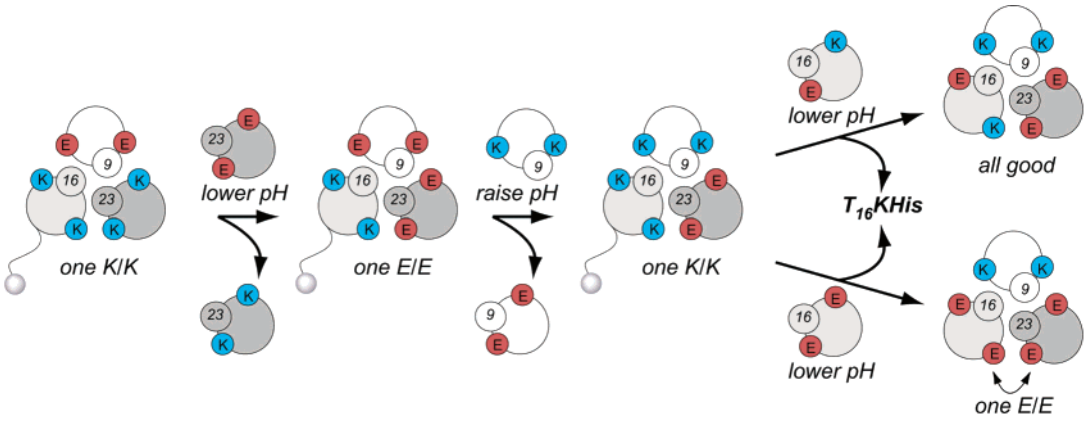

Figure 4. Three-step exchanges. Displacement of the last original peptide ($T_{16}K_{His}$) from the product of two-step exchange (T_9K:$T_{16}K_{His}$:$T_{23}E$, Figure 3) can occur in two different ways. Treatment with $T_{16}E/K$ at pH 5.5 affords a fully matched complex; use of $T_{16}E$ (also pH 5.5) produces a heterotrimer with one Glu/Glu interface.

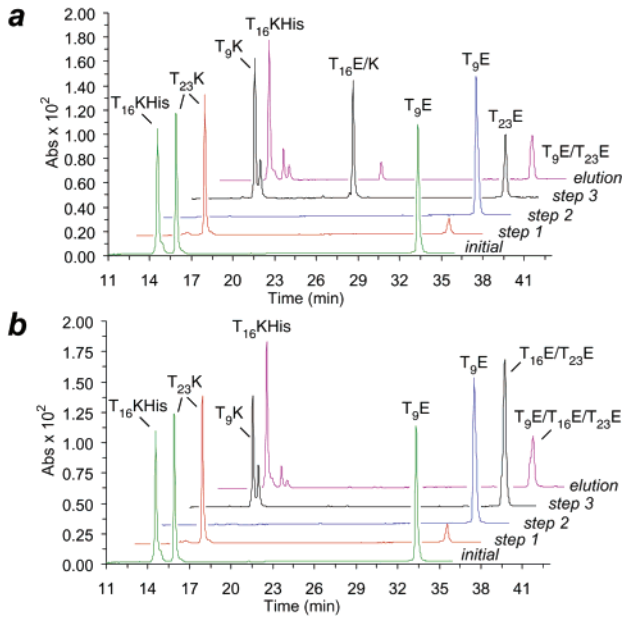

Figure 5. Three-step exchanges. The front three traces are as in the two-step process (Figure 3). Since the tagged peptide is displaced in step three, supernatants contain components of new (a) fully matched (T_9K:T_{16}-E/K:T_{23}E) or (b) single Glu/Glu (T_9K:$T_{16}E$:$T_{23}E$) complexes.

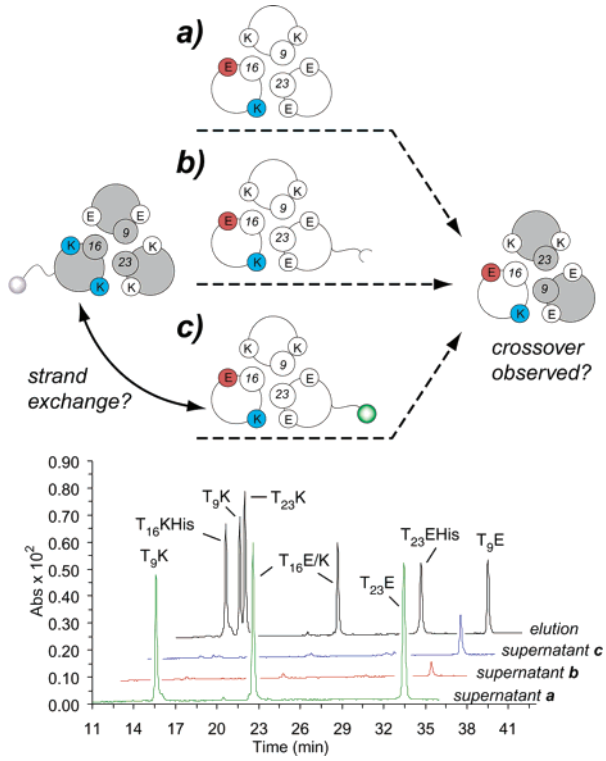

Figure 6. Crossover tests. (above) Experiment schematic. Preformed T_9E:T_{16}KHis:$T_{23}K$ heterotrimer (one Lys/Lys *e*/*g* interface) is treated with all matched complexes that either: (a) bear no affinity tag, (b) have a His tag sequence, or (c) have a tag sequence and are prebound to an independent set of agarose beads. In each case, strand exchange of T_{16}KHis for $T_{16}E$/K creates two new trimers, one of which (T_9E:$T_{16}E$/K:$T_{23}K$) contains no affinity tag. Thus appearance of T_9E and $T_{23}K$ in supernatant fractions indicates crossover. (below) Affinity analysis. In each case, supernatant traces are essentially free of T_9E and $T_{23}K$. Since the added complex in (a) has no affinity tag, its components appear in that supernatant trace. The given elution fraction is representative for traces (b) and (c).

components of the crossover product (i.e., T_9E, $T_{23}K$) are not present. In each of the other cases, where both complexes have

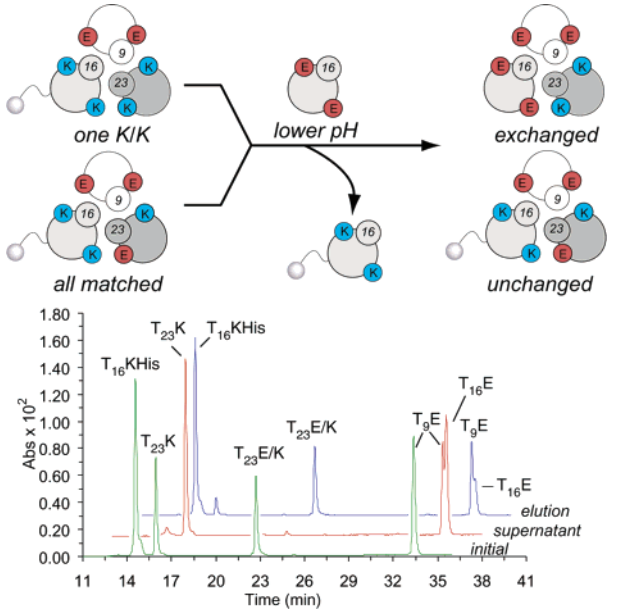

Figure 7. Strand exchange in heterogeneous systems. (above) Experiment schematic. Triggered exchange of $T_{16}E$ for T_{16}KHis in only the one Lys/Lys complex results in formation of a new, soluble heterotrimer (T_9E:$T_{16}E$:$T_{23}K$). (below) Affinity analysis. The supernatant fraction contains $T_{23}K$ and essentially no $T_{23}E$/K. Thus selective displacement of $T_{16}K_{His}$ has occurred only from the desired complex. All traces are normalized to the same height.

an affinity tag, the supernatant contains little or no material. Since the crossover product does not have an affinity tag, it should be observed in the supernatant if it forms. Together these experiments suggest that once formed, independent heterotrimers can coexist without background mixing of their components.

Having demonstrated the baseline fidelity of heterogeneous systems, we began to address selective strand replacement in these contexts (Figure 7). Given an equimolar mixture of all matched and one Lys/Lys heterotrimers, components of the Lys/Lys complex should be selectively exchangeable, as the fully matched system is relatively unaffected by pH modulation.

Affinity analysis of this more sophisticated exchange supports the intended result. A mixture of T_9E:$T_{16}K_{His}$:T_{23}E/K (all matched) and T_9E:$T_{16}K_{His}$:$T_{23}K$ (one Lys/Lys) was treated with $T_{16}E$ at pH 5.5. Since replacement of $T_{16}K_{His}$ by $T_{16}E$ removes the His tagged component from either trimer, any new complexes should appear in the supernatant. In particular, the T_{23} derivatives are unique to each initial complex, so appearance of $T_{23}K$ or $T_{23}E$/K would indicate intended (one Lys/Lys) or unintended (all matched) exchange, respectively. The observed supernatant HPLC trace contains each component (T_9E:$T_{16}E$:$T_{23}K$) of the intended complex, and virtually no $T_{23}E$/K (Figure 7). This result demonstrates essentially total selectivity for strand exchange in one of the two complexes. The ability to replace or ignore the *same peptide* in two different heterotrimers bodes well for more complicated future applications.

We have previously reported the potential for peptidic cross-linking of heterotrimeric coiled coils using bifunctional disulfide linked monomers, in which an N-terminal Gly-Gly-Cys sequence is used to couple two recognition interfaces.[5d] The

(8) Significantly longer equilibration times do lead to considerable exchange (up to ~33% after 24 h). See supporting information for details.

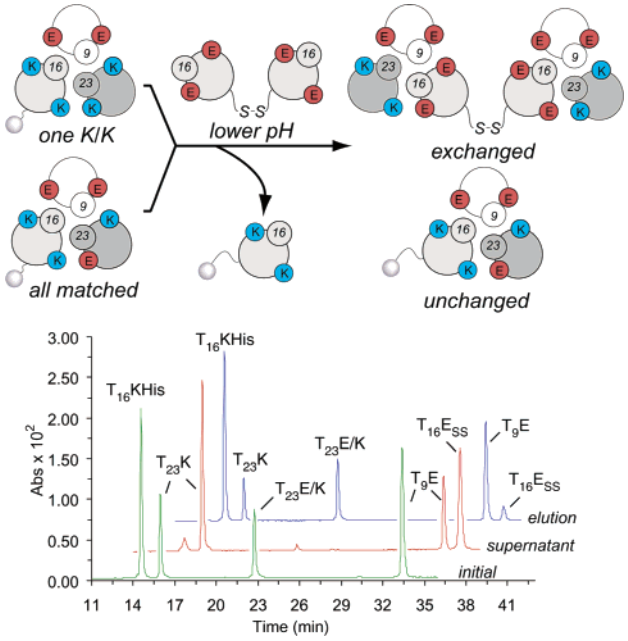

Figure 8. Selective cross-linking. (above) Experiment schematic. Displacement of $T_{16}K_{His}$ by bifunctional peptide $T_{16}E_{SS}$ should occur only in the T_9E:$T_{16}K_{His}$:$T_{23}K$ (single Lys/Lys) complex. The identical peptide in the T_9E:$T_{16}K_{His}$:$T_{23}E/K$ (fully matched) complex is left untouched. (below) Affinity analysis. Appearance of $T_{23}K$ and not $T_{23}E/K$ in the supernatant trace indicates successful selective exchange. All traces are normalized to the same height.

introduction or dissolution of such cross-links by pH adjustment has also been demonstrated. To ensure the continued viability of these strategies in more intricate environments, the same initial mixture of heterotrimers outlined above was treated with a disulfide-linked dimer of $T_{16}E$ ($T_{16}E_{SS}$). In principle, this peptide should also displace $T_{16}K_{His}$ from only the one Lys/Lys complex, resulting in formation of a soluble pentameric species consisting of two cross-linked T_9E:$T_{16}E$:$T_{23}K$ heterotrimers (Figure 8).

Successful formation of the desired cross-linked system was confirmed by the Ni-NTA methods described above (Figure 8). The expected outcome in terms of supernatant versus elution composition is identical to that in the simpler experiment (Figure 7), except that $T_{16}E_{SS}$ should of course appear in place of $T_{16}E$. This is precisely what is observed. Again, the supernatant trace contains no $T_{23}E/K$, supporting the exchange selectivity.

Conclusions

The work described above supports the idea that dual-interface design strategies are appropriate for specific and sequential exchange of single heterotrimer components, even in reasonably complex circumstances and without significant background exchange of stable complexes. The previously demonstrated pH-triggered exchange can be extended to two and even three sequential exchanges in which only the intended peptide is displaced at each step. The three-step process can be terminated by formation of either a fully matched complex that is resistant to further exchange or one that maintains the opportunity for future modification, while completely inverting each electrostatic interface. Finally, a single peptide can be displaced from one of two different heterotrimers, while leaving the identical peptide untouched in the other complex. This strategy can also be extended to formation of cross-linked heterotrimers and represents a significant advance in the capacity for triggered strand exchange. Taken together these data suggest that dual-interface exchange can be used to control composition and stimulus response in reasonably complicated constructs. Its application to more sophisticated systems, such as peptide hydrogels, is currently being investigated.

Experimental Section

All peptides were prepared and characterized as described previously.[5] Ni-NTA agarose beads were purchased as a 50% slurry from Qiagen.

Ni-NTA Affinity Analysis of Exchange Experiments. General Procedure. Ni-NTA agarose slurry (1 mL) is added to a 1.5-mL Eppendorf tube and centrifuged for 1 min, followed by supernatant removal (discarded). Initial peptide solution (1 mL, 20 μM total peptide concentration) is added to the beads, followed by in situ pH adjustment with 1 M NaOH or HCl (<1 μL). The Eppendorf tube is repeatedly inverted for 5 min and centrifuged for 1 min, followed by supernatant removal (initial fraction). The beads are then washed with 1 mL of PBS buffer (10 mM phosphate, 150 mM NaCl) of appropriate pH by repeated inversion for 30 s, centrifugation for 1 min, and supernatant removal (wash fraction). Peptide exchange solution (1 mL, 6.66 μM total peptide concentration, PBS buffer at appropriate pH) is added and final pH is adjusted as above. Exchange is effected by repeated inversion of the Eppendorf tube for 5 min, centrifugation for 1 min, and supernatant removal (supernatant fraction). The wash/exchange procedure is repeated as necessary. Following the final wash/exchange sequence, all remaining bound peptides are eluted by addition of PBS buffer at pH 2.5 (elution fraction). In some cases where the final wash step was run at high pH (8−10), additional acid (<1 μL 1 M HCl) was needed to elute all material left on the beads. Each fraction is analyzed by reverse-phase HPLC: C-18 column, linear gradients of solvent A (1% acetonitrile in water, 0.1% v/v CF_3CO_2H) and solvent B (10% water in acetonitrile, 0.07% v/v CF_3CO_2H).

Two-Step Exchange. Initial solution equimolar T_9E:$T_{16}K_{His}$:$T_{23}K$ (pH 9.1). First exchange solution $T_{23}E$ (pH 5.5); second exchange solution T_9K (pH 9.3).

Three-Step Exchange. Initial solution equimolar T_9E:$T_{16}K_{His}$:$T_{23}K$ (pH 9.1). First exchange solution $T_{23}E$ (pH 5.5); second exchange solution T_9K (pH 9.4); third exchange solution either $T_{16}E$ (pH 5.5) or $T_{16}E/K$ (pH 5.5).

Mixed Complex Exchange. Initial solution 1:1 T_9E:$T_{16}K_{His}$:$T_{23}K$ to T_9E:$T_{16}K_{His}$:$T_{23}E/K$ (2:2:1:1 T_9E:$T_{16}K_{His}$: $T_{23}K$: $T_{23}E/K$, pH 9.0). Exchange solution either $T_{16}E$ (pH 5.5) or $T_{16}E_{SS}$ (pH 5.5).

Crossover Experiments 1 and 2. Initial solution equimolar T_9E:$T_{16}K_{His}$:$T_{23}K$ (10 μM total peptide concentration, pH 9). Exchange solution 1: equimolar T_9K:$T_{16}E/K$:$T_{23}E$ (10 μM total peptide concentration, pH 9). Exchange solution 2: equimolar T_9K:$T_{16}E/K$:$T_{23}E_{His}$ (10 μM total peptide concentration, pH 9)

Crossover Experiment 3. Ni-NTA agarose slurry, 0.5 mL, was added to two separate 1.5-mL Eppendorf tubes, each were centrifuged for 1 min, and the supernatants were discarded. Initial solution 1 (1 mL of equimolar T_9E:$T_{16}K_{His}$:$T_{23}K$, 10 μM total peptide concentration, PBS buffer pH 9) was added to one set of beads; initial solution 2 (1 mL of equimolar T_9K:$T_{16}E/K$:$T_{23}E_{His}$ 10 μM total peptide concentration, PBS buffer pH 9) was added to the other set of beads. Both solutions were pH adjusted to 9.3, repeatedly inverted for 5 min and centrifuged for 1 min, followed by supernatant removal (discarded). PBS buffer (0.5 mL, pH 10) was added to each tube and the two sets of beads were mixed together via syringe (final pH = 9.5). The mixed beads were then repeatedly inverted for 5 min and centrifuged for 1 min, followed by supernatant removal (supernatant fraction). Following a

wash step (PBS buffer, pH 9), the bound material was eluted with PBS buffer (pH 2).

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Supporting Information Available: Details of high salt and longer term experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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