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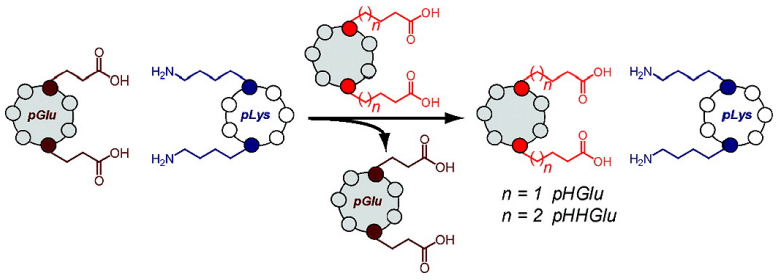


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# Variable Stability Heterodimeric Coiled-Coils from Manipulation of Electrostatic Interface Residue Chain Length

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***Abstract:*** The design of variable-stability coiled-coil heterodimers is described. The electrostatic interface between helices, formed by contact between side chains in heptad *e*/*g* positions, is manipulated to produce complexes ranging in stability from ones that are essentially unstructured to those that cannot be thermally denatured. The tuning is accomplished by incremental extension or contraction of parent glutamic acid and lysine side chains by single methylene units, producing peptides that bear either carboxylic acids or amines separated from the peptide backbone by one to four $CH_2$ groups. Detailed examination of all homodimers and electrostatically compatible heterodimers generates interesting combinations, particularly those in which longer-chain acids are incorporated into peptides paired with lysine-bearing ones. The discovery of very stable dimers allows exchange experiments in which one strand of an original heterodimer is specifically replaced by an added one, even in cases where the original complex features the native-like glutamic acid/lysine pairing. The reported results add significantly to the available design templates for coiled-coil construction and enable the future implementation of various triggered-recognition strategies.

## Introduction

Noncovalent assembly of various biopolymers underlies much of both functional and pathological biology. Accordingly, considerable recent attention has focused on dissecting and manipulating such events. Investigation of simple model assembly systems can contribute long-term impact by unearthing fundamental phenomena upon which to construct future designs and can provide short-term impact by facilitating the directed assembly of tethered cargos. The α-helical coiled-coil, derived from intertwining of two or more helical peptide strands, is a particularly attractive scaffold for inquiry.

The extensive cellular occurrence of coiled-coils has engendered considerable scrutiny of their structure−function profile.[1,2] The impressive stability of most natural and designed systems owes largely to formation of a densely packed hydrophobic core, whose constituent residues establish a repeating 4−3 pattern in the peptide sequence, contributing the *a* and *d* elements of a so-called heptad repeat (*abcdefg*). The other significant positions in this arrangement are the *e* and *g* slots, whose side chains are sufficiently juxtaposed in the folded complex to permit interhelical contact, typically involving matched electrostatic interactions (see Figure 1 for an example). Not surprisingly, glutamic acid (Glu)/lysine (Lys) pairs, observed even in early structural descriptions of a prototypical coiled-coil from GCN4, have dominated the study of natural and synthetic complexes.[2p]

Early work from the Kim lab established that persubstitution of *e*/*g* positions with either Glu or Lys, and inclusion of a buried core asparagine (Asn), reliably favored heterodimer formation from equimolar mixtures of the resultant peptides (termed acid-p1 and base-p1).[2o] Although some controversy ensued about stability gains from surface salt bridges in the heterodimer, destabilization of the competing homodimers (presumably through electrostatic repulsion) was clearly demonstrated.[3] The utility of the acid/base heterodimer in the programmed assembly of desired components has subsequently been established in numerous contexts.

The evident value of this design, and related ones based on other Glu/Lys contact patterns, suggests that increasing the available diversity of specific electrostatic recognition partners should allow for still more sophisticated applications. In particular, tuning of complex stability without sacrificing

(1) Reviews: (a) Woolfson, D. N. *Adv. Protein Chem.* **2005**, *70*, 79−112. (b) Lupas, A. N.; Gruber, M. *Adv. Protein Chem.* **2005**, *70*, 37−78. (c) Oakley, M. G.; Hollenbeck, J. J. *Curr. Opin. Struct. Biol.* **2001**, *11*, 450−457. (d) Micklatcher, C.; Chmielewski, J. *Cur. Opin. Chem. Biol.* **1999**, *3*, 724−729. (e) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779−819, and references therein.

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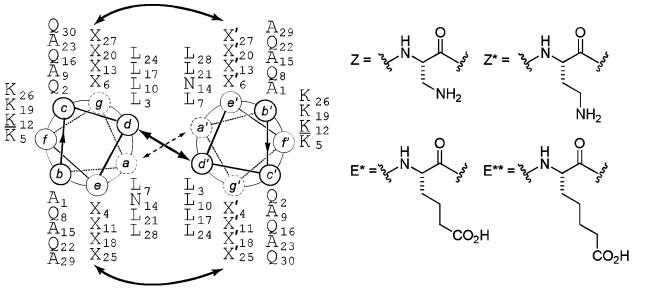
(3) (a) Lumb, K. J.; Kim, P. S. *Science* **1996**, *271*, 1137−1138. (b) Lavigne, P.; Soennichsen, F. D.; Kay, C. M.; Hodges, R. S. *Science* **1996**, *271*, 1136−1137. (c) Lumb, K. J.; Kim, P. S. *Science* **1995**, *268*, 436−439. (d) Zhou, N. E.; Kay, C. M.; Hodges, R. S., *J. Mol. Biol.* **1994**, *237*, 500−512.




| | |
|---|---|
| **pAsp** | Ac-AQLDKDLQALD<u>K</u>DNAQLDKDLQALDKDLAQ-$NH_2$ |
| **pGlu** | Ac-AQLEKELQALE<u>K</u>ENAQLEKELQALEKELAQ-$NH_2$ |
| **pHGlu** | Ac-AQLE*KE*LQALE*<u>K</u>E*NAQLE*KE*LQALE*KE*LAQ-$NH_2$ |
| **pHHGlu** | Ac-AQLE**KE**LQALE**<u>K</u>E**NAQLE**KE**LQALE**KE**LAQ-$NH_2$ |
| **pDap** | Ac-AQLZKZLQALZ<u>K</u>ZNAQLZKZLQALZKZLAQ-$NH_2$ |
| **pDab** | Ac-AQLZ*KZ*LQALZ*<u>K</u>Z*NAQLZ*KZ*LQALZ*KZ*LAQ-$NH_2$ |
| **pDapt** | Ac-AQLOKOLQALO<u>K</u>ONAQLOKOLQALOKOLAQ-$NH_2$ |
| **pLys** | Ac-AQLKKKLQALK<u>K</u>KNAQLKKKLQALKKKLAQ-$NH_2$ |

***Figure 1.*** Peptides used. Helical wheel projection (above left) demonstrates interactions in homodimers (X = X′) and heterodimers (X ≠ X′). Interhelical *e*/*g* contacts are indicated by double-headed arrows. Sequences of each peptide are given below, with nonstandard amino acids represented by the following code (structures of each given above right): Z = 2,3-diaminopropionic acid, Z* = 2,4-diaminobutyric acid, E* = homoglutamic acid, E** = homohomoglutamic acid. The letter O represents 2,5-diaminopentanoic acid (Dapt, ornithine). Underlined lysine side chains are capped with acetamidobenzoyl groups as spectroscopic labels. All peptides were prepared via standard solid-phase methods. Unnatural monomers for pHGlu and pHHGlu were prepared via an olefin cross metathesis method reported previously.[5]

specificity would permit assembly in varied contexts and potentially provide a design hierarchy to enable controlled interconversion of different assemblies. We have been interested in the impact of unnatural side chain structures on coiled-coil stability and assembly specificity and report here the study of Glu/Lys analogues that fulfill these objectives. In particular, variation in side chain length is shown to dramatically impact the stability of both homo- and heterodimeric coiled coils. Examination of peptides whose *e*/*g* residues contain 1−4 methylene units between backbone and polar terminus (ammonium or carboxylate) illuminates many new very stable dimers and allows for specific heterodimer exchange to form new complexes. Especially interesting is the capability to exchange a native-like Glu/Lys interface to a designed one, which may lead to eventual increased control over natural systems.

## Results and Discussion

The acid-p1/base-p1 sequence is the logical context in which to test the impact of *e*/*g* residue chain length. In addition to its broad application, preliminary evidence from disulfide-linked 2,3-diaminopropionic acid (Dap) and 2,4-diaminobutyric acid (Dab) homodimers (and acid-p1/Dap, acid-p1/Dab linked heterodimers) in the original report[2o] suggested that dimer stability was a sensitive function of side chain length. Fairman and coworkers demonstrated a similar effect in tetrameric systems, examining aspartate vs glutamate and ornithine vs lysine.[2k] The single buried asparagine is expected to enforce dimeric parallel structures.[4] Our template dimer structure is thus derived from the acid/base structure, containing leucine (Leu) residues at all but the central hydrophobic core position (occupied by Asn),

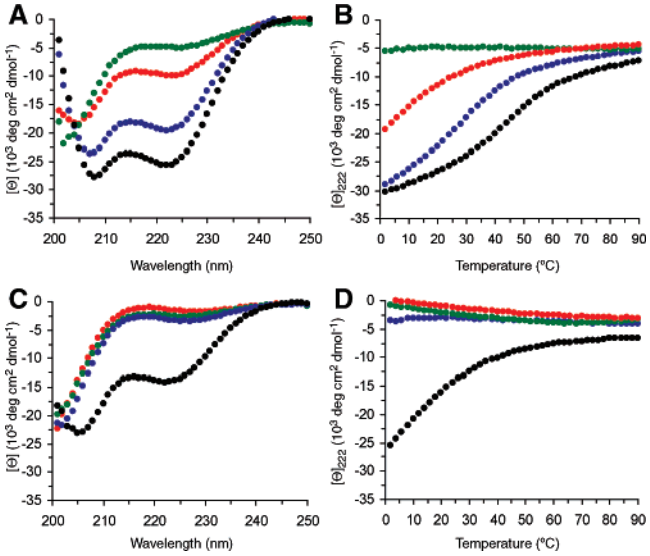



***Figure 2.*** Investigation of homomeric complexes. Wavelength (A, C) and thermal denaturation (B, D) CD spectra for pure solutions of (A, B): pAsp (green), pGlu (red), pHGlu (blue), pHHGlu (black) and (C, D): pDap (green), pDab (red), pDapt (blue), pLys (black).[8]

as well as helix-promoting/solubilizing alanine (Ala), glutamine (Gln), and Lys residues in the solvent-exposed *b*, *c*, and *f* positions (Figure 1). The *e*/*g* residues are then substituted with one of four acidic or basic amino acids, bearing side chains having one to four methylenes between the backbone $C_{alpha}$ and the terminal carboxylic acid (pAsp, pGlu, pHGlu, pHHGlu) or amine (pDap, pDab, pDapt, pLys). The resulting eight homodimers and sixteen heterodimers (excluding the like-charged ones) have been investigated by a variety of biophysical methods, as described below.

Each peptide except pHGlu and pHHGlu was prepared via standard solid-phase methods using commercial amino acids.[9] The requisite unnatural Glu homologues were prepared using our previously reported route.[5] With the peptides in hand, we began our efforts with an examination of homodimer structure and stability. Circular dichroism (CD) spectra of each pure peptide solution in neutral buffer reveal dramatic differences in chain length effects (Figure 2). In keeping with known results, neither pGlu nor pLys solutions exhibit much helical structure or unfolding cooperativity (based on thermal denaturation), although the pLys signal is clearly more significant.[6] However, while all the shorter amino-terminated side chains give rise to peptides with virtually no structure, both pHGlu and pHHGlu solutions display strong helical signatures at room temperature and are moderately stable to thermal unfolding ($T_m$ = 45 °C and 49 °C). Both complexes are significantly more stable at neutral pH than even the reported disulfide-linked acid-p1/acid-p1 homodimer ($T_m$ < 25 °C at pH 7). The behavior of pHGlu and pHHGlu, when compared with pDapt and pLys, suggests that at equivalent methylene spacing, carboxylates are much preferred to ammonium ions for homodimer formation.[7]

(4) (a) Kim, B.-M.; Oakley, M. G. *J. Am. Chem. Soc.* **2002**, *124*, 8237−8244. (b) Zeng, X.; Herndon, A. M.; Hu, J. C. *Proc. Nat. Acad. Sci. U.S.A.* **1997**, *94*, 3673−3678. (c) Lumb, K. J.; Kim, P. S. *Biochemistry* **1995**, *34*, 8642−8648.
(5) Ryan, S. J.; Zhang, Y.; Kennan, A. *Org. Lett.* **2005**, *7*, 4765−4767.
(6) Since isolated coiled coil peptides tend to adopt random coil conformations, sample helicity correlates with degree of complex (i.e., dimer) formation and hence stability.

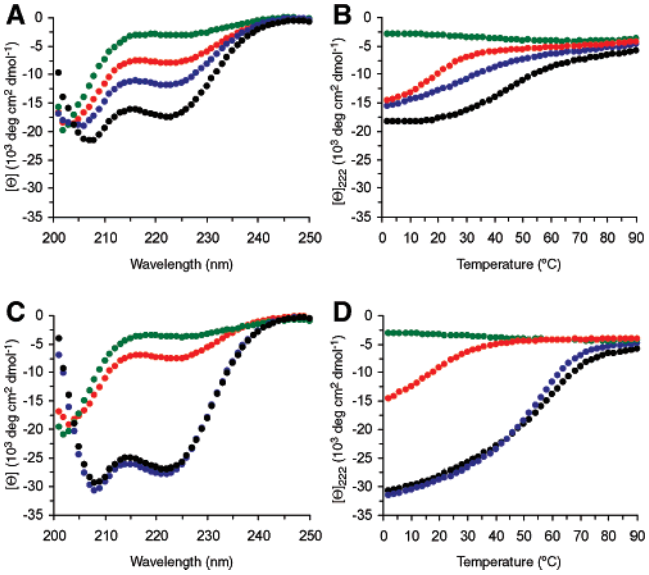



***Figure 3.*** Investigation of pDap and pDab heterodimers. Wavelength (A, C) and thermal denaturation (B, D) CD spectra for equimolar solutions of pDap (A, B) or pDab (C, D) with pAsp (green), pGlu (red), pHGlu (blue), and pHHGlu (black).[8]

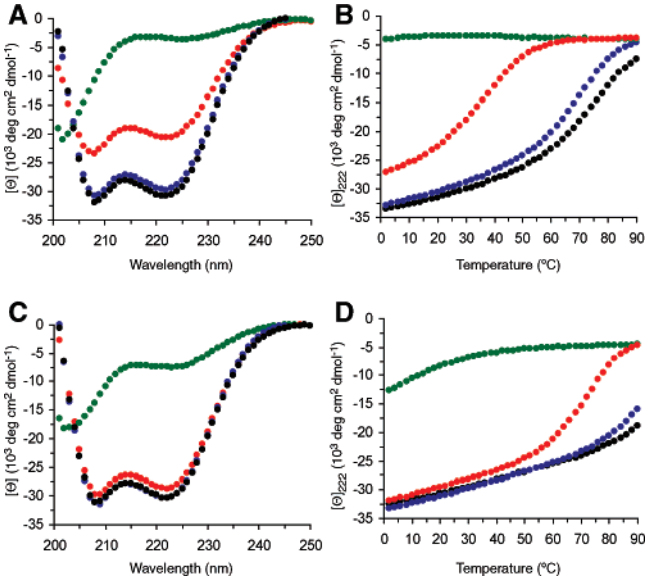



***Figure 4.*** Investigation of pDapt and pLys heterodimers. Wavelength (A, C) and thermal denaturation (B, D) CD spectra for equimolar solutions of pDapt (A, B) or pLys (C, D) with pAsp (green), pGlu (red), pHGlu (blue), and pHHGlu (black).[8]

Emboldened by these results, we moved on to the question of specific heterodimer formation.

Preliminary screening of heterodimer stabilities was conducted by CD analysis (Figures 3 and 4, Table 1). Equimolar solutions of pDap and each of the acidic peptides demonstrate that heteromeric complexes are also stabilized by increasing acid chain length, although even the pDap/pHHGlu solution shows only moderate helicity (and it is not much in excess of the component weighted average spectrum).[9] More dramatic are the

(7) Clearly the two termini are not isosteric, and also differ in relative charge dispersion. Detailed explanations for this phenomenon await more high-resolution structural information.
(8) All wavelength spectra were recorded at 25 °C, and solutions for all CD experiments contained 10 $\mu$M total peptide in PBS buffer (150 mM NaCl, 10 mM phosphate, pH 7.4).
(9) See Supporting Information.

***Table 1.*** Observed Melting Temperatures[a]

| sample | $T_m$ (°C) | sample | $T_m$ (°C) |
|---|---|---|---|
| pHGlu | 47 | pDapt/pHGlu | 75 |
| pHHGlu | 49 | pDapt/pHHGlu | 83 |
| pDap/pHHGlu | 54[b] | pLys/pGlu | 72 |
| pDab/pHGlu | 61 | pLys/pHGlu | >90 |
| pDab/pHHGlu | 67 | pLys/pHHGlu | >90 |
| pDapt/pGlu | 42 | | |

[a] All solutions contain 10 $\mu$M total peptide in PBS buffer at pH 7.4. All mixtures are equimolar (i.e., 5 $\mu$M in each peptide). [b] The trace for this mixture is not much in excess of the weighted component average spectrum (see Supporting Information).

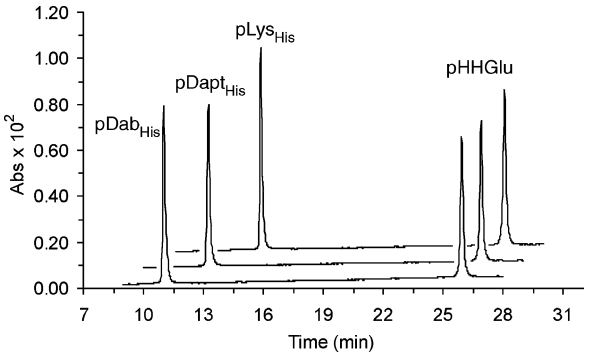



***Figure 5.*** Sample Ni-NTA analysis of heterodimer formation. HPLC traces from 1:1 mixtures of his-tagged basic peptides (pDab$_{His}$, pDapt$_{His}$, pLys$_{His}$) with pHHGlu, after exposure to Ni-NTA agarose beads, washing, and elution with imidazole buffer.

effects of pairing any of the longer amine side chains with the acidic ones. Although mixtures using pAsp as the acidic component gave uniformly poor outcomes, and pGlu is only a mediocre partner for even pDapt ($T_m$ = 42 °C versus 72 °C with pLys), solutions of pDab and either pHGlu or pHHGlu display strongly helical signatures and thermal stabilities approaching those of the parent pGlu/pLys complex ($T_m$ = 61 °C, 67 °C). Even more stable are the combinations of pDapt with pHGlu, pHHGlu ($T_m$ = 75 °C, 83 °C). Finally, the combination of pLys with either pHGlu or pHHGlu produces a very stable structure with melting temperatures in excess of 90 °C. Together these data support the idea that wide stability variations can be tuned by adjustment of side chain length and confirm the existence of heterodimers with stabilities well in excess of the parent Glu/Lys interaction.

Since CD is a poor tool for distinguishing oligomerization states, we conducted Ni-NTA (nitrilotriacetic acid) affinity experiments to support heterodimer formation in mixtures that exhibited reasonable stability: pGlu with pDapt or pLys, and pHGlu or pHHGlu with pDab, pDapt, or pLys (Figure 5).[9] Tagged versions of each basic peptide (pDab$_{His}$, pDapt$_{His}$, pLys$_{His}$) were prepared by derivatization with an N-terminal Gly-Gly-(His)$_6$ sequence, and 1:1 mixtures of each derivative with the appropriate acidic peptide were exposed to Ni-NTA agarose beads. For all but the weakest complex (pGlu/pDapt), material eluted from the beads was equimolar in both tagged and untagged components, as expected for heterodimers (Figure 5).

More extensive support for general heterodimer formation was obtained from analytical ultracentrifugation experiments (Table 2). Equimolar mixtures of pDab, pDapt, and pLys with pHGlu or pHHGlu all gave observed $M_r$ values consistent with those calculated for the appropriate heterodimer, as did a pDapt/

***Table 2.*** Molecular Weights from Sedimentation Equilibrium[a]

| sample | $M_r$(calcd) | $M_r$(obsd) | sample | $M_r$(calcd) | $M_r$(obsd) |
|---|---|---|---|---|---|
| pHGlu | 7560 | 6839 | pDapt/pGlu | 7216 | 6819 |
| pHHGlu | 7786 | 7152 | pDab/pHHGlu | 7329 | 7818 |
| pDab/pHGlu | 7216 | 7603 | pDapt/pHHGlu | 7441 | 7914 |
| pDapt/pHGlu | 7328 | 7886 | pLys/pHHGlu | 7553 | 7837 |
| pLys/pHGlu | 7438 | 8137 | | | |

[a] $M_{r\ obs}$ values were obtained from fits using an average partial specific volume for nonstandard amino acids.[9] $M_{r\ calc}$ values reflect expected dimer molecular weights.

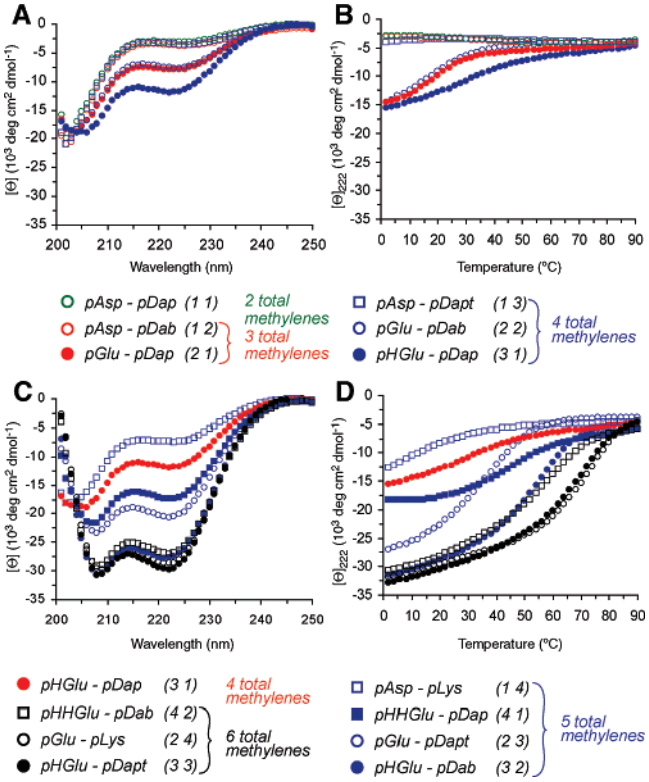



***Figure 6.*** Comparison of complexes with two to six total methylene spacer units. Wavelength (A, C) and thermal denaturation (B, D) CD spectra for equimolar solutions as indicated. The most helical/stable trace (red) from A, B is included in C, D as a reference point.[8]

pGlu mixture. Similar results were obtained from pure solutions of pHGlu or pHHGlu, supporting homodimer formation in those cases.

To look for general trends in optimal side chain lengths, comparisons of the above CD data were made according to total methylene count (the combined number of methylenes present on both side chains). Perhaps not surprisingly, there is no clear optimal number, although it seems that complexes whose *e*/*g* side chains bear less than five total methylenes are disfavored (Figure 6A, B). Substantial stability variance is observed in structures with five or six total methylenes (Figure 6C, D), with few clear trends. Although the most stable dimers contain seven or eight spacer units (Figure 7), the pHHGlu/pDapt complex unfolds at 83 °C, while the pHGlu/pLys dimer does not denature, despite having an identical total side chain length. Overall, stability increases with chain length, but optimal pairings must also have additional determinants.

Having firmly established the significance of side chain length variance in stabilizing homo- and heterodimeric coiled coils, we sought to extend our control over the assembly process to include strand exchange. Specifically, we examined

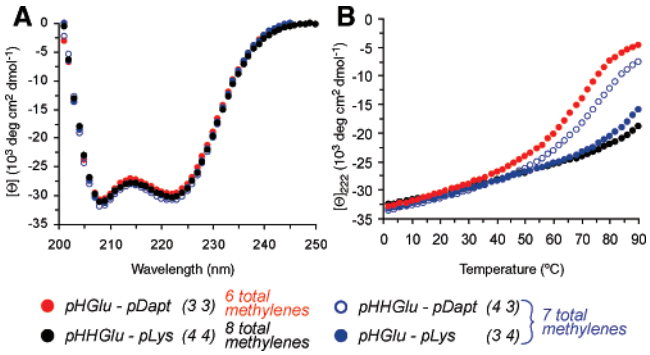



***Figure 7.*** Comparison of complexes with six to eight total methylene spacer units. Wavelength (A) and thermal denaturation (B) CD spectra for equimolar solutions as indicated.[8] The most stable/helical trace (red) from Figure 6 is included as a reference point.

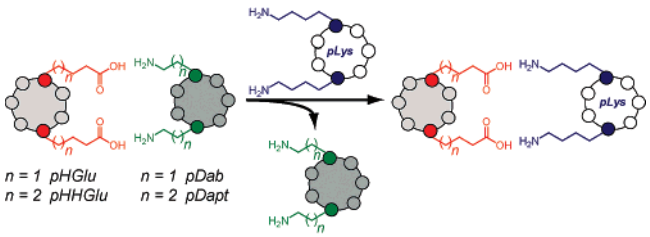



***Figure 8.*** Displacement of pDab or pDapt. Cartoons represent peptides with indicated side chains in all *e*/*g* positions. Treatment of an initial pHGlu/pDab or pHGlu/pDapt heterodimer with pLys results in exchange to form a more stable complex.

whether the extremely stable pHGlu/pLys or pHHGlu/pLys dimers could be generated from less stable precursors. Addition of pLys to either the pHGlu/pDab or pHGlu/pDapt heterodimer was expected to displace the original basic strand and form the pHGlu/pLys complex, while starting from pHHGlu/pDab or pHHGlu/pDapt should give the pHHGlu/pLys dimer (Figure 8).[10]

As in previous exchange experiments, complex formation was determined by Ni-NTA analysis.[11] Initial solutions paired pHGlu or pHHGlu with the appropriate His-tagged derivative and were treated with untagged pLys. HPLC analysis of the supernatant fractions in each experiment reveals equal concentrations of pLys and the acidic peptide (pHGlu or pHHGlu), while the elution fractions are dominated by $pDab_{His}$ or $pDapt_{His}$ (Figures 9 and 10). In each case, displacement of the original tagged basic strand results in formation of a more stable untagged complex with pLys, which migrates to the supernatant fraction. The minimal untagged material remaining in the elution fractions demonstrates the exchange efficiency. This process provides a mechanism for controlled switching of stable coiled-coil dimers, potentially useful in the assembly of more complicated aggregates.

Given the numerous applications of Glu/Lys electrostatic interfaces in both natural and designed systems, conversion of the pGlu/pLys heterodimer to a new one could be a particularly useful control mechanism. To test whether this native interaction could be disrupted in similar fashion we again relied on exchanges to form the most stable complexes discovered above, namely pHGlu/pLys and pHHGlu/pLys (Figure 11).[12] Here again, we employ Ni-NTA methods to document the outcome,

(10) The pDab/pDapt complexes with pHHGlu and pHGlu are stable to the assay conditions in the absence of pLys, as shown in Figure 5 and Figure S-1 (Supporting Information), respectively.

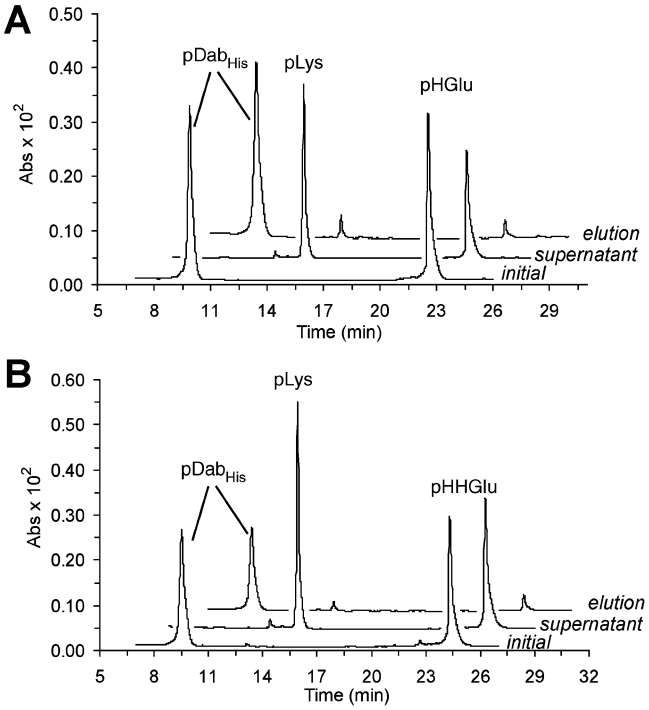



***Figure 9.*** Ni-NTA analysis of pDab$_{His}$ displacement experiments depicted in Figure 8.[9] An initial His-tagged pDab$_{His}$/pHGlu (A) or pDab$_{His}$/pHHGlu (B) heterodimer is converted to a pLys/pHGlu (A) or pLys/pHHGlu (B) one by addition of untagged pLys. Removal of the tagged peptide by strand exchange forces the product complex into the supernatant fraction.

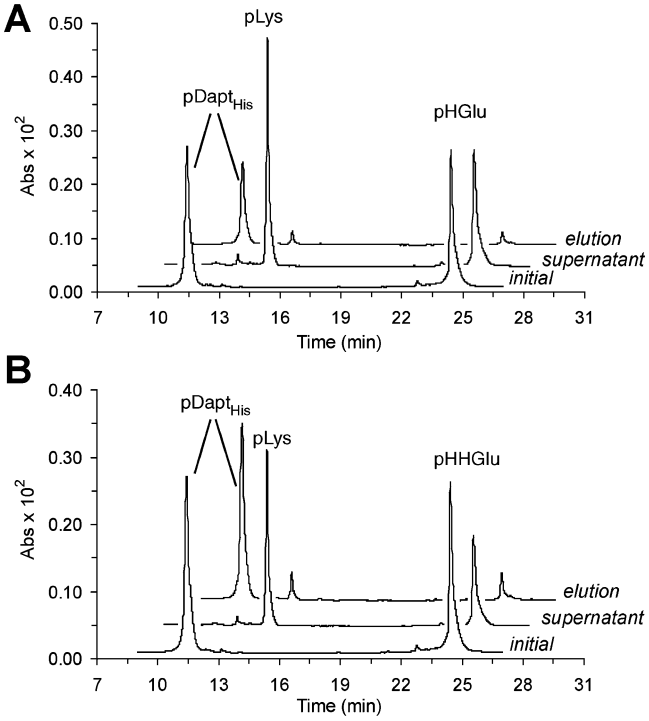



***Figure 10.*** Ni-NTA analysis of pDapt$_{His}$ displacement experiments depicted in Figure 8.[9] An initial His-tagged pDapt$_{His}$/pHGlu (A) or pDapt$_{His}$/pHHGlu (B) heterodimer is converted to a pLys/pHGlu (A) or pLys/pHHGlu (B) heterodimer by addition of untagged pLys. Removal of the tagged peptide by strand exchange forces the product complex into the supernatant fraction.

but since the pLys$_{His}$ derivative had already been prepared it was used as the tagged strand in the original heterodimer with pGlu. Treatment with either pHGlu or pHHGlu should then

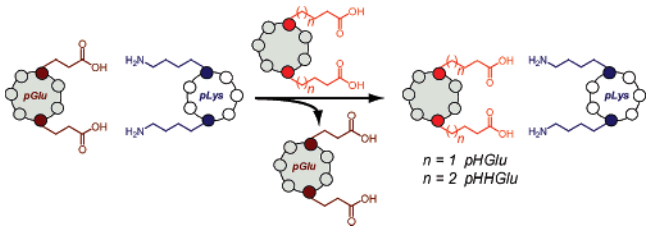



***Figure 11.*** Disruption of a native-like interface. Treatment of an initial pGlu/pLys hetrodimer with pHGlu or pHHGlu results in exchange to form a more stable complex.

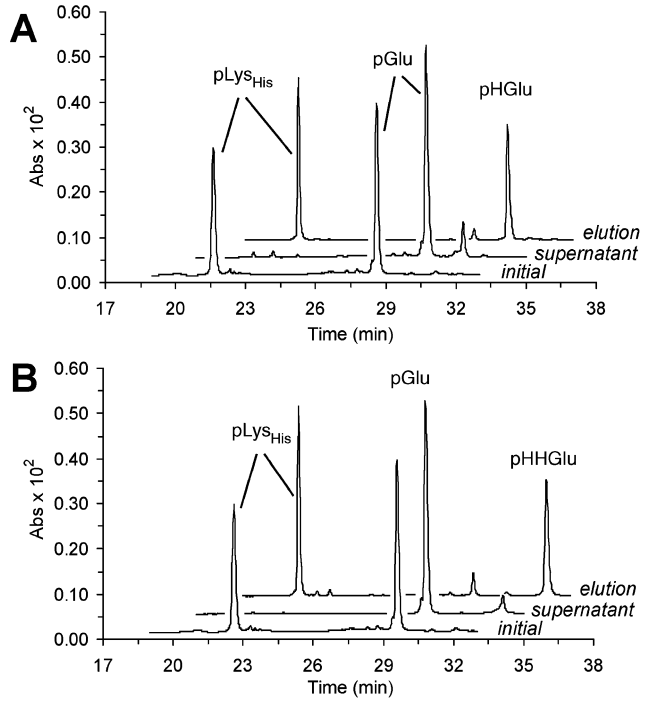



***Figure 12.*** Ni-NTA analysis of pGlu displacement experiments depicted in Figure 11.[9] An initial His-tagged pGlu/pLys$_{His}$ heterodimer is converted to a pLys/pHGlu (A) or pLys/pHHGlu (B**)** heterodimer by addition of the appropriate untagged acidic peptide. Since the displaced pGlu has no tag, it appears in the supernatant, while the added component is specifically retained in the elution fraction by binding to pLys$_{His}$.

displace the pGlu, in an exchange of acidic strands (as opposed to the basic strand exchange above). Since the tag remains in the final dimer, success here is demonstrated by retaining 1 equiv of the added acidic peptide in the elution fraction, as is observed (Figure 12). In this case, the original pGlu peptide is banished to the supernatant fraction, with only residual amounts appearing in the elution fraction. As before, the exchange efficiency is high and confirms that the stability of each final complex is sufficient to disrupt a more biologically relevant interaction.

## Conclusions

The work described above emphasizes the importance of subtle alterations to side chain structure in tuning the stability of dimeric coiled-coils. Although there is no clear optimal total chain length, the general trend of increasing complex stability with chain length is quite powerful. Substitutions of HHGlu

(11) (a) Schnarr, N. A.; Kennan, A. J. *Org. Lett.* **2005**, *7*, 395−398. (b) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* **2004**, *126*, 14447−14451.
(12) In the absence of added pHGlu or pHHGlu the experiment reveals formation of the pGlu/pLys heterodimer (Figure S-1, Supporting Information).

(or even HGlu) for Glu, or Lys for Dab/Dapt, give drastic effects. Clearly optimization of electrostatic contact by positioning of polar termini is only part of the effect, particularly given the associated entropic costs that can in some cases render complexes with such interactions overall less stable than neutral counterparts. It may well be that additional hydrophobic core shielding, especially relevant for somewhat porous dimer structures, is significant (an explanation previously advanced for early chain length experiments). Regardless of the underlying causes, the capability to tune dimer stability with side chain structure expands the landscape of available applications. In particular, the ability to rationally and reliably convert one dimer into another by simple strand exchange techniques, especially when the original structure involves commonly applied Glu/Lys contacts, supports the feasibility of future triggered-recognition events.

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**Supporting Information Available:** Detailed experimental procedures. Additional CD, Ni-NTA, and analytical ultracentrifugation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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