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CGGEIRALKYEIARLKQAKQAKIRALEQKIAALEGGC

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Sequence and Structural Duality: Designing Peptides to Adopt Two Stable Conformations

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Abstract: To improve our understanding of conformational transitions in proteins, we are attempting the de novo design of peptides that switch structural state. Here, we describe coiled-coil peptides with sequence and structural duality; that is, features compatible with two different coiled-coil motifs superimposed within the same sequence. Specifically, we promoted a parallel leucine-zipper dimer under reducing conditions, and a monomeric helical hairpin in an intramolecularly disulfide bridged state. Using an iterative process, we engineered peptides that formed stable structures consistent with both targets under the different conditions. Finally, for one of the designs, we demonstrated a one-way switch from the helical hairpin to the coiled-coil dimer upon addition of disulfide-reducing agents.

Introduction

Large conformational transitions within proteins—by which we mean large relative movements of, or changes in secondary structure—are increasingly being recognized as a means through which protein function (and dysfunction) is elicited and mediated. For example, in the serpins, which include a family of suicide inhibitors of serine proteases, cleavage of the scissile bond leads to the transformation of a native loop into an internal strand of a β-sheet;[1,2] this conformational change cements interactions between the cleaved serpins and their targets. Peptides and proteins that rearrange to amyloid-like structures represent another type of transition in which β-structure is formed;[3] in these cases, unfolded and folded polypeptides, that are not necessarily β-structured, transform to fibers rich in β-sheet. The archetypal example of conformational change involving the rearrangement of largely helical structure is the switching of influenza hemagglutinin into an active form competent for virus-host membrane fusion.[4] On a similar theme, the assembly of certain SNARE-protein complexes, which mediate the fusion of vesicle membranes, is inhibited by the native conformation of one component that must open prior to SNARE-complex assembly.[5,6] Finally, transitions in structures that are accompanied by changes in protein oligomer state and, in turn, protein function include the release and assembly of the trimerisation region and subsequent DNA binding of certain heat shock transcription factors,[7,8] and the dimer-tetramer switch in IF_1 that regulates the mitochondrial ATP synthase.[9] Inspired

by such natural examples, we are attempting the de novo design of peptides that switch structural state.

The examples given above may be regarded as proteins with internal structural conflicts; that is, protein sequences with different accessible folded states. This appears to run against Anfinsen's basic tenet of protein folding that one sequence determines one structure; or, in other words, that proteins adopt the thermodynamically most-stable state.[10] However, the idea is not new: for example, β-sheet-based structures, such as the cross-β structure of amyloid fibers, have long been suspected, and are increasingly being considered, as general solutions for minimizing the energy of any polypeptide chain;[11,12] native influenza hemagglutinin is described as a "spring-loaded, metastable" conformation that is trapped awaiting a trigger to switch to the fusogenic form;[13] the native serpins are referred to as "stressed" states, cleavage of which by the targeted protease brings about a conformational transition to the relaxed state;[1,2] and the inactive forms of certain SNAREs are referred to as "closed" conformations in which the SNARE oligomerization motif is rendered inaccessible.[5,6] In these cases, of course, the structures that are adopted depends not only on the protein sequences—and, in some cases, modifications to these—but also on the prevailing conditions.

Developing this idea, one way to consider designing conformational switches is to set up a structural conflict within a peptide or protein by superimposing motifs for two different

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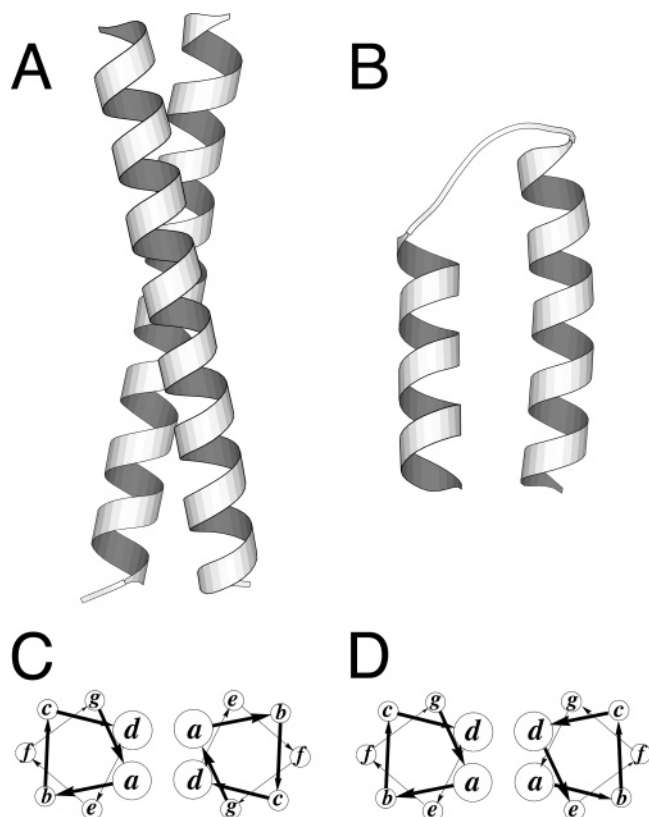
Figure 1. Ribbon structures of the leucine zipper (**A**) a dimeric coiled coil (pdb identifier 2zta[55]) and the helical arm from the seryl tRNA synthase (**B**) an antiparallel, two-stranded coiled coil (pdb identifier 1sry[61]). The latter has been cropped to a region of similar length to the leucine-zipper peptide. These figures were generated using Molscript.[62] Panels **C** and **D** show how the heptad-repeat sequence, ***abcdefg***, maps onto these two types of structure, i.e. parallel and antiparallel two-helix structures, respectively.

structures in a single sequence. The sequence may then be altered in some reversible way and/or the conditions changed to bring about a structural transition. In this way, Anfinsen is not contravened, it is simply that one sequence motif will be 'frustrated' when the alternative structure is adopted. We refer to such polypeptides as having sequence and structural duality. We introduced and tested this concept previously with the design of a leucine-zipper peptide—that is, a sequence that forms a parallel helical dimer, Figure 1A—in which amino acids were substituted to favor an alternative β-hairpin structure.[14] In this case, the switch between the helical-dimer and β-structured states was brought about by heat and resulted in the formation of amyloid-like fibrils.

On related themes, Minor and Kim have designed a chameleon peptide sequence that adopts different secondary structures in different contexts within the same protein;[15] Regan and co-workers have transmuted one α/β protein fold into an $\alpha\alpha$ fold while maintaining ~50% of the original protein sequence;[16] and others have either engineered, or discovered by serendipity peptide systems that switch conformation.[17−22] Most recently,

Schneider and co-workers have engineered pH-responsive hydrogels, which centers on a peptide that switches state from unfolded to an amyloid-like assembly;[23] Schnarr and Kennan show that the helix composition of trimeric coiled-coil systems is sensitive to pH;[24] and Kammerer and colleagues have designed a second coiled-coil system that converts to amyloid-like structures upon heating.[25]

The goal of the study described herein was to design a peptide that switched conformation upon simple chemical modification. Our target conformations were a parallel coiled-coil dimer and an antiparallel two-helix coiled coil (helical hairpin), Figure 1, and the trigger for the switch was an intramolecular disulfide bridge: cysteine residues placed at the termini of the sequence could either be left reduced to favor a parallel coiled-coil dimer, or oxidized intramolecularly to form a hairpin and favor the antiparallel structure. Thus, if achieved, a switch would change both the helical content and the oligomer state of the peptide. While good rules are available that allow confident rational designs of parallel coiled-coil peptides (particularly leucine zippers),[14,26−28] similar rules for antiparallel coiled coils are only just being explored;[29−31] although successful designs for antiparallel coiled-coil peptides are being reported.[29,30,32−35] Because of this, our approach in the design of a switch was iterative: a parent peptide, Coiled-coil-Switch Peptide-1 (CSP-1), was designed as a canonical dimeric leucine zipper; small changes were then made sequentially in an attempt to stabilize the helical-hairpin form, while minimizing the destabilization of the parallel (leucine-zipper) state. Various iterations were characterized in solution using circular dichroism (CD) spectroscopy and analytical ultracentrifugation (AUC). In this way, we were able to incorporate and test modifications designed to favor one state or the other and evolve the design. This process culminated in designs with characteristics fully compatible with the desired target structures in the appropriate states: in the reduced and oxidized states, the peptides formed helical dimers and monomers, respectively; the structures were predominantly α-helical; and both folded states showed sigmoidal thermal unfolding transitions consistent with the formation of unique, cooperatively folded species. Finally, for one of the later designs, we demonstrated another objective of this project by effecting a switch from the oxidized, helical hairpin to the reduced, coiled-coil state by adding of disulfide reducing agents and monitoring by CD spectroscopy.

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Table 1. Sequences of the CSP Designs. Mutations between Successive Iterations Are Highlighted Bold

		Peptide sequence and underlying coiled-coil repeat
Design iteration	Peptide name	*gabcdefgabcdefghijkabcdefgabcde*
1	CSP-1	CGGEIRALKYEIARLKQ----KKRALEQKIAALEGGC
2	CSP-2	CGGEIRALKYEIARLKQ**GGGG**KKRALEQKIAALEGGC
3	CSP-3	CGGEIRALKYEIARLKQ**AKQAKI**RALEQKIAALEGGC
4	CSP-4	CGGEIRALKYEIARLKQ**AKQAEI**RAL**K**Q**E**IAAL**K**GGC
5	CSP-5	CGG**K**IRAL**EY**KIARL**E**Q**AKQAKI**RALEQKIAALEGGC
6	CSP-6	CGGEIRALKYEIARLKQ**AAQAKI**RALEQKIAALEGGC
7	CSP-7	CGGEIRALKYEIARLKQ**AAQAKK**RALEQKIAALEGGC

Results and Discussion

Design Principles. The hydrophobic cores of the coiled coils are made up in layers. The usual heptad sequence motif, ***abcdefg***, of the coiled coil contributes every 1st and 4th site (***a*** and ***d***) to the core, Figure 1C. In parallel, homodimeric coiled coils—such as the targeted leucine-zipper structure—each layer comprises a symmetrical pair of side chains from each strand; i.e., both ***a***, or both ***d***. The choice of residues at these sites largely dictates oligomer-state selection.[26,28] Therefore, CSP-1 was designed as a canonical leucine zipper with all of its ***d*** sites Leu, and all but one of the ***a*** sites Ile, Table 1; the central ***a*** site was made Lys, which is also compatible with parallel coiled-coil dimers, but not with higher-order oligomers.[28]

Sequence-to-structure relationships are less well established for antiparallel two-stranded coiled coils. These structures contain mixed layers of ***a*** and ***d*** residues in which ***a:d*** and ***d:a*** pairs alternate in the core, Figure 1D;[30] n.b. Our distinction between ***a:d*** and ***d:a*** pairs is simply that the first-named position indicates the residues that appears first in the linear sequence. The same combination of Leu at ***d*** and predominantly Ile at ***a***, which favors parallel coiled-coil dimers, also appears to be compatible with antiparallel two-helix structures.[31] The ***a:d*** alignment had clear consequences for our initial design: using a canonical heptad repeat, the turn in the oxidized form of CSP-1 could coincide with an ***abcd*** or a ***defga*** sequence. To give the possibility for the slightly more flexible turn, we targeted the latter, Table 1, which gave alternating ***d:a*** and ***a:d*** layers emanating from the turn. We aimed to achieve this register by appropriate turn design and placement of the Cys residues at the termini. For CSP-1, the turn design built upon the Lys at the central ***a*** site; that is, to destabilize helical structure in this region further, the local sequence was made basic, Table 1.

Inter-helix charge—charge interactions were also incorporated in the designs. In parallel structures, ***g*** and ***e*** residues of successive heptad repeats are brought close in space and flank the core residues, Figure 1C. On this basis, many designs have used oppositely charged ***g:e*** pairs to achieve specific helix-helix interactions;[36−40] four such ***g:e*** pairs were included in the CSP-1 design. In antiparallel coiled coils, however, ***g:g*** and ***e:e*** pairs flank the core, Figure 1D.[29,30,32] To allow for this and to promote

favorable inter-helix charge—charge interactions in both target structures, we simply swapped the polarity of ***g:e*** pairs between the N and C-terminal ends of CSP-1; i.e., from the N to the C-terminus the ***g:e*** pairs read Glu:Lys, Glu:Lys, Lys:Glu, Lys:Glu.

Finally, for each peptide the coiled-coil sequence was flanked by Cys-Gly-Gly and Gly-Gly-Cys tripeptides at their N- and C-termini, respectively, Table 1. This allowed the termini to be linked intramolecularly by a disulfide bond to promote the helical-hairpin state.

The names of the peptides were derived as follows: all were termed CSP for *C*oiled-coil *S*witch *P*eptide; all were given a number, CSP-#, to indicate the design iteration; all were suffixed by "r", "ra", or "o" to indicate whether they were studied with the terminal Cys residues reduced, reduced and alkylated with iodoacetamide, or oxidized as an intramolecular disulfide bridge, respectively.

Characterization of the Parent Design, CSP-1. The parallel coiled-coil template design, CSP-1r, was confirmed as follows. At pH7 and 100 μM peptide concentration, CSP-1r was almost fully helical as judged by circular dichroism (CD) spectroscopy, Figure 2A; compared with the $[\theta]_{222}$ value of $\sim$$-36\,000$ deg cm^2 dmol-res^{-1} for the archetypal leucine-zipper peptide, GCN4-p1,[41] the $[\theta]_{222}$ value for CSP-1r suggests $\sim$90% helix, which is consistent with the central coiled-coil sequence of the peptide being fully folded. Thermal unfolding of the peptide followed by CD spectroscopy was reversible and gave a sigmoidal curve indicative of a cooperative transition, Figure 2B. The transition midpoint, T_M, was 41 $\pm$ 2 °C. Consistent with an oligomerizing system, the T_M was concentration-dependent and decreased at lower peptide concentrations; specifically, at 10 μM peptide the T_M reduced by 12 °C. Sedimentation equilibrium analysis of CSP-1r using analytical ultracentrifugation (AUC) at pH 7 and 5 °C—for these long experiments, the free Cys residues of CSP-1r were alkylated by treatment with iodoacetamide (to give CSP-1ra) to prevent oxidation—revealed a monomer—dimer equilibrium, Table 2: a weighted fit of the data assuming a single ideal species gave a molecular weight of 6885 compared with the monomer M_r 3761; a fit to a monomer—dimer model gave a dissociation constant of 33 μM, Table 2. These data are all consistent with CSP-1r forming a stable coiled-coil dimer as designed.

n.b. CD and AUC measurements with the reduced (but not alkylated) and the alkylated versions of the peptide compared well (data not shown).

Intramolecular oxidation of the Cys residues to give the monomeric, disulfide-cross-linked hairpin, CSP-1o, was confirmed by hplc, mass spectrometry and sedimentation equilibrium analysis. The weighted fit of the sedimentation equilibrium data assuming a single ideal species gave a molecular weight of 3260 compared with the monomer M_r 3643, Table 2. CD measurements indicated limited, partial folding for a 100 μM sample of CSP-1o, consistent with only marginal helix, Figure 2A. This signal changed little upon heating, (Figure 2B) consistent with little, or at best ephemeral structure, and this behavior did not change with peptide concentration. These data indicate that although CSP-1o is monomeric as designed, but that its helical content is low; i.e., CSP-1o does not form a good helical hairpin.

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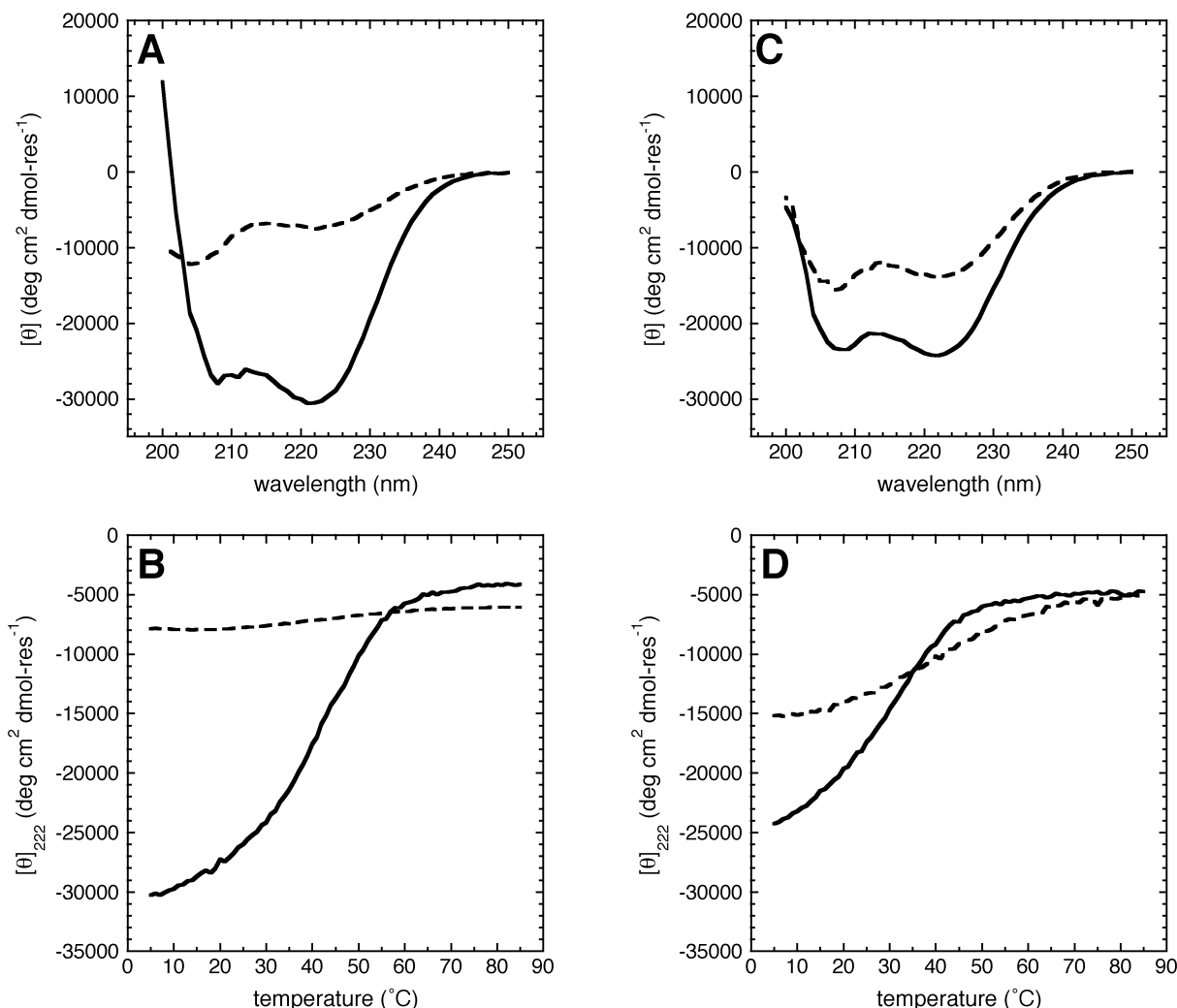
Figure 2. CD spectra and thermal unfolding curves for CSP-1 (panels **A** and **B**), and CSP-3 (panels **C** & **D**) in the reduced (solid lines) and intramolecularly oxidized forms (broken lines). For all of these experiments the peptides were at a concentration of 100 μM.

Table 2. Solution-Phase Molecular Weights and Association Constants for the CSP Designs. Key: NA, Not Applicable; ND, Not Determined

peptide	M_r	MW in solution (Da) (95% confidence limits)	K_D (μM) (95% confidence limits)
CSP-1ra	3761	6885 (6639, 7127)	33 (22, 48)
CSP-1o	3643	3260 (3018, 3495)	NA
CSP-2ra	3988	3901 (3708, 4088)	ND
CSP-2o	3872	3811 (3642, 3975)	NA
CSP-3ra	4143	6652 (6372, 6925)	191 (142, 256)
CSP-3o	4027	3522 (3308, 3731)	NA
CSP-4ra	4143	6949 (6715, 7180)	77 (57, 103)
CSP-4o	4027	3768 (3563, 3968)	NA
CSP-5ra	4143	6819 (6336, 7286)	72 (48, 106)
CSP-5o	4027	3810 (3591, 4023)	NA
CSP-6ra	4086	7148 (6931, 7361)	34 (24, 48)
CSP-6o	3970	3665 (3535, 3791)	NA
CSP-7ra	4101	5481 (5199, 5755)	506 (382, 670)
CSP-7o	3985	3295 (3137, 3449)	NA

Redesigning the Turn Region. One possibility for the poor folding of CSP-1o is that the turn region is too tight. One way to provide more freedom is simply to increase the loop length. However, increased loop lengths tend to destabilize proteins.[42,43]

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Furthermore, in our case, an insertion had also to be compatible with the parallel two-stranded coiled-coil target. Therefore, we chose to add four residues in the middle of the CSP-1 sequence. Regarding the fully helical dimeric target, this potentially added an additional helical turn, and replaced the central heptad of CSP-1 with an eleven-residue (hendecad) repeat. Multistranded, parallel coiled coils are known to tolerate such hendecad inserts.[44−47] Indeed, recently we have shown that for inserts of between 1 and 6 residues in otherwise heptad-based coiled coils, 4-residues inserts are the least destabilizing.[48]

An additional advantage of using mixed heptad-hendecad sequences is that they are should further specify the parallel dimer state by designing against alternative intermolecular antiparallel dimers. This was not a concern for CSP-1 because of the combination of Ile and Lys at *a* and Leu at *d* used in the design.[26,28] However, for canonical heptad repeat sequences with only hydrophobic residues at *a* and *d* (see CSP-3 to CSP-6 below) there is the potential for both parallel and antiparallel

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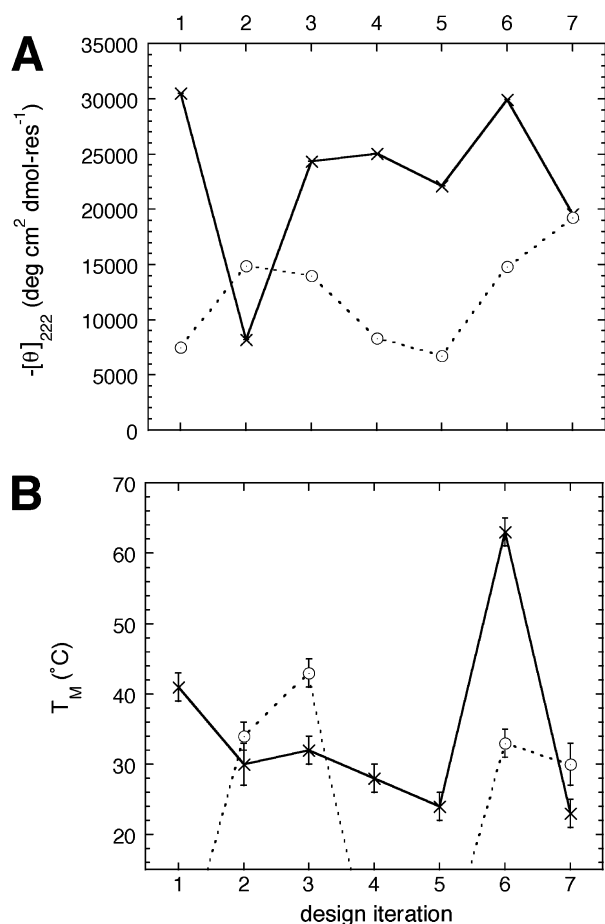
Figure 3. Summary of the helicities and stabilities of the reduced and oxidized variants of the CSP peptides through the design process. (**A**) Helicities, expressed as the absolute value of the CD signal at 222 nm ($-[\theta]_{222}$). (**B**) Stabilities measured as the midpoints of the thermal unfolding curves (T_M). Crosses and solid lines are for the reduced peptides, circles and broken lines are for the oxidized peptides. The solid and broken lines are added simply to guide the eye.

alignment of the resulting hydrophobic layers. The introduction of a hendecad repeat, however, disrupts the contiguous 3,4-hydrophobic spacing and, thus, reduces the possibility for intermolecular antiparallel assembly. Thus, the use of hendecad repeats introduces further negative design,[49,50] which helps discriminate alternate states and select the desired structures.

To begin testing this idea, we inserted tetraglycine after the *f* position of the second heptad of CSP-1 to give CSP-2, otherwise the sequence was unchanged, Table 1. First, we characterized the disulfide-cross-linked version of this iteration, CSP-2o. Sedimentation equilibrium analysis indicated a monomeric species in solution with an effective molecular weight very close to that expected for the monomeric peptide, Table 2. Encouragingly, under the same buffer conditions used for CSP-1o, CD spectra of CSP-2o indicated approximately double the helical signal ($[\theta]_{222}$) of the parent peptide Figure 3A. From this we estimated that CSP-2o was ~40% helical.

As regards thermal unfolding, CSP-2o gave a sigmoidal transition with a midpoint of 34 ± 2 °C, Figure 3B, which was concentration independent. Thus, the tetraglycine insert im-

proved the structure and stability of helical-hairpin state as desired.

The reduced form of the peptide (CSP-2r), on the other hand, was only partially folded as judged from CD spectra (consistent with only ~20% helix), Figure 3A; its thermal unfolding transition showed the latter part of a broad sigmoidal curve, from which we estimated a T_M of 30 ± 3 °C, Figure 3B, but this must be considered a very approximate value; sedimentation equilibrium analysis returned a molecular weight close to that expected for monomer, Table 2, and fits to monomer−dimer models were poor and gave weak dissociation constants in the 10−100 mM range.

Thus for CSP-2, the helical-hairpin form was stabilized, but this was at the expense of the helical-dimer state. Presumably, tetraglycine favors hairpin formation because it breaks the contiguous helical stretch; but, by the same token, it destabilizes the helical dimer because of its flexibility and low helix propensity. Therefore, the next step of the design required us to improve the stability of the helical dimer in the reduced state while keeping sufficient flexibility in the central region to maintain the integrity of the helical hairpin in the oxidized state.

Engineering the Hendecad Insert. A simple step to tighten up the targeted turn region and to improve helical propensity would be to replace the tetraglycine insert with tetraalanine. This works in another of our design systems,[48] but still destabilizes parallel dimers considerably. Therefore, we chose only to replace the two outer glycines with Ala. The second position of the insert is potentially a buried *h* site of the eleven-residue repeat, ***abcdefghijk***.[45,51] On the basis of earlier work on peptides with consensus heptad-hendecad-heptad sequences drawn from a cytoskeleton protein from *Giardia lamblia*,[45] we placed Lys at this site. We made the third position, which should be a solvent-exposed site, Gln to improve solubility and helix propensity. Thus, the final insert chosen for CSP-3 was AKQA. In addition, having introduced another potentially buried lysine in the helical dimer − i.e., at the new *h* site − we replaced the Lys at the *a* site of the original design with Ile, Table 1.

Compared with CSP-1r, CD spectroscopy of CSP-3r indicated slight reductions in helicity, Figure 2C, and thermal stability, Figure 2D. Nonetheless, the thermal unfolding curve was sigmoidal with a T_M of 32 ± 2 °C, Figure 2D. The helix content and stability of CSP-3r was concentration dependent and sedimentation equilibrium analysis revealed a monomer−dimer equilibrium with a dissociation constant of ~200 μM. These data are fully consistent with our previous studies on nonheptad inserts into otherwise canonical leucine-zipper peptides, which are usually destabilizing.[48]

In its oxidized form (CSP-3o), however, the peptide showed considerable improvements in both helicity (compared with CSP-1o) and thermal stability (compared with both CSP-1o and CSP-2o): compare Figure 2A&B with 2C&D; and see Figure 3A&B. Specifically, the CD spectrum, Figure 2C, indicated approximately 40% helix on the basis of the leucine-zipper-peptide metric; and the thermal unfolding curve was sigmoidal with a midpoint of 43 ± 2 °C, which was not concentration dependent. Sedimentation equilibrium analysis confirmed that CSP-3o was monomeric, Table 2.

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Thus, with three iterations of the design process we had achieved our goal; namely to create a peptide sequence that could adopt two different cooperatively folded structures.

It is worth noting that we also oxidized CSP-3r under folding conditions. This did not produce aggregates, but a distinct covalent dimer as confirmed by MALDI-TOF mass spectrometry. This form of the peptide gave a helical CD spectrum ($[\theta]_{222}$ = −28 500 deg cm^2 dmol^{-1}), and a sigmoidal thermal unfolding curve with a single midpoint (T_M = 62 °C). Thus, the covalent dimer is more helical and more stable than the noncovalent dimer. CSP-3r. On this basis, we assume that the covalent dimer is a cross-linked parallel coiled-coil dimer consistent with the design, although this will have to be confirmed by high-resolution structural work.

Influence of the Designed Inter-Helix Salt Bridges. At this stage, having established a reasonable design sequence (CSP-3) in which both target structures could be accessed, Figure 2C&D—for instance, the ΔT_M between the dimer and helical-hairpin forms was approximately 10 °C at 100 μM peptide, Figure 3B—we chose to test the interactions that we had used in the designs. A potential advantage of our system is that the effect of mutations on both the parallel dimers and antiparallel hairpins can be assessed within very similar sequence contexts and, so, compared directly.

To assess the contribution of core-flanking charged residues to the thermal stability of the hairpin state, we prepared control peptides in which pairings at *e* and *g* were swapped to disfavor an antiparallel coiled coil (compare Figure 1C and 1D). Recall that in the parent peptide four oppositely charged *g:e* pairs were introduced to favor parallel dimer formation, and that these were made compatible with the helical hairpin by swapping their polarity halfway through the peptide; i.e., the *g:e* pairs read Glu:Lys, Glu:Lys, Lys:Glu and Lys:Glu along the sequence. To keep these compatible with the parallel dimer design, but to make potentially repulsive *e:e* and *g:g* interactions in the hairpin we swapped polarity to make all the *g:e* pairs either Glu:Lys (CSP-4), or Lys:Glu (CSP-5), Table 1. Otherwise, the sequences were the same as CSP-3. Thus, the compositions of CSP-3, 4 and 5 were identical; we call such peptides *anagram mutations*.

The reduced forms of the peptides, CSP-4r and CSP-5r, were dimeric with helicities closely similar to that of the parent, CSP-3r peptide, Figure 3A. Similarly, although the anagram mutants were less stable than the parent, both showed sigmoidal unfolding transitions from which T_M's could be calculated, Figure 3B. The T_M values for CSP-3r, CSP-4r, and CSP-5r spanned the range 32−24 ± 2 °C and, therefore, were similar. The order of thermal stability was CSP-3r > CSP-4r > CSP-5r. At present, we can offer no explanation for this order.

n.b. The maintained helicities and stabilities of CSP-4r and CSP-5r compared with CSP-3r is also important in light of the early discussion of the possible formation of the alternative intermolecular antiparallel dimer forms of these peptides: the rearranged *e* and *g* positions of CSP-4r and CSP-5r would also lead to all repulsive *e:e* and *g:g* pairings in any intermolecular antiparallel dimers; the fact that these peptides are neither less folded, nor significantly destabilized with respect to CSP-3r, lends further support to the robustness of our design principles.

The effects of the mutations on the oxidized forms of the peptides, CSP-4o and CSP-5o, however, were altogether different: first, both CSP-4o and CSP-5o behaved effectively as

monomers in AUC, Table 2; second, by CD spectroscopy, both CSP-4o and CSP-5o showed only approximately half the helical content of CSP-3o, Figure 3A; third, the unfolding transitions of the two anagram mutations were weak and broad, and it was not possible to determine reliable T_M values from these data.

These results for CSP-4 and CSP-5 are fully consistent with the anagram mutations, which were designed to affect the helical-hairpin (oxidized) states more than the helical dimer (reduced) states: first, the helicities and stabilities of the reduced dimeric forms of the peptides are comparable with those of the parent, CSP-3r; second, and by contrast, the folding and stabilities of the oxidized forms of the peptides are considerably compromised by the mutations. This second finding confirms that one of key design principles that we used to engineer the CSP peptides—namely, the use of oppositely charged pairs at *e:e* and *g:g* to stabilize the oxidized, hairpin forms—does exert a considerable influence on peptide structure and stability.

Further Engineering of the Dual-Structure Peptides. In an attempt to stabilize the parallel dimer, CSP-3r, we decided to replace the lysine at *h* with a hydrophobic residue. Although different types of residue are observed at *h* positions,[44,45] there are very few examples of high-resolution structures for dimeric coiled coils that encompass noncanonical hendecad regions to help guide our choice of residue at *h* more definitively; structures include the nucleotide exchange factor GrpE (PDB identifier 1dkg),[52] and the 2B region of the intermediate filament protein vimentin (PDB identifier 1gk4).[53] In both cases, the $\alpha-\beta$ bonds of the side chains at the *h* positions point directly toward each other. This contrasts with knobs-into-holes packing at *a* and *d* sites of canonical coiled-coil dimers, where side chains form complementary side-by-side interactions.[26,31,54,55] Potentially butting side chains at *h* are not ideal; indeed, SOCKET analyses[31] of the GrpE and vimentin coiled coils indicated that the packing around the noncanonical units is considerably relaxed compared with that in canonical coiled-coil interfaces. For these reasons we chose the small (and helix-favoring) residue Ala for the replacement of the *h* position in the CSP-3 to CSP-6 design iteration, Table 1. Thus, all of the prescribed *a*, *d*, and *h* positions of CSP-6 were hydrophobic. Consistent with this, the helicity and thermal stability of CSP-6r were considerably increased over the other hendecad-containing designs CSP-2r and CSP-3r, Figure 3A&B. Furthermore, the helicity of CSP-6r was comparable to that of the first-generation, pure-heptad-based design, CSP-1r, and its stability was much higher. Importantly, CSP-6r was dimeric like the foregoing designs, Table 2. Interestingly, however, within experimental error both the helicity and stability of disulfide cross-linked form, CSP-6o, were the same as those determined for CSP-3o, Figure 3A&B and compare Figure 2C&D with Figure 4A&B. As a result the difference in thermal stability between the reduced and oxidized forms of these peptides measured at 100 μM peptide, ΔT_M, increased from −11° for CSP-3 to +30 °C for CSP-6.

Our ultimate goal is to engineer tunable conformational switches. This will require low ΔT_Ms between the target states.

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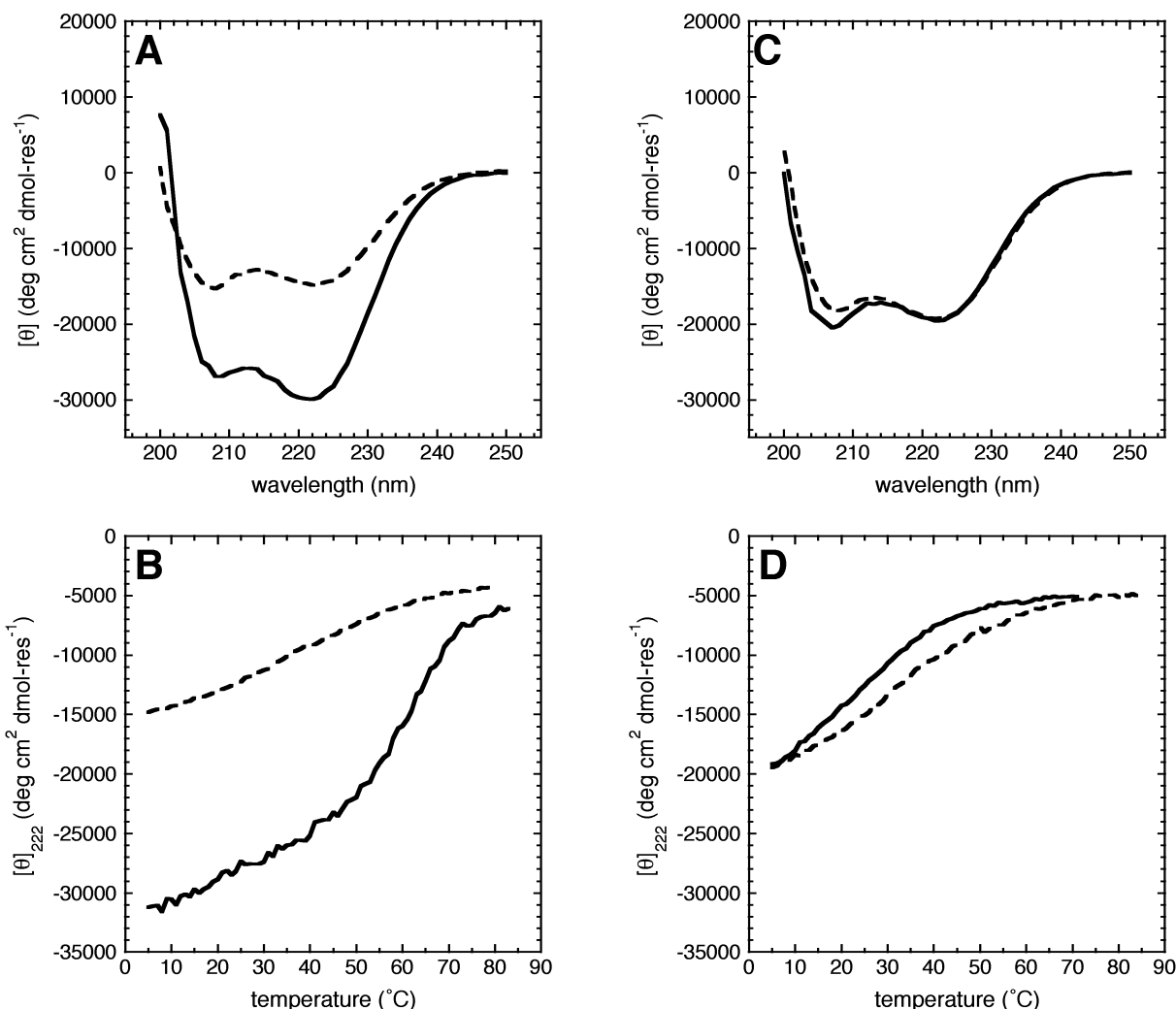(55) O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber, T. *Science* **1991**, *254*, 539−44.

Figure 4. CD spectra and thermal unfolding curves for CSP-6 (panels **A** and **B**), and CSP-7 (panels **C** & **D**) in the reduced (solid lines) and intramolecularly oxidized forms (broken lines). For all of these experiments, the peptides were at a concentration of 100 μM.

Because the reduced peptides dimerize their thermal stabilities can be tuned by changing the concentration; indeed, at the lower peptide concentration of 10 μM CSP-6r remained a fully helical dimer, but with a lowered T_M of 52 $\pm$ 2 °C. To reduce the ΔT_M for CSP-6 by redesign, we attempted to destabilize the parallel dimer form by replacing the Ile at the **a** site immediately following the hendecad unit with Lys—which, as described earlier, is allowed at the **a** sites of parallel coiled-coil dimers—to give CSP-7, Table 1. As expected, this substitution reduced both the helicity and the thermal stability of CSP-7r, Figure 3A&B and Figure 4C&D. The stability of the oxidized form (CSP-7o), however, was affected less. As a result, ΔT_M between the two states was reduced from +30 for CSP-6 to −7° for CSP-7. However, closer inspection of the CD and AUC data for CSP-7r revealed that the peptide was only weakly dimerized: the unfolding curve recorded at 100 μM peptide (solid line, Figure 4D) showed only the later part of a sigmoid suggesting only partial folding at 5 °C, which is consistent with the K_D of high hundreds of μM from the AUC, Table 2.

Thus, as judged by the high helical contents and sigmoidal thermal unfolding curves determined by CD spectroscopy, and by the oligomerization states determined by AUC, designs CSP-3, Figure 2C&D and CSP-6 Figure 4A&B, fulfill our design

prerequisite of peptide sequences that can adopt two distinct and cooperatively folded structures.

Effecting and Monitoring the Conformational Switch. Finally, as our overall objective was to create a conformational switch, we tested the ability of CSP-6 to switch from its oxidized, helical hairpin to its reduced, coiled-coil dimer form. There were two primary reasons for choosing CSP-6 rather than CSP-3 for these experiments: first, both forms of CSP-6 are folded to higher temperatures than the corresponding forms of CSP-3; second, the difference in helical CD signal between CSP-6o and CSP-6r is greater than that for CSP-3o and CSP-3r. Both of these points aided the following experiment, which used CD spectroscopy to follow the switch between the two forms.

100 μM CSP-6o was incubated at 20 °C in the CD spectropolarimeter. Aliquots of disulfide-reducing agent, DTE (dithioerythritol) or TCEP (tris-[2-carboxylehthyl]-phosphine), were added, both using rapid and manual mixing, and the change in $[\theta]_{222}$ was monitored, by stopped-flow and conventional CD spectroscopy, respectively. Typical kinetic traces for these experiments (with TCEP) are shown in Figure 5. Upon addition of the reducing agent there was a rapid gain in CD signal indicative of an increase in helicity. This is consistent with reduction of the disulfide bond of CSP-6o followed by a switch
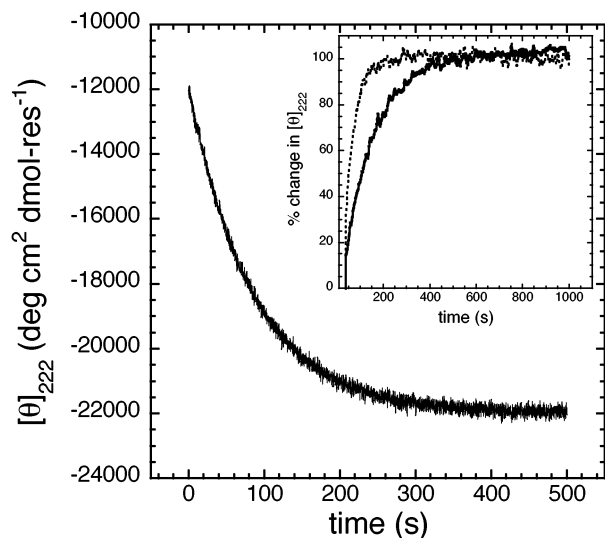
Figure 5. Folding curve following the change in CD signal at 222 nm accompanying the switch from CSP-6o to CSP-6r upon reduction by 2 mM TCEP. This kinetic was followed by stopped-flow CD spectroscopy. The inset shows normalized data for the reaction of CSP-6o with 2 mM (solid line) and 8 mM (broken line) TCEP. These data were from manual-mixing experiments followed by conventional CD spectroscopy.

to the more-helical CSP-6r. Indeed, spectra recorded prior to, and after reduction overlayed with the equilibrium spectra for CSP-6o and CSP-6r, respectively, confirming that the transition was between these two states.

The switch kinetics depended on both the nature and the concentration of reducing agent used: first, TCEP was more efficient than DTE. The half-life for the process at 20 °C with 2 mM reducing agent was 85 s for TCEP, and 16 min for DTE. Second, the rate of the reaction increased with increasing concentration of either reducing agent as is shown for TCEP in the inset to Figure 5. The rates of reaction also increased with peptide concentration. These data indicate that the rate-limiting step in the switching process is the bimolecular reaction of reducing agent with peptide, and that the conformational change itself from the (reduced) hairpin to the dimeric coiled coil is relatively rapid. These conclusions are consistent with literature reports on the kinetics of disulfide-bond reduction by TCEP, and leucine-zipper folding, respectively.[56,57] As a control, we reduced CSP-2o in the CD spectropolarimeter and observed a decrease in $[\theta]_{222}$ intensity, as expected for a transition from folded CSP-2o to the unfolded CSP-2r. The overall rate of this ring-opening and unfolding process was similar (rates were within a fator of 1.5) to the ring-opening and folding process observed for CSP-6o. Furthermore, Arrhenius analyses of the change in rate constant for the CSP-2o and CSP-6o switches with temperature (at 5 mM TCEP) gave similar enthalpies of activation of 42.4 and 34.8 kJ mol^{-1}. These data are again consistent with the ring-opening reduction step being rate limiting.

Summary

Using straightforward sequence-to-structure rules and an iterative design approach we have succeeded in engineering

peptides with sequence and structural duality for a helical dimer and a monomeric, helical hairpin; that is, sequences in which two patterns are superimposed, one compatible with a dimeric leucine zipper and the other for an antiparallel coiled coil. The helical-hairpin state was favored by cross-linking the termini with a disulfide bridge, while the alternative, coiled-coil dimer was favored by keeping the terminal Cys residues reduced. The starting point in the design process was a canonical leucine-zipper template, CSP-1, which was constructed de novo based on established design rules. This peptide formed a dimeric helical structure as expected in the reduced state, but was only poorly folded in the oxidized state. In the next step, we introduced a central nonheptad unit into the peptides (CSP-2 and above). This was a key feature in the success of our design process. The eleven-residue (hendecad) units allowed more flexibility for the turn in the helical hairpin, but were also compatible with the helical-dimer state. Some engineering of the hendecad/turn unit was required to optimize the structures and stabilities of the targeted motifs, but this was achieved in a small number of iterations.

Comparison of the stability data for CSP-3, 6 and 7 is interesting: while the reduced, dimeric forms displayed a broad range of thermal stabilities (the T_Ms were 32, 63 and 23 ± 2 °C, respectively), those for the oxidized states were tighter (43, 33 and 30 ± 2 °C, respectively). Thus, once the design was made compatible with the helical-hairpin target by introducing the hendecad insert, the stability of this structure was less sensitive to sequence changes than the alternative helical dimer. A plausible explanation for this is that in the hairpin state the hendecad acts mostly as a turn to tether the two helical regions together, and possibly contributes little to the hydrophobic core of the structure. In the dimeric form, however, residues at the *a*, *d,* and *h* sites of the hendecad almost certainly do contribute to the hydrophobic interface and, thus, to the overall stability of the structure. A full understanding of this awaits high-resolution structures for the folded designs. Finally, we used the CSP-6 design to demonstrate successfully the switch between the oxidized "closed" form of the peptide and its reduced "open" form. A full kinetic analysis of these switches is in progress and will appear elsewhere.

Our current results suggest that switches between the two structures that we describe could be made, and that these might be tuned by focusing on interactions in the dimer state. With this in mind, CSP-6 displays the biggest difference in thermal stability between the hairpin and dimeric forms (the latter being the more stable). Thus, the peptide in its oxidized, hairpin state might be considered as the most stressed or frustrated of all of the current designs; in other words, there is more stabilization energy to be gained in switching from the oxidized to the reduced form than in the other designs. At this stage, our designs are for a redox-based switch with a disulfide bond as the trigger. Thus, the difference in stabilities of the oxidized and reduced states may provide a route for modulating the redox potential of this bond. In turn, this might allow the development of tunable redox sensors for applications in cell biology.[58] The designs presented here may also offer prospects for engineering conformational switches that respond to more subtle changes in solution conditions such as pH,[4,9,59] and small-ligand binding,[60]

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which potentially open routes to novel peptide-based sensors.[34] In turn, the molecules that we describe could prove useful as "reporter" components of novel biosensors if the conformational switch that we have engineered can be coupled to some optical read-out such as a change in fluorescence.

Experimental Section

Peptide Synthesis. Peptides were made on a Pioneer Peptide Synthesis System (Perspective Biosystems) using standard Fmoc chemistry. They were purified by reversed-phase HPLC and their identities confirmed by MALDI-TOF mass spectrometry. Purified peptides were stored at pH 2, or as lyophilized powders at −20 °C. In the second case, fresh stock solutions were prepared on the day of each experiment. Peptide concentrations were estimated in solution by UV absorption at 280 nm (ϵ_{280} =1490 M^{-1} cm^{-1}). Oxidized peptides were prepared by agitation of a 100 μM peptide solution at room-temperature overnight in 0.1 M Tris-HCl pH 8.5 containing 6 M guanidine hydrochloride. Oxidized peptides were purified by RP-HPLC and the oxidation state confirmed by mass spectrometry and negative Ellman's assays. For the latter, 50 μL aliquots of Ellman's reagent (DTNB at 4 mg ml^{-1}) were mixed with peptide solutions (0.1 M phosphate buffer at pH 8) of appropriate concentration for the assay; after 10-min incubations, absorbances at 412 nm were read and corrected with blanks (ϵ_{412} (DTNB) 13600 M^{-1} cm^{-1}). Alkylated peptides were prepared by (1) incubation of a 1 mM peptide solution at 40 °C for 1 h in 0.6 M Tris-HCl pH 8.6 containing 1.25% (v/v) 2-mercaptoethanol, 8 M urea, 5 mM EDTA; (2) mixing the resulting reduced peptide solutions with 0.75 mL fresh 0.36 M iodoacetamide solution and incubating at room temperature for 15 min in the dark; and (3) dialysis against water. Chemical modification of cysteines was confirmed by mass spectrometry.

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were conducted at 5 °C in a Beckman-Optima XL-I analytical ultracentrifuge fitted with an An-60 Ti rotor. 100 μL peptide solutions at 100−500 μM in 25 mM K phosphate buffer pH 7 containing 0.1 M KCl were equilibrated for 48 h at three speeds in the range from 40 000 to 60 000 rpm. Data were fitted simultaneously assuming either a single ideal species or a monomer−dimer equilibrium and fixed monomer molecular weight, using routines in the Beckman-Optima XL-A/XLI−I data analysis software (v4.0). The molecular weights (Table 2) and partial specific volumes (range 0.7417 to 0.7461) of the peptides were calculated from their amino acid sequences, and the viscosity of the buffer at 5 °C was taken to be 1.008 mg ml^{-1}.

Circular Dichroism Spectroscopy. CD measurements were made using a JASCO J-715 spectropolarimeter fitted with a Peltier temper-

ature controller. Peptide solutions were prepared in 25 mM potassium phosphate buffer at pH 7 and were examined in 1 or 5 mm quartz cuvettes. Spectra were recorded at 5 °C using 1 nm intervals, a 1 nm bandwidth and 8 or 16 s response times. After baseline correction, ellipticities in mdeg were converted to molar ellipticities (deg cm^2 dmol-res^{-1}) by normalizing for the concentration of peptide bonds. Percent helicities were estimated for each peptide in one of two ways: either, simply by comparison with the $[\theta]_{222}$ value for folded GCN4-p1,[41] the archetypal leucine-zipper peptide, which is ≈−36 000 deg cm^2 dmol-res^{-1} Thermal unfolding curves were recorded through 1 °C min^{-1} ramps using a 1 nm bandwidth, averaging the signal for 16 s every 1 °C intervals. Several different methods were used to estimate the midpoints (T_{MS}) of these curves, notably, taking first and second derivatives of the curves, or fitting the curved to sigmoid functions. The reversibility of the unfolding transitions was tested by recording CD spectra and analytical RP-HPLC before and after thermal unfolding; in all cases except for CSP-2 and CSP-5 the transitions were completely reversible within the sensitivity of the two measurements; for CSP-2 and CSP-5 reversibility was >70% and >80%, respectively.

Switching Experiments. Switch experiments were performed for CSP-6o, and for CSP-2o as a control. Some experiments were done by rapid-mixing of 1:1 ratios of CSP-6o and DTE, dithioerythritol, as the reducing agent to give final concentrations of peptide and DTE of 100 μM and 2 mM, respectively. These experiments were performed at 20 °C using an Applied Photophysics π*−180 system (Leatherhead, UK). Otherwise, the switch was initiated by manual addition of reducing agent to preincubated buffered peptide samples. The switches were monitored by CD spectroscopy using the change in $[\theta]_{222}$ with time. Two reducing agents were used: DTE; and TCEP, tris-[2-carboxyle-hthyl]-phosphine. Experiments were done in which the final concentration of reducing agent was varied in the range 1 to 12 mM; the temperature was varied in the range 5 to 30 °C; and at final peptide concentrations of 10, 50, and 100 μM. Kinetic traces were analyzed using single-exponential fits to obtain rates and half-lives of the processes.

Supporting Information Available: Analytical ultracentrifugation data for the CSP-1, -3, and -6 designs are given. This material is available free of charge via the Internet at http://pubs.acs.org.

JA045568C

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