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A β/γ Motif to Mimic α-Helical Turns in Proteins

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This scientific work is dedicated to Professor Hans-Jörg Hofmann on the occasion of his 65th birthday.

The attempt to construct nature's architecture from nonnatural building blocks has challenged scientists for many decades. One goal of this field of study is to overcome the intrinsic protease susceptibility of natural peptides as it limits their clinical use. Peptides composed of homologous amino acids, those that have additional backbone methylene units compared to the natural α-amino acids, are at present among the most widely studied biomimetic oligomers that adopt well-defined conformations (foldamers).[1a] The wide variety of specific secondary structures that can be adopted by β- and γ-peptides[1] becomes especially valuable for the design of higher levels of organization such as tertiary or quaternary structures. Previous efforts towards this goal employing β-amino acid building blocks led to the discovery of both homomeric and heteromeric helix bundles[2] and helical inhibitors of protein–protein interactions;[3] however, there are significant differences between the packing observed in these artificial quaternary assemblies and that in the corresponding natural assemblies. This phenomenon has thus far impeded the combination of both classes into compact protein-like chimeric structures.

The aim of the current study was to identify extended sequences of β- and γ-amino acids that can be incorporated into an α-helical coiled coil to produce artificial chimeric folding motifs. Such artificial motifs with their orthogonal structural elements are great candidates for incorporation into natural helical proteins.[4] Because protein–protein interactions involving helical domains determine specificity for important biological processes such as transcriptional control, cellular differentiation, and replication, selective disruption should be an excellent strategy for drug discovery. We were inspired by previous reports in which the principle of "equal backbone atoms" was suggested.[5] Those designs were based on either unsubstituted or conformationally constrained amino acids.[5b–d] In particular β/γ-hybrid peptides appear to be well-suited to mimic an α-helical conformation,[5a] thus we focused on preserving the natural side chains for the purpose of accurately imitating the natural packing in order to lend stability to the assembly.

The α-helical coiled coil is a well-conserved and versatile folding motif that can serve as a model for tertiary and quaternary protein structures.[6] This motif features a canonical heptad repeat, $(abcdefg)_n$, in which hydrophobic residues occupy the a and d positions; these side chains make up the hydrophobic core of the interhelical interface.[7] Charged residues at e and g generally form the second molecular recognition motif by interhelical ionic interactions. One such characteristic heptad, comprising three 13-atom hydrogen-bonded turns of the helix, can be substituted by a pentad repeat of alternating β- and γ-amino acids with retention of the helix dipole and the formation of two 13-membered helix turns.

The peptide model system described here comprises a basic α-peptide "Base-pp" which has a high propensity for heterooligomerization to an α-helical coiled coil in the presence of the acidic peptide "Acid-pp" (Figure 1A). Heterooligomerization is driven by the burial of hydrophobic surface area, primarily contributed by Leu, and is directed by electrostatic interactions between Lys and Glu residues that flank the hydrophobic core. To evaluate β/γ-hybrid peptides as α-helix mimics, the two central turns of Base-pp (positions 15–21) were replaced by a pentad of alternating β- and γ-amino acid residues in the chimera B3β2γ (Figure 1B and C). CD spectroscopy (Figure 2A) indicates random coil and mostly unfolded conformations for B3β2γ and Acid-pp, respectively, as was expected based on the design of positions e and g. In contrast, an equimolar mixture of B3β2γ and Acid-pp shows significant α-helical structure formation with two well defined minima at 208 and 222 nm. Analysis of the ellipticity at 222 nm as a function of the mole fraction of B3β2γ reveals a global minimum at 0.5 (inset in Figure 2A), which corresponds to the presence of a heteromeric assembly between Acid-pp and B3β2γ with 1:1 stoichiometry.

Size exclusion chromatography (SEC) was performed to characterize the oligomerization states of the peptides described above. Comparison of retention times with the peptides GCN4-p1, GCN4-pII, and GCN4-pLI as investigated by Harbury et al.[8] suggests the presence of monomeric species (64 min) for the individual peptides Acid-pp, Base-pp and B3β2γ, but the formation of four-helix-bundles (57 min) in the equimolar mixtures Acid-pp/Base-pp and Acid-pp/B3β2γ (Figure 2B). Also,

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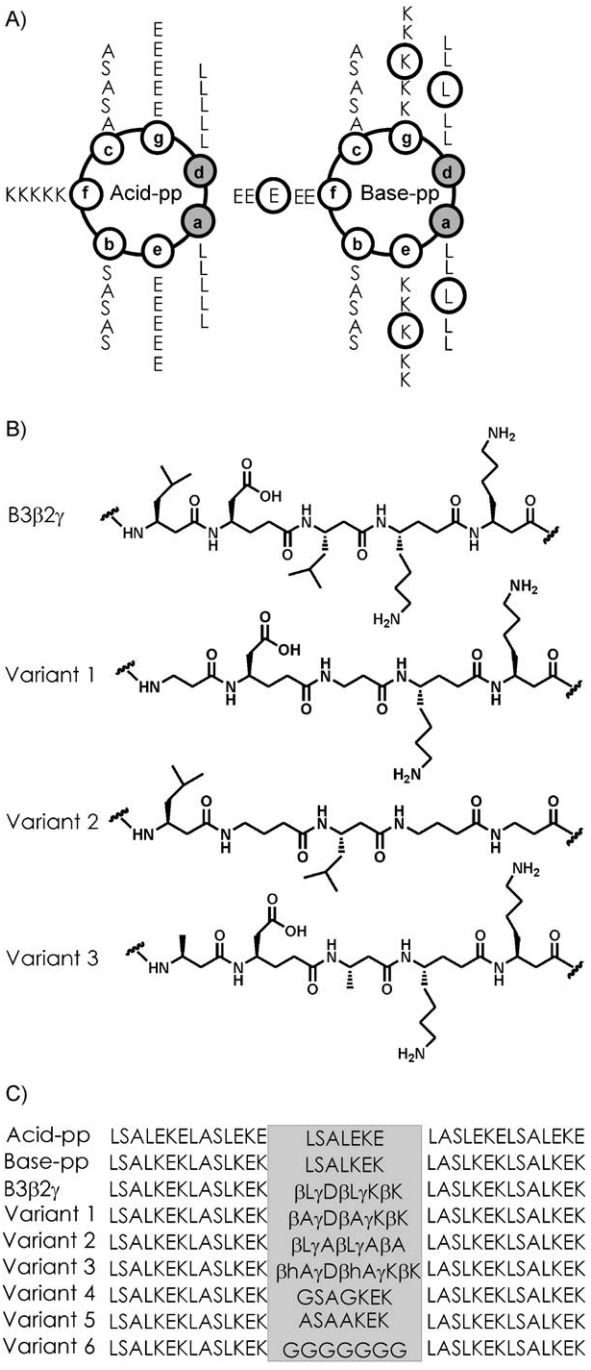

Figure 1. A) Helical wheel representation of α-peptides Base-pp and Acid-pp. Open circles in the Base-pp sequence indicate backbone modification in the form of β- and γ-amino acids. B) Structures of the β/γ-segment in chimeric peptides. C) Sequences of α- and α/β/γ-peptides. The central α-heptads and β/γ-pentads are shaded in gray.

a small fraction of dimeric (62 min) Acid-pp/B3β2γ is indicated by its SEC chromatogram. Further investigations by analytical ultracentrifugation (AUC) clearly show monomeric states for the peptides B3β2γ and Acid-pp individually. AUC results for the equimolar mixture of B3β2γ with Acid-pp (10 μM in each) are consistent with the major amount of species with molecular weight expected for a heterotetramer (see the Supporting Information). However, other species consistent with a higher order of oligomers can be observed; this has been also indicated by SEC at about 40 min. The very similar SEC and AUC results of both systems (Acid-pp/Base-pp and Acid-pp/B3β2γ) suggest that the structural properties of the heteromers have been maintained after the insertion of the β/γ-pentad into the α-peptide and a tetramer is the main oligomerization species.

Modeling and molecular dynamics (MD) simulations were conducted for tetrameric oligomerization states of the heterooligomers. Parallel and antiparallel heterotetrameric alignment of Acid-pp/Base-pp is supported by these calculations (Supporting Information). With regards to the quality of the packing in the hydrophobic core, the tetrameric topologies are very favorable. Similar to the parent system, possible structural arrangements in Acid-pp/B3β2γ containing the β/γ-segment are derived from a parallel or antiparallel alignment of heterotetramers. The results of the MD simulation and modeling on the tetrameric Acid-pp/B3β2γ system are shown in Figure 2C and D.[9] The four-helix bundles remain stable and form a densely packed hydrophobic core for the 20 ns simulation time. Both possible alignments, parallel and antiparallel, are of similar stability. The orientation of the helices towards each other and the backbone conformation of the α-peptide parts remain in the spatial configuration expected for both the coiled-coil motif and the α-helical secondary structure. The two β/γ-hybrid turns of the helix are well accommodated by the global structure, and contribute to the hydrophobic core formed between the four individual helices. A helical starting conformation of the isolated peptide B3β2γ is not stable in the MD simulation; it rapidly unfolds and adopts a random coil conformation (Supporting Information).

We performed thermal and chemical denaturation experiments to investigate the structural stability of the four-helix bundles. Our data reveal a higher stability of Acid-pp/Base-pp compared to the chimeric helix bundle (see Figure 2E, F and the Supporting Information). However, the Acid-pp/B3β2γ heterooligomer is still extremely thermostable and not fully denatured at 100 °C (Table 1). The pronounced fold stability of Acid-pp/B3β2γ points to a prodigious complementarity in side-chain packing between the β/γ segment and its α interaction partner that leads to successful integration of the artificial fragment into an otherwise native-like α-helical coiled-coil structure.

Table 1. T_m and $D_{50\%}$ values of equimolar mixture of Acid-pp with basic peptides and chimeras determined by chemical and thermal denaturation.

Peptides	$D_{50\%}$ [M] GndHCl	T_m [°C]
Acid-pp/Base-pp	3.4	>100
Acid-pp/B3β2γ	2.6	≅100
Acid-pp/variant 1	1.8	68
Acid-pp/variant 2	–	90
Acid-pp/variant 3	–	76
Acid-pp/variant 4	2.2	88
Acid-pp/variant 5	–	>100
Acid-pp/variant 6	1.6	56

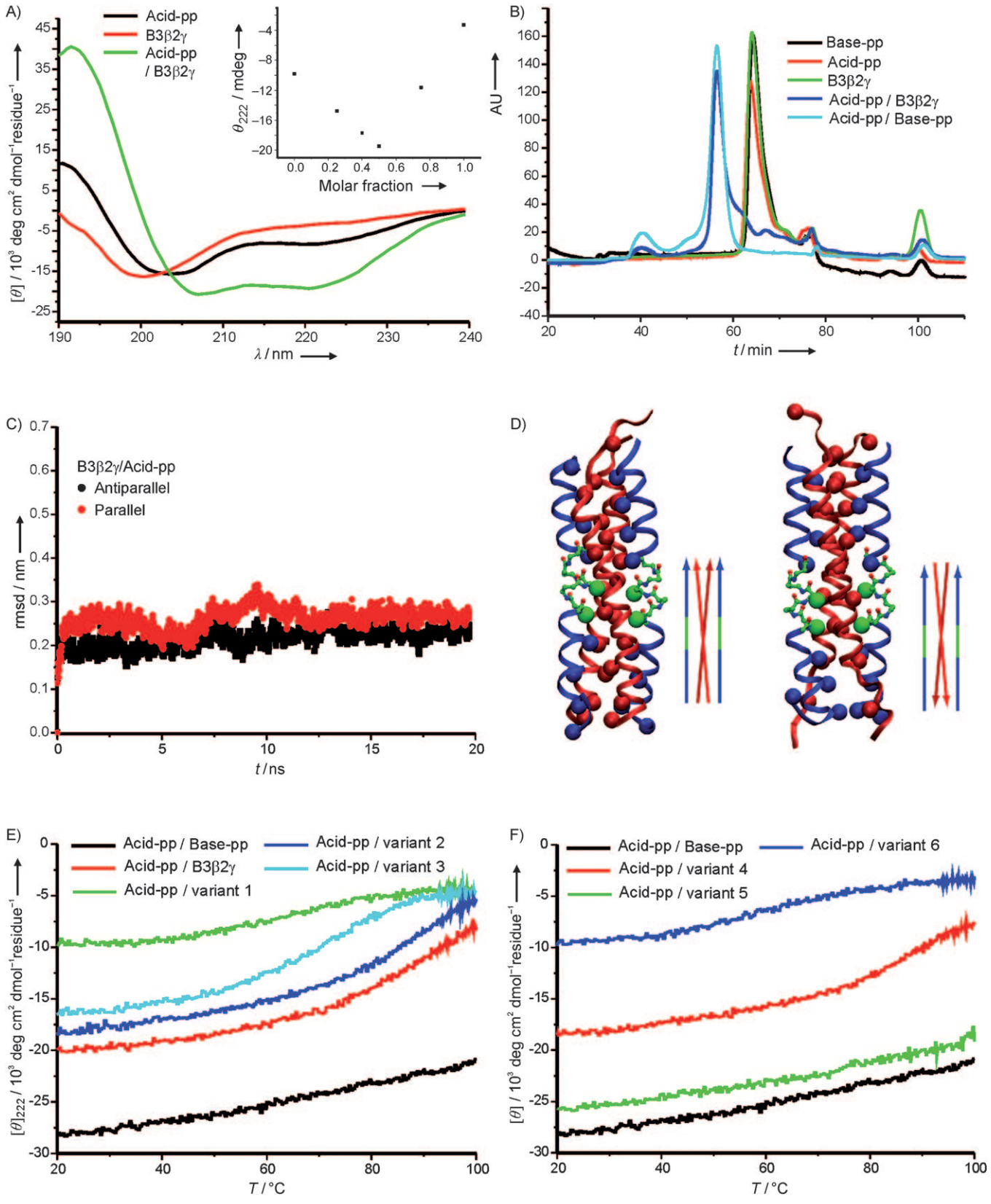

Figure 2. A) CD spectra of B3β2γ at 20 μM, Acid-pp 20 μM, and an equimolar mixture (10 μM in each peptide) in Tris/HCl buffer solution at pH 7.4. Inset is a plot of the ellipticity of the mixture at 222 nm as a function of the molar fraction of B3β2γ. B) SEC of the B3β2γ , Acid-pp and Base-pp alone and equimolar mixture of Acid-pp/B3β2γ and Acid-pp/Base-pp at overall peptide concentration 20 μM. The peak at 100 min is the internal reference. C) Plot of the rmsd values as a function of simulation time of the system Acid-pp/B3β2γ (parallel and antiparallel tetramer). D) Molecular models of chimera B3β2γ (blue ribbon) engaged in parallel (left) and antiparallel (right) coiled coil interaction with Acid-pp (red ribbon). The β/γ segment is shown in green. E) and F) Thermal denaturation of the equimolar mixtures of Acid-pp with Base-pp, B3β2γ, and the variants at overall peptide concentration 20 μM.

We prepared several side-chain mutants of B3β2γ to probe the specific contributions of the β- and γ-amino acid side chains to the formation of the hydrophobic and electrostatic interfaces that drive the heteromeric oligomerization. The stability of all variants was studied by using temperature-dependent CD spectroscopy and selected variants were further studied by applying chemical denaturation (Figure 2E, F and the Supporting Information). In variant 1 the two hydrophobic β^3-homoleucine residues were substituted with β-alanine thereby removing four side chains from the hydrophobic core of the four-helix-bundle. In variant 2 the three cationic and anionic β- and γ-residues were replaced with β-alanine and γ-amino butyric acid, respectively, impairing the charged interface (Figure 1C). Both peptides form heteromeric coiled coils with Acid-pp, as judged by CD spectroscopy (Supporting Information), although these show lower helical stability than the parent Acid-pp/B3β2γ pair (Table 1). According to the thermal melting points T_m of Acid-pp/variant 1 (68 °C) and Acid-pp/variant 2 (90 °C), the hydrophobic positions of the β/γ-segment make a greater contribution to fold stability in the chimeric helical bundle compared to the charged side chains at the electrostatic interface. This phenomenon is well known for natural α-helical coiled-coil assemblies.[6] To investigate the contribution of hydrophobic core packing in the β/γ-segment to fold stability of the chimeric coiled coils in more detail, we replaced the isopropyl groups of the βLeu side chains by methyl (βhAla), resulting in variant 3 (Figure 1B and 1C). Comparison of T_m values of the heteromeric bundles reveals less destabilization of Acid-pp/variant 3 compared to the Acid-pp/variant 1 mixture; this points to the contribution of the hydrophobic side chains of the chimeric segment to fold stability (Table 1).

To compare these destabilization effects in the hydrophobic core of the chimeric system with the situation in native helix bundles, we generated comparable variants of Base-pp. Variant 4 contains two glycine residues instead of the leucines in the central heptad repeat of Base-pp, equivalent to variant 1, while variant 5 has two alanines instead, equivalent to variant 3. As judged by the T_m values of the respective heteromers (Table 1), complete side-chain removal in Acid-pp/variant 4 led to greater destabilization compared to Acid-pp/variant 5. In both the natural system of regular α-heptads and the chimeric folding motif containing a β/γ-pentad, we observed comparable gradual destabilization of the heterooligomeric structures by gradual degradation of side chain length at two positions in the hydrophobic core. In a further peptide, variant 6, we substituted all seven amino acids of the central heptad in Base-pp by glycine presenting the most drastic deletion of side chains possible. The T_m and $D_{50\%}$ values of an equimolar mixture of Acid-pp/variant 6 show an extreme loss in structural stability caused by the Gly variations (Table 1). The observed destabilization is stronger than that observed for the chimeric Acid-pp/variant 1 in which the leucine side chains removed from the hydrophobic core of the helical bundle. This control experiment confirms that the pentad-sequence of alternating β- and γ-amino acids contributes to fold stability in the four-helix bundle Acid-pp/B3β2γ by specific orientation of hydrophobic and charged amino acid side chains as well as by maintenance of an overall α-helix-like backbone conformation.

The present study represents the first example of the substitution of an entire heptad repeat in an α-helical coiled-coil protein motif by a nonnatural fragment consisting of five alternating β- and γ-amino acids with retention of global conformation and the stability of the fold. The assembly of this chimera into a heteromeric leucine zipper with an exclusively natural oppositely charged α-peptide is driven only by noncovalent interactions and the resulting folded structure is highly thermostable. The side chains of the β/γ fragment participate in the formation of the characteristic interaction domains of the α-helical coiled-coil folding motif similar to those of the natural system. Our results facilitate a new direction in protein engineering toward modular replacement of extended α-helical segments with β/γ fragments in biologically important helical protein domains. The large number of protein–protein interactions that utilize α-helical interfaces for recognition and binding highlights the significance of this new class of chimeras, which we have shown is highly potent in imitating α-helices and quaternary structure formation.

Experimental Section

Peptide synthesis and characterization: All α-amino acids were coupled by standard automated Fmoc solid-phase synthesis by using a SyroXP-I peptide synthesizer (MultiSyn Tech GmbH, Witten, Germany) on 0.05 mM scale by using HOBT/TBTU activation. All couplings were performed two times with fourfold excess of amino acids and coupling reagents. Manual coupling of β- and γ-amino acids was carried out by HOAT/DIC activation without the addition of base to prevent racemization. The molar excess of amino acid and coupling reagents was reduced for β- and γ-residues to twofold for the first and onefold for the second coupling. Completion of these couplings was indicated by a negative Kaiser test. Prior to each deprotection step, capping of the possibly nonacylated N termini was carried out by treatment with acetic anhydride (10%) and DIEA (10%) in DMF (3×10 min). To determine the concentration by UV/Vis spectroscopic analysis, the peptides were N-terminally labeled with anthranilic acid. Finally, the resin was treated with a cleavage cocktail composed of water (1% (*v*/*v*)), triisopropylsilane (5% (*v*/*v*))and of TFA (95% (*v*/*v*)) for 3 h. The peptides were precipitated by addition of ice-cold ether. Each peptide was purified by reverse-phase HPLC (Phenomenex Luna C8, 10 μm, 250 mm×21.2 mm), solvent A: H_2O, CF_3CO_2H (0.1% (*v*/*v*)); solvent B: CH_3CN, CF_3CO_2H (0.1% (*v*/*v*)). Peptides were characterized by analytical HPLC (Phenomenex Luna C8, 5 μm, 250 mm×4.6 mm) and ESI-TOF mass spectrometry.

Circular dichriosm spectroscopy: Measurements were carried out with a J-810 spectropolarimeter (Jasco GmbH, Gross-Umstadt, Germany), by using a quartz cuvette with 0.1 cm path length. The spectra (Figure 2A in the main text and Figure S1) are the average of three scans obtained by collecting data from 190 to 240 nm at 0.2 nm intervals, 2 nm bandwidth, and 1 s response time. Ellipticity data in mdeg were converted to conformation parameters, by the following equation: $[\theta]=[\theta]_b \times mrw/10 \times l \times c$, in which $[\theta]_b$ is the ellipticity measured in degrees, *mrw* is the mean residue molecular weight (molecular weight of the peptide divided by the number of amino acid residues), *c* is the peptide concentration in g mL^{-1}, and *l* is the optical path length of the cell in cm.

Thermal denaturation: Thermal melts were carried out in 0.5 °C intervals with a heating rate of 3 °C min^{-1}. For quantitative comparison of helical stability the T_m values, the transition midpoint (maximum of the first derivative) of the temperature-dependent CD spectra, were determined.[10]

Chemical denaturation: The signal at 222 nm was recorded for solutions of constant peptide concentration with guanidine hydrochloride concentrations that varied from 0–6 M. Data were collected with 30-minute equilibration times. GndHCl was purchased from Fluka. The denaturation curves were fitted by using the program Sigma Plot 10.0 (sigmoidal-5 parameter). For quantitative comparison of helical stability, the $D_{50\%}$ values, the concentration of GndHCl at which 50% of the peptide remains folded, were determined.

Analytical ultracentrifugation: The partial specific volume of the samples was determined in a density oscillation tube (DMA 5000, Anton Paar, Graz, Austria) to be 0.760 mL g^{-1} for B3β2γ-Abz and 0.681 mL g^{-1} for acid-Abz (10 mM TRIS, pH 7.4) by using 300 μM overall peptide concentrations. The partial specific volume of the equimolar mixture of both peptides was selected as the arithmetic average to be 0.721 mL g^{-1}. Analytical ultracentrifugation (AUC) was performed on a XL-I (Beckman-Coulter, Palo Alto, CA, USA) ultracentrifuge at 25 °C applying the UV/Vis absorption optics at 230 nm and using titanium 12 mm double sector center pieces (Nanolytics, Potsdam, Germany). Sedimentation velocity experiments were performed at 60000 rpm. The samples were dissolved in Tris buffer (10 mM, pH 7.4; $\rho = 0.997624$ g mL^{-1}, $\eta = 10009$ cP both at 25 °C).

Size-exclusion chromatography (SEC): Size-exclusion chromatography was accomplished with a VWR-Hitachi Elite LaChrome system (Pump L-2130, UV Detector L-2400, VWR, Darmstadt, Germany) equipped with a Superdex 75 PC 3.2/30 column from Amersham Biosciences. Elution buffer was sodium phosphate (100 mM, pH 7.4) and the flow rate was 0.025 mL min^{-1}. Peptide absorbance was registered at 220 nm. For molecular-weight calibration the Gel Filtration Calibration Kit LMW form GE Healthcare Life Sciences was used. The retention times are corrected with internal and external references. Anthranilic acid labeled Gly is used as internal reference. GCN4-p1, GCN4-pII, and GCN4-pLI were applied as references for dimeric, trimeric and tetrameric coiled coils,[8] the monomer size of which is comparable to the model system used in this study.

Molecular-dynamics simulations: The MD-simulations were carried out with the Gromacs suite[11] using the Gromos 53a6 force field.[12] A twin range cut-off for van der Waals (0.9/1.4 nm) and a smooth particle mesh Ewald algorithm for Coulomb interactions (switching distance of 0.9 nm, neighbor list update every 0.01 ps).[13] Temperature (320 K) and pressure (1 bar) were kept constant by Berendsen weak coupling, with coupling constants of 0.1 ps for the temperature and 1 ps for the pressure.[14] Bond constraints were applied with the LINCS algorithm.[15] The coiled-coil dimers were solvated in dodecahedric boxes with approximately 10000 SPC water molecules and periodic boundary conditions were applied. The Gromos 53a6 topologies of the β- and γ-amino acids are available from the authors upon request.

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[1] a) S. H. Gellman, *Acc. Chem. Res.* **1998**, *31*, 173; b) D. Seebach, A. K. Beck, D. J. Bierbaum, *Chem. Biodiversity* **2004**, *1*, 1111; c) C. M. Goodman, S. Choi, S. Shandler, W. F. DeGrado, *Nat. Chem. Biol.* **2007**, *3*, 252; d) R. P. Cheng, S. H. Gellman, W. F. Degrado, *Chem. Rev.* **2001**, *101*, 3219; e) S. Hanessian, X. Luo, R. Schaum, S. Michnick, *J. Am. Chem. Soc.* **1998**, *120*, 8569; f) K. Möhle, R. Günther, M. Thormann, N. Sewald, H.-J. Hofmann, *Biopolymers* **1999**, *50*, 167; g) C. Baldauf, R. Günther, H.-J. Hofmann, *Helv. Chim. Acta* **2003**, *86*, 2573; h) C. Baldauf, M. T. Pisabarro, *J. Phys. Chem. B* **2008**, *112*, 7581.

[2] a) T. L. Raguse, J. R. Lai, P. R. LePlae, S. H. Gellman, *Org. Lett.* **2001**, *3*, 3963; b) R. P. Cheng, W. F. DeGrado, *J. Am. Chem. Soc.* **2002**, *124*, 11564; c) J. X. Qiu, E. J. Petersson, E. E. Matthews, A. Schepartz, *J. Am. Chem. Soc.* **2006**, *128*, 11338; d) J. L. Price, W. S. Horne, S. H. Gellman, *J. Am. Chem. Soc.* **2007**, *129*, 6376.

[3] a) J. A. Kritzer, N. W. Luedtke, E. A. Harker, A. Schepartz, *J. Am. Chem. Soc.* **2005**, *127*, 14584; b) O. M. Stephens, S. Kim, B. D. Welch, M. E. Hodsdon, M. S. Kay, A. Schepartz, *J. Am. Chem. Soc.* **2005**, *127*, 13126.

[4] R. David, R. Günther, L. Baumann, D. Seebach, H.-J. Hofmann, A. G. Beck-Sickinger, *J. Am. Chem. Soc.* **2008**, *130*, 15311.

[5] a) C. Baldauf, R. Günther, H.-J. Hofmann, *J. Org. Chem.* **2006**, *71*, 1200; b) I. L. Karle, A. Pramanik, A. Banerjee, S. Bhattacharjya, P. Balaram, *J. Am. Chem. Soc.* **1997**, *119*, 9087; c) P. G. Vasudev, K. Ananda, S. Chatterjee, S. Aravinda, N. Shamala, P. Balaram, *J. Am. Chem. Soc.* **2007**, *129*, 4039; d) S. Chatterjee, P. G. Vasudev, K. Ananda, S. Raghothama, N. Shamala, P. Balaram, *J. Org. Chem.* **2008**, *73*, 6595.

[6] a) J. A. Scheike, C. Baldauf, J. Spengler, F. Albericio, M. T. Pisabarro, B. Koksch, *Angew. Chem.* **2007**, *119*, 7912; *Angew. Chem. Int. Ed.* **2007**, *46*, 7766; b) K. Pagel, B. Koksch, *Curr. Opin. Chem. Biol.* **2008**, *12*, 730; c) E. Moutevelis, D. N. Woolfson, *J. Mol. Biol.* **2009**, *385*, 726.

[7] R. S. Hodges, A. K. Saund, P. C. S. Chong, S. A. St.-Pierre, R. E. Reid, *J. Biol. Chem.* **1981**, *256*, 1214.

[8] P. B. Harbury, P. S. Kim, T. Albor, *Nature* **1994**, *371*, 80.

[9] For the starting structures of the tetrameric coiled coils of Acid-pp/B3β2γ please see the available PDB files.

[10] D. M. John, K. M. Weeks, *Protein Sci.* **2000**, *9*, 1416.

[11] E. Lindahl, B. Hess, D. van der Spoel, *J. Mol. Model.* **2001**, *7*, 306.

[12] C. Oostenbrink, A. Villa, A. E. Mark, W. F. Van Gunsteren, *J. Comput. Chem.* **2004**, *25*, 1656.

[13] U. Essmann, L. Perera, M. L. Berkowitz, T. Darden, H. Lee, L. G. Pedersen, *J. Chem. Phys.* **1995**, *103*, 8577.

[14] H. J. C. Berendsen, J. P. M. Postma, W. F. van Gunsteren, A. Dinola, J. R. Haak, *J. Chem. Phys.* **1984**, *81*, 3684.

[15] B. Hess, H. Bekker, H. J. C. Berendsen, J. Fraaije, *J. Comput. Chem.* **1997**, *18*, 1463.

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