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Letters

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An Unusual Interstrand H-Bond Stabilizes the Heteroassembly of Helical αβγ-Chimeras with Natural Peptides

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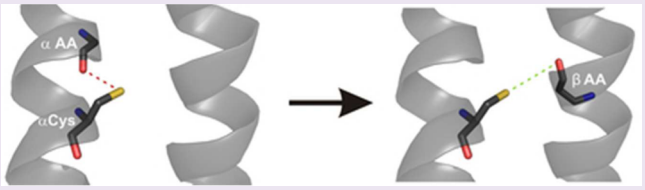
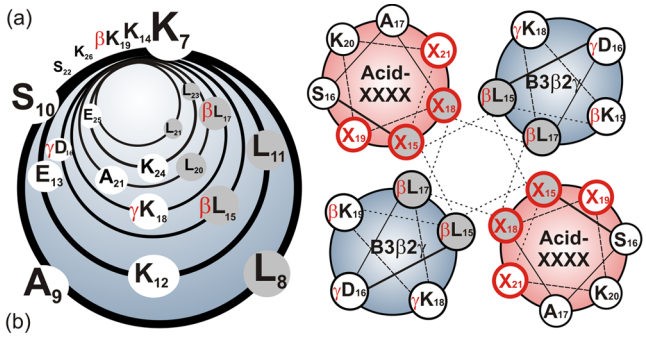
Supporting Information

ABSTRACT: The substitution of α-amino acids by homologated amino acids has a strong impact on the overall structure and topology of peptides, usually leading to a loss in thermal stability. Here, we report on the identification of an ideal core packing between an α-helical peptide and an αβγ-chimera via phage display. Selected peptides assemble with the chimeric sequence with thermal stabilities that are comparable to that of the parent bundle consisting purely of α-amino acids. With the help of MD simulations and mutational analysis this stability could be explained by the formation of an interhelical H-bond between the selected cysteine and a backbone carbonyl of the β/γ-segment. Gained results can be directly applied in the design of biologically relevant peptides containing β- and γ-amino acids.



The fidelity of nearly all life functions relies on the specificity of protein−protein interactions. To better understand as well as to perturb molecular events underlying disease, considerable effort has been devoted to the identification of molecules that disrupt protein interaction. Many disadvantages that accompany the use of natural peptide inhibitors have necessitated the design of unnatural oligomers that closely resemble both protein folding and function.[1−6] The development of heterogeneous backbone foldamers, i.e., peptide structures that contain not only α- but also β- and/or γ-amino acids, with enhanced proteolytic stability or specific biological activities highlights their potential for biomedical applications.[7−10] We herein report on the identification of a 4-amino-acid segment that selectively recognizes the βγ-unit of an αβγ-chimera.

Protein−protein interactions in which at least one partner contributes an α-helix to the protein interface are attractive candidates for the design of foldamer-based inhibitors, since a variety of helices comprising foldameric sequences can be predictably generated. However, despite the growing number of such systems, the precise mimicry of side-chain topology and recognition properties of natural α-helical sequences still remains challenging. Theoretical studies have shown that sequences composed of alternating β- and γ-amino acids are able to adopt helical structures similar in shape and H-bonding to α-helices.[11−14] However, unlike helices composed purely of α-amino acids, helical αβγ-chimera always harbor at least one non-H-bonded backbone carbonyl. Our efforts in designing a heterogeneous coiled-coil foldamer have led to the generation of the tetrameric Acid-pp/B3β2γ, which possesses a pentad of alternating β- and γ-amino acids (Figure 1).[15,16] While this



	Peptide sequences		
Base-pp	LSALKEKLASLKEK	LSALKEK	LASLKEKLSALKEK
B3β2γ	LSALKEKLASLKEK	βLγDβLγKβK	LASLKEKLSALKEK
B3β2γ-variant1	LSALKEKLASLKEK	βAγDβAγKβK	LASLKEKLSALKEK
Acid-pp	LSALEKELASLEKE	LSALEKE	LASLEKELSALEKE
Acid-XXXX	Randomized Library LSALEKELASLEKE	a'^{15} $d'^{18}e'^{19}$ g'^{21} XSAXXKX	LASLEKELSALEKE

Figure 1. (a) (Left) Helical representation of B3β2γ's 21 central residues. (Right) Central βγ-pentads of two B3β2γ molecules and the interacting heptads of two Acid-pp molecules depicted as a tetrameric helical wheel with parallel helix orientation. Amino acids at the hydrophobic core are highlighted in gray, and randomized positions in the Acid-pp library are shown as X's. (b) Primary structure of chimeric and natural sequences. Randomized positions shown as red X's.

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system tolerates the heterogeneous modification, a loss in thermal stability is observed when compared to that of its parental system Acid-pp/Base-pp. This highlights the challenge in predicting ideal core packing with matching side-chain composition and geometry between a $\beta\gamma$-foldameric binding groove and an α-peptide,[15,17] a fact that so far precluded the assembly of $\alpha\beta\gamma$-chimeras and natural peptides into stable quaternary structures. The current communication describes the application of phage display as one of the most powerful selection techniques in protein engineering,[18−23] in order to facilitate the empirical search for high-affinity binders to an $\alpha\beta\gamma$-chimeric sequence.

The peptide library construction was based on the Acid-pp (*wt*) sequence and included the randomization of the central heptad key positions (a'^{15}, d'^{18}, e'^{19}, g'^{21}), which are directly interacting with the $\beta\gamma$-segment of B3β2γ (Figure 1). The Acid-pp library was displayed on the surface of filamentous bacteriophage M13, and the chimera was N-terminally labeled with biotin to provide for loading on streptavidin-coated magnetic particles. For the evaluation of the selection outcome of interaction partners, a second foldameric sequence, B3β2γ-variant1, was examined. In this control chimera the two β^3-homoleucine residues of B3β2γ were substituted with β-alanine, thereby removing two key side chains for coiled-coil interactions.[15,16] Magnetic particles without peptide served as a negative control. B3β2γ and B3β2γ-variant1 were screened against the phage displayed Acid-pp library in six panning rounds. The selection against B3β2γ led to a significant colony enrichment and revealed two most prevalent peptide sequences (denoted Acid-CFLE and Acid-ICEF) of high similarity (Supplementary Table S2). Both sequences comprise a cysteine residue, either at position a'^{15} or d'^{18}, with a Phe next to it in position d'^{18} or g'^{21}, respectively. In addition, an aliphatic residue (Leu or Ile) occupies one of the four randomized positions. In contrast to their parent peptide Acid-pp, only one Glu residue is found in core flanking positions, instead of two. It is either located at g'^{21} (Acid-CFLE) or at position e'^{19} (Acid-ICEF). Similar to previous studies where aromatic residues were found at positions designated for charged amino acids,[23] the selection of an additional hydrophobic residue at position e'^{19} or g'^{21} indicates an extension of the hydrophobic core. The bulky hydrophobic side chains of Ile and Phe are presumably selected to provide ideal shielding from water for the binding groove of the foldamer.

The screening of B3β2γ-variant1 resulted in a low colony number comparable to that of unspecifically bound phages in the negative control. Moreover, no consensus sequence but rather a completely random distribution of amino acids occurred in all randomized positions (Supplementary Table S2), indicating that there was no specific recognition of the β-alanine backbone.

The two most prevalent Acid-pp variants were chemically synthesized, and their interaction with B3β2γ was tested in solution. Applying static light scattering (SLS), we could verify that the tetrameric oligomerization state of the parent system is retained when selected Acid-pp variants assemble with B3β2γ (Supplementary Table S3). The 1:1 mixtures of Acid-ICEF/B3β2γ and Acid-CFLE/B3β2γ formed coiled coils with an increased helical content when compared to B3β2γ/Acid-pp (Figure 2a). Also the thermal stabilities of these assemblies are significantly higher compared to that of the Acidpp/B3β2γ bundle (T_m = 61 °C). While Acid-CFLE/B3β2γ has a T_m value of 70 °C, the equimolar mixture of Acid-ICEF/B3β2γ starts melting at 74 °C, and thus its thermal stability closely resembles

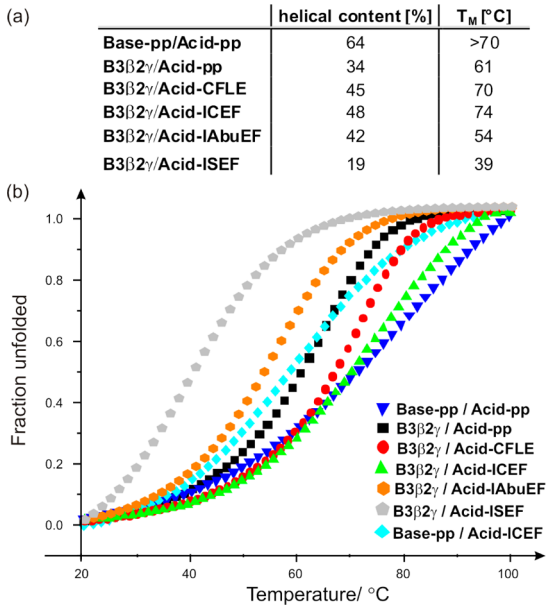

Figure 2. (a) CD data of equimolar mixtures of Base-pp/Acid-pp and its variants. (b) Thermal denaturation spectra.

that of its native parental system Acid-pp/Base-pp (T_m >70 °C), even though it appears to be less helical (Figure 2).

Moreover, the selected side-chain composition in Acid-ICEF seems to specifically interact with the $\alpha\beta\gamma$-chimera, as the identified peptide binds to its chimeric partner with a considerably higher thermal stability than to the α-parent peptide, Base-pp. Thus, the combination of a thiol side chain with a bulky residue seems to match the specific packing requirements of the $\beta\gamma$-pattern.

To determine the relative orientation of the helices in both chimeric coiled coils, Acidpp/B3β2γ and Acid-ICEF/B3β2γ, we applied a FRET assay using o-aminobenzoic acid (Abz) as the fluorescence donor and 3-nitrotyrosine (Y(NO_2)) as the acceptor.[24,25] Fluorescence quenching by resonance energy transfer from Abz to Y(NO_2) only occurs when the donor and the acceptor are in close proximity. The fluorescence spectra of both, N-terminally as well as C-terminally Abz-labeled Acid-pp (Acid-*N*Abz and Acid-CAbz, respectively), show a progressive decrease in fluorescence intensity at increasing concentrations of N-terminally YNO_2-labeled B3β2γ (B3β2γ-*N*Y(NO_2)). Control experiments in the presence of denaturant (GndHCl) demonstrated that the observed quenching is the result of specific folding rather than self-quenching (Supplementary Figure S7). Thus, the helices possess an unspecific orientation toward each other, which might be attributed to the absence of any orientation-selective element in their sequences. In contrast to this result, FRET experiments in which the fluorescence donor Abz was present at the C-terminus of Acid-ICEF show much weaker quenching than similar experiments with its N-terminally labeled analogue, and, thus, suggest that Acid-ICEF and B3β2γ preferentially form parallel hetero-oligomers. The experimental set up of phage display might have directed the selection of high-affinity binders for the chimera in parallel helix orientation. During panning B3β2γ was N-terminally biotinylated and the Acid-pp library was fused to the N-terminus of the pIII coat protein. Therefore, a parallel coiled-coil formation on the magnetic particle is feasible, whereas an antiparallel orientation is likely to be sterically hindered.

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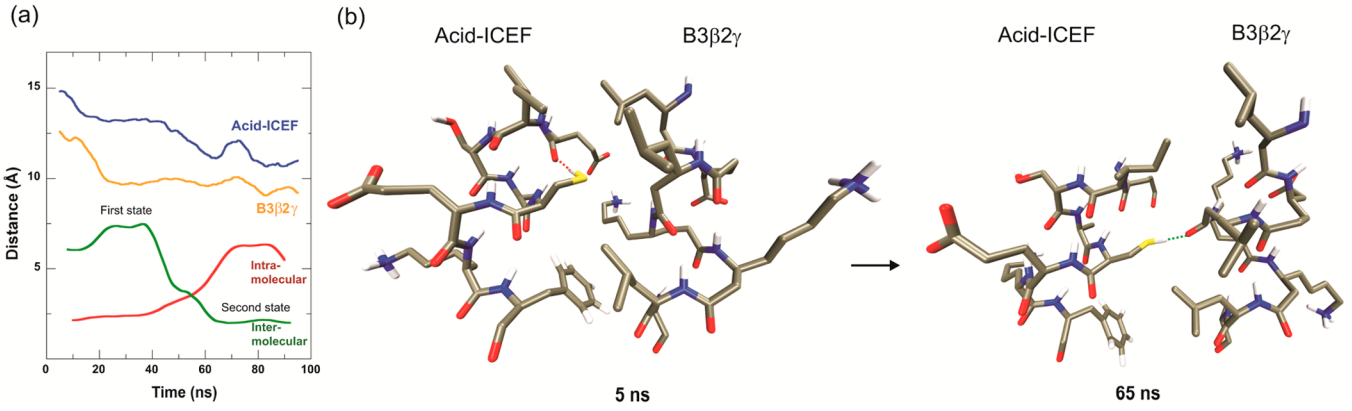

Figure 3. (a) Plot illustrating the distances between the two Acid-ICEF helices (blue) and the two B3β2γ helices (orange), SH Cys_{18}–CO Glu_{14} (red) and SH Cys_{18}–CO βL_{17} (green). (b) Snapshots of MD simulation showing amino acids 14–21 and 15–20 of one Acid-ICEF and one B3β2γ strand, respectively. Red dotted line depicts intrahelical SH Cys_{18}–CO Glu_{14} H-bond (2.3 Å). Green dotted line depicts interhelical SH Cys_{18}–CO βL_{17} H-bond (2.2 Å).

Molecular dynamics[26−28] with the B3β2γ/Acid-ICEF system further confirmed experimentally gained results (simulations were carried out using Gromacs on the High-Performance Computing system at Freie Universität Berlin (http://www.zedat.fu-berlin.de/Compute)). When simulating the tetrameric system in an antiparallel strand orientation, no significant alterations could be observed (data not shown). If simulated in a parallel strand orientation, however, three major structural changes occurred (Figure 3): (i) The centers of mass distance between the helices decreased drastically. (ii) The intramolecular H-bond network of the B3β2γ helices rearranged such that the carbonyl of βLeu17 are set free. (iii) The side-chain orientation of Acid-ICEF's Cys switches from an intramolecular interaction with the backbone carbonyl of Glu14 to an intermolecular interaction with the liberated carbonyl of βLeu17. Moreover, a root-mean-square deviation (RMSD) analysis indicated two plateaus corresponding to two main states adopted by the complex, thus further verifying the switch from intra- to interhelical H-bonding (Supplementary Figure S8). These observations suggest that a strong intermolecular interaction occurs when Acid-ICEF faces the β- and γ-residues. Moreover, they further confirm the crucial role that Cys has on the stability of the fold as it might enable an intermolecular H-bond that stabilizes the quaternary structure. Hydrogen bond formation involving Cys have been shown to contribute to structural stability of proteins to a large extent.[29,30] To rule out cysteine's oxidation and thus potential disulfide bond formation, we compared the melting curves as well as size exclusion chromatograms of 1:1 Acid-ICEF/B3β2γ mixtures prior to and post incubation with reducing agent DTT (Supplementary Figure S5). The resemblance of the curves before and after DTT treatment gave no indication for oxidation.

Considering the key function of Cys, the mutation of this residue was expected to affect the stability of the entire motif. To test this hypothesis, two control peptides in which the Cys of Acid-ICEF is substituted by either aminobutyric acid (Acid-IAbuEF) or Ser (Acid-ISEF) were generated. While 1:1 mixtures of either of these variants with B3β2γ exhibited considerably less stability in solution compared to the selected analogue, the variant containing hydrophobic Abu is slightly more stable than the variant containing the polar Ser (Figure 2a). Moreover, no stable intermolecular interaction involving serine's hydroxyl group could be observed in MD simulations with Acid-ISEF (Supplementary Figure S8). As opposed to Cys residues that are involved in disulfide bonds, free Cys residues appear to have a rather hydrophobic character and are only slightly polar.[31] Our results suggest that Cysteine's side chain is just polar enough to undergo an interhelical H-bond to a backbone carbonyl and at the same time hydrophobic enough to prevent disruption of the hydrophobic core of coiled coils. Thus, this study compliments a recent report of Burton et al., which demonstrated that non-disulfide-linked cysteine residues can be incorporated at hydrophobic core positions of a coiled coil purely composed of α-amino acids, without disrupting the assembly.[32] Here we show that the physicochemical properties of cysteine (i.e., volume, polarity, and side-chain reactivity) seem to provide for ideal core packing involving $\alpha\beta\gamma$-foldamers.

In summary, we have undertaken a broad survey for peptides that specifically match an $\alpha\beta\gamma$-chimera in a coiled-coil assembly using phage display. Two of the selected peptides bound the chimeric sequence with higher thermal stability in comparison to the α-parent peptide, while the oligomerization state is maintained. To the best of our knowledge, we here report for the first time the identification of a Cys residue within the hydrophobic core of an $\alpha\beta\gamma$-chimera coiled-coil assembly as a stabilizing element. The presence of the Cys side chain in combination with a bulky aromatic residue provides for an ideal geometric arrangement of hydrophobic core residues. The buried Cys can significantly influence the core-packing of chimeric coiled coils through the formation of an interhelical H-bond with a non-H-bonded backbone carbonyl of the $\alpha\beta\gamma$-chimera, which was shown to account for an outstanding stability. Our results show that the incorporation of a functionality that is able to involve a free backbone carbonyl of $\alpha\beta\gamma$-chimeras in an interstrand H-bond is an excellent strategy to improve the recognition specificity of α-peptides for $\beta\gamma$-foldameric sequences and may thus pave the way for the design of stable bioactive $\alpha\beta\gamma$-chimeras.

■ ASSOCIATED CONTENT

Supporting Information

Peptide synthesis, phage display, CD spectroscopy, FRET assay, light scattering, and MD simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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