β- and γ-Amino Acids at α-Helical Interfaces: Toward the Formation of Highly Stable Foldameric Coiled Coils

Elisabeth K. Nyakatura, ‡ Jérémie Mortier, ‡ Vanessa S. Radtke, ‡ Sebastian Wieczorek, ‡ Raheleh Rezaei Araghi, ‡ Carsten Baldauf, ‡ Gerhard Wolber, ‡ and Beate Koksch, ‡* ‡Institute of Chemistry and Biochemistry, Freie Universität Berlin, Takustraße 3, 14195 Berlin, Germany
‡Institute of Pharmacy, Freie Universität Berlin, König-Luisestrasse 2 + 4, 14194 Berlin, Germany
§Fritz Haber Institute, Faradayweg 4-6, 14195 Berlin, Germany

Supporting Information

ABSTRACT: Since peptides are vital for cellular and pathogenic processes, much effort has been put into the design of unnatural oligomers that mimic natural peptide structures, also referred to as foldamers. However, to enable the specific application of foldamers, a thorough characterization of their interaction profiles in native protein environments is required. We report here the application of phage display for the identification of suitable helical environments for a sequence comprising an alternating set of β- and γ-amino acids. In vitro selected sequences show that an increase in the hydrophobic surface area at the helical interface as well as the incorporation of a polar H-bond donor functionality can significantly improve interhelical interactions involving backbone-extended amino acids. Thus, our data provide insight into the principles of the rational design of foldameric inhibitors for protein–protein interactions.

KEYWORDS: Phage display, chimeric peptides, βγ amino acids

The α-helix constitutes one of the most naturally abundant structural motifs and is found at the interface of several important intracellular and pathogenic protein–protein interactions. Consequently, this secondary structure element has been of interest as a minimal unit in the development of peptides that disrupt protein–protein interactions. Unfortunately, the clinical applicability of peptide-based inhibitors is limited by their low bioavailability. One approach to solve this problem is to design unnatural oligomers that fold in ways similar to natural proteins commonly referred to as foldamers but are otherwise bioorthogonal, in the context of degradation by human proteases. Foldamers that are composed of amino acids that possess one or two additional backbone methylene units, denoted β- and γ-amino acids, respectively, have been shown to mimic the self-assembly of native peptides and thus present functional groups in a predictable manner. However, despite the growing number of such systems, precise mimicry of the surface topology and recognition properties of natural α-helical sequences remains challenging.

Inspired by theoretical studies, we previously showed that the substitution of a central heptad of α-amino acids with a pentad of alternating β- and γ-amino acids, which comprises two helical turns, is accompanied by a substantial loss in thermal stability. Aiming to stabilize this chimeric system, denoted Acid-pp/B3/2γ, we recently utilized a phage display screen to isolate optimal all-α binding partners for the chimeric peptide. This screen led to the selection of Acid-pp variants containing a cysteine that significantly improves coiled-coil core packing through the formation of an interhelical S–H···O=C H-bond with a non-H-bonded backbone carbonyl of the αβγ-chimera. Thus, we showed that the incorporation of a functionality that is able to involve a free backbone carbonyl of αβγ-chimera in noncovalent interstrand interactions is an excellent strategy to improve the recognition specificity of α-peptides for βγ-foldameric sequences. However, it also revealed that the N-terminal immobilization of the B3/β2γ chimera created a bias for the selection of parallel, as opposed to antiparallel, coiled coils formed between B3/β2γ and cysteine containing Acid-pp variants. Considering the distinct geometrical constraints of parallel vs antiparallel coiled coils, we here address the issue of whether the side of immobilization directs the outcome of the phage display screen. We assumed that the C-terminal immobilization of B3/β2γ chimera would lead to the isolation of different and perhaps even more thermally stable complexes based on noncovalent interactions under an antiparallel coiled coil regime. Therefore, B3/β2γ was immobilized on streptavidin coated magnetic particles via a C terminally attached biotin and the four central heptad positions of the Acid-pp α-peptide that directly interact with the βγ-

Received: September 3, 2014
Accepted: October 28, 2014
Published: October 28, 2014
segment of the chimeric B3/2γ peptide ($a'_{15}$, $d'_{18}$, $e'_{19}$, and $g'_{21}$) were randomized. After six rounds of panning, four sequences were isolated (Figure 1).

Two of the selected Acid-pp sequences comprise an extension of the hydrophobic core similar to previous studies, in which hydrophobic residues were selected at solvent-exposed positions.20,21 One of these two new sequences bears leucine in all four randomized positions (denoted Acid-LLLL). Likewise, a second peptide (Acid-LFYL) contains hydrophobic amino acids in three of the randomized positions; however, polar tyrosine occupies position $e'_{19}$. The remaining two sequences harbor charged amino acids in most of the randomized positions. Acid-MTER possesses two oppositely charged amino acids; position $e'_{19}$ is occupied by glutamic acid, $g'_{21}$ by arginine. Surprisingly, only one of the two randomized core positions harbors a hydrophobic residue (methionine), the other one is taken up by polar threonine. In Acid-HCAN, polar histidine occupies the hydrophobic core position $a'_{15}$. However, similar to our previous result obtained in a parallel regime,20 cysteine is found in the remaining hydrophobic core position $d'_{18}$. The presence of another polar residue at position $g'_{21}$ in combination with the rather small hydrophobic alanine at position $e'_{19}$ indicates that this assembly is stabilized by electrostatic interactions rather than being a consequence of the hydrophobic effect. Given the diversity within the selection outcome, these results differ substantially from our former screen under a parallel coiled-coil regime from which two highly similar sequences containing a cysteine in combination with a bulky hydrophobic residue in randomized positions emerged.20

To study the interaction of the here selected Acid-pp variants with B3/2γ in solution, all four variants were chemically synthesized and characterized. Size exclusion chromatography in combination with static light scattering revealed that the tetrameric oligomerization state of the parent system is retained when either of the two hydrophobic core-extended Acid-pp variants (Acid-LLLL or Acid-LFYL) assembles with B3/2γ (see Supporting Information). Interestingly, Acid-MTER primarily forms dimers with B3/2γ, while a small population of the monomeric species is observed. An equimolar mixture of B3/2γ and Acid-HCAN is found to contain largely monomers and only a small fraction of the dimeric species. Moreover, CD studies showed that although 1:1 mixtures of B3/2γ/Acid-LLLL and B3/2γ/Acid-LFYL form coiled coils with an increased helical content when compared to B3/2γ/Acid-pp, the two selected variants harboring charged amino acids in randomized positions are less helical than B3/2γ/Acid-pp (Figure 2a).

Also, the investigation of the thermal stability of these assemblies revealed fundamental differences among the selected variants (Figure 2b). Whereas significantly higher thermal stabilities are observed for B3/2γ/Acid-LLLL (90 °C) and B3/2γ/Acid-LFYL (71 °C), compared to the B3/2γ/Acid-pp bundle (61 °C), the melting points of B3/2γ/Acid-MTER (40 °C) and B3/2γ/Acid-HCAN (48 °C) are significantly lower.

To determine the relative orientation of the helices in the highly thermodynamically stable B3/2γ/Acid-LLLL and B3/2γ/Acid-LFYL bundles, we applied a fluorescence resonance energy transfer (FRET) assay using o-aminobenzoic acid (Abz) as the donor and 3-nitrotyrosine (Y(NO$_2$)) as the acceptor (see Supporting Information). These studies revealed that no particular helix orientation is preferred in either of the two investigated bundles, even though we intended to impose antiparallel coiled-coil formation by immobilizing B3/2γ C-terminally during the phage display screen. However, considering the absence of charged side chains in the

---

**Figure 1.** (a) Chemical structure of the βγ-amino acid pentad of B3/2γ. (b) Helical wheel representation of the Acid-pp heptad that directly interacts with the βγ-segment of B3/2γ. Randomized positions depicted as red X. (c) Amino acid sequences of B3/2γ, Acid-pp, and Acid-pp variants selected after the 6th panning round against C-terminally biotin labeled B3/2γ. The four randomized sites in the Acid-pp library and the βγ-segment of B3/2γ are highlighted in gray.

**Figure 2.** CD spectra (a) and thermal denaturation spectra (b) of equimolar mixtures of B3/2γ/Acid-pp and its variants. Melting points given in figure legend. Standard deviations from three independent measurements were ±0.1 °C. The total peptide concentration was 20 μM. The spectra were recorded in 50 mM phosphate buffer, containing 0.25 M GdnHCl at pH 7.4.

randomized positions that could enforce directionality, the formation of both parallel and antiparallel bundles, as observed with the parental system, is not surprising.

For the purpose of gaining insight into the intermolecular interactions that underlie the stability enhancements, each of the four novel Acid-pp variants was investigated using molecular dynamics (MD). Tetramer models were initially built with the chimeric peptide B3/βγ oriented in antiparallel fashion with either Acid-LLLL or Acid-LFYL. The previously reported model of the tetrameric Acid-pp/B3/βγ bundle served as a reference structure. Each complex was investigated for 50 ns using the Gromos 53a6 force field implemented within the Gromacs Suite. During this simulation time, the parental tetrameric Acid-pp/B3/βγ was previously shown to be stable. Similarly, and in agreement with our experimental data, the Acid-LLLL/B3/βγ and Acid-LFYL/B3/βγ systems reported here exhibit very high stabilities, which is illustrated by the root-mean-square deviations (RMSD) from the initial conformation below 4 Å (see Supporting Information). Moreover, the theoretical model of the Acid-LFYL/B3/βγ bundle shows that the voluminous side chains of the selected phenylalanine and tyrosine residues pack efficiently in the more spacious environment in the vicinity of the βγ-backbone of B3/βγ. Interestingly, after 30 ns of simulation time, the orientation of Acid-LFYL’s residue Y19 is fixed in the direction of the B3/βγ residue L20 (Figure 3a), stabilized by a persistent intermolecular H-bond between the hydroxyl group of Y19 and the backbone carbonyl of B3/βγ’s L20. Simultaneously, the distance between the aromatic ring of the tyrosine and the charged ammonium group of γK18 decreases below 6 Å, bringing these two residues in proximity and thus enabling the formation of a cation−π interaction (Figure 3b). These observations are in agreement with a recent study, which highlights that aromatic side chains can be utilized to stabilize β-peptidic helical conformations.

Since the FRET experiments described above revealed that the Acid-LLLL/B3/βγ and Acid-LFYL/B3/βγ bundles are also able to interact in a parallel orientation, the corresponding models were also constructed and investigated by MD. Again, the simulations were consistent with the in vitro experiments. Similar to the antiparallel Acid-LLLL/B3/βγ bundle, the four leucines are stabilized within the hydrophobic core in the setting of a parallel tetramer. Likewise, when the tetrameric Acid-LFYL/B3/βγ system was investigated in a parallel orientation, nearly identical interactions as those found in the antiparallel model were observed. The side chains of F18 and Y19 of Acid-LFYL form a local perimeter around the hydrophobic core, in which hydrophobic contacts on the inside and potential cation−π interactions on the outside stabilize the structure.

Finally, antiparallel dimeric models were built for Acid-MTER/B3/βγ and Acid-HCAN/B3/βγ (see Supporting Information). In agreement with our experimental results, all simulations showed that dimers are less stable than tetramers for these sequences. Although no dissociation was observed, the peptides do undergo partial unfolding of the helices in each investigated dimeric model.

In summary, we conducted a broad survey for α-peptides that bind tightly to an αβγ-chimeric peptide. Phage display experiments enabled the isolation of four sequences after six rounds of panning. With two of these peptides, the hydrophobic core of the resulting coiled coil was extended to solvent-exposed positions that are typically occupied by charged residues. These peptides maintain the parental tetrameric oligomerization state when forming bundles with the αβγ-chimeric peptide. As opposed to our previous study and in the absence of charged side chains, in neither of these two bundles a particular helix orientation is preferred. The other two sequences harbor charged amino acids in positions that are typically occupied by hydrophobic residues, which in both cases leads to the formation of dimeric bundles with the αβγ-chimeric peptide. Although the thermal stabilities of those bundles are reduced and partial dissociation as well as unfolding was observed, these assemblies represent the first foldameric dimers reported so far. Previous attempts to design such systems de novo were unsuccessful. Apparently, the greater area of the interface and more numerous noncovalent interactions that characterize the higher oligomerization state of the two hydrophobic variants provide stabilizing effects, which counteract the perturbation caused by the unnatural building blocks. This systematic study of the interaction profiles of foldameric sequences in helical protein environments provides a knowledge base for the rational design of peptide based pharmaceuticals targeting helical protein interfaces.
REFERENCES


