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Selection of a buried salt bridge by phage display

Toni Vagt[a], Christian Jäckel[a,†], Sergey Samsonov[b], M. Teresa Pisabarro[b], Beate Koksch[a,*]

[a] *Department of Biology, Chemistry and Pharmacy – Institute of Chemistry and Biochemistry, Freie Universität Berlin, Takustraße 3, 14195 Berlin, Germany*
[b] *BIOTEC, TU Dresden – Structural Bioinformatics, Tatzberg 47-51, 01307 Dresden, Germany*

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ABSTRACT

The α-helical coiled coil is a valuable folding motif for protein design and engineering. By means of phage display technology, we selected a capable binding partner for one strand of a coiled coil bearing a charged amino acid in a central hydrophobic core position. This procedure resulted in a novel coiled coil pair featuring an opposed Glu-Lys pair arranged staggered within the hydrophobic core of a coiled coil structure. Structural investigation of the selected coiled coil dimer by CD spectroscopy and MD simulations suggest that a buried salt bridge within the hydrophobic core enables the specific dimerization of two peptides.

The α-helical coiled coil is one of the most widespread structural motifs in nature and is found in motor proteins, transcription factors, viral fusion proteins, and many more.[1] Due to extensive investigations within the last decades, the coiled coil is also one of the best understood protein folding motifs.[2] The structural simplicity and distinctive coherence between sequence and structure allows the de novo design of coiled coils with special features and makes it a useful tool in protein engineering.[3,4] In such cases, one of its most valuable features is the formation of highly specific dimerization domains, which introduces the possibility of targeting viral fusion proteins or transcription factors in therapeutic applications. Furthermore, the coiled coil has proven to be an excellent model system for the systematic investigation of protein folding.[5,6]

Coiled coils typically consist of two to 5 right-handed α-helices that wrap around each other to form a left-handed superhelix. The primary structure of each helix comprises the so-called heptad repeat, a periodicity of seven residues commonly denoted $(a–b–c–d–e–f–g)_n$. Typically, nonpolar residues occupy positions **a** and **d**, forming a hydrophobic surface which initiates oligomerization under aqueous conditions. Charged amino acids in positions **e** and **g** form a second interaction domain which favors coiled coil formation by interhelical ionic interactions, while positions **c**, **b** and **f** are solvent-exposed and thus often populated by polar residues.

Despite the important role of the hydrophobic character of positions **a** and **d** for the formation and stability of the coiled coil folding motif, an analysis of protein databases revealed that in natural occurring proteins up to 20% of these positions are populated by polar and charged residues.[7] In spite of their destabilizing effect, these amino acids play a decisive role in the oligomerization state and orientation of the monomers within the coiled coil.[8,9] As the construction of specific interacting peptide domains is one of the main goals of coiled coil design, modification of the hydrophobic core by charged amino acids represents a promising strategy for the design of coiled coil heteromers.[10] Buried salt bridges have already been used successfully in the design of highly specific interacting coiled coil dimers.[11–13] However, these approaches are commonly based on lysine or arginine analogues with shortened side chains in order to minimize steric mismatches within the hydrophobic core. Even so, folding motifs which are applicable for synthesis in vivo, desirable for many applications, typically require a composition of naturally occurring amino acids. Otherwise, charged interactions within the hydrophobic core provide a promising design principle to direct oligomerization specificity in a manner which keeps the **e**- and **g**-positions free for the introduction of further specifications. To combine both features (control of heteromerization specificity by charged interactions within the hydrophobic core and accessibility by in vivo synthesis) within one system, we were interested in the determination of peptides built up of canonical amino acids which specifically interact with coiled coil strands bearing charged amino acids in hydrophobic core positions.

In contrast to the often used strategy of rational peptide design, we used saturation mutagenesis to construct an extensive library of potential coiled coil pairs which was screened using phage display technology.[14] This technique links the phenotype of a peptide which is displayed on the surface of a bacteriophage with the

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[*] Corresponding author. Fax: +49 0 30 83855644.
E-mail address: koksch@chemie.fu-berlin.de (B. Koksch).

[†] Present address: Laboratory of Organic Chemistry, ETH Zürich, CH-8093 Zürich, Switzerland.

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genotype encoding this peptide within the phage particle. While saturation mutagenesis enables generation of a phage displayed peptide library, physical linkage between phenotype and genotype enables the easy identification of individual peptides which are selected by binding preference to a given target. Phage display has already proven to be a powerful tool for the screening of protein–DNA, protein–protein, and protein–peptide interactions.[15–18] Previously, this technique was successfully applied in the determination of specific coiled coil pairing, demonstrating its suitability for peptide design.[19,20]

The starting point for our studies was the coiled coil pair VPE/VPK, reported by our group to form parallel heterodimers.[21] VPK features lysine in positions **e** and **g** while **e**′ and **g**′ in VPE are occupied by negatively charged glutamic acid to further the formation of heterodimers by ionic interactions under physiological conditions (Fig. 1a). A parallel arrangement as well as a preference for dimer formation is dictated by valine in position **a**.[22] In order to place a negatively charged side chain within the hydrophobic core, Leu_{19} in VPK was replaced by glutamic acid (Fig. 1b). As expected, this substitution results in significant destabilization of the VPK/VPE coiled coil.

Neither homodimerization of VPK-E_{19} nor the formation of VPK-E_{19}/VPE dimers could be observed by CD spectroscopy (Fig. 2). Subsequently, the four amino acid positions within VPE which directly interact with Glu_{19} in VPK-E_{19} were fully randomized and the resulting VPE-library was fused to the minor coat protein pIII on the surface of the filamentous bacteriophage M13 (Fig. 1b). The DNA fragment that encodes for the VPE-peptide, including the four randomized positions $\mathbf{a_{16}}$, $\mathbf{d_{19}}$, $\mathbf{e_{20}}$ and $\mathbf{a_{23}}$, was inserted into the phagemid vector pComb3H to the 5′-end at the gene that encodes for the C-terminal part of the truncated minor coat protein pIII.[23] After successful cloning of the randomized DNA into M13, amplified phage that present the peptide library were used in the selection for binding partners of the Glu_{19}-substituted VPK-variant. VPK-E_{19} used for the selection procedure carries an N-terminal biotin label for immobilization on streptavidin-coated magnetic beads. Coiled coil pairing selectivity was then used to determine the best binding partner in the library, which is able to compensate for the destabilizing effect of the charge within the hydrophobic domain of VPK-E_{19}.

In a control experiment, five rounds of panning against wild type VPK resulted in a variety of sequences which can be summarized as the motifs VPK-$Z_{16}L_{19}X_{20}L_{23}$ and $L_{16}L_{19}X_{20}Z_{23}$, where X indicates predominantly hydrophobic residues and Z marks aromatic residues (Table S3). Despite the surprising variety of selected VPE-variants, the hydrophobic character of the amino acids which were found in the positions of the hydrophobic core clearly correlate with the design principles of the α-helical coiled coil.

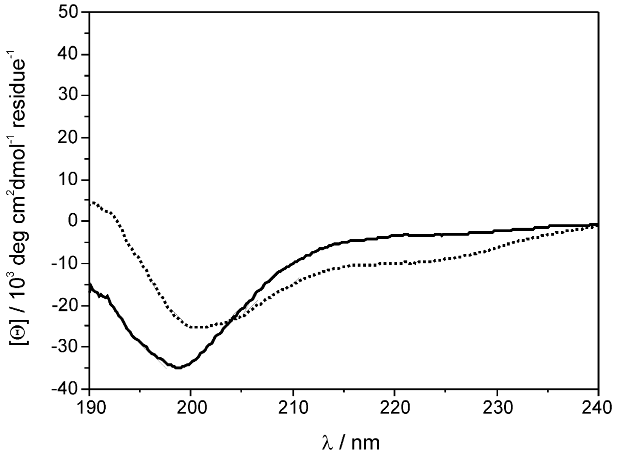

Figure 2. CD spectra of VPK-E_{19} (solid line) and an equimolar mixture of VPK-E_{19} and wild type VPE (dotted line). Spectra were recorded at 20 °C in 100 mM phosphate buffer pH 7.4 at an overall peptide concentration of 20 μM.

In contrast, panning against VPK-E_{19} resulted in greater sequence convergence. Sequencing of nine clones yielded only two different phenotypes: 8 of 9 clones matched the sequence of VPE-LLLK, in which leucine occupies positions $\mathbf{a_{16}}$, $\mathbf{d_{19}}$ and $\mathbf{e_{20}}$ and lysine position $\mathbf{a_{23}}$. The remaining clone differs only in position $\mathbf{e_{20}}$, where methionine was found instead of leucine. Overall, leucine represents the most common amino acid within the hydrophobic core of naturally occurring coiled coil peptides and its selection in positions $\mathbf{a}_{16}$ and $\mathbf{d_{19}}$ is unsurprising. The increase in hydrophobic surface area by the substitution of Glu_{20} with leucine or methionine obviously stabilizes the coiled coil structure even further.

As expected, CD analysis of VPK-E_{19} and VPE-LLLK in isolation yields CD spectra characteristic for predominantly random coil structures (Fig. 3a). In both peptides, the charged residue within the hydrophobic core, in addition to the repulsive interactions between the **e**/**g**′ and **g**/**e**′ positions which are a feature of the original design strongly discourages the formation of homodimers. In addition, a 1:1 mixture of VPK-E_{19} and wild type VPE does not result in coiled coil formation (Fig. 2). In contrast, an equimolar mixture of VPK-E_{19} and VPE-LLLK shows a strong α-helical CD signal. The melting temperature of the new heterodimer is 36.4 °C compared to 71.3 °C for the VPK/VPE wild type (Ref. 21). Plotting the mean

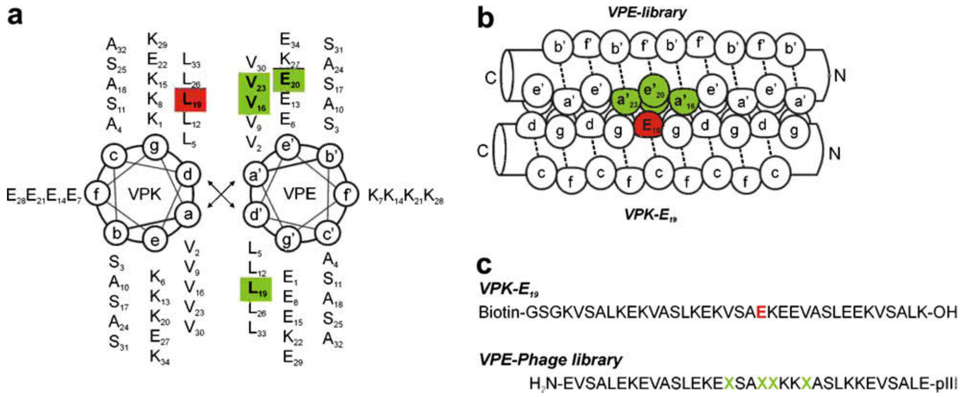

Figure 1. Helical wheel presentation of the parallel VPK-VPE heterodimer (a) and schematic side view of VPK-E_{19} and the VPE-library (b). Glu_{19} in VPK-E_{19} is highlighted in red, while the four randomized amino acid positions in VPE which directly interact with Glu_{19} are highlighted in green. (c) Amino acid sequences of VPK-E_{19} and the VPE-phage library.

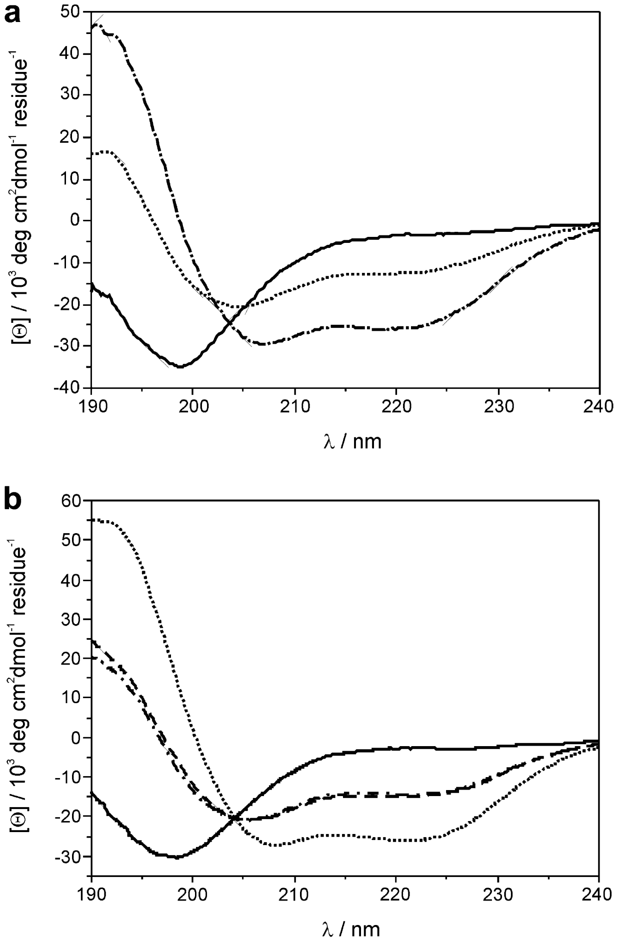

Figure 3. (a) CD spectra of VPK-E_{19} (solid line), VPE-LLLK (dotted line), and an equimolar mixture of VPK-E_{19} and VPE-LLLK (dashed-dotted line). (b) CD spectra of VPK-E_{19} (solid line) and an equimolar mixture of VPK-E_{19} and VPE-LLLL (dashed line). The sum of the single spectra of VPK-E_{19} and VPE-LLLL is presented as dashed-dotted line and the spectrum of VPE-LLLL as dotted line. Spectra were recorded at 20 °C in 100 mM phosphate buffer pH 7.4 at an overall peptide concentration of 20 μM.

residue ellipticity at 222 nm versus the mole fraction of VPE-LLLK shows a minimum at a 1:1 molar ratio of VPK-E_{19} and VPE-LLLK, suggesting the presence of heterodimers (Figure S3). An additional experiment confirmed the importance of Lys_{23} for the formation of the coiled coil heteromer. The control peptide VPE-LLLL differs from VPE-LLLK only in position $\mathbf{a_{23}}$, in which lysine was replaced by leucine. While this single substitution enables homodimerization of VPE-LLLL, no formation of heteromers in combination with VPK-E_{19} could be observed. Instead, an equimolar mixture of the two peptides shows a CD spectrum which exactly matches the sum of the CD spectra recorded for each isolated peptide (Fig. 3b). This indicates that no interaction between VPK-E_{19} and VPE-LLLL takes place. Obviously, Lys_{23} in VPE-LLLK is the key element for the formation of the heteromeric coiled coil dimer VPK-E_{19}/VPE-LLLK.

Our results suggest that the formation of an interhelical salt bridge between Glu_{19} (VPK-E_{19}) and Lys_{23} (VPE-LLLK) represents one of the driving forces for dimerization of VPK-E_{19}/VPE-LLLK and that it determines the specificity of this interaction (Fig. 4). Computer modelling supports this assumption. MD simulations (see Supplementary data for details) demonstrate that the heterodimer is stable in solution. The distances between C_β atoms of the residues in **a**- and **d**-positions in each helix do not fluctuate significantly in the simulation and the backbone dihedral angles remain close to ideal α-helical values. The distance between the carbonyl oxygen of Glu_{19} and amide nitrogen of Lys_{23} was calculated to be less than between 3 Å (95.9% of the simulation time) and 4 Å (99.7% of the simulation time), which strongly suggests the existence of a salt bridge between these functional groups. Furthermore, decomposition of the MM-GBSA energy values show that Lys_{23} contributes the most significant value of all amino acids to the overall energy of the coiled coil interaction (−5 kcal/mol, which is about 20% of the overall free energy of unfolding).

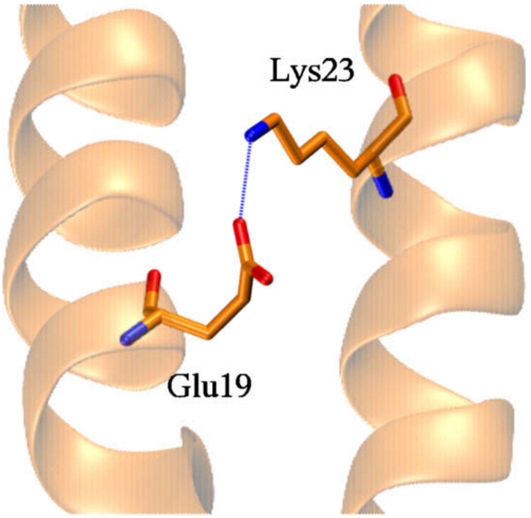

Figure 4. Structure of the proposed salt bridge between Glu_{19} of VPK-E19 and Lys_{23} of VPE-LLLK.

Buried salt bridges within the hydrophobic core of coiled coil peptides created by rational design to construct specific interacting coiled coil pairs have been shown before. Nevertheless, such structures typically are limited to directly opposed **a** and **a**′ or **d** and **d**′ positions. Due to the small distance between these positions in the parallel coiled coil, aspartic acid and positively charged non proteinogenic amino acids with shortened side chains are necessary for stable coiled coil formation.[11–13] However, by using saturation mutagenesis and selection, we were able to find a coiled coil pair characterized by a buried salt bridge between lysine and glutamic acid. Here, the charged amino acids are not placed in positions directly opposed, but staggered within the hydrophobic core. This arrangement between i and i′ + 4 positions apparently enables the formation of a salt bridge between the ammonium function of the lysine side chain and the carboxylate function of the glutamic acid side chain without disruption of the coiled coil structure. Instead, this interaction results in a highly specific dimerization domain made up of canonical amino acids which could potentially be used in protein design and material engineering.[10] The selection of VPE-LLLK not only delivers a new highly specific coiled coil heterodimer but also demonstrates the potential of the phage display-based screening system to select specific interaction partners for uncommon amino acids within the hydrophobic core of coiled coils.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.062.

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