Protein Mimetics

DOI: 10.1002/anie.201106177

Induced-Fit Binding of a Polyproline Helix by a β-Hairpin Peptide**

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The role of prolyl–π interactions in protein structure and function has only recently been recognized.[1–6] Prolyl–π interactions have been proposed to be critical to the binding of polyproline helices, which are binding epitopes in a number of different protein–protein interactions, including recognition events with SH3 domains, WW domains, EVH1 domains, GYF domains, EUV domains, and the single-domain profilin proteins.[5,6] These protein–protein interactions are involved in a wide range of signaling pathways,[6,7] and some are associated with disease states, including HIV infection, Alzheimer's, and cancer,[8–11] making them potential therapeutic targets.[12–15] The recognition domains for polyproline helices contain an aromatic cleft in which at least one proline binds.[5,6] This aromatic cleft is typically displayed on the surface of a β-sheet, as in the WW and EVH1 domains (Figure 1).[5,6] Herein we report the design of a minimalist model system for studying prolyl–π interactions in a biologically relevant context (Figure 1). To this end, we have investigated the use of well-folded β-hairpin peptides as mimics of the aromatic cradles found in oligoproline recognition domains. We find that the ability to bind polyprolines is intimately linked to the peptide structure and that binding occurs by an induced-fit mechanism. This study provides insight into the role of prolyl–π interactions in protein–protein recognition events and provides valuable information for inhibitor design.

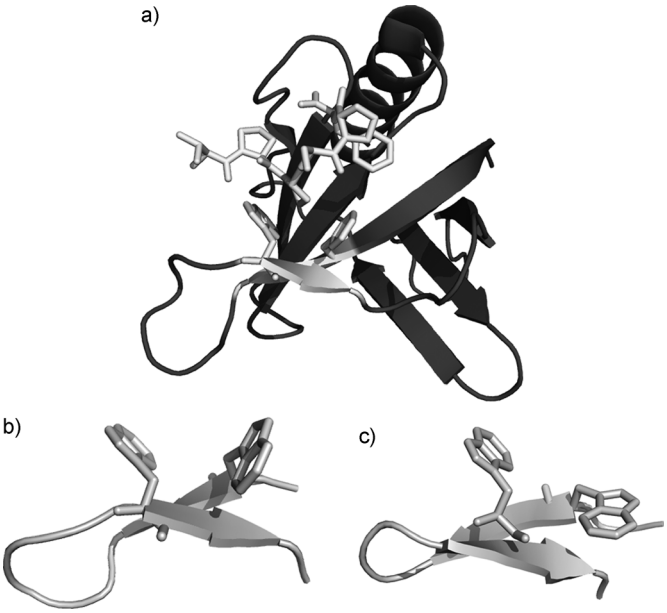

Figure 1. a) The aromatic binding cleft (light gray) of the Homer 1a EVH1 domain (dark gray) bound to the natural ligand TPPSPF (light gray; PDB: 1DDV). b) Structure of the trpzip peptide (PDB: 1LE0) as determined by NMR spectroscopy. c) Structure of the peptide WKWK as determined by NMR spectroscopy.[33]

Two reported β-hairpins, trpzip and WKWK, incorporate an aromatic cradle in their structures, and both have been estimated to be more than 95% folded (Figure 1).[16–19] WKWK has been shown to bind nucleotides with micromolar affinity.[17] In contrast, trpzip has not been shown to provide a binding cleft for a guest. Structures of trpzip and WKWK determined by NMR spectroscopy suggest that trpzip provides an optimal aromatic cradle for oligoproline helices (see overlay in Supporting Information), whereas the aromatic cradle of WKWK appears to be much flatter. Thus, we investigated both sequences as potential oligoproline receptors and found that trpzip is a competent mimic of polyproline-recognition domains, whereas WKWK is not. These studies provide insight into how sequence influences structure and function as well as into the contribution of prolyl–π interactions relative to other noncovalent interactions in protein–protein recognition.

Disulfide exchange was used to measure binding between each β-hairpin peptide and an oligoproline molecule.[20–23] The parent hairpin sequences were appended with a Cys-Gly-Gly sequence at the N terminus for disulfide exchange, and two additional Lys residues were added to the at the C terminus of trpzip to ensure water-solubility and prevent aggregation (Table 1). In addition, D-Pro-Gly was used for the turn

Table 1: Sequences of β-hairpin and oligoproline peptides.

Peptide	Sequence[a]
trpzip	Ac-C-G-G-S-**W**-T-W-E-<u>DP-G</u>-K-**W**-T-W-K-K-K-NH_2
WKWK	Ac-C-G-G-R-**W**-V-K-V-<u>DP-G</u>-O-**W**-I-K-Q-NH_2
CW	Ac-C-W-G-G-NH_2
CW-Pro5	Ac-C-W-G-G-P-P-P-P-P-NH_2
CW-Pro6	Ac-C-W-G-G-P-P-P-P-P-P-NH_2
CW-Pro7	Ac-C-W-G-G-P-P-P-P-P-P-P-NH_2
WC-Pro7	Ac-*W*-*C*-G-G-G-P-P-P-P-P-P-P-NH_2

[a] All amino acids are L-amino acids, except when specifically designated (DPro). O represents ornithine. Bold residues indicate the positions that make up the aromatic cleft. Underlined residues indicate the turn sequence. Italic residues indicate a change in the position of the Trp in the linker region.

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[**] The National Science Foundation is gratefully acknowledged for funding of this research (Grant No. CHE 0716126).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201106177.

sequence in both β-hairpins, as it enforces a strong type II′ turn while avoiding the use of a base-labile Asn residue.[24] Oligoprolines containing 5–7 proline residues were investigated as the ligands, appended with a Cys-Trp-Gly-Gly sequence at the N terminus (Table 1). The Cys was incorporated for disulfide exchange and the Trp was included for UV detection and concentration determination. All of the ligands formed type II polyproline helices, as determined from their CD spectra (see the Supporting Information).[25–28] Lastly, the peptide CW, consisting only of the linker residues, was used as a control.

In a typical disulfide exchange experiment (Figure 2), approximately equal amounts of both β-hairpin and oligoproline. In contrast, the equilibrium constant for trpzip and CW is 1.3 ± 0.15, indicating a favorable self-association of trpzip.[16,20–23] Thus, Equation (1) was used to correct trpzip–

$$\Delta G^{\circ}_{a}(\text{trpzip}) = -R\,T \ln (K_{EQ}/1.3) \quad (1)$$

oligoproline equilibrium constants for the competitive self-association of trpzip (see the Supporting Information). The control reaction of WKWK with CW showed no sign of self-association of WKWK; instead, it demonstrated that WKWK binds to the linker, giving an equilibrium constant of 14 ± 1. This nonstatistical value is likely due to binding of the Trp residue of CW in the aromatic cleft, which is not surprising as WKWK has been found to bind other aromatic molecules.[17–19] Equation (2) was used to correct WKWK–oligo-

$$\Delta G^{\circ}_{a}(\text{WKWK}) = -R\,T \ln (K_{EQ}/14) \quad (2)$$

proline equilibrium constants for the interaction of WKWK with the linker (see the Supporting Information).

Favorable hairpin–oligoproline interactions were observed in all cases with trpzip (Table 2). To confirm that the Trp residue in the linker of the oligoproline peptides is not

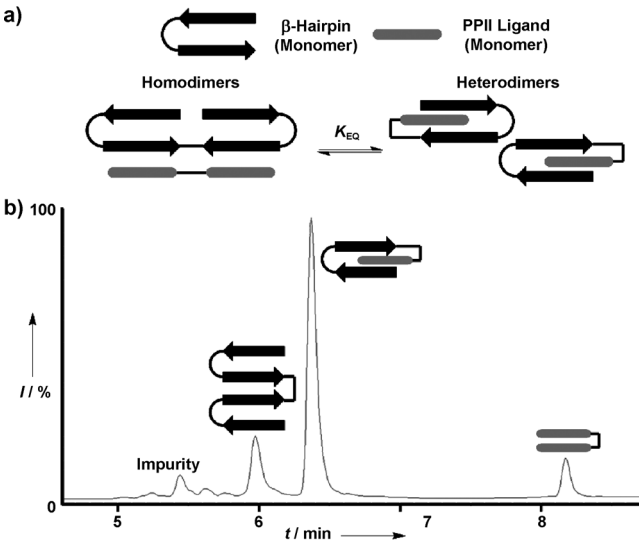

Figure 2. a) Schematic diagram for a disulfide exchange reaction. A statistical mixture should contain twice the concentration of β-hairpin–oligoproline heterodimer as compared to either respective homodimer. b) HPLC analysis of disulfide exchange reaction after equilibrium has been reached. Favorable interactions can be inferred from a non-statistical distribution of species.

lines were mixed in a basic (pH 8.1–8.5) buffer exposed to atmospheric oxygen. The peptides were allowed to oxidize to disulfides, and the reactions were monitored by LC-MS until equilibrium was reached (as verified by spiking experiments, see the Supporting Information). Equilibrium constants (K_{EQ}) were calculated for the exchange reactions based on the corrected integrations derived from the chromatograms (see the Supporting Information). For a statistical mixture, $K_{EQ} = 4$, corresponding to a 1:2:1 ratio of homodimer to heterodimer to homodimer; any deviation from that value suggests a favorable interaction between two components.

The equilibrium distributions were measured for the control peptide CW, which consists of the linker alone, with CW-Pro7 and both hairpins to verify that a statistical mixture is obtained in the absence of an appropriate partner sequence. The equilibrium constant (K_{EQ})for CW-Pro7 and CW is 4, as expected for a system lacking any favorable interactions. This result demonstrates that there is no significant contribution from the interaction of an unstructured Trp residue and the

Table 2: Equilibrium constants K_{eq} for the disulfide exchange reactions and association energies ΔG°_{a}.

Oligoproline	β Hairpin	K_{eq}[a]	ΔG°_{a} [kcal mol^{-1}]
CW-Pro5	trpzip	18 ± 1	-1.6[b]
CW-Pro6	trpzip	47 ± 2	-2.1[b]
CW-Pro7	trpzip	68 ± 3	-2.3[b]
WC-Pro7	trpzip	69 ± 5	-2.3[b]
CW-Pro7	WKWK	15 ± 2[c]	0[d]

[a] Equilibrium constants (K_{eq}) are averages from at least two separate trials. Error estimates were made based on HPLC peak resolution for the different trials. [b] Gibbs free energy change for association (ΔG°_{a}) was calculated using Equation (1). Error values estimated to be less than ± 0.1 kcal mol^{-1} based on the uncertainty value reported. [c] The equilibrium constant (K_{eq}) for WKWK interacting with the linker sequence CWGG was measured as 14 ± 1. [d] Gibbs free energy change for association (ΔG°_{a}) was calculated using Equation (2). Error value estimated to be less than ± 0.1 kcal mol^{-1} based on the uncertainty value reported.

contributing to binding, a scrambled linker sequence was also investigated, in which the Trp residue was placed N-terminal to Cys, giving *WC*-Pro7. This change in sequence had no impact on the equilibrium constant relative to CW-Pro7, indicating that the Trp residue in the oligoproline peptide does not contribute to binding. The interaction is more favorable with longer oligoproline sequences, but levels off at about seven proline residues ($\Delta\Delta G$ for Pro5−Pro6 = 0.5 kcal mol^{-1}; $\Delta\Delta G$ for Pro6−Pro7 0.2 kcal mol^{-1}). Since the CD spectra of CW-Pro5, CW-Pro6, and CW-Pro7 are identical (see the Supporting Information), the observed preference for the longer oligoprolines is not likely due to increased helicity. Instead, this trend may reflect additional contacts or more optimal positioning of the oligoproline for binding.

In contrast to trpzip, WKWK exhibits no binding to the oligoproline CW-Pro7 (Table 2). The K_{eq} for CW-Pro7 is the

same as for the CW control peptide, indicating that binding of the Trp sidechain is preferred over binding of a proline residue in this case. This result indicates that an aromatic cleft alone is not sufficient to bind a polyproline helix. This level of selectivity is impressive, given that both hairpins have the same type II′ turn sequence and both form an aromatic cleft between Trp5 and Trp12.

NMR spectroscopy studies were performed on the disulfide-linked trpzip–Pro7 heterodimer to characterize the nature of the interaction. NOEs indicate that preferential binding of one proline residue occurs between Trp5 and Trp12, which are the two internal Trp residues that make up the aromatic cleft, with NOEs primarily to a single Pro residue (Figure 3). No NOEs were observed between a Pro residue and the other two Trp sidechains, suggesting that they

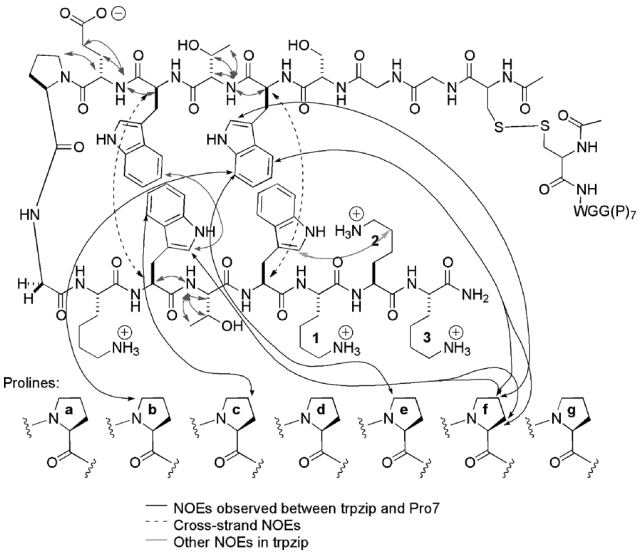

Figure 3. NOEs in the trpzip–Pro7 heterodimer. The labels for the Pro residues (**a**–**g**), do not correspond to their order in the polyproline helix, but instead to their assignments in the NMR spectra (see the Supporting Information). The order of the prolines could not be assigned.

do not contribute to binding. These results confirm that the binding interaction occurs as designed. We cannot definitively assign which Pro residue in Pro7 interacts preferentially with the aromatic cage, but we expect that it is Pro6, since longer oligoprolines form more favorable interactions.

Interestingly, comparison of the NMR spectra of trpzip alone and the trpzip–Pro7 heterodimer indicates a number of structural changes to trpzip (Figure 4). Most notably, the Hα protons of Trp5, 12, and 14 are shifted downfield, Trp 7 is upfield shifted, and the Hα protons of the neighboring Thr6 and Thr13 residues are shifted downfield. These changes in the backbone proton chemical shifts indicate that this region of the peptide changes conformation upon binding to Pro7. Importantly, these shifts are consistent with the peptide becoming more folded,[29] and cross-strand NOEs show that a β-hairpin structure is maintained. These results clearly indicate induced-fit binding of the oligoproline by the β-

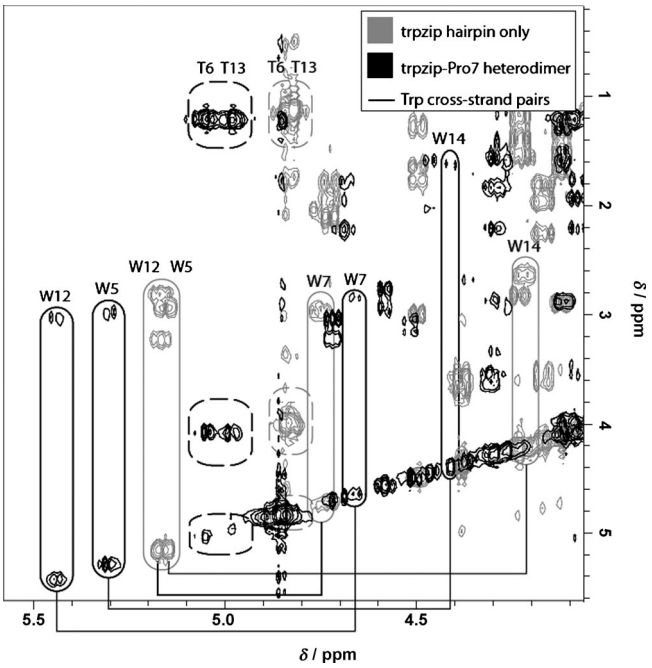

Figure 4. Overlayed 2D TOCSY spectra for the trpzip peptide (gray) and the trpzip–Pro7 heterodimer (black), showing the change in Trp and Thr proton chemical shifts in the heterodimer relative to the monomer. Black lines indicate the cross-strand pairs of Trp residues.

hairpin. This situation is in contrast to the binding of ATP by WKWK, in which no change in Hα chemical shifts were observed upon binding.[17]

Taken together, these findings indicate that trpzip forms a competent aromatic cleft for the binding of a Pro sidechain, while the shape of the cleft in WKWK is not appropriate. This system represents a minimalist example of sequence defining structure defining function, where small perturbations of the surrounding sequence drastically alter the binding properties, favoring either a proline guest or an aromatic guest depending on the sequence surrounding the aromatic cleft. It is interesting to note that the prolyl–π interaction in trpzip–Pro7 is very similar in energy to the aromatic interaction in the binding of ATP to WKWK (estimated to be -1.8 kcal mol^{-1}).[8] In addition, the trpzip–Pro7 interaction is stronger than the aromatic interaction between WKWK and Trp in the WKWK–CW heterodimer (-0.7 kcal mol^{-1}).

Zondlo and co-workers used Pro *cis–trans* isomerization in a series of tetrapeptides to measure the thermodynamic driving force for a number of aromatic–prolyl interactions and determined the energy for a Trp-Pro interaction to be about -0.9 to -1.0 kcal mol^{-1}.[30] The value we have measured for the trpzip–Pro7 interaction (-2.3 kcal mol^{-1}) implies that two prolyl–π contacts contribute to oligoproline recognition. This situation is what would be expected for a Pro bound in the aromatic cleft between two Trp residues, as indicated by the NMR spectroscopy studies above and the mode of binding observed in native protein–protein interactions (Figure 1).[5,6]

The association energy for the trpzip–oligoproline interaction is similar in magnitude to other tertiary interactions

between peptide secondary structures that have been reported recently. For example, Gellman and co-workers found that the association energy for a covalently linked coiled-coil dimer consisting of two-heptad repeats is −0.5 to −3.0 kcal mol^{-1}, depending on the exact sequence.[31] Furthermore, Roy and Case have recently reported a number of covalently linked 2.5-heptad repeat trimeric coiled coils with association energies up to −1.8 kcal mol^{-1}.[32] The association energies in several of the coiled-coil systems are weaker than the β-hairpin–oligoproline interaction reported herein, even though more contacts are made in the coiled coils. For example, in a typical 2-heptad coiled coil, eight hydrophobic residues interdigitate to make up the core. Coiled-coil formation involves a large degree of binding-induced folding of the helices, while both the β-hairpin and oligoproline are somewhat preorganized for binding. This situation may explain the relatively large binding affinity for trpzip–Pro7 relative to the coiled-coil association energies. Indeed, the rigid nature of the type II polyproline helix has often been invoked as the reason why it is a common mediator of protein–protein interactions.[1–6]

In conclusion, we have shown that the trpzip peptide serves as a minimal model system for oligoproline-binding domains. These studies provide an estimate of the contribution of prolyl–π interactions to the overall binding affinity between oligoprolines and their binding domains, which may have application to inhibitor design for such protein–protein interactions. In addition, these studies demonstrate that while an aromatic cleft can provide substantial binding affinity for oligoproline recognition, this recognition is sensitive to small perturbations in structure, emphasizing the connection between sequence, structure, and function that provides specificity in biological systems. Lastly, this is a rare example of induced-fit binding in a minimal model system, mimicking the type of binding that is common in protein–protein interactions.

Experimental Section

Peptides were synthesized by automated solid-phase peptide synthesis on a Thuramed tetras synthesizer using standard fmoc protection strategies. Peptides were purified by reversed-phase HPLC. Disulfide exchange experiments were conducted by mixing approximately equal proportions of β hairpin and ligand peptides in 10 mM phosphate buffer, pH 8.1–8.5. Equilibration typically required several days. Integrated areas were corrected for the number of Trp residues before calculations were made.

Received: August 31, 2011
Published online: October 28, 2011

Keywords: peptide structure · prolyl–π interaction · protein mimetics · protein–protein interactions · β hairpins

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