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Stabilizing and Understanding a Miniprotein by Rational Redesign

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Supporting Information Placeholder

ABSTRACT: Miniproteins reduce the complexity of the protein-folding problem allowing systematic studies of contributions to protein folding and stabilization. Here, we describe the rational redesign of a miniprotein, PP α , comprising a polyproline-II helix, loop and α helix. The redesign provides a *de novo* framework to interrogate non-covalent interactions. Optimized PP α has significantly improved thermal stability with a midpoint unfolding temperature (T_M) of 51 °C. Its NMR structure indicates a higher density of stabilizing non-covalent interactions than the parent peptide, specifically increased CH- π interactions. In part, we attribute this to improved long-range electrostatic interactions between the two helical elements. We probe further sequence-to-stability relationships in the miniprotein through a series of rational mutations.

The primary sequence of a protein determines its three-dimensional structure and function. Understanding sequence-to-structure relationships is known as the protein-folding problem.¹ Studying miniproteins reduces the complexity of the problem, allowing contributions to protein folding and stability to be dissected more easily.^{2, 3} Prominent examples of miniproteins include: the Trp-cage,⁴ $\beta\alpha\beta$ motifs,⁵ villin headpiece,⁶ pancreatic peptides⁷⁻¹⁰ and the Trp-Plexus.¹¹ The formation of many weak and cooperative non-covalent interactions is central to protein stability, allowing the loss of conformational entropy upon folding to be overcome. This is particularly important for miniproteins with fewer interactions possible.¹²

α Helices are common building blocks in proteins. They are often stabilized by tertiary and quaternary interactions, as exemplified in α -helical coiled coils, a ubiquitous folding motif comprising two or more α helices supercoiled around each other.¹³ Most coiled-

coil sequences have a seven-residue heptad repeat, **abcdefg**, with hydrophobic residues at **a** and **d**, and polar residues at the remaining sites. This pattern leads to the association of helices *via* “knobs-into-holes” packing interactions, where side chains of one helix dock into diamond-shaped holes of another.^{14, 15} α Helices can also be stabilized by the interdigitation of proline (Pro, P) from polyproline-II helices between aromatic residues on α helices, which are analogous to knobs-into-holes packing.^{2, 7, 16}

Elsewhere, we describe the fragment-based design of a 34-residue miniprotein, PP α .¹⁷ This comprises a polyproline-II helix, loop and α helix adapted from natural proteins (Figure 1A,B).^{7, 16} PP α is monomeric in aqueous solution and unfolds reversibly with a T_M of 39 °C. A solution NMR structure shows the stabilizing effect of Pro and tyrosine (Tyr, Y) interdigitation and associated CH- π interactions. In the latter, protons of C-H bonds interact with aromatic rings.¹⁷⁻²⁰ Here, we explore the optimization of PP α by rational redesign, revealing sequence-to-stability relationships for the miniprotein fold and delivering a *de novo* framework of significantly enhanced thermal stability.

In the design of α -helical coiled coils, charged residues often flank the hydrophobic core and form inter-helical Coulombic interactions.² We reasoned that PP α might be stabilized by introducing similar interactions between the **2** and **3** positions of the polyproline-II helix and the **e** and **g** positions of the α helix (Figure 1A,C).

There are two extreme possibilities for pairing charged residues in PP α : a lysine (Lys, K)-based polyproline-II helix and a glutamic acid (Glu, E)-based α helix or *vice versa* (PP α -KE and PP α -EK, respectively, Table 1). We optimized helix propensity for the solvent-exposed face of the α helix by placing alanine (Ala, A) at **b** and **c** and Lys at **f** in both designs.

Otherwise, the helix-helix interface was maintained, with Pro at **1**, leucine (Leu, L) at **a** and Tyr at **d**, along with the PP α loop. We adopted the coiled-coil heptad nomenclature for the α -helical sequence as it has a 7-residue repeat.

Figure 1. Helical wheel representations for the two helices in PP α (A) and PP α -KE (oPP α) (C). Leaf tips indicate the direction of the C α -C β bond vectors. Lowercase letters on the α helix and numbers on the polyproline-II helix denote the helical register. C and N refer to the helix termini nearest the viewer. Dashed black lines: long-range interhelical electrostatic interactions. Representative models from the NMR ensembles of PP α (B, PDB entry 5LO2, model 14) and PP α -KE (D, PDB entry 6GWX, model 8). Dashed yellow lines: CH- π interactions. Key: Lys (blue), Glu/Asp (red), and Tyr/Pro/Leu (green), Gln (orange).

PP α -KE and PP α -EK were synthesized by solid-phase peptide synthesis, purified by reverse-phase HPLC, and confirmed by MALDI-TOF mass spectrometry (Figure S1A,B). Circular dichroism (CD) spectroscopy revealed both peptides were 50% α helical at 5 °C like PP α (Figure 2A). Sedimentation equilibrium analytical ultracentrifugation (AUC) showed that both peptides were monomeric (Figure S3A,B). PP α -KE had cooperative and reversible thermal unfolding transitions when followed by far- and near-UV CD (Figure S2U,V), and the T_M (51 °C) was substantially increased over PP α (39 °C). However, PP α -EK was less stable than PP α -KE by 10 °C (Figure 2B). We posit that the reduced stability of PP α -EK stems from the large

charge on the α helix—+6 compared to -2 for PP α -KE—and different propensities of Glu and Lys for α and polyproline-II helices (Table S1,2).^{21,22}

High-resolution NMR spectroscopy was used to determine the solution-phase structure of PP α -KE (Figure 1D), with 95.8% of the ¹H NMR resonances assigned. The core of the structure was well defined, while several of the solvent-exposed residues were more dynamic. The root-mean-square deviations (RMSDs) across the 20 structures of the ensemble were 0.52 ± 0.13 Å (backbone) and 1.05 ± 0.15 Å (all atom), comparable to those for the structure of PP α (Table S3).¹⁷ A representative structure matched PP α with backbone and all-atom RMSDs of 0.5 Å and 0.9 Å, respectively.

The average distances between N ϵ of Lys and C δ of Glu introduced in PP α -KE were 8.7 ± 1.8 Å and 11.5 ± 2.4 Å for **2:e** and **3:g**, respectively. These fall outside of any accepted definition of salt-bridge interactions,²³ suggesting longer-range electrostatic interactions may stabilize the folded state of PP α -KE. To test this, the thermal stability of PP α -KE was measured at different salt concentrations in phosphate buffer (Figure S2C,D). With increased concentrations of NaCl from 0 to 700 mM the T_M fell from 61 to 57 °C (Figure S2W), indicative of an electrostatic component to thermostability.

As PP α is stabilized by CH- π interactions,¹⁷ we searched for these in PP α -KE. This revealed 87 CH- π interactions across the ensemble (4.35 per structure), an increase over PP α (68 CH- π interactions; 3.4 per structure) (Figure 1B,D, Table S5 and Figure S8). A shift in CH donors between the two miniproteins was also observed. In PP α -KE fewer CH donors emanated from Pro residues (PP α -KE, 5; PP α , 22), but more arose from Leu (PP α -KE, 25; PP α , 4) and Lys residues (PP α -KE, 57; PP α , 15). On closer inspection, the side chains of Lys4 and Lys7 of PP α -KE lie across the faces of Tyr27 and Tyr20, respectively, leading to CH- π interactions between the C α and C β protons of the former and the aromatic rings of the latter. To test for CH- π interactions experimentally, we replaced all of the electron-rich Tyr residues in PP α -KE by more-electron-poor phenylalanine (Phe, F) giving PP α -KE-Phe (Table 1). As with PP α ,¹⁷ this reduced the T_M by approximately 20 °C (Figure S2E,F). This indicates that CH- π interactions are at play in PP α -KE in addition to van der Waals' forces.

Overall, we posit that the improved stability of PP α -KE arises from increased long-range electrostatic interactions between the polyproline-II and α helix,²⁴⁻²⁷ which lead to an increased density of shorter-range CH- π interactions.

Tyr residues at the **d** sites of the 7-residue repeats of PPα and related natural folds are critical for folding and stability.^{7, 16, 17} These residues form two vertices of 4-residue ‘holes’ defined by side chains at successive **d**, **a**, **g**, and **d** sites, which accommodate Pro ‘knobs’ from the polyproline-II helix. In PPα and PPα-KE, the **g** site is fixed as aspartic acid (Asp, D) or Glu,

respectively. The **a** sites are all Leu, which is the preferred residue in natural sequences.^{7, 28} To explore possible substitutes for Leu at **a**, we made mutants with all three **a** sites replaced by: β-branched, isoleucine (Ile, I) or valine (Val, V); charged,

Table 1. Designed peptides and summary of biophysical data.

Peptide	Sequence and helical register	TM / °C	AUC *
	<i>321321321321321</i> <i>efgabcd efgabcd efgabcd efgabcd efgabcd</i>		
Parent PPα	PPTKPTKP GDNAT PEKLAKY QADLAKY QKDLADY	39	0.9
PPα-KE/oPPα	PPKKPKKP GDNAT PEKLAAY EKELAAY EKELAAY	51	1.0
PPα-EK	PP EEPEEP GDNAT PEKLAAY KKKLAAY KKKLAAY	40	0.9
PPα-KE-Phe	PPKKPKKP GDNAT PEKLA AF EKELA AF EKELA AF	33	1.0
PPα-KE-E@a	PPKKPKKP GDNAT PEK E AAY EK E AAY EK E AAY	-	-
PPα-KE-A@a	PPKKPKKP GDNAT PEK A AAY EK A AAY EK A AAY	-	1.0
PPα-KE-K@a	PPKKPKKP GDNAT PEK K AAY EK K AAY EK K AAY	19	1.0
PPα-KE-I@a	PPKKPKKP GDNAT PEK I AAY EK I AAY EK I AAY	40	1.0
PPα-KE-V@a	PPKKPKKP GDNAT PEK V AAY EK V AAY EK V AAY	34	1.0
PPα-KE-A@g	PPKKPKKP GDNAT PEKLA EY EK ALA EY EK ALA EY	21	1.0
PPα-KE-L@g	PPKKPKKP GDNAT PEKLA EY EK L LA EY EK L LA EY	76	1.2
PPα-KE-a↔g	PPKKPKKP GDNAT PEKLAAY A A L E K EY A A L E K EY	19	1.1
oPPα-2	PPKKP GDNAT PEKLAAY EKELAAY	19	0.9
oPPα-4	PPKKPKKP PKKP GDNAT PEKLAAY EKELAAY EKELAAY EKELAAY	66	1.0
oPPα-5	PPKKPKKP PKKP PKKP GDNAT PEKLAAY EKELAAY EKELAAY EKELAAY EKELAAY	72	1.0
oPPα-5-skip	PPKKPKKP PKKP PKKP GDNAT PEKLAAY EKELAAY EKELAAY EKELAAY EKELAAY	68	1.0

Peptides were capped with N-terminal acetyl and C-terminal amide groups. *Oligomeric state (× monomer mass) as determined by AUC. Mutations to the PPα-KE/oPPα interface are shown in bold.

Glu or Lys; or Ala (Table 1).

AUC confirmed that these PPα-KE-X@a mutants were monomeric in solution (Figure S3). However, CD spectroscopy revealed a range of stabilities. PPα-KE-E@a was not folded, possibly due to the proximal glutamates at **e** if the α helix were folded. The stabilities of PPα-KE-A@a and PPα-KE-K@a were compromised compared to PPα-KE (Figure 2C,D). By contrast, both PPα-KE-I@a and PPα-KE-V@a were folded, albeit with reduced thermal stabilities compared with PPα-KE. This suggests that the extra steric bulk of the β carbon may hinder docking of Pro into the hole, although we note that α-helical propensities of both Ile and Val are also lower than that of Leu.²¹

We also probed the **g** position of the hole, initially *via* an Ala mutation to determine the contribution of this residue to stability. To maintain the introduced electrostatic interactions between the helices, Glu was shifted to the **c** position, although this may introduce

favorable K_i→E_{i+4} interactions. The resulting PPα-KE-A@g was monomeric, but significantly less stable than PPα-KE (Figure S2I,J). Next, we mutated the **g** position to Leu, giving a fully hydrophobic hole. The thermal unfolding transition of PPα-KE-L@g was broad, with a T_M of 76 °C (Figure S2K,L). However, the CD signal was concentration dependent, indicating aggregation (Figure S2M,N). AUC experiments returned a mass of 1.2 × monomer mass, suggesting PPα-KE-L@g forms higher-order species, which could involve the wider hydrophobic interface. Our interests lie in fully monomeric miniproteins, so the **g** positions were not probed further.

As a final exploration of hole-residue preferences, we swapped the **a** and **g** residues (PPα-KE-a↔g). This tests an alternate helical register, LXXYXXX, as opposed to LXXYXXX in PPα-KE. Residues at **e** and **c**, and **b** and **f** were swapped to avoid introducing charged interactions in the α helix of PPα-KE-a↔g not present

in PP α -KE. PP α -KE- $\alpha\leftrightarrow g$ was less folded and less thermally stable (T_M 19 °C) than PP α -KE. Thus, PP α -KE possesses the optimal arrangement of residues in the diamond-shaped hole.

The results of this mutagenesis study reveal that Leu at **a**, Tyr at **d** and Glu at **g** provide the optimal 'hole' to accommodate Pro in PP α -like miniproteins. On this basis, we renamed PP α -KE as optimised-PP α , oPP α .

Using oPP α , we investigated the effect of helix length on stability. Variants were synthesised with 1-, 3- and 4-unit polyproline-II-helical repeats and corresponding 2-, 4- and 5- heptad α -helical repeats (Table 1, Figure S15). Like oPP α , which completes this series with two polyproline-II and three

Figure 2. Folding and stability of PP α -KE and mutants. Far-UV CD spectra at 5 °C for: (A) parent PP α (black, dash), PP α -KE (black), and PP α -EK (purple); and (C) PP α -KE-E@a (red), PP α -KE-A@a (sky blue), PP α -KE-K@a (blue), PP α -KE-I@a (green), PP α -KE-V@a (orange), and PP α -KE (black). (B&D) Temperature dependence of the mean residue ellipticity (MRE) at 222 nm, with the key as for panels A&C. Conditions: 100 μ M peptide, PBS, pH 7.4.

α -helical repeats, these peptides were monomers (Figure S3M,N,O). However, only oPP α , oPP α -4 and oPP α -5 were appreciably and stably folded (Figure 3), with the T_M increasing from 51 to 66 to 72 °C, respectively. As observed in α -helical coiled coils, folding and stability increases in a non-linear, cooperative manner.^{29, 30}

Figure 3. Folding and stability of oPP α variants of different lengths. (A) Thermal unfolding curves for oPP α -2 (blue), oPP α (black), oPP α -4 (green), and oPP α -5 (pink). (B) Plot of heptad length vs T_M (blue) and MRE₂₂₂ at 5 °C (red). Conditions: 100 μ M peptide, PBS, pH 7.4.

There is a periodicity mismatch between the 3- and 7- residue sequence repeats of polyproline-II and α helices: the former span $\approx$ 9.3 Å and the latter $\approx$ 10.5 Å. For oPP α -5 this could result in a $\approx$ 6 Å discrepancy over the lengths of the helices in the interface. The bacterial adhesin AgI/II from *S. mutans* overcomes this with Pro insertions that break the PXX repeat.¹⁶ Therefore, we introduced an extra Pro to the middle of the polyproline-II repeat of oPP α -5. This peptide, oPP α -5-skip, was monomeric, folded and stable (Figure S2S,T) but had a slightly lower T_M than the parent. This suggests that the helix-helix interface of the oPP α fold is plastic and accommodates minor mismatches in periodicities, perhaps not possible for longer, fibrous assemblies.

In summary, a miniprotein fold has been optimized by rational protein redesign to give oPP α , a completely *de novo* framework with significantly enhanced thermal stability relative to the parent design. We propose that complementary long-range electrostatic interactions and increased shorter-range non-covalent interactions are responsible for the enhanced stability. The specific sequence-to-structure/stability relationships that we have explored should help guide further designs of PP α -like miniproteins. oPP α should be a useful addition to the toolkit of synthetic peptide building blocks for constructing more-complex systems through modular assembly.^{31, 32}

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details and characterization data.

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Notes

The authors declare no competing financial interest.

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