Engineering a Monomeric Miniature Protein

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The stability, size, and structure of avian pancreatic polypeptide (aPP) makes it a useful starting point for the design of miniature proteins that bind with high affinity and specificity to DNA[1−4] and proteins[5−14] and inhibit their interactions, both with high affinity and specificity, both in vitro[1−6,8,9,11−14] and in mammalian cells and extracts.[7,10] The utility of these molecules in a cellular context could be diminished, however, by self-association: aPP forms a dimer at $10^{−6}$ M concentration,[15,16] a property shared by some, but not all, aPP-based miniature proteins.[8,9,17−19] Interestingly, two miniature proteins, p007, which binds DNA,[2,4] and pGolemi, which binds EVH1 domains,[7] remain monomeric and well-folded at $10^{−4}$ M concentration despite primary sequence differences that exceed 50% (Figure 1). This observation suggests that it should be possible to identify aPP variants that are both monomeric and well-folded. Here we systematically isolate, characterize, and remove two structural elements responsible for aPP dimerization and install a new element—a proline switch—that single-handedly repacks aPP's signature fold. The result is a monomeric and well-folded miniature proteins that can serve as a starting point for the *in vitro* and *in vivo* applications of these molecules.

The structures of pancreatic fold proteins (PP) are composed of an N-terminal type II polyproline helix that folds upon a C-terminal α-helix to generate a stable, well-packed hydrophobic core.[21−24] The first evidence of aPP self-association was the X-ray structure, which revealed an antiparallel dimer (Figure 1A).[20] Molecular sieve chromatography indicated that formation of the aPP dimer was pH- and temperature-dependent, with an equilibrium dissociation constant (K_d) between 400 pM and 5 μM.[15,16] However, not all PP-fold proteins self-associate; the NMR structure of peptide YY (PYY), an aPP ortholog, shows an aPP-like hydrophobic core but no evidence of dimerization.[22,23,25] Subsequent sedimentation equilibrium experiments confirmed that PYY remains monomeric in the micromolar concentration range, forming a dimer only at a very high concentration ($K_d = 21$ mM).[21]

Visual inspection of the aPP structure identifies three potentially stabilizing interactions at the dimer interface (Figure 1A). An intermolecular π-stacking interaction between Y7 side chains is evident (Figure 1B), with the orientation of each Y7 side chain defined by an intramolecular edge-to-face interaction with F20 (Figure 1B); an intermolecular π-stacking interaction between Y21 side chains is also observed (Figure 1C). Comparison of the sequences of natural and designed PP-fold miniature proteins reveals that all self-associating molecules contain tyrosine at position 7, whereas the nonassociating pGolemi, p007, and PYY do not (Figure 1D). The identities of the side chains at positions 20 and 21, however, do not correlate with self-association; most miniature proteins (including pGolemi) contain phenylalanine at position 20, and the residue at position 21 varies widely across miniature proteins in the PP family.

To determine the extent to which these residues contribute to aPP dimer stability, we prepared aPP variants containing alanine
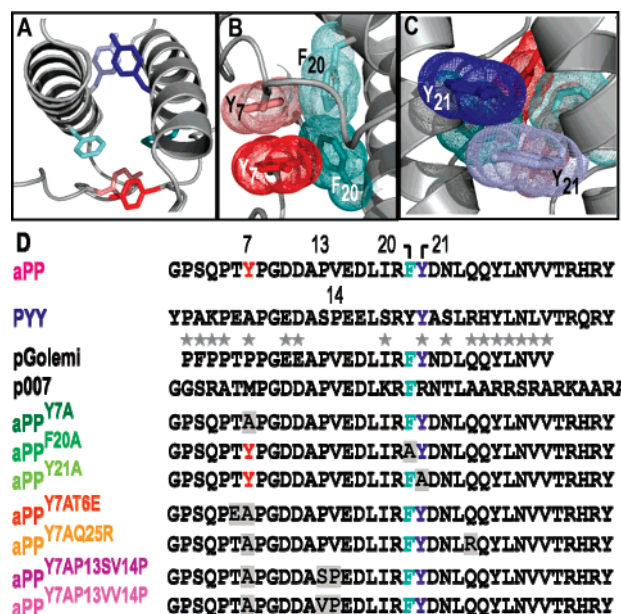

Figure 1. (A) Ribbon diagram of the aPP dimer[20] highlighting pairs of Y7 (red, pink), F20 (teal, light teal), and Y21 (blue, light blue) side chains. (B) Close-up of intermolecular network comprising Y7 and F20 from both monomers. (C) Close-up of intermolecular π-stacking interaction between Y21 side chains. In panels B and C, equivalent residues on each monomer of the aPP dimer are distinguished by black and white residue labels. (D) Alignment of natural and designed PP-fold proteins and variants prepared as part of this work. Residues that differ from wt aPP are shaded; those that differ between p007 and pGolemi are starred.

in place of each of the residues present at the dimer interface (aPP[Y7A], aPP[F20A], aPP[Y21A]) and characterized them using circular dichroism (CD) and analytical ultracentrifugation (AU). aPP[Y7A] and aPP[F20A] both assemble into tetramers, with K_d values of $3.9 \times 10^{−12}$ and $7.6 \times 10^{−11}$ M³, respectively. Because of the stoichiometry of self-association, however, aPP[Y7A] and aPP[F20A] are tetrameric only at very high concentration: at 30 μM more than 97% of the molecules remain monomeric. By contrast, aPP, with $K_d = 4.1 \times 10^{−6}$ M, exists predominantly (>94%) in the dimer state at 30 μM. Although less prone to self-association than aPP, as monomers neither aPP[Y7A] nor aPP[F20A] assembled into the hairpin fold that characterizes PP-fold proteins, as judged by minimal negative ellipticity at 222 nm and a shift in the 208 nm minima to 205 nm (Figure 2A). By contrast, aPP[Y21A] formed a modestly *more* stable dimer than did aPP, as judged by analytical ultracentrifugation ($K_d = 1.3 \times 10^{−6}$ M) and CD (16 500 and 14 700 deg·cm²·dmol⁻¹ at 208 and 222 nm, respectively). These data suggest that Y7 and F20 of aPP contribute to both dimer stability and maintenance of the characteristic aPP fold.[26] Y21, although positioned at the dimer interface in the X-ray structure, contributes modestly to dimer stability.
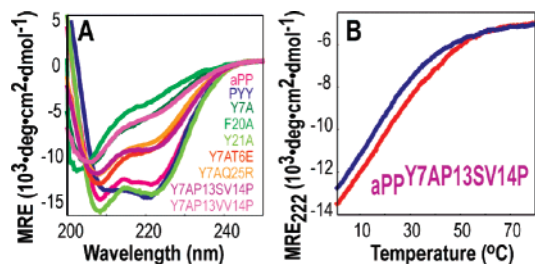
Figure 2. (A) Wavelength-dependent circular dichroism (CD) spectra of aPP variants. (B) Temperature-dependent change in the ellipticity at 222 nm of aPP^{Y7A,P13S,V14P} as the temperature is raised (red) and then lowered (blue). All spectra were acquired at 30 μM concentration in Tris-Cl buffer (25 mM Tris, 50 mM NaCl, (pH 8.0)).

We next explored two strategies to stabilize and rebuild the hairpin fold of monomeric aPP^{Y7A}. The first strategy builds on the observation of Bjornholm *et al*. that PYY possesses a stronger dipole moment (449 D) and a larger electrostatic stabilization energy (−10 kcal·mol^{-1}) than does aPP (430 D and −7 kcal·mol^{-1}, respectively).[27] To evaluate whether macrodipole stabilization would increase the stability of monomeric (but poorly folded) aPP^{Y7A}, we prepared a second set of variants containing two additional residues from PYY: a glutamic acid at position 6 (near the α-helix N-terminus) and an arginine at position 25 (near the C-terminus). Sedimentation equilibrium experiments showed that neither aPP^{Y7A,T6E} nor aPP^{Y7A,Q25R} was appreciably monomeric at 30 μM, with 45% and 46% of the solution forming tetramers, respectively. The CD spectra of both molecules show minima at 208 and 222 nm that are likely due to the large fraction of molecules assembled into well-folded tetramers at this concentration. These results indicate that macrodipole stabilization alone is insufficient to refold aPP^{Y7A}.

A more subtle difference between aPP and PYY is a proline residue whose location effectively demarcates the N-terminus of the α-helix. This proline is highly conserved among PP-fold family members; however, its position varies between residue 13 and 14 (Figure 1). By specifying where the α-helix begins, this proline defines the relative orientation of the PPII and α-helices and thus the precise packing structure of the hydrophobic core. Indeed, Zerbe and co-workers recently reported that altering the residues at positions 13 and 14 in PYY dramatically destabilizes the PP-fold.[28] To determine whether this "proline switch" could increase the stability of poorly folded but monomeric aPP variants, we synthesized two variants of aPP^{Y7A} containing the sequences VP and SP at positions 13 and 14, in place of the natural PV sequence. aPP^{Y7A,P13V,V14P} remained predominantly monomeric (90%) at 30 μM concentration[29] but lacked a well-defined conformation, as judged by CD. aPP^{Y7A,P13S,V14P}, however, displayed significant minima at both 208 (12 400 deg·cm^2·dmol^{-1}) and 222 nm (9 700 deg·cm^2·dmol^{-1}) (Figure 2A). Temperature-dependent CD studies revealed that, like PYY, aPP^{Y7A,P13S,V14P} underwent a cooperative unfolding transition with a T_m of 20 °C (Figure 2B). We also conducted AU experiments at temperatures from 5 to 30 °C (5 °C increments) to fully characterize how the extent of self-association varies with temperature.[29] At 25 °C, the data for aPP^{Y7A,P13S,V14P} fits a monomer/dimer/tetramer model with K_D values of 5.3 × 10^{-4} M and 6.8 × 10^{-12} M^3, respectively, which corresponds to 90% monomer, 9% dimer, and 1% tetramer at 30 μM.

In conclusion, here we show that some, but not all, side chains located at the aPP dimer interface contribute significantly to dimer stability. Although substitutions at these positions reduced dimer stability at the expense of tertiary structure, insights gleaned from comparisons among natural and designed PP-fold family members identified a key "proline-switch" that restored the signature aPP fold. The result is a well-folded miniature protein that is monomeric at concentrations above that where aPP-derived miniature proteins associate with their macromolecular targets. We expect this improved platform to accelerate *in vitro* and *in vivo* studies of functional miniature proteins.

Supporting Information Available: Materials and experimental methods; CD and AU data. This material is available free of charge via the Internet at http://pubs.acs.org.

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