Stabilization of a miniprotein fold by an unpuckered proline surrogate

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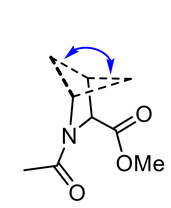
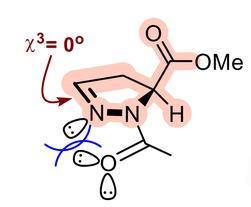
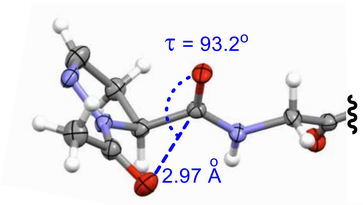
BACKGROUND and OBJECTIVES

The installation of unnatural monomers into proteins can augment their thermal stability, resistance to proteolysis, and biological activity.[1,2] Proline (Pro) is a unique proteinogenic residue due to isoenergetic *cis/trans* amide rotamers and constrained backbone torsions. Given its ability to modulate protein folding and dynamics, there is considerable interest in the development of unnatural and tunable Pro surrogates.[3,4] We recently described an unpuckered Pro surrogate, γ,δ-dehydro-δ-azaproline (ΔaPro), with unusually high *trans* amide rotamer bias, low amide isomerization barrier, and backbone torsions typical of a polyproline II (PPII) fold.[5] We hypothesized that ΔaPro could enhance miniprotein stability upon incorporation into PPII and loop domains.

Study Objectives: [1] Develop an efficient synthetic protocol to incorporate ΔaPro into peptides and proteins; **[2]** Evaluate the effect of ΔaPro substitution on the folding and stability of the avian pancreatic polypeptide (aPP).

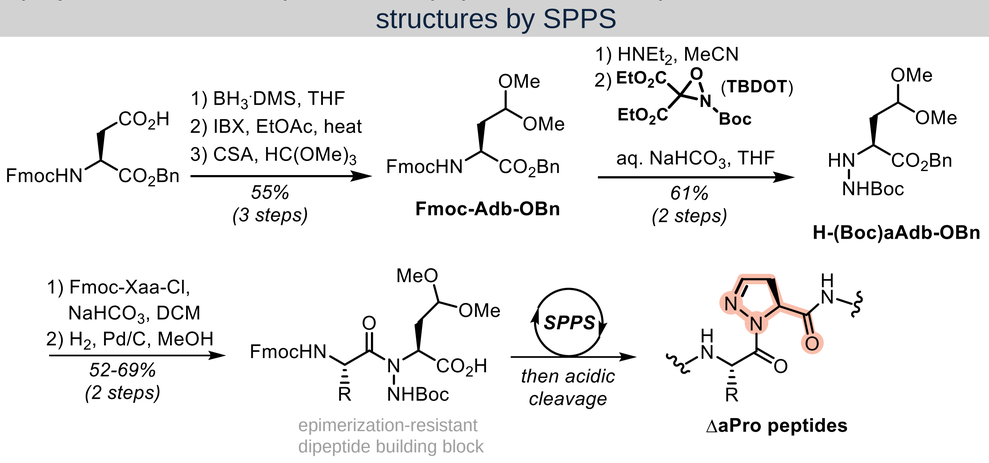




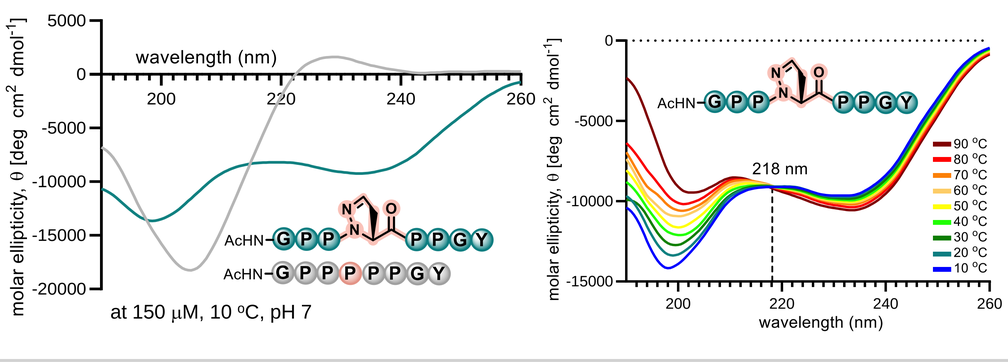


RESULTS

1) Synthesis of ΔaPro precursor dipeptides and incorporation of ΔaPro into host structures by SPPS

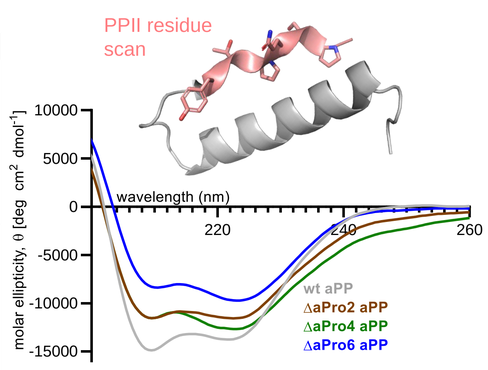


2) A single-strand PPII model peptide featuring an ΔaPro guest residue gives an anomalous CD signature but undergoes two-state thermal transition

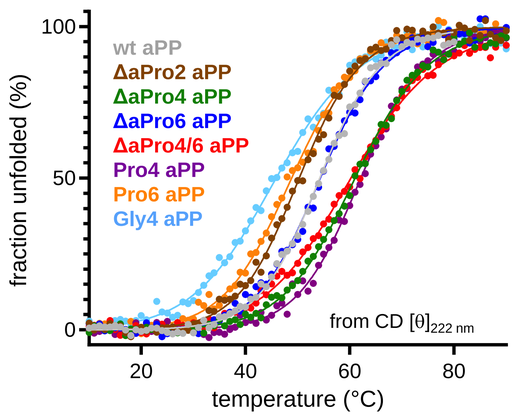


3) aPP miniproteins featuring ΔaPro substitution in the PPII helix adopt wild-type tertiary structure by CD

sequence (X = ΔaPro)	yield %
GPSQPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	8
GXSQPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	6
GPXQPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	6
GPSXPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	7
GPSQXTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	8
GPSQPXYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	6
GPSQPTXPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	14
GPSQPTYXGDDAPVEDLIRFYDNLQQYLNVVTRHRY	3
GPSXPXYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	13
GPSPPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	10
GPSQPPYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	7
GPSGPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	10

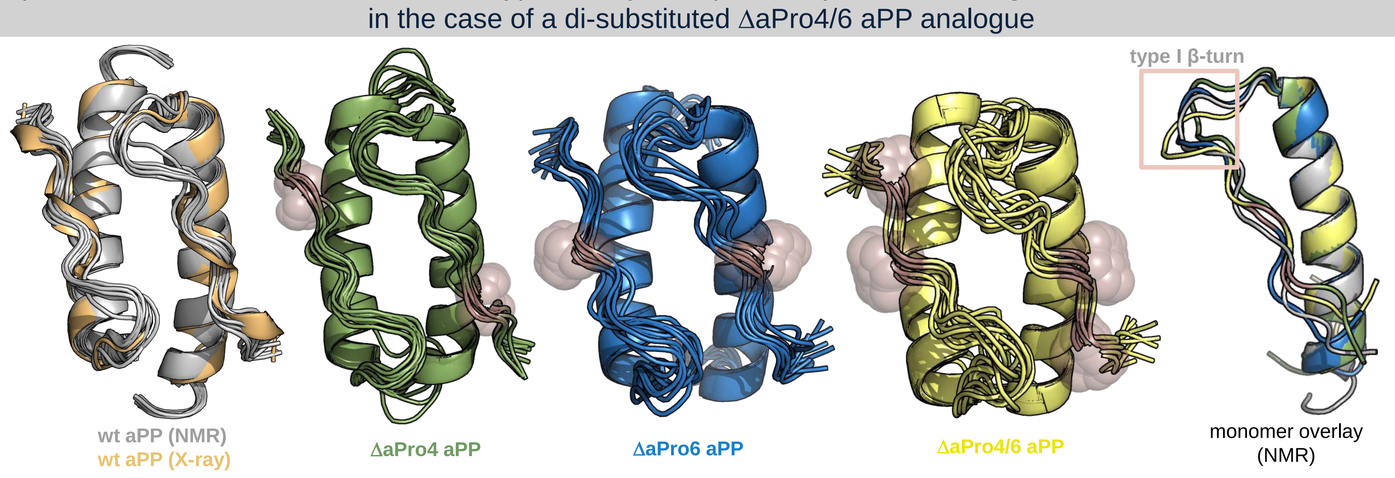


4) ΔaPro4 and ΔaPro6 substitutions in the aPP PPII helix maintain or enhance thermal stability

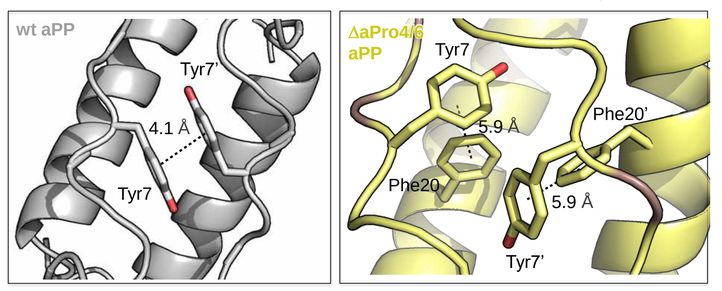


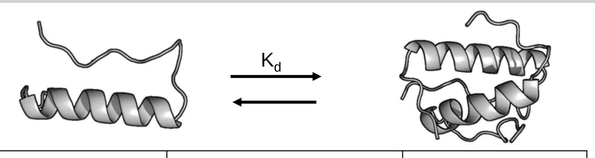
peptide	T_m (°C)
wt aPP	54.2 ± 0.8
ΔaPro2 aPP	49.7 ± 0.9
ΔaPro4 aPP	61.9 ± 1.2
ΔaPro6 aPP	53.6 ± 1.1
ΔaPro4/6 aPP	60.9 ± 0.9
Pro4 aPP	62.7 ± 0.6
Pro6 aPP	47.7 ± 1.6
Gly4 aPP	47.6 ± 2.4

5) NMR-derived ensembles reveal wild-type tertiary and quaternary structure but greater conformational disorder in the case of a di-substituted ΔaPro4/6 aPP analogue



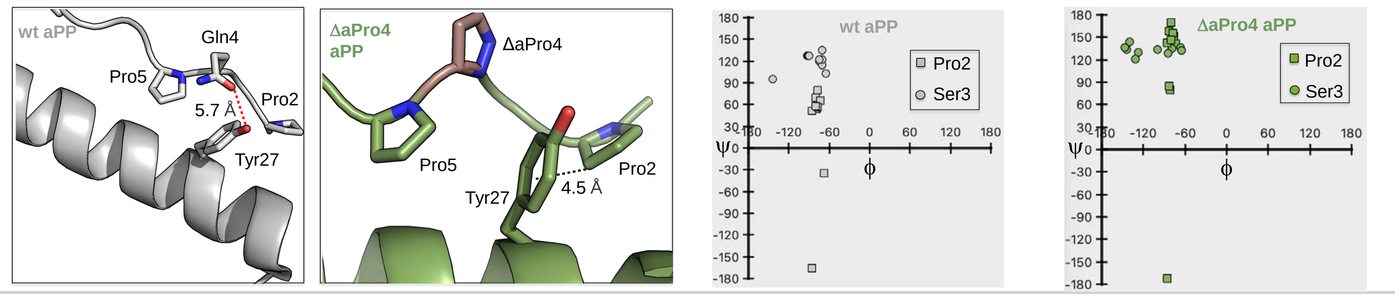
6) ΔaPro4/6 aPP exhibits loss of a dimer-stabilizing π-π interaction but gains intramonomer π-π interactions





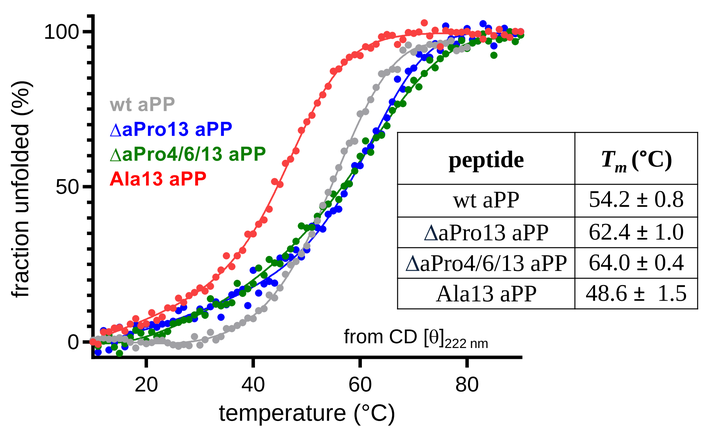
peptide	K_d (× 10^{-6} M) from AUC	% monomer at 30 μM
wt aPP	1.31	19%
ΔaPro4/6 aPP	4.71	33%

7) Loss of a wild-type intramonomer H-bond in ΔaPro4 aPP allows for Pro2-Tyr27 CH-π interaction and a more ordered PPII strand



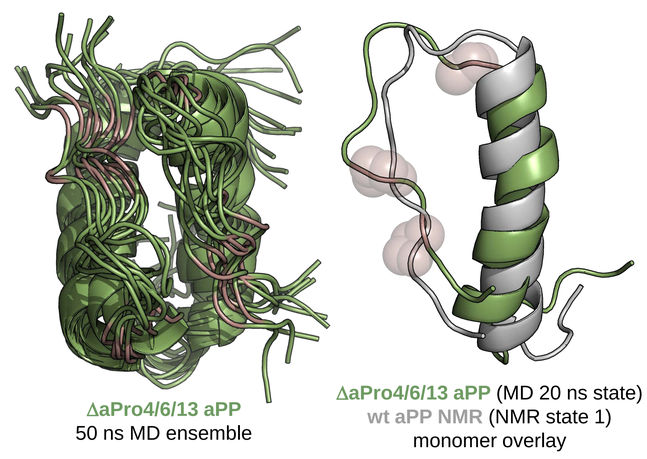
8) Substitution of the loop Pro13 residue for ΔaPro significantly enhances thermal stability

sequence	yield %
GPSQPTYGGDDAXVEDLIRFYDNLQQYLNVVTRHRY	6
GPSQPTYGGDDAAVEDLIRFYDNLQQYLNVVTRHRY	6
GPSXPXYGDDAXVEDLIRFYDNLQQYLNVVTRHRY	5



peptide	T_m (°C)
wt aPP	54.2 ± 0.8
ΔaPro13 aPP	62.4 ± 1.0
ΔaPro4/6/13 aPP	64.0 ± 0.4
Ala13 aPP	48.6 ± 1.5

9) ΔaPro13 substitution stabilizes the aPP tertiary fold despite reduced dimer population



peptide	K_d (× 10^{-6} M) from AUC	% monomer at 30 μM
wt aPP	1.31	19%
ΔaPro13 aPP	24.4	58%
ΔaPro4/6/13 aPP	58.2	73%

CONCLUSIONS

We developed a convenient method for the incorporation of a novel, unpuckered proline surrogate into peptides and proteins. ΔaPro substitution of solvent-exposed PPII residues, and the key "Pro switch" residue in the loop of aPP, can stabilize the miniprotein while substitutions in the hydrophobic core destabilize the fold. NMR-derived structures reveal that ΔaPro readily adopts canonical PPII backbone torsions in all cases. Our most stable analogue, whose melting temperature is 10°C higher than wild-type aPP, features incorporation of three ΔaPro residues (positions 4, 6, and 13). Notably, this stabilization of tertiary structure is attended by a significant increase in monomer population. Our results suggest that ΔaPro is an effective conformational surrogate of Pro for use in the design of thermostable proteomimetics.

ACKNOWLEDGEMENTS

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