Engineering Stable Miniproteins: The Journey from Computational Design to Tertiary Structure Stability

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https://doi.org/10.17952/37EPS.2024.P1285

BACKROUND

Recent advances in miniproteins containing β-amino acid residues have changed the understanding and manipulation of helical structures, facilitated by the use of cycloalkane-based β-building blocks. These α/β-peptides exhibit unique structural features and have demonstrated promising biological activities, such as antimicrobial properties and protein interaction inhibition[1]. Additionally, miniproteins containing β-residues, have been studied extensively for their well-defined three-dimensional structures, making them attractive candidates for various applications due to their precise conformation control and resistance to enzymatic proteolysis[2].

While secondary structure studies have been successful, research on more extended systems is limited, especially of peptide foldamers composed of *cis*-aminocyclopentanecarboxylic acid. Initially, miniproteins were designed by naturally excreting proteins. Since then, however, the field has evolved into a rational design approach that respects not only biochemical and physicochemical principles but also empirical data. Nowadays, the use of computational methods that rely on fragment-based design is crucial in *de novo* design of most miniproteins[3].

OBJECTIVES

De novo design of conformationally stable miniproteins that fold cooperatively in an absence of cross-links and binding metal using computational methods and the rational approach.

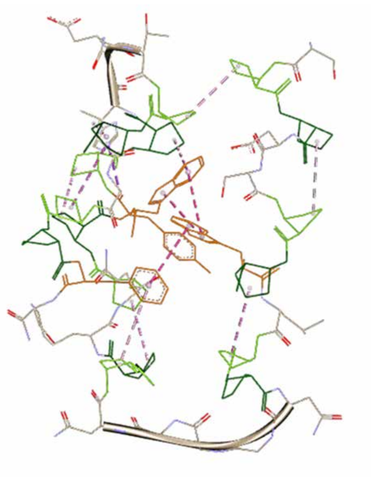
Synthesis of the series of miniproteins composed of three helices, that have well-defined tertiary structure and high thermal stability.

Inclusion of unnatural *cis*-(1R,2S)-ACPC and *cis*-(1S,2R)-ACPC residues as building blocks to induce the folding due to their torsion angles and rigidity.

HHH MINIPROTEINS

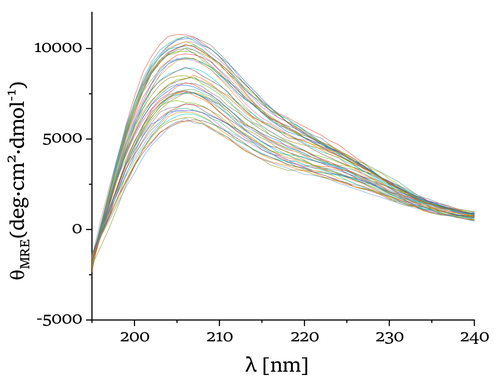
The structural design of the miniproteins, composed of three helices (HHH), ensures their stability solely due to the precise arrangement of the hydrophobic core.

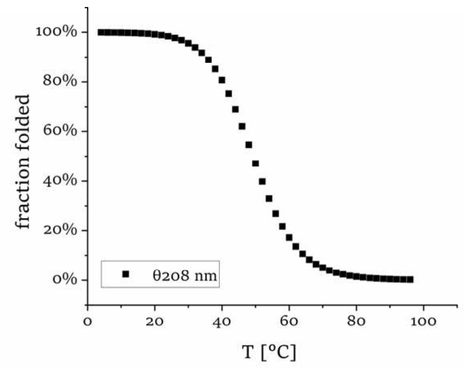
Helical fragments based on the 9/12/9/10-helix structure[4] were designed maintaining the ααββ motif. This was achieved by alternating L- and D-α-amino acid residues with cyclic β-amino acid building blocks of different stereochemistry (*cis-(1R,2S)*-ACPC *and cis-(1S,2R)*-ACPC) following the stereochemical patterning approach[5]. The α-residues were selected using the Rosetta FastDesign protocol[6] to optimise the packing of the hydrophobic core.



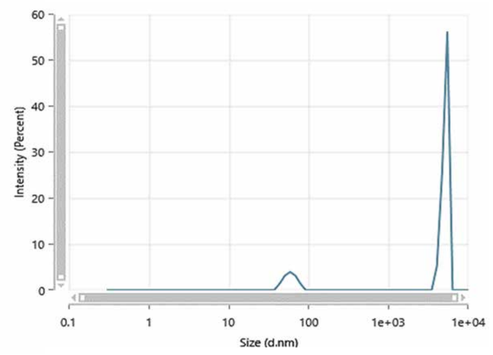
Model of **c3H1**

c3H1 miniprotein*:* s ⬟ ⬟ D s ⬟ ⬟ W l ⬟ ⬟ N GGGn ⬟ ⬟ Kq ⬟ ⬟ Wy ⬟ ⬟ VEGT ⬟ ⬟ Ha ⬟ ⬟ Fs

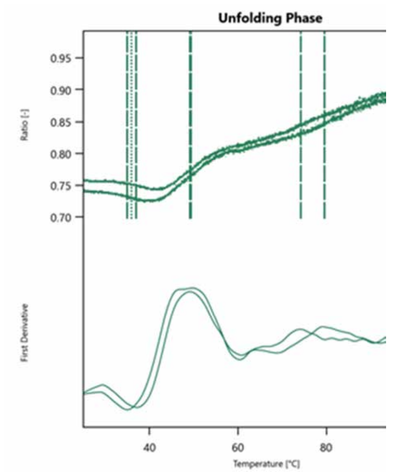




Thermal denaturation plot of **c3H1**, water pH 10.5



DLS analysis of **c3H1,** water pH 10.5



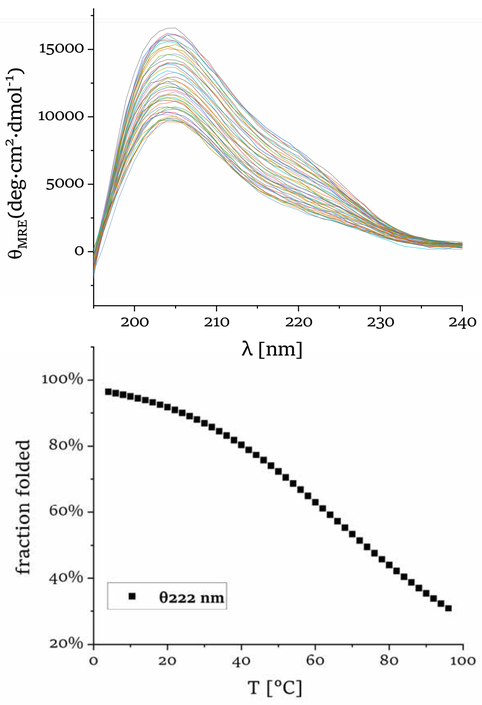
nanoDSF analysis of **c3H1,** water pH 10.5

The CD and nanoDSF measurements confirmed that the designed miniprotein folds cooperatively and indicates high thermal stability – the estimated melting temperature is close to 50°C.

Since the presence of hydrophobic building blocks inducing peptide folding significantly reduces its solubility, initially designed peptides tend to aggregate and precipitate in the pH value lower than 10.5. Thus, the designed miniproteins were subsequently modified to improve the physicochemical properties.

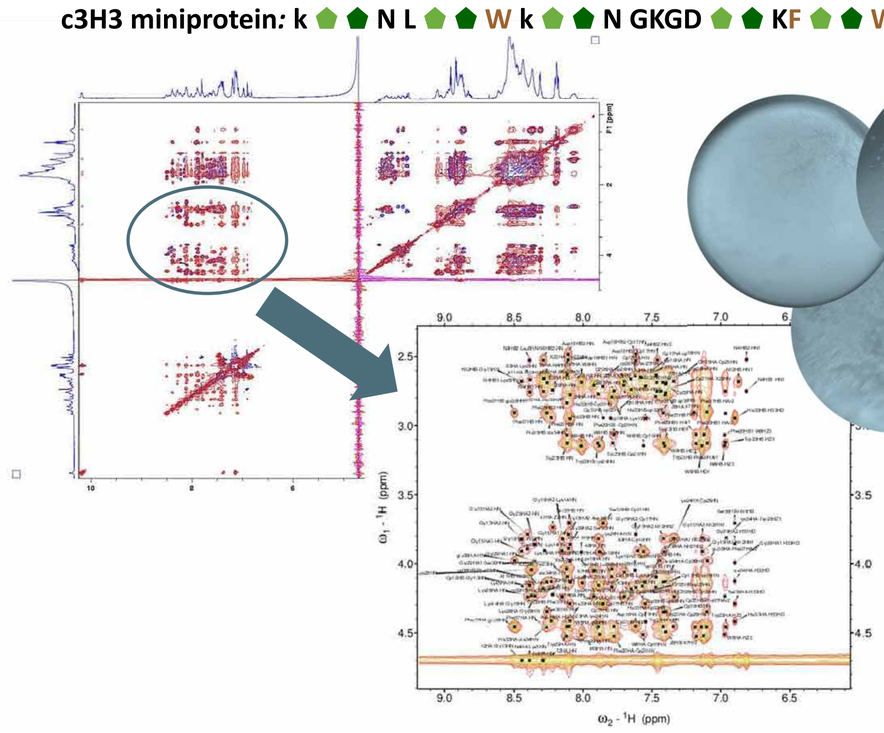
⬟ (*1R,2S*)-aminocyclopentanecarboxylic acid [*cis-(1R,2S)*-ACPC]

STRUCTURE OPTIMISATION



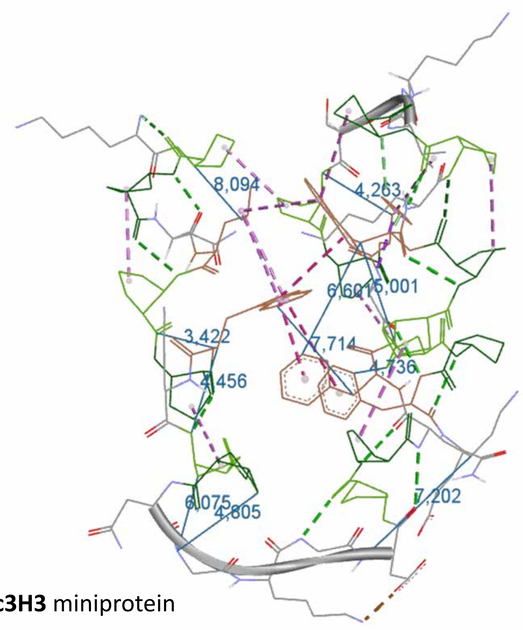
Thermal denaturation plot of **c3H3**, potassium phosphate buffer 25 mM pH 7.5

c3H3 miniprotein*:* k ⬟ ⬟ N L ⬟ ⬟ W k ⬟ ⬟ N GKGD ⬟ ⬟ KF ⬟ ⬟ Wk ⬟ ⬟ VK GS ⬟ ⬟ Ha ⬟ ⬟ Fe



NMR spectrum of **c3H3**, potassium phosphate buffer 25 mM pH 7.5

Crystalline products of **c3H3** miniprotein



Model of **c3H3** miniprotein

⬟ (*1S,2R*)-aminocyclopentanecarboxylic acid [*cis-(1S,2R)*-ACPC]

The sequence of **c3H3** miniprotein was resolved and all the signals have been assigned. Thirty four long-range contacts were found, selected few are marked on the model above.

SUMMARY

✓ The use of fragment assembling has proven to be an excellent tool for the design of miniproteins. The series of stable oligomers that fold cooperatively was successfully designed and synthesised. Obtained peptides are accessible through the solid phase synthesis, facilitating reachable analysis.

✓ The possibility of controlling the folding process of the synthesised miniproteins, as well as their rigidity and specific physicochemical, and pharmacokinetic properties, such as high proteolytic stability and biocompatibility, make the obtained oligomers attractive scaffolds for drug design and other biomedical applications.

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Research was financed by the National Science Centre
GRANT ID.: 2021/43/B/ST4/01837