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Protein cocrystallization molecules originating from *in vitro* selected macrocyclic peptides

Christopher J Hipolito, Nasir K Bashiruddin and Hiroaki Suga

Transmembrane proteins are intractable crystallization targets due to their low solubility and their substantial hydrophobic outer surfaces must be enclosed within a partial micelle composed of detergents to avoid aggregation. Unfortunately, encapsulation within a partial micelle diminishes specific protein-to-protein contacts needed for crystal lattice formation. In addition, the high conformational flexibility of certain transmembrane proteins reduces sample homogeneity causing difficulty in crystallization. Cocrystallization ligands, based on either antibody scaffolds or other proteinaceous non-antibody scaffolds, have greatly facilitated the crystallization of transmembrane proteins. Recently, *in vitro* selected macrocyclic peptide ligands have been shown to facilitate protein crystallization as well. In this review, we discuss selection strategies used for the discovery of macrocyclic peptide ligands and the three-dimensional crystal structure of the transporter PfMATE in complex with *in vitro* selected macrocyclic peptides.

Addresses
Department of Chemistry, Graduate School of Science, The University of Tokyo, Tokyo 113-0033, Japan

Corresponding author: Suga, Hiroaki (hsuga@chem.s.u-tokyo.ac.jp)

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Introduction

Transmembrane proteins function as a means of communication between the interior and exterior environments of cells and organelles. Those located on outer cell membranes, are important targets for drug discovery, and structural information obtained by X-ray crystallography is invaluable for drug development as well as the understanding of protein function, or in some cases, malfunction. Despite the importance of this class of biomolecule, their poor representation in the database of solved crystal structures underscores the difficulty of elucidating membrane protein structure [1]. The difficulty in transmembrane protein crystallization is caused by two innate characteristics. The first characteristic is the significant amount of hydrophobic surfaces that can lead to protein instability and precipitation. The necessary use of detergents to keep proteins in solution causes new difficulties by producing a large partial micelle around the protein, which potentially obscures the hydrophilic surfaces of the protein needed for crystal contacts. The second problematic characteristic is the flexibility of certain transmembrane proteins. To deliver a signal or small molecule cargo from one side of a lipid bilayer to the other, a protein may undergo a conformational change that can span its entire length. Additionally, if the energy barrier of interconversion is low, there will be more conformational heterogeneity in a protein sample thus decreasing the chance of uniform crystallization.

Crystallization additives may assist with overcoming these problems [2]. Small molecule additives can be added to supplement the surface of the protein with charge that can be used to form protein-to-protein contacts. However, discovery and optimization of the correct cocktail of small molecules is often done by trial and error and requires testing of numerous conditions to identify the key small molecules and their optimal concentrations. Cognate ligands such as enzyme substrates and products, co-factors, inhibitors, agonists, antagonist, and fragments of natural binding partners may prove to be useful for stabilizing a single conformation of the target protein if protein-ligand affinity is high enough.

When no suitable cognate ligands for cocrystallization are available, one can engineer a synthetic binding ligand. Selection for conformation-specific proteinaceous ligands has facilitated the cocrystallization of various difficult targets [1,3•]. Although antibody-based ligands, such as Fab (Fragment antigen binding) fragments and nanobodies [3•], continue to be a popular choice for the generation of novel cocrystallization ligands, proteinaceous ligands based on non-antibody scaffolds such as anticalins [4,5], DARPins (designed ankyrin repeat proteins) [6], affibodies [7], and monobodies [8] from highly diverse synthetic libraries provide ease of production and functional diversity [9,10]. One benefit of using Fab fragments is that it can supplement the target protein with a larger amount of hydrophilic surface area for complex-to-complex contact than smaller cocrystallization ligands. However, the ease of production and application of diverse synthetic libraries *in vitro* underscores the advantage of minimizing ligand size. In addition, both DARPins and monobodies, despite their smaller sizes, are still capable of mediating crystal contacts [11,12]. Although the hydrophobic nature of DARPins that

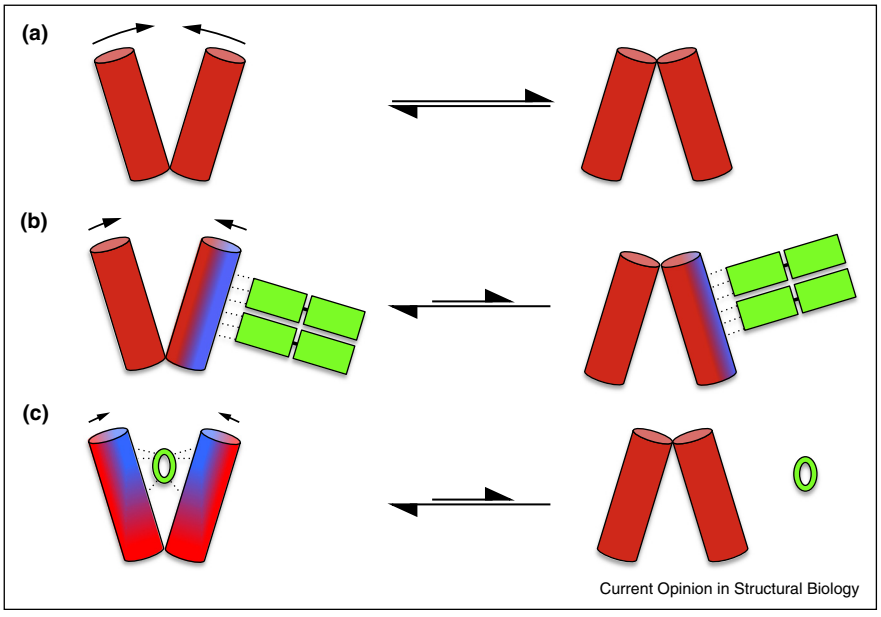
have been isolated from *in vitro* selections against transmembrane proteins proved to be problematic [13,14], recent improvements to the naïve DARPin library design have been made to increase the potential of isolating more hydrophilic DARPins [15].

Small macrocyclic peptides without predetermined, proteinaceous secondary structures have recently been shown to facilitate crystallization [16••,17••]. To the best of our knowledge, the macrocyclic peptide backbone is the first selectable, non-proteinaceous scaffold to be successfully utilized for facilitating cocrystallization. Despite the fact that the macrocyclic peptides' smaller size reduces the chances of forming extensive contact with a flat or convex protein surface, the smaller scaffold could be used to make extensive interactions with the concave surface of a protein's substrate-binding site (Figure 1). Binding in this manner may reduce interconversion between states as well as stabilizing residues buried within the target protein. Also, the small size of the macrocyclic peptide (~2 kDa) improves ligand solubility even if a substantial number of the peptide's side chains are hydrophobic. In this review, we limit our discussion to the valuable lessons learned from recent reports of cocrystallization employing macrocyclic peptides identified using the RaPID system and other selected ligands relevant to the discussion.

Macrocyclic peptide ligands identified using the RaPID system

Macrocyclic scaffolds serve as natural product-like peptidomemetics for use in the development of therapeutic drugs [18–20]. With regards to drug discovery, the macrocyclic scaffold is a crucial non-standard element intented to improve binding affinity, resistance to degradation by proteases and membrane permeability of peptides. Researchers have managed to combine translation machinery with chemical reactions for the production of combinatorial macrocyclic peptide libraries [21••]. By further combining the ribosomal production of macrocyclic peptides with mRNA display [22,23], macrocyclic peptides with high affinity for a chosen target can be identified by the appended genetic tag. The genetic material recovered *via* the successful binding of macrocyclic peptides to the target can be used to produce a new RNA library that is enriched with genes coding for high affinity macrocyclic peptides. This selection process can be repeated until the library is sufficiently enriched and individual clones with the most desirable property or properties can be identified.

Figure 1



Possible methods of stabilization of a dynamic protein structure by cocrystallization ligands. **(a)** Schematic representation of an unbound dynamic transmembrane protein. If the energy barrier of interconversion between the two states is low and the two state are similar in energy, rapid interconversion will take place. **(b)** Schematic representation of a dynamic transmembrane protein bound from the exterior of the protein by a proteinaceous ligand. A proteinaceous ligand, represented by the green boxes, that binds to the exterior of the target can stabilize one conformational state and the target residues in contact with the ligand. However, if the ligand can bind the alternative conformational state, the target may still be capable of interconversion. **(c)** Schematic representation of a dynamic transmembrane protein stabilized from within by a relatively small non-proteinaceous cocrystallization ligand. The small ligand, represented by a green oval, physically impedes interconversion between states by acting as a wedge and interconversion will only occur after full dissociation of the complex. The curved arrows represent the relative range of motion for the subdomains of the target protein. The red colour indicates regions of high flexibility. Stabilized regions are coloured blue. Intermolecular interactions are represented by dashed lines.