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Metal-Assisted Folding of Prolinomycin Allows Facile Design of Functional Peptides

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Abstract

Cyclic peptides have been proposed as privileged scaffolds that may mimic the folding and function of natural proteins. However, simple cyclic peptides typically cannot fold into well-defined structures. Herein, we describe a foldable cyclic peptide scaffold, on which functional side chains can be displayed for targeted recognition of biomolecules. The foldable scaffold is based on prolinomycin, a proline-rich analogue of valinomycin. We report synthetic mutants of prolinomycin that retain the metal-assisted folding behavior under physiological conditions. The predictable structure formation of prolinomycin makes it a powerful platform to develop synthetic receptors for biomolecules of interest. We demonstrate the potential of this scaffold by creating prolinomycin mutants that selectively bind anionic vesicles and bacterial cells.

Graphical Abstract

Prolinomycin, a proline-rich analogue of valinomycin, was found to tolerate various mutations, which enables predictable display of side chains for target binding. Prolinomycin presents a versatile scaffold for developing functional peptides.

Selective molecular interactions underlie essentially every aspect of biology, for which nature has evolved various proteins to bind signaling molecules with exquisite specificity. Such targeted molecular interactions are realized with proteins that fold into well-defined structures, allowing multiple side chains to preorganize for substrate binding. It has been a long-standing goal of chemists to develop low-molecular-weight mimics of large proteins for specific molecular recognition.^{1–6} Small peptides and peptidomimetics have become increasingly attractive in this pursuit and cyclization of peptides has been commonly performed to favor structure formation.^{7–11} However, monocyclic peptides are typically too

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floppy to fold¹² and multicyclization can bring synthetic challenges, as well as unpredictable consequences to the structure and function of the peptides.

This contribution describes a new strategy toward accessing functional cyclic peptides by taking advantage of the metal nucleated folding of the prolinomycin scaffold (Figure 1). Prolinomycin is a twelve-residue, head-to-tail cyclized peptide. It was first reported as a structural analogue of valinomycin,¹³ a potent K⁺ transporter. Replacing the six hydroxy-acids in valinomycin with prolines gives prolinomycin (Figure 1a), which was found to be a poor K⁺ transporter in comparison to valinomycin.¹⁴ The inferior performance originates from the slow kinetics of K⁺ chelation and release, which results from the restricted “breathing” motion of the proline-rich scaffold. Nevertheless, prolinomycin displays a stable structure (Figure 1b) with six carbonyl oxygens chelating K⁺, while the other six form intramolecular hydrogen bonds with the NHs of the non-proline residues.^{15,16} While the prolines are known to be critical for prolinomycin folding,¹⁴ we envision that multiple mutations can be tolerated at the non-proline positions, thus allowing various functional groups to be grafted for substrate binding. It is important to note that prolinomycin has been previously characterized only in organic solvents, not in aqueous media possibly due to its poor water solubility. Incorporation of polar side chains may afford water solubility, thus enabling biological applications. Encouragingly, preliminary Density Functional Theory (DFT) calculation revealed a minimal difference in the peptide conformational energy change upon binding K⁺ when all six Val residues were mutated to Ala (Figure S1).

To test our hypothesis, we have first developed an efficient strategy for the synthesis of prolinomycin mutants. Specifically, we have devised an intramolecular proline ligation protocol,¹⁷ which enables cyclization with an N-terminal thioproline (tPro) and a C-terminal thioester (Figure 2a). The linear precursor peptides were synthesized on the Dawson Dbz resin¹⁸ with tPro installed at the N-terminus. A D-Ala was installed at the C-terminus instead of D-Val to ensure high yields of cyclization (Figure S2). Additionally, the cyclized product displays a free thiol for facile labeling with fluorophores. A series of prolinomycin mutants were generated by replacing subsets of the Val residues with both natural and unnatural amino acids (Figure 2b). For ease of discussion, the tPro residue is designated as residue number 1 for the linear precursors and the final cyclic peptides (Scheme S3).

To confirm the K⁺ induced folding, several prolinomycin mutants were analyzed using NMR spectroscopy (Figure 3 and Figures S5–S14). To ensure solubility at high concentrations, the samples were prepared in a 1:1 mixture of a sodium phosphate buffer (pH 4.0) and CD₃OH. Without KCl, the peptide RRR-4W afforded no amide resonances and the observed peaks were broad and poorly dispersed, indicating the lack of a unique structure. Upon KCl addition, the peptide afforded much better dispersed peaks (Figure 3). Importantly, six new peaks emerged in the amide region, consistent with the six intramolecular hydrogen bonds observed in the crystal structure (Figure 1b). The WT-like folding behavior of RRR-4W is quite remarkable considering the peptide has five out of the six Val residues replaced with non-β-branched amino acids. Plotting the peak intensity at 7.48 ppm versus KCl concentration yields a titration curve. Curve fitting gives a *K_d* value of ~0.5 mM (Figure S5), which falls below the physiological concentrations of K⁺.

Similar to RRR-4W, KCl addition into other prolinomycin mutants elicited the appearance of new peaks in the amide region, as well as sharpening of the aliphatic resonances (Figure S6, S8–10). Decreasing the K^+ concentration through dilution resulted in the rapid disappearance of select amide peaks of RRR, indicating that the peptide binds K^+ in a highly dynamic manner (Figure S7). Further structural analysis was secured through 2D NMR experiments of RRR (Figure S11, S12), which revealed distinct cross peaks indicative of a beta-turn in the metal-chelated structure (Figure 1b). The KCl titration experiment for RRR and KKK yielded K_d values of 2 and 8 mM respectively, which are comparable to that of VVV (~5 mM) (Figure S6, S8b, S9b). These results suggest that mutation of the Val residues does not dramatically change the K^+ binding and folding behavior of prolinomycin. We note that an earlier report documented a lower K_d (10 μ M) for the WT prolinomycin binding to K^+ .¹⁴ However, this value was extracted from a two-phase partition experiment as the poor solubility of prolinomycin prohibited direct measurement in aqueous solutions. Given the high similarity of our VVV mutant to the wild type prolinomycin, we submit the contrasting K_d values likely result from the distinct experimental settings for their determination.

For the polar mutants RRR and RRR-4W, we attempted to assess their K^+ binding and folding behavior in pure aqueous solutions. While reducing the percentage of methanol caused peak broadening for RRR (Figure S13, S14), presumably due to aggregation, the peptide did show the signature peaks of K^+ binding in a purely aqueous solution. Similarly, KCl addition into RRR-4W elicited the appearance of six amide peaks and the characteristic resonances in the aliphatic region (Figure 4), analogous to what we observed with the sample in 50% methanol. We note that this spectral change is specific to the presence of K^+ , as no change was observed with the addition of NaCl.

Our structural characterization shows that the prolinomycin mutants are capable of folding into well-defined structures at physiological concentrations of K^+ . For biological applications, we have evaluated the prolinomycin mutants for selective binding to anionic membranes, which is a prominent feature of bacterial cells in comparison to their mammalian counterparts.^{19–21} We envisioned that the drum-like structure of prolinomycin could readily preorganize multiple residues to bind anionic membranes (Figure 5a). Indeed, a fluorescein-labeled RRR was found to bind anionic small unilamellar vesicles (SUVs, POPC/POPG 1:1) in a K^+ -dependent manner (Figure 5b, Figure S18): KCl addition elicited a gradual increase in membrane partitioning of RRR. In sharp contrast, NaCl addition caused a slight decrease in fluorescence anisotropy of the peptide, presumably due to the salt screening effect of the electrostatic attraction between the peptide and the membrane. Fitting the data into a hyperbolic equation gives an EC_{50} value of 4.6 mM, which is consistent with the K_d value obtained by NMR (Figure S6b). In contrast to the anionic vesicles, SUVs made of POPC alone elicited no RRR association, regardless of the salts in the media. Further, a linear control of RRR (Figure S19) showed no membrane binding under all experimental conditions examined. Not surprisingly, the mutant KKK also binds anionic vesicles in a K^+ dependent manner (Figure S20). Further, RRR-4W showed potassium-enhanced membrane partitioning with comparable K^+ sensitivity to RRR (Figure 5c). Interestingly, the mutant VRR-4R displayed improved membrane binding only at low concentrations of KCl, which at high concentrations caused the dissociation of the peptide from the vesicles (Figure 5c). This

is particularly noteworthy given that VRR-4R and RRR have identical side chain compositions and only differ in the location of the arginines. Furthermore, RRR and VRR-4R display comparable K_d values for binding K^+ according to our NMR titration results (Figure S6 and S10). The distinct behavior of these two peptides highlights the critical importance of the cooperative action of side chains for membrane binding.

We further examined the potential of the prolinomycin mutants to target bacterial cells, which are highly enriched in anionic lipids.^{19–21} Despite the strong binding of RRR to anionic SUVs, it showed little staining of *S. aureus* (Figure 6a). This is perhaps not surprising as SUVs are known to induce high affinity binding of peptides due to their large membrane curvature,²² therefore serving as a suboptimal model of bacterial membranes. To enhance membrane binding, we have synthesized two additional mutants RR(ObK) and RR(TpK), which incorporate a 4-octylbenzoyl-lysine (ObK) and a terphenylcarbonyl lysine (TpK), respectively (Scheme S2). The modified lysines were included to enhance the hydrophobic driving force for membrane insertion. Both peptides were found to effectively stain *S. aureus* cells, as expected, only in the presence of K^+ (Figure 6a).

We examined the bacterial cell selectivity of RR(ObK) by treating a co-culture of *S. aureus* and Jurkat cells. The microscopic images (Figure 6b) show that, with K^+ present, RR(ObK) stains the bacteria but not the Jurkat cells. The bacterial staining potential of the peptides was quantitatively assessed via flow cytometry (Figure S21). In the absence of K^+ , neither peptide elicited significant staining of *S. aureus*. With 30 mM KCl, RR(TpK) displayed a modest increase in the mean fluorescence intensity of the cells, while RR(ObK) afforded dramatic increases in *S. aureus* staining. Fitting the concentration profile yielded an EC_{50} value of 1.0 μ M (Figure S21). These results demonstrate the robustness of the K^+ nucleated folding of the prolinomycin scaffold, even in the complex environment of cell surfaces.

In summary, this contribution describes our characterization of a series of prolinomycin mutants toward the goal of developing peptides that mimic the folding and functional properties of large proteins. Our results show that the metal-assisted folding of prolinomycin tolerates various side chain mutations, even simultaneously at multiple positions. The mutants readily adopt the WT-like structure under physiologic concentrations of K^+ , allowing predictable display of side chains for target binding. We present the proof-of-concept application by developing peptides that selectively target bacterial membranes. The scaffold of prolinomycin should be applicable to the design of functional peptides for a wide array of biological applications. The K^+ induced folding behavior of prolinomycin may be useful for designing sensors to probe the physiological and pathological roles of K^+ homeostasis as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Structures of prolinomycin. a) Comparison of prolinomycin to valinomycin, with a Pro residue and the corresponding hydroxy-acid highlighted in red. b) Side view (left) and top view (right) of the prolinomycin-K⁺ complex. C: green; N: blue; O: red; K: purple.

Figure 2.

Synthesis (a) and sequences (b) of the prolinomycin mutants. The underlined residues are in the D-configuration. ObK: 4-octylbenzoyl lysine; TpK: terphenylcarbonyl lysine. Structures are shown in Scheme S2.

Figure 3. K^+ -nucleated folding of RRR-4W monitored by ^1H -NMR. The prominent emerging peaks upon K^+ addition are highlighted with an asterisk.

Figure 4. K^+ induced folding of RRR-4W in purely aqueous solutions. The prominent emerging peaks upon K^+ addition are labeled with an asterisk.

Figure 5.

K^+ -dependent membrane binding of the prolinomycin mutants. a) Illustration of the K^+ assisted folding of RRR and binding to anionic membranes. b) Titration experiments highlighting the differential effects between KCl and NaCl on RRR partition into membranes. c) Comparison of the K^+ -induced membrane binding of several prolinomycin mutants.

Figure 6.

K^+ dependent staining of *S. aureus* by the prolinomycin mutants. a) Microscopy images showing cell staining only in presence of K^+ ; (A) Phase contrast with no KCl; (B) FITC channel with no KCl; (C) Phase contrast with 30mM KCl; (D) FITC channel with 30mM KCl. b) Microscopy images of a Jurkat and *S. aureus* co-culture showing bacterium selective staining of RR(Obk) in the presence of K^+ ; (A) Phase contrast; (B) FITC channel. All samples were prepared in a pH 7.0 Tris buffer with 100 mM NaCl.