Synthesis and Conformational Analysis of Macrocyclic Peptides Consisting of Both α-Helix and Polyproline Helix Segments

Sung-ju Choi,[1] Soo hyun Kwon,[1] Tae-Hyun Kim,[2] Yong-beom Lim[1]

[1] *Translational Research Center for Protein Function Control and Department of Materials Science & Engineering, Yonsei University, Seoul 120-749, Korea*

[2] *Department of Chemistry, Incheon National University, Incheon 406-840, Korea*

Received 29 April 2013; revised 18 June 2013; accepted 9 July 2013
Published online 19 July 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bip.22356

ABSTRACT:

Macrocycles are interesting molecules because their topological features and constrained properties significantly affect their chemical, physical, biological, and self-assembling properties. In this report, we synthesized unique macrocyclic peptides composed of both an α-helix and a polyproline segment and analyzed their conformational properties. We found that the molecular stiffness of the rod-like polyproline segment and the relative orientation of the two different helical segments strongly affect the efficiency of the macrocyclization reaction. Conformational analyses showed that both the α-helix and the polyproline II helix coexisted within the macrocyclic peptides and that the polyproline segment exerts significant effect on the overall helical stability and conformation of the α-helical segment. © *2013 Wiley Periodicals, Inc. Biopolymers 101: 279–286, 2014.*

Keywords: α-*helix; polyproline; macrocycles; peptides*

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

Additional Supporting Information may be found in the online version of this article.

Correspondence to: Yong-beom Lim; e-mail: yblim@yonsei.ac.kr or Tae-Hyun Kim; e-mail: tkim@incheon.ac.kr

Contract grant sponsor: Research Institute of Industry-Academic Cooperation Foundation Incheon National University Research Grant (2010)

INTRODUCTION

Peptides have been used, as isolated monomeric molecules or as basic building blocks for self-assembly into nanostructures, to mimic the functions of natural proteins. Peptides have several advantages in that they can be relatively easily synthesized and have broader chemical diversity than natural proteins.[1,2] However, peptides typically have unstable molecular conformations due to their lack of the multiple stabilizing interactions provided by a folded protein environment.[3,4] Such conformational instability can significantly limit the affinity and selectivity of peptide epitopes for target receptors. Therefore, the maintenance and stabilization of the actively folded form of peptides are important for increasing their biomacromolecular recognition capabilities.

The α-helix is a secondary structure element of proteins and is well known to play an important role in specific biomacromolecular recognition processes, such as protein–protein, protein–RNA, and protein–DNA interactions. α-Helical structures are stabilized within the context of well-folded protein structures; however, when isolated from the intact protein, the stability of the helical motif is compromised and is rarely helical in solution.[3] Because the stabilization of the active molecular conformation of a peptide is a crucial factor for maintaining the unique functions of a protein, extensive research into stabilized α-helical peptides has been conducted. Such studies include side chain crosslinking, hydrogen-bond surrogates, metal coordination, salt bridge formation, and synthetic α-helix receptor approaches.[5–8]

Macrocycles, which have a cyclic structure, are interesting molecules because their topological features significantly affect their chemical, physical, biological, and self-assembling properties.[9–13] Macrocycles have superior proteolytic stability and often display enhanced biological activity because of their increased affinity toward target receptors. Macrocycles can maintain more rigid and

conformationally constrained structures than their linear counterparts. It has been shown that that macrocyclization of the N- and C-termini of an α-helical peptide with a rigid crosslinker helps stabilize an otherwise unstable α-helix.[14] One notable example of the stabilization of an α-helical peptide by macrocyclization is the peptide stapling method.[5,15] In this approach, side chains at the same face of an α-helix are crosslinked by covalent bonds, and the formation of this intramolecular covalent bridge can decrease the conformational entropy of the unfolded state. This approach yields a stabilized α-helical peptide in monomeric form. In addition to this single molecule approach, there are multimeric approaches in which supramolecular peptide assemblies are constructed.[16] It has been reported that nanostructures with multiple stabilized α helices can be fabricated by using macrocyclic peptides.[17,18] The formation of multiple stabilized helices was made possible by the self-assembly-mediated coil-to-rod transition in the self-assembling β-sheet segment within the macrocyclic peptide, which constrains and subsequently stabilizes the α-helices.

The polyproline helix is one of the common secondary structures found in natural proteins. Proline oligomers tend to adopt the polyproline II (PPII) conformation in aqueous solution.[19,20] The PPII conformation is characterized by a left-handed helix with three residues per turn, i.e., every third residue is stacked on top of one another. Amide bonds in the PPII conformation exist in a *trans* conformation. The PPII conformation has been recognized to form rigid rod-like structures in aqueous solutions.[21–23] In organic solvents, polyproline adopts the polyproline I (PPI) conformation, a right-handed helix with all amide bonds in a *cis* conformation.

Here, we report the synthesis of macrocyclic peptides consisting of both an α-helix and a polyproline segment and the analysis of their conformational properties. Syntheses of macrocycles from large peptides are often extremely difficult because of the entropic penalty associated with intramolecular cyclization. We found that rigid polyproline-containing peptides display an orientation-dependent structural effect during the head-to-tail cyclization reaction. Macrocyclic peptides containing both an α-helix and a polyproline helix represent a unique class of cyclic molecules because two different types of peptide helices are contained within a single constrained cyclic scaffold. We analyzed whether the rigid PPII helix could assist the stabilization of the potential α-helix segment and examined how these two different helices affect each other (Figure 1C). To accomplish this goal, we employed an α-helix moiety derived from the Rev protein of the human immunodeficiency virus type-1 (HIV-1).[24]

RESULTS AND DISCUSSION

Design and Synthesis of Macrocyclic Peptides

The macrocyclic peptides designed for this study consist of a segment with high α-helix propensity, a PPII helix, and two linker segments that connect the two different helical segments (Figure 1). The α-helix segment is derived from the HIV-1 Rev protein. A short stretch of amino acids within Rev, called the arginine-rich motif (ARM; approximately 14–17 amino acids), is known to mediate specific binding to the cognate Rev-response element (RRE) RNA in an α-helical conformation.[25,26] Although Rev-ARM has a high propensity to form α-helical structures, it is not structured under ambient conditions, similarly to most short peptides.[27] Because both helical segments are rigid and are contained in the constrained cyclic structure, it was of interest to investigate how they mutually influence each other. Moreover, we envisioned that the rigid PPII helix might stabilize an otherwise unstructured Rev-ARM segment by further constraining and thereby lowering the conformational entropy of the unfolded state of Rev-ARM.

Linear peptides were synthesized on Rink amide MBHA resin. A cysteine protected with highly acid labile methoxytrityl (Mmt) was placed at the C-terminus to enable an orthogonal deprotection scheme. Because we aimed to obtain macrocyclic peptides with very large rings of 32–34 amino acid residues, the cyclization reaction was a challenging one (Figure 1A). As an initial attempt, we synthesized the α-helical segment first from the resin and then subsequently elongated the linker and polyproline segments. Then, bromoacetic acid was coupled to the N-terminal portion of the peptide chain. To achieve a pseudo-dilution effect, the cyclization reaction was performed while the protected peptide was still bound to the resin.[28] Following selective deprotection of the Mmt group using 1% trifluoroacetic acid (TFA) while side chains for other amino acids were protected, an intramolecular S_N2 reaction (S-alkylation of a cysteine thiol for cyclic thioether formation) was commenced by adding a base, *N*,*N*-diisopropylethylamine (DIPEA), in *N*-methyl-2-pyrrolidone (NMP). A small portion of the resin was sampled occasionally and treated with cleavage cocktail. Monitoring the progress of the reaction by mass spectrometry revealed that the cyclization reaction did not occur under these conditions, even after several days of extensive reaction.

The N- and C-termini must meet for effective intramolecular cyclization to take place. We speculated that the long persistence length of the rigid polyproline rod might reduce the total possible degrees of freedom for reactive encounters (Figure 1B; left). Therefore, we examined whether reversing the placement of the two helical segments could alleviate this problem. We speculated that if the stiff polyproline segment is

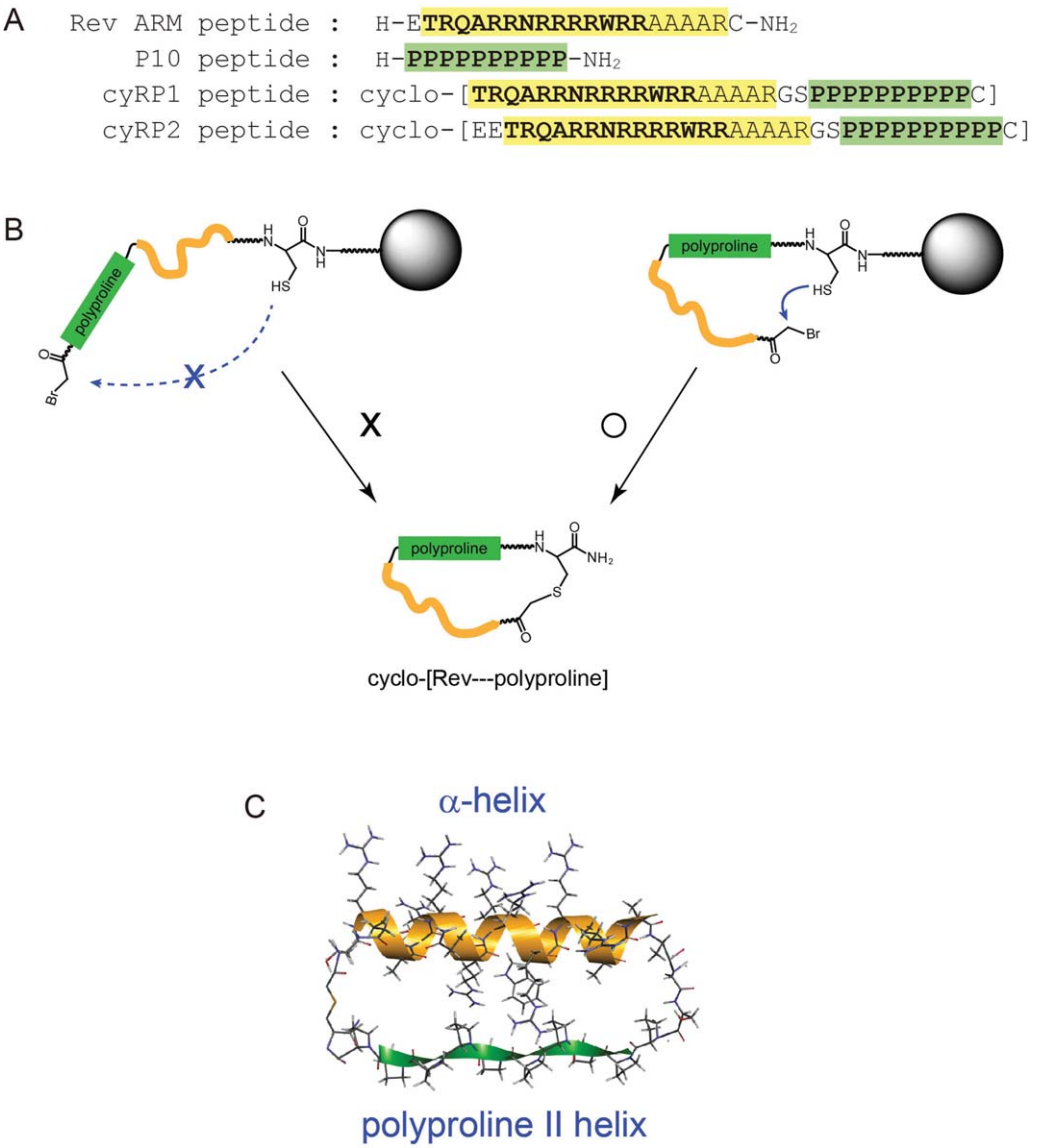

FIGURE 1 A: Sequences of the peptides used in this study. B: Effect of the segmental arrangement of the rigid polyproline rod on the efficiency of intramolecular S-alkylation reactions. C: Molecular model depicting the ideal structure of cyRP1.

located near the resin, the other flexible segments might move freely until head-to-tail reactive encounters have taken place (Figure 1B; right). Indeed, investigation of the reaction progress revealed that the cyclized peptide could be obtained when this reverse approach was employed (Supporting Information Figure S1). Therefore, the results indicate that molecular stiffness strongly affects the efficiency of the macrocyclization reaction.

The cyRP1 peptide contains a minimal Rev-ARM sequence of 14 amino acids. The cyRP2 peptide has two additional glutamic acid residues at the N-terminus of Rev-ARM. These negatively charged glutamic acids were attached to stabilize the helix macrodipole.[26] Moreover, a short stretch of alanines was appended to the C-terminus of Rev-ARM for both the cyRP1 and cyRP2 peptides to exert an additional α-helix stabilization effect.[26,29]

Conformational Analysis of the Macrocyclic Peptides

We examined the secondary structures of the synthesized peptides using circular dichroism (CD) spectroscopy. As shown in Figures 2A and 2B, the P10 peptide displayed a CD profile typical of a conventional PPII helix, with a strong negative minimum of ellipticity at 205 nm and a weak positive maximum of

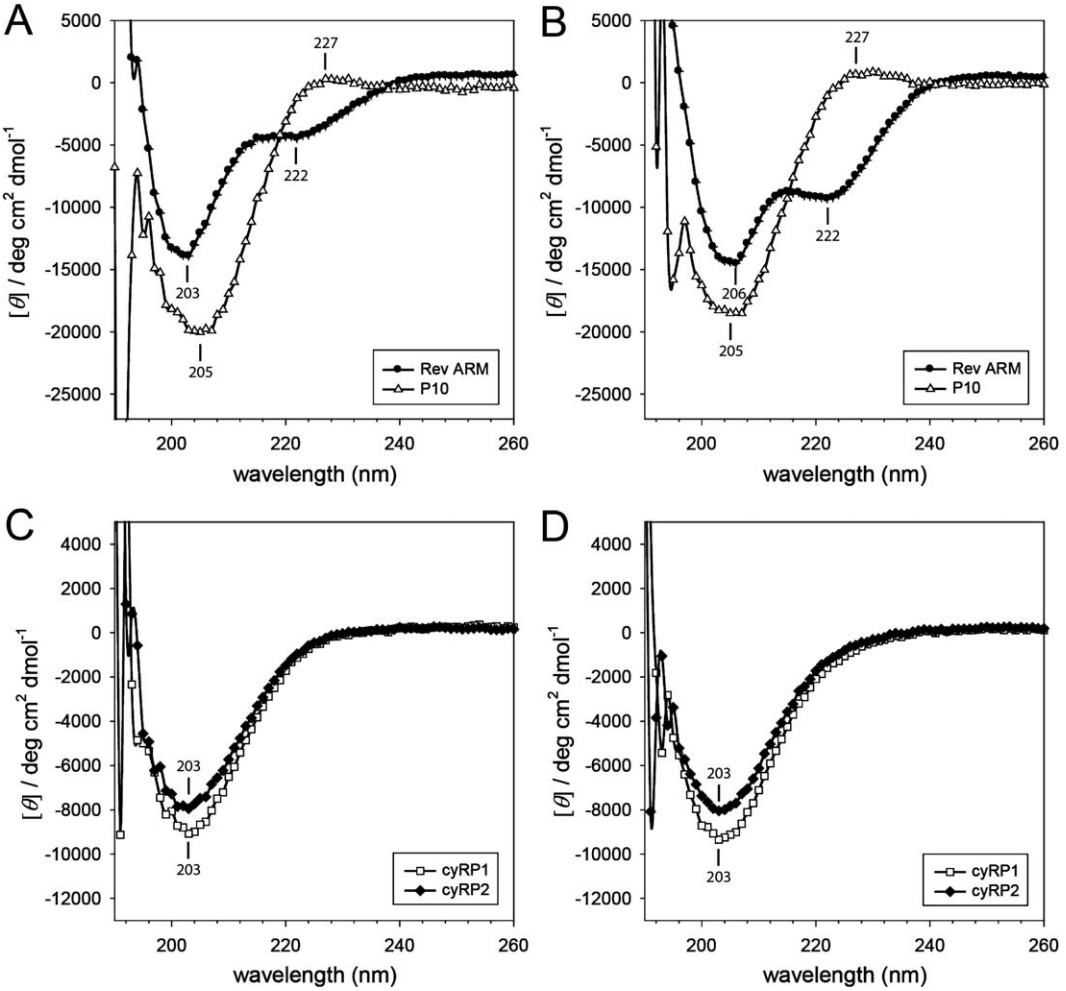

FIGURE 2 CD spectra of various peptides at (A) 25°C, (B) 4°C, (C) 25°C, (D) 4°C. Peptides concentration was 20 μM in 20 mM potassium phosphate, 150 mM KF, pH 7.4. The widely oscillating data at the lower wavelength range (less than 197–198 nm) are unreliable due to the strong UV absorption of salt ions.

ellipticity at 227 nm, regardless of the applied temperature (25°C or 4°C). In contrast, the Rev-ARM peptide displayed temperature-dependent conformational changes. At ambient temperature (25°C), the Rev-ARM peptide was mostly unstructured, with a strong negative band at 203 nm, although a weak negative band at 222 nm indicated that a small portion of α-helical structure exists in solution (Figure 2A). α-Helix formation is an enthalpy-driven process, where the helicity increases with decreasing temperature.[29] Because the Rev-ARM peptide possesses a high helix propensity, it showed a CD profile closer to that of a typical α-helix at 4°C (negative minima at 206 and 222 nm and a $[\theta]_{222}/[\theta]_{208}$ ratio that increased from 0.49 at 25°C to 0.72 at 4°C, Figure 2B). The $[\theta]_{222}/[\theta]_{208}$ ratio is sensitive to backbone dihedral angles and has been used as a measure of α-helicity, which increases as the helicity increases.[30] The $[\theta]_{222}/[\theta]_{208}$ ratio for the fully stabilized α-helix is approximately 0.86.[31] We then investigated the conformation of the macrocyclic peptides. The CD spectra of both cyRP1 and cyRP2 are characterized by a single strong negative band at 203 nm (Figure 2C). The characteristics of the PPII helix (maximum at 227 nm) and the α-helix (minimum at 222 nm) that appeared in the respective CD spectra of the P10 and Rev-ARM peptides could not be observed in those of cyRP1 and cyRP2. This suggests that the positive maximum of the PPII helix at 227 nm and the negative minimum of the α-helix at 222 nm cancel each other out to zero. The signature of the α-helix at 222 nm could not be observed even when the measurements were performed at 4°C, possibly due to the strong cancellation of the signal by the PPII helix. Therefore, it was difficult to assess whether the rigid PPII rod might assist the stabilization of the Rev-ARM α-helix and/or how the two differential helix segments affect each other using CD measurements alone.

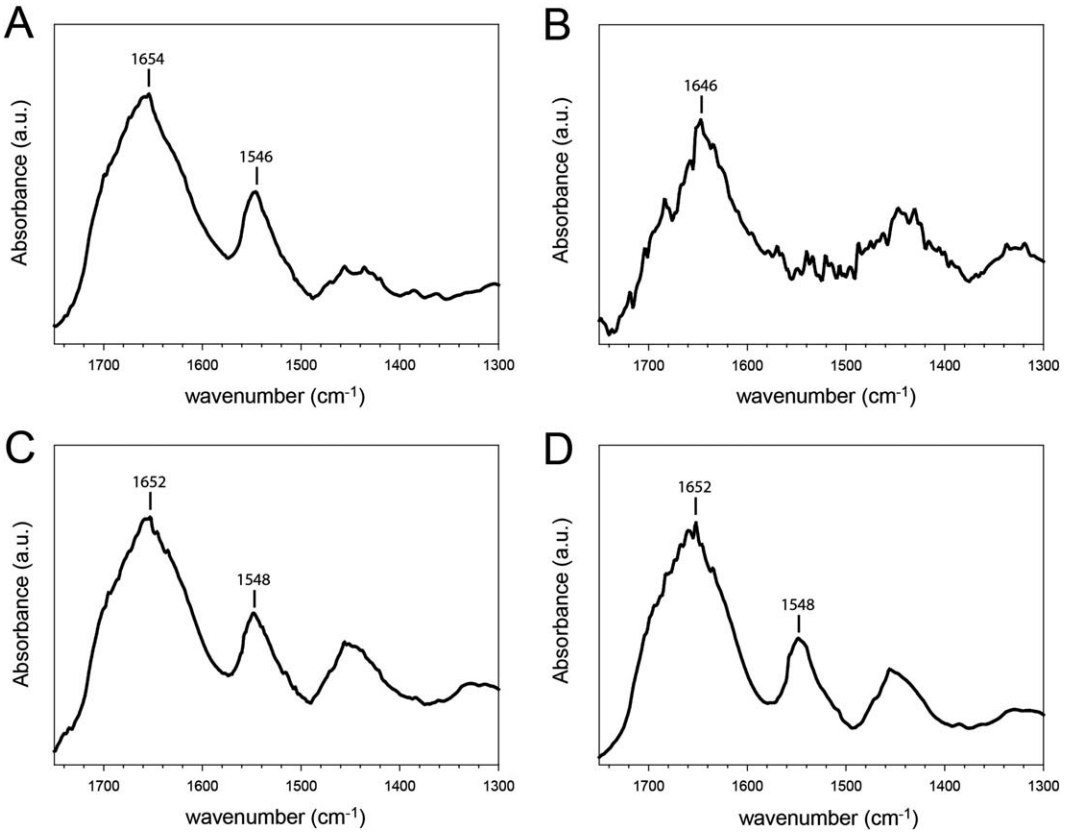

FIGURE 3 FTIR spectra of (A) Rev-ARM peptide, (B) P10 peptide, (C) cyRP1 peptide, (D) cyRP2 peptide.

Based on the results described above, we questioned whether the α-helix conformation is disrupted rather than stabilized by the rigid PPII rod. Moreover, we asked whether it is possible to differentiate the α-helix and PPII signals in a macrocycle that contains both helical segments. The FTIR spectrum of the Rev-ARM peptide displayed the intense absorptions at 1654 cm^{-1} (amide I) and 1546 cm^{-1} (amide II) that correspond to the signature of an α-helix (Figure 3A).[32] The P10 peptide displayed the signature of a PPII conformation with a strong absorption at 1646 cm^{-1} (Figure 3B).[33] The FTIR spectrum of the cyRP1 peptide revealed strong bands at 1652 cm^{-1} and 1548 cm^{-1}, confirming that both α-helix and PPI helix structures are simultaneously present in the macrocyclic peptide (Figure 3C). Similar to cyRP1, the spectrum of cyRP2 is characterized by bands at 1652 cm^{-1} and 1548 cm^{-1} (Figure 3D). Taken together, although it is difficult to quantitate the relative abundance and/or stability of both helical species, FTIR studies confirmed that α-helix and PPII helix coexist in the macrocyclic peptides.

Differential Sensitivity to Helix Stabilizers

Organic solvents can induce conformational changes in peptides. For example, alcohols such as trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP) can stabilize α-helical conformations in unfolded proteins and peptides, and HFIP is usually more effective than TFE.[34] The PPI conformation is typically preferred over PPII in organic solvents and in many alcohols, including 1- and 2-propanol.[19,20] However, PPII is favored in TFE, benzyl alcohol, and organic acids, and there is also a chain-length dependence of the conformational stability.[35] The conformational preference of polyproline in HFIP is not well documented to our knowledge. Because HIFP is a strong stabilizer of α-helix conformation, we hypothesized that the Rev-ARM and P10 segments in the macrocyclic peptide might have differential susceptibility to HFIP, which might help deduce additional structural information about the relative stability of the α-helix and the polyproline helix within the macrocycle.

As the concentration of HFIP increased, there was a gradual increase in α-helix content for the Rev-ARM peptide as expected (Figure 4A). Unexpectedly, the P10 peptide showed unusual behavior upon increasing the HFIP concentration (Figure 4B). The spectrum with a negative band at 205 nm did not change at all when the HFIP concentration was increased to 4%; however, there was an abrupt decrease in overall signal intensity at HFIP concentrations of 8 and 10%. A further

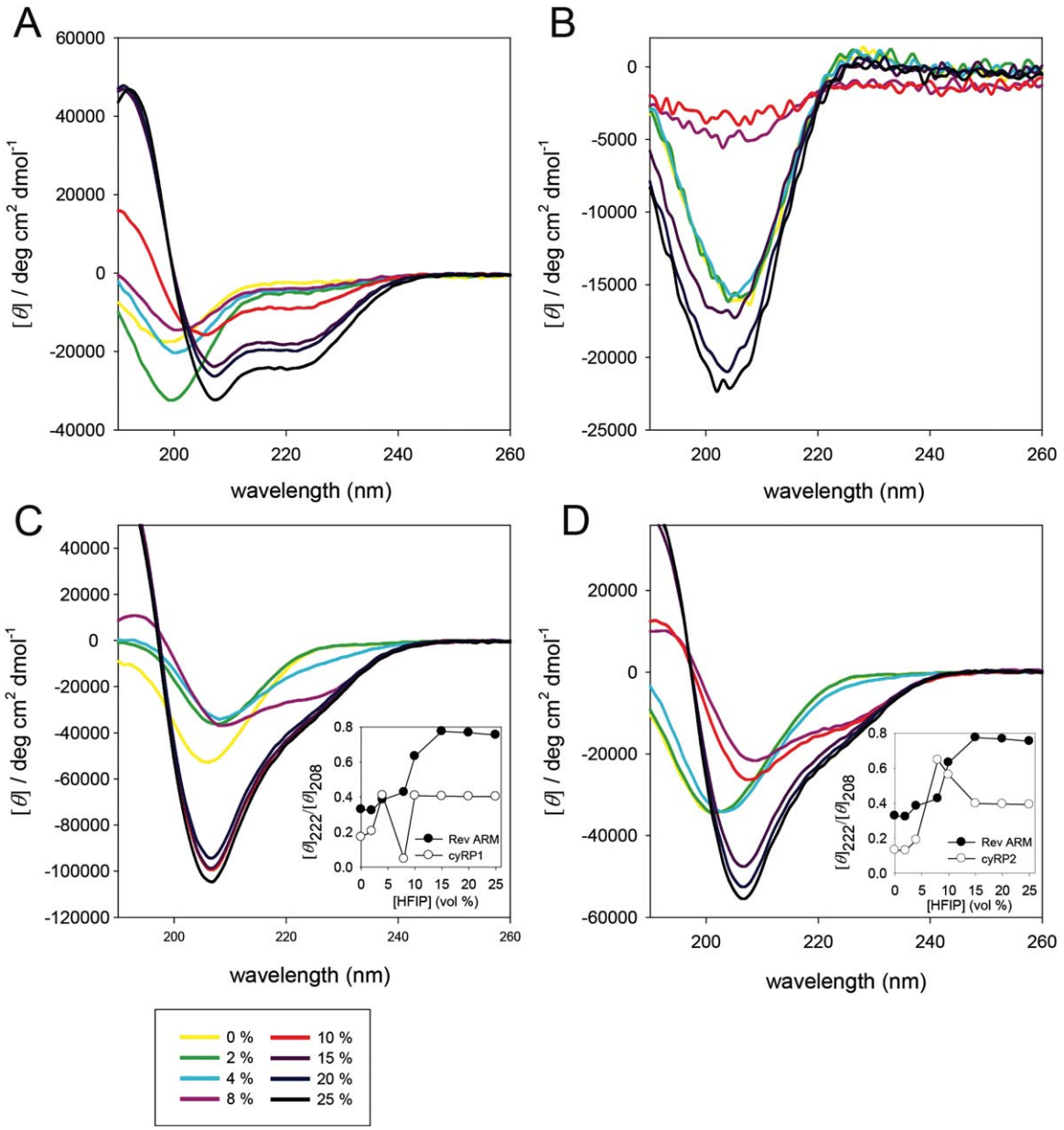

FIGURE 4 HFIP titration experiment. CD spectra of (A) Rev-ARM peptide, (B) P10 peptide, (C) cyRP1 peptide, and (D) cyRP2 peptide at various HFIP concentrations. Plots of $[\theta]_{222}/[\theta]_{208}$ as a function of HIFP concentration are shown as insets in (C) and (D). Spectra were recorded at peptide concentrations of 10 μM at 25°C in 150 mM KF.

increase in HFIP concentration to 15–25% led to the reappearance of the strong negative band, and there was a concomitant blue shift of the negative minimum to 203 nm. This peculiar behavior was reconfirmed by the independent experiments. Therefore, this result suggests that there is a certain conformational change in the P10 peptide around HFIP concentrations of 8–10%. A detailed investigation of the HFIP-dependent conformational change in polyprolines should be the subject of further investigation.

In sharp contrast to the Rev-ARM peptide, both macrocyclic peptides showed discrete changes in CD spectra upon increasing the HFIP concentration (Figures 4C and 4D). To systematically interpret these unique phenomena, we plotted the $[\theta]_{222}/[\theta]_{208}$ ratio as a function of HFIP concentration (insets in Figures 4C and 4D). As expected, there was a gradual increase in the $[\theta]_{222}/[\theta]_{208}$ ratio for the Rev-ARM peptide when the HFIP concentration was increased, which then plateaued at high HFIP concentrations. For the macrocyclic

peptides cyRP1 and cyRP2, the $[\theta]_{222}/[\theta]_{208}$ ratio initially increased, but a discontinuous change around an HFIP concentration of 8–10% was observed, which coincides with the HFIP concentration at which the abrupt change in the CD spectra of the P10 peptide arose. Taking all the data into consideration, the polyproline segment in the context of macrocyclic peptides exerts a significant effect on the overall helical stability of the Rev-ARM segment.

CONCLUSIONS

In this study, we have synthesized peptides that contain both a potential α-helix and a polyproline helix segment in a single macrocyclic scaffold. We expected that the rigid PPII helix might assist the stabilization of the potential α-helix segment when they are located within a single macrocyclic scaffold. Because the macrocycles were very large and have a rigid polyproline segment, the head-to-tail cyclization reaction was a challenging one. We found that the molecular stiffness and the relative orientation of two different helical segments strongly affect the efficiency of macrocyclization reaction. Conformational analyses showed that both the α-helix and the PPII helix coexisted in the macrocyclic peptides. We found that there existed a previously unnoticed conformational transition in the polyproline peptide at HFIP concentrations of 8 and 10%. More in-depth structural studies are necessary to uncover this peculiar behavior of polyprolines. Although it was not possible in this study to quantify the extent of the α-helix stabilization effect exerted by the rigid PPII rod segment, it was revealed that the polyproline segment has a significant effect on the overall helical stability and conformation of the Rev-ARM segment. Because this study demonstrated the possibility of controlling the conformational stability of an α-helix using a rigid polyproline rod, further fine-tuning the structure of the macrocyclic peptide might lead to the development of highly stabilized bioactive α-helical peptides that can control biomacromolecular interactions.

MATERIALS AND METHODS

Materials

Fmoc-amino acids and resins for peptide synthesis were purchased from Novabiochem (Germany) and Anaspec (USA). All other reagents and chemicals are commercially available.

Peptide Synthesis and Cyclization

The linear peptide was synthesized on Rink Amide MBHA resin LL (Novabiochem) using standard Fmoc protocols in a Tribute peptide synthesizer (Protein Technologies, Inc).[36] Standard amino acid protecting groups were employed except for cysteine, for which an acid-labile Mmt group was used for orthogonal deprotection. For cyclization, the resin attached with a fully synthesized linear peptide (20 μmol of N-terminal amine groups) was swollen in NMP for 30 min. Bromoacetic acid was then coupled to the N-terminal portion of the resin-bound peptide. Before addition to the resin, a mixture of bromoacetic acid (28 mg, 200 μmol) and *N*,*N*′-diisopropylcarbodiimide in NMP was incubated for 10 min for carboxyl activation. Following the addition of this mixture to the resin, the reaction was allowed to continue for 1 h with shaking at room temperature in a 6-mL polypropylene tube with a frit. The resin was then washed successively with NMP, *N*,*N*-dimethylformamide (DMF), and dichloromethane (MC). For orthogonal removal of the Mmt protecting group from the cysteine, the resin was treated with 1% TFA/5% triisopropylsilane (TIS) in MC several times (1 min × ~7 times). The intramolecular cyclization reaction was performed in 3 mL of 1% DIPEA in NMP with shaking at room temperature for 2 days. The resins was then successively washed with NMP, DMF, and tetrahydrofuran (THF) and dried under reduced pressure. For cleavage and final deprotection, the resin was treated with a cleavage cocktail (95:2.5:2.5 TFA:1,2-ethanedithiol:thioanisole) for 3 h and was triturated with *tert*-butyl methyl ether (TBME). The peptides were purified by reverse-phase high-performance liquid chromatography (HPLC; water–acetonitrile with 0.1% TFA). The molecular weight was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The peptide concentration was determined spectrophotometrically using the molar extinction coefficient of tryptophan (5500 M^{-1} cm^{-1}) at 280 nm.

Circular Dichroism Spectroscopy

CD spectra were acquired using a Chirascan™ Circular Dichroism Spectrometer equipped with a peltier temperature controller (Applied Photophysics). Spectra were recorded from 260 nm to 190 nm using a 2-mm path length cuvette. Scans were repeated five times and averaged. Molar ellipticity was calculated per amino acid residue. Peptide samples were clearly soluble in distilled water, potassium phosphate-buffered saline solution, and in HFIP at least up to 25%.

Fourier Transform Infrared Spectroscopy

Prior to Fourier transform infrared (FTIR) spectroscopy analysis, the lyophilized peptide was dissolved in D_2O. Then 50 μL of the peptide sample (150 μ*M*) was cast onto a ZnSe window. FTIR spectra were acquired on a Bruker Vertex 70 FTIR spectrometer.

REFERENCES

1. Lim, Y. B.; Moon, K. S.; Lee, M. Chem Soc Rev 2009, 38, 925–934.
2. Robinson, J. A. J Pept Sci 2013, 19, 127–140.
3. Rizo, J.; Gierasch, L. M. Annu Rev Biochem 1992, 61, 387–418.
4. Sia, S. K.; Carr, P. A.; Cochran, A. G.; Malashkevich, V. N.; Kim, P. S. Proc Natl Acad Sci USA 2002, 99, 14664–14669.
5. Walensky, L. D.; Kung, A. L.; Escher, I.; Malia, T. J.; Barbuto, S.; Wright, R. D.; Wagner, G.; Verdine, G. L.; Korsmeyer, S. J. Science 2004, 305, 1466–1470.
6. Henchey, L. K.; Jochim, A. L.; Arora, P. S. Curr Opin Chem Biol 2008, 12, 692–697.
7. Woolley, G. A. Acc Chem Res 2005, 38, 486–493.

8. Azzarito, V.; Long, K.; Murphy, N. S.; Wilson, A. J. Nat Chem 2013, 5, 161–173.
9. White, C. J.; Yudin, A. K. Nat Chem 2011, 3, 509–524.
10. Barker, P. L.; Bullens, S.; Bunting, S.; Burdick, D. J.; Chan, K. S.; Deisher, T.; Eigenbrot, C.; Gadek, T. R.; Gantzos, R.; Lipari, M. T.; Muir, C. D.; Napier, M. A.; Pitti, R. M.; Padua, A.; Quan, C.; Stanley, M.; Struble, M.; Tom, J. Y. K.; Burnier, J. P. J Med Chem 1992, 35, 2040–2048.
11. Wong, C. T.; Rowlands, D. K.; Wong, C. H.; Lo, T. W.; Nguyen, G. K.; Li, H. Y.; Tam, J. P. Angew Chem Int Ed 2012, 51, 5620–5624.
12. Maksimov, M. O.; Pelczer, I.; Link, A. J. Proc Natl Acad Sci USA 2012, 109, 15223–15228.
13. Choi, S. J.; Jeong, W. J.; Kang, S. K.; Lee, M.; Kim, E.; Ryu, D. Y.; Lim, Y. B. Biomacromolecules 2012, 13, 1991–1995.
14. Zhang, F.; Sadovski, O.; Xin, S. J.; Woolley, G. A. J Am Chem Soc 2007, 129, 14154–14155.
15. Bernal, F.; Tyler, A. F.; Korsmeyer, S. J.; Walensky, L. D.; Verdine, G. L. J Am Chem Soc 2007, 129, 2456–2457.
16. Han, S. H.; Lee, M. K.; Lim, Y. B. Biomacromolecules, 2013, 14, 1594–1599.
17. Lim, Y. B.; Moon, K. S.; Lee, M. Angew Chem Int Ed 2009, 48, 1601–1605.
18. Choi, S. J.; Jeong, W. J.; Kim, T. H.; Lim, Y. B. Soft Matter 2011, 7, 1675–1677.
19. Kumin, M.; Sonntag, L. S.; Wennemers, H. J Am Chem Soc 2007, 129, 466–467.
20. Crespo, L.; Sanclimens, G.; Montaner, B.; Perez-Tomas, R.; Royo, M.; Pons, M.; Albericio, F.; Giralt, E. J Am Chem Soc 2002, 124, 8876–8883.
21. Sahoo, H.; Roccatano, D.; Hennig, A.; Nau, W. M. J Am Chem Soc 2007, 129, 9762–9772.
22. Yoon, Y. R.; Lim, Y. B.; Lee, E.; Lee, M. Chem Commun 2008, 1892–1894.
23. Sato, S.; Kwon, Y.; Kamisuki, S.; Srivastava, N.; Mao, Q.; Kawazoe, Y.; Uesugi, M. J Am Chem Soc 2007, 129, 873–880.
24. Pollard, V. W.; Malim, M. H. Annu Rev Microbiol 1998, 52, 491–532.
25. Battiste, J. L.; Mao, H.; Rao, N. S.; Tan, R.; Muhandiram, D. R.; Kay, L. E.; Frankel, A. D.; Williamson, J. R. Science 1996, 273, 1547–1551.
26. Tan, R.; Chen, L.; Buettner, J. A.; Hudson, D.; Frankel, A. D. Cell 1993, 73, 1031–1040.
27. Han, S.; Kim, D.; Han, S. H.; Kim, N. H.; Kim, S. H.; Lim, Y. B. J Am Chem Soc 2012, 134, 16047–16053.
28. Roberts, K. D.; Lambert, J. N.; Ede, N. J.; Bray, A. M. J Pept Sci 2006, 12, 525–532.
29. Marqusee, S.; Robbins, V. H.; Baldwin, R. L. Proc Natl Acad Sci USA 1989, 86, 5286–5290.
30. Wang, D.; Chen, K.; Kulp III, J. L.; Arora, P. S. J Am Chem Soc 2006, 128, 9248–9256.
31. McNamara, C.; Zinkernagel, A. S.; Macheboeuf, P.; Cunningham, M. W.; Nizet, V.; Ghosh, P. Science 2008, 319, 1405–1408.
32. Bandekar, J. Biochim Biophys Acta 1992, 1120, 123–143.
33. Wellner, N.; Belton, P. S.; Tatham, A. S. Biochemical J 1996, 319, 741–747.
34. Hirota, N.; Mizuno, K.; Goto, Y. Protein Sci 1997, 6, 416–421.
35. Kakinoki, S.; Hirano, Y.; Oka, M. Polym Bull 2005, 53, 109–115.
36. Jeong, W. J.; Lim, Y. B. Macromol Biosci 2012, 12, 49–54.

Reviewing Editor: Stephen Blacklow