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Peptide Recognition: Encapsulation and α-Helical Folding of a Nine-Residue Peptide within a Hydrophobic Dimeric Capsule of a Bowl-Shaped Host

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Abstract: A dimeric capsule of coordination bowl **1** encapsulated a nine-residue peptide (Trp-Ala-Glu-Ala-Ala-Ala-Glu-Ala-Trp; **2**) within the large hydrophobic cavity in water, and stabilized the α-helical conformation of bound **2**. An NMR titration experiment revealed that monomeric bowl **1** recognized two Trp residues at the both terminals of **2** through **1**/**2** = 1:1 to 2:1 complexation. The 1:1 and 2:1 species exist in equilibrium even in the presence of excess **1**. It was found that the formation of the 2:1 complex, in which two bowls of **1** wrapped the whole of **2**, became dominant by the addition of $NaNO_3$ due to the fact that the enhanced ion strength increased the hydrophobic interaction between Trp residues and the cavity of **1**. The α-helical conformation of **2** within the dimeric capsule of **1** was elucidated from detailed NOESY analysis.

Keywords: helical structures · host–guest systems · NMR spectroscopy · peptides · self-assembly

Introduction

Peptide engineering has enabled the realization of the artificial construction of molecules and materials with sophisticated functions by utilizing the latent nature of peptides to be spontaneously folded into their secondary structures.[1] This research field is rapidly developing through the understanding of the mechanism and design principle of peptide folding, which is still of major interest in peptide chemistry. Extensive studies have been made on the formation of peptide secondary structures from de novo designed oligopeptides.[2,3] Unlike those incorporated in a protein scaffold, however, short peptide fragments cannot form stable secondary structures.[4] Hence the folding of secondary structures of peptides has been often achieved by cross-linking of side chains by means of covalent bonding and metal coordination.[5,6]

Another approach for the stabilization of secondary structures is peptide encapsulation within a hydrophobic cavity. This strategy is reminiscent of nature's system, since the secondary structures are sustained only by weak interactions in the cavity. However, oligopeptide encapsulation by synthetic hosts is a difficult task, because most of host molecules, such as cyclodextrins and calixarenes, have relatively small cavity that can bind only one small organic molecule. In previous examples of peptide recognition, two binding sites had to be covalently linked.[7] For example, Breslow and co-workers prepared cyclodextrin dimers and demonstrated the stabilization of the α-helix conformation of a peptide containing unnatural amino acids as recognition sites.[7b]

The most efficient way to provide cavities large enough to bind peptides is the construction of large hosts by molecular self-assembly. Cages, bowls, and capsules with extraordinarily large cavities have been prepared by self-assembly through hydrogen or coordination bonds.[8] Atwood and Rebek, Jr. reported the assembly of a nanocapsule from six molecules of resorcin[4]arene through 60 hydrogen bonds, though the recognition of large molecules by the nanocapsule has been not achieved.[9] Recently, Rebek, Jr. and coworkers demonstrated the helical folding of linear alkanes by encapsulation within a cylindrical capsule that assembled

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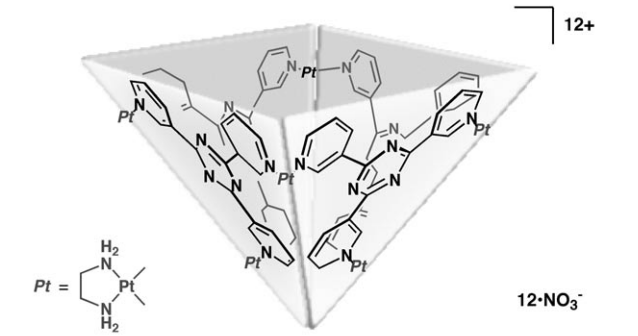
from two cavitands.[10] We[11] and others[12] have shown the remarkable potential of metal-directed self-assembly for the construction of discrete host compounds with large cavities. Among them, Pd^{II}- or Pt^{II}-linked roughly spherical M_6L_4-type cages bind a variety of large neutral organic guests in a surprisingly efficient fashion.[13] Quite recently, peptide recognition by the M_6L_4 cage was examined and oligopeptides were recognized by the cage in a sequence-selective fashion.[13h]

The metal-linked M_6L_4 bowl **1** also shows unique host properties.[14] It dimerizes into a hydrophobic capsule that can bind up to four large guests. For example, encapsulation of four molecules of *m*-terphenyl or *cis*-stilbene have been confirmed by X-ray crystallographic analysis and NMR spectroscopy.[14b] In expectation of binding peptides within the dimeric capsule, we recently examined the recognition of nine-residue peptide with the bowl **1**. Although the dimeric capsule was not formed, the bowl **1** induced an α-helical conformation of the peptide in an aqueous solution in the presence of $CHCl_3$ (1 v/v%) to give a 1:1 complex.[15] In this complex, the hydrophobic face in the α-helix was accommodated deeply in the cavity of **1**. The solvent $CHCl_3$ seems to be co-enclathrated with the peptide. In the absence of $CHCl_3$, the conformation of the peptide was ambiguous and not readily analyzed.



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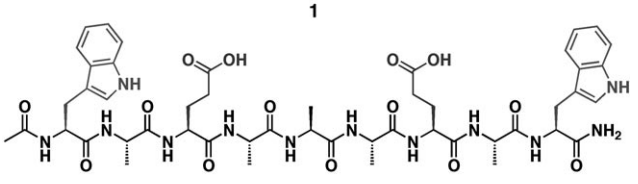


2

To encapsulate the peptide within the dimeric capsule of **1**, we examined a salt effect, because the host–guest hydrophobic interaction is expected to increase with increasing ion strength of the solution. Accordingly, we describe here the encapsulation of nine-residue peptide **2** within the large hydrophobic cavity of Pt^{II}-bowl **1** dimer in $NaNO_3$ aqueous solution. A detailed NMR study shows that 1) the dimerization of bowl **1** is induced by complexation with two Trp residues at the both ends of **2**, 2) the formation of the dimeric structure occurs stepwise through a 1:1 complex, and 3) the peptide accommodated in the dimeric capsule adopts an α-helical conformation.

Results and Discussion

Site-selective recognition of nine-residue peptide: The association of the nine-residue peptide (**2**) with Pt^{II}-bowl **1** was studied by NMR titration. The sequential assignment of **2** in **1** was carried out by TOCSY and NOESY measurements. Upon the addition of **1** into the aqueous solution of **2**, the colorless solution turned yellow, indicating the charge-transfer interaction between the electron-rich Trp residues and the electron-deficient bowl.[13h] It was found that the binding of the two Trp residues (Trp1 and Trp9) with the bowl was not simultaneous, but sequential. First, Trp9 at the C terminus was bound to give a 1:1 complex and, second, the complexation of Trp1 at N terminus followed to give a 2:1 complex.

The ^1H NMR titration showed that, at a **1**/**2** ratio of 1:1, the Hβ signal of Ala8 was shifted significantly up-field relative to the other Ala residues (Figure 1). Upon further addition of **1**, the up-field shift of this signal was saturated at a **1**/**2** ratio of about 2:1, while the Hβ signals of other the Ala residues were not saturated. The signal of Hβ proton of Ala2 near the N terminus was shifted up-field to a much lesser extent. This suggests that the C terminus of **2** was preferentially recognized to give a 1:1 complex as an intermediate. Association constant for the 1:1 complexation was estimated to be roughly 10^4–$10^5\,M^{-1}$ from NMR titration. This site selectivity was supported by two control experiments. The association of **1** with Trp9/Ala9-mutated peptide, Ac-Trp-Ala-Glu-Ala-Ala-Ala-Glu-Ala-*Ala*-NH_2 (**3**), and Trp1/Ala1-mutated peptide, Ac-*Ala*-Ala-Glu-Ala-Ala-Ala-Glu-Ala-Trp-NH_2 (**4**), was examined. UV/Vis titrations showed that peptide **4** was recognized five time more strongly than **3** (Table 1), consistent with the C-terminal-selective recognition of **2**. We also prepared Glu7/Lys7-mutated peptide. The complexation at the C terminus should be disturbed, since the positive charge of Lys7 repels the cationic bowl **1**. As a result, affinities at both termini were comparable, as indicated by simultaneous up-field shifting of Ala2 and Ala8 (see the Supporting Information).

Encapsulation of 2 within a dimeric capsule of 1: As mentioned above, 2:1 complexation was observed in the presence of excess **1**. The Hβ signals of not only Ala8, but also Ala2 were shifted up-field in the presence of five equivalents of **1** (Figure 1d), suggesting that both termini were bound within the cavity of **1**. To confirm the formation of 2:1 species, we estimated the molecular size of complexes from DOSY measurements. From DOSY, the diffusion coefficient of control peptide **4** in the presence of four equivalents of bowl **1** was $1.26\times10^{-10}\,m^2s^{-1}$. This value should reflect the 1:1 complex, because peptide **4** has only one Trp residue and can form only 1:1 complex with **1**. The diffusion coefficient of peptide **2** in the presence of four equivalents

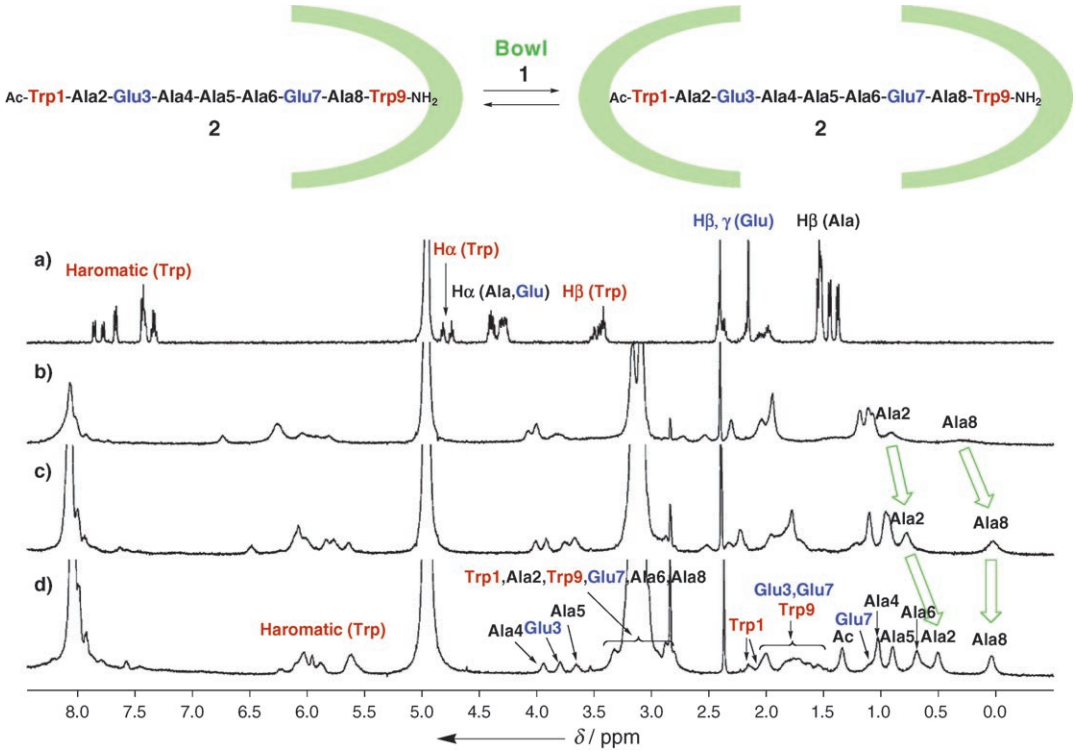


Figure 1. ^{1}H NMR spectra (500 MHz, D_2O, 27 °C) of **2** ([**2**] = 2 mM) in 100 mM phosphate buffer (pH 6.8) and **1**: a) [**1**] = 0 mM, b) 2 mM, c) 4 mM, and d) 10 mM.

Table 1. Association constants of **1** with control peptides in water.

Peptides	K_a [M^{-1}][a]
Ac-**Trp**-Ala-Glu-Ala-Ala-Ala-Glu-Ala-Ala-NH$_2$ (**3**)	1.6×10^4
Ac-Ala-Ala-Glu-Ala-Ala-Ala-Glu-Ala-**Trp**-NH$_2$ (**4**)	8.6×10^4

[a] Measured by UV/Vis titration at 20 °C in 10 mM phosphate buffer (pH 6.8).

of bowl **1** (1.06×10^{-10} m^{2}s^{-1}) was smaller than that of **4** (Figure 2). This result indicated the formation of a complex larger than the 1:1 complex; namely, a 2:1 species is suggested.

The Job plot obtained by UV/Vis measurements indicated that both the 1:1 and 2:1 species existed in equilibrium even in the presence of excess **1**.[16] The plot exhibited a peak at approximately 0.6 (Supporting Information), which is smaller than 0.66, the ideal value for 2:1 complexation. Assuming that the maximum up-field shift of Ala2 is comparable to that of Ala8, we estimated the ratio of the 1:1 and 2:1 complexes to be about 40:60 from $\Delta\delta_{Ala2}/\Delta\delta_{Ala8}$ ratio at [**1**] = 10 mM and [**2**] = 2 mM (Figure 1d).

Stabilization of the 2:1 complex by addition of $NaNO_3$: To stabilize the 2:1 complex formation, we studied a salt effect, since the host–guest hydrophobic interaction is expected to

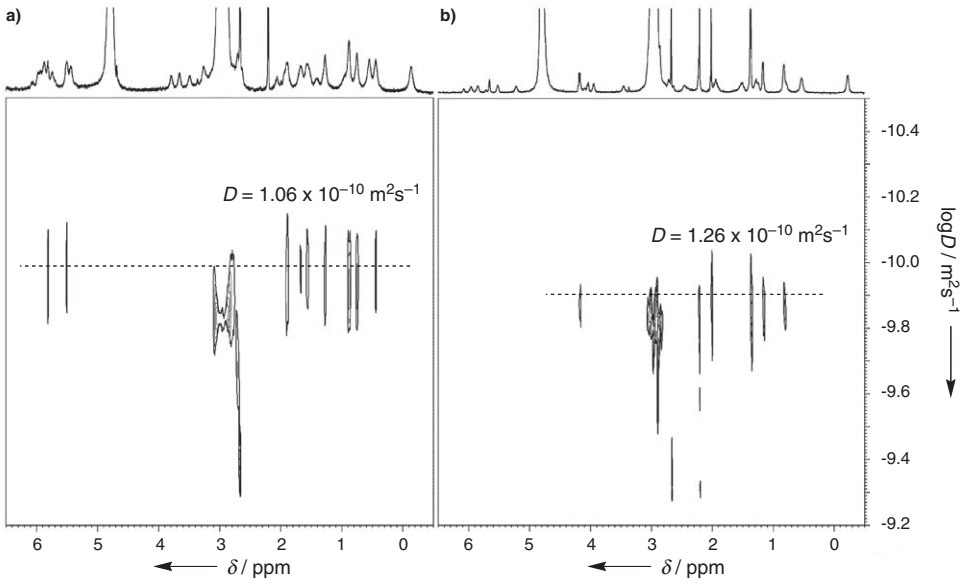


Figure 2. DOSY spectra (500 MHz, D_2O, 27 °C) of four equivalents of **1** in 100 mM phosphate buffer (pH 6.8) and a) peptide **2** ([**2**] = 1.9 mM) and b) peptide **4** ([**4**] = 1.8 mM).

increase with increasing ion strength.[17] The addition of $NaNO_3$ induced a further up-field shift of the Hβ proton signal of Ala2 in **2** (Figure 3), suggesting that formation of the 2:1 complex became dominant. Under the conditions used to produce the spectra shown in Figure 3c ([**1**] = 10 mM, [**2**] = 2.5 mM; [$NaNO_3$] = 400 mM), the ratio of the 1:1 and the 2:1 complexes was estimated to be about 20:80.

Stabilization of the α-helical structure of 2 within the dimeric capsule of 1: In the absence of $NaNO_3$, the conformation of peptide **2** was difficult to analyze, because of rapid equilibration on the NMR timescale between two different conformers in the 1:1 and 2:1 complexes and also because of the relatively weak host–guest interaction. Upon the addition of $NaNO_3$ (400 mM), the 2:1 complex dominated and the conformation of the peptide was fixed. Thus, the secondary structure of peptide **2** in the 2:1 complex was analyzed by NOESY experiments. Circular dichroism (CD) spectrometry, generally used for the characterization of peptide secondary structures, was not useful because of fatal interference of CD of **2** by strong absorption of **1** around 200–250 nm.

In the NOESY spectrum, we observed sequential NOEs $d_{NN}(i,i+1)$ and several medium range NOEs: for example, $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$, and $d_{\alpha N}(i,i+4)$. The observation of these NOEs is characteristic of a typical α-helix conformation (Figure 4).[18] Only a few unreasonable NOEs for the α-helical conformation were observed due to the co-existence of some minor conformers. Since CD measurement showed the unordered structure of free peptide **2** in water, the α-helical conformation of **2** was evidently induced by the encapsulation of **2** within the dimeric capsule of **1**.

Proposed structure of helical peptide 2 and the dimeric capsule of 1: To estimate the geometry of **2** in the 2:1 complex, chemical shifts of the residues were compared with those of free **2**. All signals of **2** exhibited negative $\Delta\delta$ values from −0.6 to −2.3 ppm (Figure 5). This observation indicates that the whole of **2** is covered by the dimeric capsule of **1**. The Hβ protons near terminal Trp residues were shifted up-field to a much greater extent than the others, indicating the tight and deep accommodation of the residues near both termini within bowl **1**. For example, $\Delta\delta$ values of the Hβ signals of Ala2 (−1.20 ppm) and Ala8 (−1.38 ppm) were larger than those of Ala4 (−0.62 ppm), Ala5 (−0.83 ppm), and Ala6 (−0.87 ppm). The up-filed shift of the middle residues also indicates the folding of the peptide into a compact form. Based on these observations, we proposed the encapsulation of peptide **2** in an α-helical conformation within the hydrophobic cavity of the dimeric capsule as shown in Figure 6.

Dynamic host–guest assembly controlled by media: Dynamic feature of the bowl–peptide complexation deserves attention. In the aqueous solution of **2** (2.5 mM) and **1** (7.5–10 mM), the peptide conformation and the complexation ratio are ambiguous. Addition of $NaNO_3$ induced the dominant formation of the 2:1 complex in which peptide **2** is encapsulated in the dimeric capsule of **1** in an α-helical conformation, as described above. On the other hand, we have previously reported the dominant formation of a 1:1 complex in the presense of $CHCl_3$, in which peptide **2** is co-enclathrated with $CHCl_3$ in the monomeric bowl in an α-helical conformation.[15] There are many examples of dynamic molecular assembly in which guests induce host frameworks.[19] In contrast, the present host–guest system varies the assembly manner depending on media conditions ($CHCl_3/H_2O$ vs $NaNO_3/H_2O$; see Scheme 1). Such a dynamic host assembly responsive to media provides a prototype for a new type of external-stimuli-responsive molecular assembly.

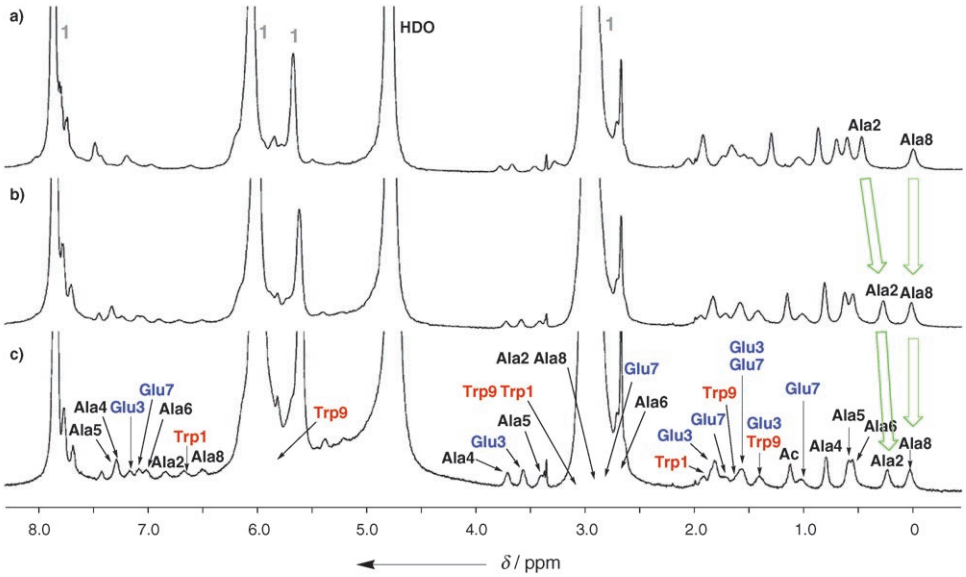


Figure 3. ^1H NMR spectra (500 MHz, H_2O/D_2O = 9/1, 27 °C) of **1** ([**1**] = 10 mM) and **2** ([**2**] = 2.5 mM) in 100 mM phosphate buffer (pH 6.8) in the presence of $NaNO_3$ a) 0 mM, b) 200 mM, and c) 400 mM.

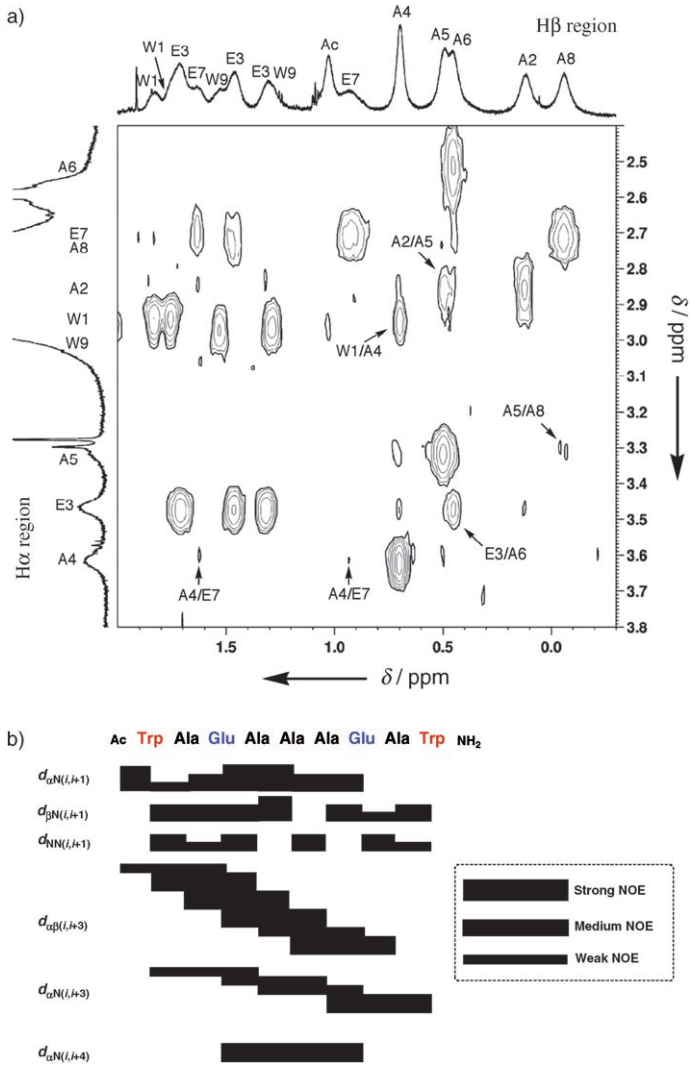


Figure 4. a) Selected NOESY spectrum (600 MHz, $H_2O/D_2O = 9/1$, 27 °C) of **1** ([**1**] = 10 mM) and **2** ([**2**] = 2.5 mM) in 100 mM phosphate buffer (pH 6.8) at the presence of 400 mM $NaNO_3$. b) NOE correlations for the bound peptide **2** under the same conditions.

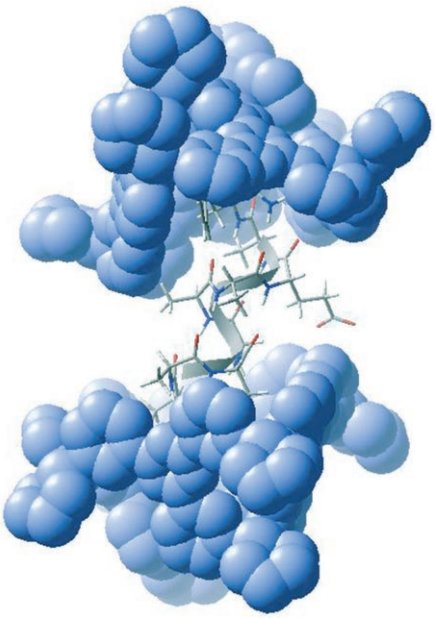


Figure 6. The proposed structure of **2** and dimeric capsule of **1**. Dimeric capsule of **1** and peptide **2** are represented by space-filling and cylindrical models, respectively.

Conclusion

In conclusion, we demonstrated that nine-residue peptide **2** was encapsulated within the large hydrophobic cavity of a dimeric capsule assembled from two coordination Pt^{II} bowl complexes **1**. The driving forces behind the formation of the dimeric capsule are the hydrophobic and charge-transfer interactions between **1** and Trp residues at both termini of peptide **2**. Furthermore, we observed the stabilization of the α-helical conformation of **2** within the large hydrophobic cavity of the dimeric capsule from NOESY experiments. Most interestingly, the approach in this work for the stabilization of secondary structure mimics biological systems. Thus, other secondary structures, such as β-strand, β-hairpin,

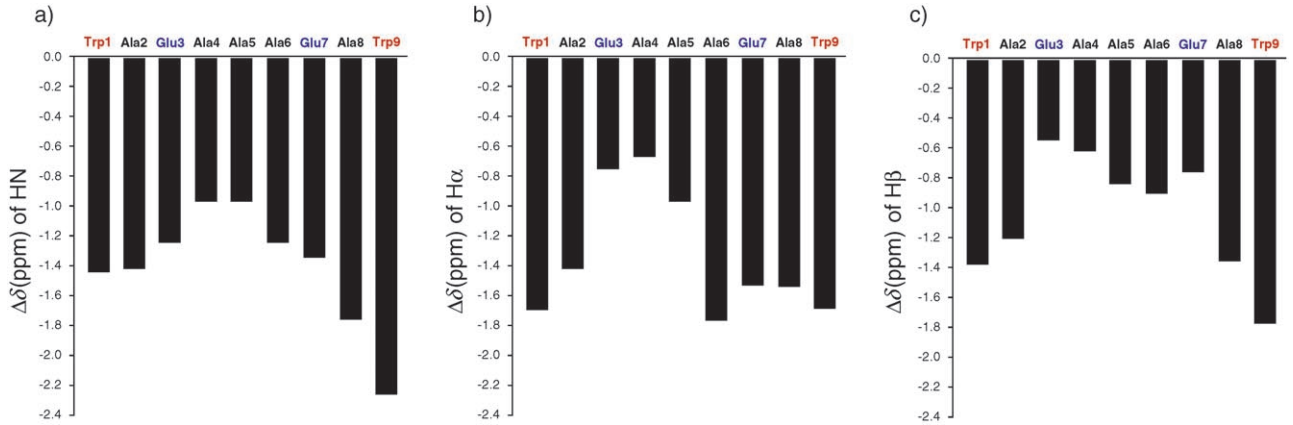
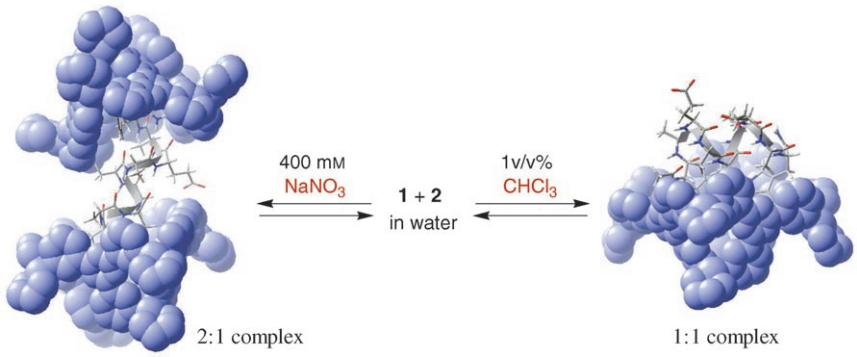


Figure 5. Up-field chemical shifts for **2** ([**2**] = 2.5 mM) with **1** ([**1**] = 10 mM) in 100 mM phosphate buffer (pH 6.8) in the presence of 400 mM $NaNO_3$. The values for each residue were compared with chemical shifts of extended chain.



Scheme 1.

and turn structures, have been stabilized within hydrophobic pocket of various coordination structures synthesized by our group.[11] Moreover, when designing larger cavities that could to accommodate a whole or part of an aromatic-rich protein molecule, this strategy is expected to be applicable to not only the stabilization of ephemeral tertiary structure, but may also be used to study the pathway of protein folding.

Experimental Section

Materials: Organic solvents and reagents were purchased from TCI, WAKO Pure Chemical Industries, and Aldrich Chemicals. Deuterated solvents were acquired from Cambridge Isotope Laboratories. Fmoc amino acids and some reagents for the peptide synthesis were purchased from Watanabe Chemical Industries. The Pt^{II} bowl compound **1** was prepared according to a procedure reported earlier.[15]

Peptide synthesis: Peptides **2**, **3**, and **4** were synthesized by an automated peptide synthesizer (ABI 433 A, Applied Biosystems) by using the standard Fmoc-based FastMoc coupling chemistry (0.1 mmol scale). Peptides were cleaved from the resin with trifluoroacetic acid (TFA; 10 mL) containing 5% (v/v) water and 5% (v/v) 1,2-ethanedithiol as a scavenger at room temperature for 3 h. Free peptides were washed from the resin subsequently with TFA (3 mL) and dichloromethane (5 mL). After evaporation, a large amount of Et_2O was added to the residue and the precipitate was collected by filtration. Crude peptides were purified by reversed-phase HPLC on an Inertsil Peptides C18 (GL Sciences) semipreparative column (20 mm × 250 mm) by using 10 mM ammonium hydrogencarbonate solution with a 0.05% TFA (pH 6.0) and acetonitrile gradient. Then the white powder of peptides was obtained by lyophilization. Characterization of peptides was carried out by ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry (Voyager-DE STR, Applied Biosystems).

UV titration: A peptide was dissolved in water under sonication and the solution was filtered by a disk filter to remove trace amount of insoluble impurities. The concentration of peptide was determined by the absorption of Trp residues.[20] The aqueous solution of the peptide (3.0 mL) was placed in a 1 cm quartz cell. The aqueous solution of **1** (5 mM) was added in portions by means of a microsyringe to the cell. Absorption spectra were recorded on a SHIMADZU UV-3150 spectrometer. All titrations were carried out at room temperature under 10 mM phosphate buffer conditions (pH 6.8). Since **1** and the peptide itself have no absorption at 430 nm, the absorption appeared at 430 nm was monitored for the titration. The change in volume due to the addition of a **1** was less than 4%. The association constant was calculated by a nonlinear curve-fitting procedure.[21]

Job plot: Equimolar solutions of **1** and **2** (2.0 mM) were mixed in various ratios. Absorption spectra were measured and charge-transfer band at 430 nm were analyzed by Job's method.[16,21]

NMR spectroscopy: For preparing NMR sample, peptide **2** was dissolved in 100 mM phosphate buffer solution (H_2O/D_2O = 90:10) and the solution was filtered through a disk filter. The concentration of **2** was determined by the absorption of Trp residues.[20] Powdered **1** was added to an aqueous solution of **2** (0.6 mL) and, then, the solution was shaken until **1** dissolved completely. After about 1 h, NMR measurements were carried out. NMR spectra were recorded on Bruker Avance 600 spectrometer and Bruker DRX 500 spectrometer at 300 K. TOCSY and NOESY were measured in the phase-sensitive mode. Mixing times of the TOCSY and NOESY were 80 ms and 250 ms, respectively. For water signal suppression, a WATERGATE solvent suppression scheme[22] was applied to most NMR experiments. DOSY spectra were recorded on a Bruker DRX 500 spectrometer at 300 K with a z axis gradient amplifier. All spectra were processed by using XWINNMR (Brucker).

Acknowledgements

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