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Helical Templating of Oligopeptides by Cyclodextrin Dimers

David Wilson, Lisa Perlson and Ronald Breslow*

Department of Chemistry, Columbia University, New York, NY 10027, USA

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Abstract—β-cyclodextrin-based receptors were synthesized and tested for their ability to induce a helical fold in peptides bearing hydrophobic amino acid residues in the i, i + 11- or i, i + 14-positions. Circular dichroism experiments revealed that a dimeric β-cylodextrin receptor synthesized from a [1,1′-biphenyl]-4,4′-dithiol core demonstrated an ability to fold a designed peptide bearing the artificial amino acid L-*p*-*t*-butylphenylalanine in the i, i + 11-positions, while other dimeric and monomeric receptors failed to do so. Titration studies were performed using both circular dichroism and calorimetry, the analysis of which yielded an apparent K_a on the order of 10^4–10^5 M^{-1}. However, no evidence could be obtained for helical folding with a peptide carrying tryptophan residues in place of the *p*-*t*-butylphenylalanine units. Our studies suggest that receptors of this type may be useful in molecular recognition of hydrophobic, already α-helical peptides in aqueous solution.

Introduction

The hydrophobic effect has been implicated in a variety of biological phenomena, in particular protein–protein interactions essential to the viability of basic cellular processes.[1] For example, hormone-receptor binding, intracellular signaling, and protein oligomerization events all appear to involve this important form of molecular recognition.[2–4] Structural studies of proteins have revealed that hydrophobic interactions contribute to folding of a peptide or protein into its native structure, via packing of hydrophobic residues in coiled-coil or bundle structures, or via molecular chaperonins which assist in folding and transport of newly assembled proteins in the cytoplasm.[5,6] Thus, it is of great interest to develop receptors that can recognize the hydrophobic surfaces of proteins selectively, especially surfaces with well-defined secondary or tertiary structure.

We have reported that certain linked dimers of cyclodextrins can selectively bind the hydrophobic side chains in polypeptides,[7] and apparently bind to hydrophobic patches of proteins so as to disrupt protein aggregation.[8] In efforts to develop sequence-selective receptors, we examined the possibility that cyclodextrin-based dimers could bind selectively to hydrophobic residues on the face of an α-helical peptide via helical templating experiments. We reasoned that chelate binding of two hydrophobic side chains in an oligopeptide might be able to induce helicity in the peptide if binding occurred to side chains that were appropriately spaced. Since an α-helix has 3.5 residues per turn, the side chains would be presented in more or less the same direction if the spacing were 3–4 residues, or 7–8, 10–11, or 14–15 residues. Our molecular models suggested that only with a 10-residue separation or more would two bulky bound β-cyclodextrins be able to fit the same face of an α-helix. Thus we have examined the ability of some β-cyclodextrin dimers to induce helicity in oligopeptides with hydrophobic side chains at positions i and i + 11, or i and i + 14.

To start, we introduced the well-bound *p*-*t*-butylphenyl groups of (L)-*p*-*t*-butylphenylalanine into the oligopeptides. This is of course an unnatural amino acid, but *p*-*t*-butylphenyl groups typically bind into β-cyclodextrin rings in water with a K_a of approximately 10^4 M^{-1},[9] much higher than the affinity of natural amino acid side chains such as Phe or Trp. We then examined the folding of these peptides in water by circular dichroism (CD) in the presence of various synthetic receptors. The binding was further investigated by titration calorimetry.

Prior efforts to develop α-helical templates have involved nucleation via N-terminal capping,[10–12] porphyrin and bipyridine-based templates,[13,14] metal binding via the

*Corresponding author. Tel.: +1-212-854-2170; fax: +1-212-854-2755; e-mail: rb33@columbia.edu

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artificial amino acid aminodiacetic acid,[15,16] and template-assembled synthetic proteins (TASPs).[17–20] More recently, Zn(II) dipicolylamine-based artificial receptors were used to selectively bind and stabilize the α-helix conformation of peptides via recognition of histidine residues.[21] To our knowledge, our approach is the first to utilize the hydrophobic effect to induce folding in designed oligopeptides.

Results and Discussion

Synthesis

Peptides **1**–**4** (Fig. 1) were synthesized using Fmoc-protected amino acids on a solid-phase synthesizer, using side-chain protection of Lys with *t*-Boc and of Glu with *O*-*t*-Bu. The crude peptides were purified by reversed-phase HPLC chromatography, their identities confirmed by laser desorption mass spectrometry, and their purity assessed by analytical HPLC.

The cyclodextrin receptors examined, compounds **1**–**8**, are shown in Figure 1. Dimers **1**–**4** were prepared by reacting 6-deoxy-6-iodocycloheptaamylose with 1,4-benzenedithiol, [1,1′-biphenyl]-4,4′-dithiol, [1,1′:4′,1″-terphenyl]-4, 4″-dithiol, and 1,6- hexanedithiol, respectively, in dimethylformamide with potassium carbonate. Dimer **5** was prepared by acylating 6-amino-6-deoxycycloheptaamylose with the bis-pentafluorophenyl ester of the linker diacid, as described previously.[8] Monomers **7** and **8** were synthesized by reacting 6-deoxy-6-iodocycloheptaamylose with an excess of benzenethiol and [1,1′-biphenyl]-4-thiol, respectively, in dimethylformamide with sodium methoxide. The compounds were purified on a LOBAR C-18 reversed-phase column. All of the compounds had the expected NMR and mass spectra for the structures assigned.

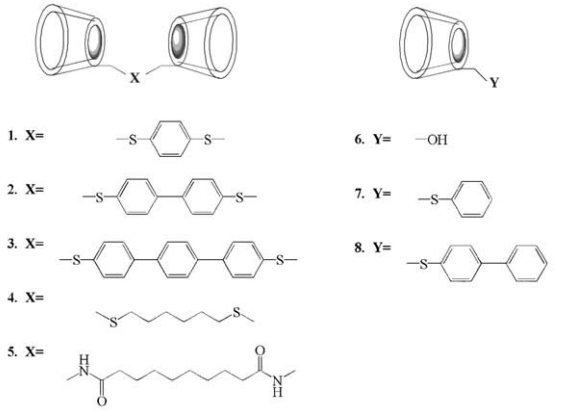

Figure 1. Cyclodextrin-based receptors and peptides studied.

Folding studies

Prior to CD studies of the putative peptide/receptor complexes, the propensity of peptides **1**–**4** to fold into an α-helical conformation was investigated by studies in which the concentration of trifluoroethanol was varied from 0 to 50% with the peptide concentration at 50 μM in 2 mM phosphate buffer (pH = 7.0). The resulting CD spectral change confirmed qualitatively that all four designed peptides had substantial capacity for additional folding (data not shown).

Peptides **1**(**F**,**F**) and **4**(**F**,**F**) were constructed incorporating the artificial amino acid L-*p*-*t*-butylphenylalanine since the binding constant K_a of this hydrophobic moiety to β-cyclodextrin has been determined to be on the order of 10^4 M^{-1} for a monomeric binding event. In contrast, the binding constant of tryptophan to β-cyclodextrin has been estimated by NMR studies to be on the order of 50–100 M^{-1}.[21]

The results of CD studies for peptides **1**–**4** with cyclodextrin-based receptors are shown in Table 1. An initial screen of peptide **1**(**F**,**F**) and peptide **4**(**F**,**F**) with cyclodextrin-based receptors **1**–**8** revealed that only the biphenyl-derived receptor **2** displayed marked ability to induce an α-helical conformation in peptide **1**(**F**,**F**), as indicated by intensification of the characteristic negative Cotton peak at 222 nm. Receptor **2** did not template helix formation for peptide **4**(**F**,**F**), nor was any other receptor studied able to do so. Based on this initial screen, peptides **2**(**W**,**F**) and **3**(**W**,**W**) were constructed in order to investigate the sensitivity of this system to substitution with weaker-binding tryptophan. These studies revealed that receptor **2** was unable to induce a helical fold for these two peptides, even in the presence of 10 equivalents of the receptor (final concentration = 500 μM). For peptide **3**(**W**,**W**), infrared spectroscopy (IR) studies at even higher concentrations ([peptide] = 1 mM and [receptor] = 5 mM) failed to indicate a change in the intensity or distribution of peaks in the amide III (1220–1320 cm^{-1}) region of the spectrum that would indicate helix formation (data not shown).[22]

These results are consistent with modeling studies which suggest that the intercavity distance for dimeric receptor

Table 1. Changes in molar ellipticity on mixing of peptides with various receptors

	1	2	3	4	5	6	7	8
Peptide 1(F,F)	1.08	−5.00	−0.68	0.73	0.11	1.51	1.40	1.54
Peptide 2 (W,F)	—	0.39	—	—	—	—	—	—
Peptide 3 (W,W)	—	0.20	—	—	—	—	—	—
Peptide 4(F,F)	2.23	1.68	0.29	0.69	0.66	0.74	0.51	0.98

The difference $\Delta[\theta]_{222}$ ($\Delta[\theta]_{222} = [\theta]_{complex} - [\theta]_{peptide} - [\theta]_{receptor}$) was calculated based on separate CD experiments. In each case the $[\theta]_{222}$ measured for the peptide and receptor alone were subtracted from the $[\theta]_{222}$ observed for the complex. Values reported in the table correspond to $\Delta[\theta]_{222} \times 10^{-3}$. [Peptide] = 50 μM for all studies. [Receptor] = 50 μM (for dimeric receptors 1–5) and 100 μM (for monomeric receptors 6–8) for peptides 1(F,F) and 4(F,F). For peptides 2(W,F) and 3(W,W), [Receptor] = 500 μM. In each case the final buffer concentration (phosphate, pH 7.0) was 2 mM and the temperature 25 °C.

2 corresponds well with the distance between t-Bu-Phe residues when the conformation of peptide **1**(**F**,**F**) is assumed to be 100% α-helical. Dimeric receptors **1** and **3**, in which the linker has been varied from **2** by a phenyl unit, appear to be too short and too long respectively to induce helix formation. Receptors **4** and **5**, which modeling studies suggest could adopt a conformation in which the β-cyclodextrin cavities are separated by the optimal distance, appear to have too much flexibility for the desired templating effect. In the majority of cases, particularly for monomeric cyclodextrin receptors **6**–**8** and for peptide **4**(**F**,**F**), CD studies suggested that there was a substantial decrease in helical content observed upon addition of receptor. This suggests that for this system hydrophobic guest/receptor interactions are inhibitory to helix formation in general.

The qualitative changes in CD spectrum upon addition of receptor **2** were studied for peptide **1**(**F**,**F**) by performing a titration with the initial [peptide] = 25 μM and the [receptor] varied from 0 to 50 μM, as depicted in Figure 2. Two negative Cotton peaks at 208 and 222 nm, characteristic of α-helix formation, were intensified upon addition of receptor **2**. These qualitative spectral changes were not observed for any other peptide/receptor complex. It should be noted that peptide **1**(**F**,**F**) in the absence of receptor displays a CD spectrum more consistent with a beta-conformation than with a random coil. This suggests the possibility that this particular peptide is able to form a stable inter-molecular antiparallel β-sheet, in which there is excellent hydrophobic and charge complementarity. This observation is consistent with findings in the laboratory of DeGrado for peptides of similar structure.[23]

In order to calculate a binding constant and determine the stoichiometry for this interaction of **1** with **2**, we conducted careful CD titration experiments and constructed a Job's plot, as shown in Figure 3. For the titration data, the concentration of the peptide was 50

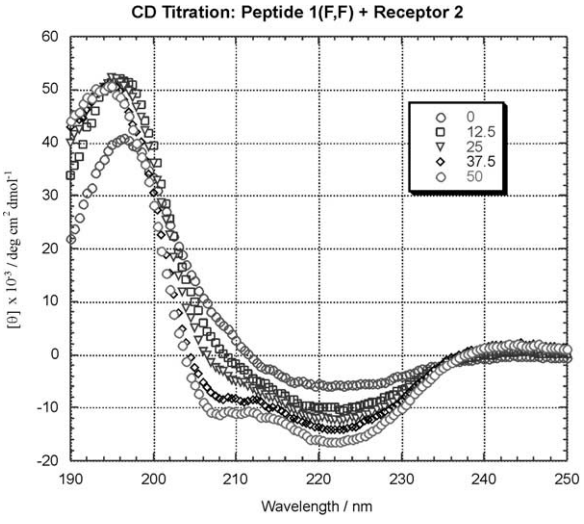

Figure 2. CD titration for peptide **1**(**F**,**F**) and β-cyclodextrin-based receptor **2** performed with [peptide] = 25 μM and [receptor] varied from 0 to 50 μM. In each case the final buffer concentration (phosphate, pH 7.0) was 2 mM and the temperature 25 °C.

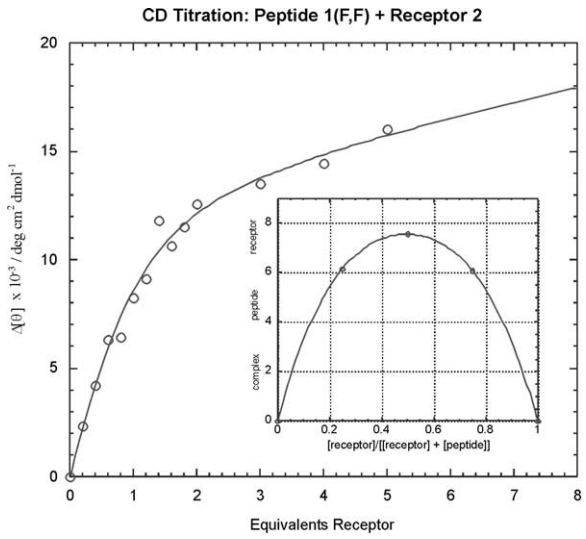

Figure 3. CD titration curve of peptide **1**(**F**,**F**) with β-cyclodextrin-based receptor **2** performed with [peptide] = 50 μM and [receptor] varied from 0 to 250 μM. Inset: Job's plot of this combination (the total concentration is 100 μM). In each case the final buffer concentration (phosphate, pH 7.0) was 2 mM and the temperature 25 °C.

μM and the receptor concentration was varied from 0 to 250 μM. For the Job's plot, [peptide + receptor] was held constant at 100 μM. From these data, the binding stoichiometry was determined to be 1:1 and the CD titration curve plotted at 222 nm, which displays typical saturation behavior, yielded an apparent binding constant K_a of 6.9×10^4 M^{-1}.

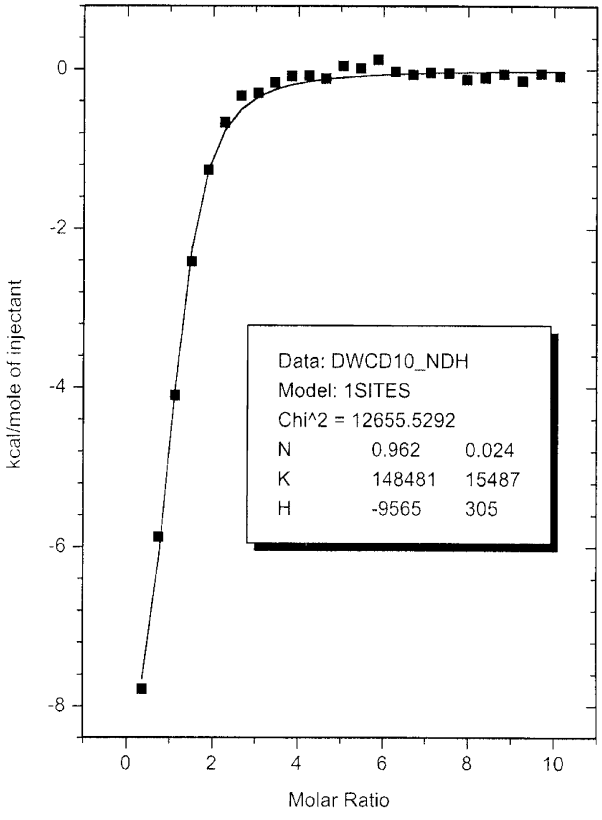

Figure 4. Titration curve of peptide **1**(**F**,**F**) with β-cyclodextrin-based receptor **2** obtained by calorimetry. This study was performed with [peptide] = 40 μM (in the cell) and [receptor] = 2 mM (in the syringe).

Table 2. Binding constants of peptide/ receptor complexes derived from titration calorimetry data

Peptide	Receptor	K_{ass}
Peptide 1(**F**,**F**)	1	$1.9\pm0.4\times10^5$ M^{-1}
Peptide 1(**F**,**F**)	2	$1.5\pm0.2\times10^5$ M^{-1}
Peptide 1(**F**,**F**)	3	$5.5\pm4.9\times10^4$ M^{-1}
Peptide 1(**F**,**F**)	4	$6.9\pm1.9\times10^4$ M^{-1}
Peptide 1(**F**,**F**)	5	$4.8\pm0.8\times10^4$ M^{-1}
Peptide 1(**F**,**F**)	6	$6.7\pm3.2\times10^4$ M^{-1}
Peptide 2(**W**,**F**)	2	$4.4\pm0.4\times10^4$ M^{-2}
Peptide 3(**W**,**W**)	2	(Not detectable)

For peptide 1(**F**,**F**) the concentration was 40 μM and the receptor concentration 2.0 mM. For binding studies with peptide 2(**W**,**F**) and peptide 3(**W**,**W**), the peptide concentration was 0.2 mM and the receptor concentration 2.0 mM. All experiments were conducted in 50 mM phosphate buffer (pH = 7.0) at 25 °C.

The titration data derived from CD studies showed reasonable concordance with those obtained by calorimetry for this complex, depicted in Figure 4. For titration calorimetry experiments, a 2-mM receptor **2** solution in buffer was injected into a sample-cell compartment containing a 40 μM solution of peptide **1**(**F**,**F**) at 25 °C. Using a non-linear least-squares fitting algorithm, the binding constant K_a was determined to be 1.7×10^5 M^{-1} and the stoichiometry of complex formation 1:1. Studies of other potential complexes were performed, with solutions of receptors **1**–**6** in buffer at identical concentrations injected into the peptide solutions. In each case the observed stoichiometry was 1:1, with the exception of simple cyclodextrin **6** for which the stoichiometry was 1:2 (peptide/receptor). Studies were not performed on receptors **7**–**8** due to lack of solubility at the desired concentration. The results of these experiments are shown in Table 2. For tryptophan-containing peptides **2** and **3**, a 2 mM solution of receptor **2** was injected into a sample cell containing a 0.2-mM solution of peptides **2**(**W**,**F**) and **3**(**W**,**W**), respectively. For peptide **2**(**W**,**F**) the binding constant K_a was determined to be 4.4×10^4 M^{-2} with a stoichiometry of 2:1 (peptide/receptor), whereas binding was not detectable at these concentrations for peptide **3**(**W**,**W**) (Fig. 5).

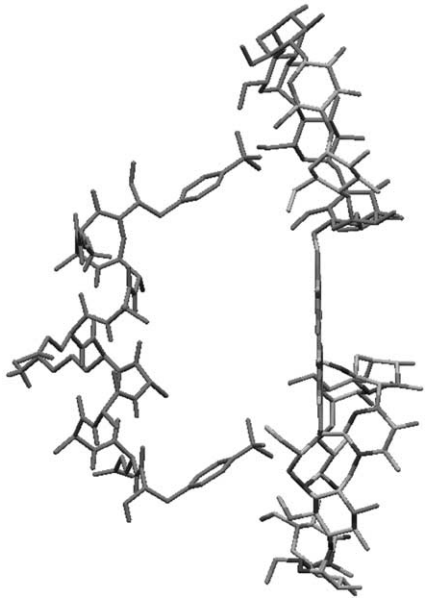

Figure 5. Schematic representation of helical templating capacity of β-cyclodextrin based receptor **2**. The peptide is shown in helical form, and in this form its two *t*-butylphenyl groups would be able to bind into the cavities of the cyclodextrin dimer. For clarity, the bound structure is not shown, but would involve simply pushing the two components together.

Conclusions

These studies demonstrate that while there is substantial binding between peptides bearing the *t*BuPhe sidechain and β-cyclodextrin-based receptors, this relatively tight binding induces a helical fold only if the binding geometry is correct to do so. Interestingly, we did not observe large difference between binding constants observed for monomeric and dimeric host/receptor interactions. For the monotopic binding interaction between a β-cyclodextrin unit and a *t*-Bu-phenyl group with K_a $\sim10^4$ M^{-1}, we might expect ditopic binding with K_a on the order of 10^8 M^{-1}, but we do not observe this.

Previous studies in this laboratory have suggested that ditopic (or tritopic) binding is only possible with extremely rigid, pre-organized substrate and receptor structures for which the loss of entropy upon binding is relatively small.[24,25] Perhaps this 'entropy problem' helps to explain why helical templating is not possible for peptides **2**(**W**,**F**) and **3**(**W**,**W**) given the relatively weak enthalpic gain in accommodating a tryptophan residue in a β-cylodextrin host. Future studies will focus on expanding hydrophobic α-helical recognition to tritopic receptors, as well as determining binding constants for receptors of interest to already fully folded helical structures.

Experimental

Chemical synthesis of peptides: general procedure

Fmoc-protected amino acids Glu, Ala, Lys, Trp, and *p*-*t*BuPhe were acquired as free acids from Bachem. The peptides **1**–**4** were synthesized on a continuous flow Applied Biosystems Pioneeer solid phase synthesizer using the Fmoc/^{t}Bu protection strategy with PAL-PEG-PS resin at 0.2 mmol scale. Single extended coupling cycles of 60 min using OH/HBTU activation chemistry were employed for all amino acids. The side chain protecting groups used are as follows: Lys (^{t}Boc); Glu (O^{t}Bu). After peptide assembly the N-termini were manually acetylated using 1:1 acetic anhydride:pyridine for 30 min followed by thorough washing with DMF, isopropanol and CH_2Cl_2. Each peptide was cleaved from the resin and simultaneously deprotected using 90:8:2 (v/v/v) trifluoroacetic acid/ethanedithiol/water for 3 h. Crude peptides were precipitated and washed with cold ether, followed by dissolution in water (0.1% v/v TFA), lyophilization and reversed phase C_{18} HPLC purification to homogeneity using aqueous-acetonitrile gradients containing 0.1% (v/v) TFA. After lyophilization, the identities of the resulting peptides were

confirmed with laser desorption mass spectrometry and their purity assessed by analytical HPLC.

Chemical synthesis of β-cyclodextrin receptors

The cyclodextrin receptors examined, compounds **1**–**8**, are shown in Figure 1. Dimers **1**–**4** were prepared by reacting 6-deoxy-6-iodocycloheptaamylose with 1,4-benzenedithiol, [1,1′-biphenyl]-4,4′-dithiol, [1,1′:4′,1″-terphenyl]-4, 4″-dithiol, and 1,6 hexanedithiol, respectively, in dimethylformamide with K_2CO_3. Dimer **5** was prepared by acylating 6-amino-6-deoxycycloheptaamylose with the bis-pentafluorophenyl ester of the linker diacid. Monomers **7**–**8** were synthesized by reacting 6-deoxy-6-iodocycloheptaamylose with an excess of benzenethiol and [1,1′-biphenyl]-4-thiol in dimethylformamide with sodium methoxide. In each case, the receptor was precipitated by slowly adding the reaction contents into anhydrous ether, and redissolved in a minimum amount of water. After filtration, the filtrate was loaded on a LOBAR C-18 reversed-phase column and chromatographed with MeOH/H_2O (linear gradient 0–80% MeOH). The fractions which contained the desired product were concentrated under reduced pressure, and the residual aqueous solution was lyophilized. All of the compounds had the expected ^{1}H NMR and mass spectra for the structures assigned.

Circular dichroism spectropolarimetry

CD spectra were recorded on an Jasco 810 spectropolarimeter using rectangular quartz cells of 2-mm path length with a 10-s averaging time. Peptide concentrations were between 25 and 50 μM as determined by amino acid analysis via a Hewlett Packard AminoQuant System (performed by the protein chemistry laboratory at Texas A&M University). Cyclodextrin-receptor concentrations were between 0 and 500 μM as determined by ^{1}H NMR using terepthalic acid as an internal standard.

Titration calorimetry

Calorimetric titrations were performed on a Microcal, Inc. (Northampton, MA, USA) Omega titration calorimeter. Peptide concentrations were between 40 and 200 μM as determined by amino acid analysis. The receptor concentration was 2 mM. Both peptide and receptor were dissolved in 50 mM phosphate buffer (pH = 7.0). The peptide solution (2.5 mL) was placed into the sample-cell compartment of the microcalorimeter, while the receptor solution was loaded in a 250-μL syringe and assembled onto the calorimeter. The setup was equilibrated until RMS error was less than 5×10^{-3} with the syringe spinning at 400 rpm. The receptor solution was then injected into the cell in 25 injections (10 μL, 7 s per injection, with a 4-min interval between injections). These data were analyzed by ORIGIN software, with the data fit using a non-linear least-squares fitting algorithm. Fits provide apparent binding constant K_a, ΔH^o, and stoichiometry of complex formation.

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