Peptide Complexes

Assessment of Cooperativity in Ternary Peptide-Cucurbit[8]uril Complexes

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Abstract: Evaluating cooperativity for cucurbit(8)uril (CB[8])-mediated ternary complexation is required for understanding and advancing designs of such ternary self-assembled systems. A key issue is to dissect the contributions of the binding steps of the first and second guest molecules to the overall ternary complex formation energy. This is addressed by performing concentration-dependent titrations between CB[8] and guests by means of concentration-dependent calorimetric and 1H-NMR titrations. The sensitivity of the fitting of the cumulative heat of complexation of the calorimetric titrations is evaluated in terms of fitting error and enthalpy-entropy compensation and, together with the NMR spectroscopic analysis of the separate species, non-cooperative binding is conceived to be the most probable binding scenario. The binding behavior of CB[8] homoternary complexes is similar to CB[8] heteroternary complexes, with an enthalpy-driven tight fit of the guests in the CB[8] cavity overcoming the entropic penalty. Also for these types of complexes, a non-cooperative binding is the most probable.

Specific molecular recognition properties between ligands (guests) and receptors (hosts) allow non-covalent synthesis of artificial receptor–ligand complexes to occur.[1–4] Cucurbit[n]urils (CB[n]) form a new class of macrocyclic hosts that show remarkable molecular recognition properties in water.[5] The highest affinities between CB[n]s and their guests occur when high energy solvation water molecules are released from the cavity, which generates an enthalpic gain upon complexation.[6] CB[8] is the first homologue large enough to promote binding of two equivalents of guest forming a ternary complex.[6–7] For example, a heteroternary complex forms through the well-defined sequential binding of two different guests inside the CB[8] cavity and this can drive the self-assembly of copolymers,[8] hydrogels,[9] particles,[10–11] and monolayers.[12] Also homoternary complexes can be used for such purposes, in particular as demonstrated for the binding of N-terminal aromatic amino acidic residues such as tryptophan (Trp) or phenylalanine (Phe) to CB[8].[13] This type of CB[8]-peptide complex extends the application of CB[8] assemblies into the biological arena.[14–16]

A ternary complex offers the opportunity for tuning the assembly properties by cooperativity. Cooperativity describes the relationship between the affinities of binding of the first and second equivalent of guest by the host.[17] In comparison to the affinity of the first guest molecule, the binding of the second guest can either be favored, unfavored, or unaffected (i.e., positive, negative, or non-cooperative, respectively). The principle of cooperative interactions is common in living systems and modulates the function of a receptor by the concentration of the ligands. For example, the binding of oxygen to the four pockets of hemoglobin is a positive cooperative process resulting in an increase of the binding affinity of hemoglobin for the substrate oxygen upon each molecule of oxygen bound.[20] Proper design of the stability and dynamics of self-assembled systems based on ternary interactions requires a thorough understanding of the, possibly cooperative, binding behavior of the ternary complex interaction motif. In a systematic study of the sequence-specific recognition of peptides by CB[8], the homoternary complex between PheGly, and CB[8] was proposed as a synthetic, positively cooperative receptor–ligand interaction.[13] An overall ternary binding constant \( K_{\text{sw}} \) of \( 1.5 \times 10^7 \text{M}^{-2} \) was reported for the complex CB[8]-PheGly2.[13] The positively cooperative nature of this complex was suggested on the basis of 1H-NMR experiments, but the extent of cooperativity was not quantified.[18] Here, we assess the degree of cooperativity for ternary complexes of CB[8] and two peptides both with an N-terminal phenylalanine, followed by either two (PheGly2) or six glycine (PheGly6) residues. Isothermal titration calorimetry (ITC) and 1H-NMR titrations were used to study the dependence of the affinity of CB[8] on the concentration of the guest. A key issue is to dissect the contributions of the bindings of the first and second guest molecules to the overall ternary complex formation. This is addressed by performing concentration-dependent titrations, an evaluation of the error sensitivity in the ITC experiments, and by a spectroscopic analysis of the separate species by 1H NMR spectroscopy.

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Figure 1 shows the first and the second binding events between the host CB[8] (H) and the peptide guest (G), leading to the formation of the 1:1 complex HG and the homoternary 1:2 complex HG₂, respectively. The first equilibrium binding constant K₁ arises from the interaction of a single guest G with the host H. For the second binding step, the dissociation of a guest is associated with a pre-factor 2 (2*K₂), to account for the presence of two identical guest molecules in the cavity. Overall, the degree of cooperativity, defined by the ratio K₂/K₁, governs which of the three scenarios, positive, negative, and non-cooperativity, applies, depending on whether K₂ is larger than, smaller than, or equal to K₁, respectively.

An important aspect for the assessment of the degree of cooperativity is to work in an as wide as possible range of concentrations of H and G to make use of the different concentration dependencies of the binding constants for the formation of HG and HG₂. For a given overall binding constant Ktot, different degrees of cooperativity are expected to give different species distributions. This means that the distributions of the concentrations of H, HG, and HG₂, while keeping the initial concentrations of host and guest constant, correspond to unique scenarios of K₁/K₂. To be able to accurately determine the ratio K₁/K₂, different distributions of H, HG, and HG₂ can be measured starting from different initial concentrations of host and guest. A proper working range of concentrations was determined to be between 1 and 50 μM (see the Supporting Information for details). ITC studies were performed to determine the ratio between K₁ and K₂ for the ternary complexes of CB[8] with the peptides PheGly₂ and PheGly₄. The simultaneous fitting of the ITC data sets measured at three different host concentrations provided a restricted range of physically acceptable K₁/K₂ ratios. Specifically, with the optimal range of concentrations, CB[8] was loaded in the cell at concentrations between 10 and 50 μM and titrated with a solution of the peptide guest. The enthalpograms obtained for each host–guest complex are given in Figure 2a, e. A mathematical model was used to fit the experimental heats with a least-squares minimization routine (see the Supporting Information for details). Briefly, the heat of complex formation was expressed as a function of the species concentrations, and the thermodynamic parameters K₁, K₂, ΔH₁, ΔH₂, and ΔTₛ were used as fit parameters. Heats of dilution for each set of initial concentration were also included in the model, and calculated values were confirmed by reference experiments. The best fits provided the optimal four parameters ΔH₁, ΔH₂, K₁, and K₂ and the optimal K₁/K₂ ratio for each peptide guest. K₁/K₂ values of around 2 were found for both peptides (K₁/K₂ = 2.1 ± 0.8 for PheGly₂ and 1.8 ± 0.4 for PheGly₄, Table 1), which agrees with a non-cooperative binding scenario.

To evaluate how sensitive the fit error is to variations of the K₁/K₂ ratio, the least-squares error was calculated for different degrees of cooperativity. Thus, the parameters ΔH₁, ΔH₂, and K₁ (correlated to K₂) were optimized for chosen values of K₁/K₂. Figure 2 shows the dependence of the fit error (Figure 2b, f) on the ratio K₁/K₂ and the correlated enthalpies (Figure 2c, g) and entropies (Figure 2d, h). Figure S4 in the Supporting Information shows the changes in fit of the ITC titrations at very high and very low K₁/K₂. The trends in fit show, in short, that:

(a) a much higher K₁/K₂ should be visible by a plateau of Q at low [Gtot] and [Htot] combined with a clear inflection at [Gtot]/[Htot] = 100.
Table 1. Thermodynamic binding constants for complexes of CB[8] and PheGly₀

|          | ITC PheGly₀/H₂O | ITC PheGly/H₂O | ¹H NMR PheGly₀ | ¹H NMR PheGly/H₂O |
|----------|-----------------|----------------|----------------|-------------------|
| Kᵣ/Kₛ   | 2.1 (0.8)       | –              | 1.8 (0.4)      | 0.5               |
| Kᵣ [M⁻¹] | 2.2 (1.1) x 10⁹ | –              | 8.7 (0.6) x 10⁸ | 3.8 x 10⁵         |
| Kₛ [M⁻¹] | 1.0 (0.2) x 10⁹ | –              | 5.1 (1.3) x 10⁸ | 7.8 x 10³         |
| ΔHᵣ [kcal mol⁻¹] | –11.6 (0.3) | –29.6 (0.2) | –8.3 (0.2) | –                   |
| ΔSᵣ [kcal mol⁻¹ K⁻¹] | –13.7 (1.7) | –14.7 (2.5) | –                | –                  |
| ΔGᵣ [kcal mol⁻¹] | –7.2 (0.3) | –15.4 (0.1) | –6.7 (0.1) | –7.6               |
| ΔGₛ [kcal mol⁻¹] | –6.8 (0.1) | –6.4 (0.2) | –8.0            | –6.7              |
| TΔSₛ [kcal mol⁻¹ K⁻¹] | –4.3 (0.5) | –14.2 (0.3) | –1.5 (0.2) | –                  |
| TΔSₗ [kcal mol⁻¹ K⁻¹] | –6.9 (2.2) | –8.3 (3.3) | –                | –                  |

Standard deviations are given in parentheses. [a] Concentration of CB[8] was spectrophotometrically determined. [b] See Figure 2 and text for details. Data obtained at 25 °C in PBS (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4). [c] Data as reported for the overall ternary complex H[G]. [d] See Figure 2 and text for details. Data obtained at 25 °C in D₂O [e] Product of Kᵣ and Kₛ gives Kᵣₛ. [f] Difference ΔG and ΔT gives TΔSᵣ H₂O

1, and (b) a much lower Kᵣ/Kₛ should lead to a rather shallow slope (Figure 5a) in the two higher concentrations, and in Figure 5b, at the two lower concentrations, which clearly conflict with the observed data. An evaluation of all thermodynamic parameters presented in Figure 2 allowed for the determination of a range of possible degrees of cooperativity (indicated in green in Figure 2). Values of the fit error within 20% from the minimum error were defined as acceptable. This 20% cut-off value was selected based on the variability of the minimum error observed in triplicate calorimetric experiments. Therefore, the upper boundary of the range of acceptable degrees of cooperativity was set at values of Kᵣ/Kₛ equal to 6 for PheGly and to 3.5 for PheGly₀. For higher values of Kᵣ/Kₛ (strongly negative cooperativity), the fit errors became quickly unacceptably high (Figure 2b, f). Regarding the thermodynamic parameters, such high Kᵣ/Kₛ ratios gave more exothermic enthalpies and less favorable entropies for the second step (Figure 2).

The lower limit of the range was determined considering that, even though the fit errors did not rise as quickly as at the upper limit, the binding enthalpies and entropies for the first and second binding events diverged more and more for values of Kᵣ/Kₛ lower than 0.5. Specifically, an inversion of the signs of ΔHₛ and ΔHₗ as well as of TΔSₛ and TΔSₗ was observed for values of Kᵣ/Kₛ below 0.2 for PheGly and below 0.1 for PheGly₀ (Figure 2). Under these conditions, the second binding event became less enthalpically favored (and more entropically favored) than the first step. Both steps would thus be associated with large enthalpy–entropy compensation effects and opposite driving forces, that is, strongly enthalpy-driven for the first step and strongly entropy-driven for the second. In particular, the unfavorable positive enthalpy contribution (Figure 2c, g) and the highly favorable entropy contribution (Figure 2d, h) for the second step are not realistic considering that CB[8] complexation is known to be enthalpically driven and entropically unfavorable.[21–28] Overall, the considerations made in terms of fit error and of enthalpy–entropy compensation determined a range of acceptable Kᵣ/Kₛ ratios between 0.2 and 6 for PheGly and between 0.1 and 3.5 for PheGly₀, which are highlighted in green in Figure 2. For both peptides, these ranges indicate either a non-cooperative or a weakly, negative or positive cooperative system.

For both PheGly and PheGly₀, the second binding event has a larger enthalpic gain than the first, as well as a larger entropy loss (Table 1). This indicates a tighter fit for the second guest in the CB[8] cavity, which is logical as it involves interaction with an already partially filled cavity. It is also in agreement with studies performed by Biedermann and co-workers[22] that show, in the case of heteroternary complexes, a more favorable enthalpy for the second aromatic guest correlates with a less favorable entropy contribution. Similar to what was shown for the heteroternary complexes, this can be expected also in the case of the homoternary complexes studied here; the first guest reduces the cavity volume of CB[8] in such a way that the potential energy of the residual cavity water molecules is increased, thus leading to a stronger enthalpic response upon release of these water molecules upon the binding of the second guest. In contrast, the tightly packed ternary complex reduces the degrees of freedom of both guests and therefore brings an additional unfavorable entropy contribution.[22]

Another observation from our calorimetric results is that when comparing the thermodynamic data for the two peptides, a stronger binding affinity was found for PheGly, with respect to PheGly₀, arising from differences for both the first and second guest binding steps. In particular, the first PheGly seems to have a weaker interaction with the host (less favorable ΔHₛ).

Moreover, our results reveal a slightly weaker overall binding than the one reported in the literature[13] for the overall ternary complexation of the peptide PheGly with CB[8] (see Kᵣₛ in Table 1), which can be explained by a higher concentration of cations competing with the guest for the binding to the host in our buffer.[23] The crystal structure of the complex[13] shows that the shorter PheGly₂ can assume a circular conformation to maximize its dipole–dipole interactions of the amided protons with the carbonyl on the CB[8] rims. This cannot be achieved for a longer chain in the case of PheGly₀ which may explain...
the observed difference in affinity. Unfortunately, the X-ray structure of the complex CB[8]-PheGly$_2$ is not available to confirm this hypothesis. Our observations are in agreement with calorimetric experiments on heteroternary complexes of CB[8], paraquat and TrpGly$_2$ or TrpGly, that have shown a tighter binding for the short peptide compared to the long one.\cite{22}

Taken together, the calorimetric data indicate that the most realistic scenario is the non-cooperative binding of the peptides.

However, further narrowing the range of possible $K_i/K_f$ values could not be achieved by ITC alone, due to both the restricted operative concentration range (see above) and the convolution of the heat effects arising from the first and the second binding events. To overcome the latter limitation, $^1$H-NMR was used to provide direct spectroscopic insight into the (relative) concentrations of all participating species separately. This technique has a relatively low sensitivity, so fairly high concentrations are preferred; however, to prevent precipitation of CB[8], experiments were performed at 50 µM, which contrasts an earlier study that used CB[8] at a concentration that exceeded the solubility limit.\cite{13} A titration experiment was performed at a constant total CB[8] concentration (in D$_2$O) of 50 µM, while titrating from 0.5–4 equivalents of the peptides (Figure 3a, d and see full spectra in Figure S3 of the Supporting Information). The three species G, HG, and HG$_2$ were distinguished based on the signals of the aryl protons of the guests.\cite{13} Upon the first complexation, the upfield shifts of the phenyl protons of the Phe residue verified the shielding of the surrounding CB[8] host molecule. With the second complexation, the interaction among the two guests in the cavity of the CB[8] caused an additional upfield shift.\cite{26} Under non-saturation conditions for CB[8], the HG complex is well visible at low concentrations for both peptides, thus excluding a strongly positive cooperative system, in contrast to what has been described in an earlier study.\cite{13} By monitoring the signals of the aromatic protons (Figure 3a, d), the distributions of all species G, HG, and HG$_2$ were determined for each titration step (Figure 3b, e). These distributions were fitted to a model expressing the calculated distributions of species as a function of the fitting parameters $K_i$ and $K_f$ (see the Supporting Information for details). The calculated data are shown as lines in Figure 3b, e for the peptides PheGly$_2$ and PheGly$_4$, respectively. Table 1 summarizes the values found for the optimized parameters $K_i$, $K_f$, the corresponding free energies $\Delta G^0_i$, $\Delta G^0_f$ (see also Figure S7), and the overall binding constant $K_{solv}$. Higher overall binding affinities ($K_{solv}$ in Table 1) were found as expected because the cations in the PBS solutions used for ITC can compete with the guest for the interaction with the host, thus destabilizing the complex \cite{25} whereas these salt effects are absent in the solvent (D$_2$O) used for the $^1$H-NMR experiments.

In agreement with ITC, CB[8] binds more strongly with the shorter peptide PheGly$_4$ (3.0 × 10$^{11}$ M$^{-1}$) than the longer PheGly$_6$ (7.1 × 10$^{10}$ M$^{-1}$, Table 1). The optimal fits gave $K_i/K_f$ = 0.5 and 1.2, for PheGly$_4$ and PheGly$_6$, respectively, indicative of non-cooperative or slightly positive cooperative binding.

To assess the sensitivity of the degree of cooperativity, the graphs in Figure 3c, f were obtained by optimizing $K_i$ (and the correlated $K_f$) at chosen values of the ratio $K_i/K_f$. The values of the least-squares error for each $K_i/K_f$ ratio are reported for each peptide (Figure 3c, f). A cut-off value of 20% from the minimum fit error was arbitrarily chosen to find the acceptable range of degree of cooperativity. The values of $K_i/K_f$ are in a range between 0.2 and 1 for the shorter peptide PheGly$_4$ and between 0.6 and 10 for the longer PheGly$_6$. Notably, the minima by $^1$H NMR are within the range of $K_i/K_f$ obtained by calorimetry, indicating a non-cooperative system. Taken togeth-

![Figure 3](image-url)
er, these results confirm a most probable scenario in which the ternary complexation between the peptides and CB[8] is non-cooperative.

It should be noted that these ternary CB[8]-peptide complexes cannot be compared directly to, for example, the cooperativity observed in hemoglobin, because in the former case, the first guest does not occupy one of two identical, well-spaced binding sites, but resides somewhere in the same cavity to which also the second one binds in the next step. As a result, the second guest experiences interactions with the first guest directly, as witnessed by the correlation between enthalpy and entropy.

In conclusion, combining the pieces of evidence from calorimetric and $^1$H-NMR titrations shown in this work, the most probable scenario to describe the homoternary complexation of phenylalanine-based peptides by CB[8] is a non-cooperative mode of interaction. This is independent of the tail length of the peptides studied in this work. Remarkably, whereas the second guest experiences a stronger interaction with the host after the first complexation step, there appears to be a counter-balancing entropic contribution that leads to an overall non-cooperative behavior in affinity. This contrasts the normal non-cooperative behavior of well-separated binding sites, in which case the binding enthalpies of all steps are equal, and entropy differences arise solely from differences in statistical pre-factors. The binding behavior of the homoternary peptide complexes resembles that observed for heteroternary complexes. The PheGly binding motif offers the synthetic flexibility and bioconvertability of peptides, and can have an active role in natural functional structures as well, such as in nuclear membrane pores. The insights in the complexation between peptides and CB[8] allow for a rational design of more complex self-assembled systems built on this powerful interaction motif.

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Conflict of interest

The authors declare no conflict of interest.

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