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A Novel Approach for the Evaluation of Positive Cooperative Guest Binding: Kinetic Consequences of Structural Tightening

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Abstract: Cooperativity is one of the most relevant features displayed by biomolecules. Thus, one of the challenges in the field of supramolecular chemistry is to understand the mechanisms underlying cooperative binding effects. Traditionally, cooperativity has been related to multivalent receptors, but Williams et al. have proposed a different interpretation based on the strengthening of noncovalent interactions within receptors upon binding. According to such an interpretation, positive cooperative binding operates through structural tightening. Hence, a quite counterintuitive kinetic behavior for positively cooperative bound complexes may be postulated: the more stable the complex, the slower it is formed. Such a hypothesis was tested in a synthetic system in which positive cooperative binding was previously confirmed by calorimetric experiments. Indeed, a linear correlation between the thermodynamics ($\Delta G°$) and the kinetics ($\Delta G^{\neq}$) of guest binding confirmed the expected behavior. These distinctive kinetics provide solid evidence of positive cooperative guest binding, which is particularly useful bearing in mind that kinetic experiments are frequently and accurately carried out in both synthetic and biological systems.

Keywords: cooperative effects · host–guest systems · kinetics · linear free energy relationships · noncovalent interactions

Introduction

Cooperative binding is vital in supramolecular chemistry and biology.[1] It forms the basis underlying essential phenomena such as the regulation of ligand affinity and the emergence of collective properties that are not present in the individual molecular components. Traditionally, cooperativity has been correlated to multivalency, whereby receptors display several binding sites, and binding on one of the available sites affects subsequent binding in another site. Indeed, the seminal papers of Monod, Wyman, and Changeux on positive cooperative binding,[2] and of Koshland et al. on negative cooperative binding,[3] consider receptors displaying multiple ligand binding sites. In this usage, cooperativity is positive when ligands that bind successively are bound with increasing affinities and negative when successively binding ligands are bound with decreasing affinities. However, Williams et al. have proposed a slightly different and more general interpretation of cooperative binding:[4] positive cooperative effects arise from the mutual reinforcement between two or more noncovalent interactions and, as a result, the global free energy is greater than that expected from the addition of the individual contributions. Conversely, it is considered that two or more noncovalent interactions cooperate negatively when they are mutually disfavored. According to such an interpretation, part of the driving force for guest binding comes from the strengthening of existing interactions within the host, which is correlated with a tightening within the receptor system.[4a,5] Indeed, because motion opposes bonding, the restriction of the internal motions upon guest binding should result in strengthening of the intrareceptor noncovalent interactions; such strengthening causes not only a more strongly bound complex but also induces the structural tightening of the receptor system. This structural tightening of noncovalent complexes as a function of thermodynamic stability is supported by considerable experimental evidence[6] and it appears to be of some generality.[4a,7]

The structural tightening upon binding mentioned above has a clear thermodynamic consequence: an imprecise compensation of enthalpy and entropy. Particularly, a more favorable enthalpy of association and a partial compensation for the less favorable entropy of association.[4,5a] Such an experimental outcome is measurable by isothermal titration calorimetry and it has been traditionally proposed to represent solid evidence of positive cooperative guest binding.[8]

Herein, we postulate that there must be another experimental outcome in any positive cooperative binding event

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that has been overlooked so far: the more stable the complex, the slower it should be formed. Such kinetic behavior is quite counterintuitive because traditionally the increasing exothermicity of a reaction is correlated with increasing rates, in what is long known as the Bell–Evans–Polanyi principle (BEP).[9] However, such a principle has constantly neglected a possible tightening concomitant to stabilization. Examining the potential energy profile in detail, the strength of a complex is related to the deepness of its potential, and the tightness of a complex with the constriction of such potential.[10] According to Marcus theory,[11] reactants and products nest in parabolic potential energy wells, and the crossing between them is an approximate representation of the transition state. Thus, in case there is no tightening, a more stable complex will display a deeper potential although an analogous steepness, and thus the activation energy of association will be lower as the complex becomes more stable (Figure 1a, $\Delta G^{\neq}_{a2} < \Delta G^{\neq}_{a1}$). This corresponds to the BEP principle. However, a positively cooperative strongly bound complex will display not only a deep but also a narrow potential energy (Figure 1b, complex 2), whereas a loosely bound complex will show a broad, shallow potential (Figure 1b, complex 1). Thus, providing the potential is increasingly steep as the complex is more stable, the crossing with the energy potential curve of the host will be higher in energy, which corresponds to a more energetic transition state both for the association and the dissociation reactions.[12] It can clearly be seen in Figure 1 that the steeper potential of complex 2 implies a crossing with the potential of the host at a higher point than complex 1, and this results in a higher activation energy of association ($\Delta G^{\neq}_{a2} > \Delta G^{\neq}_{a1}$).

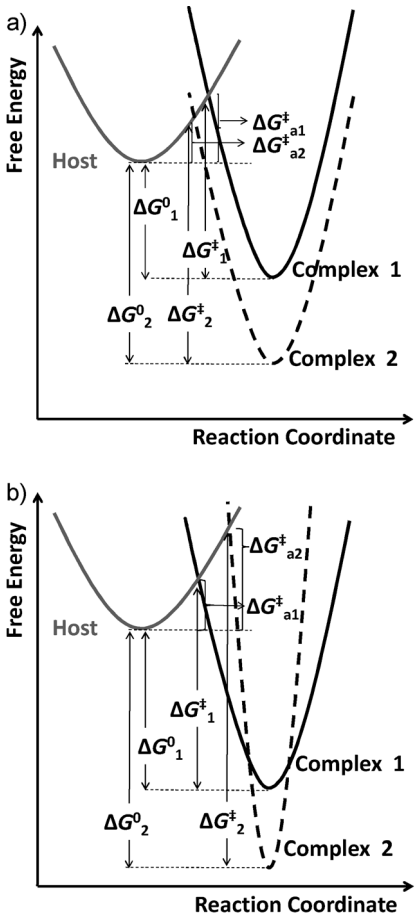

Figure 1. Potential energy diagrams. a) Traditional kinetic behavior based on the BEP principle: the more stable the complex, the faster it is formed. b) Kinetic behavior of positive cooperative binding: the more stable the complex, the slower it is formed. Indeed, the potential energy well of complex 2 is steeper than that of complex 1. Thus, it will cross with the potential well of the host at a higher point and, consequently, the activation energy of association will be higher, even when complex 2 is more stable.

Some reported data obtained from analysis of protein receptors provide a hint that such distinctive kinetic behavior may be correct, although no rationalization has been made so far. On the one hand, there are some examples of protein receptors that associate slower with guests that bind tighter;[13] on the other hand, it is known that some mutated proteins display higher affinity for the ligand than the wild type, but also display a slower rate of association.[14] It is worth mentioning that traptavidin,[15] a variant of streptavidin in which two residues are mutated, binds biotin with a higher association constant than streptavidin, although with a slower rate of association. This example is very significant because streptavidin is a paradigm of positive cooperative binding with concomitant tightening.[4b]

An evaluation of the suggested kinetic effect in complex biological systems might, however, be hard to accomplish because of nontrivial mechanisms of binding, aggregation effects, and several other experimental difficulties. Thus, herein we employ a synthetic host-guest system as a model to confirm the postulated kinetic behavior of a positive cooperative binding. By simply correlating the thermodynamics and the kinetics of guest binding through a linear free energy relationship (LFER), it is possible to verify the predicted structural tightening and thus evaluate the positive cooperative guest binding.

Results and Discussion

We decided to test positive cooperativity in receptor **1** (Figure 2),[16] due to its similarity to other systems for which positive cooperativity between intrareceptor interactions and guest binding has been recently confirmed by our group.[17]

As mentioned above, an excellent procedure to confirm positive cooperative guest binding is to perform isothermal titration calorimetry (ITC), because this technique directly yields the enthalpy and entropy of association.[8] Thus, any imprecise compensation of enthalpy and entropy is clearly obtained. The approach also clarifies whether cooperativity is enthalpic or entropic in origin. The ITC experiments were performed for several complexes of **1** with methyl esters of amino acid ammonium salts (Table 1). The data clearly confirm a positive cooperativity governed by the enthalpic

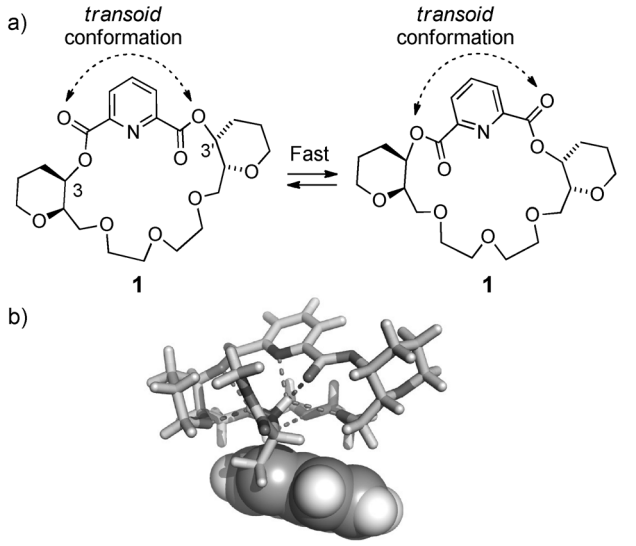

Figure 2. Receptor **1** and one of its complexes. a) Receptor **1**; involved in a fast equilibrium between two degenerate *transoid*-folded conformations. b) Complex of **1** with D-Trp-OMe$^+$; the folded receptor and the *transoid* conformation can be clearly seen.

Table 1. Thermodynamic parameters of the complexes of host **1** at 298 K (in kJ mol^{-1}) obtained by ITC in CH_3CN.

Complex[a]	$-\Delta G°$	$-\Delta H°$	$-T\Delta S°$
1·D-Trp-OMe$^+$	25.48 ± 0.11	32.01 ± 0.25	6.49 ± 0.36
1·D-Phe-OMe$^+$	25.00 ± 0.10	30.38 ± 0.16	5.35 ± 0.26
1·D-Leu-OMe$^+$	24.87 ± 0.25	30.41 ± 0.50	5.50 ± 0.75
1·D-Val-OMe$^+$	23.33 ± 0.09	26.75 ± 0.15	3.39 ± 0.24
1·D-2-Aba-OMe$^+$[b]	23.08 ± 0.11	27.30 ± 0.21	4.20 ± 0.32
1·D-Ala-OMe$^+$	23.06 ± 0.17	27.70 ± 0.39	4.62 ± 0.56
1·L-Leu-OMe$^+$	21.24 ± 0.62	24.18 ± 2.50	2.92 ± 3.12

[a] Picrate is the anion in all cases. [b] Ammonium salt of D-2-aminobutyric acid methyl ester.

factor, with a linear correlation between enthalpy and entropy of association (Figure 3a) and a slope larger than 1. In other words, for increasingly stable complexes, the enthalpic term is progressively more important than the entropic term (Figure 3b).[18] This reflects that the increase in free energy is composed of an entropic penalty and an overwhelming benefit in enthalpy.

Once positive cooperative guest binding was confirmed, the kinetic consequences of structural tightening were analyzed. As mentioned above, binding kinetics should reflect the restriction of internal motions expected for the structural tightening,[19] which additionally should be proportional to the binding strength. Therefore, to further confirm the positive cooperative guest binding, a sensible approach is to correlate the kinetics of the complexation with the thermodynamic stability. Any linear correlation between the standard free energy change ($\Delta G°$) and the free energy of activation ($\Delta G^{\neq}$) is called the linear free energy relationship (LFER) and this correlation has been quite useful to analyze the mechanism of reactions[20] and to evaluate electronic aspects of noncovalent interactions.[21] Herein, the observation of a

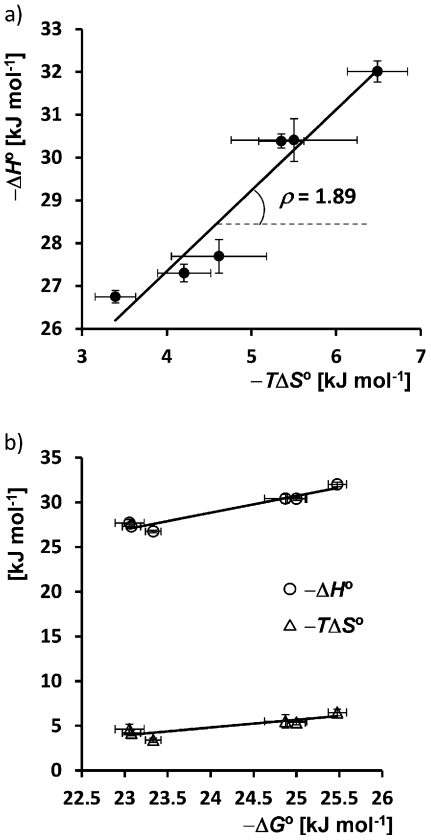

Figure 3. Relationship between the enthalpy and the entropy of association. a) Plot of $-\Delta H°$ vs. $-T\Delta S°$. b) Overlapped plot of $-\Delta H°$ and $-T\Delta S°$ vs. $-\Delta G°$ in kJ mol^{-1}.

LFER would shed some light on the synergy between guest binding and structural tightening in receptor **1**.

The activation energy of guest dissociation can be determined directly by NMR spectroscopic analysis. However, such direct measurements are not accurate in this case due to peak overlap (see Figure S18 in the Supporting Information), moreover, calculating all the rate constants at 298 K by this method is very complex. To reliably obtain the guest dissociation rate, we can take advantage of the fact that complexes of **1** switch between two equivalent and degenerate folded situations termed A and B (Figure 4a), as confirmed by 2D-EXSY spectroscopy (Figure 4b).[22] Such an interconversion process must imply guest dissociation and host unfolding to a postulated flat transition state, which can fold and bind to the guest again on the opposite face (Figure 4a).[23] Guest dissociation is clearly the rate determining step because the interconversion of free host is so fast that it is barely observable by ^{1}H NMR spectroscopic analysis (Figures 2a, 4a, and Figure S1 in the Supporting Information). Hence, the measured $\Delta G^{\neq}$ of the overall dissociation–flipping–reassociation process corresponds to the free energy of activation of guest dissociation.[24] Thus, interconversion equilibrium between the two degenerate folded complexes was evaluated through a total band-shape analysis of the proton in position 3 (H3/H3′) in CD_3CN (Fig-

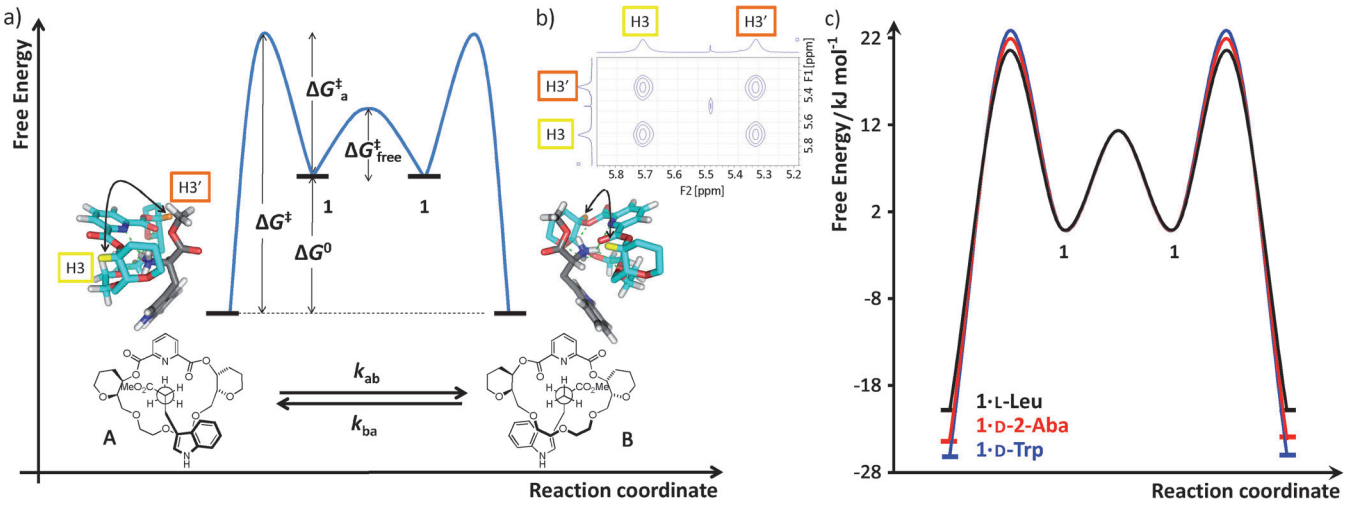

Figure 4. Reaction coordinate diagrams. a) Interconversion process between two equivalent and degenerate complexes A and B. The rate determining step is guest dissociation and thus the observed activation energy of interconversion ($\Delta G^{\ddagger}$) is the same as the activation energy of guest dissociation. b) Part of 2D-EXSY NMR spectroscopy proving the interconversion between A and B, in this case with D-Trp-OMe$^+$ as a guest (500 MHz, CD_3CN, 237.5 K, $m\tau = 50$ ms). c) It is clearly seen that for increasingly stable complexes, the activation energy of association is higher (only three complexes shown).

ure 4 a),[25] by using the WinDNMR-Pro software.[26] Finally, the corresponding $\Delta G^{\ddagger}$ was extracted from Eyring plots (Table 2), and confirmed by 2D-EXSY spectroscopy.

Table 2. Thermodynamic and kinetic parameters of the complexes of host **1** at 298 K (in kJ mol^{-1}) obtained by NMR in CD_3CN.

Complex[a]	$-\Delta G^{\circ}$[b]	$\Delta G^{\ddagger}$[c]	$\Delta H^{\ddagger}$	$T\Delta S^{\ddagger}$	$\Delta G^{\ddagger}_a$[d]
1·D-Trp-OMe$^+$	25.91 ± 0.10	48.18 ± 0.01	57.25 ± 0.36	9.07 ± 0.35	22.27 ± 0.11
1·D-Phe-OMe$^+$	25.69 ± 0.08	47.98 ± 0.15	51.72 ± 0.60	3.74 ± 0.75	22.29 ± 0.23
1·D-Leu-OMe$^+$	25.05 ± 0.16	47.19 ± 0.02	52.95 ± 0.06	5.76 ± 0.09	22.14 ± 0.18
1·D-Val-OMe$^+$	24.51 ± 0.08	46.65 ± 0.06	51.14 ± 0.05	4.49 ± 0.01	22.14 ± 0.14
1·D 2-Aba-OMe$^+$[c]	24.09 ± 0.07	45.55 ± 0.03	48.98 ± 0.67	3.43 ± 0.70	21.46 ± 0.10
1·D-Ala-OMe$^+$	23.07 ± 0.11	44.31 ± 0.13	48.44 ± 0.33	4.13 ± 0.44	21.24 ± 0.24
1·L-Leu-OMe$^+$	20.76 ± 0.06	40.96 ± 0.01	44.81 ± 0.02	3.85 ± 0.01	20.20 ± 0.07

[a] Picrate is the anion in all cases. [b] Calculated from the association constants obtained by ^{1}H NMR titration. [c] Calculated from $k_{A/B}$ obtained by NMR line-shape simulations. [d] Free energy of activation for the association process, $\Delta G^{\ddagger}_a = \Delta G^{\ddagger} - (-\Delta G^{\circ})$.

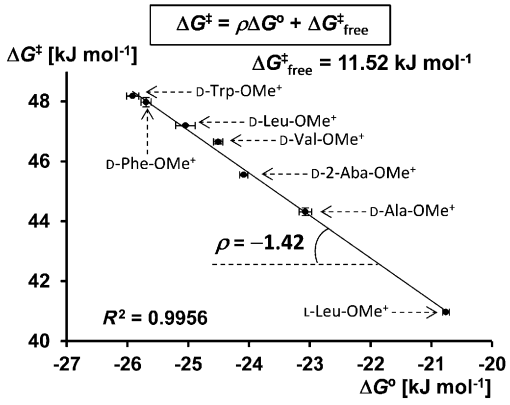

Figure 5. Plot of the activation energy ($\Delta G^{\ddagger}$) vs. the binding energy (ΔG°), showing a linear free energy relationship.

On the other hand, binding energies (ΔG°) of receptor **1** towards different guests were calculated from the association constants measured by NMR titration in CD_3CN at 298 K (Table 2). When the host-guest affinities (ΔG°) were plotted against the corresponding activation energies ($\Delta G^{\ddagger}$), a well-correlated linear relationship was observed (Figure 5), which means that there exists a LFER between the two sets of effects. In our case, the linear correlation was fitted to the following equation:

$$\Delta G^{\ddagger} = \rho \Delta G^{\circ} + \Delta G^{\ddagger}_{free}$$

The slope obtained was $\rho = -1.42$, which corresponds to the tendency of the free energy of activation of dissociation ($\Delta G^{\ddagger}$) to respond to the change in guest affinity of **1** (ΔG°). The intercept of the fitted line, which we denote as $\Delta G^{\ddagger}_{free}$, yields a value of $\Delta G^{\ddagger}_{free} = 11.52$ kJ mol^{-1}, and represents the activation barrier of interconversion of the complex for a guest having a binding energy of $\Delta G^{\circ} = 0$ kJ mol^{-1}. In other words, $\Delta G^{\ddagger}_{free}$ correlates to the activation barrier of the folding/unfolding equilibrium of free receptor **1** in CD_3CN.

Additionally, according to the microscopic reversibility principle, the free energy of activation for the association process ($\Delta G^{\ddagger}_a$) is linearly correlated with the free energy difference ΔG°, displaying also a negative slope ($\rho = -0.42$):

$$\Delta G^{\ddagger}_a = \Delta G^{\ddagger} - (-\Delta G^{\circ}) = -0.42\,\Delta G^{\circ} + \Delta G^{\ddagger}_{free}$$

Therefore, the association of host **1** with a guest for an increasingly stable complex is increasingly slow. In other words, the more exergonic the reaction, the slower it is (Figure 4 c).

A linear correlation between the activation free energy $\Delta G^{\neq}$ for a process with a free energy difference $\Delta G°$ for that process is known as the BEP principle.[9] As mentioned above, this principle rationalizes the increasing rates of many reactions with increasing exothermicities and, in this case, exactly the opposite is observed: the more stable the complex, the slower it is formed. This counterintuitive kinetic behavior is exactly what we postulated for positive cooperative binding. There are some reported exceptions to the BEP principle,[27] although, as far as we are aware, this is the first exception reported in a supramolecular reaction. Indeed, traditionally, the slope of the linear correlation is expected to change only from 0 to 1,[28] which is in sharp contrast to the slopes obtained herein ($\rho = -1.42$ and -0.42).

As mentioned in the introduction, this kinetic behavior can be rationalized on the basis of the steepness of the potential energy profile (Figure 1b).[29] Because the potential energy profile is mostly related to the vibration of bonds,[30] the steepness of such potential is correlated to the relative motion of the associating molecules. Thus, a steeper potential corresponds to a tighter association. A positive cooperative strongly bound complex will also be tight, and therefore the potential will be deep and narrow. As a rule, a deep, narrow potential will cross with the potential energy well of the host at a higher point than a broad, shallow potential. Therefore, the activation energy of guest association for an increasingly stable complex will be increasingly high. According to our experimental results, the more stable the binding, the higher the activation energy of association, and hence the tighter the final complex. As mentioned above, such tightening is a direct consequence of a positive cooperative binding. In addition, it is axiomatic that[31] guest binding is necessarily improved in any receptor that becomes tighter upon guest binding. Thus, this distinctive kinetic behavior can serve as solid evidence of positive cooperative guest binding.

Conclusion

Although positive cooperative guest binding is a thermodynamic concept and it directly affects to thermodynamic parameters, kinetics can help to evaluate its existence by confirming the concomitant structural tightening. Indeed, a restriction of the internal motion is an essential consequence of any positive cooperative guest binding governed by the enthalpic factor, and a steeper potential energy well is expected for improved binding. As a consequence, unexpectedly high activation barriers must be found. We postulate that this distinctive kinetic behavior is characteristic of any positive cooperative binding, and thus it may be used as solid supportive evidence for any putative cooperative binding. This approach seems particularly useful considering that kinetic measurements are commonly carried out, both in simple and complex systems, by several different methods with a notable accuracy. Thus, such a simple procedure to evaluate cooperativity based on kinetic experiments can be used as an efficient tool in many other artificial and natural cooperative binding systems.

Experimental Section

Materials and general methods: See the Supporting Information for full experimental details on the measurement methods. See our previous publication[16] for the synthesis and characterization of receptor **1** and complexes used in this study.

Association constants: Association constants were determined by using standard ^{1}H NMR titration protocols. The host solution **1** was prepared at a concentration between $6–12\times10^{-4}$ M in CD_3CN. The amino acid methyl ester salt (guest) ($1–4\times10^{-2}$ M) solution was prepared by dissolving the guest in a sample of the host stock solution, so that there was no dilution of the host during the titration. On addition of aliquots (5, 10, 15, 20, 25, 30, 40, 50, … μL) of the guest solution, the NMR tube was thoroughly shaken to mix the two solutions, and a ^{1}H NMR spectrum was recorded (Figure S2 in the Supporting Information). The temperature of the NMR probe was maintained at 298 K. The observed changes in chemical shift were analyzed, and the association constants were obtained by a nonlinear least-squares fitting method using the program provided by Dr. J. M. Sanderson.[32]

^{1}H NMR line-shape simulation: The rate constants $k_{A/B}$ for the A/B interconversion of the complexes of **1** were obtained by simulating the lineshapes of H3 and H3′ signals at various temperatures (WINDNMRPro, version 7.1.14)[26] in the presence of guest molecules in excess (30 molar equivalents) in CD_3CN to transform the process into a pseudo first order system (Figure S4 in the Supporting Information). The excess of the guests (ammonium salts) decreases the melting point of the CD_3CN (m.p. 227 K) by several degrees, which enables NMR experiments to be conducted below the standard melting point of CD_3CN.

It is worth mentioning that according to the mechanism of interconversion proposed for the system, only half of the processes actually lead to an exchange of spins. Indeed, during the interconversion process, the host molecules unfold and one half of them will refold again to the same face whereas the other half will fold to the other face. Thus, only half of the processes in each direction will imply an exchange of spins and, therefore, a statistical correction is usually required for cases such as this:[33]

$$k_{A/B} = 2 \cdot k_{ab}$$

in which k_{ab} is the rate constant measured by exchange of spins by NMR spectroscopic analysis for the forward process.

However, WinDNMR-pro yields a rate constant that is the sum of the forward and reverse processes ($k_{ab}+k_{ba}$) and therefore, in a degenerate process such as this, such a value can be taken as the real rate constant $k_{A/B}$. Indeed, it is known that at equilibrium:

$$X_A \cdot k_{ab} = X_B \cdot k_{ba}$$

in which X_A and X_B are the mole fractions of complex A and B respectively, and k_{ab} and k_{ba} are the rate constants for the forward and reverse processes respectively, measured by exchange of spins by NMR spectroscopic analysis. For a degenerate process the equilibrium constant of the process is $K_{eq}=1$ and therefore:

$$X_A = X_B = 0.5$$

$$k_{ab} = k_{ba}$$

Hence it follows that:

$$k_{A/B} = 2 \cdot k_{ab} = (k_{ab}+k_{ba})$$

Finally, the free energy of activation $\Delta G^{\neq}$ at 298 K was calculated from

the Eyring plot for each system (298 K, Figure S5–S11 in the Supporting Information). These values are the average of at least two independent measurements.

^{1}H NMR EXSY spectroscopic measurements: A solution of receptor **1** and an excess of guest (30.0 molar equivalents) in CD_3CN were placed in an NMR tube, which was cooled to the temperature indicated in Figure S12–S17 in the Supporting Information and allowed to equilibrate for 20 min. A series of NOESY experiments were then run with a relaxation delay of 6 s and mixing times ($m\tau$) of 50 ms and 100 ms, for which the cross-peaks were clearly resolved. Each of the 128 F1 increments was the accumulation of 8 scans. The peak amplitudes were determined using Topspin 2.1 software from Bruker, after phase and baseline corrections in both dimensions. Thus, the exchange rate is calculated from a single 2D EXSY NMR spectrum by the cross peak to diagonal peak intensity ratio [Eq. (1)]. The rates must be observable on the NMR timescale, in other words, the rates cannot be so slow that the relaxation process occurs during the mixing time, thus erasing memory of the exchange process, or so fast that the exchanging resonance cannot be resolved. For this reason, 2D EXSY is most accurate when the exchange rate is between $k \approx 10^2$–10^{-2} s^{-1}.

To calculate the rate constant, the following equation was applied:[34]

$$k_{A/B} = 2 \cdot k_{ab} = (k_{ab}+k_{ba}) = (1/m\tau)\ \ln[(r+1)/(r-1)] \quad (1)$$

in which

$$r = \{4X_AX_B(I_{AA}+I_{BB})/(I_{AB}+I_{BA})\}-(X_A-X_B)^2$$

if A and B are degenerate, then $X_A = X_B = 0.5$ and:

$$r = \{(I_{AA}+I_{BB})/(I_{AB}+I_{BA})\}$$

in which $k_{A/B}$ is the real rate constant for the interconversion process; k_{ab} and k_{ba} are the forward and reverse rate constant measured by exchange of spins by NMR; $m\tau$ is the mixing time, which is related to the relaxation times (T_1s) of the exchanging hydrogen atoms; X_A and X_B are the mole fraction of complex A and B respectively; and I_{AA}, I_{BB} and I_{AB}, I_{BA} are the raw volume intensities of diagonal and cross peaks observed in the 2D-EXSY experiments, respectively.

It is worth mentioning that this equation yields the sum of the forward and reverse rate constants measured by exchange of spins ($k_{ab}+k_{ba}$), as calculated in the WinDNMR-pro software (see previous section). As mentioned above, the mechanism of this interconversion implies that only one half of the unfolded molecules of the receptor will fold to a different face whereas the other half will refold again to the same face. Thus, only half of the processes in each direction will imply an exchange of spins and therefore a statistical correction is usually required for cases like this one ($k_{A/B} = 2 \cdot k_{ab}$). However, as we have just said, Equation (1) yields the sum of the forward and reverse rate constants measured by exchange of spins ($k_{ab}+k_{ba}$), and it can be proved that, in a degenerate system, this sum is equal to the real rate constant $k_{A/B} = (k_{ab}+k_{ba})$ (see previous section for a detailed explanation).

Finally, the free energy of activation can be calculated from the exchange rate by using the Eyring equation.

Isothermal titration calorimetry (ITC): ITC titrations were performed with an isothermal titration microcalorimeter Microcal VP-ITC. All experiments were conducted at 298 K. A solution of receptor **1** (1–2 mM) in acetonitrile was loaded into the titration cell. The syringe was loaded with 250 μL of guest (28 mM) also in acetonitrile. The syringe was positioned in the calorimeter and the following parameters were set: injection size, 5 μL; injection duration, 10 s; temperature, 25 °C; injection interval, 85 s; cell feedback, 15 μcal; and the stirring speed was 300 rpm; 40–50 injections were recorded. In each case, heats of dilution determined in the absence of receptors were subtracted from the titration data prior to curve fitting. Additionally, an initial 5 μL injection was discarded from each dataset to remove the effect of titrant diffusion across the syringe tip during the equilibration process. Titrations were repeated until they were reproducible. Curve fitting was undertaken in Origin v. 7.0 by using the standard noninteracting one-site model supplied by MicroCal (Figure S19 in the Supporting Information).

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Cooperative Effects

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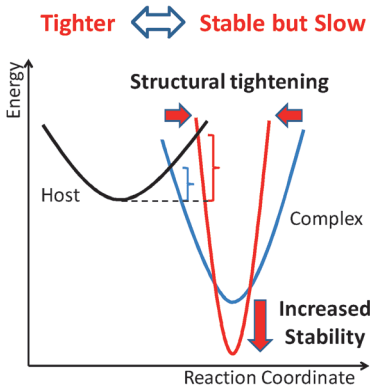
A Novel Approach for the Evaluation of Positive Cooperative Guest Binding: Kinetic Consequences of Structural Tightening



Tight and slow: A linear correlation between the thermodynamics ($\Delta G°$) and the kinetics ($\Delta G^{\ddagger}$) of guest binding in a synthetic receptor has been found. Such correlation reveals a quite counterintuitive behavior: the more stable the complex, the slower it is formed. This peculiar kinetics can be rationalized by an increasing structural tightening as the binding is improved, which is solid evidence of positive cooperative guest binding (see figure).