Case study

Solving complex interactions with AFFINImeter:

Competing ligands binding to a multiple site receptor

AFFINIMETER

Solving complex interactions with AFFINInimeter: Competing ligands binding to a multiple site receptor

Eva Muñoz and Juan Sabin, Software for Science Developments

Isothermal Titration Calorimetry (ITC) is a versatile technique with the potential of deconvoluting the various binding events that may coexist in complex interactions. In this sense, a major drawback has been the lack of mathematical models and computational tools to properly analyze such experiments. AFFINImeter counts with an advanced functionality, the "Model Builder" with which researchers can easily design their own binding models through the combination of distinct (coupled) binding equilibria, to obtain thermodynamic and structural information from complex ITC experiments.

Introduction

The molecular recognition of biomolecules is central for most physiological and pathological phenomena. Nowadays the characterization of such processes represents a research area of extremely high interest since it is directly related with the development of new therapeutic and diagnostic methods. The difficulty of studying biomolecular recognition processes frequently arises from the inherent complex structure of biomacromolecules such as proteins, nucleic acids or polysaccharides that often exhibit multiple – equivalent or non-equivalent; independent, dependent or even cooperative – binding sites for a given ligand. Moreover, in a biological environment the existence of two or more ligands competing to bind a receptor (biomacromolecule) is not rare.

The appropriate physicochemical analysis of such complex interactions requires the use of experimental techniques able to deconvolute the overall individual binding events. In this sense, Isothermal Titration Calorimetry (ITC) represents an attractive alternative; in an ITC standard experiment involving the formation of a 1:1 complex the heat per mole of titrant generated or absorbed upon interaction is plotted against the titrant/titrate molar ratio to yield a sigmoidal binding isotherm. The presence of various binding equilibria characterized by different association constant (K_A) and/or binding enthalpy (ΔH) values is typically reflected in a deviation of the traditional sigmoidal shape of the corresponding isotherm (leading to steeped sigmoidal curves, significant curvature changes or to a shift in the inflection point). These non-standard isotherms are rich in thermodynamic information of the different coexisting interactions, which can be extracted through fitting to the right binding model. In this sense, a major drawback has been the lack of mathematical models and computational tools able to describe and properly analyze such experiments. [1]

AFFINImeter counts with a friendly and powerful functionality, the "Model Builder", with which researchers can easily design their own binding models through the combination of distinct (coupled) reactions. This tool permits the generation of suitable models to describe complex processes of molecular recognition, including high order competitive coupled reactions.

Competitive binding of two ligands to a receptor with two sites

As a demonstration of the potential of AFFINImeter and its "Model Builder", the analysis of an ITC experiment involving a receptor "M" (biomacromolecule) with two different binding sites (s1 and s2) that may accommodate two competing ligands ("A" and "B") is presented here. It will be assumed that "A" binds both sites albeit with different efficacy (different K_A and ΔH) and in a sequential manner– following the reaction scheme illustrated in Fig. 1 – that is, binding of a first "A" molecule with M(s1) is a requisite for binding of the second "A" with M(s2). This particular dependency between equilibria can be found in multivalent chemical[2] and biological[3] interactions. Conversely, "B" may bind just one of the receptor sites (s1 or s2).

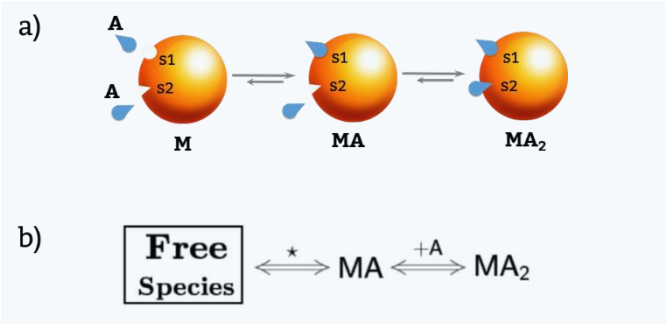

Fig 1. a) Graphical representation of a receptor "M" binding to a ligand "A" through two different sites "s1" and "s2" in a sequential manner; b) Sequential model generated with AFFINImeter that describes these binding events.

According to this scenario a series of three ITC isotherms (I-III) were generated with the AFFINImeter simulation tool. These isotherms correspond to the following experiments: the titration "A" in the syringe into "M" in the calorimetric cell (Fig. 2a); the titration of "B" in the syringe into "M" in the cell (Fig 2b); the titration of a "A" in the syringe into a solution of "M" + "B" in the cell (Fig 2c). The thermodynamic parameters and experimental conditions used to simulate the curves are summarized at the end of this document.

From a visual inspection of isotherm I (Fig. 2a) it is easily deduced that there is more than one equilibrium participating in the interaction between "A" and "M". Since it is known that "M" has two potential binding sites, this isotherm was fitted to the sequential binding model described in Fig. 1b.

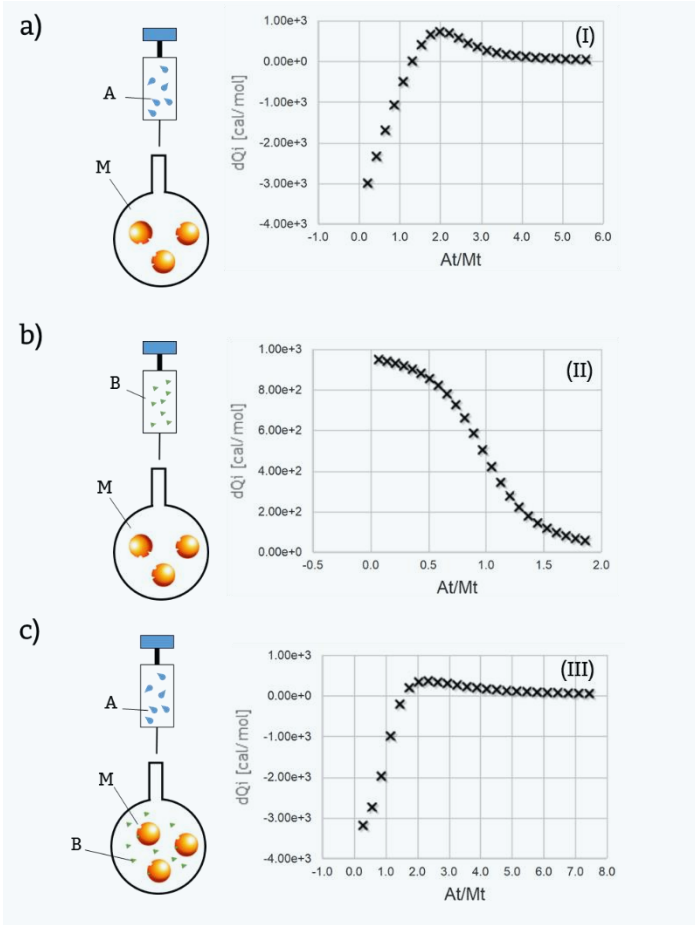

Fig 2. Simulated ITC isotherms of a) titration of "A" into "M"; b) titration of "B" into M"; and c) titration of "A" into "M" + "B" (the concentrations employed are summarized at the end of this document).

Isotherm II (Fig. 2b) follows a sigmoidal shape and it was fitted to a 1:1 binding model. Here, a scaling parameter[4] for the concentration of M "r_M" was also allowed to be fitted. The value obtained for r_M was exactly 1, as expected for a 1:1 complex between "B" and "M". However, it is unclear whether "B" binds to s1 or to s2. A common strategy to get this information is to monitor the interaction of "B" with mutants of

"M" that selectively lack one of the binding sites.[1] However, this alternative is frequently costly and time consuming as requires the development and purification of the appropriate mutants. Instead, we designed a competitive ITC experiment consisting of a titration of "M" with "A" in the presence of "B" (see Fig. 2c). The "Model Builder" functionality of AFFINImeter was used to draw two different reaction schemes according to the potential possibilities of binding (Fig. 3).

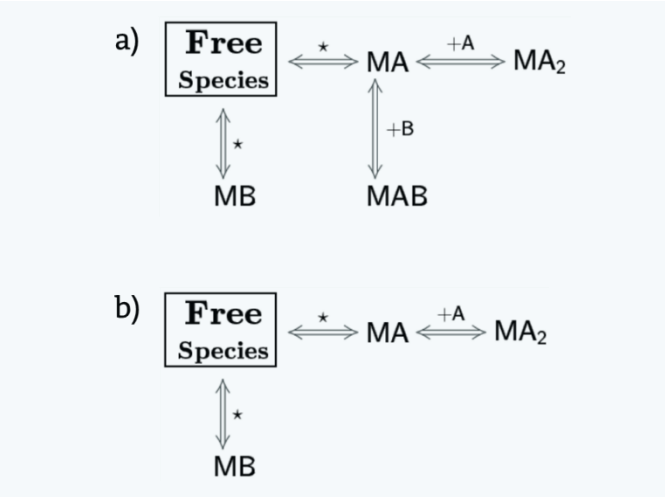

Fig 3. Sequential binding models created using the AFFINImeter "Model Builder" in order to describe the competitive experiment of Fig. 2c where a) "B" binds to s_2 of "M"; b) "B" binds to s_1 of "M".

The first model (Fig 3a) describes the competition between "A" and "B" considering that "B" binds to the second site of "M" (s_2) while the second model (Fig 3b) considers that "B" binds to the first site (s_1). Here, the main difference resides in that, in the first case "A" and "B" compete with each other to give "MA_2" or "MAB". However, in the second case both ligands compete to form "MA" or "MB". Isotherms I, II and III were globally analyzed with AFFINImeter, using the models described in Fig 3 to fit curve III, and sequential binding and 1:1 binding model to fit curves I and II, respectively. Here, K_A and ΔH of the formation of "MA"and "MA_2" were set as common parameters among curves I and III. Similarly, K_A and ΔH of the formation of "MB" were common parameters in curves II and III. The goodness of the fit corresponding to each model can be easily deduced from a visual inspection of graphs in Fig. 4. It is directly concluded that model a) is better than model b) to simultaneously describe the three isotherms. Hence, "B" binds to the second site (s_2) of the receptor. **This example shows that, by designing the right ITC experiments it is possible to get structural information of what binding site of "M" is recognized by "B". It requires essaying different binding models as well as high quality measurements.** The thermodynamic information obtained from both global fittings is shown in Table I. The K_A and ΔH values corresponding to the formation of "MA" and "MB" obtained through fitting to model a) coincide well with those obtained from the individual fitting of curves I and II to standard models (sequential 1:1 + 1:2, and 1:1 respectively). On the contrary, K_A and ΔH from fitting to model b) were far from the thermodynamic data obtained from the individual fitting (Table I).

Table I. Thermodynamic data of M binding to A and B

Curves/binding model		Species	K_A (10^7 M^{-1})	ΔH (kcal/mol)
I	Sequential binding model	MA	2.01	–3.50
		MA_2	0.50	3.00
II	1:1 model	MB	0.02	1.00
I, II, III	Global fitting using model "a"	MA	2.14	–3.45
		MA_2	0.50	2.93
		MB	0.02	1.00
		MAB	0.02	1.00
I, II, III	Global fitting using model "b"	MA	8.00	–2.72
		MA_2	0.19	2.14
		MB	0.02	1.00

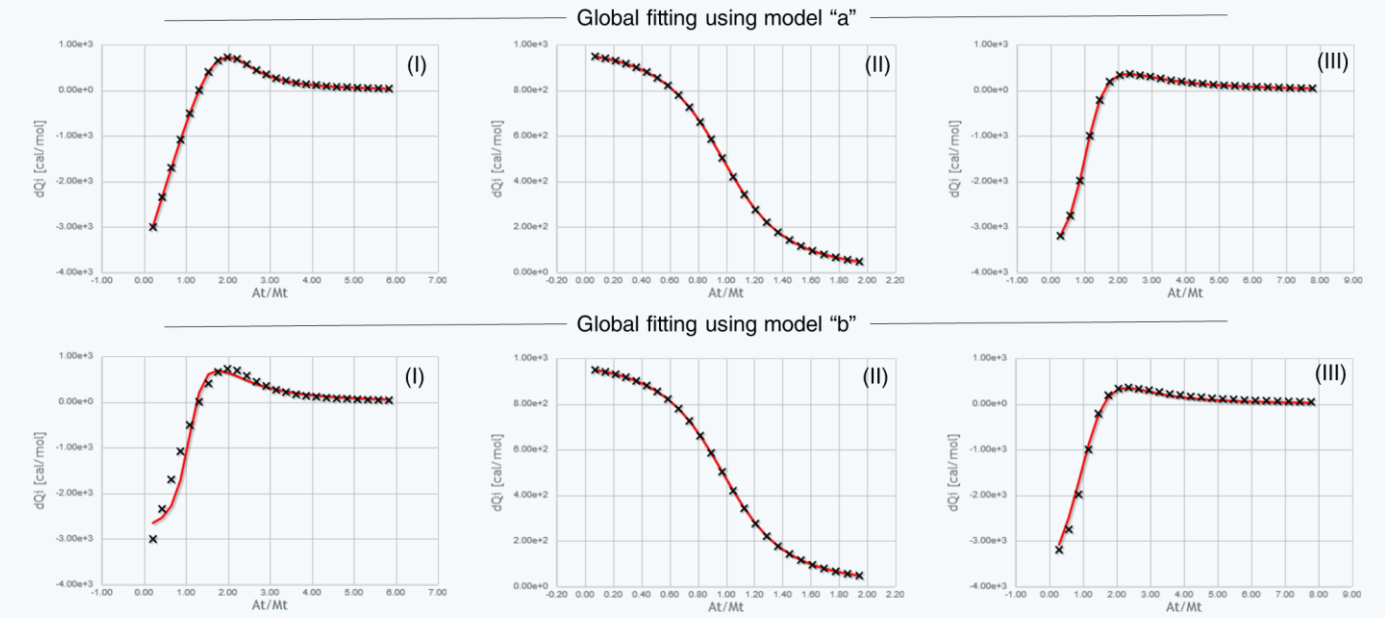

Fig 4. Global fitting of curves I-III obtained using AFFINImeter applying the models described in Fig 3 to fit curve III. The "link" function available in the "Fit Settings" form was used to share fitting parameters between curves: (K_A, ΔH) corresponding to the formation of "MA" and those for "MA_2" of curves I and III; the same treatment was applied to (K_A, ΔH) for "MB" of curves II and III.

Notably, the global fitting to model a) yielded further relevant information: K_A and ΔH of the "MAB" complex are equivalent to those of the "MB" complex and this demonstrates that the interaction between "M(s2)" and "B" is independent of the interaction between "M(s1)" and "A". Additionally, **AFFINImeter provides a diagram with the distribution of the species present in the sample cell throughout the titration** (Fig. 5). This diagram eases the understanding of this complex binding phenomena.

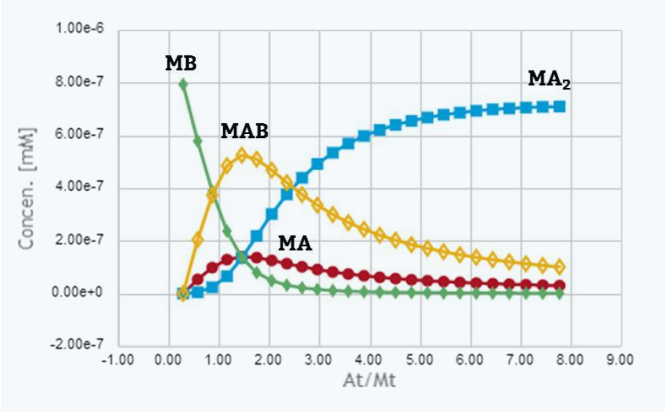

Fig 5. Distribution of species formed throughout the competitive binding experiment described in Fig. 2c. Results obtained from the fitting to the model represented in Fig. 3a.

Conclusions

This case study exemplifies the potential of AFFINImeter that, by using the "Model Builder" tool together with the global fitting functionality, makes possible the resolution of complex binding events only via ITC experiments. In this particular case we dealt with the competitive binding of two ligands to a two site receptor. The global analysis of three isotherms to different binding models has permitted the acquisition of accurate thermodynamic data of all binding equilibria, structural information of the complexes formed and quantitative information of the species distribution as a function of the nominal concentration of titrant and titrate in the sample cell.

Thermodynamic and experimental parameters used to simulate the isotherms I-III (Fig 2)

THERMODYNAMIC PARAMETERS

M + A ↔ MA:

$K_A = 2 \cdot 10^7\ M^{-1}$; $\Delta H = -3.5$ kcal/mol

MA + A ↔ MA_2:

$K_A = 5 \cdot 10^6\ M^{-1}$; $\Delta H = 3.0$ kcal/mol

M + B ↔ MB:

$K_A = 2 \cdot 10^5\ M^{-1}$; $\Delta H = 1.0$ kcal/mol

EXPERIMENTAL CONDITIONS

Instrument: ITC calorimeter microcal VP; cell volume = 1.4 mL.

Injection volume (all isotherms) = 10 μL

The initial concentration in the cell of M ($[M]_0$) or B ($[B]_0$) and the concentration in the syringe of A ([A]) or B ([B]) for the different isotherms are:

I $[M]_0 = 1$ μM; [A] = 30 μM;

II $[M]_0 = 100$ μM; [B] = 1 mM;

III $[M]_0 = 1$ μM; [A] = 40 μM; $[B]_0 = 20$ μM

References

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[2] P. Brocos, X. Banquy, N. Díaz-Vergara, S. Pérez-Casas, Á. Piñeiro, M. Costas, A Critical Approach to the Thermodynamic Characterization of Inclusion Complexes: Multiple-Temperature Isothermal Titration Calorimetric Studies of Native Cyclodextrins with Sodium Dodecyl Sulfate. J. Phys. Chem. B, **2011**, 115 (49), pp 14381–14396.

[3] N. Popovych, S. Sun, R.H. Ebright, C. G. Kalodimos, Dynamically driven protein allostery. Nature Structural & Molecular Biology. **2006**, 13, pp 831 – 838.

[4]The parameter "r_M" of AFFINImeter scales the concentration of "M" in the calorimetric cell and it should be fitted when [M] is imprecise. r_M can also be employed to support that the proposed model is correct, as in the present document.

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www.affinimeter.com

S4SD - AFFINImeter,
Edificio Emprendia, Campus Vida,
15782 Santiago de Compostela
Spain

info@affinimeter.com