

Case study

Global Analysis in ITC Displacement Titrations with AFFINImeter



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Eva Muñoz and Juan Sabin, Software for Science Developments

Isothermal titration calorimetry (ITC) displacement titrations offer an attractive alternative to standard ITC assays when working with ultra high- or ultra low- affinity interacting systems. The method requires the fitting of at least two isotherms that share various adjustable parameters. AFFINImeter counts with advanced tools, like the global fitting of multiple dataseries, which can facilitate the analysis and expand the range of applications of ITC experiments like the displacement titration.

Introduction

ITC is one of the most commonly used approaches to obtain affinity and thermodynamic data of molecular interactions and has become a routine method in the pharmaceutical industry.¹ It is applicable to a wide variety of interacting systems, as long as a detectable heat change is produced during complexation, covering an important range of binding affinities. Nevertheless, standard ITC experiments present some limitations when the interaction is of very low or very high affinity (in the millimolar or nanomolar range, respectively). High affinity interactions are characterized by square-shaped ITC isotherms whose fitting yield accurate values of the binding enthalpy (ΔH) but only estimates of the association constant (K_A). Attempts to recover a sigmoidal shape requires the use of very low concentrations of the interactants that, in most cases, is not feasible in the practice (the minimum concentration that will typically cause a confidently measurable heat change for a 1:1 interaction is about 10 μM). On the opposite, low affinity interactions need to be evaluated at high concentrations and this requirement is often a serious limiting step

due to various potential reasons: the limited solubility and/or availability of the sample molecule, or the existence of aggregation processes at the required concentration. In both high- and low-affinity systems these experimental drawbacks can be circumvented with the ITC displacement titration.^{2,3} Here, the receptor is titrated with a high affinity ligand, but in the presence of a second, weaker interactant in the sample cell that competes for binding to the receptor. With this experimental set up the apparent affinity of the strong ligand is “artificially” lowered, obtaining a sigmoidal isotherm that yields more precise binding data. This protocol is taking special relevance in affinity based hit discovery approaches within the drug discovery process, where many candidates to bind the same receptor are available.^{3,4} When the goal is to obtain the thermodynamic parameters of an ultra high- affinity system, a titration with the weaker binder is performed first, to obtain the corresponding affinity constant and enthalpy ($K_{A\text{-weak}}$ and H_{weak}). These values are required for the analysis of the displacement experiment, where a competitive binding model is used to estimate the thermodynamic parameters of the tight binding ($K_{A\text{-tight}}$ and ΔH_{tight}). Analogously,

when the goal is to obtain information of an ultra low- affinity system a direct ITC titration of the receptor alone with a ligand of higher affinity is performed. The resulting $K_{A\text{-tight}}$ and $\Delta H_{\text{-tight}}$ are then incorporated into the analysis of the isotherm from the displacement assay.

AFFINImeter can be used as an efficient tool in the analysis of ITC displacement assays as it offers the possibility of simultaneous, global fitting of various isotherms to different binding models. Moreover, it counts with a “link” function to correlate parameters between isotherms.

Thermodynamic characterization of the interaction between HIV-protease and indinavir



As an illustration of the use of AFFINImeter in displacement assays we have reproduced the protocol described by A. Velazquez-Campoy and E. Freire to determine the thermodynamics of HIV-protease (receptor) with indinavir, a high affinity binder ($K_A > 10^9 \text{ M}^{-1}$). Acetyl-pepstatin, a generic protease inhibitor is used as the weak ligand.²

Three binding isotherms were generated using the package –AFFINImeter simulation– representing: (I) HIV-protease (in the calorimetric cell) binding to indinavir (in the syringe); (II) HIV-protease (in the calorimetric cell) binding to acetyl-pepstatin (in the syringe); (III) HIV-protease + acetyl-pepstatin (in the calorimetric cell) binding to indinavir (in the syringe). The simulations, shown in Fig. 1, were made using the thermodynamic parameters and experimental conditions described in the original protocol and are summarized at the end of this report.

The interaction of HIV-protease with indinavir yields a squared-shape isotherm whose fitting provides only an estimate of the association constant (calculated $K_{A\text{-tight}}$ was $3.69 \cdot 10^9 \text{ M}^{-1}$, in contrast to the theoretical value of $2.10 \cdot 10^9 \text{ M}^{-1}$ used to simulate the curve). However, the interaction of HIV-protease with acetyl-pepstatin is described by a well-defined sigmoidal isotherm (Fig. 1 a and b)

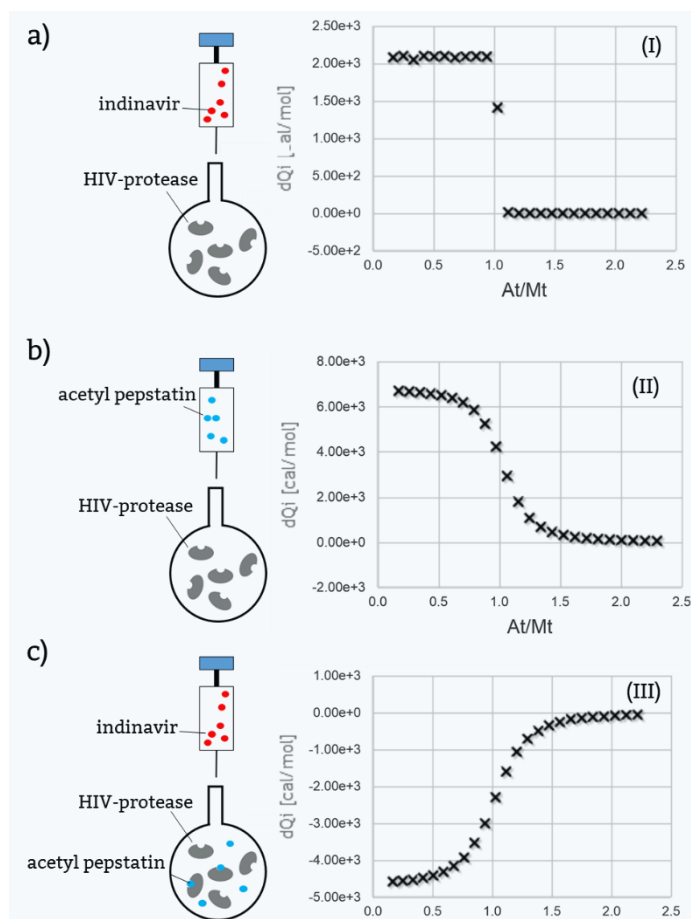


Fig 1. Graphical representation of a standard ITC Displacement Assay. (I), (II) and (III) are simulated curves of (a) the high affinity interaction, (b) low affinity interaction and (c) displacement experiment, respectively.

The standard displacement assay consists on a first fitting of isotherm II (acetyl-pepstatin vs HIV-protease) to a 1:1 binding model to obtain $K_{A\text{-weak}}$ and $\Delta H_{\text{-weak}}$ followed by fitting of isotherm III (indinavir vs HIV-protease + acetyl-pepstatin) to a competitive binding model in which the calculated $K_{A\text{-weak}}$ and $\Delta H_{\text{-weak}}$ are introduced into the equations of the competitive model as constant parameters. The same analysis can be executed in one single step with AFFINImeter and its advanced tool of global analysis. Moreover, it allows simultaneous fitting of more than just two curves making possible the global fitting of curves I, II and III. Accordingly, we performed the simultaneous analysis of I – III that were fitted to a 1:1 binding model (I and II) and to a competitive binding model (III). The function “link parameters” was used to match thermodynamic parameters among curves: $K_{A\text{-tight}}/\Delta H_{\text{-tight}}$ were common values on I and III

during the fitting process while II and III shared $K_{A\text{-weak}}/\Delta H_{\text{-weak}}$. Using this procedure the expected binding constants and enthalpies were obtained, simultaneously, for both tight and weak interactions. Curve I contains accurate information of $\Delta H_{\text{-tight}}$ and its incorporation into the global analysis increases the robustness of the displacement assay.

An additional interesting advantage of using AFFINImeter in displacement assays is in the marked flexibility of the software to describe and fit original experimental designs. For instance, the traditional displacement experiment where the competitor is mixed with the receptor in the calorimetric cell (see Fig. 1 c) can be replaced by a competitive assay where both ligands are mixed in the syringe. To illustrate this functionality an additional isotherm was simulated, to represent a titration of HIV-protease with indinavir + acetyl-pepstatin, both in the syringe (Fig. 2).

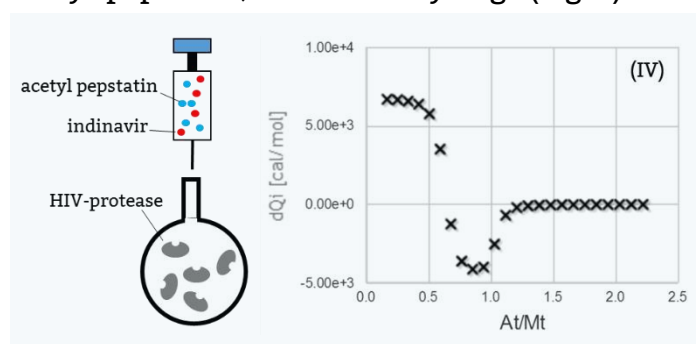


Fig 2. Graphical representation of an ITC Displacement experiment where both ligands are mixed in the syringe. (IV) is the corresponding simulated isotherm.

Finally, the full potential of AFFINImeter for global analysis of multiple curves was exploited for the simultaneous fitting of isotherms I, II, III and IV as follows: I and II were fitted to a 1:1 model; III and IV were fitted to a competitive model; $K_{A\text{-tight}}/\Delta H_{\text{-tight}}$ were common values in curves I, III and IV during the fitting process; similarly, $K_{A\text{-weak}}/\Delta H_{\text{-weak}}$ were common in II, III and IV (Fig. 3 and Table I).

Table I. Thermodynamic data of HIV-protease binding to indinavir and acetyl-pepstatin

	Indinavir – HIV-protease		acetyl-pepstatin – HIV-protease	
	$K_{A\text{-tight}} (10^9 M^{-1})$	$\Delta H_{\text{-tight}} (Kcal/mol)$	$K_{A\text{-weak}} (10^6 M^{-1})$	$\Delta H_{\text{-weak}} (Kcal/mol)$
Global Fitting	2.07	2.09	4.11	6.80
Theoretical value	2.10	2.10	4.10	6.80

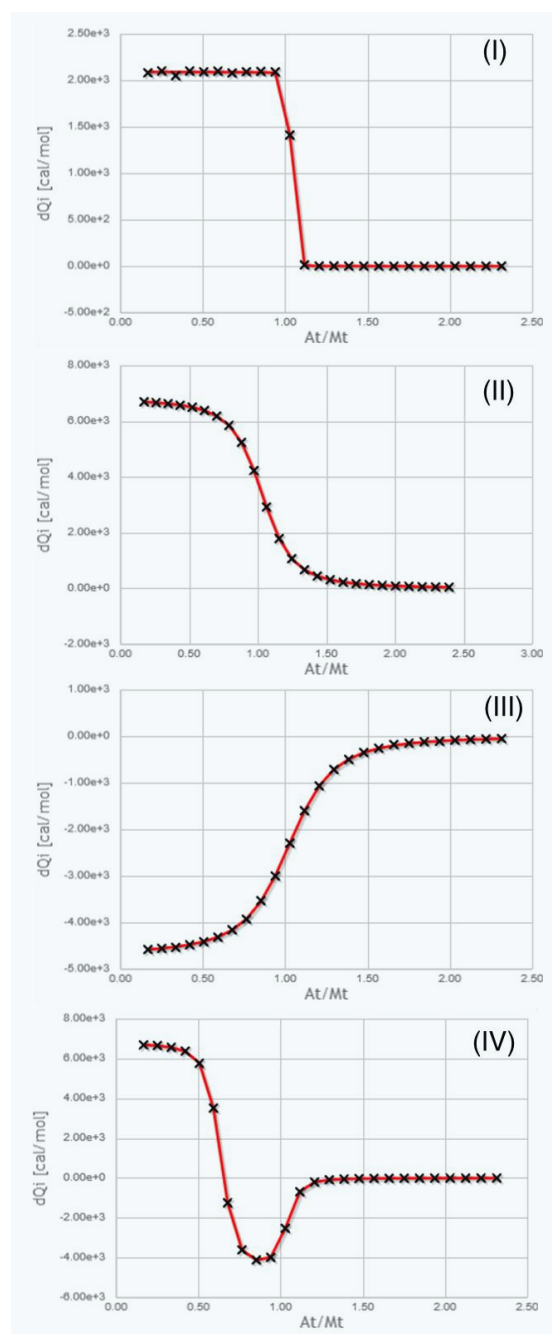


Fig 3. Global fitting of curves I-IV with AFFINImeter

Conclusions

This case study exemplifies the potential advantages of using AFFINImeter in ITC displacement assays. The software offers unique advanced tools, like the global fitting of multiple data series sharing various thermodynamic parameters and the analysis of isotherms registered under unusual experimental design. Altogether, these tools enhance the robustness of the method and makes it more versatile, facilitating the acquisition of reliable thermodynamic data from ultra-high of ultra-low affinity systems. Thus, it opens a door for new applications of the displacement assay.

Thermodynamic parameters and experimental conditions used to simulate isotherms I-IV

Thermodynamic parameters:

HIV-protease – indinavir:

$K_A = 2.1 \cdot 10^9 \text{ M}^{-1}$; $\Delta H = 2.1 \text{ Kcal/mol}$

HIV-protease – acetyl-pepstatin:

$K_A = 4.1 \cdot 10^6 \text{ M}^{-1}$; $\Delta H = 6.8 \text{ Kcal/mol}$.

Experimental conditions:

ITC calorimeter microcal VP, cell volume

1.4 mL

T=298 K

Injection volume = 10 μL .

Isotherm (I) $C_{\text{HIV-protease}} = 0.02 \text{ mM}$, $C_{\text{indinavir}} =$

0.29 mM; Isotherm (II) $C_{\text{HIV-protease}} = 0.02 \text{ mM}$,

$C_{\text{acetyl-pepstatin}} = 0.30 \text{ mM}$; Isotherms (III) and (IV)

$C_{\text{HIV-protease}} = 0.02 \text{ mM}$, $C_{\text{indinavir}} = 0.29 \text{ mM}$,

$C_{\text{acetyl-pepstatin}} = 0.20 \text{ mM}$

A slight noise was added to the simulated curves prior fitting

References

¹ G. Holdgate, S. Geschwindner, A. Breeze, G. Davies, N. Colclough, D. Temesi, L. Ward, Biophysical Methods in Drug Discovery from Small Molecule to Pharmaceutical. Protein-Ligand Interactions. In *Methods in Molecular Biology* **2013**, 1008, pp 327-355.

² A. Velazquez-Campoy and E. Freire, Isothermal titration calorimetry to determine association constants for high-affinity ligands. *Nature protocols* **2006**, 1, pp 186-191.

³ W. B. Turnbull, Divided we fall? Studying low-affinity fragments of ligands by ITC. *GE Healthcare Life Sciences protocol*, **2011**, pp 1-11.

⁴ H. Ohtaka, A. Velazquez-Campoy, D. Xie, and E. Freire. Overcoming drug resistance in HIV-1 chemotherapy: the binding thermodynamics of amprenavir and TMC-126 to wild-type and drug-resistant mutants of the HIV-1 protease. *Protein Sci.* **2002**, 11, pp 1908-1916.

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www.affinimeter.com

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

info@affinimeter.com

