Methods 76 (2015) 99–115

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

A unified framework based on the binding polynomial for characterizing biological systems by isothermal titration calorimetry

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ARTICLE INFO

Article history:
Received 31 July 2014
Accepted 29 September 2014
Available online 8 October 2014

Keywords:
Isothermal titration calorimetry
Binding polynomial
Macromolecular interactions
Ligand binding
Homotropic and heterotropic chemical linkage
Allosteric and polysteric linkage

ABSTRACT

Isothermal titration calorimetry (ITC) has become the gold-standard technique for studying binding processes due to its high precision and sensitivity, as well as its capability for the simultaneous determination of the association equilibrium constant, the binding enthalpy and the binding stoichiometry. The current widespread use of ITC for biological systems has been facilitated by technical advances and the availability of commercial calorimeters. However, the complexity of data analysis for non-standard models is one of the most significant drawbacks in ITC. Many models for studying macromolecular interactions can be found in the literature, but it looks like each biological system requires specific modeling and data analysis approaches. The aim of this article is to solve this lack of unity and provide a unified methodological framework for studying binding interactions by ITC that can be applied to any experimental system. The apparent complexity of this methodology, based on the binding polynomial, is overcome by its easy generalization to complex systems.

1. Introduction

Isothermal titration calorimetry (ITC) has become a standard laboratory technique worldwide. ITC allows the simultaneous determination of the association equilibrium constant, the binding enthalpy and the binding stoichiometry in a binding process where non-covalent interactions are established in solution. Regarding its applicability, researchers employ ITC for characterizing biomacromolecular interactions involving proteins, nucleic acids, carbohydrates, lipids, low-molecular-weight compounds, and even cellular organelles and whole cells. The practical use of ITC ranges from the study of the interaction between two natural binding partners or the elucidation of binding cooperativity phenomena underlying allosteric regulation, to the optimization of lead compounds in drug discovery and development.

Just a few decades ago calorimetry was considered a marginal technique in the applied chemistry field. Nowadays, the widespread use of ITC for biological systems, facilitated by technical developments (e.g. increase in sensitivity, miniaturization and automation) and the availability of user-friendly commercial calorimeters, has led to its incorporation in many biophysical/biochemical labs, as well as its consideration as the gold-standard for molecular interaction studies.

Among the advantages in ITC, these can be highlighted:

- Complete thermodynamic characterization in a single experiment.
- Broad practical range for binding affinity determination.
- No need for molecular labeling.
- No need for molecular immobilization.

At the same time, among the disadvantages in ITC, these can be highlighted:

- Time and sample consumption.
- Inability to reliably determine binding affinities higher than nanomolar (and lower than millimolar) applying a simple direct set-up.
- Complexity of data analysis for non-standard models.

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http://dx.doi.org/10.1016/j.ymeth.2014.09.010
1046-2023/

The last point is even applicable to standard models (e.g. protein with a single binding site), because of many ITC users lacking the appropriate experimental training and knowledge about fundamental concepts associated with binding and, in particular, calorimetry. Thus, it is not uncommon to observe misinterpretations and systematic errors in published work. In addition, commercial software packages for ITC analysis contain only a few binding models and, consequently, users must either develop their own fitting routines for specific binding models or rely on the capability to apply standard models to more complex systems with the appropriate assumptions (under certain circumstances a complex model can be reasonably substituted by a simple, approximate model).

Many models for studying macromolecular interactions can be found in the literature: protein with a single binding site, protein with several independent binding sites, protein with several binding sites exhibiting cooperative interactions (chemical homotropic and heterotropic linkage), oligomeric protein dissociation, protein self-association/dissociation coupled to ligand binding (polysteric linkage). Reviewing a representative set of these publications it is easy to conclude that each experimental scenario requires its own specific hypotheses, strategies, and approximations, leading to a rather heterogeneous set of approaches lacking unity.

The aim of this article is to provide a unified mathematical formalism for studying binding interactions by ITC that can be applied to any experimental system. This methodology is based on the binding polynomial, the partition function of the system containing all relevant information [1–3]. As it occurs with many general methodologies, it may seem artificially complex for simple systems (i.e. like "using a sledgehammer to crack a nut"), but the easy and straightforward generalization to complex systems represents a remarkable benefit. Providing a unified framework does not preclude the user for acquiring proficient training and knowledge about experimental and theoretical aspects of binding interactions, as well as data analysis. The methodology and tools described here are directed to the appropriate description of the binding model, its implementation and its connection with the real system regarding the estimation of the thermodynamic binding parameters. The strategies for obtaining additional information (e.g. performing experiments at different temperatures, ionic strengths, pH's, co-solutes, etc. in order to estimate the binding heat capacity, the net number of protons/ions/molecules exchanged upon binding, etc., or adopting different experimental approaches), as well as technical descriptions and protocols for ITC experiments, can be readily found elsewhere. Specific details about the data pre-analysis (e.g. baseline correction, integration of peaks, etc.) are expected to be already known to the ITC user.

Before advancing to the following sections two important observations must be made. First, the set of binding equations does not imply any restrictive assumption (at least, other than dilute solutions are considered and, therefore, activities can be replaced by concentrations; alternatively, the activity coefficients can be considered included in the equilibrium constants) and it is valid for any technique suitable for binding studies (e.g. circular dichroism, fluorescence spectroscopy, nuclear magnetic resonance). The basic difference consists of the final observable employed in each technique (e.g. heat, ellipticity, fluorescence intensity, chemical shift). Second, ITC is not a differential technique, but a finite-difference technique. Thus, finite changes in the excess molar enthalpy of the system (heat evolved at constant pressure) triggered by finite changes in the composition of the system are monitored. Therefore, cumulative data must not be employed (minimizing systematic errors) and differential equations should not be employed for modeling the system. Differential equations-based approaches are fine for academic purposes, but they really do not have added value compared to finite-differences-based approaches and, furthermore, do not resemble the actual scenario.

2. The binding polynomial

For a biological macromolecule, P, with several binding sites for a given ligand, L, different liganded states can be considered: $\{PL_i,\ \ i = 0, \ldots, n\}$. This ensemble of states is in equilibrium and overall association constants, β_i, or step-wise association constants, K_i, can be defined:

$$\begin{aligned} \beta_i &= \frac{[PL_i]}{[P][L]^i} \\ K_i &= \frac{[PL_i]}{[PL_{i-1}][L]} \end{aligned} \tag{1}$$

The meaning of these equilibrium constants is simple and rather ambiguous at the same time, since they are not associated with specific binding sites, but with subsets of macromolecule-ligand complexes. The constant β_i reflects the binding of i ligand molecules to any i binding sites of the n possible binding sites (no matter their binding affinity and the specific location of the binding sites) to form the PL_i complex (that is, it is associated to a maximum number of $\binom{n}{i}$ liganded states), whereas the constant K_i reflects the binding of the ith ligand to the PL_{i-1} complex to form the PL_i complex (no matter the binding affinity and the specific location of the binding site).

These two sets of equilibrium constants are equivalent and are interrelated:

$$\begin{aligned} \beta_i &= \prod_{j=1}^{j=i} K_j \\ K_i &= \frac{\beta_i}{\beta_{i-1}} \end{aligned} \tag{2}$$

From now on, and for the sake of simplicity, we will only employ the overall association constants. These association constants constitute a set of macroscopic phenomenological model-free parameters governing the binding equilibrium (i.e. statistical distribution of the macromolecule into the different liganded states), and no mechanistic interpretation should be inferred from them. However, based on these association constants, appropriate binding models based on site-specific microscopic association constants and, if necessary, cooperativity constants, can be discriminated.

The partition function of the system, Z, is defined as:

$$Z = \sum_{i=0}^{n} exp(-\Delta\widehat{G}_i/RT) \tag{3}$$

where R is the ideal gas constant, T is the absolute temperature and $\Delta\widehat{G}_i$ is the Gibbs energy difference between the liganded state with i ligands bound and a reference state. The ligand-free macromolecule, the state with higher energy (since ligand binding always lowers the Gibbs energy of the system), is taken as the reference state, and each exponential term is equal to the relative population ratio of the corresponding liganded state:

$$exp(-\Delta\widehat{G}_i/RT) = \frac{[PL_i]}{[P]} = \beta_i[L]^i \tag{4}$$

The Gibbs energy $\Delta\widehat{G}_i$ does not coincide with the usual definition for the Gibbs energy $\Delta G_i = -RT \ln \beta_i$, but it contains an additional term $-iRT\ln[L]$ accounting for the additional stabilization effect (i.e. reduction in Gibbs energy) of entropic nature induced by the presence of the ligand.