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Multithermal titration calorimetry: A rapid method to determine binding heat capacities

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Abstract

Herein a new method that allows binding ΔCp to be determined with a single experiment is presented. Multithermal titration calorimetry (MTC) is a simple extension of isothermal titration calorimetry (ITC) that explicitly takes into account the thermal dependences of ΔH and the binding constant. Experimentally, this is accomplished by performing a single stepwise titration with ITC equipment, allowing temperature re-adjustments of the system at intermediate states of the titration process. Thus, from the resulting multitherm, ΔCp can also be determined. The experimental feasibility of MTC was tested by using the well-characterized lysozyme–chitotriose complex as a model system.

Keywords: Isothermal titration calorimetry; Molecular recognition; Binding energetics

1. Introduction

An old maxim of chemistry and molecular biology states that in molecular recognition processes, structure underpins function [1]. In due course, this picture was complemented with the thermodynamic certainty that complex formation depends, in turn, on the energetics that makes it a spontaneous event. As a consequence, the thorough and precise characterization of the energetics of molecular complex formation, including its dependence on relevant environmental variables, became widely agreed to be of major concern, constituting nowadays one of the most vigorous branches of research in molecular sciences.

Thermodynamically, the stability of a complex is defined by the difference in free energy between the complex and the free molecular partners (ΔG). The quantitative interpretation of this state function in terms of its microscopic components continues to be one of the major challenges of current molecular sciences. In particular, it has proven to be difficult for systems of moderate or high complexity, as in the case of the vast majority of biomolecular processes. A first step towards the dissection of ΔG is the evaluation of its enthalpic (ΔH) and entropic (ΔS) components. Many contemporary models aimed at establishing structure–function correlates consider separately these functions, capturing different, though interrelated, properties of the system. For instance, in the case of molecular recognition reactions occurring in an aqueous medium, ΔH is approximately equal to the change in internal energy of the solutes and the solvent, which in turn results from changes in electrostatic and van der Waals interactions. In contrast, ΔS arises from the change in the degrees of freedom of the solutes (conformational and mixing entropy) and the solvent (solvation entropy). In the thermodynamic characterization of molecular binding, the heat capacity change (ΔCp) is another key function to be considered. ΔCp is not only indispensable to account for the thermal dependence of the distribution of species, but is also an important sensor of the re-arrangement of the intermolecular interactions network elicited mostly by changes in the exposition of solutes to the solvent [2–4].

Isothermal titration calorimetry (ITC) is one of the most powerful and precise techniques currently available to characterize the energetics of molecular interactions (e.g., Refs. [5] and [6] and references therein). Heat effects have proven to be a widely spread property in molecular recognition processes, which ITC measures directly. Since the heat evolved is proportional to the moles of complex formed, the binding constant (K_B) can also be solved by conducting a conventional

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titration protocol. Thus, ITC allows the binding functions ΔG, ΔH and ΔS to be determined with a single experiment. It also permits an accurate determination of the reaction stoichiometry, or alternatively, the actual concentration of the active form of one of the chemical species [7,8]. The current approach for determining ΔCp by ITC involves the acquisition of binding isotherms at different temperatures. Thus, ΔCp can be evaluated from the first derivative of the enthalpy with respect to temperature. This is an enormous advantage in accuracy in relation to non-calorimetric techniques, where ΔCp is calculated as a second derivative of the equilibrium constant.

With the advent of commercially available high-precision ITC equipments, there has been a boom in the number of reports dealing with the calorimetric characterization of molecular complexes over the last fifteen years. For instance, a search for "isothermal titration calorimetry" in the *SciFinder Scholar* (V. 2004) database yielded a total of 203 papers published just in 2004, i.e., 75% of the total number of papers published during the entire decade of the 1990s. However, the number of studies reporting ΔCp has remained relatively small even nowadays (about 1 out of every 10 papers published in 2004). Since ΔCp is a fundamental binding function, the scarcity of data must often be rooted in reasons of economical and practical order. In fact, ITC typically requires large amounts of chemicals (up to several milligrams). Furthermore, although ITC is a non-destructive technique, many receptors and ligands cannot be recycled inexpensively, thus requiring a new sample for each temperature to be evaluated.

2. Multithermal titration calorimetry: description of the model

Herein we present a novel method, denominated multithermal titration calorimetry (MTC), which was developed to reduce the costs associated with the calorimetric determination of ΔCp. The central idea underlying MTC is illustrated in Fig. 1. Fig. 1A shows simulated isotherms for a hypothetical complex at three different temperatures. Since ΔH and K_B remain constant throughout each titration process, the analysis of isotherms at different temperatures is imperative for the determination of ΔCp by conventional ITC. However, one can envision a single titration curve built up as the sum of partial segments of these isotherms (Fig. 1B). Hence, the resulting multi-temperature titration curve (binding multitherm) would contain not only information on K_B and ΔH, but also on the temperature dependence of these thermodynamic parameters, allowing ΔCp to be determined.

The performance of an MTC experiment is based on a conventional stepwise titration. Nevertheless, at an intermediate state of the titration process the injection program is halted to allow the system to be equilibrated at a new temperature. This process of partial titration followed by a temperature jump is repeated to sample all the temperatures desired, ensuring a high degree of receptor saturation (>80%) at the highest temperature [9]. Analytically, the multitherm can be described by classical titration expressions, explicitly considering their dependence on temperature. At any given temperature T, the heat evolved (Qp(T)) depends on $\Delta H(T)$ and the concentration of complex attained ([RL](T)):

$$\mathrm{Qp}(T) = \Delta H(T) V_0 [\mathrm{RL}](T) \tag{1}$$

where V_0 is the effective cell volume of the calorimeter. In turn, the complex concentration is a function of $K_B(T)$ and the total receptor ($[R]_T$) and ligand ($[L]_T$) concentrations:

$$[\mathrm{RL}](T) = f\left(K_B(T), [R]_T, [L]_T\right). \tag{2}$$

The thermal dependences of $K_B(T)$ and $\Delta H(T)$ are formally described by the van't Hoff and Kirchoff relationships, respectively:

$$K_B(T) = K_B(T_R)\exp\left(\int_{T_R}^{T} \frac{\Delta H(T)}{RT^2} dT\right) \tag{3}$$

$$\Delta H(T) = \Delta H(T_R) + \int_{T_R}^{T} \Delta C p dT \tag{4}$$

where T_R is a reference temperature and R is the gas constant.

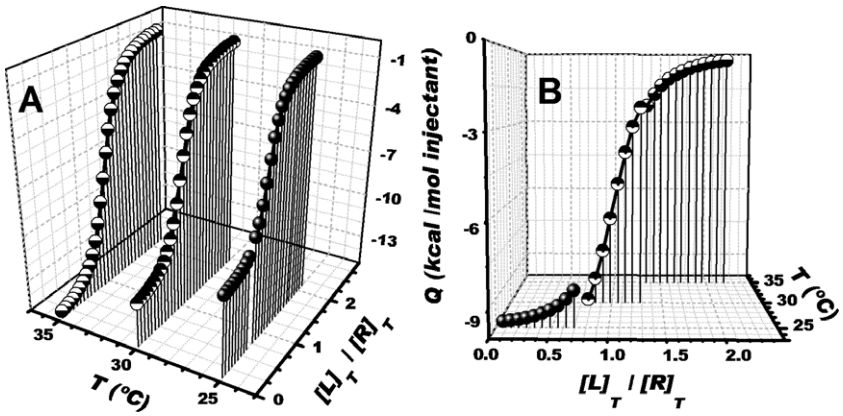

Fig. 1. (A) Simulated binding isotherms for a hypothetical 1 : 1 complex of moderate affinity (K_B (25 °C)=10^5, ΔH (25 °C)=−10 kcal/mol, ΔCp=−500 cal/mol K) at three temperatures. Determination of ΔCp by conventional ITC involves acquisition of the whole isotherms, each obtained from an independent titration experiment. (B) Simulated binding multitherm constructed as the sum of partial segments of the three binding isotherms in panel A. In contrast with ITC, in an MTC experiment the stepwise titration is carried out in a temperature range, allowing temperature re-adjustments of the system at intermediate states of the titration process.

3. Multithermal titration calorimetry: experimental validation

Although the MTC method may appear simple and conceptually well-grounded, its experimental feasibility with modern isothermal calorimeters needs to be proven. To address this issue, we used a VP-ITC equipment (MicroCal Inc., USA), one of the most popular and precise calorimeters commercially available nowadays. In the first step, different equilibration/heating regimes were tested to establish appropriate working conditions for the calorimeter. Once these conditions were met (see Fig. 2 legend for details), the method was tested by using the lysozyme–chitotriose complex as a model system. Lysozyme is an enzyme that cleaves the glycosidic bonds of chitin $(GlcNAc\beta1\rightarrow4GlcNAc)_n$ and cell-wall mucopolysaccharides $(GlcNAc\beta1\rightarrow4MurNAc)_n$. Chitotriose, the trimer of GlcNAc, is the largest chitin-oligosaccharide that binds to lysozyme without being hydrolyzed, thus acting as a competitive inhibitor of the enzyme. This experimentally "well-behaved" adduct has been extensively characterized (Ref. [10] and references therein), and its binding energetics, including ΔCp, has been determined by ITC [11].

Fig. 2A shows a raw MTC thermogram obtained from the progressive titration of lysozyme with chitotriose. Three temperatures were sampled in the course of the whole titration. The corresponding binding multitherm (constructed as the heat evolved in the *i*th injection per mol of ligand added vs. the molar ligand/receptor ratio, $X_T=[L]_T/[R]_T$) after subtraction of the heats of ligand dilution is shown in Fig. 2B. Although the overall shape of a multitherm may resemble that of an isotherm, an important difference is evident upon a closer inspection: the MTC trace is not as smooth as an ITC curve. It may present breaks between adjacent temperatures. On the one hand, this is due to the redistribution of the molecular species triggered by the change in the magnitude of the binding constant upon temperature variation. In the case of the exothermic formation of the lysozyme-inhibitor complex, the equilibration of the system at a higher temperature leads to the dissociation of a fraction of the complex formed after the last addition at the lower temperature (Fig. 2C). On the other hand, since ΔCp for this

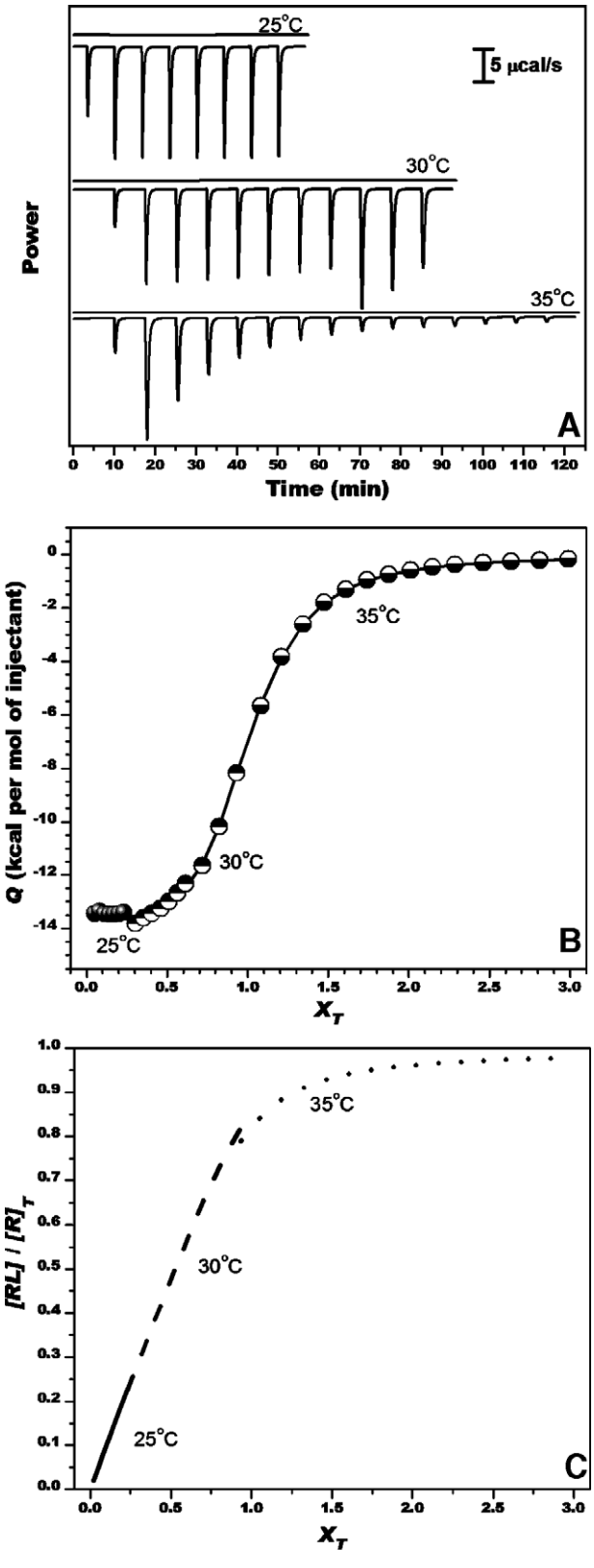

Fig. 2. Multithermal titration calorimetry (MTC) of lysozyme (0.402 mM) with chitotriose (5.75 mM) in 50 mM acetate buffer, pH 4.7. Samples were prepared as described in Ref. [11]. Throughout the whole experiment, the syringe was kept inside the reaction cell to avoid any mixing with the solution displaced out of the cell during previous injections. The experiment was started by equilibrating the sample-loaded equipment at 25 °C. After isothermically carrying out an injection program of one 2 μl (dummy) and 7 × 3 μl ligand additions, the temperature was re-adjusted to 30 °C. The equipment was then allowed to thermostat at this new temperature for 45 min before one 2 μl (dummy), 7 × 5 and 3 × 10 μl ligand injections were applied. Finally, this procedure was repeated at 35 °C, applying one 2 μl (dummy), 11 × 12 μl and 3 × 15 μl ligand injections. The appropriate equilibration/heating regime for our VP-ITC equipment was established by carrying out electronic and chemical calibrations. It was found that after the second temperature jump, at least 45 min were needed to thermostat the equipment at the new temperature. Otherwise, the instrument seemed to fall into a "false" equilibration state, yielding measurements with large deviations from the expected (electronically or chemically supplied) heats. (A) Raw potential difference traces vs. time at different temperatures. The figure also shows the traces corresponding to the dilution heats of the ligand obtained under the same injection schedule as in the binding experiment. (B) MTC curve constructed as the heat evolved normalized per mol of ligand injected vs. chitotriose/lysozyme molar ratio (X_T). The solid line represents the best fit obtained from an independent and identical binding sites model (Eqs. (5)–(7)). (C) Lysozyme's saturation degree ($[RL]/[R]_T$) as a function of X_T at different temperatures.

Table 1
Energetics of lysozyme–chitotriose interaction at 35 °C determined by MTC and ITC[a]

Experiment	η	ΔH (kcal/mol)	K_B	$-T\Delta S°$ (kcal/mol)	$\Delta G°$ (kcal/mol)	ΔCp (cal/mol K)
MTC 1	1.01±0.00	−15.00±0.07	68,300±1500	8.18	−6.82	−120±8
MTC 2	1.00±0.00	−15.18±0.15	64,000±1500	8.40	−6.78	−121±15
MTC 3	1.04±0.01	−15.45±0.19	64,300±2500	8.67	−6.78	−123±12
Average	1.02	−15.21	65,500	8.42	−6.79	−121
S.D.[b]	0.02	0.23	2400	0.25	0.02	2
ITC[c]	0.97±0.01	−15.20±0.10	68,700±2300	8.38	−6.82	−119±3

[a]Uncertainties correspond to regression standard errors, unless otherwise stated.
[b]Standard deviation of the mean of three MTC experiments.
[c]Taken from Ref. [11]. 1 cal=4.184 J.

complex is negative, a temperature increase yields a larger negative molar enthalpy, changing therefore the ratio between the heat evolved and the moles of complex formed upon ligand addition (Eq. (1)).

To analyze the binding multitherm of the lysozyme–chitotriose complex, an independent and identical binding sites model was used [8,11]:

$$\frac{\partial Qp(T)}{\partial [L]_T}\frac{1}{V_0} = \Delta H(T)\left[\frac{1}{2} + \frac{1-\frac{X_T}{\eta}-K_B(T)\eta[R]_T}{2\sqrt{\left(1+\frac{X_T}{\eta}+K_B(T)\eta[R]_T\right)^2-4\frac{X_T}{\eta}}}\right] \quad (5)$$

where η is the reaction stoichiometry. Although ΔCp is known to be a function of temperature, its actual variation is small and typically beyond the resolution of modern ITC equipment. Assuming ΔCp as temperature-independent, the integrated forms of Eqs. (3) and (4) become:

$$K_B(T) = K_B(T_R)\exp\left[\frac{1}{R}\left((\Delta H(T_R)-T_R\Delta Cp)\left(\frac{1}{T_R}-\frac{1}{T}\right)+\Delta Cp\ln\frac{T}{T_R}\right)\right] \quad (6)$$

$$\Delta H(T) = \Delta H(T_R) + \Delta Cp(T-T_R). \quad (7)$$

It is to be noted that adaptation of the MTC method to more complex binding models is as straightforward as in the case of a single-independent sites model, since only the inclusion of Eqs. (6) and (7) into the particular binding model is required. The solid line in Fig. 2B corresponds to the best fitting of Eqs. (5)–(7) to the experimental data. Table 1 summarizes the results for the binding functions obtained from three independent MTC experiments. As can be seen, variations among experiments are rather small, exhibiting the reliability of the determinations. Table 1 also shows the magnitudes previously obtained for the same system by conventional ITC [11]. Overall, the excellent agreement between the two methods for each binding function demonstrates that the MTC method indeed preserves the advantages and robustness inherent to ITC.

The feasibility of the MTC method has been shown here by using a complex of moderate affinity, relatively large ΔH and small ΔCp. Satisfactory results have also been obtained in our laboratory for the titration of concanavalin A with methyl-α-mannose (data not shown), a low-affinity complex (K_B ~9000) that exhibits smaller ΔH and ΔCp (−7.7 kcal/mol and −52 cal/mol K, respectively) as compared to those of the lysozyme–chitotriose complex. For both complexes, large calorimetric signals were obtained in relation to the minimum detection threshold of the calorimeter (<1 μcal), as a result of the use of protein concentrations required to establish appropriate experimental conditions (i.e., $c=\eta K_B M_T>1$). In part, this situation made possible a successful analysis of both complexes through the MTC model, even though the corresponding ΔCp magnitudes were small. However, caution should be taken when studying the formation of adducts yielding small heat signals, e.g., systems with small molar enthalpies and/or systems with large affinity constants or poor solubility that require to be studied at very low concentrations. In fact, although this limitation is common to MTC and ITC methods, it should be noted that MTC also requires precise recordings to be obtained at higher temperatures, where the saturation degree is relatively high and therefore smaller heats are generated upon new ligand additions.

ITC represents a benchmark in the development of tools for measuring the thermodynamic properties of molecular complexes. In contrast to all indirect methods (surface plasmon resonance, fluorescence, ultracentrifugation, ELISA, etc.), ITC allows a straightforward measurement of the binding enthalpy. MTC may be seen as a new step in this direction. By performing temperature jumps at intermediate states of a titration process, a multitherm binding curve can be built from which ΔCp can be evaluated with a single experiment. An enormous advantage achieved from this simple experimental variation is that the amounts of chemicals required can be brought down significantly. Furthermore, an MTC experiment is just slightly longer than a single-temperature ITC experiment, implying therefore a drastic reduction in time for the determination of ΔCp. This time could be further reduced if ITC equipments are optimized to carry out MTC measurements, especially by decreasing the time of temperature re-adjustment and by fully automatizing the process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2005.09.022.

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