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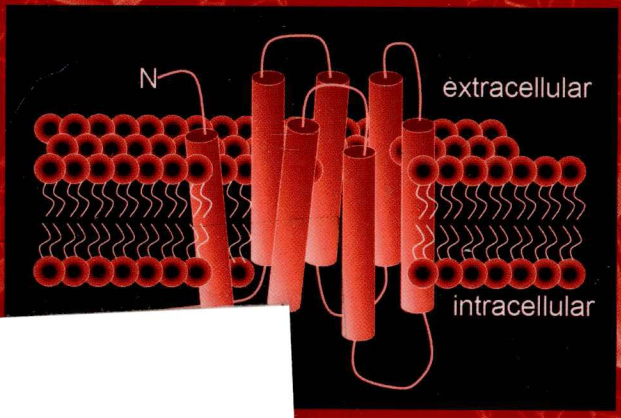
Protein–Ligand Interactions

Methods and Applications

Edited by

G. Ulrich Nienhaus



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Preface

The genomes of several organisms have been sequenced in recent years, and the efficient exploration of interactions among tens of thousands of gene products has moved to center stage in our quest for a detailed understanding of life at the molecular level. Molecular recognition and binding of ligands (atoms, ions, molecules) by proteins with high sensitivity and selectivity is of central importance to essentially all biomolecular processes. Therefore, a thorough understanding of protein–ligand interactions is of key importance for the basic and applied sciences. Techniques to study protein–ligand interactions have been established and refined for many years. They continue to be improved by the development of new reagents, protocols, and instrumentation. A variety of powerful experimental and theoretical tools have become available in recent years, and novel techniques are continually being introduced to meet new demands.

Protein–Ligand Interactions: Methods and Applications features a collection of methods for studying the interaction between proteins and ligands, including biochemical/bulk techniques, structure analysis, spectroscopy, single-molecule studies, and theoretical/computational tools. The presen volume aims to provide the researcher with technical background information that will enable him or her to develop strategies for characterizing protein–ligand interactions in the most effective way. Life scientists in both academia and industry will find hands-on information regarding both established and novel approaches for the study of protein–ligand interactions. We have attempted to present a broad selection of widely applicable techniques. We hope that *Protein–Ligand Interactions: Methods and Applications* will provide a good starting point from which to embark on other, more specialized techniques.

I wish to thank all contributing authors for their hard work and considerable patience. I greatly appreciate the high quality of their presentations that made compiling this volume a particularly pleasurable experience.

Gerd Ulrich Nienhaus

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Contents

1

Isothermal Titration Calorimetry

Edwin A. Lewis and Kenneth P. Murphy

Summary

Isothermal titration calorimetry is an ideal technique for measuring biological binding interactions. It does not rely on the presence of chromophores or fluorophores, nor does it require an enzymatic assay. Because the technique relies only on the detection of a heat effect upon binding, it can be used to measure the binding constant, K, the enthalpy of binding, ΔH° and the stoichiometry, or number of binding sites, n. This chapter describes instrumentation, experimental design, and the theoretical underpinnings necessary to run and analyze a calorimetric binding experiment.

Key Words: Binding; thermodynamics; proton linkage; enthalpy; heat capacity; data analysis.

1. Introduction

Titration calorimetry was first described as a method for the simultaneous determination of K and ΔH about 40 yr ago by Christensen and Izatt *(1,2)*. The method was originally applied to a variety of weak acid-base equilibria and to metal ion complexation reactions *(3–5)*. These systems could be studied with the calorimetric instrumentation available at the time that was limited to the determination of K values less than about 10^4 to 10^5 M^{-1} *(6)*. The determination of larger association constants requires more dilute solutions and the calorimeters of that day were simply not sensitive enough.

Beaudette and Langerman published one of the first calorimetric binding studies of a biological system using a small volume TRONAC titration calorimeter *(7)*. Their data for the titration of an enzyme, bovine liver glutamate dehydrogenase (GDH), with an inhibitor, adenosine diphosphate (ADP), are shown in **Fig. 1.** In 1979, Langerman and Biltonen published a description of microcalorimeters for biological chemistry, including a discussion of available

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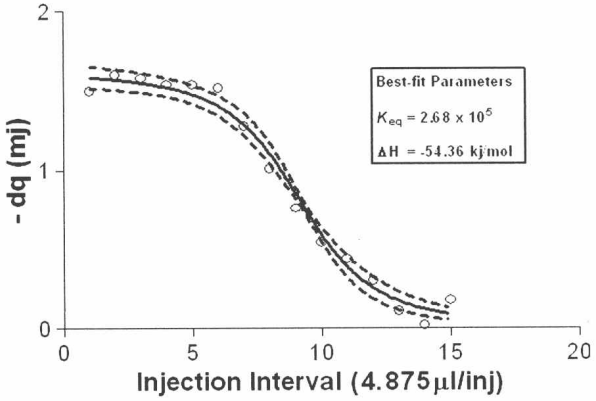

Fig. 1. Titration of 2.00 mL of 0.1340 m*M* GDH with 6.17 m*M* ADP at pH 7.6 and 25°C. (Data taken from **ref. *8***.)

instrumentation, applications, experimental design, and data analysis and interpretation ***(8,9)***. This was really the beginning of the use of titration calorimetry to study biological equilibria. It took another 10 yr before the first commercially available titration calorimeter specifically designed for the study of biological systems became available from MicroCal ***(10)***.

Isothermal titration calorimetry (ITC) is now routinely used to directly characterize the thermodynamics of biopolymer binding interactions ***(11–13)***. This is largely a result of improvements in the ITC instrumentation and data analysis software. Modern instruments, like the MicroCal and Calorimetry Sciences Corporation ITCs, make it possible to measure heat effects as small as 0.4 µJ (0.1 µcal) allowing the determination of binding constants, *K*'s, as large as 10^8 to 10^9 M^{-1}.

In order to take full advantage of the powerful ITC technique, the user must be able to design the optimum experiment, understand the nonlinear fitting process, and appreciate the uncertainties in the fitting parameters *K*, ΔH, and n. ITC experiment design and data analysis have been the subject of numerous papers ***(14–17)***. This chapter reviews the planning of optimal ITC experiments, guides the reader through a sample experiment, the titration of RNase A with 2'-cytidine monophosphate (2'-CMP), and reviews theory underlying the nonlinear fitting of ITC data and the interpretation of ITC results.

2. Instrument Description

A schematic diagram of an isothermal titration calorimeter is shown in **Fig. 2.** The essential components of the ITC instrument are: (a) a matched pair of sample and reference cells contained within a thermostatted environment,

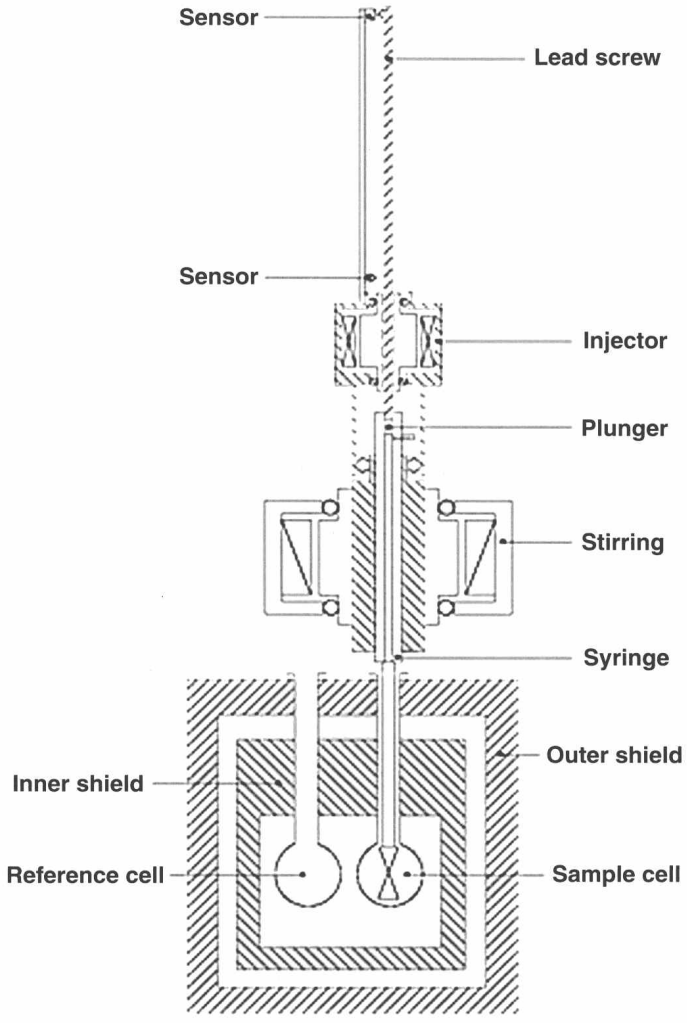

Fig. 2. Diagram of the MicroCal VP-ITC measuring unit (taken from the MicroCal website, http://www.microcalorimetry.com/).

(b) a stepper-motor-driven syringe for injecting titrant (ligand solution) into the sample cell, (c) a stirrer for keeping the contents of the sample cell homogeneous, and (d) a means for compensating (and measuring) the heat flow to the sample cell so that it is maintained at the same temperature as the reference cell. In modern ITC instruments, the cell volumes are nominally 1.5 mL, the temperature of the thermostat can be set from about 5 to 80°C, the injected volume can range from about 1 to 20 μL, and heats as small as 0.4 μJ (0.1 μcal) can be measured.

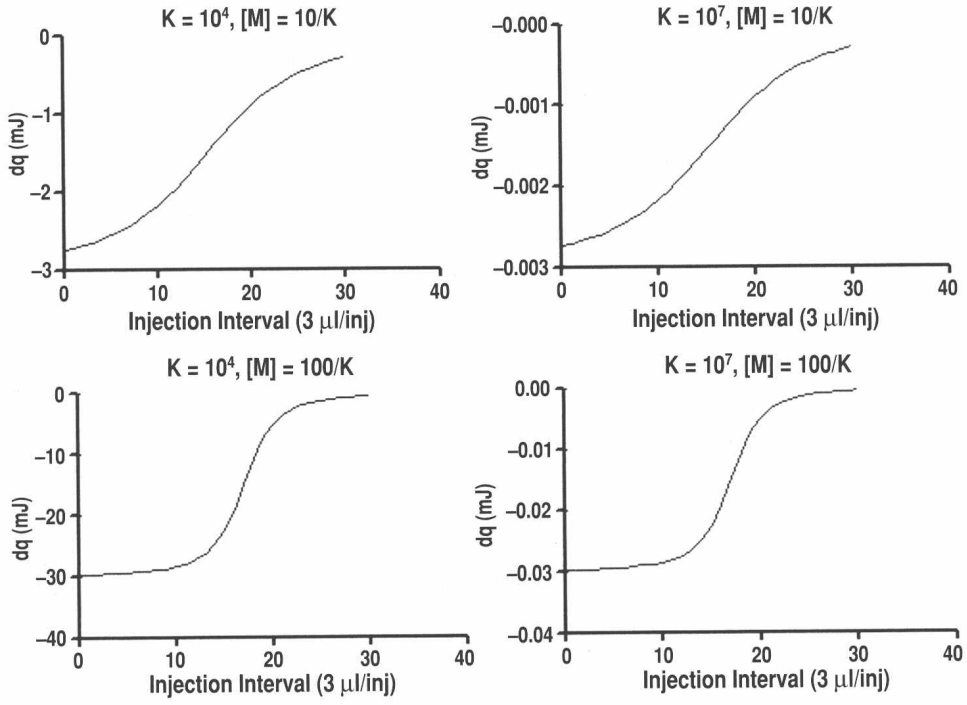

Fig. 3. Relationship between thermogram curvature and the c parameter, ([M] × K), for two different values of c, 10 and 100.

The ITC signal is dependent on the concentrations of the macromolecule, [M], and the ligand, [L]; the cell volume; the injected volume; and the values of K, ΔH, and n (or a larger set of parameters for binding models more complicated than the n independent sites model). In order to obtain an estimate for K, the ITC experiment must yield a curved thermogram. Furthermore, of course, the ITC experiment must also be done under conditions that produce detectable amounts of heat for each titrant addition. These points are illustrated graphically in **Fig. 3.** The upper panels in **Fig. 3** show the curvature that would be observed in two experiments for systems with different binding constants, 10^4 and 10^7 M^{-1}, if the concentration of the macromolecule was chosen to be $(10/K)$, i.e., [M] = 1 mM for data shown in the upper left panel, and [M] = 1 µM for the data shown in the upper right panel. The lower two panels in **Fig. 3** show thermograms for the same two systems with the exception that the macromolecule concentration was increased to be $(100/K)$. The first point that can be made from the data in **Fig. 3** is that the curvature is the same as long as the product of macromolecule concentration and K is held constant. It has been

widely reported that the c parameter, ([M] × K), must be between 1 and 1000 in order to produce a thermogram with the curvature required for the simultaneous determination of K and ΔH ***(10)***. The authors of this chapter believe that the best experiments will be done with the c parameter having a value between 10 and 100.

At first glance, each of the simulated thermograms in **Fig. 3** would seem to be representative of an experiment in which K and ΔH could be accurately determined. On closer inspection, it is apparent that two of the experiments shown are less than optimal. The experiment shown in the upper right panel would yield heats that are too small to be determined accurately, even for the first injections where the largest heats would only be about 2.7 mJ (0.65 mcal), whereas the experiment shown in the lower left panel yields heats that are too large, approx 7000 mcal for the first injections, and the experiment would require excessive amounts of reagents. Clearly, simulations are important in optimizing the ITC experiment and in achieving a balance between detectable heats and curvature in the thermogram.

3. Methods

There are seven steps to running the ITC experiment. These are: 1) planning the experiment (simulations), 2) preparing the L and M solutions, 3) collecting the raw ITC data, 4) collecting the blank (L solution dilution), 5) correcting the raw ITC data, 6) nonlinear regression of the corrected titration data to provide estimates of the thermodynamic parameter values, and 7) interpretation of the model data. Each step will be discussed herein.

In our discussion of running the ITC experiment, we will use the binding of cytidine-2'-monophosphate, 2'-CMP, to bovine ribonuclease A, RNase, as a test system ***(10,18,19)***. These chemicals are available from Sigma Aldrich (St. Louis, MO) in suitable purity and have been widely used as a test system by ITC manufacturers. The approximate thermodynamic parameters for the 2'-CMP/RNase system are $K \approx 6 \times 10^4\ M^{-1}$ and ΔH ≈ – 45 kJ/mol with a stoichiometry of 1 at 25 °C ***(19)***. An alternative test system is the binding of Ba^{+2} ion by the cyclic poly ether, 18-crown-6 ***(19,20)***.

3.1. Planning the Experiment

The first step in running the ITC experiment is to determine the concentrations for the macromolecule and ligand solutions. If the objective of the ITC experiment is only to determine the binding enthalpy change, ΔH, then the only consideration is that the concentration of the ligand will be large enough that an accurately measurable heat effect, ≥ 40 μJ (10 μcal), will be observed and that the macromolecule concentration will be in excess. In the case of our test system, the binding of 2'-CMP to RNase, these conditions would be met

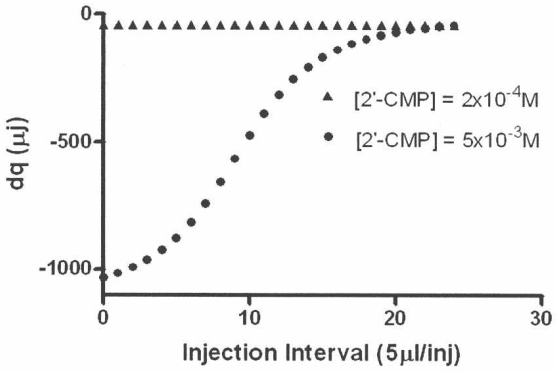

Fig. 4. Simulated experiments for the titration of a 1.7×10^{-4} *M* solution of RNase with two different titrant solutions. (2'-CMP at a concentration of either 2×10^{-4} or 5×10^{-3} *M*.)

with [2'-CMP] = 2×10^{-4} *M*, and [RNase] = 1.7×10^{-4} *M*. With an injection volume, v_{inj}, of 5 µL, the heat per injection would be given by **Eq. 1** below and there would be no curvature in the thermogram:

$$q_{inj} = \Delta H \times [L] \times v_{inj} = -45 \text{ kJ/mol} \times \left(2 \times 10^{-4}\right) \text{M} \times \left(5 \times 10^{-6}\right) \text{l} \approx -45 \text{ µJ} (-11 \text{ µcal}) \quad (1)$$

If the concentration of the L (2'-CMP) is increased to 5×10^{-3} *M*, the thermogram would show curvature similar to that shown in the lower panels of **Fig. 3** (c = 10) and an endpoint would be reached after approx 20 (5 µL) injections. The integrated heat values for the first injections would now be more than –1000 µJ. Increasing the concentration of RNase to 1.7×10^{-3} *M* (c = 100) and the ligand concentration to 5×10^{-2} *M* would yield a thermogram showing the same curvature as that shown in the upper panels of **Fig. 3**. In this last case, the heat observed in the early injections would be too large, more than –10,000 µJ. **Fig. 4** shows simulated ITC data for experiments done under the first set of conditions where only ΔH would be determined and under the second set of set of conditions where both *K* and ΔH would be determined.

3.2. Solution Preparation and Handling

The final results of the ITC experiment depend on exact knowledge of the titrate and titrant solution concentrations, so it is imperative that the concentrations be determined as accurately as possible. Perhaps the ITC solutions can be made by volumetric dilution of stock solutions that were made up by weight. Whenever possible the concentrations should be verified by another analytical procedure (e.g., absorbance, kinetic activity, other analysis, etc.). As will be

noted later, it is especially important that the L concentration be known precisely, as errors in this value will affect the determination of both K and ΔH.

It also is extremely important that the two solutions be matched with regard to composition, e.g., pH, buffer, salt concentration, etc. If the two solutions are not perfectly matched, there may be heat of mixing (or dilution) signals that overwhelm the heat signals for the binding reaction. It is typical that the solution of the macromolecule is dialyzed against a large volume of the buffer. The artifact heats of mixing can be minimized by using the dialysate from preparation of the macromolecule solution as the *solvent* for preparation of the ligand solution.

3.3. Correcting the Raw ITC Data

Obviously, the dialysis/dialysate approach will virtually eliminate the mixing or dilution effects for all solute species in common between the macromolecule and ligand solutions. The exception is that the heat of dilution for the ligand itself must be measured in a blank experiment. In this blank experiment, the ligand solution is titrated into buffer in the sample cell. The heat of dilution of the macromolecule should also be measured in a second blank experiment. This is done by simply injecting buffer from the syringe into the macromolecule solution in the sample cell. Usually the heat of dilution of the macromolecule measured in this way is negligible. To be completely rigorous, a third blank experiment should also be done. This buffer into buffer experiment may be thought of as an instrument blank. The equation to correct the heat data for dilution effects is:

$$Q_{corr} = Q_{meas} - Q_{dil,\ macromolecule} + Q_{instrument\ blank} \qquad (2)$$

The blank corrections are for the same injection volumes as used in the collection of the actual titration data. In the case of the 2'-CMP/RNase titration experiment shown in **Fig. 5**, the only significant correction is for the dilution of the titrant (the results of the 2'-CMP dilution blank experiment are also shown in **Fig. 5**).

Another complicating reaction encountered in many biological binding experiments results from the release (or uptake) of protons as binding occurs. The released protons are taken up by the buffer conjugate base, and there are contributions to the heat both from binding protons to buffer and from the heat of removing protons from the macromolecule ***(17)***. The treatment of this complicating reaction requires knowledge of the number of protons released (or taken up) and the heat of ionization of the buffer. The measured enthalpy is given by:

$$\Delta H_{meas} = \Delta H_0 - \Delta H_{ion} \times n_p \qquad (3)$$

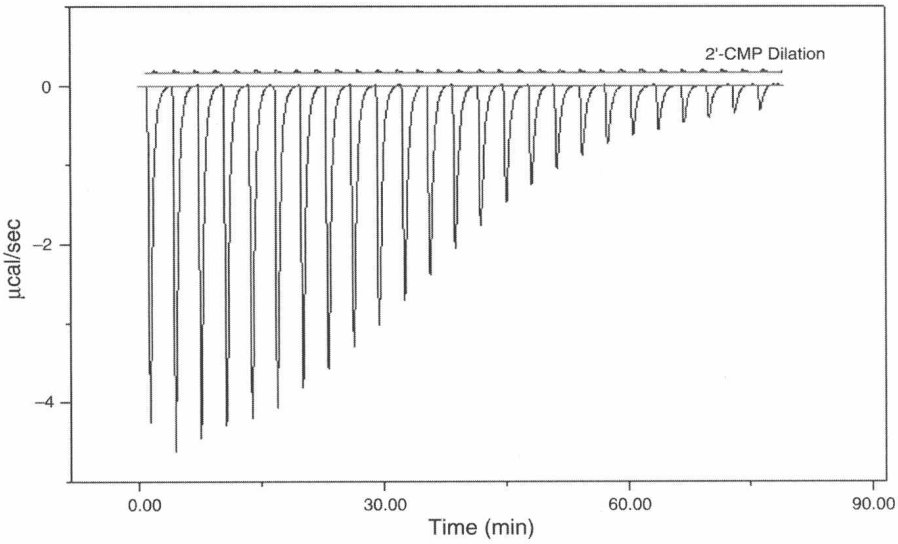

Fig. 5. The raw ITC data (power vs time) are shown for two titrations. The larger heat pulses are from the titration of 1.4 mL of a 1.55×10^{-4} *M* RNase solution with a 3.19×10^{-3} *M* solution of 2'-CMP. The smaller heat pulses are for the dilution of 5 mL of the 2'-CMP titrant into 1.4 mL of the acetate buffer. The power is given in units of mcal/s (where 1 mcal/s = 4.184 mJ/s = 4.184 mW).

where ΔH_0 is the enthalpy of binding in the absence of a heat from protons binding to the buffer, ΔH_{ion} is the heat of proton ionization for the buffer and n_p is the number of protons released on binding 1 mole of L. The value of n_p is determined from titrations done in at least two buffers with different heats of ionization. It should be emphasized that ΔH_0 includes the heat of protons being released from the protein upon binding and, as such, does not represent the *intrinsic* heat of the protein L interaction ***(17)***. Instead, it simply removes the contribution of the buffer. This phenomenon also provides an approach to manipulating the heat signal for a reaction that is accompanied by proton release. By simply using a buffer with a large heat of ionization, the heat signal can be enhanced. Alternatively, the use of a buffer with a small ΔH_{ion} (≈ 0) could be used to minimize the *artifact signal*.

Finally, because the generation of bubbles in the sample (or reference) solutions during an ITC experiment will generate spurious heat signals, the solutions should be degassed prior to filling the cell and injection syringe. The ITC manufacturers provide vacuum degassing accessories for this purpose. Precautions need to be taken to avoid boiling the solutions and changing the concentrations. Also, the ITC manufacturers supply cell loading syringes and