

CHAPTER 11

Isothermal Titration Microcalorimetry

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1. Introduction

Isothermal microcalorimetry is the generic term for a range of versatile and, in principle, nondestructive techniques suitable for direct measurement of the energetics of biological processes in samples ranging from homogeneous macromolecules up to complex and heterogeneous systems, including intact organisms (1–6). We shall concentrate here on the use of calorimetry in the titration mode (7–10) to study the thermodynamics of ligand binding to macromolecules and related processes, since this is central to current efforts to understand the fundamental basis of macromolecular recognition processes and their applications in biotechnology.

It is worth emphasizing from the start that biomolecular binding experiments usually present much more of a calorimetric challenge than typical DSC measurements (*see* Chapter 10) and require considerably more sample material. This arises not only because noncovalent ligand-binding energies are intrinsically smaller than conformational transition enthalpies, for instance, but also because the total energy must usually be liberated gradually during progressive titration of a macromolecular binding site. Furthermore, ligand addition has additional heat effects (dilution, mixing) for which correction must be made, and which are frequently comparable to the binding heat effects.

Consider a possible estimate of the heat effects that one might wish to measure. Enthalpies of noncovalent binding of small molecules to protein sites are typically of the order 20 kJ/mol (5 kcal/mol; 1 cal =

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4.184 J).^{*} A 1- to 2-mL sample solution containing a few milligrams of protein (10^{-7} moles) would consequently liberate about 2 mJ (0.5 mcal) of heat energy during saturation binding. In practice, this would not normally be observed all at once, but rather as the accumulation of a series of heat effects during a thermal titration experiment involving successive ligand addition in which heat effects in any individual measurement might be 10% or less of the total. Measurement of this with an experimental accuracy of 10% or better would require instrumental sensitivity and noise levels down to 20 μ J (5 μ cal) or less. This, corresponding to temperature changes in the sample of just a few millionths of a degree, is about the limit of current instrumentation, and is comparable to the inevitable heat effects of dilution, mixing, and stirring required in any binding experiment.

2. Instrumentation

Of the range of microcalorimeters available from various manufacturers, only those originating from LKB (now Thermometric) and, more recently, from Microcal Inc. have a reasonable track record for biophysical applications. These instruments are based on slightly different principles, which are worth describing separately.

2.1. LKB/Thermometric Thermal Activity Monitor (TAM)

Contact address: Thermometric AB, Spjutvägen 5A, S-17561 Järfälla, Sweden, or Thermometric Ltd., 10 Dalby Court, Gadbrook Business Park, Northwich, Cheshire, CW9 7TN, UK. The original LKB reaction microcalorimeters (batch and flow versions) still in use in many laboratories are based on designs by Wadsö (7,9) utilizing the so-called "heat leak principle." The Thermometric TAM is the direct successor based on the same principle but with updated electronics and improved temperature control (using a recycling water bath instead of air thermostat).

In outline, pairs of reaction vessels (cell) are contained within a relatively heavy and carefully temperature-controlled heat sink (Fig. 1). Between each vessel and the heat sink, and in good thermal contact with both, are solid-state thermopiles, which generate a voltage proportional to the rate of heat energy flux across them. The thermopiles

^{*}See the comment regarding energy units in Chapter 9.

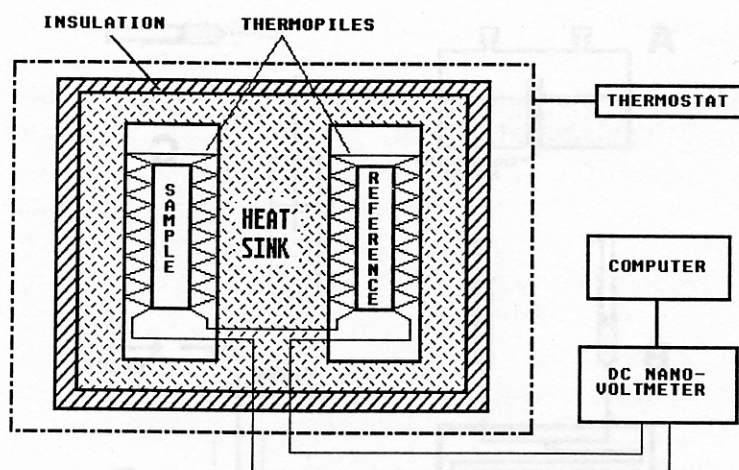


Fig. 1. Basic layout of a typical isothermal reaction microcalorimeter using the "heat-leak" principle. Sample and reference thermopiles are connected back-to-back in series to compensate for global temperature fluctuations and give differential measurements.

for each vessel are connected back-to-back in series for differential measurements, and one vessel would normally act as reference. For small temperature differences, ΔT , between any cell and the heat sink, both the heat flux across a thermopile and the potential (V) it generates are proportional to ΔT :

$$(dQ/dt) \approx k_1 \Delta T \text{ and } V \approx k_2 \Delta T \quad (1)$$

Hence

$$(dQ/dt) = k \cdot V \text{ and the heat of reaction } Q = k \int V \cdot dt \quad (2)$$

where k is the calibration constant. Integration over a heat pulse or measurement of the voltage offset during a steady-state experiment yields the desired heat.

This essentially passive measurement system has the advantage of simplicity and reliability, although these instruments tend to be rather slow to equilibrate and use. Simplicity of construction means that they are quite versatile and relatively easy to adapt for special purposes. A range of reaction vessels is available, or special cells may be constructed for different purposes (Fig. 2). Simple titration cells are usually fitted with motor-driven microliter syringes for delivery of ligand into fixed

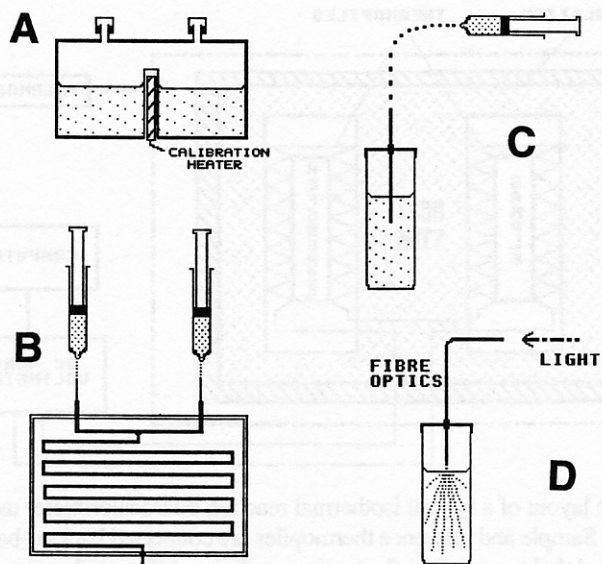


Fig. 2. Examples of cell configurations for different experiments in isothermal reaction microcalorimetry. All vessels will normally include electrical calibration heaters, shown here only in **A**. Vessels may be of glass or inert metals (Au, Pt, tantalum, Hastelloy™, and so on), and typical cell volumes are 1–10 mL. (**A**) Batch cell for single mixing experiments. Reactant solutions are loaded on either side of the dividing wall and, after thermal equilibration, are mixed by inversion/rotation of the entire calorimetric unit. (**B**) Flow cell for continuous or stopped-flow mixing experiments using either peristaltic pumps or, better, precision syringe pumps for delivery of samples. (**C**) Titration cell for *in situ* thermal titrations by microsyringe injection of ligand. Mixing is by inversion, as in **A**, or more recently using built-in stirrers. (**D**) Photocalorimeter cell for light-induced reactions. Samples are irradiated via flexible fiber optics light guides.

volumes of macromolecule solution. These syringes may be mounted in the thermostat or, alternatively, incorporate a heat exchanger coil in the delivery tube, so as to minimize spurious heat effects owing to lack of temperature equilibration of injected solutions.

2.2. Microcal Omega Rapid Titration Calorimeter

Contact address: Microcal Inc., 22 Industrial Drive East, Northampton, MA 01060. The Omega titration microcalorimeter is a more recent development utilizing a somewhat more complex active feedback system for determination of reaction heats (8). It may be purchased as

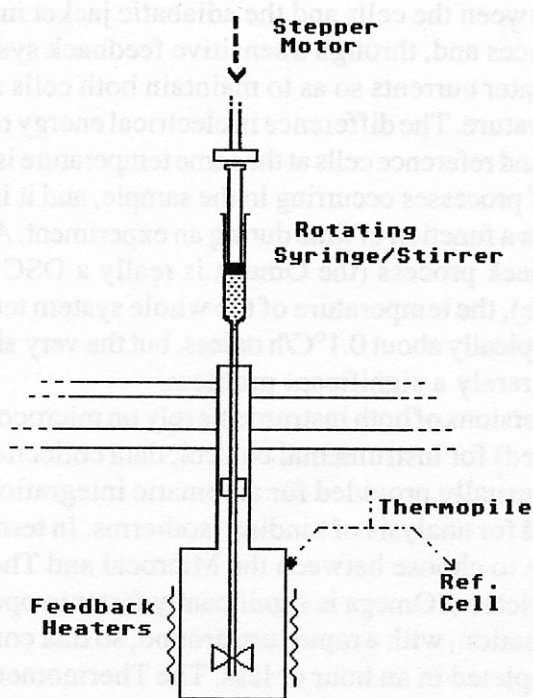


Fig. 3. Schematic of the Microcal Omega titration calorimeter cell. Each cell is suspended by its narrow filling tube in an evacuable adiabatic chamber. The reference cell (not shown) is connected to the sample cell by sensitive thermopile devices, and thermocouples also monitor the cell-jacket temperature difference. The delivery syringe, which is removed for filling and cleaning, is driven by a stepper motor drive and also rotates continuously during titration to provide stirring (the end of the needle is shaped as a narrow paddle).

a stand-alone instrument or as an attachment to the DSC instrument from the same manufacturer.

The reaction vessel consists of a simple cell fitted with a rotating syringe that acts as both delivery device and stirrer (Fig. 3). The reference cell is just a blank, unstirred and with no delivery system, usually kept filled with water. The principle of operation is very similar to the DSC from which it has been developed. Both cells are suspended on the ends of their filling tubes in an adiabatic chamber. (This chamber may be evacuated, although this is only usually necessary at low temperatures to prevent condensation.) Thermopiles mounted between both

cells and between the cells and the adiabatic jacket measure temperature differences and, through a sensitive feedback system, control the feedback heater currents so as to maintain both cells and jacket at the same temperature. The difference in electrical energy required to maintain sample and reference cells at the same temperature is a measure of the energetics of processes occurring in the sample, and it is this energy that is recorded as a function of time during an experiment. As a consequence of the feedback process (the Omega is really a DSC running at very low scan rate), the temperature of the whole system tends to rise slowly with time, typically about $0.1^{\circ}\text{C}/\text{h}$ or less, but the very slow temperature variation is rarely a significant problem.

Current versions of both instruments rely on microcomputer interfacing (PC-based) for instrumental control, data collection, and analysis. Software is usually provided for automatic integration of experimental peaks and for analysis of binding isotherms. In terms of sensitivity, there is little to choose between the Microcal and Thermometric systems. The Microcal Omega is significantly faster in operation: typically 2–3 min/injection, with a rapid turn around, so that complete titrations may be completed in an hour or less. The Thermometric TAM, on the other hand, requires several hours for each equilibration and titration sequence. However, it has the advantage of greater versatility over the Omega, with a wide range of cells available for different applications and good long-term stability.

3. Calorimetric Titration Method

Whichever instrumental system one uses, a typical calorimetric titration experiment consists of injection of a series of aliquots of ligand solution (with mixing) into a known volume of macromolecule solution. Each injection produces a heat pulse, which when corrected for ambient baseline and integrated over time, gives the total heat generated (or absorbed) in the event. There are four major sources of heat in such a process:

- A. Macromolecule–ligand interaction (usually what we want);
- B. Dilution of ligand on injection into the (normally) larger volume of sample;
- C. Dilution of macromolecule by added ligand solution; and
- D. Mechanical mixing effects.

In general, four separate measurements will be needed to discriminate them:

Titration	Heat Effects
1. Macromolecule/ligand	A + B + C + D
2. Macromolecule/buffer	C + D
3. Buffer/ligand	B + D
4. Buffer/buffer	D

Consequently, the required heat (A) for each addition of ligand is given by:

$$A = (1) - (2) - (3) + (4) \quad (3)$$

Sample preparation is usually quite straightforward provided that all solutions are equilibrated in exactly the same buffer, since even minor differences in buffer salt concentrations can give heats of mixing in excess of the ligand-binding heat required. The best procedure is normally to dialyze the macromolecule solution against a large volume of appropriate buffer, and to use aliquots of the final dialysis buffer to make up the ligand solution and for dilutions. Actual procedures and optimal concentration ranges will depend somewhat on the system under investigation (8), but the following example will give guidance to the uninitiated.

3.1. Example: Lysozyme/*N*-Acetylglucosamine Binding

The binding of the simple monosaccharide, *N*-acetylglucosamine (NAG), to the specific site in the binding cleft of hen egg-white lysozyme (EC 3.2.1.17) provides a convenient and reliable test experiment for calorimetric titrations. This reaction has been well characterized (11,12), and the materials are cheap and readily available. The binding is quite weak and exothermic with:

$$\Delta H = -5.81(\pm 0.2) \text{ kcal/mol } (-24.3[\pm 0.8] \text{ kJ/mol}) \quad (4)$$

and binding constant

$$K = 41(\pm 3) \text{ M}^{-1} (K_d = 25 \pm 2 \text{ mM}) \quad (5)$$

3.1.1. Materials

Hen egg-white lysozyme (Sigma), *N*-acetylglucosamine (Sigma, St. Louis, MO). Buffer: 0.1M Na acetate adjusted to pH 5.0 with acetic acid (2–3 L).

3.1.2. Procedure

1. Prepare a lysozyme solution in buffer (about 10 mL, 5–10 mg/mL are adequate), and dialyze overnight in the cold against 2–3 L of buffer. Retain

this dialysis buffer for later dilutions. (Some protein samples may require centrifugation to remove insoluble material at this stage.)

2. Meanwhile, prepare the NAG solution, about 500 mM in the same buffer, and allow to stand for 3 h or more at room temperature to establish mutarotation equilibrium. (The α - and β -anomers of *N*-acetylglucosamine bind somewhat differently to lysozyme [11].)
3. Prior to the experiment, briefly degas the lysozyme, NAG, and a sample of buffer solution. This is best done by placing the samples in small tubes or vials in a vacuum desiccator on a magnetic stirrer, evacuated with a water aspirator pump. Take care not to let the samples boil—just long enough for small air bubbles to be expressed.
4. Prepare the calorimeter for titrations at 25°C. Each titration will involve about 20 injections of NAG solution, about 5 μ L each, into lysozyme or buffer solutions (1.5–2 mL).
5. Determine the concentration of the lysozyme solution by measuring the absorbance at 280 nm of accurately diluted aliquots (20–30-fold dilution normally required). A 1 mg/mL solution has an A_{280} of 2.65. Take the lysozyme mol wt to be 14,600.

The following procedures are specific for the Omega titration calorimeter, since this is currently the instrument of choice for simple binding studies, but the description will serve as a useful guide for other systems. Bear in mind that some other systems are more versatile in that heats of dilution may be (partially) compensated directly by use of simultaneous titrations into a reference cell containing just buffer.

6. Totally fill the Omega sample cell with lysozyme solution, being careful to remove any bubbles that would produce noisy data. About 2 mL will be required. This is best done using a 2-mL disposable syringe fitted with tubing long enough to reach to the bottom of the cell (small animal cannula with Luer fitting is ideal). Load the syringe with solution, and express any bubbles. Insert the tube down the filling port of the calorimeter cell until it just touches the bottom. Inject the sample slowly, but firmly. The last few microliters should be injected rapidly to dislodge any bubbles, and this last step should be repeated several times.
7. Allow the sample cell to regain temperature equilibrium. This may take some time, especially if the sample is substantially cooler than the calorimeter. Equilibration may be accelerated by warming the sample to near the required temperature before loading—e.g., by using the heat of the hand around the loading syringe.
8. Meanwhile, totally fill the 100- μ L titration syringe with NAG solution, avoiding bubbles. Rinse the syringe tip with buffer, and wipe dry.

9. Insert the full titration syringe into its rotating mount, and insert the assembled unit into the calorimeter. Care should be taken not to bend the needle or displace the plunger as the syringe tip is inserted into the sample. The unit should be pushed down firmly to form a good seal against the upper O-rings.
10. Swing the stepper motor and stirrer drive assembly back over the syringe, and attach the stirrer drive belt. Clamp the assembly down, and lower the stepper motor drive until it barely touches the syringe plunger. Attach the holding clip between drive piston and plunger. Switch on the stirrer motor, and adjust to about 400 rpm.
11. Subsequent thermal equilibration should be quite rapid (5 min). Use the software options to select an injection schedule of $20 \times 5 \mu\text{L}$ injections, each of 30-s duration, 3–4 min between each. Start this injection schedule after a stable, noise-free baseline is obtained (50 $\mu\text{cal/s}/200 \mu\text{W}$ range). Typical experimental results are shown in Fig. 4.
12. Repeat this procedure using the cell/syringe combinations: lysozyme/buffer (to give protein dilution heats), buffer/NAG (NAG dilution), and buffer/buffer (mixing effects).
13. Data analysis: Integrate each of the peaks using the software options provided. This is usually straightforward, but some care is necessary in the choice of appropriate baselines for noisy or drifting data. In the present example (Fig. 4), the exothermic lysozyme/NAG titration peaks range from 560 down to 270 μcal (2.34–1.13 mJ) during the titration (Fig. 4A). The NAG dilution heat (Fig. 4C), although roughly constant, decreases consistently from about 260 to 230 μcal (1.09–0.96 mJ) under identical conditions, and the best results are obtained by subtracting each peak from its corresponding titration partner. Additions of buffer to lysozyme solution or buffer alone (Fig. 4B) give identical, negligible heat effects in this case. The resulting thermogram (total heat effect vs total ligand concentration) may then be analyzed using standard least-squares procedures to give K and ΔH . For weak binding situations such as this, it is usually necessary to fix the number of binding sites ($n = 1$) to give sensible results, since the shallow curvature of the thermogram (Fig. 4) means that the stoichiometry of the binding is poorly defined.

3.2. Calibration

Electrical calibration involving standard heater resistors is provided with most instruments, and is quite accurate and satisfactory for routine calibration of thermal sensitivity. More complete calibration checks, which test the complete system including sample volume and

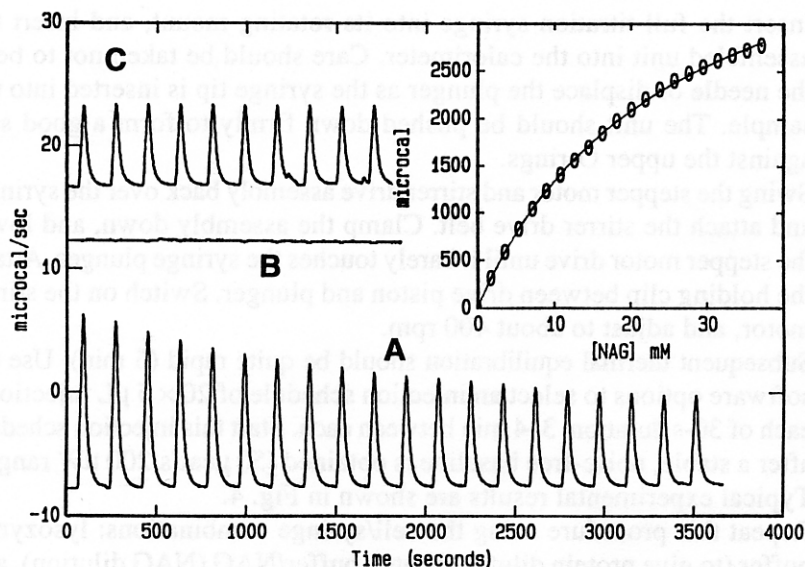


Fig. 4. Calorimetric titration of hen egg-white lysozyme (0.597 mM, 1.3963 mL) with *N*-acetylglucosamine (NAG), 0.1M acetate buffer, pH 5.0, 25°C using the Omega system. (A) 20 \times 5 μ L injections, 30-s duration, 3 min separation. (B) Lysozyme dilution, 10 \times 5 μ L injections of buffer into protein solution. (C) NAG dilution, 10 \times 5 μ L injections of 500 mM NAG into buffer solution. Insert: Integrated heat effects, corrected for dilution heats. The solid line is the least-squares fit for these data, calculated assuming a single binding site with $K = 40.2M^{-1}$, $\Delta H = -5.86$ kcal/mol (-24.5 kJ/mol).

titrant injection volumes, involve the use of standard reactions. The binding of *N*-acetylglucosamine to hen egg-white lysozyme (described in Section 3.1.) is now sufficiently well characterized (11,12) to act as a convenient test system. Simpler calibration reactions, giving larger heat effects, involve addition of standardized aqueous HCl to dilute Tris or NaOH solutions. For example, using the heat of ionization data at 25°C from ref. 13, each 5- μ L injection of 0.1M HCl into excess (10 mM) Tris base or NaOH solution should give exothermic heat effects of 23.7 mJ (5.67 mcal) and 27.9 mJ (6.67 mcal), respectively.

4. Additional Notes

1. Significant improvement in performance of earlier versions of both the Omega and LKB systems can be obtained by replacing the first-stage amplifier in the cell feedback circuit with a more stable and sensitive

system. Microcal originally recommended the Keithley Instruments Model 181 null detector, but a much more satisfactory (and considerably cheaper) amplifier is the Model N2a DC nanovoltmeter from EM Electronics (Brockenhurst, Hampshire SO42 7SJ, UK). Use of such an amplifier on the 1-mV range (i.e., preamplifier gain of 1000) reduces the RMS noise on the calorimetric signal (Omega, 2-s filter) to $<0.005 \mu\text{cal/s}$. Fitting of the alternative amplifier is straightforward provided one takes care to avoid introducing spurious thermal emfs—use only pure copper connectors, *never* solder, and so on.

2. Room temperature fluctuations can cause baseline distortions in all kinds of microcalorimeters, either directly by affecting thermostat temperature and uniformity, or indirectly by producing spurious thermal emfs in the sensing circuitry. Place the instrument away from drafts and windows. Temperature control of the instrument room can help, but temperature fluctuations of up to $\pm 1^\circ\text{C}$ can occur during the normal duty cycle of most air-conditioner systems and can cause cyclic fluctuations in calorimetric baselines.
3. Electrical interference is a major problem, especially with the passive heat-leak type of instrument (LKB, and so forth), where the calorimetric signal is normally in the microvolt range. This usually shows up as spikes associated with switching of refrigerators, heaters, and other inherently noisy electrical equipment—even fluorescent lighting, on occasions. Microcomputers (PCs, and so on) can also be very noisy, usually producing continuous background interference (“grass” rather than “spikes”). Stabilized power supplies, “spike filters,” screening of cables, and resiting of equipment can all help. Computer-based interference can sometimes be eliminated by switching the PC to another clock frequency.
4. Avoid β -mercaptoethanol in sample buffers. The slow air oxidation of this sulfhydryl reagent has a large associated heat effect that can cause large baseline offsets and drift. We have seen particularly large effects in calorimeters fitted with platinum reaction vessels, apparently because of Pt-catalyzed oxidation. Dithiols (DTT, DTE) present less of a problem since they are less prone to air oxidation.
5. Surprisingly large heat effects can arise from relatively minor microbial contamination of samples—the metabolic heat causing baseline offset and drift. Adopt clean working practices, and use Na azide in buffers if necessary. (**Caution:** Sodium azide is highly toxic. It also decomposes in acidic solutions liberating potentially harmful gas.)
6. Spurious and erratic behavior in continuous and stopped-flow reaction calorimeters can arise from temperature gradients in the system. This is a particular problem with older LKB-type instruments with air-bath ther-

- mostats, where it is virtually impossible to achieve sufficient thermal stability and uniformity for sensitive binding experiments (although these instruments are ideal for less demanding experiments). Performance of these older instruments can be improved dramatically by mounting them in a water bath thermostat, $\pm 0.001^\circ\text{C}$ or better, with efficient stirring.
7. Evaporation from poorly sealed sample or reference cells (LKB batch type) can cause erratic behavior. This is not usually a problem with total-fill-type cells (Omega), where the liquid meniscus is at the top of the inlet tube and away from the thermally active part of the cell.
 8. Long periods of equilibration with the injection syringe in place can lead to leakage or diffusion of ligand into the sample before the titration experiment starts. This effectively dilutes the first injection in the series and results in a lower than expected heat pulse. This can be minimized by delaying insertion of the loaded syringe until the sample cell has come almost to equilibrium after loading a new sample. Even so, trial experiments indicate that, in our hands, the first heat pulse of a titration series using the Omega 100- μL injection syringe might be up to 5% too low. Fortunately, the Omega software allows for this possibility with an option to compensate for the effect. Similar problems in LKB and other titration systems may be minimized by use of very fine bore delivery tubing.
 9. Heats of dilution depend on the change in concentration during the mixing process. This means that, in general, the dilution correction may be different for each injection in a titration series. The best procedure is to repeat each titration protocol with an identical injection sequence (omitting ligand or macromolecule, as appropriate) and to correct each binding heat pulse with its equivalent dilution partners in the sequence.
 10. In experiments where binding is sufficiently tight that saturation is achieved during the titration (as indicated by the constancy of heat pulses from later injections), these later heat effects may be taken as an estimate of the combined dilution and mixing effects. This is recommended only as an approximate procedure, since it does not correct for possible concentration dependence during the titration (*see* Note 9).
 11. Impurities: In theory, calorimetric experiments can tolerate any amount of contaminating material provided it does not interfere with the reaction of interest. This can be particularly useful with turbid samples, which might be difficult to study by spectroscopic methods. It is, however, necessary to know exactly the amount of active macromolecule in the sample, and unless unavoidable, extraneous impurities should be avoided. Also bear in mind that, at the relatively high protein concen-

- trations usually required, even trace contamination with other enzymes (proteases, nucleases, phosphatases, and so on) may catalyze unwanted side reactions with ligand and produce spurious heat effects.
12. Isothermal microcalorimetry is in principle nondestructive, and samples may be recovered and recycled after use. However, some denaturation of proteins (for example) may occur during long periods of stirring and/or equilibration in the calorimeter, and samples should be tested for viability after the experiment.
 13. Optimization of macromolecule and ligand concentration ranges for titration experiments is discussed in ref. 8.

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