ITC-Derived Binding Constants
Using Microgram Quantities of Protein

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CONTENTS

Isothermal Titration Calorimetry (ITC) has gained wide acceptance in drug discovery and development laboratories throughout the world. Every major pharmaceutical company and most biopharmaceutical companies and major research institutions are now utilizing ITC. Modern, highly sensitive ITC instruments are being applied in the drug discovery and development process for applications such as:

1. Selection of small molecule "hits" following primary and secondary screening of chemical libraries against protein targets of interest,
2. Optimization of small molecule leads based on elucidation of the binding mechanism and binding characteristics and development of structure-activity-relationships (SAR) that are used in the optimization process, and
3. Selection and optimization of therapeutic protein variants.

Since ITC directly measures the heat released or absorbed during a biomolecular binding event, it is the only technique which allows simultaneous determination of all binding parameters (n, K, ΔH and ΔS) in a single experiment [1]. The ITC technique provides this unique capability in an experimental environment that is completely label-free, in-solution and requires no immobilization of either the target macromolecule or ligand.

Modern instruments are highly sensitive, accurate and reproducible and unlike spectroscopic methods, the degree of optical clarity of the solutions is unimportant.

As modern ITC instrumentation has evolved, these instruments have become more sensitive, faster and easier to use. Binding parameters determined by ITC are often referred to as the "gold standard" values and are frequently used as reference standard values for other techniques.

Despite the fundamental advantages of this technique and the advances in instrumentation, use of ITC in the early, critical decision-making stages of drug discovery is often limited due to:

1. The lack of sufficient quantities of protein during those early stages and
2. The time required to perform the ITC experiments on large numbers of potential ligands (or protein constructs) of interest.

Traditionally 50 - 1500 ug of protein has been consumed to complete an ITC experiment and completing each experiment could require two hours or more.

In this Chapter we will describe the characteristics of a new miniaturized, ultrasensitive ITC that has been designed to push back these limitations allowing ITC to be effectively utilized at earlier stages of the drug discovery and development process. This new microcalorimetry system reduces the quantity of protein (or other macromolecule sample) required to obtain a complete thermodynamic profile (n, K, ΔH and ΔS) by up to 7-fold. When combined with modified experimental protocols, the protein sample requirements are reduced by as much as 70 fold as compared to traditional ITC experiments performed on

previous generations of instruments. In many instances the protein sample requirements are as low as single-digit µg quantities. With its reduced size and associated sample quantity requirements, this new ITC microcalorimeter provides a significantly faster response time allowing as many as 2-4 experiments to be completed per hour. The fully automated configuration of this new instrument provides a sample throughput of at least 50 samples per day with a capacity to process as many as 384 samples in an unattended run.

BINDING CONSTANTS AND THERMODYNAMICS FROM A SINGLE LABEL-FREE MEASUREMENT

Introduction to the ITC Experiment

ITC allows the complete thermodynamic profile (*n, K, ΔH and ΔS*) of a ligand-receptor interaction to be determined from a single label-free experiment. A titration naturally provides a systemic stepwise addition of one starting component of a ligand-receptor complex into the other starting component. During an ITC experiment, the incremental heat changes, microcalories (ucal), from each step of the titration are accurately measured and recorded. This heat data, combined with the known quantity of titrant (usually ligand) and known quantity of titrate (usually macromolecule), are fit using a non-linear least squares method to a binding model that yields the best fit values for the stoichiometry (*n*), binding constant (K) and heat of binding (enthalpy, ΔH) of the biomolecular interaction. A typical example of ITC raw data and the data fit to a binding model are shown in Figure 1.

As previously described [1], when characterizing interactions between a biological macromolecule, M, and a small ligand, X, or between macromolecules, i.e.,

$$M + X = MX$$
$$MX + X = MX_2$$
$$\vdots \qquad \vdots$$
$$MX_{n-1} + X = MX_n \qquad [1]$$

The parameters *n, K,* and *ΔH,* are the independent variables of thermodynamic interest [1]. The entropy *ΔS* and free energy *ΔG* of binding are dependent variables obtained by the calculation

$$\Delta G = -RT \ln K = \Delta H - T\Delta S \qquad [2]$$

The experimental parameter determined in the titration calorimeter is the differential heat dQ/dX_{tot} (actually $\Delta Q/\Delta X_{tot}$ for each discrete injection). The differential heat does not depend on the absolute value of M_t, but only on its value relative to K and X_{tot}.

For a reaction stoichiometry of 1:1, it can easily be shown that

$$1/V_0 (dQ/d[X]_{tot}) = \Delta H \left(\frac{1}{2} + \frac{1 - \frac{(1+r)}{2} - \frac{X_r}{2}}{\sqrt{X_r^2 - 2X_r(1-r) + (1+r)^2}} \right) \qquad (3)$$

where $[X]_{tot}$ is the total ligand concentration, free plus bound, in the reaction cell of volume V_0, Q is the heat absorbed or liberated, and ΔH is the molar heat of binding and r is defined below in Equation 4.

As mentioned above, the determination of the thermodynamic parameters from these heat data relies upon the nonlinear least squares fit of the data to an appropriate binding model. Detailed explanations of the ITC experiment, data analysis and data interpretation have been previously published and will only be touched upon briefly herein [2-7]. Figures 2 and 3 pictorially illustrate the portions of the thermogram (the fitted data) that correlate primarily to each of the independent thermodynamic variables and how changes in the thermogram are indicative of specific changes in the nature of the biomolecular interaction being studied.

Examination of equation 3 provides some useful insights into the optimal design of an ITC experiment. The right hand side of equation 3 contains two unitless parameters ($1/r$ and X_t) which depend on the total ligand concentration, $[X]_{tot}$ and the total macromolecule concentration, $[M]_{tot}$. These parameters are defined as

$$1/r = c = [M]_{tot} K \tag{4}$$

and

$$X_t = [X]_{tot} / [M]_{tot} . \tag{5}$$

The unitless parameter $1/r$ is the "c" parameter [1]. Provided that concentration is expressed as total concentration of binding sites, the shape of a binding curve for proteins with n identical sites will be exactly the same as for a molecule with a single binding site having the same K value. To account for this circumstance the c parameter is defined as

$$c = n \cdot [M]_{tot} \cdot K \tag{6}$$

The c parameter (sometimes referred to as the *stoichiometry*) may be used to pictorially represent the range of binding constants which can actually be measured quantitatively when one is forced to use low protein concentrations that challenge the sensitivity of the instrument. A general, informative plot can be made without using actual numerical values for n, K, ΔH, $[M]_{tot}$ or $[X]_{tot}$. Use of the c parameter allows generalizations to be made concerning the behavior of all binding reactions, and simplifies the process of making the best choice of the macromolecule concentration if the approximate binding constant is known [1,8,9].

Binding curves simulated from equation 3 may be generated for any selected values for c. Several examples are given in Figure 4. For very tight binding ($c = \infty$) all added ligand is bound until saturation occurs so that a rectangular curve of height ΔH is seen. The sharp transition occurs at the stoichiometric equivalence point, n, in the molar ratio. The shape of this curve is invariant with changes in K so long as the C value remains above ca. 5000. For moderately tight binding with c values between 1 and 1000 the shape of the binding isotherms are very sensitive to small changes in c values. The intercept of these curves on the ordinate is no longer exactly equal to ΔH but this parameter is still easily obtained by deconvolution from the total area under the curve and its shape. Very weak binding ($c <$

0.1) yields a nearly linear trace which again, like very tight binding, yields little information of the precise value of K. It is apparent from looking at these isotherms that their shape is sensitive to binding constant only for c values in the range $1 < c \leq 1000$. This range has been referred to as the "experimental K window". When available, the middle of the window from $c = 5$ to 500 is most ideal for measuring K. Later in this Chapter a new, highly sensitive calorimeter designed specifically to minimize protein consumption will be introduced and the useful experimental range of that calorimeter will be discussed. As will be seen, both the instrument sensitivity as well as the c value (or experimental K window) will be determinant in defining the use boundaries of this (and all other) microcalorimetric instruments.

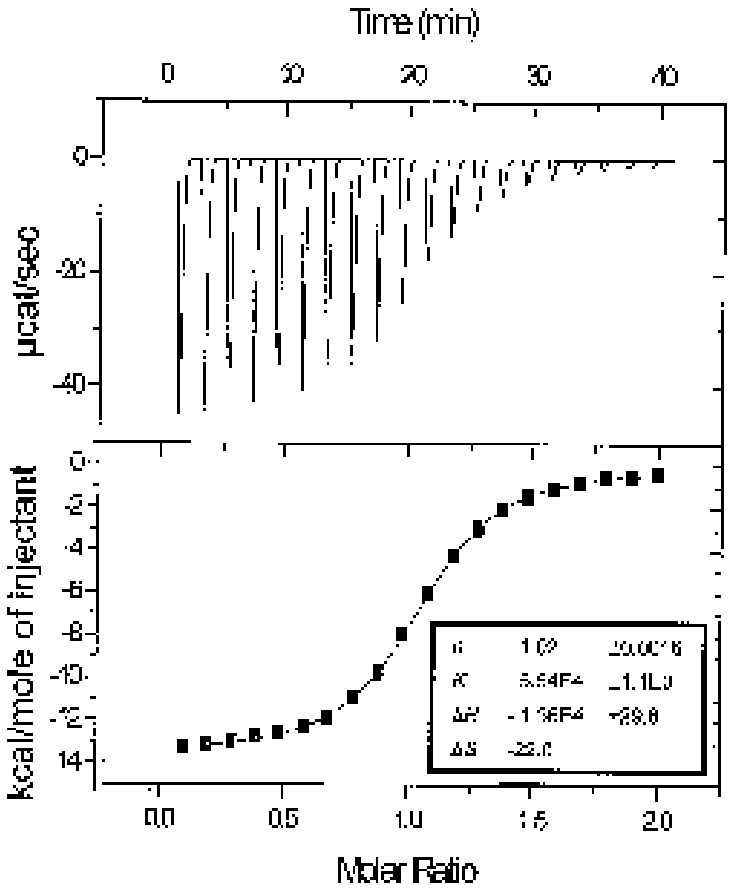


Figure 1. Top panel illustrates the raw ITC from an experiment of 20 equal injections of a ligand solution into a macromolecule solution. Bottom panel illustrates the non-linear least squares fit of the peak area data from the top panel to a binding model.

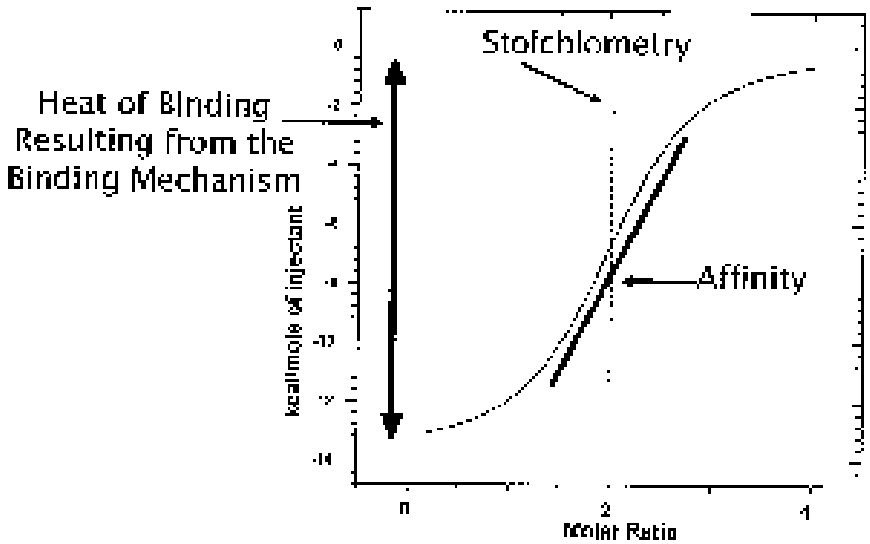


Figure 2 Pictorial representation of the correlation of the independent variables of thermodynamic interest to the fitted thermogram

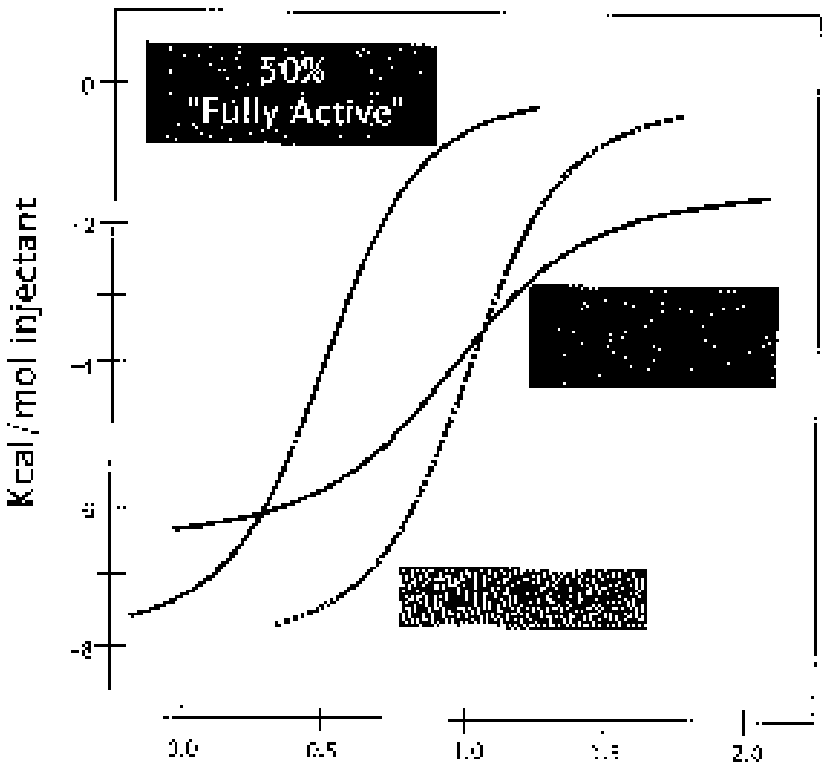


Figure 3. Observed changes in the binding thermogram for a specific macromolecule-ligand system are easily interpreted and indicative of specific changes in the specific biomolecular interaction. A representative thermogram for a "fully active" two component binding system is compared to the thermogram resulting from the same system after some loss of activity (decrease in ΔH) versus the thermogram that would result from loss of material, but where the remaining material is fully active.

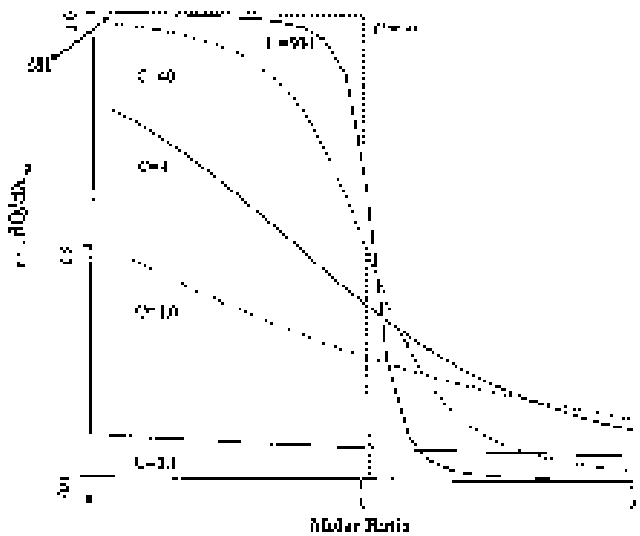


Figure 4. Scatchard binding isotherms for various values of the parameter c (equal to the product of the binding constant times the total macromolecule concentration), presented in derivative format (see text for details).

Application Example - Binding Thermodynamics of Statins to HMG-CoA Reductase

An excellent and representative example of the application of ITC to the binding characterization of a class of drugs to a protein target was recently published by Carbonell and Freire, "The Binding Thermodynamics of Statins to HMG-CoA Reductase" [10]. HMG-CoA reductase is the key enzyme in the cholesterol biosynthetic pathway that leads to hypercholesterolemia, the most important risk factor for cardiovascular disease. The statins, as a drug class, are the most widely prescribed of all drugs and have been demonstrated to be powerful inhibitors of HMG-CoA reductase. The structures of the five statins examined in this study are displayed in Figure 5. Each of these highly related drugs has a unique safety and efficacy profile. ITC was employed to dissect the binding mechanism of these compounds to the protein target. Since many issues related to a lack of selectivity are a result of nonspecific binding to unwanted targets, it is important to explore how these drugs bind to their intended targets, the nature of the forces that drive the binding reaction, and whether there are any correlations between their binding mechanism and their selectivity. Since the thermodynamic signature of an inhibitor reflects the types of interactions that drive the binding reaction, it can become an important tool for assessing the potential for a compound to associate non-specifically with other proteins.

The raw calorimetric data and binding thermograms are displayed in Figure 6 and the associated thermodynamic parameters are shown in Table 1. The proportion by which the binding enthalpy contributes to the binding affinity is not the same for all statins as illustrated in Figure 7. At 25°C, for fluvastatin, pravastatin, cerivastatin, and atorvastatin the dominant contribution to the binding affinity is the entropy change. Only for rosuvastatin does the enthalpy change contribute more than 50% of the total binding energy (76%).

The differences in the proportion by which the enthalpy and entropy changes contribute to the binding affinity reflect differences in the type of interactions established between the various statins and HMG-CoA reductase. Since the crystal structures of the complexes with rosuvastatin, atorvastatin, cerivastatin, and fluvastatin are known [11], it was possible to analyze the binding thermodynamics of these statins in terms of structure. All statins target the same site in the protein and according to the crystallographic structures do not cause differential conformational rearrangements in the enzyme that may significantly influence the binding energetics. Accordingly, differences in binding energetics can be attributed to different inhibitor-enzyme interactions. Furthermore, since the HMG region is the same in all inhibitors included in these studies, differences can be attributed to the so-called "hydrophobic region".

The binding enthalpy primarily reflects the strength of the hydrogen bonds and van der Waals forces, between the inhibitor and enzyme, relative to those with water. These forces tend to have directionality and can impose specificity as well as affinity to an interaction. On the other hand, the major favorable contributions to affinity from the entropic component originates primarily from the release of water molecules from the binding interface. This effect reflects the

repulsion of the inhibitor from the solvent rather than an attractive inhibitor-enzyme interaction. As such, it is intrinsically nonspecific and requires specificity only by combining it with shape complementarity of the inhibitor and binding cavity. Another source of binding entropy is related to changes in conformational degrees of freedom. As these changes usually involve fixing the inhibitor and certain regions of the protein in more restricted conformations, they lead to a diminished conformational entropy and therefore an unfavorable contribution to the binding affinity. This loss in conformational entropy is minimized, however, by conformationally constraining and pre-shaping the inhibitor to its bound conformation. Other entropy contributions such as those associated with translational degrees of freedom are the same for all inhibitors and do not contribute to differences in binding affinity.

Combining the thermodynamic information obtained from ITC with knowledge of the structure allows this type of detailed understanding of the binding mechanism and associated differences even among members of a very closely related class of compounds.

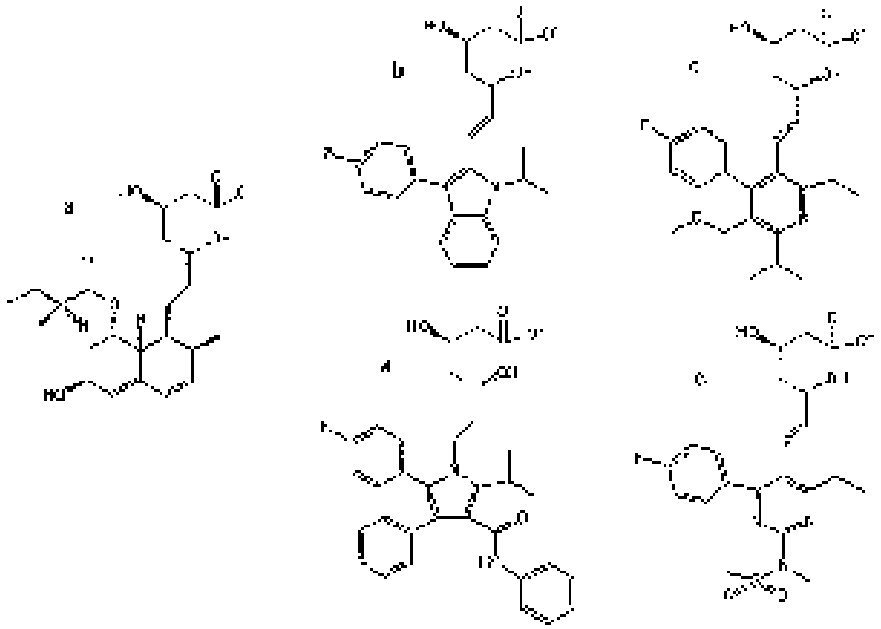


Figure 5 Chemical structures of the statins considered in these studies. (a) pravastatin, (b) fluvastatin, (c) atorvastatin, (d) simvastatin, and (e) rosuvastatin. The HMG moiety common to all statins is colored red and the variable hydrophobic region black.

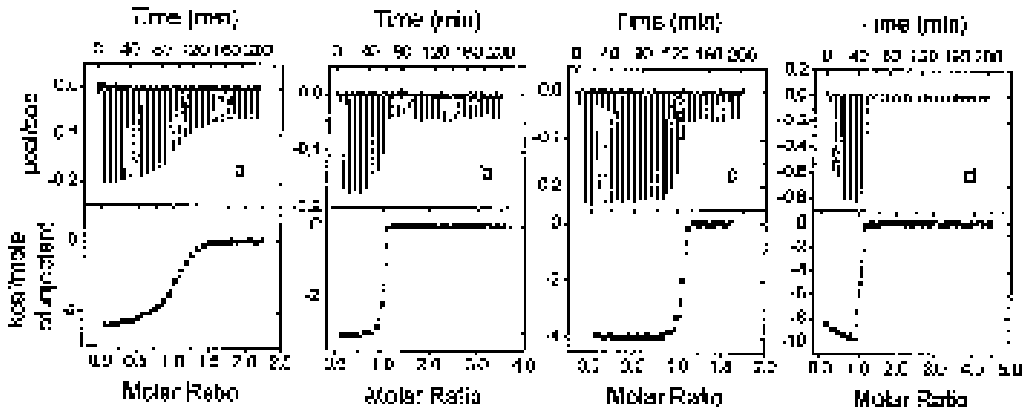


Figure 6 Calorimetric titration of HMG-CoA reductase with pravastatin (a), cerivastatin (b), atorvastatin (c), and rosuvastatin (d). The experiments were performed in triplicate at 25°C in 20 mM Tris HCl (pH 7.5), 2 mM DTT, 1 mM NADPH, and 2% DMSO.

Table 1
Thermodynamic Parameters Associated with Binding of Statin to HMG-CoA Reductase[a]

Statin	ΔG (kcal/mol)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)
Fluvastatin	-9.0 ± 0.1	-0[b]	--9.0
Pravastatin	-9.7 ± 0.4	-2.5 ± 0.1	-7.2 ± 0.4
Cerivastatin	-11.4 ± 0.4	-3.3 ± 0.2	-8.1 ± 0.4
Atorvastatin	-10.9 ± 0.8	-4.3 ± 0.1	-6.6 ± 0.6
Rosuvastatin	-13.3 ± 0.7	-0.2 ± 0.1	-13.0 ± 0.7

[a] *The values for ΔG, ΔH, and $-T\Delta S$ are quoted at 25°C.* [b] *The binding enthalpy of fluvastatin was beyond the detection limit of the instrument, suggesting that it is close to zero, in which case most of the binding energy is entropic in origin. The free energy of binding was derived from enzyme inhibition assays.* [c] *Not determined.*

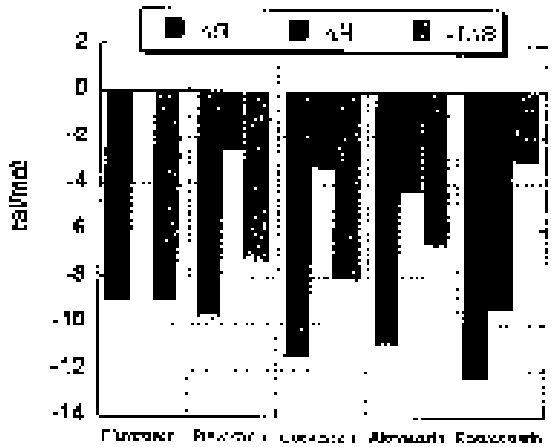


Figure 3. Proportion by which the binding enthalpy contributes to the Gibbs energy of binding for each of the statins that was studied.

ITC INSTRUMENT DESIGN – MINIATURIZATION DEVELOPMENTS

A new generation isothermal titration calorimeter, the ITC_{200}, of a design similar to that originally described by Wiseman et al has been developed [1]. Nearly a thousand of the prior generation instrument, the VP-ITC, are currently in operation. Similar to the VP-ITC, the ITC_{200} measures the heat evolved or absorbed upon mixing precise incremental amounts of a titrant into a macromolecule solution. A spinning syringe is used for injecting and mixing of reactants.

A pair of identical coin-shaped cells is enclosed in an adiabatic Outer Shield (Jacket). Access stems travel from the top exterior of the instrument to the cells. Both cells are filled with liquid during operation and both cells have a working volume of 200 µL – a seven-fold reduction in working volume from the prior generation VP-ITC instrument. The syringe volume has been correspondingly reduced to 40 µL with minimum injection volumes as low as 0.1 µL. A block diagram of the instrument is shown in Figure 8 and the ITC_{200} is shown in Figures 9 and 10.

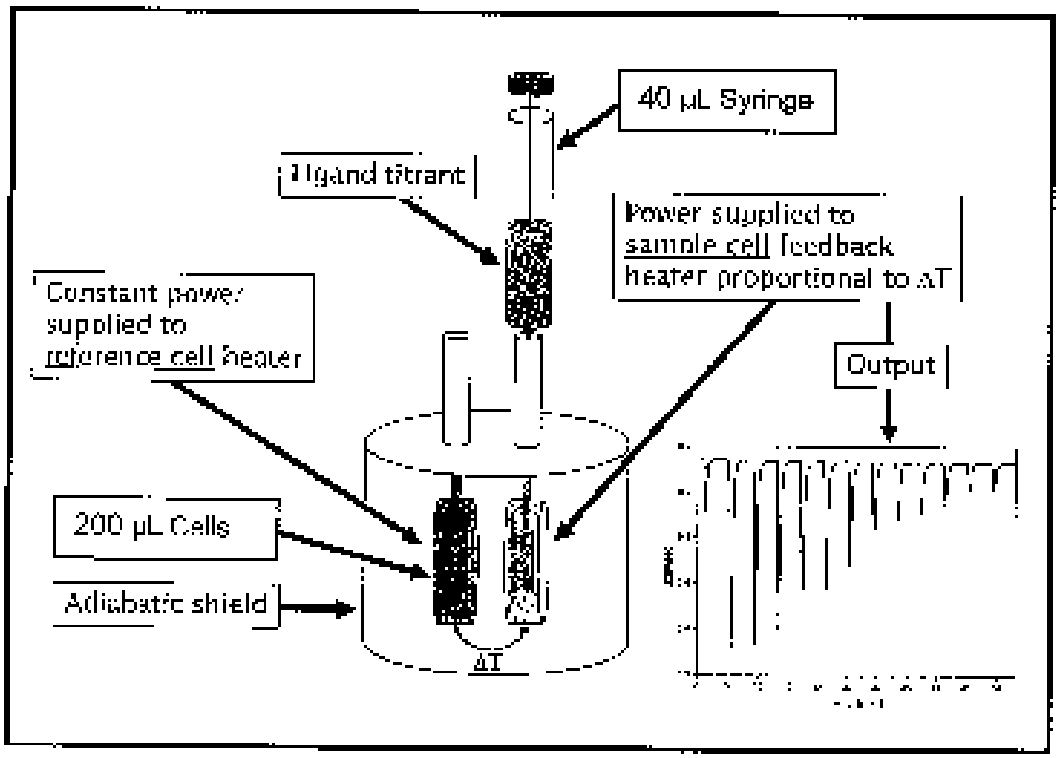


Figure 8. Block diagram of the isothermal titration calorimetry (ITC) instrument. The sample and reference cells are maintained at a constant temperature inside the inner-most shield of the adiabatic jacket. The reference cell is always kept at the experimental temperature. One of the components of the interaction is placed in the syringe and the other in the cell. When an injection is made, the change in heat associated with binding (endothermic or exothermic) results in a change in temperature in the sample cell. A change in power (heater) is required to return the cells to identical temperatures (T_r i.e. $\Delta T = 0$). This change in power is recorded as a series of injections is made. As the course of injections is completed, the binding sites on the sample in the cell are gradually saturated, and the effect becomes reduced [1, 12].

As the titration progresses, temperature differences between the reference cell and the sample cell are measured, calibrated to differential power (DP) units and then displayed and saved as raw data (see Figure 1). This signal is sometimes referred to as the "feedback" power used to maintain temperature equilibrium. Calibration of this signal is obtained electrically by administering a known quantity of power through a resistive heater element located on the sample cell.

The syringe containing a "ligand" solution is titrated into the cell containing the "macromolecule" solution. An injection resulting in the evolution of heat (exothermic) within the sample cell causes a negative change in the DP power since the heat evolved chemically provides heat that the DP feedback is no longer required to provide. The opposite is true for endothermic reactions. Since the DP has units of power, the time integral of the peak yields a measurement of thermal energy, ΔH. This heat is released or absorbed in direct proportion to the amount of binding that occurs. When the macromolecule in the cell becomes saturated with ligand, the heat signal diminishes until only the background heat of dilution is observed.

The entire experiment takes place under computer control including analysis of the ITC raw data using non-linear least squares fitting models to calculate reaction stoichiometry (n), binding constant (Ka) and enthalpy (ΔH).

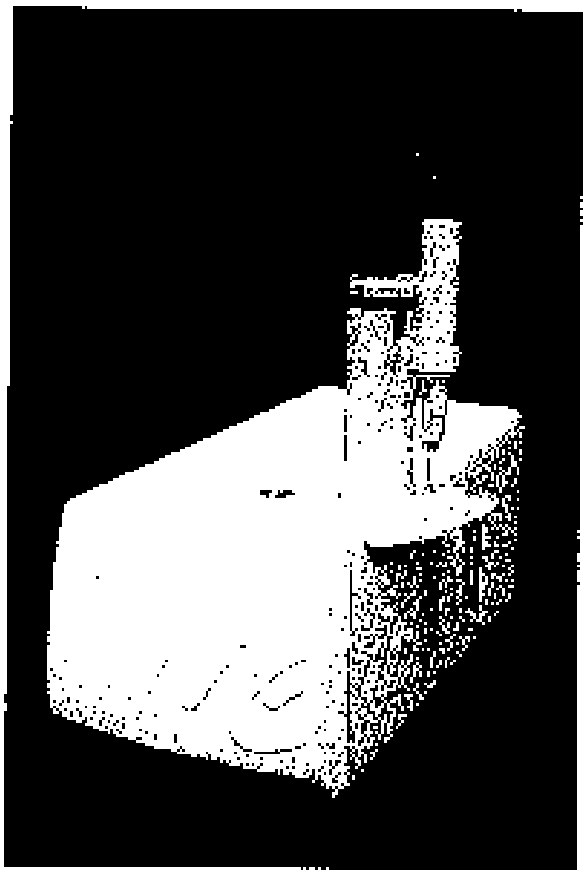


Figure 9. The ITC$_{200}$ utilizes new 200 µL sample and reference cells and a new 40 µL syringe capable of injection volumes as small as 0.1 µL. A "pipette tower" is used to accurately and reproducibly position the computer controlled pipette and syringe stirrer into the cell port, the sample loading station, and the wash station.

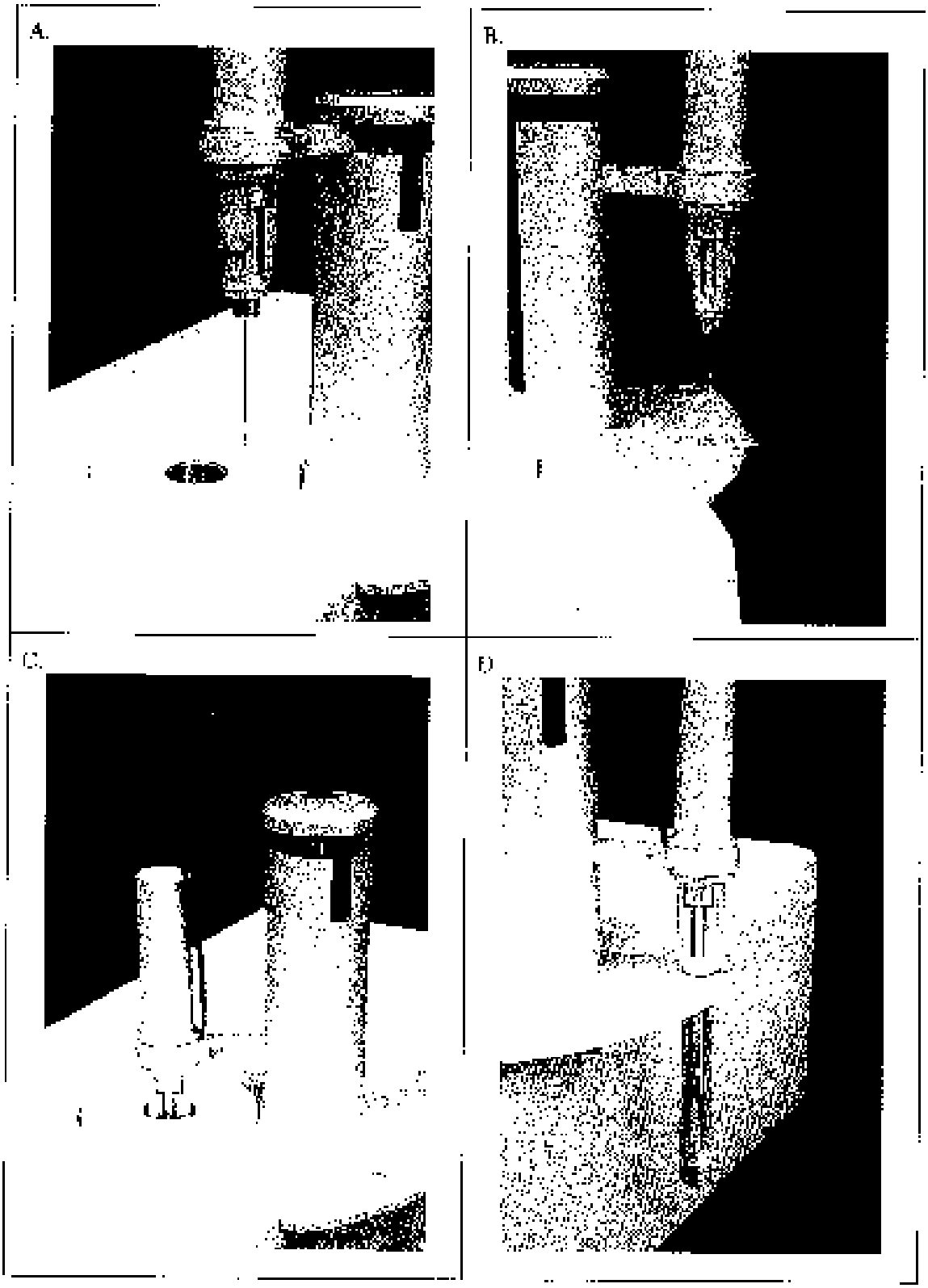


Figure 10. The computer controlled pipette and syringe stirring mechanism is totally supported by and positioned by the pipette tower for all functional operations. A. Pipette lowered to the rest position. B. Pipette moved to the sample loading station to aquire sample from a micro centrifuge tube; C. pipette positioned in the cell for the titration; D. pipette positioned in the syringe and stirrer wash station

Latest developments in ITC miniaturization

The objectives of miniaturization are twofold; first is to significantly reduce protein quantity requirements and second to increase sample throughput. The minimum amount of protein that can be used in an ITC experiment may be limited by either the minimum protein concentration required to achieve a "c value" within the experimental window, or by the mass of protein required to produce at least the minimal detectable heat signal for the ITC injections. These concepts have been well developed in the literature and their application to this new ITC microcalorimetry system will be briefly reviewed later in this Chapter [2, 8, 9, 15-18].

Reduction in sample quantity requirements

Reducing the required protein sample quantity requires first a reduction in the sample and reference cell volumes, second a reduction in the minimum precise injection volume to sub-microliter levels, and finally an increase in the absolute instrument sensitivity.

The first step in achieving this next generation ITC capability was to decrease the reference and sample titration cell volumes from 1.4 mL to 0.2 mL. To put this into perspective, a 1.4mL stainless steel (hastelloy) cell weighs approximately 8.5 g. The completely fabricated and assembled 200 µL hastelloy cell weighs approximately 0.95 g. The 1.4 ml and 200 µL cells are shown in Figures 11 and 12. As will be seen later, this reduced cell mass and reduced sample mass provide the added benefit of faster instrument response time.

Figure 11. Comparative sizes of the 1.4 mL and 0.2 mL Hastelloy cells used as sample and reference cells in the VP-ITC and iTC$_{200}$ instruments, respectively. The 0.2 mL cell weighs less than 1 g.

Figure 12. Cut-away view of the traditional 1.4 mL and new 0.2 mL cells with spinning syringe and stirrer mechanism inserted.

The second requirement for achieving reduced protein sample quantities is a reduction in the minimum precise injection volume to sub-microliter levels. The seven-fold reduction in sample cell volume is accompanied by an equivalent reduction in the ligand syringe volume and minimum injection volumes. The ITC$_{200}$ pipette performs automated injections of 0.1 -1.0 µL with an accuracy of 1 3% and injections of 1.0 µL or greater with an accuracy of better than 1%. Figure 13 and Table 2 illustrate the reproducibility of typical small volume injections.

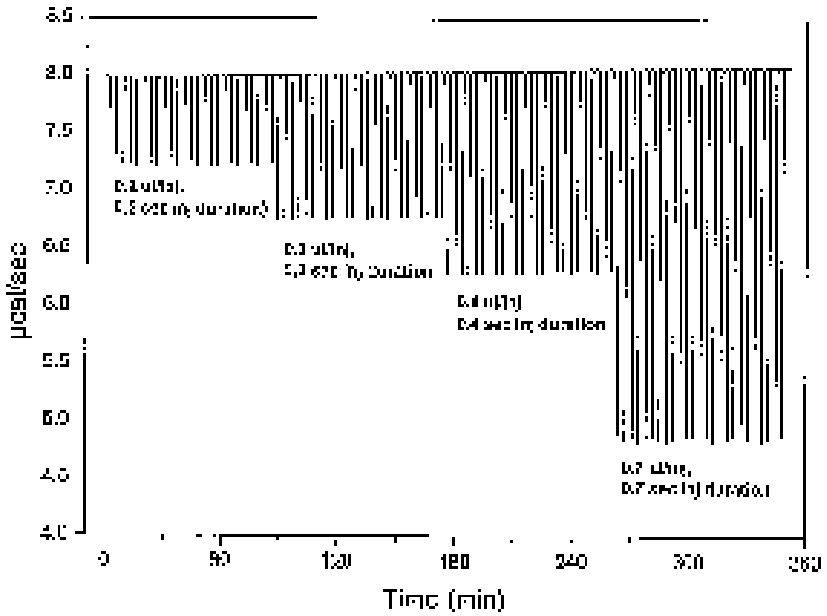


Figure 13. *Titration of 2.0 mM succinic acid into 5 mM NaOH at 30°C. Injection volumes range from 0.2 µL per injection to 0.7 µL per injection. The precision of the 0.2 µL, 0.3µL, 0.4µL and 0.7µL injections were 1.9%, 1.2%, 0.6% and 0.8% RSD, respectively.*

Table 2

ITC_{200} Injector Volume Delivery
Succinic Acid & NaOH Titration at 30°C

Injection volume (µL/inj)	*Succinic acid concentration (mM)*	*Injection Number*	*Injection Period (min)*	*Standard Deviation (%)*
0.2	2.0	25	3.5	1.5
0.3	2.0	25	3.5	1.2
0.4	2.0	25	3.5	0.6
0.5	1.0	25	3.5	1.8
0.7	2.0	25	3.5	0.7
1.0	1.0	25	3.0	0.1
2.0	1.0	20	2.0	0.4

Stirring rate: 500 rpm, 3 sec filter, 1 µL per sec injection duration, 5 mM NaOH in the cell for all titrations.

The third requirement for achieving reduced protein sample quantities is an increase in absolute instrument sensitivity.

There are two sensitivity parameters which are important when comparing calorimeters [3]. The absolute detection limit, S (µcal), is proportional to the minimum total mass of macromolecular solute which must be used to produce a detectable signal, while the volume-normalized sensitivity S/V (µcal/mL) is proportional to the minimum concentration of solute necessary to produce a detectable signal. Modern ultrasensitive microcalorimeters are hundreds to thousands of times more sensitive than conventional calorimeters and the ITC_{200} is the most sensitive microcalorimeter ever developed [3, 19-23]. It has been specifically designed to address the requirements of drug discovery and development. Previous generations of ITC microcalorimeters have S values of 0.7 – 0.1 µcal [1]. The present instrument has an S value of 0.04 µcal. Table 3 compares the sensitivities and volume-normalized sensitivities of the VP-ITC and ITC_{200} instruments.

Table 3

Comparative Sensitivities and Volume Normalized Sensitivities

Microcalorimeter	S (µcal)	V (mL)	S/V
VP-ITC	0.10	1.4	0.07
ITC_{200}	0.04	0.2	0.20

As can be seen in Table 3, the iTC$_{200}$ is approximately 2½ times more sensitive than the VP-ITC in absolute sensitivity, and has a cell volume seven times smaller. A key question is then, what are the operating and performance consequences of a 2½ times greater absolute sensitivity combined with a 7-fold smaller cell volume.

As will be described later in this Chapter, the answer to this question is found in the intended use of the instrument. These instrument parameters enable some ITC applications that would otherwise be impossible, while for other applications, an ITC with a lesser absolute sensitivity, but a superior volume-normalized sensitivity will be more appropriate.

Increases in sample throughput

Sample throughput is dependent on both the length of time required to complete the titrations and the sample change-over and re-equilibration time between experiments. The rapid calorimetric response time, and fast thermal equilibration time in the 0.2 mL cell together serve to increase overall sample throughput.

Figure 14 illustrates the comparative instrument response times as measured by the ½ time response to a 3 second heat pulse. As can be seen in Figure 15, this faster instrument response time results in higher and narrower peaks for equivalent heat signals, and a correspondingly faster return to baseline. Depending on the binding reaction kinetics of the system being studied, faster instrument response to heat signals and a faster return to baseline, may allow the time between injections and hence overall titration time to be reduced.

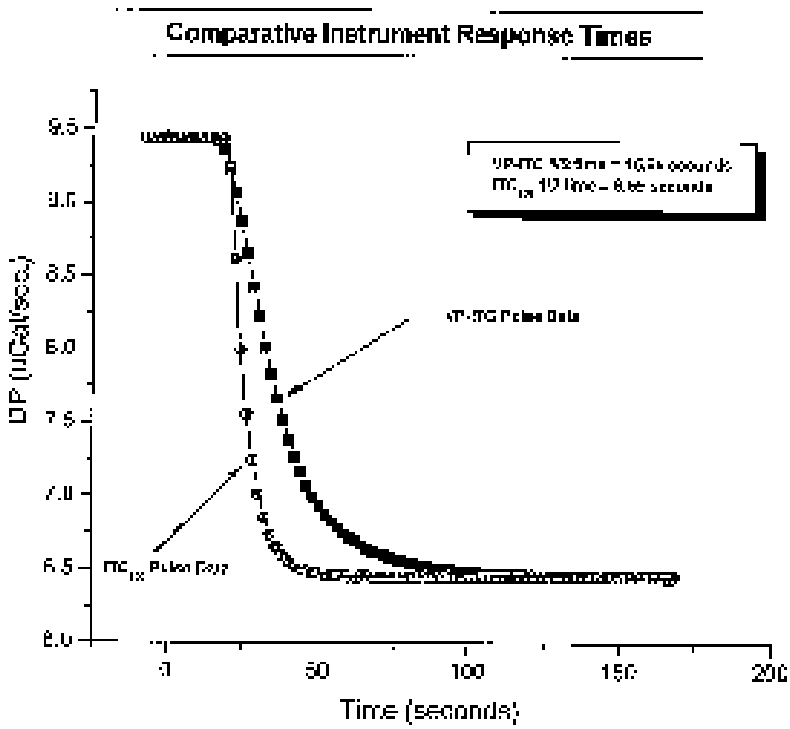


Figure 14 VP-ITC and ITC$_{18}$ calibration pulse data to compare instrument response times. Pulse Size = -2 μCal/sec., Filter Period = 2 seconds; Both pulses fit to 1st order exponential decay model. VP-ITC τ Time = 10.96 seconds; ITC$_{18}$ τ Time = 6.59 seconds.

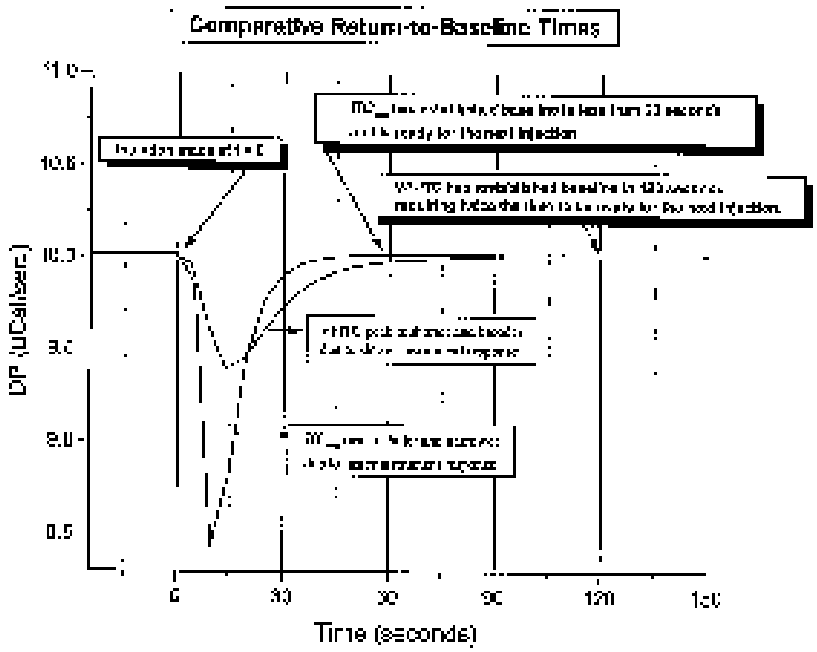


Figure 13. The ITC_{200} with its faster instrument response time returns more quickly to baseline and therefore requires less time between injections. In this example, both peaks are of equal area representing approximately 25µcal.

Faster instrument response to heat events and faster instrument return-to-baseline, reduce time required for each injection and the time delay between injections and hence allow the entire titration to proceed more rapidly. Overall sample throughput rates can be further increased by reducing the instrument equilibration time between experiments. Figure 16 illustrates the reduction in time required for the for the 0.2 mL cell to achieve a 1°C temperature change and Figure 17 illustrates the time reduction for instrument equilibration.

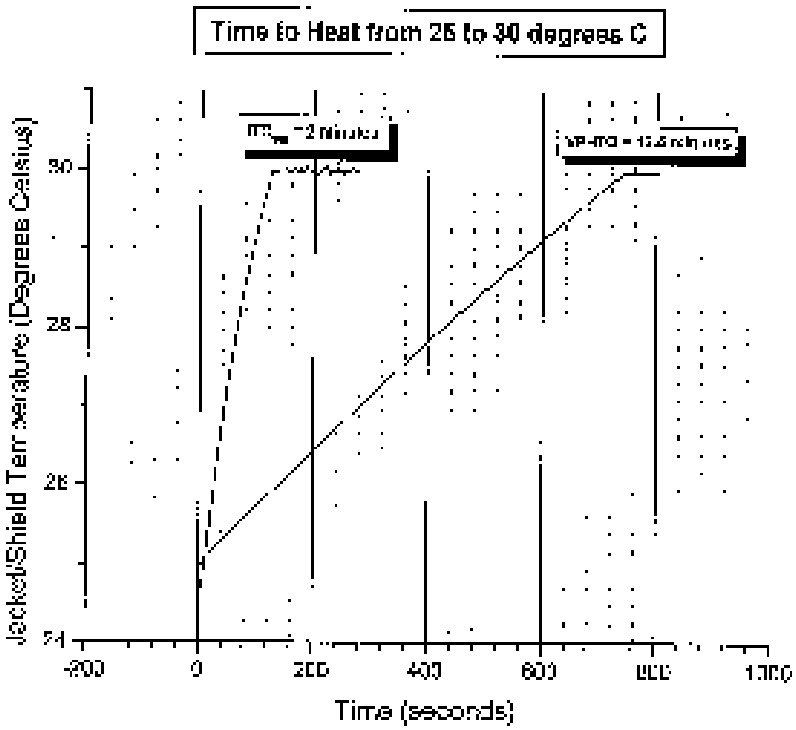


Figure 16. The $^{13}C_{13}$ with sample and reference cell masses of less than 1 g require less than 25 percent of the time as the VP-DSC to temperature stabilize following a 5°C change in temperature

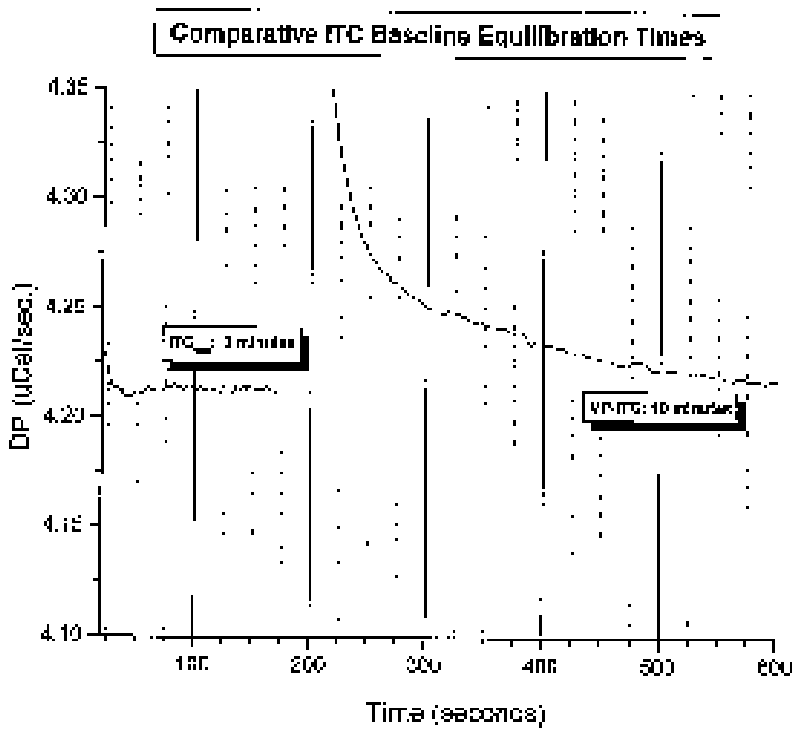


Figure 17. Comparative ITC baseline equilibration times. No temperature change required, only the time required to equilibrate after experimental temperature has been achieved.

Table 4 illustrates the effect of the faster instrument response and equilibration times on typical ITC experiment turnaround times. Sample throughput rates are doubled with the iTC_{200} and can be doubled again by reducing the number of injections from the traditional 24 to 12. With the automated instrument sample throughput rates of 48 -60 samples per day can be achieved.

Table 4

ITC Sample Processing Times

FUNCTION	VP-ITC		iTC_{200}	
# of injections	24	12	24	12
Cleaning (minutes)	8	8	6	6
Loading (minutes)	2	2	2	2
Equilibration (minutes)	20	20	4	4
Titration (minutes)	96	48	36	18
TOTAL (minutes)	126	78	50	32

Low Protein Quantity Data Comparison

For the purposes of data comparison to the VP-ITC, results are presented for the binding of cytidine 2'-monophosphate (2'CMP) to the active site of ribonuclease A.

Figure 18 depicts a direct comparison of the VP-ITC to the ITC_{200}. Due to the cell volume difference (1.4 mL for the VP-ITC and 0.2 mL for the ITC_{200}) protein usage was reduced 7-fold for the ITC_{200}. The effect of the 2½ fold increase in sensitivity is apparent in this data. Peak heights of approximately 7 µcal/sec in the VP-ITC are reduced to only about 2.8 µcal/sec the ITC_{200} even though the cell volume, and therefore total heat produced, has been reduced 7-fold.

Since the volume requirements for loading the two instruments vary similarly, both the protein usage and the ligand usage were reduced approximately 7-fold. For the VP-ITC, 2.6 mL of material was used to load the cell and at least 0.5 mL to wash the syringe. The ITC_{200} required approximately 0.3 mL to load the cell and 50 µL to load the syringe.

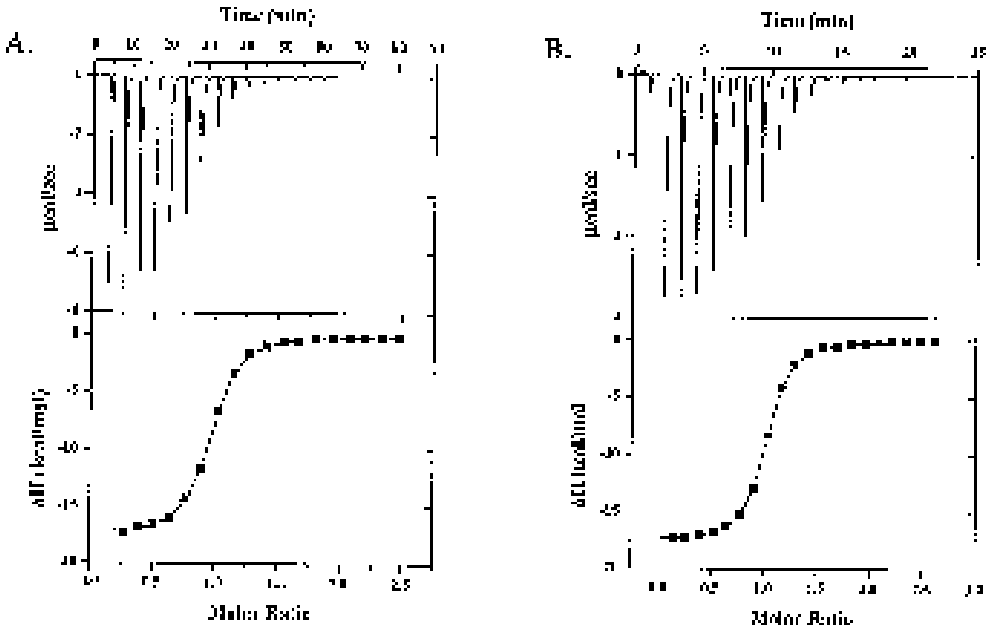


Figure 18. Comparison of the VP-ITC and ITC_{200}. Raw and processed data from the reaction of RNASE with 2'CMP in 50 mM potassium acetate buffer pH 5.5 (25° C). The VP-ITC cell volume = 1.4 mL and the syringe volume = 275 µL. The ITC_{200} cell volume = 200 µL and the syringe volume = 40 µL. Concentrations for the titrations were [RNASE] = 0.96 mM, [2'CMP] = 2.16 mM. Top panels show raw data following baseline subtraction. Bottom panels show processed data corresponding to each raw data set (solid squares) and fit obtained (solid lines). Fitted parameters are given in Table 2. A. Titration on the VP-ITC; 20 x 5 µL injections. B. Titration on the ITC_{200}; 20 x 1.7 µL injections.

Table 5 summarizes the data for the comparison of the ITC microcalorimeters using the 1.4 mL and 0.2 mL sample cells. The values shown for K and ΔH are the averages from three replicates. For the purposes of this comparison the concentrations of RNAse and 2'CMP were the same, and resulted in a c value of 84. The thermodynamic parameters, K_a and ΔH, are in good agreement with each other and with their known values. The protein usage was reduced from 1,194 µg using the 1.4 mL sample cells to 164 µg using 0.2 mL sample cells.

Table 5.

Comparison of RNASE-2'CMP ITC Results Using the 1.4 mL and 0.2 mL Sample Cells

VP ITC[1]	14.0 ± 0.8	0.82 ± 0.04	-17.5 ± 0.1	84	1194	80
iTC_{200}[2]	13.1 ± 1	0.97 ± 0.02	-17.1 ± 0.4	84	164	25

[1][RNASE] = 0.06 mM, [2'CMP] = 2.18 mM, 26 x 5 µL injections.
[2][RNASE] = 0.06 mM, [2'CMP] = 2.18 mM, 26 x 0.688 µL injections.

The key question is, how much can the protein quantity be reduced in the ITC experiment while still generating useful data? The answer, as presented earlier in this Chapter and elsewhere [1, 8, 9], is that it depends on the value of ΔH which will ultimately limit the amount of heat generated per injection, and on K which will limit the c value as the macromolecule concentration is reduced. The lowest possible protein usage for every ITC experiment will be limited by either:

1. The heat per injection, at the selected concentration and cell volume, remaining above the absolute instrument sensitivity, or
2. The c value, at the selected concentration, remaining above a minimum level to achieve a successful and unique least-squares fit to the experimental data.

ITC INSTRUMENT DESIGN – AUTOMATION DEVELOPMENTS

With its 2½-fold increase in absolute sensitivity and 7-fold reduction in sample cell volume the ITC_{200} microcalorimeter provides:

1. A significant reduction in sample quantity requirements
2. A significantly faster response time and sample throughput rate

A fully automated version of the ITC_{200} microcalorimeter is being developed that will provide a sample throughput rate of up to 50 samples per day with a capacity to process as many as 384 samples in an unattended run.

The automated system will utilize the same microcalorimeter core as the ITC_{200} allowing an ITC_{200} to be converted to the fully automated system at any time. Although not "high throughput" from a drug discovery primary screening standpoint, this automated microcalorimetry system provides for the first time access to true thermodynamic profile data using samples quantities and a throughput rate appropriate for secondary and confirmatory screening.

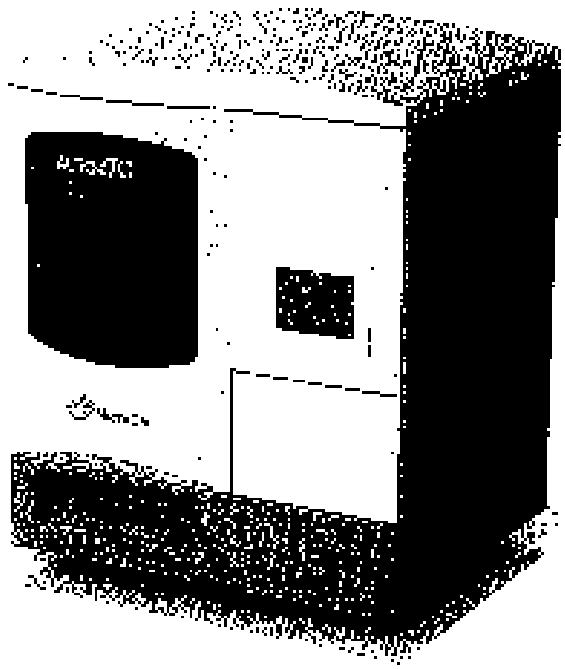


Figure 19 – The automated ITC_{200} will process up to 50 samples per day and up to 384 samples during an unattended run.

ITC EXPERIMENT DESIGN CONSIDERATIONS

It is the usual case that a determination of the thermodynamic parameters (n, K, ΔH and ΔS), or at a minimum K, are desired and their determination as desired using the minimal amount of protein. In the early, decision-making stages of the drug discovery process, the availability of target protein is usually very limited and there is a large demand for it to serve the needs of a large number of testing procedures. As has been illustrated herein, the advent of a highly sensitive microcalorimeter with a 0.2 mL cell volume offers the possibility to greatly reduce the minimum protein requirements. This possibility can best be realized by optimizing the design of the ITC experiment, the protein binding system being studied and the information desired from the experimental results.

Experiment Design Alternatives to Minimize Protein Usage

For the RNAse-2'CMP system, we have explored the impact of alternative ITC experiment designs on the experimentally determined thermodynamic parameters. Figure 20 displays three different types of experimental approaches to the (RNAse-2'CMP) experiment using the ITC$_{200}$; the traditional multiple (20 injections) injection method, a reduced injection number (5 injections) method, and the single injection method. For each experiment, only 9 µg of RNAse was loaded into the sample cell. As can be seen from the results presented in Table 6, using as little as 9 µg of RNAse (at the resultant lower c value of 5-7), the data integrity is retained and the experiment time can be reduced to as little as 5 ½ minutes by reducing the number of injections. Each data set shown is the result of three replicate titrations. The protein concentration was reduced 10-fold and the syringe concentration was reduced 10-fold from standard concentrations.

In Figure 20, Panel A displays a traditional multiple injection (20 injections) method. Panel B displays the reduced injection method (5 injections). Injection volumes and concentrations for a reduced injection method should be adjusted to cover a fractional saturation range between 20 and 80% if possible. The experimental time is reduced to 8 minutes by reducing injection number. Panel C displays the result of a single injection experiment with the same concentrations as the other two data sets. Total ligand titrated is equal to the multiple injection method value, 20µL. The experiment time is reduced to 5 ½ minutes.

The reduced injection and Single Injection Method (SIM) experiments work well here for the RNAse-2'CMP system which has an average k_a and a relatively high ΔH. Concentrations would need to be increased when ΔH is lower (to avoid injection heats falling below the instrument absolute sensitivity), or when K_a is low (to avoid being below the minimum c value to achieve a fit to the experimental data). This observation is true regardless of which method is chosen.

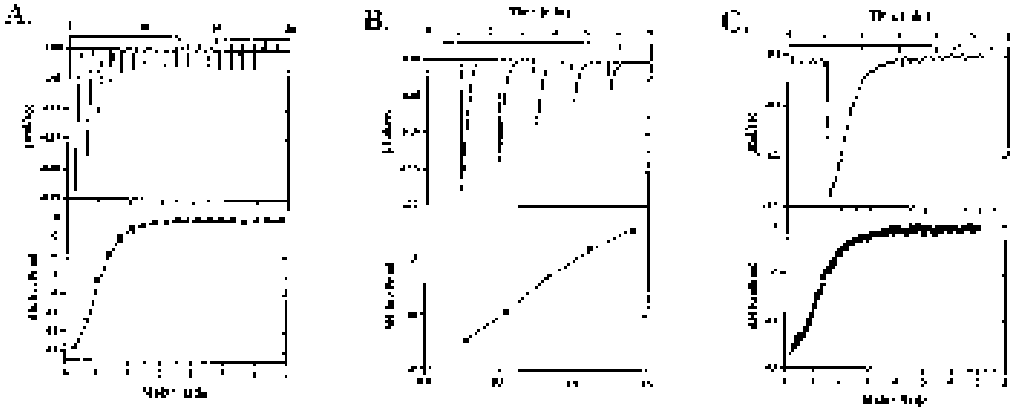


Figure 20. Comparison of Titration Methods. A. Raw and processed data from the reaction of RNASE with 2'CMP in 50 mM potassium acetate buffer pH 7.5 (25° C). The cell volume = 200 µL and the syringe volume = 60 µL. Top panels show raw data following baseline subtraction. Bottom panels show processed data corresponding to each raw data set (solid squares) and fit obtained (solid lines). Concentrations for the three data sets were [RNASE] = 0.0032 mM and [2'CMP] = 0.218 mM. Fitted parameters are given in Table 1. A. Multiple injection method; 20 x 0.5 µL injections. B. Reduced injection method; 5 x 1.5 µL injections. C. Single injection method, 1 x 20 µL injection.

Table 6.

Comparison of RNASE-2'CMP ITC Results Using the 0.2 mL Sample Cells at Higher and Lower c Values

20 Injections[2]	13.1 ± 2	0.97 ± 0.02	-17.1 ± 0.4	84	164.4	26
20 Injections[3,5]	20.4 ± 3	0.99 ± 0.02	-16.6 ± 0.3	7	5	25
5 Injections[3]	21.6 ± 1	0.34 ± 0.07	-16 ± 1	7	9	8
Single Injection[4]	16.7 ± 3	1.09 ± 0.01	-17 ± 1	5	9	0.5

[1][RNASE] = 0.06 mM, [2'CMP] = 2.18 mM, 20 x 0.639 µL injections
[2][RNASE] = 0.009 mM, [2'CMP] = 0.218 mM, 20 x 1.0 µL injections.
[3][RNASE] = 0.009 mM, [2'CMP] = 0.218 mM, 5 x 1.5 µL injection.
[4][RNASE] = 0.0032 mM, [2'CMP] = 0.218 mM, 1 x 20 µL injection.
[5]The apparent higher K value at lower c values has been previously experimentally observed [1].

Example – Binding Study of Carbonic Anhydrase II to Multiple Ligands

To further explore the ITC experiment design alternatives, when using a sample-cell volume of 0.2 mL, we utilized the carbonic anhydrase II protein with four ligands, 4-AMBS, sulfanilamide, furosemide, and acetazolamide. For each protein-ligand system we determined K_a and ΔH using a standard 20-injection method, a 5-injection method and finally the single injection method. This allowed us to compare the results over a range of protein-ligand systems and over a meaningful range of binding constants and molar enthalpies.

Table 7 summarizes the data collected for the 4 systems using the three experimental methods. Each data point is an average of at least 3 experiments and includes standard deviation values. The values for K range from 1×10^5 M^{-1} to 5×10^7 M^{-1}. Values for ΔH range from -3 kcal/mol to -21 kcal/mol. Protein concentration was determined spectrophotometrically, and ligand concentrations were determined by weight only.

Table 7.
Comparison of the Binding of Multiple Ligands to Carbonic Anhydrase II

4-AMBS	1.7 ± 0.3	-5.4 ± 0.2	7	977	39
Sulfanilamide	3.1 ± 0.3	-10.1 ± 0.5	12	220	39
Furosemide	16 ± 3	-10.0 ± 0.5	20	162	39
Acetazolamide	105 ± 35	-18.6 ± 0.5	52	162	39
4-AMBS	1.3 ± 0.3	-5.7 ± 0.3	8	122	11
Sulfanilamide	2.4 ± 0.1	-13 ± 2	2	58	11
Furosemide	19.5 ± 5	-5.4 ± 0.9	15	58	11
Acetazolamide	112 ± 33	-21.0 ± 0.4	36	29	11
4-AMBS	0.60 ± 0.2	-5.0 ± 0.3	2	174	8
Sulfanilamide	3.0 ± 0.3	-8.1 ± 0.5	3	58	8
Furosemide	24 ± 11	-11.5 ± 0.9	24	58	8
Acetazolamide	104 ± 7	-16.2 ± 0.3	32	29	8

[1]Standard experiments consisted of multiple injections (20 – 30 × 1 µL). [2]5-injection method experiments consisted of five 3–5 µL injections. [3]Single injection experiments consisted of one shot injection of 20 to 30 µL. Values for binding parameters include standard deviations determined from at least three data sets.

Each of the carbonic anhydrase ligands studied in Table 7 were first dissolved in 100% DMSO and subsequently diluted into buffer. These data illustrate the applicability of ITC for applications such as secondary drug screening. The reduced sample cell size and increased absolute instrument sensitivity permit the direct determination of thermodynamic parameters using only 10 – 20% of the sample quantity previously required. The increased instrument response and equilibration times allow 2-4 samples per hour to be completed using multi-point injection methods and up to 4-5 samples per hour using the single injection method.

These benefits in reduced sample consumption and increased sample throughput are realized without sacrificing any of the primary advantages of the ITC technique. Retained is the truly universal applicability to measure the heat of complexation between molecules completely free in-solution without requirement for any labeling, probe or other reporter mechanism in the system. No immobilization onto a surface, along with the associated method development, is required. Most importantly, the determination of the binding constant and the heat of binding are completed directly in a single experiment eliminating the uncertainty and potential errors inherent in other indirect methods that rely on unvalidated model assumptions for the specific macromolecule-ligand systems being studied.

The ITC Experiment Design Window

As discussed earlier, the two main factors needed for a successful ITC experiment, beyond protein and ligand solubility, are:

1. An appropriate *c* value. A wide range, typically 5 – 1,000, is suitable for most experiments providing a wide range of workable protein and ligand concentrations
2. Enough heat to be detected and quantified. The reduced cell volume and increased sensitivity of modern instrumentation has helped to push ever lower the heat levels that can be accurately measured and therefore fitted to a binding thermogram.

It is important to remember that both these criteria must be met simultaneously in the experiment design. That is, the smallest amount of protein that can be used in the experiment is, the greater of that required to provide a useable *c* value or that required to produce sufficient heat for detection and quantification.

In many situations, such as secondary or confirmatory screening of hits in a drug discovery program, the desire to obtain reliable, interpretable thermodynamic parameters for a series of ligands to a target is critically important, and ITC is ideally suited to the task. Often times, in the early stages of such a project, minimization of protein usage is a requirement since only a finite (and usually small) amount of protein is available.

For purposes of illustration, we have used the RNAse – 2'CMP system to consider the range of *c* values and hence the protein concentrations over which ITC data may be obtained. Table 8 illustrates the thermodynamic parameters that result from experiments with *c* values of 5, 10, 25 and 50 using both a 1.4 mL instrument cell volume and a 0.2 mL instrument cell volume. The results for *K* and ΔH are in good agreement while the quantity of protein used in these experiments ranges from a high of 685 µg to a low of 10 µg. To confirm that both experiment design requirements (*c* value and minimum heat) were met, the heats per injection were both calculated and experimentally measured. The minimum heat required per injection was somewhat arbitrarily defined as 10 times the instrument sensitivity (10 times the baseline noise level). From experience, we know that data can be successfully collected and fit to the binding model at levels that are 2–4 times lower than this "defined minimum heat per injection" requirement. This is demonstrated in this dataset for the $c = 5$ data collected using the ITC_{200} where the experimental heat per injection was only 0.7 µcal versus the defined minimal heat per injection of 1.4 µcal. Table 9 illustrates typical macromolecule usage (µg) for a 20,000 dalton protein when the minimum sample quantity is limited only by *c* value.

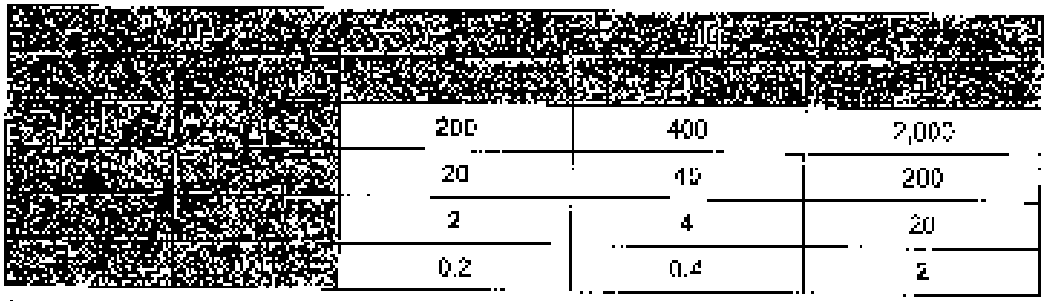
As much as Tables 8 and 9 view the experiment from the "*c* value" perspective, Table 10 views the experiment from the "minimum heat" perspective. In this case, so long as a minimum acceptable *c* value is maintained, the quantity of protein can be reduced by reducing the number of injections such that at least the minimum heat per injection is maintained. The injection volume (or concentration) of ligand can be increased accordingly to ensure completion of the titration.

Table 8.
RNAse Quantity Requirements Based on c value Requirements[1]

c value	50	25	10	5	50	25	10	5
Protein Concentration (mM)	0.035	0.018	0.0070	0.0035	0.035	0.018	0.0070	0.0035
µg of Protein	605	303	127	69	99	46	20	10
# injections	12	12	12	12	12	12	12	12
Calculated heat/injection (µcal)	62.5	31.3	12.5	6.3	8.3	4.5	1.8	0.9
Experimental heat/injection (µcal)	58.7	31.9	10.5	5.5	6.9	4.2	1.5	0.7
Required minimum heat/injection (µcal)[2]								
Experimental Results								
$K_a \times 10^8$ (M^{-1})	1.09	1.52	1.67	1.57	1.09	1.66	1.64	1.92
K_d (nM)	0.86	0.56	0.62	0.60	0.92	0.64	0.61	0.52
ΔH (kcal/mol)	-18	-17	-16	-16	-18	-17	-15	-15

[1] *Quantities calculated for the 1[1]C titration of RNAse-3'CMP assuming K_a = 1.7 x 10^8, ΔH = 15 kcal/mol, MW = 33,700.* [2] *The required minimum heat per injection = 40 x the minimum detectable heat signal (approximately 2 x baseline noise). As can be seen from the data, excellent results can be obtained even when operating at less than half of the required minimum heat per injection level.*

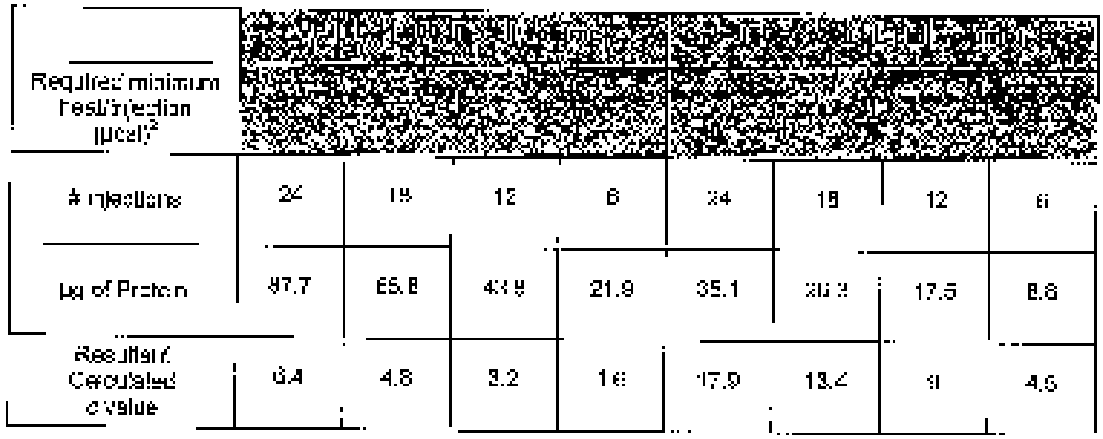
Table 9.
Typical Macromolecule Usage (µg)[1]



	200	400	2,000
	20	10	200
	2	4	20
	0.2	0.4	2

[1] *For a 30 kDa protein and an ITC sample-cell volume of 0 and, where the minimum sample quantity is limited only by c value.*

Table 10.
Minimum RNAse Quantities Based on Heat Requirements[1]



Required minimum heat/injection (µcal)[2]								
# Injections	24	15	12	6	24	15	12	6
µg of Protein	87.7	65.8	43.9	21.9	35.1	26.3	17.5	8.8
Resultant Calculated c value	0.4	4.8	8.2	16	17.9	13.4	9	4.5

[1] *Quantities calculated for the ITC titration of RNAse 2'CMP assuming K_d = 1.4 x10^{-5}, ΔH = -15 kcal/mol, MW = 13,700. [2]The retrieved minimum heat per injection = 40 x the minimum detectable heat signal (approximately 80 x baseline noise).*

In Table 11 we have attempted to quantify and compare the typical ITC experiment protein quantity requirements for a meaningful range of K_D values and ΔH values for both the 1.4 mL cell volume of the VP-ITC and the 0.2 mL cell volume of the ITC_{200}. For every given combination of K_D value and ΔH values either the c value or the minimum heat per injection value will limit the minimum quantity of protein that can be used. In Table 11 the entries in the unshaded boxes are minimum protein quantities that are determined exclusively by the c value. For any particular K_D value, so long as the c value is the limiting factor, the amount of protein required is independent of ΔH. The entries in the shaded boxes are minimum protein quantities that are determined exclusively by the minimum heat per injection requirement. So long as the minimum heat per injection is the limiting factor, the amount of protein required is dependant only on ΔH and independent of K_D.

With these experimental parameters in mind, we can begin to form a picture for the starting point of any particular ITC experiment. For example, if an IC_{50} value of 100nM was determined from a preliminary high throughput screen of a small molecule ligand to a protein target, and we assume a "typical" ΔH of -6 kcal/mole, then using the ITC_{200}, 5 µg of protein would be required, the experiment would be limited only by the minimum heat per injection and the c value would be 13. If adequate sample were available, then 11 µg could be used, the c value would be 28 and meaningful data could be gathered even if ΔH were as small as -3 kcal/mole.

Similar example scenarios are outlined below.

Example 1: A c Value-Limited Situation

A typical lead compound with a ΔH = -6 kcal/mol

	VP-ITC	ITC_{200}	
c Value	5	5	1[1]
µg Protein	140	20	5[2]

[1] A c value of 1 can be fit by least squares to the binding model if n is fixed.
[2] The lower limit for the protein quantity is reached (for this example) at 5 µg due to the heat sensitivity limitation.

Example 2: A Heat-Limited Situation

A 10 nM lead compound with a ΔH = -3 kcal/mol

	VP-ITC	ITC_{200}	
Sensitivity multiple	40 x	40 x	10 x[1]
c Value	32	100	25
µg Protein	8	4	1

[1] If a sensitivity multiple of 10 x rather than 40 x is accepted, the protein quantity can be reduced to 1 µg since the c value (25) is sufficient for the experiment.

Table 11.
Protein Quantity Usage Comparison[1]

µg of Protein Required for VP-ITC

	10.00	1.00	0.10	0.01	0.001
18.00	1,400	140	14		
15.00	1,400	140	14		
12.00	1,400	140	14		
9.00	1,400	140	14		
6.00	1,400	140			
3.00	1,400	140			

Resultant c Values for VP-ITC Protein Quantity Usage

	10.00	1.00	0.10	0.01	0.001
18.00	5	5	5		
15.00	5	5	5		
12.00	5	5	5		
9.00	5	5	5		
6.00	5	5			
3.00	5	5			

µg of Protein Required for ITC_{200}

	10.00	1.00	0.10	0.01	0.001
18.00	200	20	2		
15.00	200	20			
12.00	200	20			
9.00	200	20			
6.00	200	20			
3.00	200	20			

Resultant c Values for ITC_{200} Protein Quantity Usage

	10.00	1.00	0.10	0.01	0.001
18.00	5	5	5		
15.00	5	5			
12.00	5	5			
9.00	5	5			
6.00	5	5			
3.00	5	5			

[1]Protein quantities in the unshaded boxes are limited by the selected minimum c value of 5. Protein quantities in the shaded boxes are limited by the selected minimum acceptable heat per injection level of 40 times the instrument sensitivity. These values could be further reduced by a factor of 2-4 and still produce high quality data. The orange-shaded boxes indicate c values greater than 1,000 where fitting a binding isotherm can be problematic. It should be noted that it is very rare to have such strong binders (1 nM) with such low ΔH values.

CONCLUSIONS

As has been presented previously, ITC, as an experimental technique, provides four critically important and unique characteristics [1, 7, 18, 26-28]. First, the absorption or evolution of heat is a universal property of all chemical reactions and as such eliminates the need to use a different analytical method each time a new reaction is studied. Second, because calorimetry measures the real, model independent, enthalpy of binding and consequently allows for the determination of entropy in addition to the binding constant and stoichiometry in a single experiment. Third, the ITC technique provides this unique capability in an experimental environment that is completely label-free, in-solution and requires no immobilization of either the target macromolecule or ligand. Fourth, since the heat effect is influenced only by the *progress* of the reaction and unaffected by static situations existing in the solution, the background interference is normally very low which, allows the study of heterogeneous mixtures which may not be amenable to study by other methods.

These attributes of the ITC technique have led to its broad acceptance for the full thermodynamic characterization of biomolecular interactions. In many instances the thermodynamic parameters provide an additional level of information beyond that available from structure data alone allowing the nature of a binding event (or a SAR related series of binding events) to be more fully understood [10, 18, 29-48]. Frequently the K_D values of a series of interactions with one biomolecule are nearly equivalent. Although great initial emphasis is placed on the K_D value, and it is critically important, it alone does not provide a characterization of the mode of binding. As has been previously shown, however, within a given protein system, the distribution of the binding energy between ΔH and ΔS may differ vastly – even for a related series of ligands [19-32]. Similar K_D values and highly differentiated ΔH and ΔS values typically indicate different binding modes with correspondingly different biomolecular interactions.

This increased interest in ITC for applications such as the selection and SAR optimization of small molecule "hits" to protein targets of interest, and the selection and optimization of therapeutic protein variants has exacerbated the requirement to reduce protein sample consumption and to increase sample throughput.

Traditionally 50 –1500 µg of protein has been consumed to complete an ITC experiment and completing each experiment could require two hours or more. This latest advance in miniaturized, ultrasensitive ITC development presented herein has been specifically designed to retain all the key strengths of this technique and to address the prior sample quantity and throughput limitations. A full ITC-derived thermodynamic characterization of a biomolecular interaction can, in many instances, now be completed with as little as 5-10 µg of protein in as little as 30 minutes.

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