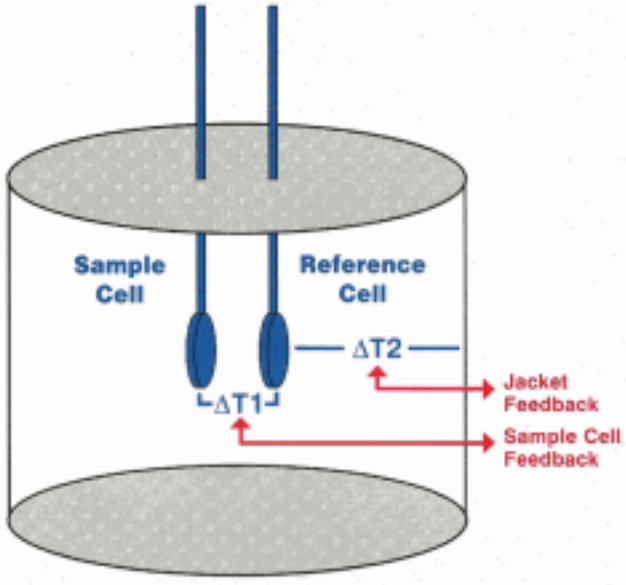
Unprecedented

High-precision CFB Network

The ITC and DSC use a cell feedback (CFB) network, pictured right, to directly and precisely measure heat. (Other instruments, such as heat flow "calorimeters," measure the decay of temperature difference between a sample cell and a reference object, then calculate the heat based on this measurement.) In both the ITC and DSC, twin coin-shaped cells are mounted in a cylindrical adiabatic shield, and communicate with the outside through long narrow access tubes. A thermoelectric device measures the temperature difference ΔT1 between the sample cell and the reference cell, while a second thermoelectric device measures the temperature difference ΔT2 between the jacket and the cells. Any nonzero ΔT1 signal activates the CFB network, thus driving ΔT1 back to zero. When exothermic reactions occur in the sample cell as a result of titrant injection or temperature scanning, less power feedback is momentarily required to null ΔT1, while endothermic reactions produce the opposite effect. This CFB signal is continuously monitored by the control unit, since its integral over time is the measure of total heat change resulting from an injection (ITC) or temperature scan (DSC). A similar feedback system is activated by the ΔT2 signal, that likewise drives a feedback circuit to the jacket, which always maintains its temperature the same as the average temperature of the cells in order to prevent heat leaks from the cells. The entire cell-jacket assembly is surrounded by 3 inches of urethane insulation to minimize heat exchange with the room. This sophisticated circuit enables precise heat measurements as small as .2 µcal by the ITC, and 10 µcal/deg by the DSC!



Superior Cell Design

After years of experience with both removable and fixed-in-place cells, our engineers have concluded that the fixed-in-place design is superior for several reasons:

(1) Fixed-in-place cells enhance baseline repeatability since the cells remain in the exact same position every time an experiment is run.

(2) Fixed-in-place cells guarantee a consistent working volume, whereas removable cells are filled on the basis of weight rather than volume, and a balance must be used to obtain this estimate.

(3) In the ITC, fixed-in-place cells provide the maximum mechanical stability necessary to eliminate noise in the baseline.

(4) Because the cells are never removed, damage to the cells or to the instrument is highly unlikely.

(5) Thanks to our cell cleaning apparatus, fixed-in-place cells are now super easy to clean!

ENGINEERING

Sophisticated Computer Automation

Because the MCS Control Unit has an on-board PC, experimentation is drastically simplified. The researcher can communicate with the ITC and DSC through the Observer™ software, eliminating the need for any knobs, dials or meters. Secondly, because the host computer need not stay in line with the MCS while experiments are in process, two experiments can be run simultaneously and asynchronously while the host computer remains free for other software applications. And since all data are automatically stored by the on-board PC, no information can be lost nor can an experiment be ruined by any activities carried out by the host computer! The MCS also has its own high speed modem, complete with Carbon Copy™ remote control software for Windows™. MicroCal can now view and operate your instrument as if we were sitting at your terminal, enabling us to provide real-time solutions to your more challenging questions.

Powerful ITC Injection System

The ITC injection system, pictured left, uses a dual photo-eye system under computer control to automatically locate and couple the stepper/injector motor to the syringe plunger and begin making injections based on prior user selections. By operating the mouse, the user can vary the number of injections, the injection size, the filter time throughout a single injection, the time interval between injections, and the stirring rate. The temperature of an experiment is achieved simply by making a single software entry, and the injection syringe containing the ligand can be thermostatted at the same or different temperature than the solution in the cell. The stirrer itself consists of a long-needle syringe with a stir paddle at its tip, which is coupled magnetically to a high-precision motor that rotates continuously throughout an experiment. This means that no correction for the frictional heat of stirring need be made!

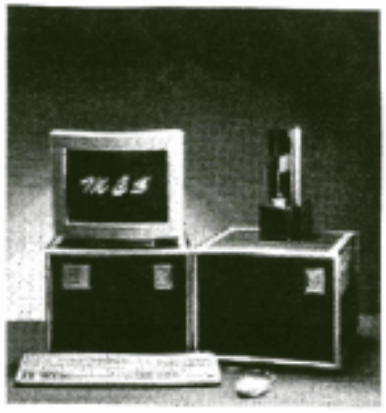
Solid Sample Accessory Cells

Solid sample cells are available for both the ITC and DSC. The ITC Solid Particle Insert Cell is ideal for studying enzyme-attached agarose or glass beads, and metabolic activities of cell cultures. The DSC Solid Sample Cell is most frequently used to study solid biological samples such as skin tissue, and to determine melting points and glass transitions of other solid materials.

From MicroCal
The Calorimetry Experts

MicroCal, Inc.
The Calorimetry Experts
MCS ITC

A new Standard of Excellence...from the Calorimetry Experts



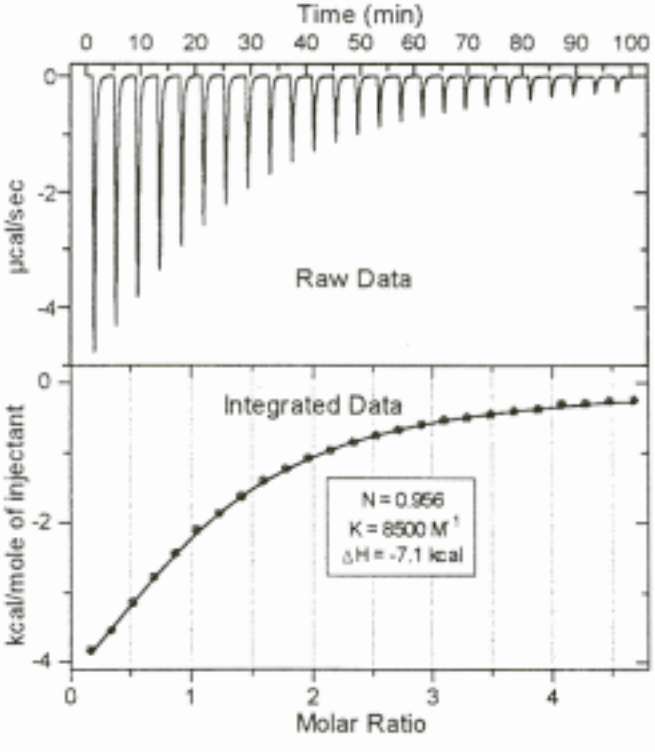
MicroCal MCS ITC

Binding of Soluble Ligands to Particle Surfaces

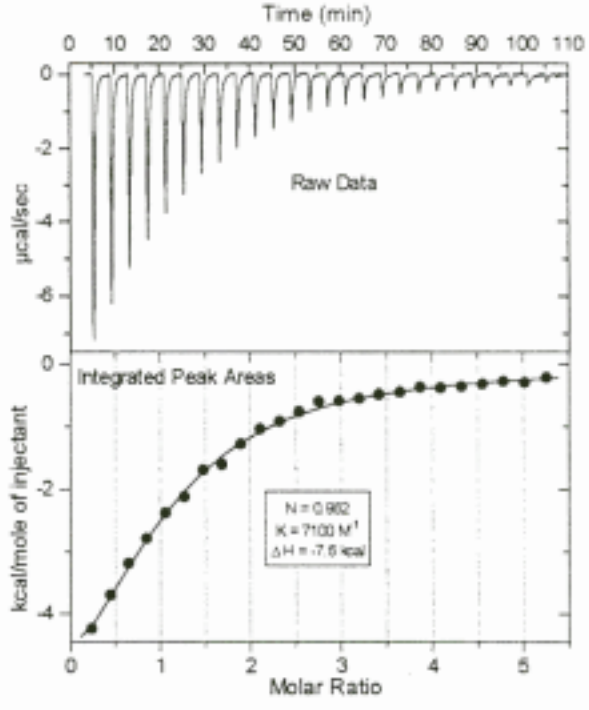
The versatility of calorimetry! Some reactions result in measurable changes in light absorption, some in light emission, others lead to measurable changes in molecular weight, solution conductivity, refractive index, reactivity of functional groups, etc. But all reactions absorb or evolve heat. The calorimeter is a universal detector.

Study of the binding of soluble ligands to particle surfaces poses a particularly difficult detection problem due to particle light scattering and to mass interference from the large size of the particles relative to the active surface groups. Recently, we attempted an experiment here at MicroCal to determine if the MCS ITC is equally capable of characterizing binding to particle surfaces as it is for reactions occuring in homogeneous solultion.

Concanavalin A (Con A) is a dimeric protein (monomer MW 26,000) which binds saccharides with 1:1 stoichiometry and varying degrees of avidity. Using soluble Con A, its interaction with the monosaccharide α-methyl D-mannoside can be quickly characterized by titration calorimetry. Shown below are raw data from twenty-four 10μl injections of 3.8 mM saccharide solution into 1.35 ml of .165 mM Con A solution, pH 5.2, 30.3 °C. The peak areas for each injection are shown in the lower frame. Because of the relatively weak interaction, the injections were carried out to a high molar ratio of ~5. The solid line through the area data shows the calculated fit curve using the best values of parameters as shown in the plot.



Con A may be covalently attached to agarose beads, and the interaction of these beads with the same saccharide ligand was studied. Shown below are the results obtained from twenty-four



10 μl injections of 5.75 mM saccharide solution into 1.35 ml of .215 mM Con A-agarose, pH 5.2, 30 °C. Again, the lower plot shows the solid line as the fit curve, with best values given for the three fitting parameters. Note the values for the K and ΔH fitting parameters (7100 M^{-1} and -7.6 kcal) obtained for agarose-immobilized Con A are extremely close to the same parameters (8500 M^{-1} and -7.1 kcal) obtained for soluble Con A interacting with the same saccharide ligand.

By J.F. Brandts, L.-N. Lin, T. Wiseman, S. Williston, and C.P. Yang

An instrument for rapid determination of binding constants for biomolecules

A LARGE FRACTION of all publications in the biochemical literature involves studies of the interaction of macromolecules, such as proteins or polynucleotides, with small solutes or with other macromolecules. The study of solute-solute interactions in the chemical literature is less pervasive, but still significant. Investigations of molecular interactions take many forms, but the single parameter which best measures the strength of interaction is the binding constant. It is unfortunate that determination of this important parameter has been so tedious and time-consuming.

When using any of the popular partitioning techniques for macromolecules (e.g., equilibrium dialysis, ultrafiltration, size-exclusion chromatography, etc.), a method of analysis must first be developed to determine concentrations. This usually involves chemical or spectroscopic methods and sometimes necessitates synthetic attachment of chromogenic or radioactive labels. Since many analyses are quite specific, the study of a series of related ligands can be further complicated by the necessity to develop more than a single analytical method. Once these are in-hand, a series of about 5-10 experiments at different concentrations should be conducted to accurately determine both the stoichiometry and the binding constant for each reaction. Sample reloading and lengthy equilibration are usually required between each experiment, which adds to the cost in both time and materials. If it is desirable to know the heat and entropy of binding as well as the binding constant, then the entire procedure must be repeated at different temperatures. Depending on the idiosyncracies of the interacting components and the extent of characterization desired, the application of partitioning methods to a single reaction may involve from days to months of research time and consume large quantities of material. These negative features restrict the use of such methods so that many important reactions have not been thermodynamically characterized to the extent they deserve.

Dr. Brandts is President, MicroCal, Inc., Northampton, Massachusetts, U.S.A., and Professor of Chemistry at the University of Massachusetts, Amherst, Massachusetts, U.S.A. Dr. Lin is Research Supervisor at the University. Mr. Wiseman, Mr. Williston, and Dr. Yang are responsible for instrument development at MicroCal, Inc.
The National Institutes of Health provided funding for the development of the titration calorimeter through Phase I (GM-35577) and Phase II (RR-03674) SBIR grants. Some of the experimental studies were partially supported by NIH grant GM-42636.

The problems described above can be avoided by working with a technique that is universally applicable to all reactions regardless of the chemical nature or size of the interacting components, which: 1) does not require sample reloading between each concentration used to define the binding curve, 2) has short equilibration and analysis times, and 3) provides an evaluation of the heat and entropy as well as the binding constant and stoichiometry in a single experiment. The only technique that has all of these advantages is titration calorimetry. Although this method has been applied to the measurement of binding constants occasionally in the past,[1-5] its success has been minimal, since instrument sensitivity necessitated that binding constants be smaller than about $10^5\ M^{-1}$, and since the complexity of instrument operation and details of the calculations restricted its use to investigators dedicated to calorimetry.

A high-sensitivity computerized titration calorimeter (MicroCal, Inc., Northampton, Massachusetts) has been designed specifically to facilitate the routine characterization of molecular interactions which exhibit binding constants as large as $10^8\ M^{-1}$. Since ease of operation by the nonspecialist was a design priority, the software package contains powerful, full-featured programs, not only for unattended instrument operation but also for automatic execution of the complex calculations that enable determination of binding constant, stoichiometry, heat, and entropy. Some of the features of the instrument, described in detail elsewhere,[6] are discussed below and experimental results are presented on some typical biochemical interactions, including the binding of a small inhibitor to the active site of an enzyme, the association reaction between two immunological proteins, and the binding of a signaling ligand to its membrane-embedded receptor.

Instrument features

A schematic illustration of the instrument is shown in *Figure 1*. The twin coin-shaped cells (1.4 mL in volume) are mounted in a cylindrical adiabatic shield, and communicate with the outside through long, narrow access tubes. A thermoelectric device measures the temperature difference $\Delta T1$ between the sample cell on the right and the reference cell on the left, while a 20-junction wire thermopile measures the temperature difference $\Delta T2$ between the jacket and the cells. A small constant power is continuously applied to the reference

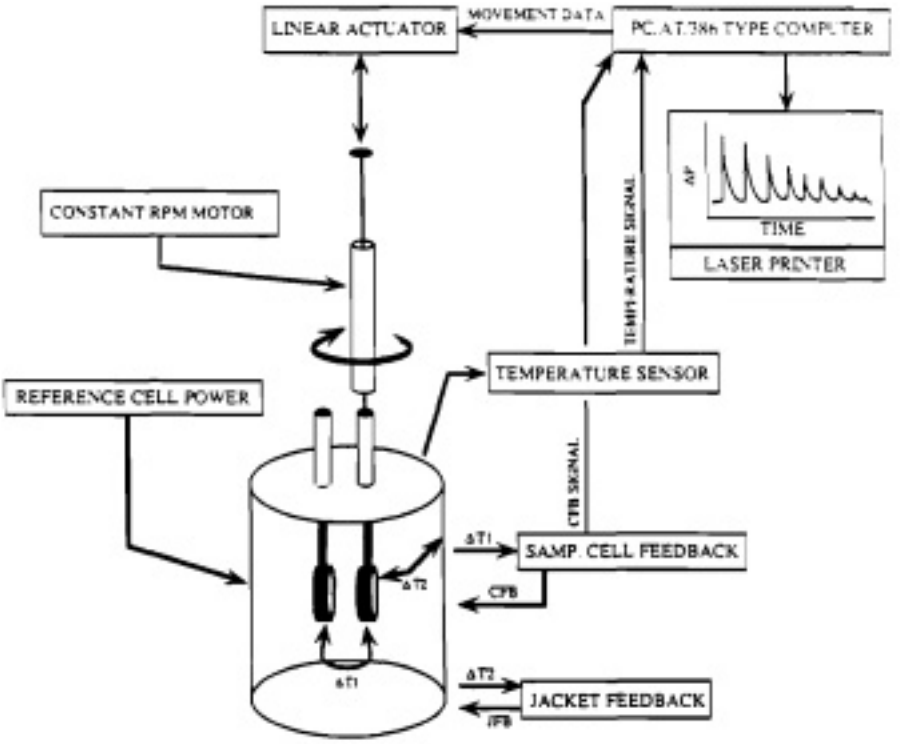


Figure 1 *Schematic diagram of the titration calorimeter.*

cell (containing buffer only) which tends to make ΔT1 negative. This nonzero ΔT1 signal activates a cell feedback (CFB) network which then supplies power to a heater on the sample cell and drives ΔT1 back to zero. When exothermic reactions occur in the sample cell as a result of titrant injection, less power feedback is momentarily required to null ΔT1, while endothermic reactions produce the opposite effect. This CFB signal is continuously monitored by the computer since its integral over time is the measure of total heat change resulting from an injection. A similar feedback system is activated by the ΔT2 signal which likewise drives a feedback circuit to the jacket that always maintains its temperature the same as the average temperature of the cells in order to prevent heat leaks from the cells. The entire cell-jacket assembly is surrounded by approx. 3 in. of urethane insulation to minimize heat exchange with the room.

The injector-stirrer assembly consists of a long-needle syringe (25-250 μL) with a stir paddle at its tip. This assembly is coupled magnetically to a high-precision motor that rotates at 400 rpm continuously throughout an experiment, which means that no correction for the frictional heat of stirring need be made. The reference cell acts only as a temperature dummy, and is not stirred. Once an experiment begins, the computer controls a linear actuator that couples to the syringe plunger and automatically carries out the injection schedule (i.e., number of injections, volume of each injection, duration of injection, and time between injections) previously set up by the operator. Data points are collected at a selectable frequency and ultimately stored on disk.

continued

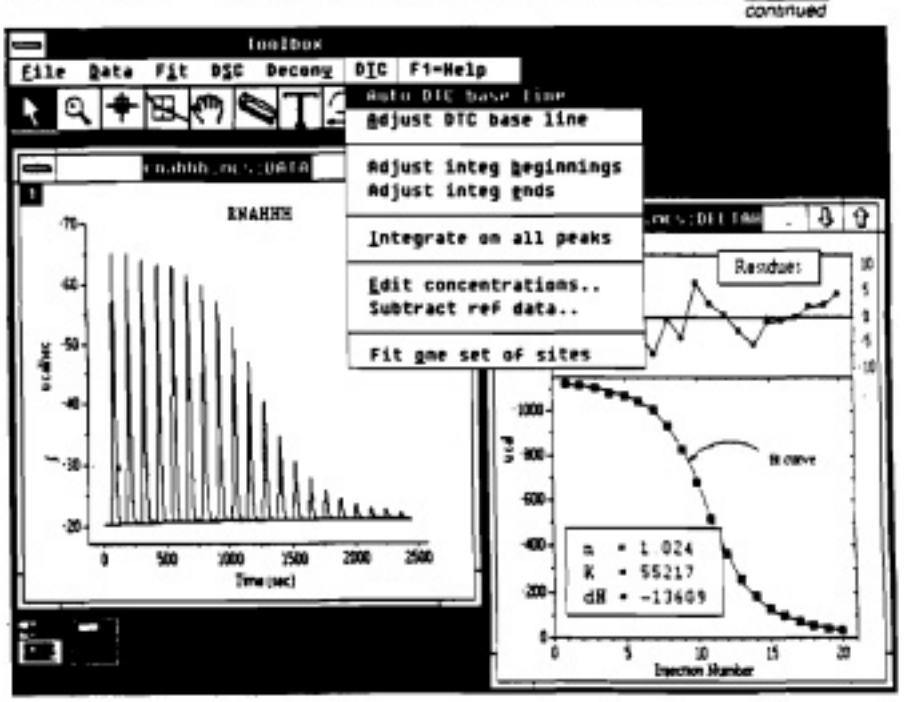


Figure 2 *Screen print from calorimeter software. Experimental data from a 20-injection sequence are shown in the left window. Using the mouse for menu selections, a baseline for the injections can be created [AUTO DTC BASELINE] and areas under each peak obtained [INTEGRATE ON ALL PEAKS]. These areas correspond to the heat change for each of the 20 injections and appear as a plot in the bottom of the right window. The best-fit curve is obtained [FIT ONE SET OF SITES] from an iterative algorithm and appears as the solid line, with residuals displayed at the top of the window. Best values for the three binding parameters are shown, and probable errors are available.*

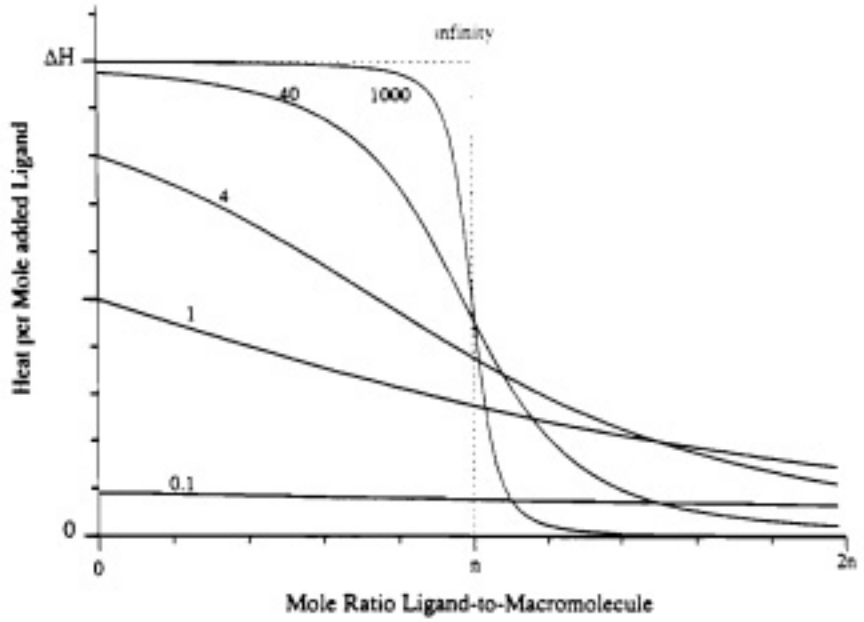


Figure 3 *Some simulated binding isotherms for different values of the* c *parameter. The* c *parameter is the product of the macromolecule concentration multiplied by the binding constant, and it controls the tightness of binding for any experimental system. The number of binding sites per macromolecule is* n.

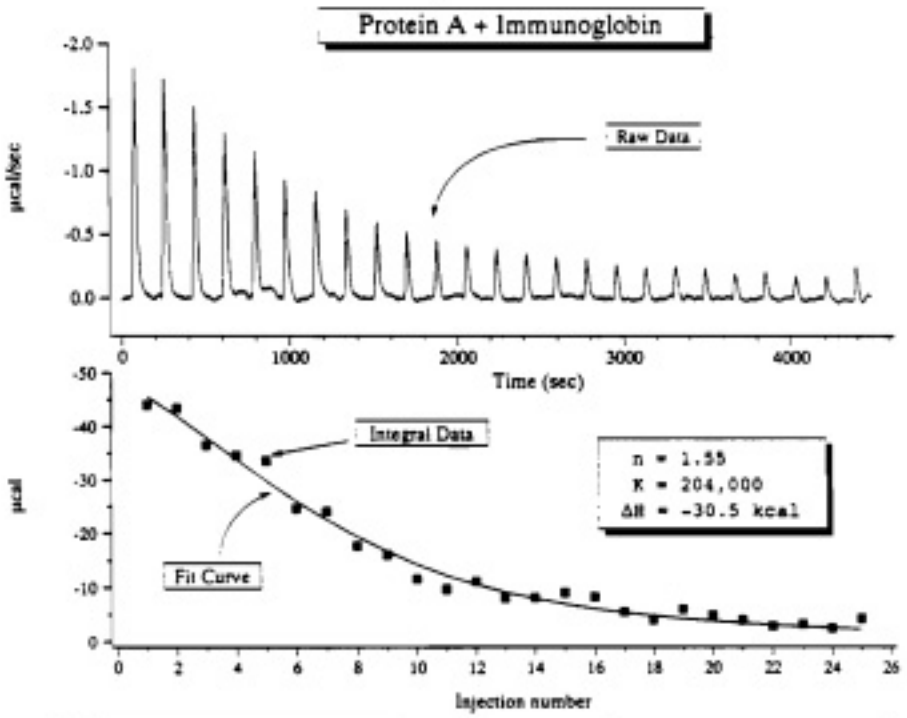


Figure 4 *Results obtained from the titration of Protein A (6.8 × 10^{-6} M) with the F_c portion of mouse IgG. The cell feedback signal (point stored every 2 sec) is shown in the upper frame for a sequence of 25 automatic injections (4 μL) spaced at 2 min. After creating a baseline and integrating all peaks, the solid symbols in the lower frame show the heat absorbed for each injection. Fitting these data by nonlinear-least-squares gives the solid curve through the points, with the best values for the three binding parameters shown in the shadow box.*

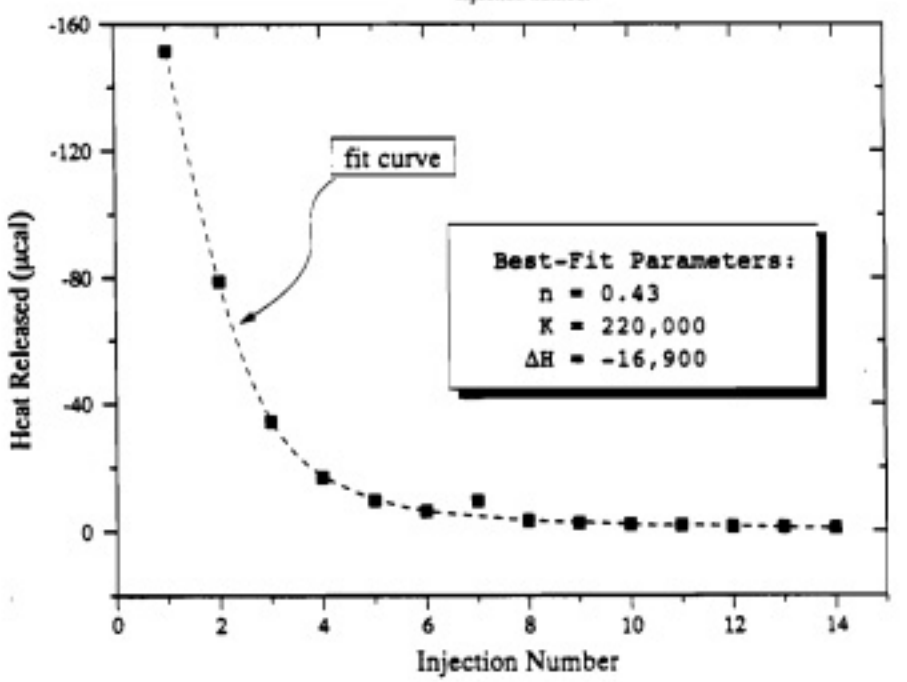


Figure 5 *The binding of L-aspartate to the aspartate receptor. The concentration of receptor in the cell was 3.2 × 10^{-6} M. Best values for the three binding parameters are shown in the shadow box.*

The equilibration time required upon loading a new sample is only about 15 min, and the fast response time (approx. 7 sec for half-time) means that only about 2 min need elapse between injections; thus an entire binding isotherm (10-20 injections) can frequently be obtained in less than 1 hr. The calorimetric sensitivity is of the order of 0.1 μcal, and the operating temperature is from 0° to 85°C. Calibration of the instrument is maintained using interactive software.

The software operates in Microsoft Windows (Microsoft Corp., Bellevue, Washington) using a mouse, and runs most effectively on a 286- or 386-based computer with a hard drive, 1 Mb RAM, math co-processor, and an EGA or VGA color monitor. A screen display is shown in *Figure 2* for data obtained from 20 injections (5 μL each) of cytidine 2'-monophosphate (2' CMP) solution into the sample cell containing a dilute solution of ribonuclease A. The raw data (μcal/sec vs sec) are shown in the window on the left. Since the injections were spaced at 2-min intervals, the total duration of this experiment was 40 min. Typical data treatment involves menu selections for automatic determination of a baseline (shown at the lower edge of the experimental data), integration to obtain the area under all injection peaks simultaneously or individually (these peak areas then appear as a separate plot, shown at the bottom of the right window), and fitting of the area curve to the appropriate parametric binding equation[6] using an iterative Marquardt algorithm. This then culminates in the appearance of the best-fit curve (solid line) through the data points, and a new plot of the residuals between the calculated curve and the experimental points (top plot in right window) appears on the screen. The best values of the binding parameters (i.e., stoichiometry [n], binding constant [K], and heat of binding [dH]; the entropy of binding is easily calculated from K and dH) are displayed and probable errors in each parameter are available. The quality of fit is quite high for the data shown in Figure 2, since the average deviation between experimental and calculated points is only approx. 0.2% of the amplitude on the area curve.

The time required for the total data analysis shown in Figure 2 is less than 30 sec, and the only operator involvement is a few seconds required for option selections using the mouse. Total documents, as shown in Figure 2, may be saved on disk for later recall, or single window plots may be printed using any output device supported by Microsoft Windows. (*Figures 3-5* were printed using the Hewlett-Packard Laserjet IIp [Palo Alto, California] with a post-script cartridge.) In addition to being a powerful scientific analysis and plotting program with all of the standard features, the software has such capabilities as curve fitting to any function entered by the user, multiple plot windows with multiple sets of axes within each window, as well as an intuitive user interface, whereby double-clicking on nearly any screen element provides control of that element to the user through a pop-up dialog box. A complete data collection and analysis package for the differential scanning calorimeter is also included, since this cell may be operated from the electronic package of the titration calorimeter and used as a companion technique. Data from noncalorimetric sources may also be imported for analysis and plotting.

Results on biochemical systems

Binding isotherms (shown in Figure 3), may be simulated using the software. The ordinate of this plot corresponds to the area under an injection peak (normalized according to the moles of ligand contained in the injection) while the abscissa is the ratio of total moles of ligand to moles of macromolecule contained in the cell subsequent to each injection. The shape of isotherms is determined by the unitless parameter c, which is the product of the binding constant multiplied by the molar concentration of macromolecule in the cell. For very tight binding (c = infinity), all added ligand is bound until saturation occurs at the stoichiometric binding ratio n, so that a rectangular curve is obtained, the height of which is equal to the heat of binding ΔH. For moderately tight binding with c values between 1 and 1000, the shape of the isotherms is very sensitive to small changes in binding constant (i.e., in c values). The intercept of these curves on the ordinate is no longer exactly equal to ΔH, but this parameter as well as the binding constant is still easily obtained from curve-fitting, as discussed earlier. Very weak binding (cf. $c = 0.1$) yields a nearly

Table 1

Best values of the binding parameters*

n	$K(M^{-1})$	$-\Delta H$ (kcal)	Std. dev. (%)
0.99	115,000	11.5	0.14
0.96	127,000	12.3	0.11
1.00	109,000	12.3	0.18
0.94	132,000	12.7	0.18
1.00	121,000	11.9	0.07
0.99	105,000	12.1	0.14
0.98 ± 0.02	118,000 ± 8500	12.1 ± 0.3	

*Resulting from six independent determinations involving 20 injections for each determination. The concentration of ribonuclease A in the 1.4-mL cell was 4.5×10^{-5} M, and the concentration of 2'CMP in the 100 μL syringe was 1×10^{-3} M (pH 5.5, 0.05 M acetate, 0.1 M KCl, 28°C).

horizontal trace which provides no useful information on the precise value of K. Although ΔH values may be easily obtained even at the largest c values, accurate estimates of the binding constant K require that the c value be within the range from about 1 to 1000 (e.g., it can be seen from visual inspection of the area curve in Figure 2, relative to the plots in Figure 3, that the c value is about 40). In order to fall within this window, strong interactions must be studied at low concentration and weak interactions at high concentration of macromolecule.

The reaction that has been most extensively characterized using the titration calorimeter is the binding of the inhibitor 2'CMP to the active site of ribonuclease A,[6] where more than 40 separate determinations of the binding constant were carried out over a 50-fold range of protein concentration at different temperatures and in different buffers, where binding constants ranged from 5×10^4 to greater than $10^6\ M^{-1}$. Each determination involved 10-20 injections (cf. Figure 2). One series of six repeat 20-injection determinations is summarized in *Table 1*, and these illustrate the high degree of precision that can be obtained. The standard deviation of the fit curves from the experimental points is of the order of 0.1%, and the precision in reproducing the binding parameters n, K, and ΔH is of the order of a few percent. The binding constants agree closely with independent literature estimates.[6]

Reactions such as this, involving the binding of drugs or inhibitors to the active site of small proteins, will normally be easy to characterize, since stoichiometry is well-defined; heats per unit mass are high, which means that solubility restrictions are likely to be minimal; and binding constants are mostly from approx. 10^4 to $10^7\ M^{-1}$, which facilitates having c values in the ideal range.

Measurements may become less routine for protein-protein or protein-polynucleotide interactions, particularly if molecular weights are high and solubilities are low. Heat signals suitable for measuring a binding constant will normally require (i.e., depending on the ΔH of binding) in excess of 1 nmol of sites in the 1.4-mL sample cell, while the concentration of ligand in the syringe (250 μL) should be 5-10 times higher if saturation is to be completed with a single loading of the syringe. The data in Figure 4 show the binding of the F_c portion (MW 50,000) of mouse IgG to Protein A (MW 45,000), where it was necessary to work with fairly small signals. The raw data are still of good quality, and the area plot for the 25-injection sequence defines a satisfactory fit curve consistent with a binding constant of 200,000 M^{-1}, ΔH of -30 kcal per mole of sites, and a stoichiometric ratio of 1.55. Ultracentrifuge studies have shown that saturation of Protein A with IgG progresses through a series of five different complexes with varying stoichiometries from 1:1 to 4:2,[7,8] and that, at equilibrium "saturation," several of these complexes persist, and the nonintegral value of 1.55 found here seems in good accord with this independent evidence.

The interaction of signal ligands with transmembrane receptors that are localized in cellular membranes is an extremely important class of reactions that is difficult to characterize by conventional binding techniques. Some results using the titration calorimeter have recently been obtained (R.M. Weis and C. Bremicker, unpublished observations) on the aspartate receptor. This receptor is responsible for the transmembrane signaling that controls bacterial locomotion toward higher concentrations of aspartic acid and other nutrients. Use of the *tar* plasmid pRK41[9] to transform *E. coli* strain RP4080 results in overproduction of the aspartate receptor (approx. 8% of the total protein).[10] Most of the receptor is embedded in the inner membrane, which can be isolated and studied by titration calorimetry. Results from the titration of the inner membrane with L-aspartate are shown in Figure 5. This sequence of 14 injections is fit nicely, assuming a binding constant of 220,000 M^{-1} and a stoichiometric ratio of 0.43. These results are in good agreement with earlier estimates for the binding constant of radiolabeled aspartate to detergent-solubilized receptor,[11] and with the observation that the functional unit of the receptor is the dimer.[12] The large heat change of -17 kcal for binding this small ligand is consistent with numerous speculations that a conformational change induced by aspartate binding is involved in the transmembrane signaling which directs bacterial locomotion.

These brief results illustrate a few applications of ultrasensitive titration calorimetry to the study of biochemical interactions. Other studies using this instrument have been conducted on the interaction of lysine with the wild-type and mutant kringle-2 domain of tissue plasminogen activator,[13] α,ω-amino acids with kringle regions of plasminogen,[14] the peptides melittin and mastoparan with the regulatory protein calmodulin,[15] cytochrome c binding to mitochondrial lipid vesicles,[16] intercalation agents ethidium and propidium with DNA,[17] and the peptide netropsin with synthetic DNAs.[18] A particularly detailed study has been carried out in J. Sturtevant's laboratory[19] on the binding of eight different variants of the S-peptide to ribonuclease-S protein, in a systematic effort to learn more about the role of hydrophobic interactions.

Summary

An ultrasensitive titration calorimeter has been developed specifically for the routine measurement of binding constants as large as $10^8\ M^{-1}$. The complete characterization of an interaction can often be accomplished in less than 1 hr, culminating in estimates for the binding constant, stoichiometry, heat, and entropy of binding. The technique is appropriate for macromolecule-small solute, macromolecule-macromolecule, or solute-solute interactions. It can be applied not only to well-characterized samples, but in many cases to samples that might be difficult (i.e., cell suspensions, and heterogeneous mixtures such as serum and opaque liquids) using other methods. Minimal operator intervention is required, and full software capabilities are available to the nonspecialist.

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