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Isothermal Titration Calorimetry of Protein–Protein Interactions

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The interaction of biological macromolecules, whether protein–DNA, antibody–antigen, hormone–receptor, etc., illustrates the complexity and diversity of molecular recognition. The importance of such interactions in the immune response, signal transduction cascades, and gene expression cannot be overstated. It is of great interest to determine the nature of the forces that stabilize the interaction. The thermodynamics of association are characterized by the stoichiometry of the interaction (n), the association constant (K_a), the free energy (ΔG_b), enthalpy (ΔH_b), entropy (ΔS_b), and heat capacity of binding (ΔC_p). In combination with structural information, the energetics of binding can provide a complete dissection of the interaction and aid in identifying the most important regions of the interface and the energetic contributions. Various indirect methods (ELISA, RIA, surface plasmon resonance, etc.) are routinely used to characterize biologically important interactions. Here we describe the use of isothermal titration calorimetry (ITC) in the study of protein–protein interactions. ITC is the most quantitative means available for measuring the thermodynamic properties of a protein–protein interaction. ITC measures the binding equilibrium directly by determining the heat evolved on association of a ligand with its binding partner. In a single experiment, the values of the binding constant (K_a), the stoichiometry (n), and the enthalpy of binding (ΔH_b) are determined. The free energy and entropy of binding are determined from the association constant. The temperature dependence of the ΔH_b parameter, measured by performing the titration at varying temperatures, describes the ΔC_p term. As a practical application of the method, we describe the use of ITC to study the interaction between cytochrome c and two monoclonal antibodies.

How are foreign molecules recognized by the immune system? How are signals mediated by small molecules translated into the expression and regulation of specific genes? These are just two of the fundamental questions currently being investigated by many research groups. Although distinctly different concepts, these two examples are fundamentally related through the necessity of forming associations between biological molecules. For the scope of this paper, we limit the discussion to protein–protein interactions. One important aspect of the study of protein–protein interactions is in the application to protein folding. The exceedingly complex process through which a polypeptide assumes its native, functional conformation shares some similarity with the process of protein–protein interactions. Both events involve the burial of solvent-exposed surface, the formation of hydrogen bonds, salt bridges, and van der Waals interactions. It should be noted though that while protein folding appears to be driven by the burial of hydrophobic residues (the hydrophobic effect) it is less clear whether protein–protein interactions are dominated by the same driving force. The energetics of the two processes also share similarities, most notable of which is the large change in C_p which is typically negative in sign. Classic transfer free energy experiments have shown that the transfer of a nonpolar solute from an aqueous solvent to a nonpolar solvent is characterized by a large negative change in C_p (1, 2). These studies have been proposed to be analogous to the sequestration of nonpolar amino acids in the interior of proteins. Thermodynamics of protein folding as studied by calorimetry as well as other indirect techniques support the proposal that a decrease in heat capacity is indicative of a decrease in exposure of hydrophobic surface (3).

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The formation of protein–protein complexes is often accompanied by a large negative change in C_p yet it is not certain whether this decrease in heat capacity can also be correlated with a decrease in the exposure of nonpolar surface. In many cases, well-resolved solvent molecules can be identified at the interface of a protein–protein complex (when a three-dimensional structure is available at atomic resolution). In cases where the observed heat capacity change cannot account for the amount of nonpolar surface buried, it has been suggested that buried solvent may be responsible for this effect (4). Although some features of protein folding and protein association are similar, it is evident that the various forces involved in stabilizing native protein structures and protein–protein complexes, such as hydrogen bonds, van der Waals interactions, and hydrophobic interactions, may have distinct roles in the two processes.

GENERAL ASPECTS OF ITC

Calorimetric methods have been an invaluable tool for understanding the forces that stabilize the folded conformations of proteins. Recently, the advent of several highly sensitive titration calorimeters has generated much interest in this technique (5–7). A thorough description of the theoretical concepts of ITC has been provided in the above and other reports (8, 9) to which the reader is referred. The following discussion represents a general description of titration calorimetry, independent of the specific instrument used.

An ITC instrument consists of two identical cells composed of a highly efficient thermal conducting material (Hasteloy or gold) surrounded by an adiabatic jacket (Fig. 1). The jacket is usually cooled by a circulating water bath. Sensitive thermopile/thermocouple circuits detect temperature differences between the two cells and between the cells and the jacket. Heaters located on both cells and the jacket are activated when necessary to maintain identical temperatures between all components. In an ITC experiment, the macromolecule solution is placed in the sample cell. The reference cell contains buffer or water minus the macromolecule. Prior to the injection of the titrant, a constant power ($<$1 mW) is applied to the reference cell. This signal directs the feedback circuit to activate the heater located on the sample cell. This represents the baseline signal. The direct observable measured in an ITC experiment is the time-dependent input of power required to maintain equal temperatures in the sample and reference cell. During the injection of the titrant into the sample cell, heat is taken up or evolved depending on whether the macromolecular association reaction is endothermic or exothermic. For an exothermic reaction, the temperature in the sample cell will increase, and the feedback power will be deactivated to maintain equal temperatures between the two cells. For endothermic reactions, the reverse will occur, meaning the feedback circuit will increase power to the sample cell to maintain the temperature. ITC instruments should be routinely calibrated by applying specified electrical pulses of approximately 5–10 μcal/s. The total heat as determined by the area under the pulse should be within 2% of the expected value (Omega ITC Manual, Microcal Inc.). The performance of the calorimeter can also be tested by measuring the heat of a standard chemical reaction, such as the protonation of tris(hydroxymethyl)aminomethane (THAM) by HCl (10). To ensure optimal performance of the calorimeter both calibration methods are recommended.

The heat absorbed or evolved during a calorimetric titration is proportional to the fraction of bound ligand. Thus, it is of extreme importance to determine accurately the initial concentrations of both the macromolecule and the ligand. For the initial injections, all or most of the added ligand is bound to the macromolecule, resulting in large endothermic or exothermic sig-

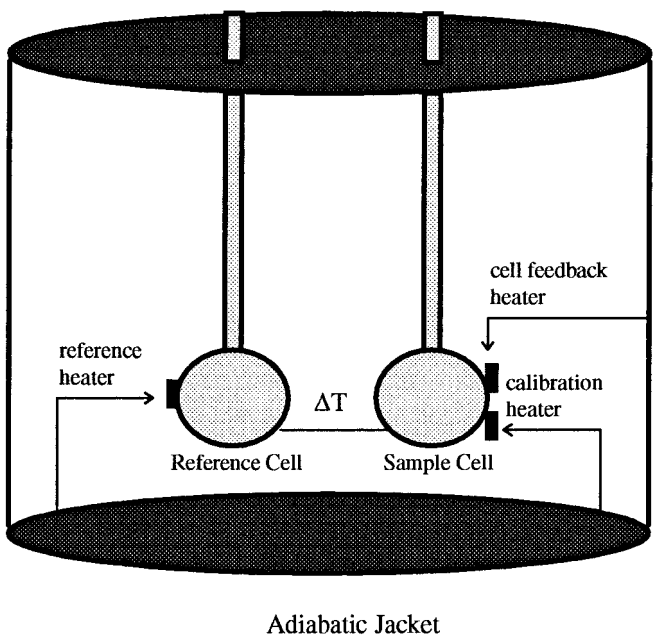

FIG. 1. Schematic diagram of an ITC instrument. Two lollipop-shaped cells are contained within an adiabatic jacket. A small continuous power is applied by the heater on the reference cell. Thermopile/thermocouple detectors sense temperature differences between the reference and sample cells. On interaction of ligand and macromolecule, heat is either taken up or evolved. Depending on the nature of the association, the feedback circuit will either increase or decrease power to the sample cell to maintain equal temperature with the reference cell. The heat per unit time supplied to the sample cell is the observable signal in an ITC experiment and a direct measure of the heat evolved on binding of a ligand to a macromolecule.

nals depending on the nature of the association. As the ligand concentration increases, the macromolecule becomes saturated and subsequently less heat is evolved or absorbed on further addition of titrant. The amount of heat evolved on addition of ligand can be represented by the equation

$$Q = V_0 \Delta H_b [M]_t K_a [L]/(1 + K_a [L]) \quad [1]$$

where V_0 is the volume of the cell, ΔH_b is the enthalpy of binding per mole of ligand, $[M]_t$ is the total macromolecule concentration including bound and free fractions, K_a is the binding constant, and [L] is the free ligand concentration. For a more general model of binding, the multiple independent sites model, the macromolecule contains multiple ligand binding sites that are noninteracting. The cumulative heat of binding can be described by

$$Q = V_0 [M]_t \Sigma (n_i \Delta H_i K_{ai} [L])/(1 + K_{ai} [L]). \quad [2]$$

A thorough discussion of the derivation of the above equations is presented by Indyk and Fisher (9). The method of analysis will be specific to the system under investigation and the reader is advised to attempt fitting according to a general model before the use of more specific models.

RUNNING A TYPICAL ITC EXPERIMENT

ITC instruments can be of either the single- or dual-injection variety (5, 6). In a single-injection instrument titrant is added to the sample cell containing the macromolecule. In a separate (control) experiment titrant is added to the sample cell in the absence of the macromolecule. In the dual-injection instrument, the titrant is simultaneously added to a reference cell containing buffer and to the sample cell containing macromolecule. The advantage of this type of instrument is that the control reaction to measure the heat of dilution of the ligand is eliminated (6). For this review, we describe the use of the single-injection Omega titration calorimeter manufactured by Microcal (Northhampton, MA) (5).

Extreme care must be taken in all aspects of an ITC experiment, from sample preparation to data analysis. In the following section we divide a typical ITC experiment into the following steps: sample preparation, sample and reference cell loading, injection syringe loading, experimental parameters, control experiments, data analysis, and troubleshooting.

1. Initial Considerations and Sample Preparation

The concentrations of macromolecule and ligand are critical, especially when one or both partners of a complex are difficult to obtain in large quantities or are of minimal solubility. Additionally, for measurement of the association constant, the initial concentrations of both the ligand and the macromolecule should be determined with a high degree of accuracy. The experimental binding isotherm can be characterized by the unitless value c, which is the product of the association constant, K_a, the concentration of macromolecule [M], and the stoichiometry of the reaction, n:

$$c = K_a [M] n. \quad [3]$$

For an accurate determination of the binding constant, a c value between 1 and 1000 is recommended (5). Large c values prohibit the determination of K_a since the transition is very sharp and too few points are collected near equivalence (saturation may be achieved in a single injection of ligand). Binding isotherms with low c values lose the characteristic sigmoidal shape and are very broad transitions that approach linearity and the equivalence point cannot be identified. For tight binding complexes such as antibody–antigen interactions with K_a values in the range 10^9–10^{10} M it is difficult to obtain accurate association constants since lowering the antibody concentration to obtain an appropriate c value extends beyond the sensitivity of the calorimeter (Fig. 2).

The choice of buffer is also critical when planning an ITC experiment. If on complex formation protons are taken up or released, the equivalent number of protons will be taken up or released by the buffer. If the selected buffer has a large enthalpy of ionization, the measured enthalpy will reflect both buffer ionization and complex formation. This can be quite informative if one wishes to determine whether protons are taken up or released on complex formation and is accomplished simply by performing equivalent experiments in a buffer with negligible enthalpy of ionization (e.g., sodium phosphate) and one with a large enthalpy of ionization (e.g., Tris–HCl). For initial experiments we recommend the use of a buffer such as sodium phosphate with a low ionization enthalpy.

Both titrant and macromolecule should be exhaustively dialyzed in buffer (preferably in the same flask) to minimize artifacts arising from mismatched buffer components. The final dialysis buffer should be saved and used for any necessary concentration adjustments of the macromolecule or titrant solutions. Macromolecule and ligand are filtered or centrifuged to remove any precipitated material. Immediately prior to loading the sample cell and injection syringe, the ligand and macromolecule solution are degassed to remove

residual air bubbles. Even the smallest air bubbles remaining in the cell or injection syringe can interfere with the feedback circuit. Air bubbles can additionally lead to poor baselines.

2. Loading the Sample and Reference Cells

The reference cell (Fig. 1) usually contains water or buffer with 0.01% sodium azide and need not be changed after every experiment. We recommend changing the solution on a weekly basis if the instrument is routinely used. The macromolecule (usually but not necessarily the larger component of the interaction) is added to the sample cell (Fig. 1) of the calorimeter using a long needle glass syringe. Typically, 1.5 to 2 ml of the solution is prepared to fill a cell with a volume of 1.3–1.5 ml. The utmost care is required to fill the sample cell without introducing air bubbles.

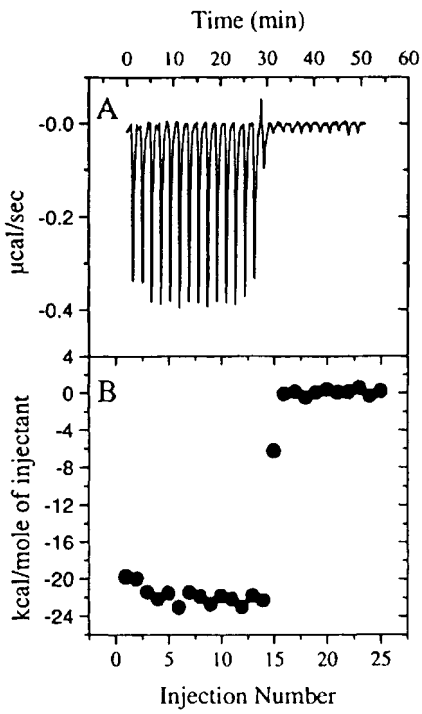

FIG. 2. Calorimetric titration of MAb 5F8 with cytochrome c in 0.1 M sodium phosphate, pH 7.0. The experiment consisted of 25 injections of 5 μl each of a 96 μM stock solution of cyt c. Cytochrome c was injected into a sample cell (volume = 1.38 ml) containing 6 μM antibody combining sites at 25°C. The injections were made over a period of 9 s with a 2-min interval between subsequent injections. The sample cell was stirred at 400 rpm. (A) Differences between the sample and reference cell containing water with 0.01% sodium azide. The heat of dilution of cyt c into buffer has been subtracted. (B) Enthalpy per mole of cyt c injected versus injection number. Since no attempt was made to obtain a binding constant from these data, it was not necessary to plot the enthalpy as a function of the molar ratio of cyt c to MAb 5F8. Reprinted with permission from C. S. Raman, M. J. Allen, and B. T. Nall (1995) *Biochemistry* **34,** 5831–5838. Copyright 1995 American Chemical Society.

Once the cell is completely filled, several rapid additions of solution will dislodge any residual air bubbles that cling to the side of the cell. Any excess solution remaining in the reservoir is removed.

3. Filling and Attachment of the Injection Syringe

Filling the injection syringe with the titrant or ligand solution also requires great care. The concentration of ligand solution should be such that the molar ratio of ligand to macromolecule, following the last injection, is approximately 2. Typically, a complete titration will involve approximately fifteen to twenty 5- to 10-μl injections of ligand. Handling of the injection syringe is extremely critical. Great care must be taken to avoid bending of the needle while the injection syringe is loaded into place. Bending of the injection syringe needle can result in some of the titrant solution being expelled into the macromolecule solution, causing the first injection to be unusable. Any minor bending in the syringe can also result in poor baselines when the injection apparatus is stirring.

4. Experimental Parameters

The parameters of the titration are input into the software program controlling data acquisition. The number, volume, and length of time of injections are critical and are discussed below. To determine accurately the enthalpy of binding, it is critical that the first several shots define a baseline region where all added ligand is bound to the macromolecule. The equivalence region should also be well defined by the concentration range spanned by the injections, to determine an accurate value of the association constant. It is necessary that concentrations be chosen so that measurable amounts of free and bound ligand are in equilibrium within the titration zone defined by the titrant injections. For characteristically tight binding affinities such as those exhibited by antibody–antigen complexes this can be an impossible task. In the tight binding limit, even when a binding constant cannot be determined, it is still possible to determine an accurate value for the binding enthalpy. Several injections should be performed after complete saturation of the macromolecule by ligand. The heat evolved or absorbed following saturation represents the heat of dilution of the titrant. The length of time of injection should be such that proper mixing is achieved. Typically, we recommend 7- to 10-s injections of ligand. To ensure proper mixing the injection syringe is fitted with a Teflon paddle and attached to a stirring motor by a rubber belt. Stirring at approximately 400 rpm should ensure good mixing.

5. Control Experiments to Determine Heats of Dilution

The observed binding isotherm is usually normalized as kilocalories per mole of ligand injected and plotted

versus the molar ratio of ligand to macromolecule. The observed heats of binding include contributions from the dilution of the titrant (ligand) and dilution of the macromolecule. A small contribution arising from stirring is also included in the observed binding enthalpy. Several control experiments must be performed to correct for the heats of dilution. The heat of dilution of the ligand is usually the most significant. This is generally true since the initial concentration and the dilution factor for the ligand are typically 10–20 times greater than those of the macromolecule. The heat of dilution of the ligand can be determined by performing an identical titration experiment in which ligand is injected into a sample cell containing buffer only (no macromolecule). The heat of dilution of the macromolecule (which is typically less significant than the heat of dilution of the ligand) is determined by titrating buffer solution into the sample cell containing the macromolecule. The two heats of dilution are used to correct the concentration-normalized binding isotherm. Since injection of ligand following saturation of the molecule is essentially a measurement of the heat of dilution of the ligand, the measured enthalpies of the last several injections can be averaged and subtracted to correct for heats of dilution. We recommend performing the control titrations for the most precise determination of the heats of dilution.

6. Data Analysis

The method of data analysis depends on the system of interest. For this article we briefly describe the procedure for fitting data to the multiple independent binding site model using the analysis software ORIGIN (Microcal, Northhampton, MA) provided with the Omega ITC. Prior to peak integration, the heats of binding are normalized as a function of ligand concentration. Additionally, a volume correction is also performed due to dilution of the macromolecule during each injection. The areas under the peaks are integrated in either a manual peak-by-peak fashion or automatically by routines provided in the software package. Baseline selection is an important factor in ITC data analysis and user input in the automated integration routine is limited. Therefore, we recommend manual peak-by-peak integration in which the operator defines the baseline regions used in the integration step. To determine n, K_a, and ΔH_b Eq. [2] is represented in terms of the binding constant and total ligand concentration $[L]_T$ to obtain the quadratic:

$$Q = (n[M]_t \Delta H V_0)/2 \{1 + [L]_t/(n[M]_t) + 1/(nK_a[M]_t) - [(1 + [L]_t/(n[M]_t) + 1/(nK_a[M]_t))^2 - 4[L]_t/(n[M]_t)]^{1/2}\}.$$

The n, K_a and ΔH_b parameters are then optimized using the standard Marquardt method with routines provided in the ORIGIN software.

7. Troubleshooting

Routine problems are expected during ITC experiments, most of which are easily corrected through practice. One common problem frequently observed is that the enthalpy of binding measured for the initial injection is less than that of subsequent injections. This is due to ligand solution slowly leaking from the injection syringe or due to the syringe plunger not being exactly flush with the driving piston. To avoid slow leakage of ligand from the injection syringe simply reduce the length of time that the injection syringe is in contact with the macromolecule prior to the first injection. Some equilibration following attachment of the injection apparatus is required. It is recommended that a baseline be initiated following the insertion of the injection syringe into the sample cell. The signal will level off on thermal equilibration of sample, reference cell, and jacket. The experiment should be started after the baseline has leveled and remained steady for several minutes. Low binding enthalpies measured after the initial injection can be observed if the injection syringe and drive piston are not exactly aligned. Aligning the drive piston exactly flush with the injection syringe can be a difficult task. The use of a magnifying glass will greatly aid in the proper alignment of the injection syringe and drive piston. Fortunately, more recent versions of the OMEGA instrument have solved this problem by the development of an automatic alignment device in which an infrared beam guides the alignment of the injection syringe and drive piston. These problems can also be corrected by injecting a small volume of the ligand for the first addition and discarding the observed data. If a preinjection is used, the concentration of added ligand must be taken into consideration during analysis.

Baseline stability is also a common problem occurring during ITC experiments and can arise for several reasons. As mentioned previously, a bent injection syringe can lead to poor baselines. Air bubbles can also result in reduced quality of baselines and can be corrected by degassing solutions longer (5–10 min should be sufficient) as well as by taking additional care in loading the sample cell. Condensation around the adiabatic jacket may also lead to poor baselines. For experiments below room temperature, the jacket must be purged with dry nitrogen prior to low-temperature equilibration.

AN ITC CASE STUDY: THE INTERACTION OF HORSE HEART CYTOCHROME *c* WITH MONOCLONAL ANTIBODIES 2B5 AND 5F8

Numerous examples of antibody–antigen binding have been characterized by isothermal titration calorimetry (11–14). Studies such as these have been invaluable for understanding the thermodynamic properties of antibody–antigen association. Additional strategies including alanine scanning mutagenesis in combination with ITC have demonstrated that although the MAb antigen interface covers a substantial surface (650–1000 Å^2) only a small number of residues contribute to the energetics of the interaction. In most cases, an accurate estimation of the binding constant for antibody–antigen binding is precluded. Despite this limitation, accurate binding enthalpies are readily determined. A complete description of the thermodynamic parameters can be obtained using association constants determined from other more sensitive methods.

Two monoclonal antibodies 2B5 and 5F8 and their association with horse heart cytochrome (cyt) c have been studied by isothermal titration calorimetry (11). Each antibody recognizes a distinct antigenic epitope on cytochrome c. Monoclonal antibody 2B5 binds to a crevice where the covalently bound heme cofactor is partially exposed. It is known that Pro 44 in cyt c is a critical residue in the epitope, since 2B5 binding does not occur in the absence of this residue. MAb 5F8 binds to the opposite side of cyt c and requires the presence of a lysine residue at position 60. Calorimetric titrations involved the addition of cyt c to the MAb contained in the sample cell (Fig. 2). Both antibodies exhibit tight associations with cyt c which precluded the determination of the association constant. Fortunately, binding constants could be determined by independent experiments. The binding constant for the MAb 5F8–cyt c interaction was determined from the association and dissociation rates ($K_a = k_{on}/k_{off}$) (15). The binding constant for MAb 2B5 was also determined experimentally from an equilibrium titration monitoring fluorescence (11). From these association constants, the free energy of binding was determined from the well-known relation

$$\Delta G = -RT \ln K_a.$$

The entropy of binding at 25°C was determined using the free energy and enthalpy of binding. The free energies and enthalpies of binding for MAb 2B5 and 5F8 were similar (Table 1). The negative signs indicate that the binding enthalpy contributes favorably to the free energy of binding. The decrease in entropy is associated with conformational restrictions of side chains in the complex and contributes unfavorably to ΔG_b. The free energies of binding of MAb 2B5 and 5F8 to cyt c are both favorable and of similar magnitude ($\Delta G_b = -12.6$ kcal mol^{-1} for 2B5 and $\Delta G_b = -13.9$ kcal mol^{-1} for 5F8). Although the thermodynamic parameters were similar at 25°C, the temperature dependences of the binding enthalpy and entropy were quite different (Fig. 3). For binding of both MAb 2B5 and 5F8 to cyt c, the Gibbs energy of binding exhibits a negligible dependence on temperature. Interestingly, the reasons for this effect are unique for each MAb. For MAb 5F8–cyt c binding, the observed temperature independence of ΔG_b is due to the temperature independence of ΔH_b and $-T\Delta S_b$. A very different situation is observed for the MAb 2B5–cyt c interaction. In this case the temperature independence of ΔG_b is due to the compensating effects of the ΔH_b and $-T\Delta S_b$ parameters. The temperature dependence of the binding enthalpy and entropy terms leads to the observed differences in the heat capacity change on binding measured for the two MAbs (Table 1, Fig. 3).

The most interesting features of 2B5 and 5F8 association with cyt c are the differences observed in both ΔC_p and protonation effects that occur on complex formation. The binding enthalpy of MAb 2B5 exhibited a strong dependence on temperature, becoming increasingly exothermic at higher temperatures. ΔC_p, described by the slope of the linear dependence of ΔH_b with temperature, was determined to be −580 cal mol^{-1} K^{-1} (Table 1). It should be noted that ΔH_b may not necessarily exhibit a linear dependence on temper-

TABLE 1

Thermodynamic Parameters for Association of Monoclonal Antibodies and Cytochrome *c*[a]

Reaction	K_a (M^{-1})	ΔC_p (cal mol^{-1} K^{-1})	ΔG^0 (kcal mol^{-1})	ΔS_b^0 (cal mol^{-1} K^{-1})	ΔH_b^0 (kcal mol^{-1})	n_{H^+}
MAb 2B5–cyt c	2×10^9	−580	−12.6	−28.2	−21.0	+0.73
MAb 5F8–cyt c	1.4×10^{10}	−172	−13.9	−26.3	−21.7	+0.02

Source. Raman *et al.* (11).

[a] Values are for 0.1 M sodium phosphate, pH 7, 25°C.

ature in which case ΔC_p is also temperature dependent. In contrast to 2B5, the enthalpy of MAb 5F8–cyt c binding exhibited only a modest dependence on temperature ($\Delta C_p = -172$ kcal mol^{-1} K^{-1}). These differences observed in ΔC_p values indicate probable differences in the binding processes for the two MAbs to cyt c.

The change in heat capacity on binding has been used to estimate the amount of polar and nonpolar surface buried on formation of the complex. Using the methods of Murphy and Freire (16) and Spolar and Record (17), the calculated apolar and polar surface was calculated for both 2B5–cyt c and 5F8–cyt c complexes (11). The two methods used gave essentially identical values of buried polar and nonpolar surface for the respective interaction. For MAb 2B5–cyt c interaction, the amount of buried apolar surface calculated was 88% of the interfacial surface. The amount of polar surface buried according to the calculation is negligible. For the interaction of cyt c with MAb 5F8, the calculated fractions of buried polar and apolar surface were very similar. Unfortunately, in the absence of the crystal structure of either MAb–cyt c complex, a comparison of buried surface and ΔC_p is only speculative. Hibbits *et al.* have shown that the heat capacity change observed on hen eggwhite lysozyme (HEL) binding to the monoclonal antibody HyHel 5 is well correlated to the amount of apolar surface buried. In this particular case the crystal structure of the HyHel–HEL complex has been determined and the interfacial surface can be well quantitated. Several groups have reported values of ΔC_p that do not correlate with the amount of buried apolar surface (18–20). The methods of calculating the amounts of apolar and polar surface from the observed ΔC_p values have been developed from the investigation of protein unfolding transitions. In contrast to protein folding, it appears that other factors must be taken into account to correlate heat capacity changes and surface exposure in protein association.

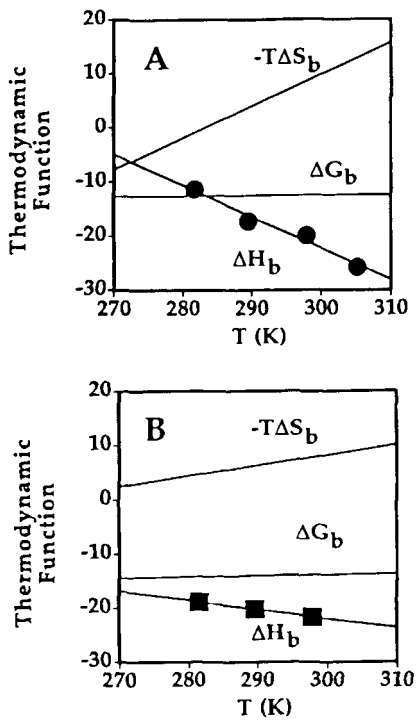

FIG. 3. Temperature dependence of the thermodynamic parameters for binding of cytochrome c to (A) MAb 2B5 and (B) MAb 5F8. The data points measured directly by isothermal titration calorimetry are included for ΔH_b. The heat capacity change associated with antibody binding to cytochrome c was determined by linear regression analysis as the slope of the plot of ΔH_b versus temperature. Values of $\Delta G_b(T)$ are calculated from the thermodynamic parameters in Table 1 using the equation $\Delta G_b(T) = (T/298)\Delta G_b^0 + [1 - (T/298)]\Delta H_b^0 - \Delta C_p[298 - T + T \ln(T/298)]$, where ΔG_b^0 and ΔH_b^0 are the thermodynamic parameters under standard conditions: 0.1 M sodium phosphate, pH 7.0, 25°C. ΔC_p is assumed to be independent of temperature. The values of $-T\Delta S_b(T)$ are calculated from $-T\Delta S_b(T) = \Delta G_b(T) - \Delta H_b(T)$. Reprinted with permission from C. S. Raman, M. J. Allen, and B. T. Nall (1995) *Biochemistry* **34,** 5831–5838. Copyright 1995 American Chemical Society.

The binding properties of the two antibodies also differed with respect to the involvement of protonation-linked equilibria. Simply by performing identical titration experiments in a second buffer system with a large enthalpy of ionization, it is possible to determine whether protonation/deprotonation events occur during complex formation (21). The enthalpy of ionization of sodium phosphate buffer is significantly less than that of Tris–HCl buffer (1 kcal mol^{-1} vs 11 kcal mol^{-1}). In 0.1 M sodium phosphate at 25°C, the binding enthalpy measured for MAb 2B5–cyt c association is -19.3 kcal mol^{-1}. In 0.1 M Tris–HCl buffer the binding enthalpy of 2B5–cyt c association is -11.7 kcal mol^{-1}. Since ΔH_b in 0.1 *M* Tris–HCl is less exothermic it can be concluded that protons are taken up on association. The apparent binding enthalpy comprises the binding enthalpies due to association and ionization of the buffer according to

$$\Delta H_b^{app} = \Delta H_b + n_{H^+}\Delta H_i. \qquad [4]$$

It cannot be determined whether protons are being taken up by cyt c or by MAb 2B5 but the total number of protons can be determined by the relation

$$n_{H^+} = \frac{\Delta H_b^{app}(P_i) - \Delta H_b^{app}(\text{Tris})}{\Delta H_i^{P_i} - \Delta H_i^{\text{Tris}}} \qquad [5]$$

where n_{H+} is the number of protons taken up, ΔH_b^{app} (P_i) is the enthalpy of binding in sodium phosphate, ΔH_b^{app} (Tris) is the enthalpy of binding in 0.1 M Tris–

HCl, $\Delta H_i^{P_i}$ is the enthalpy of ionization of sodium phosphate and ΔH_i^{Tris} is the enthalpy of ionization of Tris–HCl (21). For MAb 2B5–cyt c binding $n_{H^+} = +0.73$. For MAb 5F8, ΔH_b is identical in 0.1 M sodium phosphate and 0.1 M Tris–HCl, indicating no net changes in protonation during the association reaction. From the differences in protonation and in the observed ΔC_p it is evident that MAb 2B5 and MAb 5F8 bind cyt c in distinctly different fashions. A potential candidate responsible for protonation during MAb 2B5–cyt c binding is His-33 on cyt c. The reasons for considering His-33 are as follows. The pH chosen to study the interaction is pH 7, the pK_a of histidine is near neutral pH, and His-33 lies adjacent to Pro-44 in the crystal structure of cyt c, and as previously noted, this residue is required for MAb 2B5 binding. Also, it was recently shown for the association of porcine pancreatic elastase with the serine protease inhibitor turkey ovomucoid third domain that the pK_a of a histidine residue in the protease is shifted from 6.7 to 5.2 (22). Given this evidence it is assumed that His-33 on cyt c is the only residue undergoing a protonation change on complex formation. The contribution of His-33 protonation to the binding enthalpy is estimated by multiplying the number of protons taken up by the enthalpy of ionization of a His side chain. Using a value of +6.0 kcal mol^{-1} for $\Delta H_{i,His}$ (23) the contribution of the protonation toward MAb 2B5–cyt c binding is −4.4 kcal mol^{-1}. Unfortunately, protonation cannot fully explain the differences in the observed ΔC_p values for the two antibodies. If it is assumed that protonation of His-33 is the only ionization occurring on complex formation, then the contribution of protonation toward ΔC_p is only −40 cal mol^{-1}. Clearly, this is a small contribution given the large difference in ΔC_p observed for the two MAbs.

It is evident that an enormous amount of information about the association of biological macromolecules can be determined from isothermal titration calorimetry. Recently the crystal structure of the MAb E8-cyt *c* complex was determined at 1.8-Å resolution which paves the way for correlating structure with the energetics of binding (24). When combined with structural information obtained from X-ray crystallography or NMR spectroscopy, an understanding of the details of the association is even further enhanced.

CONCLUSIONS

The primary advantage of ITC is that the observable signal is the heat evolved or absorbed on complex formation. The only limiting requirement for study by ITC is a measurable enthalpy change on binding. This is in contrast to a number of techniques that require modification of components with fluorescent tags or require immobilization on plates. In a single ITC experiment, the binding constant and the stoichiometry and enthalpy of binding can be readily determined. For complexes that exhibit very tight affinities, the binding constant cannot be accurately determined. This restriction does not limit the determination of very precise measures of the binding enthalpy. Although easily performed, an ITC experiment requires great care in concentration determination and sample preparation. As an application of the technique, the interaction of cyt c with two MAbs was observed by ITC. On the surface, the binding parameters appeared to be quite similar. Values of ΔH_b, ΔG_b, and ΔS_b were nearly identical for the two antibodies at 25°C. Determination of the binding enthalpies as a function of temperature and buffer ionization indicated significant differences in the modes of binding. MAb 2B5–cyt c interaction was accompanied by a large ΔC_p and the net uptake of one proton. For the interaction of MAb 5F8 with cyt c, the ΔC_p was small and no changes in protonation were observed. In combination with structural information, isothermal titration calorimetry provides a thorough description of the interactions of biological macromolecules. The information obtained from such studies should aid in the development of pharmacological compounds as well in the elucidation of factors that determine the specificity of an interaction.

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REFERENCES

1. Tanford, C. (1980) The Hydrophobic Effect: Formation of Micelles and Biological Membranes, Wiley–Interscience, New York.
2. Nozaki, Y., and Tanford, C. (1971) *J. Biol. Chem.* **246,** 2211–2217.
3. Makhatadze, G. I., and Privalov, P. L. (1990) *J. Mol. Biol.* **213,** 375–384.
4. Bhat, T. N., Bentley, G. A., Boulot, G., Greene, M. I., Tello, D., Dall'Acqua, W., Souchon, H., Schwarz, F. P., Mariuzza, R. A., and Poljak, R. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1089–1093.
5. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) *Anal. Biochem.* **179,** 131–137.
6. Freire, E., Mayorga, O. L., and Straume, M. (1990) *Anal. Chem.* **62,** 950–959.
7. Gill, D. S., Roush, D. J., Shick, K. A., and Willson, R. C. (1995) *J. Chromatogr. A* **715,** 81–93.
8. Fisher, H. F., and Singh, N. (1995) *Methods Enzymol.* **259,** 194–221.

9. Indyk, L., and Fisher, H. F. (1998) *Methods Enzymol.* **295,** 350–364.
10. Hopkins, H. P., Jr., Fumero, J., and Wilson, W. D. (1990) *Biopolymers* **29,** 449–459.
11. Raman, C. S., Allen, M. J., and Nall, B. T. (1995) *Biochemistry* **34,** 5831–5838.
12. Hibbits, K. A., Gill, D. S., and Willson, R. C. (1994) *Biochemistry* **33,** 3584–3590.
13. Weber-Bornhauser, S., Eggenberger, J., Jelesarov, I., Bernard, A., Berger, C., and Bosshard, H. R. (1998) *Biochemistry* **37,** 13011–13020.
14. Murphy, K. P., Freire, E., and Paterson, Y. (1995) *Proteins* **21,** 83–90.
15. Raman, C. S., Jemmerson, R., Nall, B. T., and Allen, M. J. (1992) *Biochemistry* **31,** 10370–10379.
16. Murphy, K. P., and Freire, E. (1992) *Adv. Protein Chem.* **43,** 313–361.
17. Spolar, R. S., and Record, M. T., Jr. (1994) *Science* **263,** 777–784.
18. Frisch, C., Schreiber, G., Johnson, C. M., and Fersht, A. R. (1997) *J. Mol. Biol.* **267,** 696–706.
19. Pearce, K. H., Jr., Ultsch, M. H., Kelley, R. F., de Vos, A. M., and Wells, J. A. (1996) *Biochemistry* **35,** 10300–10307.
20. Schwarz, F. P., Tello, D., Goldbaum, F. A., Mariuzza, R. A., and Poljak, R. J. (1995) *Eur. J. Biochem.* **228,** 388–394.
21. Beres, L., and Sturtevant, J. M. (1971) *Biochemistry* **10,** 2120–2126.
22. Baker, B. M., and Murphy, K. P. (1997) *J. Mol. Biol.* **268,** 557–569.
23. Shiao, D. D., and Sturtevant, J. M. (1976) *Biopolymers* **15,** 1201–1211.
24. Mylvaganam, S. E., Paterson, Y., and Getzoff, E. D. (1998) *J. Mol. Biol.* **281,** 301–322.