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Making Cool Drugs Hot:
The Use of Isothermal Titration Calorimetry
as a Tool to Study Binding Energetics

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Ultrasensitive Calorimetry for the Life Sciences™

The Use of Isothermal Titration Calorimetry as a Tool to Study Binding Energetics

Abstract

Characterisation of the thermodynamics of binding interactions is important in improving our understanding of biomolecular recognition, and forms an essential part of the rational drug design process. Isothermal titration calorimetry (ITC) is rapidly becoming established as the method of choice for undertaking such studies. The power of ITC lies in its unique ability to measure binding reactions by the detection of the heat change duing the binding interaction. Since heat changes occur during many physicochemical processes, ITC has a broad application, ranging from chemical and biochemical binding studies to more complex processes involving enthalpy changes, such as enzyme kinetics. Several features of ITC have facilitated its preferential use compared to other techniques which estimate affinity. It is a sensitive, rapid and direct method with no requirement for chemical modification or immobilisation. It is the only technique which directly measures enthalpy of binding and so eliminates the need for van't Hoff analysis, which can be time consuming, and prone to uncertainty in parameter values.

Although ITC has facilitated the measurement of the thermodynamics governing binding reactions, interpretation of these parameters in structural terms is still a major challenge.

Introduction

Molecular recognition is a complex, but fundamental process which is essential for life. Understanding the thermodynamics that underlie such a process is of enormous interest to biochemists, but still remains a difficult challenge. Due to significant improvements in instrument sensitivity, isothermal titration calorimetry (ITC) is now becoming a routine method for the generation of thermodynamic data relating to biomolecular association. In a single experiment, ITC measures the association constant (K_a), stoichiometry (n), free energy ($\Delta G°$), enthalpy ($\Delta H°$) and entropy ($\Delta S°$) of binding. Relating these parameters to physical processes at the molecular level is more difficult, but it is in combination with structural information that ITC may help to tackle this challenge (10).

Many biochemical reactions may be started isothermally, by changing the composition of the sample by titration, leading to a wide range of potential applications of ITC. The calorimeter measures the rate of heat flow, resulting from the heat effect induced by this change in composition at approximately constant temperature. It is the only technique which directly measures enthalpy of binding ($\Delta H°$). Measurement of $\Delta H°$ values provides a method for the characterisation of proton movement which may accompany binding. However, the interpretation of $\Delta H°$ values for binding reactions is difficult and is usually only qualitative, due to the many linked equilibria that may contribute to the overall heat effect. The phenomenon of enthalpy-entropy compensation is common to many, perhaps all biochemical systems (13) and leads to an absence of correlation between $\Delta G°$ and $\Delta H°$. Meaured $\Delta H°$ values are therefore potentially valuable for the characterisation of structure-activity relationships (SAR), since large changes in $\Delta H°$ may indicate changes in binding mode, not suggested by only modest changes in $\Delta G°$. ITC allows a rapid, direct and precise measurement of affinity. There is usually no requirement for specialised reagents, and interpretation is facilitated by the absence of competing ligands. However, inclusion of other ligands during the titration can provide information on the mechanism of action of compounds, by identifying whether prior binding is required for, or prevents, binding

of the compound of interest. Information on mechanism provided by ITC can therefore help to ensure that relevant macromolecule-ligand complexes are used for structure determination and molecular design.

Here we concentrate on the utilisation of ITC, in particular with respect to drug discovery, and the application of thermodynamic and structural data to the understanding of affinity.

Measurement of Thermodynamic Parameters by ITC

ITC Instruments

Most of the commonly used isothermal titration calorimeters are based on a cell feedback network which measures the differential heat effects between a sample and reference cell. This is known as differential power compensation, and is used in both Microcal Inc. (www.microcal.com) and Calorimetry Sciences Corp. (www.calorimetrysciences.com) instruments (Figure 1A) (55). The temperature difference between these two cells is constantly monitored and a constant power is applied to the reference cell, which activates the feedback circuit to apply a variable power to the sample cell in order to maintain very small temperature difference between the cells.

This feedback power is the baseline level in the absence of any reaction. When a reaction occurs, there will be a temperature change in the sample cell, which leads to a temperature difference between the sample and reference cell. This is detected by the calorimeter, and the power applied by cell feedback is adjusted. Exothermic reactions will trigger a temporary decrease in the feedback power, and conversely, endothermic reactions will produce an increased feedback. The heat evolved, or absorbed by the reaction is then obtained by integration of these deflections from baseline, with respect to time.

Calorimeters from Thermometric (www.thermometric.com) operate by a slightly different principle, measuring the heat flow between sample cell (and reference cell) and their surroundings, a water thermostat (Figure 1B). The calorimeter measures the heat flow, and generates an electric potential proportional to this heat flow. As before, integration of the voltage offset produces the heat of reaction.
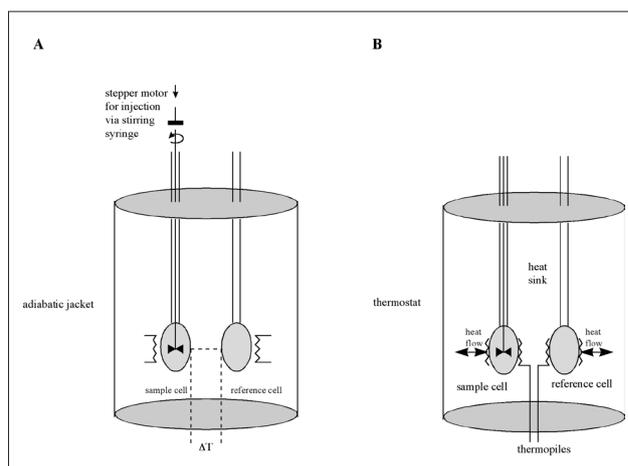

Figure 1. Schematic showing ITC instrument design. Panel A represents a differential power compensation calorimeter. Panel B represents a 'heat-leak' calorimeter.

The ITC Experiment - Design

The ITC experiment usually consists of injections of ligand into protein, with both solutions contained in exactly the same buffer. The observed heat therefore contains heat effects from several sources, the heat of binding (to be measured), the heat of dilution of protein, the heat of dilution of ligand and the heat of mixing. Strictly, control experiments are therefore needed to correct for the unwanted heat effects due to both dilutions and mixing. This involves a further 3 titrations to measure the required heats. However, the initial protein solution is usually only diluted by around 20%, leading to only small heat effects. Also, the heat of mixing is usually small, as long as buffers are matched, and can also be neglected. The dilution of the ligand however, is always larger, since the starting concentration in the calorimeter cell is zero, and the ligand is added from a high concentration in the injection syringe, until the final concentration in the cell is several times the final protein concentration. This usually leads to heats of dilution for ligands which cannot be neglected. Therefore, the heat of dilution of the ligand into buffer, at least, should be subtracted from the overall heat effect measured in the experiment.

Alternatively, subtraction of a linear regression through the last few points of the titration can often be used as an approximation for the dilution and mixing heats, without the need for further experiment.

As with any biophysical method, determination of accurate parameters depends upon correct experimental design and analysis. ITC experiments can be designed in order to determine $\Delta H°$ alone, by single injection, or to estimate K_a and stoichiometry, by carrying out a full titration.

Measurement of Enthalpy Changes

As mentioned above, information relating to enthalpy and affinity can be collected from a single experiment, consisting of several injections. However, better practice is to measure $\Delta H°$ under conditions of total association at partial saturation. Experiments are performed by titrating protein at concentrations much greater than K_d ($1/K_a$), and $\Delta H°$ values can be measured from a single injection. All of the ligand subsequently added to the calorimeter cell will bind to the available protein sites, and the heat change will depend only on the number of moles of complex formed. Measurement of $\Delta H°$ in this way requires a large excess of protein, but the experimental intercept (Figure 2) measured is close to the true $\Delta H°$. This requires a c value (c is the unitless parameter, which is the product of the binding constant times the concentration of binding sites times the stoichiometry, $K_a.M_{tot}.n$, and determines the shape of the binding isotherm) which is greater than 100.

This provides a more accurate measurement of $\Delta H°$ than experiments carried out at lower c values, and highlights the need for concentration ratios suited to the objective of the experiment.
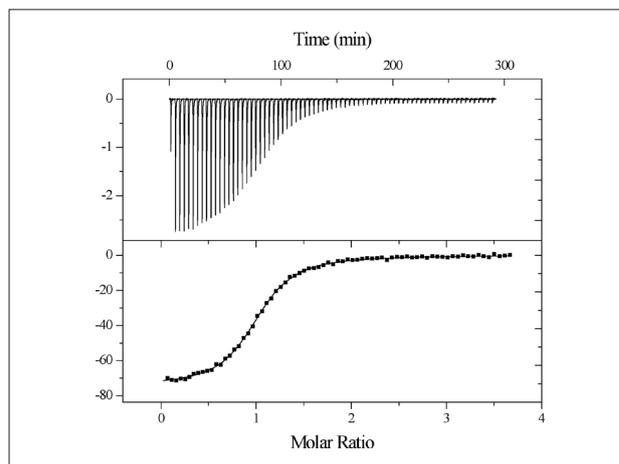

Figure 2. A typical differential binding isotherm for the binding of a triazine to the 24 kDa fragment from the Gyrase B subunit. Top: Raw data. Bottom: Binding isotherm created by plotting the integrated peaks against the molar ratio of ligand added to macromolecule present in the cell.

Measurement of Affinity

Correct choice of concentration is also important when determining affinity is the experimental objective. Low values of c lead to isotherms which are almost horizontal, indicating weak binding, and provide little information on $\Delta H°$ or K_a. Very large values of c lead to rectangular shaped isotherms, which provide information on $\Delta H°$, and n but no information on K_a.

For reliable determination of affinity, conditions should be arranged so that both the free ligands and the complex occur at significant concentrations during the experiment. In order for this to be achieved and accurate K_a values to be obtained, the concentration of protein should be around the value of K_d ($1/K_a$).

The value of c should ideally be between 5 and 100, where the shape of the isotherm is sensitive to changes in K_a. Thus, the choice of protein concentration should be chosen carefully for the experiment to be carried out.

The ligand concentration used in the syringe, as well as injection number, volume, and interval between successive

injections should also be chosen carefully to ensure that these parameters can be measured effectively.

Often, it is possible to arrange experimental conditions so that all three parameters can be measured in a single experiment. Suitable experimental design will lead to high quality data, with points at the beginning of the titration giving information on the magnitude of $\Delta H°$, points on the sloping part of the isotherm providing information relating to affinity and stoichiometry, and points towards the end of the titration estimating the effects of dilution and mixing. This leads to both accurate and precise estimates of the binding parameters.

Optimal concentrations sometimes are not possible, for example characterisation of weak binding requires high protein concentrations which may not be available or problems relating to solubility or aggregation. Equally, measuring very high affinities would require protein concentrations so low as to produce heat changes not measurable with current instruments. For this reason, the most sensitive instruments are limited to measure K_a values only below around 1×10^9 M-1 (or K_d above around 1nM).

Measurement of Stoichiometry

Assuming that the concentration of both macromolecule and ligand are accurately known (40), an important parameterthat can be determined by ITC is the stoichiometry of the biomolecular interaction. This is easily determined from the molar ratio of ligand to macromolecule at the equivalence point (Figure 2). The value of measuring stoichiometries has been shown for protein - DNA interactions, as well as receptor - growth factor interactions (29). Determination of stoichiometry also is extremely valuable in characterisation of protein preparations produced by recombinant DNA technology and supplied for functional assays and 3-D structure determination. Measurement of stoichiometry may reveal the presence of non-functional protein, which is not obvious from purity determinations, and which is not detected by other functional methods, such as binding assays or kinetics studies.

The ITC Experiment - Analysis

For each injection, in a calorimetric experiment the heat evolved or absorbed is proportional to the change in concentration of bound ligand,

(1) $q = V . \Delta H° . \Delta[L]_{bound}$

where V is the reaction volume, and $\Delta H°$ is the enthalpy of binding. For a single set of identical binding sites, indicated by equation (2)

(2) $M + n.L \rightleftharpoons ML_n$

the cumulative heat, Q, can be expressed in terms of $[L]_{total}$ to give

(3) $Q = \dfrac{(1+[M]_{total}.n.K_a+K_a[L]_{total}) - [(1+[M]_{total}.n.K_a+K_a[L]_{total})^2 - 4[M]_{total}.n.K_a^2[L]_{total}]}{2. K_a / V\Delta H°}$

Estimation of n, K and $\Delta H°$ is then achieved by fitting to Q vs $[L]_{total}$, yielding a hyperbolic saturation curve. An alternative method of data plotting and fitting has been described (55), where the incremental heat signal (1st derivative of Q with respect to $[L]_{total}$) is plotted against the molar ratio ($[L]_{total}/[M]_{total}$). This method produces the familiar sigmoid titration curve (Figure 2). It is in the capability of ITC to measure this incremental heat signal with high signal to noise, that provides the basis for the high sensitivity of the method. Both methods of data display and fitting should in principle produce the same parameter values, with differences indicative of systematic errors (4).

ITC is the only technique which allows direct measurement of the values of K_a, n, and $\Delta H°$ in a single experiment. It should be recognised that $\Delta H°$ is actually an apparent (or observed) value since the binding reaction may be accompanied by many linked equilibria yielding heat changes. For example, the observed value of ΔH must be corrected for the heat of ionisation of the buffer, if the binding interaction is associated with changes in protonation. The magnitudes of $\Delta G°$ and $\Delta S°$ are then obtained from the relationship:

(4) $\Delta G° = \Delta H° - T\Delta S° = - RT \ln K_a$

ITC also allows measurement of a central thermodynamic

parameter, ΔC_p, the change in heat capacity (also known as specific heat). ΔC_p is an important thermodynamic parameter as it governs the magnitudes of both $\Delta H°$ and $\Delta S°$:

$$(5)\ \Delta C_p = \frac{d(\Delta H°)}{dT} = \frac{T.d(\Delta S°)}{dT}$$

In order to characterise the thermodynamics of a binding interaction fully it is necessary to measure, not only $\Delta G°$, $\Delta H°$, and $\Delta S°$, but also ΔC_p, since this parameter allows the prediction of the change of the other three parameters with temperature. ΔC_p is usually measured from the change in enthalpy with temperature, using the relationship:

$$(6)\ \Delta C_p = \frac{(\Delta H°_{T2} - \Delta H°_{T1})}{(T2 - T1)} = \frac{(\Delta S°_{T2} - \Delta S°_{T1})}{\ln(T2 / T1)}$$

where T1 and T2 are two temperatures at which separate determinations have been made. Calculation of ΔC_p in this way is usually valid for narrow temperature ranges, although the situation is complicated by the fact that ΔC_p is itself temperature dependent.

Although ITC measures enthalpy directly, it is not the only method for obtaining this parameter. The van't Hoff enthalpy may be calculated from the temperature dependence of K_a, using values determined from non-calorimetric experiments. Equation (4) has often been used to calculate ΔH_{vH}, as it is an integrated form of the van't Hoff equation. However, it can be seen that there is no term for ΔC_p within this equation, and so there is an implicit assumption that ΔC_p is zero and that the enthalpy does not change with temperature. This is rarely the case, and determination of van't Hoff enthalpies should be undertaken with inclusion of a non-zero ΔC_p term:

$$(7)\ \ln K_a = \frac{\Delta H_{vHref} - T_{ref}\Delta C_p}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right) + \frac{\Delta C_p}{R}\frac{\ln T + \ln_{Ka\ ref}}{T_{ref}}$$

where $\ln K_a$ is plotted against T and the variable parameters are ΔH_{vHref} (the van't Hoff enthalpy at a reference temperature, T_{ref}), $\ln K_{aref}$ (K_{aref} is the association constant at the reference temperature), ΔC_p (the temperature independent change in heat capacity).

Again, the situation is complicated if ΔC_p changes over the experimental temperature range. In the absence of cooperativity, $\Delta H_{cal} = \Delta H_{vH}$, as both the indirect van't Hoff method and direct calorimetric method reflect the same thermodynamic system. The discrepancies that have been reported (35, 50) are likely to be due to cooperative effects, lack of precision in calculated ΔH_{vH} values or artefacts arising from the assay, such as perturbations caused by protein immobilisation (8).

Errors and Uncertainty in Measured Thermodynamic Parameters

ITC measures very small heat changes, and as a result there are many possible sources of error, resulting from heat changes caused by effects including mechanical mixing, evaporation and adsorption. Many sources of error may be eliminated via calibration and the use of reference cells. However, for a measured parameter to provide useful information on abinding interaction, the variation between compounds must be greater than the uncertainty in the magnitude of the parameter. ITC gives a more precise measure of K_a than many other methods. Of the parameters measured directly by ITC, $\Delta G°$ has the lowest signal to noise ratio, as measured values fall in a relatively narrow range. The typical range for drug-like molecules is from -30 kJ mol to -50 kJ/mol. $\Delta H°$ has a higher signal:noise than $\Delta G°$, with $T\Delta S°$ being less precise since errors are compounded as it is calculated as the difference between $\Delta H°$ and $\Delta G°$. Typical ranges of $\Delta H°$ and $T(S°$ for drug type molecules are -80 kJ/mol to +20 kJ/mol and -60 kJ/mol to +40 kJ/mol respectively. Standard errors, witin experiments, for these three measured parameters are typically around 1 kJ/mol. Changes of $\geq$ 4 kJ/mol in $\Delta\Delta G°$ or $\Delta\Delta H°$, and $\geq$ 8 kJ/mol in $T\Delta\Delta S°$ are usually significant.

A useful method to check for errors or artefacts in ITC can be to perform a second titration in reverse (usually by having the ligand in the cell instead of the syringe). If the correct model is used for data fitting, the same parameter values should be obtained for both titrations (40).

Extending the Range of Measurable Interactions

Ideal conditions for determining binding affinities sometimes cannot be met, for example low affinity binding requires high protein concentrations and therefore high ligand concentrations. Often ligand solubility can be a limiting factor. There are several methods which can be used to extend the range of compounds that can be studied by ITC, which may also be useful in extending the range of measurable affinities. These methods usually operate by increasing the aqueous solubility of a particular compound, allowing the desired concentration to be achieved, or by increasing the detectable heat signal, allowing the use of lower concentrations. A simple solution to the problem of ligands with limited solubility, may be to perform the titration in reverse to the usual situation, and place the ligand in the cell, rather than the syringe. This situation reduces the need for high ligand concentrations, but does require that the protein is available and soluble at the high concentration required for the syringe.

Many ligands of interest in drug discovery projects, especially non-polar compounds have increased solubility in organic solvents such as dimethyl sulfoxide (DMSO). However, dilution of organic solvents into aqueous buffer generally leads to large heat effects which may cause signal to noise problems. Careful matching of DMSO concentration in both protein and ligand solution therefore should be ensured. Care should also be used when interpreting the thermodynamic parameters obtained, since the change in hydrophobicity of the buffer may affect the parameter values compared to purely aqueous buffer. Solubility of compounds may also be increased by adjusting the pH of the buffer in order to increase the degree of ionisation of the compound of interest. The ligand solution may be prepared at a pH which confers greater solubility (assuming the ligand is stable at this pH), and titrated into the macromolecule solution at the pH of interest. The free ligand concentration following dilution and binding is low helping to ensure that precipitation does not occur. This method has been used successfully to study the binding of pepstatin A to endothiapepsin (19).

Competition or displacement experiments may be used to examine both weak and tight binding affinities, and may also be used to study poorly soluble ligands (25, 23, 44, 56). Competition experiments involve titrating a ligand (A), which occupies the same binding site as the compound of interest (or, more generally, for which binding is mutually exclusive with the compound of interest), with known K_a and $\Delta H°$ values, in the presence of a fixed concentration of the compound of interest (B). The presence of B will partially displace A from the complex with protein to an extent which is dependent upon the affinity and concentration of both A and B. The apparent association constant for binding of A in the presence of B is given by :

$$(8)\ K'_A = \frac{K_A}{(1+[B].K_B)}$$

The apparent enthalpy is given by:

$$(9)\ \Delta H'_A = \Delta H_A, - \frac{\Delta H_B.[B].K_B}{(1+[B].K_B)}$$

Hence, from a determination of K'$_A$ and ΔH'$_A$, it is possible to calculate the affinity and enthalpy of the compound of interest. These equations assume that the concentration of B does not change during the titration. This can be arranged if the same concentration of B is included in both the cell and the syringe. However, it is also possible to allow for the change in [B] during the titration in the data fitting (43).

One of the most powerful methods to extend the range of measurable binding affinities is to exploit the fact that free energy changes are state functions. The value of a state function is defined by the initial and final states of the system, regardless of the pathway connecting those two states. This allows affinities to be calculated under relevant conditions, from measurable values under convenient conditions, as long as the free energy changes for taking the protein (in both free and bound forms) from the relevant conditions to the convenient conditions can be measured.

For example, if the binding constant for a particular protein-ligand interaction cannot be measured at the relevant pH, the binding free energy (ΔG_b) could be measured at a convenient pH (where the affinity is measurable), if the free energy changes for protonation (ΔG_{prot}) of the free and ligand bound forms of the protein

can be determined, for taking the protein from one pH to the other (12,1) (Figure 3).

Therefore the ligand binding free energy of interest, ΔG_b^{rel}, can be calculated from the following expression:

(10) $\Delta G_b^{rel} = \Delta G_b^{con} + \Delta G_{prot}^{P} - \Delta G_{prot}^{PL} = \Delta G_b^{con} + \Delta\Delta G_{prot}$

Thermodynamic linkage can also be applied to temperature (11). From Equations 5 and 6 above, it can be seen that ΔCp controls the magnitude of $\Delta H°$, and that $\Delta H°$ is dependent on temperature. ΔC_p is rarely zero and thus, the signal from ITC can be increased, simply by choosing a temperature producing a more measurable enthalpy change. For example, for compound having a ΔC_p of around -1 kJ/K/mol (which is typical for drug-like compounds binding to proteins), the $\Delta H°$ would become 12 kJ/mol more negative on moving from 25°C to 37°C.

The utility of proton linkage to determine binding affinities has been discussed, but proton movement may be useful for extending the scope of ITC measurements in another way. By making use of buffers with varying ionisation enthalpies, it is possible to increase the apparent enthalpy of binding, and so increase the useful protein concentration range for ITC. In general the apparent enthalpy of binding ΔH_b is the sum of the intrinsic enthalpy of reaction, ΔH_r, which is independent of the choice of buffer used in the experiment, and a term proportional to the enthalpy of ionisation of the buffer:

(11) $\Delta H_b = \Delta H_r + n_{H+} . \Delta H_{ion}$

where n_{H+} is the number of protons that are released by the buffer and become associated with the protein (if $n_{H+} > 0$). If $n_{H+} < 0$, the protein releases protons to the buffer. ΔH_{ion} is the ionisation enthalpy of the buffer.

Hence, for a binding reaction involving the association of a single proton in a buffer with $\Delta H_{ion} \neq 0$, $\Delta H_b = \Delta H_r$. However, in a different buffer where ΔH_{ion} (0, the measured enthalpy is given by Equation (11), where $n_{H+} = 1$. Measurement of binding enthalpies in a range of buffers with different ionisation enthalpies also allows the calculation of the number of protons linked to the binding process.

The calorimetric effects described above for buffers can be extended to the binding or release of other ligands, which may be linked to the binding interaction of interest. For example, binding or release of divalent metal ions such as Mg^{2+}, or Ca^{2+} in the presence of EDTA or EGTA would give rise to additional heat effects associated with metal ion chelation. This emphasises the fact that calorimetric methods monitor gross heat effects in any process, as a result of thermodynamic linkage. An understanding of the conceivable coupled reactions in a system allows the possibility of exploitation of these processes to widen the scope of measurements available to ITC.
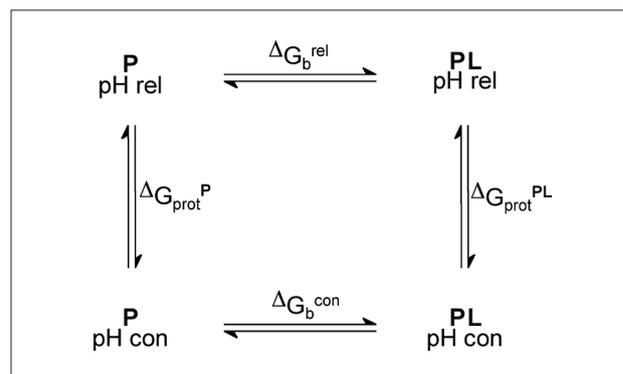

Figure 3. Thermodynamic cycle linking ligand binding free energies of a protein at two different pH conditions, relevant and convenient.

Interpretation of Binding Thermodynamics

The standard Gibbs free energy change, $\Delta G°$, is the most important energetic parameter measured by ITC. $\Delta G°$ determines the direction in which biomolecular binding equilibria will spontaneously proceed, with more negative values of $\Delta G°$ favouring higher affinity binding. It is important to realise that $\Delta G°$ and its enthalpic and entropic constituents depend upon differences between free and bound states for both of the interacting partners. It is for this reason that structural information on the free partners, as well as the protein-ligand complex, along with thermodynamic data relating to the binding process is necessary for full characterisation of the binding interaction. The standard enthalpy change, $\Delta H°$, reflects changes in the interactions between atoms. An overall

increase in bonding is associated with the release of heat, or a negative enthalpy change, and the reaction is termed exothermic. A negative value of $\Delta H°$ is favourable. Although the meaning of $\Delta H°$ appears simple, representing the changes in bond energy that have occurred in a particular binding reaction, it is actually the resultant value of formation and breakage of many individual bonds. These individual changes may produce positive or negative contributions, which means that the observed enthalpy represents the sum of many of these individual components (16). The standard entropy change, $\Delta S°$, is associated with the disorder in a system, with an increase in bonding tending to decrease disorder. A positive value of $\Delta S°$ is favourable. It can be seen that increased bonding tends to produce negative $\Delta H°$ values, and negative $\Delta S°$ values, which lead to opposing contributions to $\Delta G°$. This enthalpy/entropy compensation, which appears due to effects of perturbing the weak intermolecular bonding occurring in solvent water, tends to lead to smaller changes in $\Delta G°$ (see below). The change in heat capacity, ΔC_p controls how $\Delta H°$ and $\Delta S°$, and hence how $\Delta G°$ change with temperature. A negative value of ΔC_p indicates that $\Delta H°$ is more negative as temperature increases, and infers that there is increased bond formation (or decreased bond breaking) at higher temperatures. Understanding the various contributions to observed thermodynamics is extremely difficult, because of the wide variety of contributing factors. Even for systems where structural and thermodynamic data are available, the most reliable information is obtained when only small changes in structure are made. It should be remembered that for any binding interaction, changes in at least 3 species must be considered, both partners and the solvent water. Model systems have been used to provide a basis for interpretation of enthalpy and entropy changes, and their controlling parameter, ΔC_p, in binding reactions (48, 14).

Attempts also have been made to describe the contributions of factors such as polar and apolar surface area changes (20, 22), rotational, conformational and translational entropy (32, 39), and solvent effects to the observed thermodynamics (6, 53, 33).

Hydrophobic Interactions

The classical understanding of hydrophobic interactions is that non-polar groups associate with each other, minimising contact with solvent. Hydrophobic binding interactions often are characterised by small (frequently positive) enthalpy changes, large positive entropy changes, and often a negative contribution to ΔC_p (34, 47). This may be understood by considering the interactions of solvent water around a non-polar solute. In the presence of a non-polar compound the normal hydrogen bond network of water is reorganised. In an effort to maintain the number of hydrogen bonds, water molecules align themselves around the apolar compound. This may be explained because these apolar groups do not fulfil the hydrogen bonding capability of water, which results in the water molecules surrounding a hydrophobic molecule making stronger bonds with each other than those of bulk water. This does not cause large enthalpy changes, but the increased order is associated with a decrease in entropy. The binding thermodynamics of hydrophobic interactions are thus explained by the increase in entropy which results from the release of these relatively highly ordered water molecules surrounding the apolar surfaces of the two interacting molecules. These water molecules return to bulk where they form weaker bonds, a process which is entropically favourable and slightly endothermic (14). The negative contribution to ΔC_p is explained by the ordered shell of water around the non-polar surfaces being weaker at higher temperatures, indicating that $\Delta H°$ is less positive at these higher temperatures.

Electrostatic Interactions

Electrostatic interactions are defined here as those occurring between charged or dipolar ligands and the counter-charged binding site on a macromolecule. Energies of electrostatic interactions are difficult to determine, since they depend upon the dielectric constant (D) of the surrounding medium, as well as the inverse distance of approach of the charges, 1/Dr (the actual inverse power law for the distance being dependent on the nature of the charged groups). The dielectric constant at a binding site may be considerably lower than the

dielectric constant of bulk solvent, and would thus favour strong charge-charge interactions upon binding, but transferring a charged group to a low dielectric environment is unfavourable. This is because buried charged groups must be stabilised by local dipoles (52). Binding of charged groups to macromolecules often is entropically driven, with low values of $\Delta H°$ (38). This behaviour can in part be explained by the chelate effect, since less translationalentropy is lost by co-ordination with a polydentatemacromolecule than by co-ordination with several monodentate water molecules (14). An entropic advantage is also expected for displacement of the water moleculessolvating the free charged groups. These binding thermodynamics are also seen for chelation of metal ions by molecules having lower molecular weight, such as ATP (54). The magnitude of the entropy change is likely to be dependent on the number of co-ordination sites present on the macromolecule, and there are examples of ion binding which is enthalpically driven (36). The effect of binding of polar or charged ligands on ΔC_p is extremely difficult to assess.

Hydrogen Bonds

Hydrogen bond formation is thought to be particularly important in biological binding interactions. However, it is often overlooked that for a hydrogen bond to form between a macromolecule and ligand, similar hydrogen bonds between the macromolecular binding site and water, and also between the ligand and water will have to be broken. Thus, a hydrogen bonding group makes similar interactions as both a reactant and product, and so there may be a relatively small contribution to binding thermodynamics (15). The energy of a single hydrogen bond has been estimated as around 21 kJ/mol (5 kcal/mol), but contributions to binding thermodynamics often are much smaller than this due to the nature of hydrogen bond exchange. However, a single hydrogen bond may contribute from 10 to around 10,000 fold to affinity (15). This illustrates that it is extremely difficult to make generalisations regarding the contribution of a single hydrogen bond to binding affinity, or to characterise the enthalpy of hydrogen bond formation. Properties of both the hydrogen bond donor and acceptor, as well as their local surroundings in both the free and complexed states must be taken into account in order to assign parameterisations (5).

Conformational Changes

Ligand induced conformational changes are important in many binding interactions, and are responsible for several significant functional roles. Conformational changes may be responsible for correct orientation of active site residues involved in catalysis, or may facilitate the subsequent binding of ligands such as further substrates or allosteric effectors (14). It is likely that conformational changes occurring on ligand binding are subtle changes rather than the large changes exemplified by unfolding or refolding reactions. These subtle changes are therefore expected to exhibit relatively low magnitudes for both $\Delta H°$ and $\Delta S°$ (usually with strong enthalpy-entropy compensation), with negative values anticipated for ΔC_p (31).

The Role of Water in Binding Interactions

It is evident from the discussion above that solvent water plays a key role in governing biomolecular binding interactions. Water molecules interact with both the free partners and the complex, and changes in the number of water molecules located at a binding interface may have a dominant effect on binding affinity. Water molecules located close to binding surfaces are more structured, or ordered than those contained in the bulk solvent, and as such behave very differently. These locally structured water molecules can potentially be retained or released during a binding interaction with very different thermodynamic consequences. Release of bound waters from a binding interface isassociated with a favourable gain inentropy as these molecules are displaced back into bulk. However this is an enthalpically unfavourable process as the strong bonds made with the macromolecule or with other structural waters are replaced with the weaker bonds observed in bulk solvent. Conversely, retention of water may be associated with a favourable enthalpy resulting from increased hydrogen bonding, but an unfavourable entropy penalty. The balance between these two opposing contributions is a delicate one, but must be overcome by the medicinal chemist in order to design high affinity ligands for target macromolecules.

Several studies on the thermodynamics of water molecules localised at protein-ligand interfaces have been reported

providing valuable insight into their exploitation for ligand design (6, 7, 49, 45, 10).

For the FKBP-tacrolimus (FK506) binding interaction, displacement of 2 fewer waters when binding to the Tyr82-Phe mutant protein is associated with a negative effect on both ΔH° and $T\Delta S^\circ$, leading to more positive ΔG° ($\Delta\Delta G^\circ$ around 3 kJ/mol (0.7 kcal/mol)), with a negative contribution to ΔC_p (7). For this system, displacement of the 2 waters bound to Tyr82 is associated with a favourable entropy change driving an increase in affinity, despite an enthalpic disadvantage. In this system, the ITC data suggests that displacement of water is the preferred method for improving affinity.

Binding of the tripeptide Lys-Ala-Lys to the periplasmic transporter protein, OppA, captures 3 more waters than the binding of Lys-Trp-Lys. The immobilisation of these extra water molecules is associated with small, but favourable changes in both enthalpy and affinity. This illustrates that for this system, the favoured approach for ligand design may be to interact with the interfacial waters, rather than aiming to displace them (45).

The importance of water in biomolecular recognition is now widely appreciated and has been discussed in 2 important reviews (33, 28).

Enthalpy-Entropy Compensation

The phenomenon of enthalpy-entropy compensation appears in many, if not all biochemical thermodynamic binding studies. Enthalpy-entropy compensation is characterised by the linear relationship that is observed between the enthalpy change and the entropy change in a binding interaction. Hence, large changes in ΔH° are compensated by large and opposing changes in ΔS°, which almost cancel leading to much smaller variations in the observed ΔG°. Enthalpy-entropy compensation in these systems, seems at first to be connected to the properties of solvent water, but actually appears to be an almost inescapable general consequence of perturbing most weak intermolecular interactions (13, 18). This finding is perhaps not unexpected, since increased bonding in a biomolecular interaction will

produce a more negative ΔH°, but this will be at the expense of increased order, leading to a more negative ΔS°. Since both of these parameters are governed by ΔC_p, it may not be surprising that these two parameters are correlated. In terms of medicinal chemistry, enthalpy-entropy compensation is a difficult challenge which must be overcome in order to significantly improve the binding affinity of the compounds for the target of interest.

Although enthalpy-entropy compensation limits the effects of changed interactions on ΔG°, measured ΔH° values can provide valuable structure activity relationship (SAR) information, which may help to detect groups located at the binding interface. Several alanine scanning mutagenesis studies have revealed interfacial residues, having relatively large effects on ΔH°, but having only a moderate effect on ΔG° (17, 41, 51). A study of 2325 alanine mutants reveals that only around 5% of these residues contribute a $\Delta\Delta G^\circ$ of more than 8 kJ/mol (2 kcal/mol). It is also found that there is little correlation between $\Delta\Delta G^\circ$ and the change in solvent accessible surface area (3). Upon mutation of 12 interfacial residues on lysozyme, only 4 produce a $\Delta\Delta G^\circ$ of greater than 4 kJ/mol (1 kcal/mol) (9).

Relating Thermodynamics to Structural Changes

Data from protein folding and unfolding studies have been used to derive general relationships between ΔC_p, ΔH°, and $T\Delta S^\circ$ and changes in polar and apolar surface area (46, 19). It was thought that since the principles are the same, that these relationships would hold for biomolecular interactions involving burial of surface area at the molecular interface. However, large discrepencies between predicted and observed parameters have been shown (21, 30). It is possible that at least some of the discrepancy may be attributed to water molecules at the interface contributing to the measured thermodynamic parameter (27).

This type of approach is unlikely to be reliable when applied to medicinal chemistry, since the relationships derived from large changes in surface area relating to intramolecular folding reactions are used to predict the behaviour of thermodynamic parameters relating

to the much smaller changes in surface area encountered during ligand binding interactions. The situation is further complicated by the greater diversity of ligands produced by medicinal chemistry. The prediction of thermodynamic parameters from structural data has been attempted to validate the empirical relationships described above. Agreement has been seen for the binding of peptides to protein molecules (37, 2). However, there are many examples where agreement is not observed (24, 41, 17, 10). The use of changes in the accessible surface area to predict thermodynamic parameters should therefore be used with caution, especially since values calculated for the same system using different algorithms can be significantly different (26, 42).

Further data relating to the binding of small molecules with drug-like properties should eventually allow more reliable predictions of binding energetics thereby facilitating structure-based drug design.

Future Developments

Increased instrument sensitivity must be considered as a highly desired improvement over current technology, and some achievements are already being made towards this goal. Improved sensitivity would allow reduction in the amounts of reagents consumed, would allow higher affinity interactions to be characterised directly, and may alleviate problems associated with ligand solubility. The introduction of instrumentation capable of measuring smaller heat changeswill therefore open the way for binding interactions, not detectable by current machines, to be studied. Miniaturisation is another potential method to decrease reagent consumption, and integrated circuit calorimeters are already being built which may eventually lead to ITC being applied to micro-titre plate formats. A miniaturised differential scanning calorimetricbased high-throughput system has already been developed by 3-Dimensional Pharmaceuticals (www.3dp.com).

This type of configuration may also lead to increased throughput, which would certainly be a welcome breakthrough in the characterisation of compounds produced by medicinal chemists in the pharmaceutical industry. Current throughput is such that only the most

interesting (usually in terms of affinity) examples of chemical series are studied, which may potentially lead to missing compounds with unusually favourable enthalpy or entropy changes being used as start-points for further, improved ligand design. Compoundswith greater thermodynamic diversity may be more readily studied if throughput is increased, since this should allow even those compounds with lower affinity to be characterised.

The combination of improved sensitivity, miniaturisation and throughput may eventually lead to ITC being used as a routine tool in high-throughput screening laboratories. Indeed, novel calorimetric methods have been reported which may already have started to move the technology in this direction (www.althexis.com).

Improving our understanding of biomolecular interactions may take longer. So far it has proved extraordinarily difficult to characterise the individual contributions of non-covalent interactions (Hydrophobic, electrostatic, hydrogen bonding and van der Waals interactions) to the overall binding process. Improvement in instrument technology will lead to a growing database of thermodynamic data, with associated structural information, that will advance our understanding of these contributions.

Conclusions

ITC is a powerful technique for the accurate and precise measurement of the affinity of biomolecular binding interactions. It is also the only method for directly measuring the enthalpy of these binding reactions, and thus provides a complete thermodynamic characterisation of the reaction of interest, without the need for van't Hoff analysis. It requires no modification of reagents and is relatively rapid.

These considerations have led to ITC being the method of choice for the thermodynamic characterisation of binding interactions having affinities in the micromolar to nanomolar range. ITC thus has an important role in the determination of binding mechanism and its application to rational drug design. Improvements in our understanding of the forces governing biomolecular interactions have been aided by the use of ITC, and should continue to improve over time.

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