Direct measurement of protein binding energetics by isothermal titration calorimetry

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Of all the techniques that are currently available to measure binding, isothermal titration calorimetry is the only one capable of measuring not only the magnitude of the binding affinity but also the magnitude of the two thermodynamic terms that define the binding affinity: the enthalpy (ΔH) and entropy (ΔS) changes. Recent advances in instrumentation have facilitated the development of experimental designs that permit the direct measurement of arbitrarily high binding affinities, the coupling of binding to protonation/deprotonation processes and the analysis of binding thermodynamics in terms of structural parameters. Because isothermal titration calorimetry has the capability to measure different energetic contributions to the binding affinity, it provides a unique bridge between computational and experimental analysis. As such, it is increasingly becoming an essential tool in molecular design.

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Abbreviations
ITC isothermal titration calorimetry
PTP-1B protein tyrosine phosphatase 1B

Introduction

Isothermal titration calorimetry (ITC) measures directly the energy associated with a chemical reaction triggered by the mixing of two components. A typical ITC experiment is carried out by the stepwise addition of one of the reactants (~10 L per injection) into the reaction cell (~1 mL) containing the other reactant. A typical experiment is shown in Figure 1. The chemical reaction created by each injection either releases or absorbs a certain amount of heat (q_i) proportional to the amount of ligand that binds to the protein in a particular injection ($v \times \Delta L_i$) and the characteristic binding enthalpy (ΔH) for the reaction:

$$q_i = v \times \Delta H \times \Delta L_i \qquad (1)$$

where v is the volume of the reaction cell and ΔL_i is the increase in the concentration of bound ligand after the i^{th} injection. As modern ITC instruments operate on the heat compensation principle, the instrumental response (measured signal) is the amount of power (microcalories per second) necessary to maintain constant the temperature difference between the reaction and reference cells. The heat after each injection is therefore obtained by calculating the area under each peak. Because the amount of uncomplexed protein available progressively decreases after each successive injection, the magnitude of the peaks becomes progressively smaller until complete saturation is achieved. Once this situation is reached, subsequent injections produce similar peaks corresponding to dilution or mechanical effects that need to be subtracted from all the injection peaks before analysis. The corrected area under each peak is given by Equation 1, which is used to analyze the data. The quantity ΔL_i is the difference between the concentration of bound ligand in the i^{th} and $(i–1)^{th}$ injections, and its functional form depends on the specific binding model. For the simplest case, in which the protein has one binding site, Equation 1 becomes:

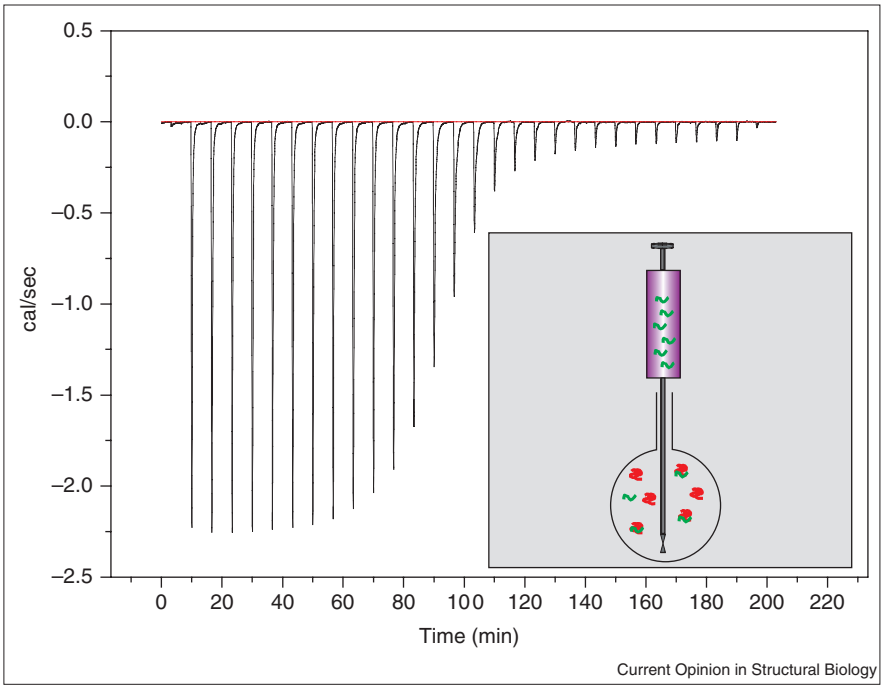
$$q_i = v \times \Delta H \times [P] \times \left(\frac{K_a[L]_i}{1+K_a[L]_i} - \frac{K_a[L]_{i-1}}{1+K_a[L]_{i-1}} \right) \qquad (2)$$

where K_a is the binding constant and [L] is the concentration of free ligand. As the known experimental quantity is the total ligand concentration, rather than the free ligand concentration, Equation 2 needs to be rewritten in terms of the total ligand concentration. The solution to this and other more complicated binding models in terms of the total ligand concentration has been published in [1,2]. Analysis of the data yields ΔH and $\Delta G = -RT/nK_a$. The entropy change is obtained by using the standard thermodynamic expression $\Delta G = \Delta H – T\Delta S$. By repeating a titration at different temperatures, it is also possible to determine the change in heat capacity (ΔC_p) associated with the binding reaction

$$\left(\Delta C_p = \frac{\partial \Delta H}{\partial T} \right).$$

During the past few years, significant advances in ITC instrumentation, data analysis and the structural interpretation of binding thermodynamic data have taken place. Together, these developments have permitted the implementation of accurate experimental protocols aimed at measuring the binding energetics of protein–ligand and protein–protein interactions, and at dissecting ΔG into the fundamental thermodynamic components: ΔH, ΔS and ΔC_p. Consequently, the range of application of ITC has been extended considerably, including systems that could not be studied by ITC before. In this review, we will discuss the application of ITC to high-affinity binding processes, processes coupled to protonation/deprotonation reactions and the development of quantitative structure/thermodynamic correlations.

Figure 1



A typical ITC experiment. The experiment shown corresponds to the titration of a phosphotyrosine peptide (TEGQpYQPQPA) with the SH2 domain of Lck (S Leavitt, E Freire, unpublished data). The experiment was performed in 10 mM Pipes, pH 7.5 at 15 C. [SH2] = 81 M and [TEGQpYQPQPA] = 0.4 mM. Analysis of the data, as described in the text, yields a binding affinity of 5.8×10^6 M^{-1} and a ΔH of −13.5 kcal/mol. The inset illustrates the configuration of an ITC reaction cell. The cell volume is 1.4 mL and is filled with the protein solution (red). The injection syringe, which also stirs the solution to assure proper mixing, is filled with the ligand solution (green). At specified time intervals, a small volume (typically 10 L) of the ligand solution is injected into the cell, giving rise to the characteristic titration heat effects. Once the protein is saturated, the residual heat effects originate from dilution of the peptide and also from mechanical effects associated with the injection. These effects need to be subtracted before thermodynamic analysis. In an ITC experiment, the quantity measured and displayed on the y-axis is the time dependence of the electric power (cal/sec) necessary to maintain constant the temperature difference between the reaction and reference cells after each injection of reactant. The area under each peak is the heat (microcalories) associated with the process.

Binding reactions with arbitrarily high binding affinities

In the past, one of the most significant limitations of ITC was the absence of protocols aimed at characterizing the thermodynamics of binding reactions with very high association constants. This limitation is especially severe in drug design, in which lead compounds, usually with micromolar affinities, are optimized to nanomolar or even higher affinities. During this optimization process, the compounds usually exceed the upper affinity limit of ITC analysis.

In 1989, Wiseman *et al.* [1] showed that the product of the protein concentration in the calorimeter cell, [P], and the binding constant, K_a, a parameter known as $c \equiv K_a \times [P]$, must be lower than 1000 for the reaction to be measured directly by ITC. In practical terms, this restriction sets an upper limit of 10^8–10^9 M^{-1} for the binding constant. Figure 2 shows the effects of increasingly higher binding affinities on the outcome of an ITC titration. As shown in the figure, beyond a certain value, the titrations lose their characteristic curvature, become indistinguishable from one another and lack the information necessary to determine the binding constant.

Recently, a solution to the problem has been obtained by the design of competition experiments in which the high-affinity ligand is titrated into protein that is prebound to a weaker inhibitor. Sigurskjold [3••] recently presented a rigorous protocol for the analysis of ligand competition experiments by displacement ITC. This approach requires three titrations: first, a titration with the weak inhibitor in order to characterize its binding thermodynamics; second, a titration with the high-affinity inhibitor to measure its binding enthalpy; and, third the displacement titration. The titration with the weak inhibitor needs to be performed only once for a particular set of conditions. The titration with the high-affinity inhibitor alone is performed to improve the robustness of the analysis, as it provides a direct measurement of its binding enthalpy.

Velazquez-Campoy *et al.* [4••] have implemented the ITC competition assay in the analysis of inhibitors of HIV-1 protease. In their implementation, Velazquez-Campoy and colleagues used a low-affinity inhibitor with a binding enthalpy of opposite sign to that of the high-affinity inhibitor, which has the added benefit of amplifying the calorimetric signal in the displacement titration. Figure 3 illustrates the implementation of this approach to HIV-1 protease.

The same principle can also be used for low-affinity ligands by monitoring the change in binding affinity of a high-affinity ligand in the presence and absence of the low-affinity ligand under examination. Zhang and Zhang

Figure 2

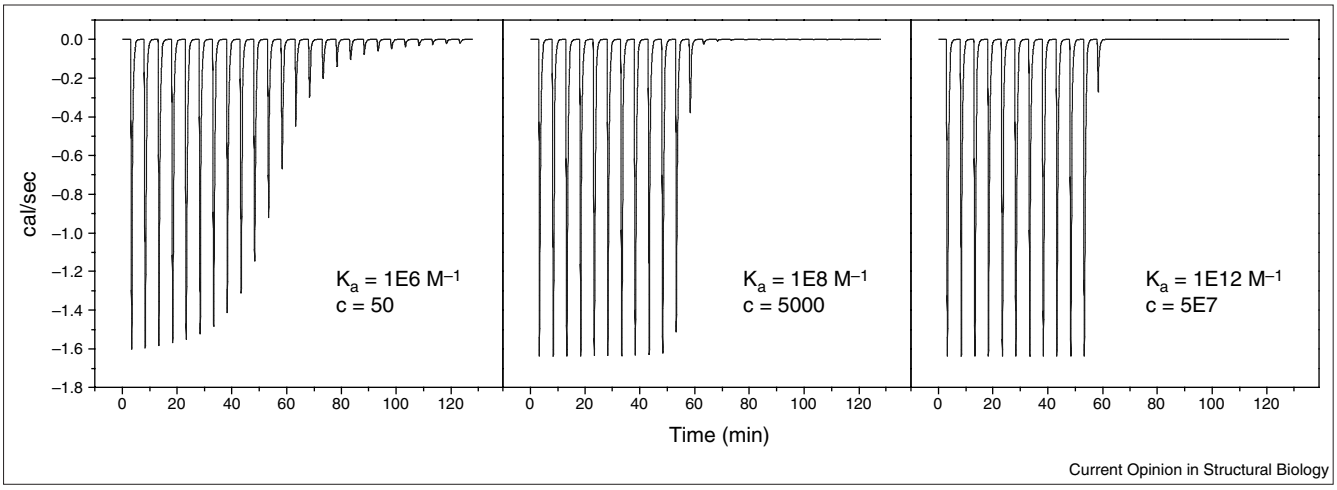


Illustration of the effect of increasing binding affinity on the ability of ITC to measure the binding constant. The ITC experiments were simulated using the following parameters: injection volume 10 L, [protein] = 0.05 mM, [ligand] = 0.6 mM, $\Delta H_b = -10$ kcal/mol, $v_{cell} = 1.4$ mL. The binding affinity (K_a) and the quantity $c \equiv K_a$ [protein] are shown in the panels. For $c > 1000$, only a lower limit for the binding affinity can be obtained, even though the binding enthalpy can be measured accurately. This situation is often encountered in drug design because the affinity of lead compounds is improved beyond the limit of ITC resolution.

[5] demonstrated this technique by studying the binding partners of protein tyrosine phosphatase 1B (PTP-1B).

Coupling of binding to protonation/deprotonation processes

Many binding reactions are coupled to the absorption or release of protons by the protein or the ligand. If this is the case, the reaction is pH-dependent and the binding enthalpy is dependent on the ionization enthalpy of the buffer in which the reaction takes place. An investigation of the binding energetics requires the dissection of the buffer-related contributions. ITC provides the most powerful method to evaluate protonation/deprotonation coupling. The first step in the analysis is the determination of the presence of protonation/deprotonation coupling, which is done by performing ITC titrations in buffers with different ionization enthalpies, ΔH_{ion} [6]. The measured enthalpy, ΔH_{app},

$$\Delta H_{app} = \Delta H_{bind} + n_H \Delta H_{ion} \quad (3)$$

is the sum of two terms: the reaction enthalpy, ΔH_{bind}, independent of the buffer used in the experiment (but pH-dependent) and another term representing the contribution of the proton ionization of the buffer, ΔH_{ion}, which is multiplied by n_H, the number of protons that are absorbed (or released if n_H is negative) by the protein–inhibitor complex upon binding. If n_H is not zero, then the reaction is coupled to protonation/deprotonation. Baker and Murphy [7] developed an experimental protocol aimed at dissecting intrinsic binding from protonation contributions to the overall energetics. This protocol has been successfully applied to different protein systems [8,9••,10].

Enthalpic versus entropic optimization of binding affinity

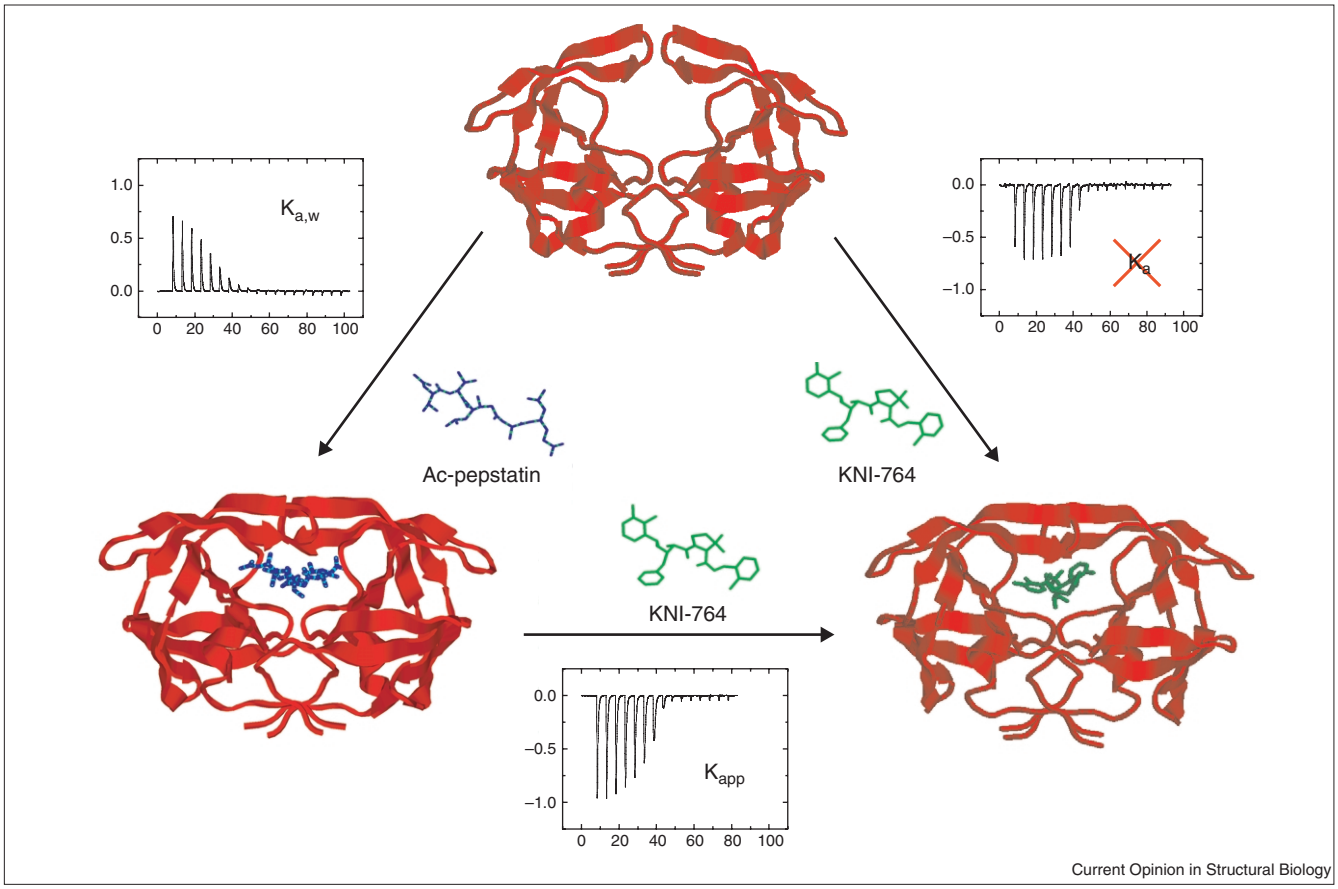
The binding affinity is dictated by the Gibbs energy of binding

$$K_a = e^{-\frac{\Delta G}{RT}} \quad (4)$$

However, ΔG is made up of two different contributions and many combinations of ΔH and ΔS values can, in principle, elicit the same binding affinity (i.e. the same ΔG and therefore the same K_a). Currently, most molecular or drug design strategies are centered around the optimization of the binding affinity; however, the behavior and the response of a ligand to changes in the environment or in the protein target are different for enthalpic or entropic molecules, even if the binding affinity is the same. These differences are not limited to different temperature dependencies. The binding enthalpy primarily reflects the strength of the interactions of the ligand with the target protein (e.g. van der Waals, hydrogen bonds, etc.) relative to those existing with the solvent. The entropy change, on the other hand, mainly reflects two contributions: changes in solvation entropy and changes in conformational entropy. Upon binding, desolvation occurs, water is released and a gain in solvent entropy is observed. This gain is particularly important for hydrophobic groups. At the same time, the ligand and certain groups in the protein lose conformational freedom, resulting in a negative change in conformational entropy. Different structural and chemical characteristics reflect themselves in different thermodynamic signatures.

Recently, the enthalpic and entropic contributions to the binding affinity of four HIV-1 protease inhibitors approved

Figure 3



Implementation of a competition titration assay for HIV-1 protease. The binding affinity (K_a) of the inhibitor KNI-764 is beyond the limit of direct calorimetric determination (right-hand side). Under these conditions, however, the ITC titration provides an accurate measurement of the binding enthalpy. The displacement titration is performed in the presence of the weak inhibitor acetyl-pepstatin ($K_{a,w} = 2.4 \times 10^6\ M^{-1}$). The selection of a weak inhibitor with a binding enthalpy of opposite sign (left-hand side) to that of the inhibitor under study produces a larger signal in the displacement titration (bottom) due to the displacement of an endothermic inhibitor by an exothermic one. In the presence of the weak inhibitor, the apparent binding constant for KNI-764, K_{app}, falls within the range required for ITC determination. K_{app} is given by the equation

$$K_{app} = \frac{K_a}{(1+K_{a,w}[X])}$$

and can be lowered to the desired level by increasing the concentration of the weak inhibitor. From these experiments, we determined a binding constant for KNI-764 of $3.1 \times 10^{10}\ M^{-1}$ at pH 5.0. These experiments were performed at 25°C in 10 mM acetate, pH 5.0, 2% DMSO. Adapted from [4].

for clinical use have been reported [11•]. In all cases, the binding affinity was entropically driven and characterized by unfavorable or only slightly favorable enthalpy changes. The thermodynamic signature of these compounds reflects an optimization process in which high binding affinity has been achieved by preshaping conformationally constrained lead compounds to the geometry of the binding site and by incorporating a high degree of hydrophobicity into the designed ligands. Conformationally constrained ligands exhibit high specificity and improved affinity compared with identical but otherwise relaxed counterparts because of a smaller conformational entropy loss upon binding. The extremely high specificity of conformationally constrained compounds limits their ability to adapt to changes in the target due to mutations or to naturally occurring amino acid polymorphisms arising from genomic diversity. This characteristic could be desirable when dealing with an immutable target, but will render a ligand susceptible to drug-resistance mutations or ineffective against a family of closely related targets. Sometimes, a certain degree of ligand flexibility is required; however, the introduction of flexibility will lower the binding affinity unless it is compensated by other favorable interactions. The hydrophobicity of a ligand and therefore its solubility are also reflected in the entropy/enthalpy balance. The achievement of super high binding affinity requires the synergy of favorable enthalpic and entropic contributions to the binding affinity [4••]. It is apparent that the selection of an enthalpic or entropic optimization influences not only the binding affinity but also other desirable properties of the ligand molecule.

Structure/energy correlations

Better strategies for structure-based drug design will require accurate ways of relating structural and thermodynamic

parameters. A recent study of the structures of protein complexes with low molecular weight ligands has revealed that, in the unbound proteins, the binding sites are characterized by the presence of regions with low structural stability [12]. Because these regions become stabilized upon ligand binding, the energetics of ligand binding is a function not only of the interactions established between ligand and protein (as depicted in the structure of the bound complex), but also of the energy required to bring the protein into its bound conformation. There is a fundamental coupling between binding and conformational stability. Accordingly, from a thermodynamic point of view, the effective binding Gibbs energy is given by:

$$\Delta G_{bind} = \Delta G^{0}_{bind} + \Delta G_{conf} \quad (5)$$

where ΔG^{0}_{bind} is the Gibbs energy of binding obtained under the assumption that the free and bound conformations of the protein are identical, and ΔG_{conf} is the Gibbs energy associated with the change in the protein in its free and its bound conformations. A similar equation applies to the enthalpy change and remaining thermodynamic functions. An accurate structural parameterization of the binding enthalpy is especially important as it will permit the identification of enthalpically favorable ligands from structural considerations. One of the main difficulties in parameterizing the binding enthalpy stems from the fact that different contributions, including the intrinsic enthalpy, the conformational enthalpy and even the protonation/deprotonation enthalpy, are of the same magnitude and need to be explicitly considered. Fortunately, the database of protein–ligand systems for which high-resolution structures and accurate thermodynamic information are available is growing, providing the basis for the development of robust statistical correlations. A first attempt at parameterizing the binding enthalpy of peptide and non-peptide ligands in terms of intrinsic and conformational components has been developed by Luque, Freire and colleagues ([13]; I Luque, E Freire, unpublished data). This parameterization, similar to the one previously developed for protein stability (see [14] for a recent review), is based on changes in the solvent accessibilities of different atom types, but involves the explicit consideration of water molecules that become buried in the protein–ligand complex. The resulting empirical equation is:

$$\Delta H(25) = \Delta H_{conf}(25) + a \times \Delta ASA_{ap} + b \times \Delta ASA_{pol} \quad (6)$$

where coefficients a and b are determined empirically by nonlinear least squares analysis of experimental binding enthalpies and ΔASA values calculated from atomic coordinates. A joint analysis of structure/thermodynamic data for HIV-1 protease, RNase T1, dihydrodipicolinate reductase (DHPR), streptavidin and pp60$^{v\text{-}src}$ SH2 indicates that this approach quantitatively accounts for the binding enthalpy, with a standard deviation of ± 0.9 kcal/mol between experimental and calculated proton-independent values at 25°C. With this limited database, the coefficients a and b are estimated to be around –4.7 ± 2.3 cal/mol × Å^2 and 31.9 ± 5 cal/mol × Å^2, respectively (I Luque, E Freire, unpublished data). It is expected that the availability of additional structural and thermodynamic data will help with the refinement and the development of more rigorous and accurate structural parameterizations of the binding energetics.

Recent applications of isothermal titration calorimetry to protein binding

Our understanding of basic biological functions, from cell development and signaling pathways to pharmaceutical efficacy against bacterial and viral infection, has been enhanced by the availability of thermodynamic data derived from ITC experiments. The thermodynamics of the binding of β-catenin to TCF4, part of the wnt signaling pathway important in gut development, was recently studied by ITC and corroborated by fluorescence and surface plasmon resonance [15]. For SH2, an important domain in signal transduction proteins, ITC has been instrumental in the identification of the specificity determinants of phosphotyrosine peptides [16,17•]. Other important biological interactions studied by ITC include the binding of FMN to apoflavodoxin [18]; SecA, a bacterial translocase, binding to nucleotides [19]; TRAF2 binding to peptides from tumor necrosis factor receptors [20]; and PCNA, proliferating cell nuclear antigen, regulation by p21 [21].

Many proteins studied are targets for pharmaceutical drug development, underscoring the prominent role of ITC in drug design. These proteins include the matrix metalloproteinase stromelysin-1 for cancer and arthritis studies [9••,22]; haematin, a target of current antimalarials [23]; PTP-1B, an important protein in signal transduction and cancer [24•]; isoleucyl-tRNA synthetase, an antibacterial target [25]; and HIV-1 protease, the most important target of clinical antiviral drugs for AIDS [4••,10,11•,26,]. In most of these cases, ITC has been used to study the contribution of enthalpy and entropy to binding, which is important when designing high-affinity ligands. Also important in drug design considerations is the effect of pH and salt concentration on ionization or protonation events, as is the case with a stromelysin-1 inhibitor that contains a hydroxamic acid that is quickly metabolized in the liver when taken orally. By understanding the effect of protonation on binding, different ionizable groups could be considered in order to increase the bioavailability of potential inhibitors.

ITC has been used extensively to study the binding of small molecules to proteins, but it can also be an invaluable tool when looking at protein–protein interactions. Advances in our understanding of these processes will provide useful information about domain–domain interaction, protein recognition, antibody–receptor binding and membrane fusion. Studying the protein–protein interface is hindered because of the large surface area that is involved. In order to look at the binding of IL-1 to its receptor, antibodies were developed to cover part of the interface and

tested by successful competition with the receptor [27]. By simplifying the interface, small ligands can be developed to block receptor activation. The interaction between CD4 and the glycoprotein (gp120) on the surface of HIV particles has also been studied by ITC. In conjunction with surface plasmon resonance and analytical ultracentrifugation, the ITC data were able to identify the magnitude of the conformational change that takes place upon gp120 binding to CD4 [28•]. The interaction of different domains in both an intramolecular and intermolecular manner plays many roles in cellular processes. The binding of kringle 4 domain to tetranectin is important in endothelial cell migration and ITC was used to identify the amino acids that are important in this interaction [29].

Conclusions

ITC is the only technique that can resolve the enthalpic and entropic components of binding affinity. Because the enthalpic and entropic components are related to structural parameters, they can be used as a guide to molecular design, as a way to validate structure-based computational predictions of binding energetics and as a way to develop rigorous structure/energy correlations. As the reliability, sensitivity and accuracy of ITC instrumentation improve, it is expected that it will play a more prominent role in molecular design in general and drug design in particular.

Acknowledgements

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