Measurements of binding thermodynamics in drug discovery

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Thermodynamics governs the process of biomolecular recognition. The steps of characterizing, understanding and exploiting binding thermodynamics have the potential to contribute to an improved rational drug design process that is more robust and reliable. It is only relatively recently that instrumentation capable of direct and full thermodynamic characterization has been improved, giving impetus to the application of thermodynamic measurements in drug discovery. This review highlights current instruments and methods that can be employed to measure binding thermodynamics and their use in studies of biomolecular recognition and drug discovery.

Measurements of binding thermodynamics can extend from determination of affinity and stoichiometry through to estimation of changes in free energy, enthalpy, entropy or heat capacity. This review explains these parameters, how they are measured and their exploitation in drug discovery. It focuses on deconvolution of binding energy into enthalpic and entropic contributions (influenced by changes in bonding and 'disorder', respectively), and on using effects on protein stability to estimate otherwise inaccessible measurements of affinity. Thermodynamic data can be exploited for the assessment of protein preparations, the evaluation of assays, characterization of protein constructs and study of target proteins. Changes in binding interactions are not often detected as changes in affinity because of enthalpy–entropy compensation, where the enthalpy released from improved bonding is offset by an entropic penalty. Measurements of binding enthalpy often reveal such changes.

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Basics of binding thermodynamics

If we consider a simple reversible bimolecular binding event, in a closed thermodynamic system [1], as representative of the interaction of a target macromolecule in its native conformations (N) with a test ligand (L), then this can be represented as:

$$N + L \rightleftarrows N \bullet L \qquad \text{(i)}$$

The change in Gibbs free energy (ΔG), under arbitrary conditions, for the formation of N • L is related to the standard Gibbs free energy change ($\Delta G°$), under defined conditions (e.g. 1 M N and 1 M L at pH 7 and 25°C), by the equation:

$$\Delta G = \Delta G^{\circ} + RT\ln\left(\frac{[N \bullet L]}{[N][L]}\right) \qquad \text{(ii)}$$

At equilibrium, under standard conditions, where $\Delta G = 0$, this becomes:

$$\Delta G° = -RT\ln\left(\frac{[N \bullet L]}{[N][L]}\right) = -RT\ln K_a = RT\ln K_d \qquad \text{(iii)}$$

where R is the gas constant, T is the absolute temperature, K_a is the equilibrium association constant and K_d is the equilibrium dissociation constant. This relationship demonstrates that the value of K_d is dependent upon the free energy of binding, $\Delta G°$.

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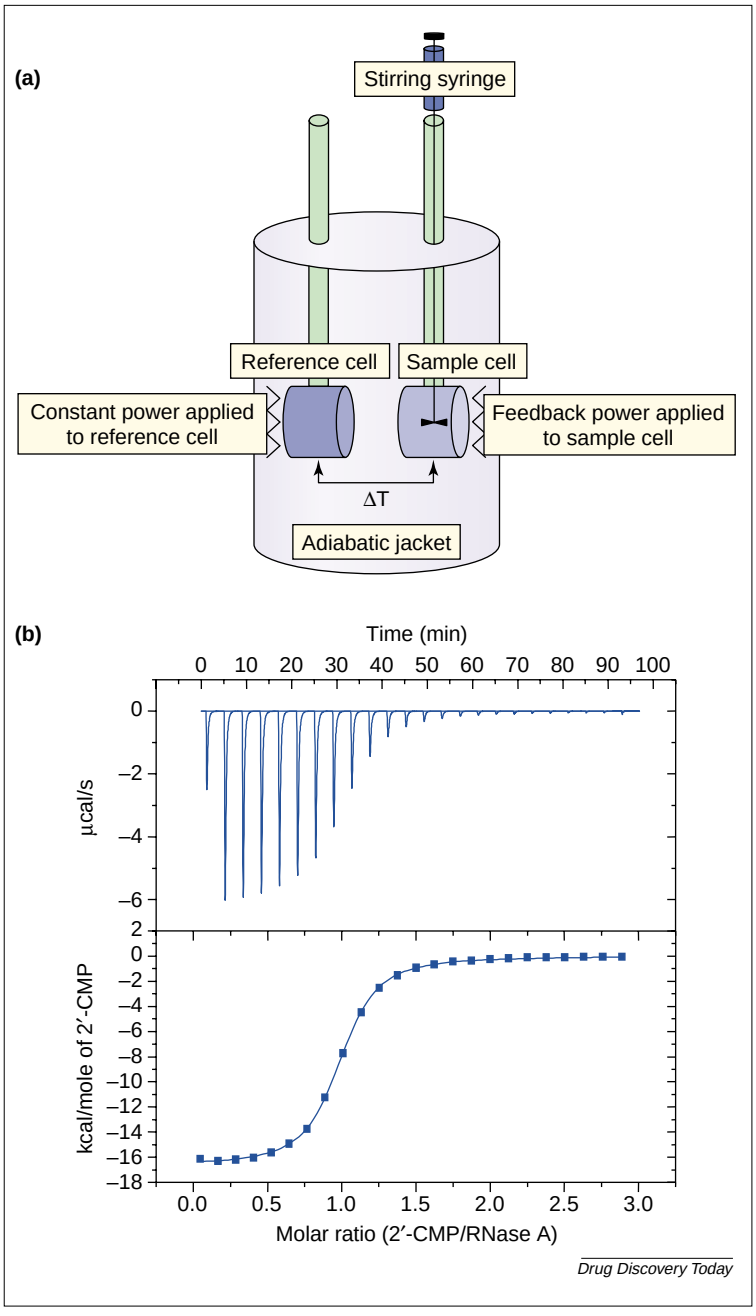

FIGURE 1

ITC measurement. (a) Schematic representation of a power compensation isothermal titration calorimeter. A constant power is applied to the reference cell, activating a feedback circuit, which applies a variable power to the sample cell. This maintains a very small and monitored temperature difference between the cells. The feedback power is the baseline in the ITC experiment. Exothermic reactions generate heat, thus triggering a decrease in the feedback power, whereas endothermic reactions increase the feedback power. **(b)** Data from an ITC experiment, where 2′-CMP was titrated into RNase A. The top panel shows the power applied by the instrument to the sample cell to minimize the temperature difference between the cells. The bottom panel shows the integrals of the peaks from the top panel, together with a line of best fit, used to estimate ΔH, K_d and stoichiometry.

The K_d value can be measured using a variety of experimental techniques, usually based on the determination of the concentration of free L when [N] = [N•L]. A full thermodynamic characterization requires that the enthalpy change, which reflects the heat released or taken up during the association event, will also be measured. There are two common ways of determining the magnitude of these thermodynamic parameters: isothermal titration calorimetry (ITC) and van't Hoff analysis. ITC is the direct and usually preferred method. Sensitive ITC instruments have become available only in the last decade and have led to an explosion of thermodynamic studies. ITC allows the values of the enthalpy change (ΔH), K_d and stoichiometry (n) to be measured in a single experiment. After correction of ΔH for the presence of any linked equilibria to obtain $\Delta H°$, the magnitudes of $\Delta S°$ and $\Delta G°$ are obtained using the relationship:

$$RT\ln K_d = \Delta G° = \Delta H° - T\Delta S° = -RT\ln K \qquad \text{(iv)}$$

where $\Delta S°$ is the change in entropy under standard conditions. An indirect alternative to ITC involves using an integrated form of the van't Hoff relationship, of which equation (iv) is an example. However, application of this particular relationship when following the temperature dependence of K_d usually is problematic. Difficulties arise from the assumption that $\Delta H°$ does not change with the temperature, which is often not the case. It is often necessary to the use an equation allowing for the temperature dependence of $\Delta H°$, characterized by a non-zero, temperature independent ΔC_p term, to describe the change in heat capacity (see below):

$$\ln\frac{K_{d1}}{K_{d2}} = \frac{\Delta H_1 - T_1\Delta C_p}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) + \frac{\Delta C_p}{R}\ln\frac{T_2}{T_1} \qquad \text{(v)}$$

Further problems are encountered because of experimental noise masking the curvature of the van't Hoff plots of ln K_d against $1/T$, and also because of the estimated values of $\Delta H°$ and ΔC_p being dependent upon each other. This has led to reported discrepancies between calorimetric and van't Hoff enthalpies [2–9] (which appear to reflect a lack of precision in the van't Hoff method) and highlights the advantage of using ITC to determine enthalpy changes.

Thermodynamic measurements using ITC

ITC is the only technique that directly measures the enthalpy change upon binding. Most ITC instruments operate a differential cell feedback system (Figure 1a), where the reference cell is filled with buffer and the sample cell usually contains the macromolecule, into which the ligand is titrated. The instrument slowly increases the temperature of both cells during each titration (less than 0.1°C per hour) in a way that approximates isothermal conditions. Injection of the ligand produces heat effects that arise from four sources: the binding interaction, dilution of ligand, dilution of the macromolecule and a heat effect due to mixing. The heat changes arising in the sample cell cause a temperature difference (ΔT) between the two cells, which is detected by the calorimeter and triggers a change in the feedback power applied to the sample cell. Exothermic reactions produce a decrease in applied power, whereas endothermic reactions produce increased feedback. The change over time in feedback power applied to the sample cell is measured (Figure 1b, top panel). The heat associated

with the binding interaction is obtained by integration of the peaks in the power-versus-time plot (Figure 1b, bottom panel) and subsequent correction for the additional heat contributions mentioned above [10]. The parameter values are then estimated by non-linear regression analysis. The range and spacing of added ligand concentrations often can be designed to enable estimations for enthalpy, affinity and stoichiometry within a single experiment [10]. Most of the added ligand becomes bound during the initial injections, because of the excess of macromolecules, and gives a measure of ΔH. The heat change becomes smaller throughout the titration as the available macromolecule sites are filled, and some of the added ligand remains free in solution. This part of the titration curve allows estimation of K_d. Assuming that the concentrations of functional macromolecule and ligand are accurately known, then the stoichiometry is easily measured from the depletion of total ligand to give a lower free-ligand concentration. Useful examinations of the sources of error within ITC experiments have been described by Tellinghuisen [11–13].

A further parameter that can be determined by ITC at different temperatures is the change in heat capacity upon binding, ΔC_p (sometimes known as 'specific heat'), which is influenced by the number of accessible energy states. This is an important thermodynamic parameter, because it controls the magnitudes of $\Delta H°$ and $\Delta S°$:

$$\Delta C_p = \frac{d(\Delta H°)}{dT} = T\frac{d(\Delta S°)}{dT} = \frac{\Delta H°_{T_2} - \Delta H°_{T_1}}{(T_2 - T_1)} \qquad \text{(vi)}$$

Each of the parameters detailed above reflects the characteristic of the complex minus that of the free partners. For example, a negative $\Delta G°$ indicates that the complex, N•L, has less free energy than the sum of free N and L. Binding is favoured under standard conditions only if $\Delta G°$ is negative, therefore negative values of $\Delta H°$ and positive values of $\Delta S°$ promote complex formation. A negative value for ΔC_p indicates that the complex has a lower heat capacity (less energy is required to cause an increase in temperature) than the free partners. ITC has been used extensively to assign thermodynamic signatures to noncovalent interactions. Observed enthalpy changes arise mainly as a result of changes in hydrogen bonding interactions [14,15], with the magnitude dependent upon bond lengths and angles and the sign dependent upon whether there is a net gain (negative) or loss (positive) of H-bond number or strength. Favourable entropy values are often associated with the release of water molecules from a binding interface, whereas unfavourable, negative entropy values are often linked to conformational restrictions [16,17]. Negative (often large) values of ΔC_p, coupled with the favourable entropy changes mentioned above, have been used as an indicator of hydrophobic interactions [18,19].

ITC in drug discovery

Characterization of protein preparations

It is essential that the quality of a protein preparation is sufficiently high to allow unambiguous interpretation of assay results. ITC is a powerful tool for the assessment of protein preparations, because it does not only give a precise measure of K_d, but also allows the measurement of binding stoichiometry and ΔH. Quality assessments can be made, without the need to develop a new assay, on protein generated using various purification and storage protocols. This approach has been used to evaluate preparations of the enzyme acyl-carrier protein enoyl reductase (ACPER) used for kinetic and structural studies [20].

Assay validation

The assays employed in hit identification and secondary screening must be capable of reliably detecting active compounds. It is the direct nature and high precision of ITC that makes it particularly valuable in the pharmaceutical industry, where the pressures for rapid throughput can lead to less rigorous assay protocols. ITC can be used as a benchmark to evaluate data from other binding assays or enzyme kinetic measurements. This is particularly useful when assessing assays that are indirect, rely upon immobilized reagents or involve model substrates [21].

Construct characterization

Often full-length, wild-type proteins are substituted with shortened constructs, sometimes containing mutations, to facilitate drug discovery. The validity of these substitutions must be verified, so that false conclusions are not drawn regarding SAR for the authentic protein [16]. ITC is well placed to test protein constructs because it does not require development of a new assay, it is precise and does not demand catalytically active protein. For example, ITC was used to verify that 24 kDa and 43 kDa fragments of DNA gyrase were valid models of the full-length enzyme [22].

Avoiding kinetic equivalence

The direct nature of ITC means that interacting proteins (for example an enzyme and its protein substrate) can be studied separately, giving an advantage over assays that require both proteins. Methods employing both proteins simultaneously are subject to the phenomenon of kinetic equivalence, where binding to either partner results in the same, or similar, dose–response equations and so prevents identification of the target protein. The value of using ITC in this way has been recognized in studies of signalling pathways and used extensively to characterize the association of phosphotyrosine analogues with SH2 domains [23–27].

Characterization of intermolecular complexes

Exploitation of crystal structures of active compounds bound to target proteins has become commonplace in drug discovery. However, these structures can be used in molecular design only if the structure is valid in the context of the mode of action of the ligand. The crystal structure should contain a molecular complex that is responsible

for the observed biological activity [28,29]. ITC can help in the identification of this complex, because it can provide information on the ability of a second ligand to influence the binding of the test compound. For example, triclosan inhibits ACPER by interacting with NAD^+ that is bound to the enzyme. A crystal structure of a binary enzyme–inhibitor complex would be misleading. ITC has also been used to study molecular recognition by the enzyme myristoyl CoA:protein N-myristoyltransferase (NMT) [20,28,30–32].

Protonation events

Performing ITC in buffers with different enthalpies of ionization allows measurement of the net number and direction of any protons moving upon complex formation. This approach has been used by Kaul *et al*. to allow the identification of amino groups involved in drug binding to 16 S rRNA [33,34].

Future potential

ITC can assist in structure-based design. The dissection of $\Delta G°$ into contributions from $\Delta H°$ and $\Delta S°$ has improved the understanding of the relationships between thermodynamics, structure and function. Thermodynamic signatures have begun to be assigned to energetic contributions from hydrogen bonding, hydrophobic interactions, conformational changes, electrostatic interactions, molecular flexibility and solvent effects [14,15,17,19,35]. However, it is often not straightforward to interpret binding thermodynamics and it is essential to realize that the measured parameters actually reflect differences between free macromolecule and free ligand compared with the complex. Thermodynamic information should be combined with high-quality structural data on both the complex and the free partners [36–39]. The combination of structural and thermodynamic data is most reliable when applied to small, conservative changes in structure.

In spite of these challenges in quantitative interpretation of enthalpy changes, qualitative interpretation can be highly informative. This is because biological systems are subject to enthalpy–entropy compensation, where the enthalpic gain from changing the structure of a compound to increase bonding interactions is offset by an entropic penalty, thus reducing the magnitude of change in free energy [16,20]. When characterizing SARs, modified interactions often can be detected as contributing to $\Delta H°$, even if K_d appears unchanged. Large changes in $\Delta H°$ might indicate a change in binding mode [16,22].

The heat change measured in a calorimetric experiment can be composed of many contributions that are difficult to deconvolute from each other. However, if the linked equilibria can be resolved, then it is possible to use this apparent limitation in an advantageous way to characterize interactions that are otherwise inaccessible [40–42]. This approach makes use of the fact that the magnitude of an energy change is defined by the initial and final states of the system, and independent of the connecting pathway. This thermodynamic linkage can be applied to extend the scope of ITC. Standard protocols usually are limited to measurements of K_d that are between 10 nM and 1 μM [16] and that require around 100-fold higher ligand solubility. Observed values of ΔH often are around –20 to +5 kcal/mol, although it can be difficult to measure values in the range –2 to +2 kcal/mol [16]. However, displacement experiments can be used to indirectly characterize compounds that fall outside the directly accessible range of affinity or solubility [16,43].

ITC instruments have also been used to measure the rates of enzyme-catalysed reactions, by following heat uptake or release [16]. This approach might be particularly advantageous for reactions that are more difficult to follow by other techniques.

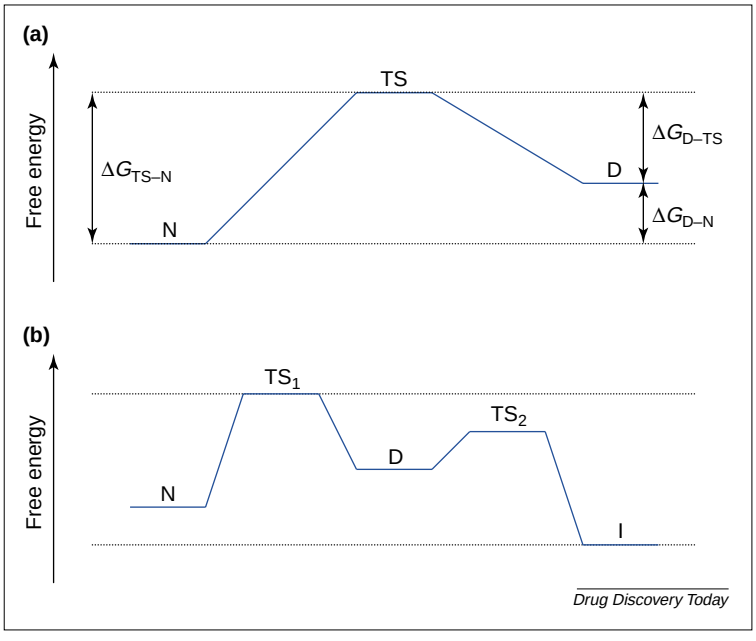

FIGURE 2

Free-energy diagrams. (a) A two-state, reversible, unfolding transition. N and D refer to native and unfolded conformations, and TS to the transition state. **(b)** A three-state, irreversible, unfolding transition for a protein that becomes rapidly inactivated (I) after unfolding.

Estimating binding thermodynamics from changes in protein stability

Thermodynamics of protein stability

Binding of a ligand to a protein occurs only if there is a release of free energy. Accordingly, the protein–ligand complex is more stable than the free partners. The extent of stabilization depends upon the magnitude of the binding energy. Comparison of stability of the complex with that of the free partners allows estimation of binding energy. This approach allows characterization of very tight binding or slowly equilibrating compounds that are not easily assessed by ITC [44]. Here, we refer to proteins that unfold and refold rapidly and reversibly, approximating to a two-state mechanism:

$$N \underset{}{\overset{K_{D-N}}{\rightleftarrows}} D \qquad \text{(vii)}$$

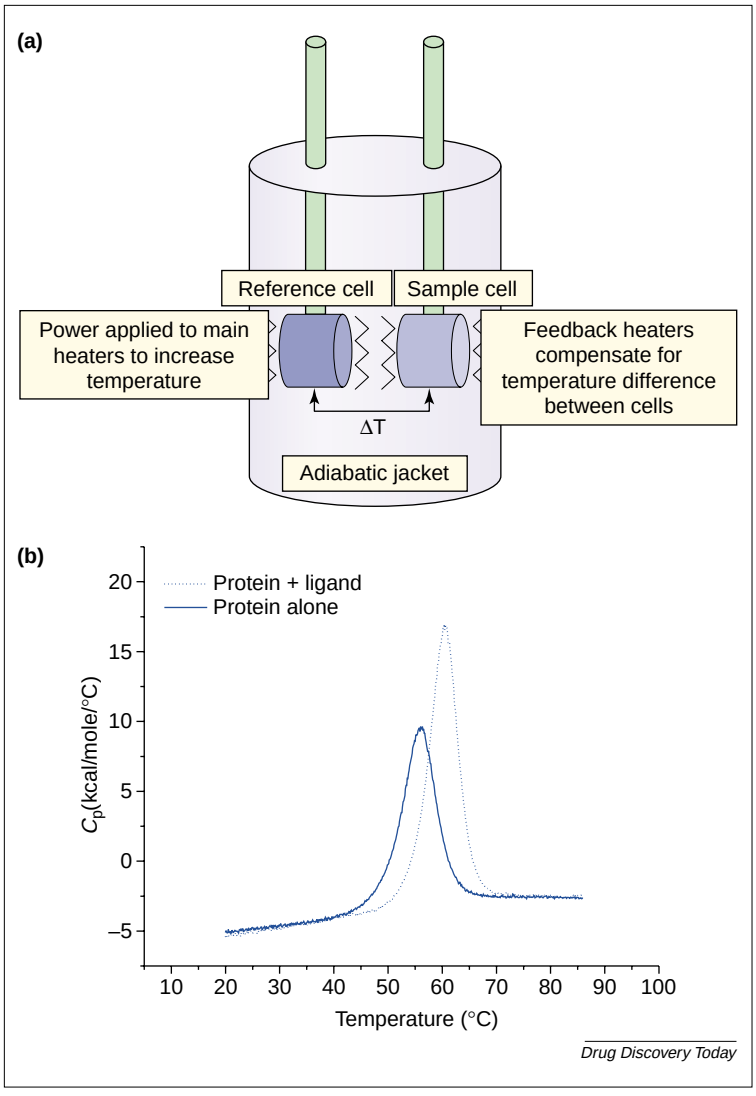

FIGURE 3

DSC measurement. (a) Schematic representation of a typical DSC instrument. In scans where the temperature is increased, power is applied to the main heaters, which causes the temperature of the cells to increase at a constant rate. The temperature difference between the sample and reference cells, ΔT, is measured by the instrument and compensated by the feedback heaters. **(b)** Normalized data output from a typical DSC experiment, showing data for protein alone (solid line) and the increase in melting temperature in the presence of ligand (dashed line). The peak defines the melting temperature (T_m), the shift in baseline defines the change in heat capacity (ΔC_p) and the area under the peak is the heat of unfolding (ΔH).

where N is the native, folded protein and D is the denatured, unfolded protein, with K_{D-N} representing the equilibrium constant for unfolding. In these systems, the stability of the protein is given by the difference in the Gibbs free energy, ΔG_{D-N}, between the native and denatured states:

$$\Delta G_{D-N} = G_D - G_N \quad \text{(viii)}$$

Larger and more positive values of ΔG_{D-N} are observed for more thermodynamically stable proteins, with typical values being of the order of +5 to +15 kcal/mol (+21 to +63 kJ/mol) (Figure 2a).

For irreversibly or slowly unfolding proteins, it is kinetic stability that is important. Irreversible inactivation can be described by the following scheme:

$$N \overset{K_{D-N}}{\rightleftarrows} D \overset{k_i}{\rightarrow} I \quad \text{(ix)}$$

where I is the inactive protein that cannot refold, and k_i is the rate constant for the irreversible inactivation process. Kinetically stable proteins have a larger free-energy barrier between the denatured and inactivated states, because it is the magnitude of this transition energy that controls the rate of inactivation. Figure 2b shows the energy profile for a rapidly inactivating protein, where, once the denatured state is reached, the energy barrier for inactivation is lower than that for refolding. Many protein stability studies reveal more than two states, inactivation or aggregation [45–51].

Measurements of protein stability

There are two commonly employed methods used to characterize the thermodynamic stability of proteins: thermal or chemical denaturation. Thermal denaturation studies can be carried out using a variety of techniques where a physical property of the protein is monitored with respect to temperature, although the widely used method of differential scanning calorimetry (DSC) offers some advantages.

A DSC instrument (Figure 3a) measures the heat capacity of a system as a function of the temperature. It can be used to determine the thermal transition or melting temperature (T_m), ΔH and ΔC_p of unfolding of the sample under study (reviewed in references [52–55]). For an equilibrium unfolding process, the change in these parameters, as a result of ligand binding, can be used to estimate binding affinity (see below).

Chemical denaturation involves monitoring changes in a physical property of the protein with respect to denaturant concentration (usually urea, or guanidine hydrochloride). The analysis of chemical denaturation data are based upon extrathermodynamic assumptions, therefore further evidence for a two-state equilibrium denaturation process should be obtained [56].

Following changes in heat capacity, intrinsic protein fluorescence, circular dichroism or absorbance of the target protein allows a direct measurement of the degree of folding [57]. Binding of a fluorophore or antibody to the denatured state gives an indirect measure. Here, caution should be employed, because binding to the unfolded state might significantly disrupt the equilibrium between native and denatured protein.

The free energy of binding is calculated as the difference between the free energy of unfolding of the unliganded protein and of the complex. The free energy change of unfolding for the apo-protein, ΔG_{D-N}, can be described by the scheme below:

$$D \underset{\Delta G_{D-N}}{\overset{\longrightarrow}{\longleftarrow}} N \quad \text{(x)}$$

In the presence of ligand, the scheme becomes:

$$D \underset{\Delta G_{D-N}}{\overset{\longrightarrow}{\longleftarrow}} N + L \underset{-\Delta G}{\overset{\Delta G}{\longrightarrow\longleftarrow}} N \bullet I \quad \text{(xi)}$$

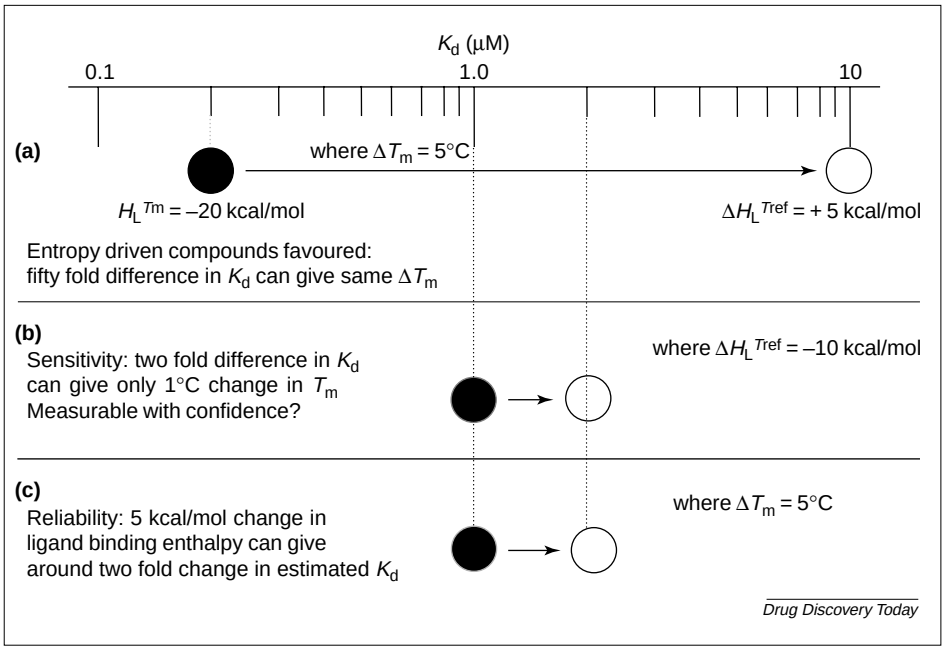

FIGURE 4

Changes in T_m do not always indicate changes in affinity. Theoretical calculations, using equations (xiv) and (xv), were performed on a model system with typical characteristics of T_m (no ligand) = 50°C, ΔH_{D-N} = 100 kcal/mol, ΔC_{pD-N} = 3.6 kcal/mol/K, [L] = 100 μM, ΔC_{pL} = –0.25 kcal/mol/K, T_{ref} = 25°C. **(a)** Compounds with fiftyfold different K_d values (at T_{ref}) can give the same change in T_m [ΔT_m = 5°C for $\Delta H°$ = +5 kcal/mol, K_d = 10 μM (open circle) and $\Delta H°$ = –20 kcal/mol, K_d = 0.2 μM (filled circle)]. **(b)** A twofold change in K_d can give a change in T_m of 1°C [$\Delta H°$ = –10 kcal/mol, ΔT_m = 5°C if K_d = 1 μM (filled circle), ΔT_m = 4°C if K_d = 2 μM (open circle)]. **(c)** A change in $\Delta H°$ of 5 kcal/mol at the same T_m gives less than a threefold change in K_d [ΔT_m = 5°C, $\Delta H°$ = –10 kcal/mol, K_d = 1 μM (filled circle); $\Delta H°$ = –5 kcal/mol, K_d = 2.2 μM (open circle)].

where ΔG refers to the free energy of binding. The observed free-energy change of unfolding for the complex, ΔG_{obs}, is given by:

$$\Delta G_{obs} = \Delta G_{D-N} - \Delta G \quad \text{(xii)}$$

which can be rearranged to give:

$$\Delta G = \Delta G_{D-N} - \Delta G_{obs} \quad \text{(xiii)}$$

Thus, the free energy change on binding is given by the free-energy change of unfolding for the free protein minus the free-energy change of unfolding for the complex. Compounds binding to the native state stabilize the folded protein and the observed free energy change for unfolding the complex will be greater than for the unliganded protein. Conversely, test compounds that act by the undesired mechanism of unfolding the target protein [58,59] are detected as decreasing the stability.

Requirements

In order for conventional analysis to be valid, several conditions should be fulfilled: (i) the ligand concentration should be much greater than the K_d value, so that almost all of the native protein exists as the complex. (ii) The transition should be reversible. Any inactivation of the denatured state complicates analysis. Truly reversible transitions are rarely observed. This problem might be overcome, or overlooked, if the equilibrium can be measured rapidly (e.g. by increasing the temperature scan rate), before inactivation occurs. (iii) The unfolding process should be described by only two states, native and denatured. If intermediates are formed as the protein unfolds, then a more complicated analysis is required. (iv) The ligand should not bind to the denatured state. If this does occur, the stability conferred on the native fold by complex formation and, hence, the affinity of the ligand for the native protein would be underestimated. (v) All measurements should be made at equilibrium. (vi) Activity coefficients are assumed to be 1.

Limitations

Even if all of the above conditions are satisfied, there are several limitations in using protein stability for the measurement of binding affinity. To compare affinities for different compounds that stabilize the protein to different extents, so producing different T_m values or concentrations of denaturant to give 50% unfolding ($[DNT]_{50}$), extrapolation to a fixed set of relevant conditions must be carried out. This often involves extrapolation to 25°C, or [DNT] = 0 M. These long extrapolations can introduce large errors.

Interpretation of thermal stability data

The validity of the extrapolation for DSC data depends upon estimation of ΔH and ΔC_{pL} for ligand binding. These values can be obtained by ITC, or can be estimated with reduced precision from DSC data.

Affinity at the melting temperature is given by:

$$K_d^{T_m} = \frac{[L]}{\exp\left\{\frac{-\Delta H_{D-N}}{R}\left(\frac{1}{T_m}-\frac{1}{T_0}\right)+\frac{\Delta C_{pD-N}}{R}\left(\ln\left(\frac{T_m}{T_0}\right)+\frac{T_0}{T_m}-1\right)\right\}-1} \quad \text{(xiv)}$$

where ΔH_{D-N} is the enthalpy of unfolding for the uncomplexed protein, T_0 is the melting temperature for the uncomplexed protein, T_m is the melting temperature for the protein–ligand complex, ΔC_{pD-N} is the change in heat capacity of unfolding for the uncomplexed protein and [L] is the free ligand concentration at T_m.

Extrapolation to affinity at a relevant temperature is then achieved by using the following equation:

$$K_d^{T_{ref}} = K_d^{T_m} \exp\left\{\frac{\Delta H_L^{T_{ref}}}{R}\left(\frac{1}{T_{ref}}-\frac{1}{T_m}\right)-\frac{\Delta C_{pL}}{R}\left(\ln\left(\frac{T_{ref}}{T_m}\right)+1-\frac{T_{ref}}{T_m}\right)\right\} \quad \text{(xv)}$$

where is the enthalpy of ligand binding at T_{ref}, ΔC_{pL} is the heat capacity change of ligand binding (assumed to be temperature independent), T_{ref} is a relevant reference temperature [44].

Affinity estimates derived from thermal stability measurements are informative, but should be used with caution (Figure 4). The magnitude of the T_m shift observed for different test compounds with the same affinity at the relevant temperature is dependent on the contributions of enthalpy and entropy to binding. Larger T_m shifts are observed for more entropically driven (e.g. hydrophobic) binding. Similarly, a given T_m shift is not unique to a given binding affinity, because a range of different affinities, with different entropic and enthalpic components, might give rise to the same change in T_m. Competing effects could also mask ligand binding. For example, a test compound binding tightly and enthalpically to the native state could be masked by weaker, entropically driven binding to the denatured state. It is even possible that the thermal stability study would show a decrease in T_m in the presence of ligand, even though the binding to the native state is of higher affinity. These effects of the entropic and enthalpic contributions to the binding free energy mean that interpreting T_m shifts is difficult for ligands that have very different physicochemical properties [60].

Interpretation of chemical stability data

Here, the validity of the extrapolation to fixed conditions relies upon the approximate linearity between ΔG_{obs} in equation (xii) and [DNT], so that the free energy of ligand binding can be described by the following equation:

$$\Delta G_{obs} = m[DNT]_{50} - m^{L}[DNT]_{50}^{L} \quad \text{(xvi)}$$

where $[DNT]_{50}$ is the concentration of denaturant giving 50% unfolding (no superscript: in the absence of ligand; superscript L: in the presence of saturating ligand) and m is a constant of proportionality, which can be estimated as the gradient at the midpoint of a plot of fraction of unfolded against concentration of denaturant [56]. It is usually assumed that m is approximately equal to m^L, often a reasonable approximation. Chemical denaturation methods have been applied to high throughput measurements of protein stability [61], illustrating the potential for application in measuring binding affinities.

Protein stability and binding thermodynamics in drug discovery

These methods have value in measuring affinities that are out of reach of other techniques. Characterizing slow or tight-binding ligands often is difficult with standard rate or equilibrium methods. Thermodynamic analysis of protein stability can be employed, as has been demonstrated by the work of Kroe *et al.* [62] in the characterization of slow binding, high affinity p38α MAP kinase inhibitors. DSC, CD and UV thermal melting approaches were used in combination with ITC to calculate ligand-binding affinities from the observed shifts in T_m. This approach showed good agreement with a fluorescence ligand exchange assay, which allowed estimation of K_d from the association and dissociation rate constants for ligand binding. This study illustrates that specialized equipment is not required to employ these methods [62]. Thermal denaturation has also been used in a less quantitative manner, in high throughput hit identification assays [63,64], with approaches being developed by Anadys and Johnson and Johnson (formerly 3-DP). These assays monitor protein unfolding using dyes that change in fluorescence after binding to denatured protein. Hits are identified as compounds that produce a significant increase in T_m.

Future prospects

In order for measurements of binding thermodynamics to be utilized much more widely within drug discovery, technical advances in instrumentation are required. Development in terms of sensitivity and throughput would allow these methods to be further integrated into the hit identification and hit-to-lead processes. Recently, attempts have been made to address these issues with automated ITC and DSC instruments, now commercially available. Additionally, chip-based calorimeters [65–67], potentially able to further minimize sample consumption and increase throughput, are currently in development.

Calorimetry provides a widely applicable method for monitoring molecular interactions in a label free manner and so is a potentially attractive technology for high-throughput assays. The large decrease in sample consumption allowed by chip-based calorimeters, as well as the parallel nature of data collection, means that these developing instruments might have wide application in drug discovery. These advances would lead to larger databases of thermodynamic measurements. Careful interpretation would bring greater understanding of the benefit that thermodynamic information can bring to decision-making within drug discovery. Even so, quantitative explanation of the magnitudes of observed enthalpy and entropy changes remains a formidable challenge for computational chemistry.

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