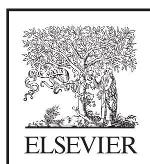
Multi-Scale Approaches in Drug Discovery

From Empirical Knowledge to *In Silico* Experiments and Back

Edited by

Alejandro Speck-Planche



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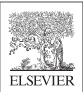

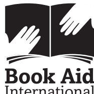

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Chapter 1

Profiling Drug Binding by Thermodynamics: Key to Understanding

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1.1. THERMODYNAMICS: A CRITERION TO PROFILE PROTEIN–LIGAND BINDING

Lead optimization seeks for conclusive parameters beyond affinity to profile drug–receptor binding. One option is to use thermodynamic signatures since different targets require different binding mechanisms. Since thermodynamic properties are influenced by multiple factors such as interactions, desolvation, residual mobility, dynamics, protein adaptations, or changes in local hydration structure, careful analysis of why a particular signature is given can provide some insights into the binding event and help to define how a lead structure can be optimized.

In medicinal chemistry a given lead scaffold, possibly discovered by a fragment-based lead discovery campaign, is optimized from milli- to nanomolar binding (Wermuth, 2003; Blundell et al., 2002; de Kloe et al., 2009) either by "growing" the initially discovered scaffold into a binding site or by exchanging functional groups at its basic skeleton by other, purposefully selected bioisosteric groups. These modifications are intended to increase the binding affinity of the small-molecule ligand toward its protein receptor, and this usually results in an increase in the molecular mass of the candidate molecules to be improved.

To quantify this optimization process, ligand binding to its target protein is measured in terms of a binding constant (see in the following section), which is logarithmically related to the Gibbs binding free energy ΔG. This entity itself partitions into an enthalpic (ΔH) and an entropic ($T\Delta S$) binding contribution (Klebe, 2013; Chaires, 2008).

Since both properties ΔH and $T\Delta S$ contribute additively to the affinity ΔG, a desirable design strategy would optimize both properties in parallel. Is this, however, accomplishable at will, and of advantage in all cases? Without doubt,

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different targets require ligands with different binding mechanism, which will possibly be mirrored by their thermodynamic profiles. A central nervous system drug needs different properties when compared with a drug addressing an extracellular target, e.g., a protease in the bloodstream. High target selectivity can be of utmost importance to avoid undesirable side effects; by contrast, promiscuous binding to several protein family members can be essential to completely downregulate a particular biochemical pathway, e.g., in case of kinases, or to achieve a well-balanced binding profile at a G-protein coupled receptor. Rapid mutational changes of viral or bacterial targets can create resistance against potent ligands. Pathogens follow this strategy by creating, e.g., steric mismatch or changes in the protein dynamics to diminish affinity of the bound active agent (Weber and Agniswamy, 2009; Ali et al., 2010). As the molecular foundations of these mechanisms are quite distinct, well-tailored thermodynamic signatures can be important to escape resistance. Freire et al. suggested improved resistance susceptibility for enthalpically optimized ligands exhibiting sufficient flexibility to evade geometrical modifications of the mutated target protein (Freire, 2008; Ohtaka and Freire, 2005). Equally well, ligands bind with entropic advantage (Fernandez et al., 2012) owing to high residual mobility, which allows the adaptation of multiple binding modes that may be beneficial to escape resistance development. This has been demonstrated by the superior resistance susceptibility of dapivirine or etravirine over other human immunodeficiency virus (HIV) reverse transcriptase inhibitors (Das et al., 2005). To apply such strategies in future projects we first need reliable access and interpretation of thermodynamic data. We therefore want to review in this contribution first the importance of different thermodynamic properties and how they can be determined by experimental methods. Furthermore, not only the accuracy and reliability but also the limitations of the experimental approaches to record thermodynamic data will be considered in the following. Subsequently, on the basis of these insights, we will discuss the thermodynamic signature across congeneric series of protein–ligand complexes to illustrate correlations between structure and thermodynamic profiles.

1.2. QUANTIFYING BINDING AFFINITY IN PROTEIN–LIGAND COMPLEX FORMATION

A binding constant can be determined either as a dissociation constant K_d, or inversely, as an association constant K_a under chemical equilibrium conditions (Ajay and Murcko, 1995). At a given concentration, it indicates which portion of a ligand is bound to the protein according to the underlying law of mass action. For enzymes, the so-called inhibition constant K_i is usually determined in a kinetic assay. The turnover of an appropriate substrate is recorded in a concentration-dependent manner. At low substrate concentration, it determines the dependence of the inhibitory concentration on the change in the turnover rate of the enzymatic reaction. Although K_i is not exactly defined as a

dissociation constant, $K_{i,}$ $K_{d,}$ and K_a are usually referred to interchangeably and represent a kind of strength of the interaction between protein and ligand. Frequently, instead of the binding constant the so-called IC_{50} value is recorded. This value is characterized by the ligand concentration at which the protein activity has decreased to half of the initial amount. In contrast to K_i, IC_{50} values depend on the concentrations of enzyme and substrate used in the enzymatic reaction. The obtained values are affected by the affinity of the substrate for the enzyme, as substrate and inhibitor compete for the same binding site. Using the Cheng–Prusoff equation, IC_{50} values can, in principle, be transformed into binding constants (Cheng and Prusoff, 1973).

Under constant pressure and standard conditions (see later) the binding constant can be transformed into the Gibbs free energy of binding ΔG, which partitions into enthalpy ΔH and entropy ΔS, according to the equation $\Delta G = \Delta H - T\Delta S$. Spontaneously occurring processes are characterized by a negative free energy. At equilibrium, ΔG attains a minimum. The enthalpy reflects the energetic changes of interactions and desolvation associated with the various steps of protein–ligand complex formation. However, enthalpy changes are not the entire answer to why a complex is actually formed. In addition, it is important to consider changes in the ordering parameters. This involves how a particular amount of energy distributes over the multiple degrees of freedom of a given molecular system, composed of the ligand and protein prior to complex formation, the formed protein–ligand complex, and all changes that occur with water and the various components solvated in the water environment. Only if the system together with its surroundings transform into a less-ordered state, which corresponds to a situation of increased entropy, a particular process such as the formation of a protein–ligand complex will occur. Important enough the entropic component is weighted with temperature. It matters a great deal, whether the entropy of a system changes at low temperature, where all particles are largely in an ordered state, or whether it occurs at a high temperature at which the disorder is already significantly enhanced. Energetically favorable, exothermic processes are defined by a negative enthalpy contribution. If entropy increases, a positive contribution is recorded; however, because the entropic term TΔS is considered with a negative sign, an increase in the entropy will cause a decrease in the Gibbs free energy and therefore an increase in binding affinity.

If binding affinity is discussed in terms of equilibrium thermodynamics, two aspects have to be kept in mind. Thermodynamics consider equilibria only; they do not tell anything about the kinetics, that is, how fast or whether even at all a particular equilibrium can be reached. Furthermore, biological processes occur, e.g., in the bloodstream or in a cellular environment where local concentrations constantly change with time. Such systems are only in first approximation in a steady-state situation of constant concentration. Finally, they have to be described as open systems, using nonequilibrium thermodynamics, which are by far more complex.

In principle, the thermodynamic properties are denoted with the superscript "°" to indicate that the values refer to standard states; however, this sign is often omitted. The necessity to refer to a standard state is to achieve comparability between measurements on a common scale for a mutual comparison (Krimmer and Klebe, 2015). Energies can only be measured as relative differences between two states, comparable with the determination of heights, e.g., of mountains. Usually, we refer the height of mountains relative to sea level to define a common and convenient reference point. In case of thermodynamic data, the binding free energy is referred to a standard state. This is defined as the conversion of 1 mol protein and 1 mol ligand to 1 mol of protein–ligand complex in a hypothetical ideal ("infinitely diluted") solution at constant pressure of $p^\circ = 10^5$ Pa. Such a solution has an activity coefficient of 1 ("activity" replaces "concentration" in real mixtures and the "activity coefficient" is a measure of the "effective concentration" of the species in a mixture, thus it describes the deviation from the originally weighted-in concentrations) (Pethica, 2015). The temperature is not part of the standard state and therefore has to be specified. The dissociation K_d and ΔH° are determined experimentally, e.g., in an isothermal titration calorimetry (ITC, see later) experiment, ΔG° is calculated using the relationship $\Delta G^\circ = RT \ln K_d$. The equation contains the natural logarithm of K_d, which is then used as a unitless value. Therefore, formally a standard concentration c° is used, which is set by convention as 1 M.

1.3. METHOD OF CHOICE TO ACCESS THERMODYNAMIC DATA: ISOTHERMAL TITRATION CALORIMETRY

The method of choice to obtain thermodynamic data is ITC (Chaires, 2008; Ladbury and Chowdhry, 1996; Ladbury, 2001; Velazquez-Campoy and Freire, 2005). ITC allows highly accurate determination of thermodynamic parameters without further requirement for chemical modifications such as labeling or immobilization. After an appropriate correction of superimposed effects such as the heat involving the exchange of protons with the surrounding buffer, the directly measured heat signal of an ITC experiment at a given temperature on titrating two compounds (e.g., protein and ligand) of known concentration provides the binding enthalpy ΔH and binding stoichiometry. From the shape of the titration curve the equilibrium binding constants (K_a or K_d) are determined and allow to directly calculate ΔG via $\Delta G = RT \ln K_d$. From an ITC experiment, ΔH and ΔG result simultaneously, and entropy is calculated as the numerical difference between ΔH and ΔG using the equation $\Delta G = \Delta H - T\Delta S$.

If protons are released either from the protein or the ligand or picked up from the buffer, a heat of ionization of the functional groups involved in the protein, ligand, and buffer substance(s) will be overlaid to the total heat signal (Jelesarov and Bossard, 1999; Baker and Murphy, 1996; Falconer and Collins, 2011). Whether such steps are involved can be determined performing the titration

experiments at buffer conditions of varying heat of ionization, or with protein variants where a particular functional group has been exchanged (e.g., Tyr/Phe, Asp/Asn). Depending on the applied pH conditions, the molar ratio of entrapped or released protons is available from the Henderson–Hasselbalch equation. Quantifying the amount of protons exchanged between the involved functional groups requires knowledge of their pK_a values. This will also help to define where the protons actually go (Czodrowski et al., 2007). Such pK_a values can be measured, taken from tabulated data, or computed by programs. Yet, an important complication has to be regarded: pK_a values change with environment, thus substantial pK_a shifts may occur during complex formation, easily ranging over several orders of magnitude. As a result, some effort might be required, including mutagenesis of the likely involved residues, to trace which of the putative titratable groups of the system are actually responsible for the protonation (Neeb et al., 2014). This has to be taken into account while correcting for superimposed protonation effects. Even tricky cases can occur where protons are internally shifted between ligand and protein functional groups largely to the same amount (Baum et al., 2009a). As a result, no net protonation effect is observed, even though important changes do occur. Remarkably, heat-of-ionization effects are minor for oxygen functionalities and rather large for nitrogen-containing functional groups. The same holds for buffer compounds based on oxygen or nitrogen functional groups (Goldberg et al., 2002). Therefore, as a strategy to avoid at least major effects arising from the buffer, ITC experiments can be performed, e.g., in phosphate or acetate buffer where the buffer's heat of ionization is rather small; however, contributions from the functional groups of either the ligand or protein will still not be corrected. It is essential to correct for superimposed protonation steps in ITC experiments as heat effects will be superimposed to the actual binding signal. If remaining uncorrected, these heat effects will distort the enthalpic signal assigned to the ligand binding. As entropy is not measured but calculated from the numerical difference between ΔG and ΔH, a false partitioning of enthalpy and entropy will inevitably result. If such uncorrected data are used to interpret thermodynamic signatures, ill-defined correlations must be the consequence.

Overlaid protonation steps can also provide the chance to record thermodynamic data of entropic binders (Simunec, 2007). Binding of the latter ligands does not lead to any measurable heat signal. Only, if a protonation step is superimposed, the binding event may result with an exothermic or endothermic heat signal. Subsequently, the purely entropic binding profile only becomes apparent if the required correction is performed. Furthermore, it is essential to compare ITC experiments run at the same temperature. As ligand binding is predominantly related to a negative heat capacity change (see later), all binding signatures become enthalpically more favorable with increasing temperature (Jelesarov and Bossard, 1999). Accordingly, data collected at different temperatures can hardly be compared conclusively.

As mentioned, ITC experiments performed without control of possibly overlaid proton exchanges will be rather meaningless and false interpretation

will likely result. If protonation changes occur within a congeneric series of protein−ligand complexes quite uniformly because, e.g., the protonation site is rather remote from the site where the congeneric ligands are actually modified, a comparison of the relative differences of the thermodynamic data is still justified and can be conclusively interpreted.

1.4. ISOTHERMAL TITRATION CALORIMETRY VERSUS VAN'T HOFF DATA TO ACCESS THERMODYNAMIC PROPERTIES

As mentioned, ITC experiments have the important advantage that two thermodynamic properties ΔH and ΔG result from one experiment, performed at the same temperature. Frequently, van't Hoff evaluations are performed to access thermodynamic parameters. In this case, the binding event is observed, usually via an easily recordable signal [e.g., photometric absorption, spectroscopic data, nuclear magnetic resonance (NMR), surface plasmon resonance (SPR), etc.] across a temperature range. To evaluate the measured data, the mostly rather inadequate assumption is made that the thermodynamic properties such as ΔH are temperature independent and may be determined by plotting in linear fashion the binding constants measured at different temperatures against the reciprocal temperature. For this, the so-called linear form of the integrated van't Hoff equation is used, which assumes ΔH to be temperature independent (Krimmer and Klebe, 2015). If the studied temperature range is rather small, an approximate linear correlation might be suggested and (inadequately) the slope of this linear correlation is extrapolated to assign a binding enthalpy. However, biological processes strongly depend on temperature; in consequence also the thermodynamic properties of these processes are temperature dependent. Thus, the van't Hoff equation cannot be straightforward integrated by assuming ΔH to be temperature independent over the considered temperature range. Instead, a nonlinear fit has to be at least applied (Liu and Sturtevant, 1995; Horn et al., 2001; Mizoue and Tellinghuisen, 2004). Furthermore, the van't Hoff evaluation assumes that the binding event follows a two-state transition between free and bound state and that the recorded signal change used to determine the binding constant reflects the entire population of free and bound molecules (Jelesarov and Bossard, 1999). As the correctness of this assumption is difficult to estimate, particular if the binding event passes through multiple states, the van't Hoff evaluation is even more difficult to justify. These considerations strongly argue to be very careful in using van't Hoff data as a source of thermodynamic binding information, at least when they are taken from a linear extrapolation. ITC appears, after appropriate corrections, as the more reliable information basis.

1.5. DETERMINATION OF HEAT CAPACITY CHANGES ΔCp

Another property, from a theoretical point of view a very informative one, is the change in heat capacity, ΔCp, at constant pressure of a biological system.

It indicates how well a system can absorb or release heat, thus it provides a crude idea how many degrees of freedom are available in the system to dissipate or store heat. Experimentally, ΔCp is available from ITC titrations performed at different temperatures (Jelesarov and Bossard, 1999). However, this evaluation and the subsequent interpretation run into similar complications as the van't Hoff evaluation. The considered multicomponent system as the formation of a protein–ligand complex is so complex, that even across a temperature range of 20–30K major structural changes will occur (e.g., already in the ubiquitously present bulk water phase) that make ΔCp interpretations extremely challenging. Consequently, it is usually rather problematic to discuss straightforward ΔCp changes of a protein–ligand complex system on molecular level, even though convincing examples have been reported (Stegmann et al., 2009).

1.6. THE ACCURACY AND RELEVANCE OF ISOTHERMAL TITRATION CALORIMETRY DATA

An important aspect addresses the accuracy of thermodynamic measurements (Tellinghuisen, 2012; Tellinghuisen and Chodera, 2011). Above all, the recorded data depend on the buffer composition and ionic strength of the ions used. Control experiments have been performed using the same biological system across different laboratories or different devices to estimate accuracy (Myszka et al., 2003; Baranauskienėm et al., 2009). Purity of the ligands, stability of the proteins, constant water content, and avoidance of protein self-degradation or autoprotolysis have to be regarded.

Thus, how accurate can we expect ITC experiments to be? First, repetitive experiments have to be performed and averaged. Error propagation across interdependent properties has to be regarded. Besides calibration of the instrument, thorough control of the concentrations of the prepared solutions is important. Proteins are fragile compounds, and their activity depends on the way they were prepared, purified, and stored before usage. If protein solutions are prepared from solid material, the actual water content of the samples can be crucial. To achieve reliable results, it is highly advised to use material from the same batch for all experiments and to prepare solutions always freshly. Particularly proteases can decompose in concentrated solutions from autoprotolysis.

Usually, the ligand is titrated from a syringe with highly concentrated solution in a dropwise fashion into a large volume of the protein solution. In principle, this experimental setup can be reversed; however, limited solubility and availability or restricted stability of the proteins at high concentration impede the dropwise addition of a highly concentrated protein solution to the diluted ligand solution. Using the setup with the ligand added from the syringe, particularly the purity of high-affinity ligands is crucial for the accuracy of determining thermodynamic parameters. This results from the sigmoidal shape of the titration curve. For potent ligands, all injected

molecules find in the beginning of the titration an unoccupied binding site. In due course of the experiment and after binding stoichiometry has been past, the heat signals reduce within a small amount of injections to the baseline when only the heat of dilution is still recorded. Uncertainties in the protein concentration will shift the binding isotherm leading to deviations in the expected stoichiometry, which are usually corrected for, assuming a 1:1 binding model. Thus, only minor deviations in the free energy determination are experienced. A much larger error will affect the determination of ΔH, which results from the integration over all heat signals. Ligand impurities can reduce these signals significantly and lead overall to smaller integrated ΔH values. As a result, an overestimated enthalpy/entropy compensation will be calculated. In case of weak binders, sigmoidal titration curves are hardly possible to record. Since fragment binding, particularly at high concentration, does not necessarily result in a stoichiometry of 1:1 (Mondal et al., 2014; Schiebel et al., 2016; Radeva et al., 2016a,b), the integration of the heat signals can become very inaccurate and such data are difficult to evaluate to reveal a reliable thermodynamic signature. Instead, displacement titrations can be used to make calorimetric analysis accessible for such ligands (Krimmer and Klebe, 2015; Zhang and Zhang, 1998; Rühmann et al., 2015a). They are also applicable to very strong binding ligands where the titration curve for the direct titration degenerates from sigmoidal to steplike shape making assignment of a K_d value unreliable (Valezques-Campoy and Freire, 2006).

Considering all these factors properly including the correction for superimposed protonation events, an evaluation across a series of congeneric compounds in terms of relative differences will cancel out most of the systematic errors. In favorable cases, the accuracy can amount to about 1 kJ/mol, particularly if relative comparisons of two related ligands are performed (Krimmer and Klebe, 2015).

The ITC experiment records all changes involving heat effects going from the individually solvated protein and ligand to the newly formed protein–ligand complex. Besides conformational and configurational changes of the binding partners, protein and ligand, this process involves also substantial changes in the water structure. However, the binding event is a multistep process; all modifications are finally compressed into the three thermodynamic parameters ΔG, ΔH, and −TΔS, and they represent the entire complex formation process. Subsequently, we are tempted to relate the changes of these parameters with the binding event and solely focus rather naively on the newly formed protein–ligand interface. However, much more is involved that might reflect changes in the protein structure, e.g., activation/deactivation of conformational, vibrational, or rotational degrees of freedom of protein side chains remote from the binding site or rearrangements of the water structure across the surface of the involved components, i.e., ligand, uncomplexed protein, and newly formed protein–ligand complex. Even compensating entropy–entropy effects have been reported involving locally deviating

activation and attenuation of rotational degrees of freedom of methyl group side chains (Homans, 2005; Kasinath et al., 2013). All these contributions will have an impact on the thermodynamic signature of the binding event. As will be shown in the following sections of this review, the presence or absence of a single water molecule next to the protein–ligand interface can easily shift the thermodynamic profile in enthalpy and entropy by 5–7 kJ/mol in either direction. Usually, within a series of congeneric ligands, we tend to interpret effects of this magnitude as significant and they might give rise to a contrary interpretation of the binding event, even though, unexpectedly, only the difference of one involved water molecule is responsible for the distinct thermodynamic profile. This can easily lead to misinterpretation, particularly if, rather superficially, a particular drug candidate is assessed as superior, e.g., for its more enthalpic profile (Ladbury et al., 2010). To reduce the danger of misconception, the consideration of complementary information is of utmost importance. In consequence, interpretation of thermodynamic data, even across a very narrow congeneric ligand series, will hardly be meaningful without monitoring the structural properties of every formed protein–ligand complex simultaneously. Such information is available from high-resolution crystallography, and the concomitant survey of the produced complexes by crystal structure analyses (or/and NMR) is an inevitable requirement for the meaningful interpretation of thermodynamic signatures.

Even in such ideal cases where the corresponding crystal structures are available, some caveat is given. ITC data are recorded at ambient temperature in a buffered solution. Structural data, however, are collected in the crystalline phase often enough at liquid nitrogen temperature. Thus, can any correlation between solution and crystalline state be expected? Recent comparative diffraction studies performed at ambient and low temperature revealed differences in the scatter of side chain torsion angles (Fraser et al., 2011; Fenwicka et al., 2014). Supposedly, these molecular degrees of freedom are soft enough to still allow motion and adjustment under the flash cooling protocol applied to freeze protein crystals for diffraction experiments. They will adjust with temperature. Other motions involving larger rearrangements cannot occur in the crystalline phase, for example the complete rearrangements of water surface layers. Here, flash-cooled crystals will likely mirror the situation at ambient temperature. In several of our investigated compound series, we observed a qualitative correlation of the B-factors, which are attributed to residual thermal motion in a crystal with entropic effects monitored by ITC in solution (Baum et al., 2010; Baum et al., 2009b; Neeb et al., 2016). Therefore, at least qualitatively, a correlation between ITC and crystal structure data seems to be given allowing for a conclusive discussion of structures along with thermodynamics. This estimation matches well with the conclusions of Nakasako (2004), who compared solvation patterns of water molecules observed in crystal structures under cryo conditions with other physicochemical measurements and found high consistency. These findings make us confident that crystal structures are actually relevant for the interpretation of ITC data.

1.7. PROTEIN–LIGAND COMPLEX FORMATION: WHAT CAN THERMODYNAMIC DATA TELL ABOUT A GOOD STARTING POINT FOR OPTIMIZATION?

Subsequent to these general considerations about the access and accuracy of thermodynamic data, we want to discuss possible strategies how to make use of thermodynamic signatures with respect to lead optimization. As discussed previously, an ITC experiment gives access to the simultaneous determination of ΔH and ΔG. Using the fundamental equation $\Delta G = \Delta H - T\Delta S$, the entropic contribution is calculated from the numerical difference between ΔH and ΔG.

What appears on first sight as a very convenient access to enthalpy and entropy bears an important caveat. Since both entities do not result from independent experiments, but from a numerical difference, they are inevitably correlated and will automatically compensate to match with the measured free energy. This means that any undetected systematic error or uncorrected effect in the measured enthalpy will automatically be reflected in a compensatory change of the entropic contribution (Krimmer and Klebe, 2015; Sharp, 2001; Olsson et al., 2011; Chodera and Mobley, 2013). Nonetheless, enthalpy/entropy compensation also occurs as an intrinsic physical phenomenon (see later) (Dunitz, 2003), but it has to be kept in mind that experimental deficiencies, inappropriate data corrections, or reference to inadequately defined standard states required to perform global comparisons will give rise to some inevitable, from a physical point of view, irrelevant enthalpy/entropy compensations.

Prior to complex formation, both, the protein and ligand, are separately solvated and move freely in the bulk solvent phase. Upon complex formation, the two independent particles merge into one species. On achieving this, they sacrifice rotational and translational degrees of freedom as two independent particles reduce to one (Murray and Verdonk, 2002). This entropic loss was calculated to amount to about 16 kJ/mol and is associated with a price in Gibbs free energy. Experimentally, this value is nicely confirmed by Nazare et al. (2012) and Borsi et al. (2010), who studied the merging of two nonoverlapping fragments binding to factor Xa or matrix metalloproteinase-12. Comparing the affinity of the two individual fragments with that of the merged "supermolecule" reveals values of 14–15 kJ/mol, which match well with the theoretically determined entropic cost for the loss of degrees of freedom for merging two particles into one.

This also sets a lower affinity limit for complex formation to about −15 kJ/mol, as reflected in a thermodynamic data compilation of Olsson et al. (2008). The authors have collected published ITC data and plotted the information on $\Delta H/-T\Delta S$ diagram (Fig. 1.1). The main diagonal in this plot corresponds to the observed data scatter in Gibbs free energy, which covers a range from approx. −15 to −60 kJ/mol. This distribution reflects the range accessible to medicinal chemists for ligand optimization from millimolar to subnanomolar

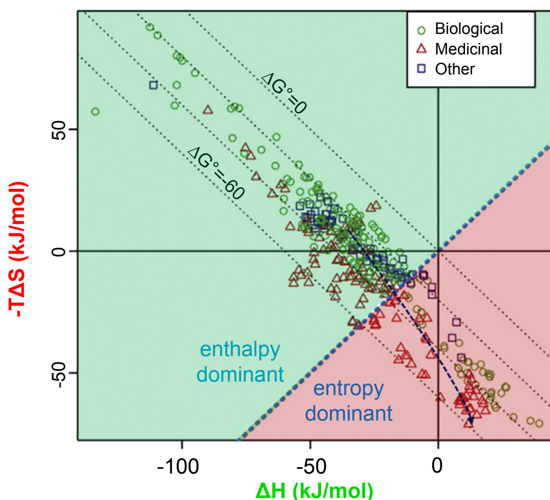

FIGURE 1.1 Published isothermal titration calorimetry data plotted onto a ΔH/−TΔS diagram. The diagram has been split into areas where enthalpy (green) or entropy (red) dominates the Gibbs free energy of binding (ΔG). Along the main diagonal the scatter in ΔG from about −15 to −60 kJ/mol is found corresponding to the range accessible to medicinal chemistry. Perpendicularly, the mutual scatter in enthalpy and entropy with opposing contributions to ΔG is shown. It spreads across a very large range indicating intrinsic enthalpy/entropy compensation. Data are classified for ligands coming from different sources; optimization resulting from medicinal chemistry programs tend to improve for entropic reasons (*blue dashed arrow*). *The figure was taken with permission from Olsson, T.S.G., Williams, M.A., Pitt, W.R., Ladbury, J.E., 2008. The thermodynamics of proteinligandinteractions and solvation: insights for ligand design. J. Mol. Biol. 384, 1002−1017 with slight modifications.*

affinity. The secondary diagonal, perpendicular to the ΔG distribution, displays the mutual scatter in enthalpy and entropy with opposing contributions to ΔG. As this distribution spreads over a very large range, it reveals an inherent enthalpy/entropy compensation, leading to the rather small scatter in ΔG.

The enthalpy/entropy diagram can be dissected into one area where enthalpic (green) and another where entropic contributions (red) dominate. Remarkably, when compared with biomolecules, ligands originating from medicinal chemistry optimization tend to display in this diagram an enhanced entropic binding profile with growing potency (blue arrow). This observation has provoked in the past the question as to whether a more enthalpically or entropically driven binding is desired (Freire, 2008; Ladbury et al., 2010) and whether such a ligand binding profile can be designed at will (Freire, 2009)? The intrinsic enthalpy/entropy compensation suggests that both properties are interdependent in reciprocal manner, thus can they be optimized independently or both in parallel? The latter would optimize ΔH and −TΔS simultaneously to achieve the most efficient ΔG enhancement, but is such a strategy achievable in light of the enthalpy/entropy compensation? Even though there is no physical law arguing for mutual enthalpy/entropy compensation,

considerations on molecular level suggest that both opponents will at least partially cancel out (Dunitz, 2003). Summarily, strong enthalpic interactions will fix a ligand at the binding site, which is entropically unfavorable. By contrast, pronounced residual mobility in the bound state is entropically beneficial, as a smaller number of degrees of freedom is lost upon complex formation. However, the quality of the formed interactions will be less efficient leading to a minor enthalpic contribution.

The correlation diagram shown in Fig. 1.1 [and similar evaluations that have been published in literature (Olsson et al., 2008; Hann and Keserü, 2011; Ferenczy and Keserü, 2010, 2016; Reynolds and Holloway, 2011)] implies that the published ITC data would have all been properly corrected for superimposed protonation changes and refer to a common standard state. Supposedly, this is in most cases not given making any global comparison of such data quite questionable and the conclusions drawn can be easily misleading (Krimmer and Klebe, 2015). The aforementioned comparison with the height of mountains should be consulted again to explain this issue. As mentioned, the height of mountains is conveniently referred to sea level; however, also other scales could be imagined, e.g., the midpoint of the earth, the lowest depression on a continent, or the deepest point found in any of the oceans. Many such reference points are imaginable. However, if everyone would decide to use a different reference point to measure heights, it would be impossible to compare mountain heights globally on a comparative map. For a mountain climber who plans to ascent a next summit and to estimate on the required resources, it is only important to know the relative height of the surrounding summits with respect to the point from where he starts his trip. Microcalorimetric measurements are setup under very different conditions, mostly optimized for the system studied (added salts, buffers, detergents, added cofactors or co-substrates, DMSO, etc.) but hardly adhering to standard conditions, particularly with respect to concentrations and the assumed "ideal solution" conditions (see earlier). This makes global comparison of such data nearly impossible, and falsely inevitable enthalpy/entropy compensations will affect the data to be correlated. We studied some systems under varying salt, DMSO, or detergent concentrations with and without added cosubstrate and observed that the Gibbs free energy is only little affected; however, the measured heat signal changes fairly strongly, thus affecting the partitioning of the derived ΔH and $-T\Delta S$ values on absolute scale. Nevertheless, what matters in this case is the important observation that within a congeneric series of ligands the *relative* differences in the changes of enthalpy and entropy ($\Delta\Delta H$ and $-T\Delta\Delta S$) remain virtually unaffected by the applied conditions. This underscores that the comparison of thermodynamic data taken from ITC measurements should only be performed across congeneric series on *a relative scale*. Considering our mountain climber, his decision regarding which summit to climb with the resources available to him can be taken considering the relative difference of the summit heights whereas the absolute heights are not important for this decision.