Published in final edited form as:
*Annu Rev Biophys*. 2013 ; 42: 121–142. doi:10.1146/annurev-biophys-083012-130318.

# Entropy-enthalpy compensation: Role and ramifications in biomolecular ligand recognition and design

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## Abstract

Recent calorimetric studies of small molecule interactions with biomolecular targets have generated renewed interest in the phenomenon of entropy-enthalpy compensation. In these studies, entropic and enthalpic contributions to binding are observed to vary substantially and in an opposing manner as the ligand or protein is modified while the binding free energy varies little. In severe examples, engineered enthalpic gains can lead to completely compensating entropic penalties, frustrating ligand design. Here, we examine the evidence for compensation, as well as its potential origins, prevalence, severity, and ramifications for ligand engineering. We find the evidence for severe compensation to be weak in light of the large magnitude of and correlation between errors in experimental measurements of entropic and enthalpic contributions to binding, though a limited form of compensation may be common. Given the difficulty of predicting or measuring entropic and enthalpic changes to useful precision, or using this information in design, we recommend ligand engineering efforts instead focus on computational and experimental methodologies to directly assess changes in binding free energy.

### Keywords

entropy-enthalpy compensation; isothermal titration calorimetry; small molecule ligand engineering

## INTRODUCTION

In recent years, numerous studies of protein-ligand association invoke some form of "entropy-enthalpy compensation" to explain the observed thermodynamic partitioning of binding free energy between entropic and enthalpic components [1–11]. A recent meta-analysis of the binding thermodynamics of an aggregated set of ~100 protein-ligand complexes selected from the BindingDB database [12] concluded that compensation is "clearly evidenced" [13]. Several groups have reported a *severe* form of compensation, in

**DISCLOSURE STATEMENT**
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which small ligand modifications (such as the introduction of a hydrogen bond partner) result in a favorable enthalpic gain that is completely offset by equivalent loss in entropy, resulting in no net gain in affinity [7,11,14]. Additionally, drug resistance mutations can apparently cause large, nearly compensatory changes in the enthalpies and entropies of inhibitor binding, suggesting important changes in ligand recognition mechanism [2]. In light of such observations, it has been proposed that entropy-enthalpy compensation should be a major concern during lead optimization in drug discovery [11,15–20].

A pervasive, severe form of entropy-enthalpy compensation would pose obvious problems in the engineering of high affinity ligands [15,17]. Complete compensation would mean that modifications made with the intent of improving the enthalpy of interaction (such as the introduction of a hydrogen bond donor) would be counterbalanced by unfavorable entropic contributions, leading to no net gain in affinity. Complete compensation would also imply that modifications made with the intent of reducing unfavorable entropic contributions to binding (such as the removal of rotatable bonds or addition of internal ligand constraints) would lead to equivalent enthalpic penalties, again resulting in no net gain in affinity. Obvious questions arise: Is compensation a real phenomenon? If so, how pervasive is it? And how can we know in advance which ligands or scaffolds will be difficult to optimize as a result?

Here, we review the concept of entropy-enthalpy compensation and critically evaluate experimental evidence for the existence of this phenomenon, discussing possible alternative explanations. We also highlight proposed physical mechanisms for compensation, and conclude by discussing implications of this work for rational ligand design.

## ENTROPY-ENTHALPY COMPENSATION

The Gibbs free energy change[1], $\Delta G$, of a ligand binding reaction can be written

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

where $\Delta H$ denotes the *enthalpic* contribution and $-T\Delta S$ the corresponding *entropic* contribution to binding. In thermodynamic terms, the enthalpic component quantifies the change in heat associated with binding, while the entropic component quantifies a change in disorder of the overall system (including the ligand, receptor, and surrounding solvent).

In ligand binding, the term *entropy-enthalpy compensation* generally means that a ligand modification results in a change in the enthalpic contribution to binding, $\Delta\Delta H \equiv \Delta H_2 - \Delta H_1$, which is partially or fully offset by a similar change in the entropic component of binding, $T\Delta\Delta S \equiv (T\Delta S_2) - (T\Delta S_1)$. This implies that $\Delta\Delta H$ and $T\Delta\Delta S$ share the same sign if compensation occurs and, for a strong form of compensation in which the net change in binding affinity $\Delta\Delta G \approx 0$, we must have $\Delta\Delta H \approx T\Delta\Delta S$.

In the literature, evidence of compensation is often presented in the form of a graph in which $T\Delta S$ is plotted against $\Delta H$ and fit with a linear regression (as in Fig. 3b), often with a slope

[1]We refer to standard state quantities [21–24] throughout, omitting the ° superscript for simplicity.

near unity. Alternatively, the graph may depict $\Delta S$ (rather than $T\Delta S$) plotted against $\Delta H$, with the slope of linear regression—termed the *compensation temperature*—is often ascribed physical meaning [25–27].

In the discussion of evidence for compensation, numerous questions have been raised: What is the origin of this phenomenon? Is this a universal phenomenon in thermodynamics? In ligand binding? If compensation is not universal, does its appearance in a congeneric series provide some form of "extra-thermodynamic information" about the system that could be exploited? Or is there a more mundane explanation for why we often see this correlation between enthalpic and entropic contributions to binding? We attempt to address some of the most provocative aspects of these questions in the course of this review.

## EXPERIMENTAL EVIDENCE FOR COMPENSATION

We begin by reviewing experimental evidence supporting the notion that entropy-enthalpy compensation exists, is pervasive, and can be severe. As many reviews have already been devoted to this topic [25–39], we restrict ourselves to a brief review of experimental evidence for compensation in general before focusing on evidence of compensation specifically in protein-ligand binding.

### Entropy-enthalpy compensation appears in many thermodynamic phenomena

Many experiments suggest the existence of a "weak" form of entropy-enthalpy compensation in response to the variation of a thermodynamic parameter, such as temperature or pressure. For example, in the transfer of neopentane from its neat phase to water (depicted in Fig. 1a), the enthalpic and entropic components vary over a large range with temperature, but appear to compensate so that the overall free energy of transfer varies by much less over the same temperature window [40]. Similarly, the unfolding of myoglobin in water (Fig. 1b) is accompanied by large changes in the entropic and enthalpic components but small changes in the overall free energy of unfolding over a wide range of experimental temperatures [41]. Protein-protein association (Fig. 1c) also often demonstrates similar behavior with temperature [35].

Since entropy-enthalpy compensation appears in so many different contexts, it has been proposed by some to be a *ubiquitous* thermodynamic phenomenon [25,31]. While a number of critical reviews have cautioned that the relatively narrow temperature range in which this behavior is observed can lead to misleading conclusions about the significance of the observed correlation between enthalpy and entropy [26, 30,36,42], the general consensus is that this form of compensation—in which entropy and enthalpy change in response to changes in temperature, sometimes called "thermodynamic homeostasis"—can be a simple consequence of processes that possess a finite heat capacity $\Delta c_p$ [43]. Ironically, it is the universality of this form of compensation that limits its utility, since different classes of interactions cannot be distinguished on the basis of their thermodynamic signatures alone [35].

### Calorimetric studies show apparent evidence of compensation in ligand binding

In principle, entropic and enthalpic contributions to ligand binding could be extracted from a van't Hoff plot of ln $K_a$ (where $K_a$ is the association constant) as a function of temperature, but the challenging nature of these studies has prompted interest in alternative approaches. A van't Hoff analysis requires multiple measurements across a wide range of temperatures, which is both time consuming and costly, and technical challenges are faced in robust data analysis [44]. In contrast, the widespread availability of sensitive modern microcalorimeters has made isothermal titration calorimetry (ITC) an attractive approach for the study of binding thermodynamics [45,46]. A single ITC experiment can provide estimates of both $K_a$ and $\Delta H$ (and hence also $\Delta G$ and $T\Delta S$) from a single experiment [47]. As of this writing, BindingDB [12], a database of binding data extracted from the literature, contains over 1,180 reported ITC measurements of binding thermodynamics, a testament to its widespread popularity.

Numerous ITC studies have reported evidence of entropy-enthalpy compensation. A series of early measurements of $Ca^{2+}$ binding to calcium-binding proteins reported that the observed linear relationship between $\Delta H$ and $T\Delta S$ with a slope of near unity (shown in Fig. 3a) was characteristic of entropy-enthalpy compensation, and suggested this might indicate binding was linked to a folding-like process given the similarity of this correlation to earlier lysozyme unfolding measurements [48]. A detailed study of a series of related *para*-substituted benzamidinium inhibitors of the serine protease trypsin (such as those shown in Fig. 2b) found nearly all ligands in the series exhibited entropy-enthalpy compensation in that the free energy of binding remained almost unchanged despite large observed changes in $\Delta H$ and $T\Delta S$ [14].

In some cases, a severe form of compensation appears to completely negate expected affinity gains. Freire and co-workers found that introducing a hydrogen bond acceptor into an HIV-1 protease inhibitor (Fig. 2a) resulted in a 3.9 kcal/mol gain in the enthalpic contribution to binding but was completely offset by a corresponding loss in the entropic contribution, resulting in no net change in affinity [11]. The authors suggested that this was a manifestation of the cancellation of entropic and enthalpic contributions for forming a hydrogen bond suggested earlier by Dunitz [31], concluding "it is apparent that structuring associated with hydrogen bonding formation can significantly compensate for any improvement in binding affinity" [11]. If plots of enthalpic and entropic contributions are to be believed, this severe form of compensation may be pervasive: A meta-analysis of the ITC measurements for a set of ~100 protein-ligand complexes selected from the BindingDB database [12] concluded that entropy-enthalpy compensation produced a plot of $\Delta H$ vs $T\Delta S$ which shows a slope of nearly unity (reproduced as Fig. 3b) [13], suggesting a form of severe compensation in which enthalpic changes are completely offset by corresponding entropic changes.

Calorimetric studies of a congeneric series of thrombin ligands concluded that competing entropic and enthalpic responses to chemical modifications of the ligand scaffold could be responsible for apparent non-additive effects [10]. A different study of pairs of trypsin ligands in which a nonpolar ring is expanded in size with the addition of a benzo group

(depicted in Fig. 2c) also demonstrated apparent entropy-enthalpy compensation, attributed by the authors to solvent ordering effects [8]. Recent work has also found that receptor mutations can cause minimal changes in the overall free energy of binding and minimal structural changes in X-ray or NMR structures of bound ligands but extreme changes in the enthalpic and entropic contributions to binding, interpreted as suggesting large changes in the mechanism of binding [1,2,6].

## CRITICAL EXAMINATION OF EXPERIMENTAL EVIDENCE

Before we can conclude there is a universal (and possibly severe) thermodynamic effect at work behind observations of entropy-enthalpy compensation, we must consider other explanations for the observed effects. Below, we survey some of the most important concerns that have been raised in the literature.

### ITC measurements can have large, underreported errors in $\Delta H$ and $T\Delta S$

As most evidence for entropy-enthalpy compensation in ligand binding comes from ITC experiments, it is important to understand the sources and magnitude of error in these measurements. In a typical ITC experiment (shown schematically in Fig. 5a), one component of the reaction (generally the macromolecule) is loaded into a sample cell inside an adiabatic thermal jacket that ensures minimal heat exchange with the environment. The other component (generally the ligand) is loaded into a syringe inserted into the sample cell. During the course of the experiment, small quantities of titrant are injected into the sample cell, and the quantity of heat liberated (or consumed) during each injection is measured by integrating the power that must be applied to the sample cell to keep the temperature equal to that of a reference cell heated by constant known power (Fig. 5b). A nonlinear fit of the injection heats by a thermodynamic binding model is used to obtain the thermodynamic parameters $K_a$ (the association constant), $\Delta H$ (the enthalpy of association), and $n$ (the stoichiometry parameter[2]), from which the free energy of binding ($\Delta G = -k_B T \ln K_a$) and entropic contribution ($T\Delta S = \Delta H - \Delta G$) are obtained.

The various sources of error contributing to an ITC measurement are now well understood [51–64]. Despite this, the errors or uncertainties of ITC measurements are consistently underreported [49,62]. In the most striking illustration of this, a large-scale laboratory assessment by the Molecular Interactions Research Group of the Association of Biomolecular Resource Facilities (ABRF-MIRG'02) [49] distributed identical aliquots of ligand and protein for a standard 1:1 association reaction—4-carboxy-benzenesulfonamide (CBS) binding to bovine carbonic anhydrase II (CAII)—to 14 different member laboratories with ITC expertise, asking them to report measurements of $K_a$ and $\Delta H$. These measurements, conducted independently by different laboratories, are a true assessment of the accuracy of the technique. Shockingly, the reported affinities and enthalpies of binding both reflected an RMS error of ~24%, with reported enthalpies of binding spanning a 10.7 kcal/mol range [49,61,62]. While each group also reported standard errors of the

[2]The stoichiometry or site parameter $n$ has been shown to also include contributions from errors in the stated sample cell volume [51], macromolecule concentration [52], and macromolecule purity.

thermodynamic parameters, the reported errors nearly universally underestimated the true error (reflected in the inter-laboratory variation) by one to two orders of magnitude [49,62].

The main source of measurement error was found to be a failure to accurately quantitate the ligand (titrant) concentration used [49,62]. While errors in the macromolecule concentration are absorbed into a site parameter $n$ in standard ITC data fitting procedures [52], these procedures generally assume the ligand concentration is known *exactly*, so an error of 10% in the ligand concentration will lead directly to 10% errors in $K_a$ and $\Delta H$ [52,62]. Moreover, the errors reported in the parameter fit reflect only the uncertainty in the nonlinear fit, completely omitting the effect of concentration uncertainties. These fit errors alone that are typically reported with ITC measurements, leading to consistent (and often drastic) underreporting of errors [62].

While some groups report standard deviations over repeated measurements as estimates of measurement error, if these measurements are performed starting from the same ligand stock, this error neglects the contribution from the uncertainty in the ligand stock concentration. While good analytical laboratory practice can consistently achieve 2% accuracies in titrant concentration, the ABRF-MIRG'02 study found that concentration errors are more typically ~10%, limiting the overall accuracy of ITC measurements to at least this much even if there is no measurement error during titration [49]. It is certainly possible to perform accurate ITC measurements if great care is exercised throughout all precision-limiting steps, and characterized titrant concentration errors can be included in a straightforward manner [62,64]. However, a publication lacking specific, documented evidence of both of these critical steps *must* be assumed to suffer from the same degree of error in $K_a$ and $\Delta H$ found in the ABRF-MIRG'02 assessment, as there is no evidence better precision has been achieved.

### Correlated errors in $\Delta H$ and $T\Delta S$ can produce apparent compensation

Because calorimetry directly determines $K_a$ and $\Delta H$ from nonlinear fitting to the injection heats, $\Delta G$ and $T\Delta S$ are computed from these values. How do typical errors observed in ITC measurements propagate into these quantities? A typical error of ~20% in $K_a$ translates into a rather small absolute error in $\Delta G$—about 0.1 kcal/mol[3]. For the CBS-CAII binding reaction[4] considered in the ABRF-MIRG'02 study, the RMS error in $\Delta G$ was only 0.13 kcal/mol, a relative error of only ~1.6%. Compared with a 2.5 kcal/mol error in $\Delta H$ (~23%), the error in $\Delta G$ is negligible. When the entropic contribution to binding $-T\Delta S = \Delta G - \Delta H$ is computed, the uncertainty in $\Delta H$ dominates (as the correlation between $\Delta H$ and $K_a$ is negligible here [64]), resulting in an *equal and opposite* error in $T\Delta S$. This immediately suggests an critical issue: Repeated, independent ITC measurements can give reliable free energies of binding, but large equal and opposite errors in the enthalpic and entropic contributions to binding that may cause the appearance of entropy-enthalpy compensation *even if none exists*!

[3]Straightforward first-order Taylor series error propagation gives $\delta\Delta G = k_B T\,|\delta K_a / K_a|$.
[4]Using $\Delta G = 8.24 \pm 0.02$ kcal/mol and $\Delta H = -11.11 \pm 0.04$ kcal/mol from Ref. [62].

Indeed, this is precisely what is observed in the ABRF-MIRG'02 dataset. Figure 3c depicts the enthalpic and entropic components of independent measurements made *for the same protein-ligand binding reaction* of CBS binding to CAII, conducted with identical source material. The striking similarity of this plot to both Figure 3a and Figure 3b—both of which purport to show experimental evidence of the existence of entropy-enthalpy compensation—cannot be avoided. Clearly, Figure 3c is not evidence of compensation, since the data comes from repeated measurements using identical samples of protein and ligand, simply measured by different labs. We are left with no conclusion except that it is meaningless to plot $\Delta H$ and $T\Delta S$ versus one another due to their large correlated errors, unless extreme care is taken to minimize, quantify, and propagate these errors is rigorously demonstrated.

While correlations between enthalpies and entropies computed from the same experimental data have been pointed out repeatedly in the literature, this issue still appears to not be widely appreciated. Exner [42] and contemporaries [29,30,65] pointed out similar problems leading to erroneous analysis of activation in kinetic studies. Apparent linear correlation between $\Delta H$ and $T\Delta S$ is often still presented as evidence of compensation [13] despite Exner's aptly-named follow-up, "How to get wrong experimental results from good experimental data: A survey of incorrect applications of regression" [32]. In view of these issues, several studies attempted to control for the effects of statistical correlation and errors and test for remaining correlations. This work generally concluded that there is indeed some residual correlation between entropy and enthalpy, but it falls far short of severe compensation in which $\Delta H$ and $T\Delta S$ nearly completely compensate [3,5,66].

To sum up, while $\Delta G$ can be measured robustly with good precision by standard practitioners, $\Delta H$ can be subject to errors in excess of 20% unless extraordinary care is taken, resulting in comparably large errors in $T\Delta S$. Thus, repeated measurements of $\Delta H$ and $T\Delta S$ for the *same system* can show apparent entropy-enthalpy "compensation" if plotted against each other.

## A "window effect" restricting the range of $\Delta G$ can cause apparent compensation

Apparent correlation between $\Delta H$ and $T\Delta S$ over multiple measurements can also arise because measured values of $\Delta G$ tend to occupy a restricted range while $\Delta H$ (and hence $T\Delta S$) can vary over a much wider range—a phenomenon sometimes termed the "window effect" [3,5,27,31,32,34,35,39,67]. Sharp [27] illustrated in a simple graphical way: He chose *random* enthalpies drawn from the range of reported $\Delta H$ values, then computed $T\Delta S$ values from these based on the experimental $\Delta G$ values. Plotting the resulting $\Delta H$ and $T\Delta S$ showed strong correlation essentially indistinguishable from the purported calorimetric evidence of compensation shown in Fig. 3a.

Why are free energies small in magnitude while enthalpies can be large? Several explanations have been put forth.

## Instrumental limitations

Experimental constraints of ITC generally limit measurable binding affinities to a range in which the calorimetric constant $c \equiv K_a[M_0]$ is restricted to $1 < c < 1000$ [45]. This naturally

appears to induce a linear correlation between enthalpic and entropic contributions to binding (Fig. 3d) [5]. While protocols for measuring thermodynamic parameters for tight-binding [68] or weak-binding [60] ligands have been developed, the vast majority of calorimetric measurements do not make use of these techniques, effectively restricting the great majority of available measurements to a narrow range of $\Delta G$ [5].

**Data selection bias—**Several claims of evidence for compensation examine the $\Delta\Delta H$ and $T\Delta\Delta S$ of matched pairs of ligands [8,69], but it has been cautioned that data selection bias can lead to the appearance of compensation, non compensation, or even *anticompensation*, depending on how the pairing was selected [33,34].

**Publication bias—**Useful or interesting biomolecular ligands have affinities within a relatively narrow range. For example, good initial hits from high-throughput screening efforts might have dissociation constants ($K_d$) in the mM–μM range, and good lead compounds in the μM–nM range. Enthalpies (and hence entropies) have no such expectations or restrictions placed on them. Indeed, examination of affinities compiled from publications into a public p$K_i$ database (not necessarily determined calorimetrically, so free of ITC measurement limitations) shows the central 95% of reported p$K_i$ s (in a curated set of 7,667 measurements) span a range of roughly 6.5–15.2 kcal/mol in equivalent binding free energy [50]. The distribution also shows significant skew toward tighter free energies, suggestive that apparent inhibition constants of tight-binding molecules are reported more frequently [50].

**Fundamental physical limitations of affinity—**It is also possible that a fundamental physical limitation restricts the affinities accessible by ligands, possibly even due to the existence of real entropy-enthalpy compensation that is inescapable at high affinities [70]. Indeed, it has been speculated for some time that thermodynamic factors limit the maximum affinity achievable for noncovalent ligands of macromolecular targets, though the exact nature of these factors remains uncertain [71].

## Choice of standard state can alter entropy-enthalpy decomposition

In order to standardize reporting of binding free energies, they are typically expressed with respect to a standard state, and presented as *standard binding free energies* [21–24]. Because the association constant $K_a = [PL]/[P][L]$ has units of inverse concentration, a choice of units and standard concentration $C_0$ must be made in order to convert this to a unitless quantity $K_a^\circ = K_a C_0$ and obtain a standard binding free energy $\Delta G^\circ = -k_B T \ln K_a^\circ$.

While the standard concentration $C_0$ is in principle arbitrary, convention is to report $\Delta G^\circ$ values where concentrations are expressed in terms of *molarity* and $C_0$ is taken to be 1 M. This choice which has certain advantages, in that it removes the translational component of the entropy from the standard entropy of binding $\Delta S^\circ$ [21]. While changing the choice of concentration units or standard concentration $C_0$ would not affect *relative* differences in enthalpies or entropies of binding, it could indeed affect the enthalpic and entropic components of a single binding affinity measurement, necessitating care in the handling and interpretation of thermodynamic signatures.

## PHYSICAL ORIGIN OF COMPENSATION

If some degree of entropy-enthalpy compensation is possible, what physical mechanism might underly this phenomenon? While numerous mechanisms have been proposed [1,11,15,38,72–79], we highlight some of the most popular proposals.

### Solvent reorganization may be a ubiquitous source of compensation

Numerous groups have suggested that solvent reorganization on binding could be responsible for compensation behavior. Lumry suggested compensation behavior was a fundamental property of processes occurring in water [25]. A statistical mechanical model of solvent reorganization attempts to demonstrate how nearly all reactions in solvent should lead to compensation behavior [80]. A two-state model of water (in which hydrogen bonds are either broken or unbroken) also has been shown to lead to severe compensation in hydrophobic hydration [81].

### Conformational restriction of bound states is not universally compensating

The simplest physical picture one might propose is that increasing favorable protein-ligand interactions in the bound state might cause additional conformational restriction of the bound ligand, narrowing or restricting the populations of the energy wells in the bound state and diminishing its conformational entropy, thus causing the entropy change upon binding to become more unfavorable [11,31,70]. While this makes some intuitive sense, it does not appear to be a universal cause of compensation. Consider, for example, the idealized protein-ligand binding reaction depicted in Fig. 6, in which a spherically symmetric ligand interacts with a protein partner via a Morse potential (Fig. 6, left). The free energy along the protein-ligand separation coordinate $r$ has a well-defined separation between bound and unbound states (Fig. 6, middle). When the protein-ligand interaction is modulated to make the interaction tighter, the free energy of binding becomes more favorable in a manner that is almost linear with the enthalpy due to the simplicity of the system. When the decomposition into entropic and enthalpic components is examined (Fig. 6, right), some compensation between entropy and enthalpy is evident, but this compensation is very weak, and certainly does not achieve the slope of unity expected from severe compensation. In fact, the maximum slope attained is near the *weakest* enthalpies (and hence free energies) of binding, the opposite of what is observed experimentally when severe compensation is claimed for very tight binders [11].

While this simple numerical model does not rule out this mechanism of compensation for all simple models, it suggests this mechanism cannot cause *universal* compensation. It should be noted that related models with different parameter choices do demonstrate the potential for near-complete compensation in a very narrow range of interaction energies [31,70], and that other theoretical treatments of weak association find both compensation and noncompensation behaviors can be observed [38].

### Receptor flexibility may be a source of compensation

A simple model of a ligand associating with a flexible macromolecule demonstrates how the free energy change on perturbation of the ligand or protein can be small, but larger (and

equal) compensating changes in entropy and enthalpy can occur [72,73]. Detailed atomistic investigation of a simple host-guest system found no correlation between the *depth* of an energy well and its narrowness, though an accurate accounting of the changes in the widths of energy wells upon binding was essential to reproducing experimental binding free energies [74]. Interestingly, subsequent investigations of a different set of host-guest systems revealed that compensation can be overcome by extremely tight-fitting guest molecules that appear to make up losses in conformational flexibility through liberating hydrating solvent molecules [15].

### The decomposition of the free energy is not unique

Another complication is that the decomposition of free energy changes into entropic and enthalpic contributions is not necessarily unique. In computational studies, the *resolution* of the model employed (i.e. the choice of which degrees of freedom are explicitly represented and which are implicitly modeled) can modulate the entropic and enthalpic components of thermodynamic processes, even if the overall free energy is preserved [82]. For example, a recent study of model ligand-cavity association with atomistic and coarse-grained potentials found that, while the overall free energy as a function of intermolecular distance was robust to model resolution, the entropic and enthalpic contributions were not [83]. Surprisingly, the experimental interpretation of entropy and enthalpy can also depend on the measurement technique or definition of the bound state, even when the binding free energy is robust to this choice [75].

## RAMIFICATIONS FOR LIGAND ENGINEERING

### Making inferences about driving forces of binding can be difficult

Recent proposals suggest enthalpic and entropic contributions to binding should play a key role in guiding ligand design [3,11,17–20]. But is there real practical value to this information? Assuming enthalpic and entropic contributions could be accurately measured, do these contributions to binding give us additional insight? And is this insight useful in making engineering decisions?

Biophysicists have numerous “rules of thumb” regarding the thermodynamic signatures of elementary molecular interactions: hydrogen bonds are enthalpically driven; hydrophobic association is entropically driven; liberating waters from a binding site increases entropy; sterically constraining a ligand by eliminating rotatable bonds reduces the entropy of the unbound state; and so on [84]. The statistical mechanics governing the behavior of the system makes no such distinction between enthalpy and entropy, nor do these elementary interactions necessarily act in an additive manner. The fundamental quantity modulated by changes to a ligand is the potential energy of the system, $U(x)$, where $x$ denotes the microscopic configuration of the system (including receptor, ligand, and surrounding solvent degrees of freedom). At equilibrium, the distribution of configurations observed within a specific volume of the cell or test tube is given by the Boltzmann distribution [85],

$$p(x) \propto e^{-U(x)/k_B T} \quad (2)$$

where $k_B$ is the Boltzmann constant and $T$ the temperature. The entropy and enthalpy are not fundamental quantities, but rather are *both* functions of the microscopic distribution,

$$\begin{aligned} H &\equiv \int dx U(x) p(x) \\ ST &\equiv -k_B T \int dx p(x) \ln p(x) \end{aligned} \quad (3)$$

Because of this dependence on the microscopic distribution, any perturbation to $U(x)$ resulting from a ligand modification will in general perturb *both* the enthalpy and entropy. That is, entropy and enthalpy are inherently intertwined, and modifications which only change one but not the other are the exception, not the norm. Thus, these rules of thumb fail to hold up when specific examples are scrutinized [84].

Furthermore, determining how to make useful modifications based on entropic and enthalpic patterns in a ligand series is not straightforward. Even rationalizing the enthalpic and entropic behavior in an extremely simple host-guest system—such as $Ca^{2+}$ binding to simple organic chelating agents related to EDTA—appears hopelessly complex [86]. There, the authors find that increasing the number of chelating arms of the host—strongly opposed by conformational entropy—increases the affinity. Without a model that includes *favorable* entropic contributions as chelating arms are added, the authors note that it is impossible to construct a simple self-consistent model of binding for this ligand series [86].

## True compensation can frustrate interpretation of thermodynamic signatures

Ironically, real compensating enthalpic and entropic contributions can actually *obscure* the true driving forces in ligand association, complicating the interpretation of thermodynamic signatures from reliable calorimetry experiments. For example, a recent computational study of the association of a spherical hydrophobic ligand with a hemispherical cavity found that association was thermodynamically favorable but *enthalpically* driven, rather than entropically driven as one would expect in hydrophobic association [77]. While it was postulated that this effect was due to a net favorable enthalpy of liberated water making additional hydrogen bonds upon returning to the bulk [77], an alternative explanation is more likely: entropy-enthalpy compensation in reorganizing water hydrogen bonds to accommodate newly liberated waters gives almost perfect compensation, resulting in a process that is net neutral in free energy [79]. Instead, the increase in configurational-translational entropy of water due to the burial of hydrophobic surface area (an entropically dominant effect) is more likely the net driving force, but this effect is masked due to the larger magnitude of the hydrogen bond reorganization event [79].

Thus, interpreting fundamental driving forces can be highly nontrivial since numerous effects contribute to observed enthalpies and entropies of binding. Other observations support this conclusion. For example, a joint calorimetric and X-ray study of a congeneric series of trypsin ligands found that, despite having nearly identical thermodynamic profiles, many ligands have different binding modes, highlighting the difficulty of drawing useful conclusions about the mechanism of binding from thermodynamic profiles [7]. In another system, the opposite was found to be the case: vastly different thermodynamic profiles resulted from essentially invisible (sub-Ångstrom) differences in binding geometry [76].

### Designing for enthalpic improvements has limited utility

Computer-aided schemes for rational ligand design that go beyond simple molecular visualization (such as virtual screening [87] and endpoint simulation methods [88]) heavily rely on the estimation of binding enthalpies. However, the optimization of binding interactions by this route presents many challenges that may explain its limited success [89]. Precise computation of the enthalpy of association is inherently difficult because of simple statistics; in effect, its estimation requires taking a small difference in the means of two distributions that are orders of magnitude broader than the difference between their means. Estimating the enthalpy of transfer by molecular simulation thus requires extremely long simulations to ensure the mean enthalpies of the bound and unbound states are estimated with sufficient precision to compute a reliable enthalpy difference [90]. Computation of entropies is similarly difficult [91].

These issues have presented difficulties for so-called *endpoint methods* that attempt to the free energy of binding by computing separate estimates of the enthalpic and entropic contributions [88,92,93]. As an alternative, docking and rescoring approaches [94] assume only the neighborhood of a single minimum energy configuration contributes to the enthalpy of binding, which introduces additional error into the computed enthalpies of interaction [95].

Worse yet, it appears that enthalpies—even if they can be accurately predicted—are only weakly correlated with binding free energies. The earliest calorimetric measurements of protein-small molecule interactions hinted that enthalpies are not necessarily predictive of binding free energies [96]. This has been confirmed by recent large-scale calorimetric database assessments that find enthalpies are only weakly correlated with free energies of binding (Fig. 4b), with a few notable exceptions [13]: HIV-1 protease and aldose reductase, in particular, which may explain why rational drug design and virtual screening efforts have found unusually high success rates in these targets [97–99]. This poor correlation does not appear to be due to the complexity of the binding landscape, as even simple host-guest systems appear to show poor correlation between enthalpy and free energy of binding [74].

Poor correlation between enthalpy and free energy of binding also may explain why endpoint methods have a great deal of difficulty with most protein targets. These methods must either use a crude model of of ligand entropy with poor accuracy and convergence properties [91–93] or ignore differences in ligand binding entropies altogether and assume enthalpies alone are predictive of affinity, a point contested by both experimental [6,7] and computational [74] studies (also recently reviewed [84]).

In summary, even if we could accurately predict specific interactions which would yield desired enthalpy changes, it seems unclear that doing so would actually yield corresponding improvements in binding affinity. Furthermore, accurate estimation of changes in binding enthalpy seems beyond the reach of current methods and, as we will see, can be difficult to validate experimentally.

### Designing for improvements in affinity directly is likely to be more productive

Through steady progress in computer simulation techniques, protein-ligand binding free energies can now be computed directly, without relying on separately estimating enthalpic and entropic components. This avoids difficulties in dealing with the large and often correlated errors and near cancellations in separate estimates of entropy and enthalpy. Alchemical methods in particular [22,100–104] directly compute the free energy of decoupling the ligand from its environment. These techniques, originally introduced nearly three decades ago [100], have advanced to the point where both the binding $\Delta G$ of individual ligands and $\Delta\Delta G$ of ligand modifications can be calculated very precisely [102–104]. In the absence of large protein conformational changes, these methods have demonstrated the ability to compute ligand binding free energies with errors of 1–2 kcal/mol [105–107]. Alternative approaches can compute free energies of binding of even large, charged ligands by estimating the free energy for direct ligand dissociation along an unbinding pathway [108].

Moreover, the computational effort required to compute precise estimates of free energy differences is often orders of magnitude less than that required to compute enthalpy differences to the same precision, even for simple solvated systems [90]. Slow protein conformational changes [109] and changes in protonation [110] or tautomeric states [111] still pose a challenge for these calculations. However, these same challenges plague estimation of enthalpies, and binding free energy calculations, unlike enthalpy calculations, give correct estimates of affinity when these issues are handled properly, at least to the accuracy achievable by the forcefield. Computations of binding free energy also have the advantage of being more easily validated against experimental data, due to the small error in typical calorimetrically-determined binding free energies determined (0.1 kcal/mol) compared to enthalpies (2.0 kcal/mol[5]). Public databases from other experimental techniques are also more plentiful and trust-worthy if only free energies of binding are of interest; a recent analysis of public $K_i$ data found the effective RMS error is ~0.75 kcal/mol[6] [50].

How can these computational tools be useful in design? Historically, standard practice in ligand engineering has been to propose, synthesize, and test small, synthetically feasible modifications, such as introducing additional hydrogen bonding partners, improving steric complimentarily, or reducing ligand conformational flexibility. However, improvements in computational power and software have now made it feasible to computationally evaluate proposed modifications to these compounds prior to synthesis and testing [112–114]. Going further, it is not hard to foresee computational schemes being routinely used to *propose* modifications likely to lead to affinity improvements, decoupling this process from human intuition altogether. Inklings of this future already exist: simulation techniques such as multi-site lambda dynamics [115] and Monte Carlo based methods [116,117] allow the evaluation of many potential changes within a single simulation. Clever schemes involving

[5]To obtain these typical error estimates, we used the standard deviation among independent experimental ITC measurements from Ref. [49], ~20% in both $K_a$ and $\Delta H$. This gives an RMS error in $\Delta G$ of $kT$ ln 1.2 ≈ 0.1 kcal/mol for $T \approx 298$ K. The error in $\Delta H$ was estimated from the RMS average error assuming the distribution of enthalpies in the BindingDB [12] was representative.

[6]Ref. [50] quotes a standard deviation of 0.54 p$K_i$ units, which we convert to free energy $\Delta G = kT \ln 10^{pK_i}$ assuming $T \approx 298$ K.

the post-processing of simulations of non-chemical species [118] also show promise for automatically proposing chemical derivatives leading to enhanced affinity or selectivity.

## Acknowledgments

J.D.C. is supported by a QB3-Berkeley Distinguished Postdoctoral Fellowship. D.L.M. acknowledges funding from the National Institutes of Health (1R15GM096257-01A1) and startup funding from the University of California, Irvine. J.D.C. is grateful to Kim Branson (Hessian Informatics), Sarah E. Boyce (Gilead), Phillip L. Geissler (UCB), Brian K. Shoichet (UCSF), Joel Tellinghuisen (Vanderbilt), and Veena Thomas (Stanford) for stimulating discussions on this topic.

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## SUMMARY

- While a weak form of entropy-enthalpy compensation is likely common, evidence of severe or pervasive form of compensation is poor.
- Measurement and calculation of enthalpies and entropies is more difficult than measuring or computing free energies.
- Entropic and enthalpic contributions are difficult to interpret, and are unlikely to be useful in rational ligand design.
- When intuition fails in proposing modifications that lead to affinity gains, schemes that compute binding free energies directly are poised to be of high utility.

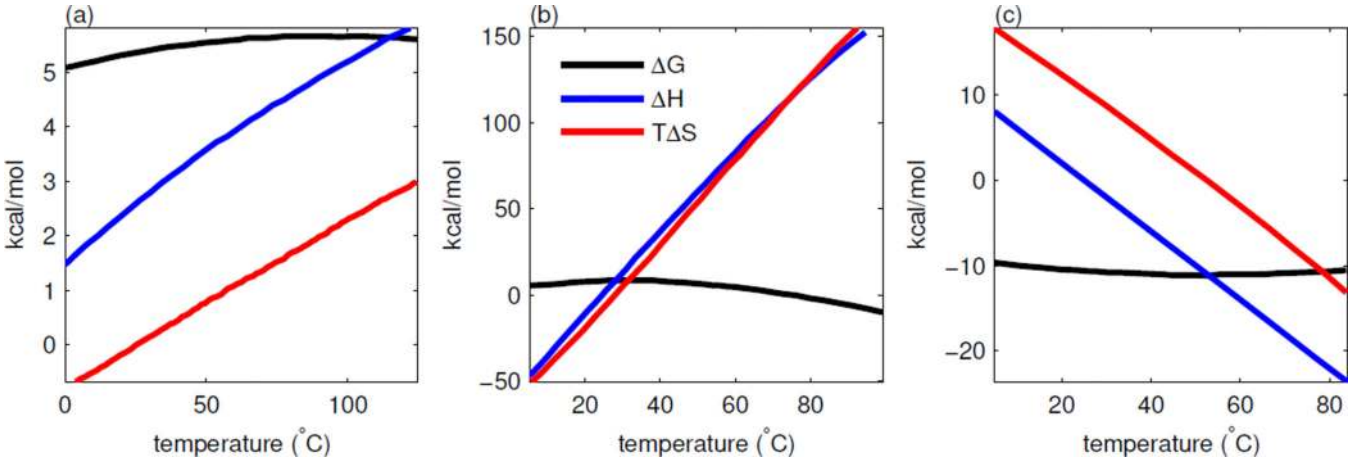
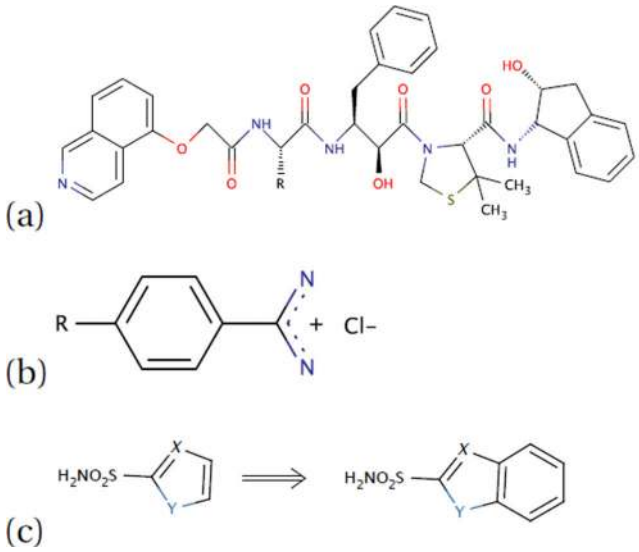



**Figure 1. Entropy-enthalpy compensation as a general phenomenon in thermodynamics**

Three examples of compensating entropic and enthalpic contributions to the free energy as a function of temperature in general thermodynamic phenomena. The free energy ($\Delta G$) of the process as a function of temperature is shown, along with enthalpic ($\Delta H$) and entropic ($T\Delta S$) contributions. *(a)* transfer of neopentane from neat phase to water (data from Fig. 3 of Ref. [40]); *(b)* myoglobin unfolding (data from Table 2 of Ref. [41]). *(c)* protein association (data from Fig. 3b of Ref. [35]). In all three cases, $\Delta H$ and $T\Delta S$ change substantially while $\Delta G$ remains almost constant, suggesting substantial entropy-enthalpy compensation.




| R group | kcal/mol $\Delta G$ | $\Delta H$ | $T\Delta S$ |
|---|---|---|---|
| –S–$CH_3$ | -14.87(9) | -8.2(2) | -6.67(9) |
| –$SO_2$–$CH_3$ | -14.6(2) | -12.1(6) | -2.5(2) |

| R group | $\Delta G$ | $\Delta H$ | $T\Delta S$ |
|---|---|---|---|
| –H | -6.35(5) | -4.5(1) | 1.8(1) |
| –Et | -6.07(5) | -3.3(1) | 2.7(2) |
| –*n*-Bu | -6.26(5) | -2.4(1) | 3.9(2) |

| X | Y | $\Delta\Delta G$ | $\Delta\Delta H$ | $T\Delta\Delta S$ |
|---|---|---|---|---|
| N | NH | 1.1(2) | 4.2(4) | 3.1(4) |

**Figure 2. Examples of severe compensation reported in the calorimetry literature**

Several cases where ligand modifications lead to large changes in the enthalpic and entropic contributions to binding while the overall binding free energy remains essentially unchanged. *(a)* Severe compensation in HIV-1 protease inhibitors (data from Table 1 of Ref. [11]); *(b) para*-substituted benzamidinium trypsin inhibitors binding to trypsin (data from Table 1 of Ref. [14]); *(b)* nonpolar ring expansions in arylsulfonamide trypsin inhibitors (data from Table S3 of Ref. [8]). Quantities in parentheses denote one standard error of last significant digit.

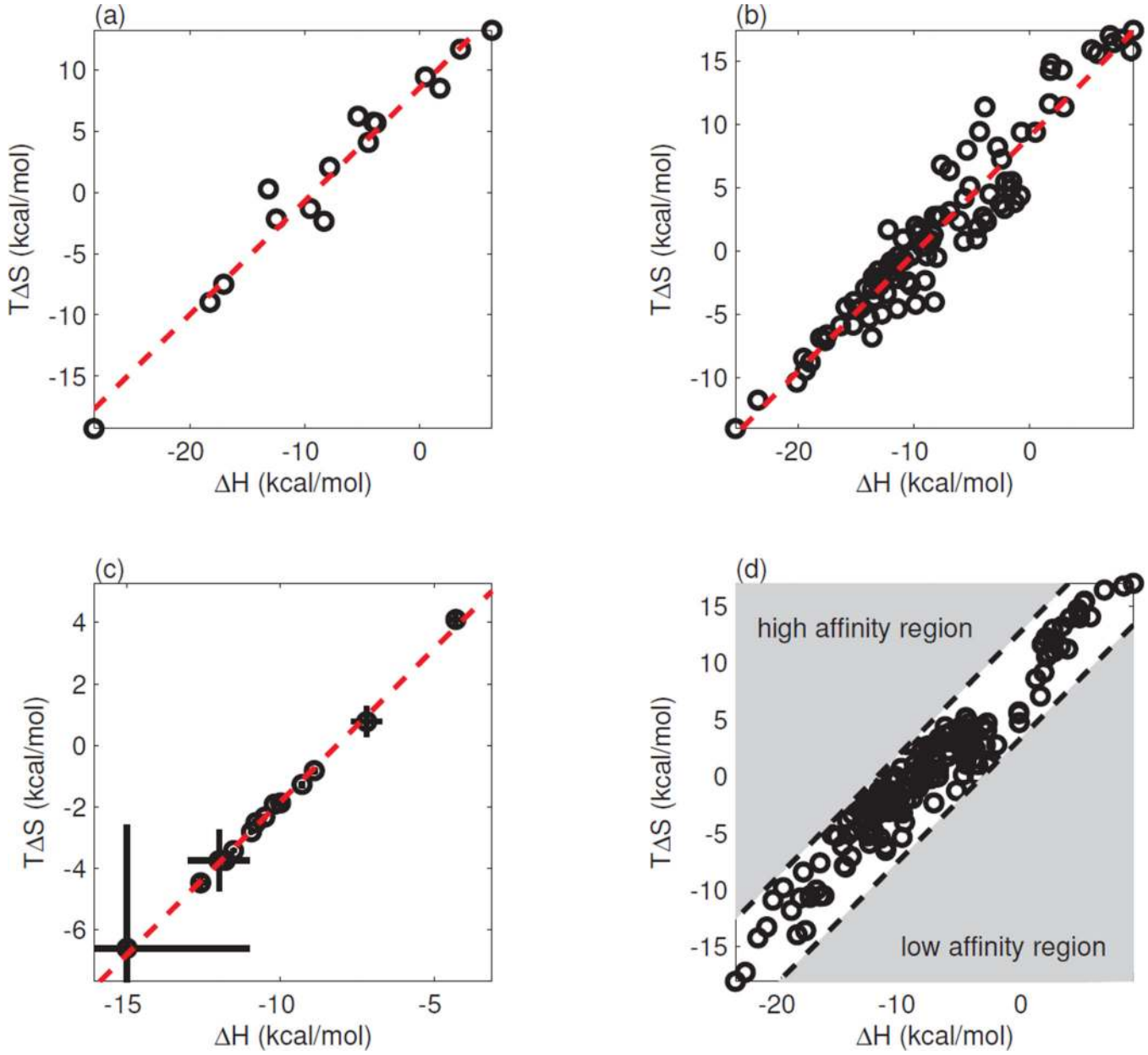



**Figure 3. Compensation behavior in calorimetry data**

All plots show *apparent* compensation behavior between enthalpic ($\Delta H$) and entropic ($T\Delta S$) components of free energy of binding. *(a)* Apparent compensation behavior from ITC measurements of $Ca^{2+}$ to calcium-binding proteins (black circles) with linear fit (red dashed line, slope = 0.92(5), $R^2$ = 0.96(3) by bootstrap) (data from Fig. 3 of Ref. [48]); *(b)* Meta-analysis of ITC measurements of protein-ligand complexes (black circles) selected from the BindingDB database [12] with linear fit (red dashed line, slope = 0.93(3), $R^2$ = 0.91(2)) (data from Fig. 1 of Ref. [13]); *(c)* Independent ITC measurements performed in different laboratories using *identical* samples of ligand ligand (CBS binding to bovine carbonic anhydrase II) from the ABRF-MIRG'02 assessment [49] shows apparent (but fallacious) compensation over a wide range of energies, and error bars (representing one standard error)

much smaller than the actual variation among independent measurements (computed from Table 3 of Ref. [49]). Horizontal and vertical bars denote *reported* measurement errors, which significantly underestimate the true inter-experiment variation. Linear fit denoted by red dashed line (slope=0.99(2), $R^2 = 0.997(1)$). *(d)* Instrumental limitations on binding affinities measurable by ITC restrict the measurable range of $\Delta G$ (but not $\Delta H$) to the unshaded region, inducing a linear correlation in $\Delta H$ and $T\Delta S$ due to the "window effect" (data from Fig. 1 of Ref. [5]).

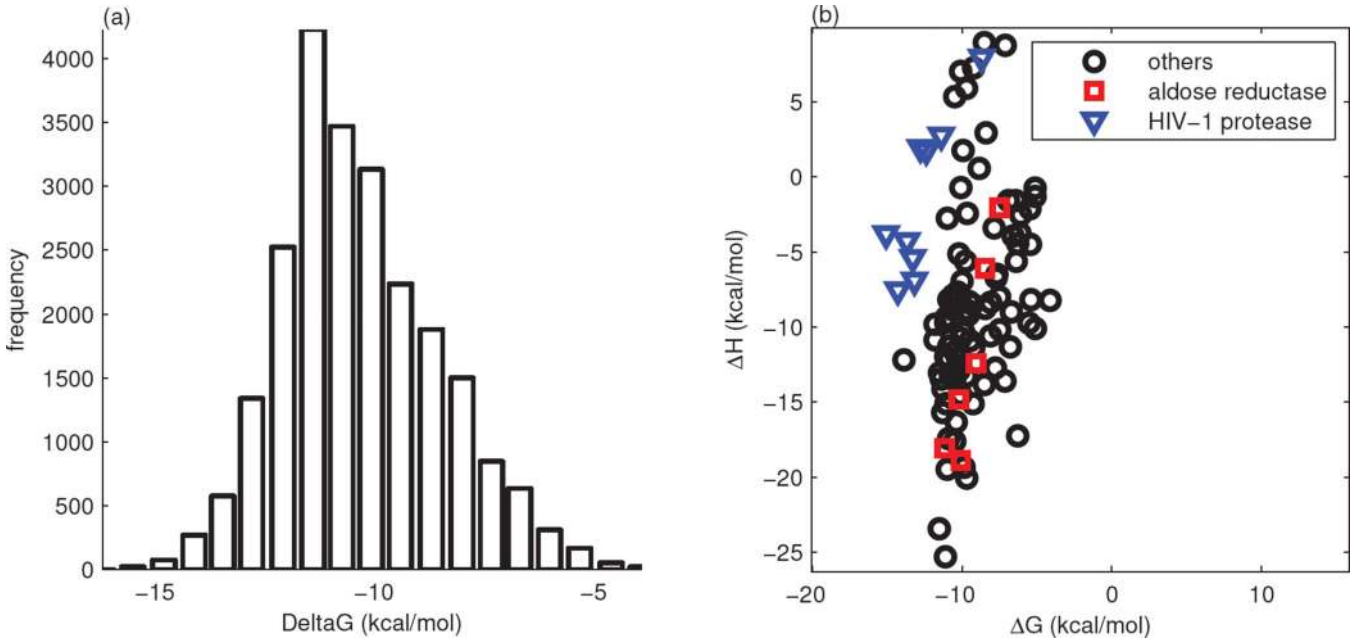



**Figure 4. Distribution of published binding free energies and correlation with enthalpy**

*(a)* Distribution of binding free energies computed from ChEMBL p$K_i$ activity data (data from Fig. 4 of Ref. [50]); *(b)* Poor correlation of enthalpy ($\Delta H$) with free energy ($\Delta G$) of binding from meta-analysis of ITC measurements [13] selected from the BindingDB database [12] (data from Fig. 2a of Ref. [13]). While aldose reductase (red squares) and HIV-1 protease (blue triangles) show some correlation between enthalpy and free energy of binding, correlation is generally poor for other complexes, and enthalpies span a much broader range than free energies.

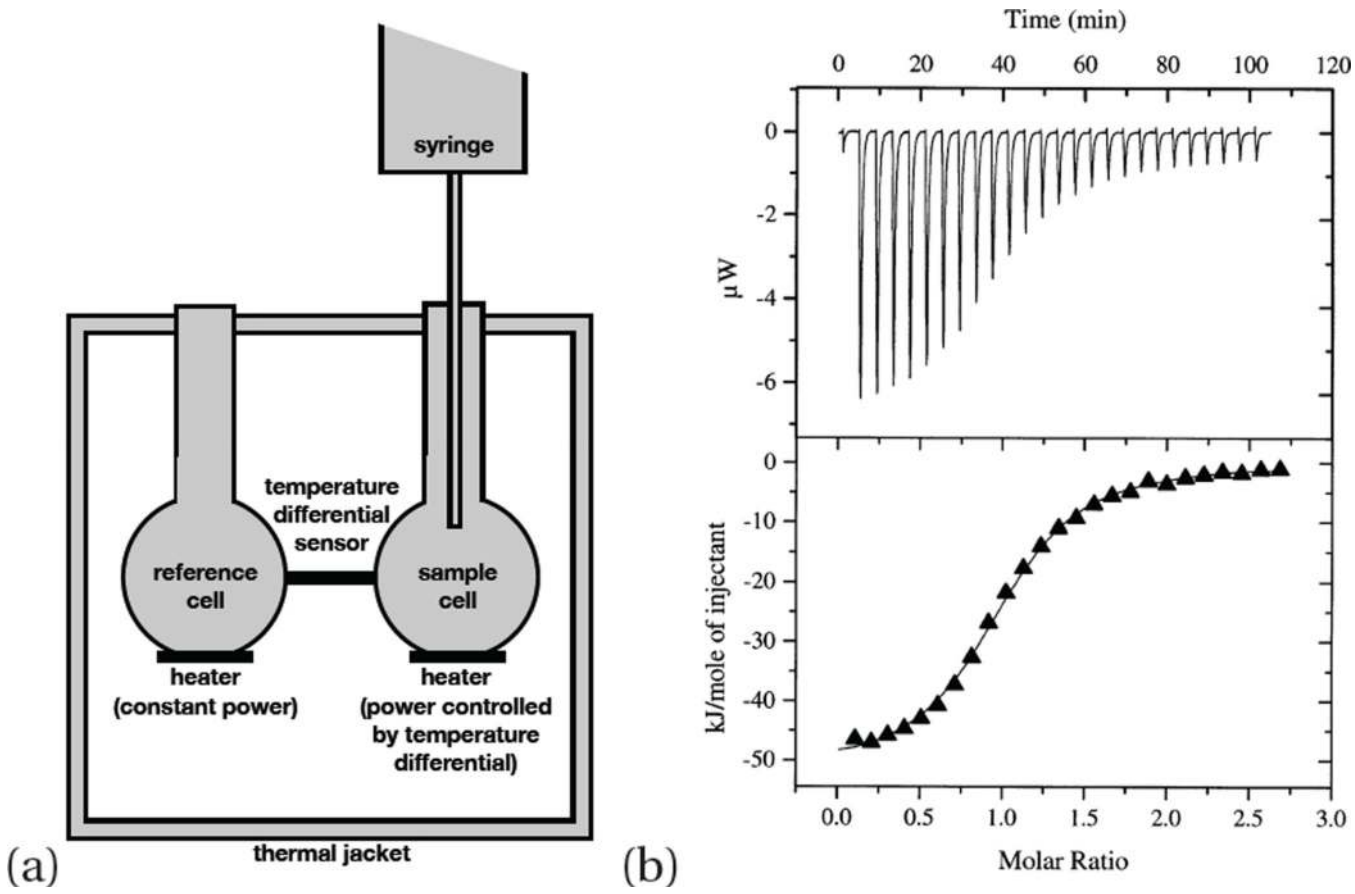



**Figure 5. Typical ITC experimental configuration and data**

*(a)* A typical experimental configuration for power-compensating isothermal titration calorimetry (ITC). *(b)* Typical data from an ITC experiment showing applied power as a function of time (*top*) and integrated heats of injection with fit to thermodynamic parameters (*bottom*) (reproduced with permission from Fig. 2 of [35]).

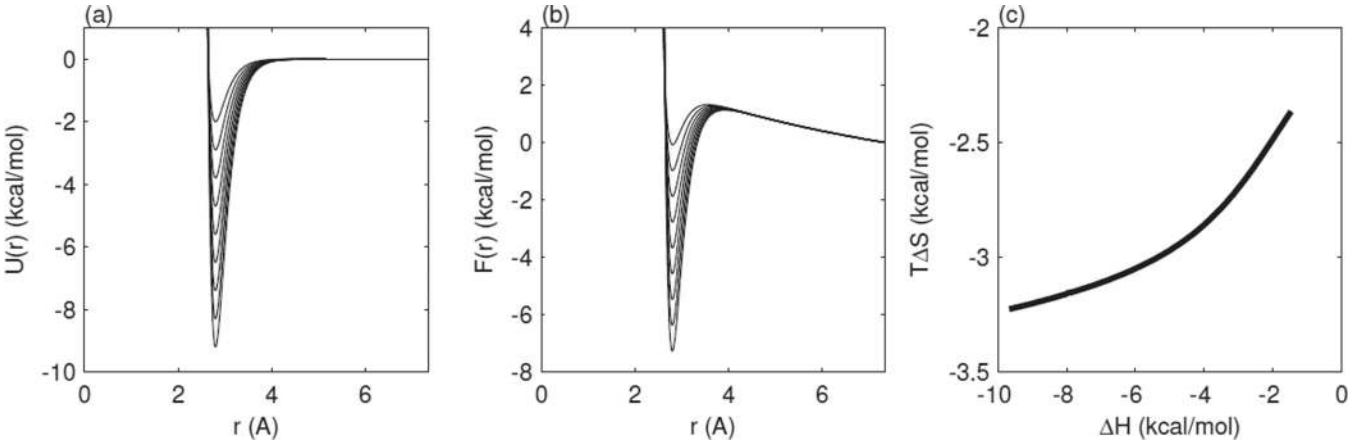



**Figure 6. Simple model system illustrating weak entropy-enthalpy compensation**

An idealized protein and ligand interact via a Morse potential that is strengthened or weakened to simulate ligand modifications. *(a)* Intermolecular Morse potential $U(r) = D_e[1-e^{-a(r-r0)}]^2$, with $r_0 = 2.8$ Å, $a = 1/(0.5$ Å), and well depth $D_e$ varying from 2–10 kcal/mol. *(b)* Potential of mean force $F(r) = U(r) - k_BT \ln 4\pi r^2$ between protein and ligand as a function of intermolecular distance $r$ for temperature $T = 25$ C. *(c)* Standard entropic ($T\Delta S$) and enthalpic ($\Delta H$) contributions to the binding free energy for different well depths $D_e$, computed from classical statistical mechanics. Note that, while some entropy-enthalpy compensation is apparent, it is not linear.