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Abstract

Elucidation of the energetic principles of binding affinity and specificity is a central task in many branches of current sciences: biology, medicine, pharmacology, chemistry, material sciences, etc. In biomedical research, integral approaches combining structural information with in-solution biophysical data have proved to be a powerful way toward understanding the physical basis of vital cellular phenomena. Isothermal titration calorimetry (ITC) is a valuable experimental tool facilitating quantification of the thermodynamic parameters that characterize recognition processes involving biomacromolecules. The method provides access to all relevant thermodynamic information by performing a few experiments. In particular, ITC experiments allow to by-pass tedious and (rarely precise) procedures aimed at determining the changes in enthalpy and entropy upon binding by van't Hoff analysis. Notwithstanding limitations, ITC has now the reputation of being the "gold standard" and ITC data are widely used to validate theoretical predictions of thermodynamic parameters, as well as to benchmark the results of novel binding assays. In this paper, we discuss several publications from 2007 reporting ITC results. The focus is on applications in biologically oriented fields. We do not intend a comprehensive coverage of all newly accumulated information. Rather, we emphasize work which has captured our attention with originality and far-reaching analysis, or else has provided ideas for expanding the potential of the method.
A survey of the year 2007 literature on applications of isothermal titration calorimetry

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Elucidation of the energetic principles of binding affinity and specificity is a central task in many branches of current sciences: biology, medicine, pharmacology, chemistry, material sciences, etc. In biomedical research, integral approaches combining structural information with in-solution biophysical data have proved to be a powerful way toward understanding the physical basis of vital cellular phenomena. Isothermal titration calorimetry (ITC) is a valuable experimental tool facilitating quantification of the thermodynamic parameters that characterize recognition processes involving biomacromolecules. The method provides access to all relevant thermodynamic information by performing a few experiments. In particular, ITC experiments allow to bypass tedious and (rarely precise) procedures aimed at determining the changes in enthalpy and entropy upon binding by van’t Hoff analysis. Notwithstanding limitations, ITC has now the reputation of being the “gold standard” and ITC data are widely used to validate theoretical predictions of thermodynamic parameters, as well as to benchmark the results of novel binding assays. In this paper we discuss several publications from 2007 reporting ITC results. The focus is on applications in biologically-oriented fields. We do not intend a comprehensive coverage of all newly accumulated information. Rather, we emphasize work which has captured our attention with originality and far-reaching analysis, or else has provided ideas for expanding the potential of the method.

Keywords: thermodynamics, calorimetry, molecular recognition, ligand binding, enthalpy, entropy, heat capacity
INTRODUCTION

Present-day large-scale genomics, proteomics, interactomics and other initiatives, and efforts to establish system-oriented approaches are expected to provide a global understanding of biological processes. However, many aspects of the intimate molecular mechanisms involved in biological function remain obscure. Macromolecular recognition is a typical example. Notwithstanding the serious progress that has been achieved over the past three decades, many details about the mechanistic, energetic, and kinetic principles of binding affinity and specificity remain vaguely understood. Part of the problem is that, at least at the structural level, there are no obvious unifying principles in the architecture of protein-protein and other protein/ligand complexes. Binding interfaces span hundreds and thousands of square angstroms, yet point mutations can severely impair binding affinity. Chemically unrelated ligands can effectively compete for the same binding site. It is still very difficult to achieve high affinity and specificity of a designed molecule for a target pocket by rational design and optimization. This is why methodologically-rigorous biophysical studies of diverse protein/ligand complexes are an indispensable endeavor toward better understanding of biological function. The ultimate goal is to find links between molecular structure, energetics, and dynamics, and to discover “rules” guiding the prediction of the energetic response of a particular complex to structural changes in the binding partners. In research programs combining biophysical and structural approaches isothermal titration calorimetry (ITC) has evolved as a valuable tool.

The theoretical background, experimental design, and practical aspects of the ITC experiment are discussed in detail in refs. [1-7]. Here, only a brief description of the technique is given outlining the essential features of the method. We consider the simplest case of a 1:1 binding reaction. The ITC experiment consists of additions of molecule L (ligand), which is placed in the injection syringe, to molecule R (receptor), which is contained in the reaction cell. The injection syringe rotates, thus facilitating rapid mixing of the reactants. A reference cell that is identical in shape and volume to the reaction cell is filled with water. Both cells are placed in an insulated jacket and are equilibrated prior the experiment at the desired temperature. The power compensation principle is implemented in most of the titration calorimeters used in biologically oriented studies nowadays. Constant power is applied to the reference cell as to maintain a minute temperature difference between the cells (ΔT). Upon binding of L to R, heat is released (exothermic reaction) or absorbed (endothermic reaction). Thermopile/thermocouple circuits detect the resulting change of ΔT. The feedback circuit decreases or increases the power supplied to the reaction cell in order to keep ΔT constant throughout the experiment. Since the power changes (differential power; units of J s⁻¹) are monitored continuously, a peak-shaped
deflection from the thermal baseline is observed. Integration of the differential power peak over time yields the heat, \( q \) (units of J), released or absorbed upon binding of \( j \) mol L to R. If \( j \) is known the ratio \( q/j \) corresponds at constant temperature and pressure to the molar enthalpy of binding, \( \Delta H \) (units of J mol\(^{-1}\)). In practice, however, the number of bound moles L \( (j \equiv [L]_{\text{bound}}) \) is unknown if the binding constant, \( K_A \), is unknown. Therefore, a titration experiment is required to determine \( K_A \) and \( \Delta H \). A series of additions of L to R is performed, so that the ratio of the total concentrations \( [L]_{\text{tot}}/[R]_{\text{tot}} \) increases from < 0.1 to > 2-3 (or more). The observed heats monitor the extent of binding as the degree of saturation increases. After corrections for the unspecific heats of dilution, for the changes in concentrations of L and R, and the displacement of part of the reactants from the active volume of the cell, the heat detected in each injection is proportional to the molar enthalpy according to:

\[
q = V_{\text{cell}} \Delta H [R]_{\text{tot}} (Y_i - Y_{i-1})
\]  

(1)

\( V_{\text{cell}} \) is the cell volume and \( Y = [RL]/[R]_{\text{tot}} \) is the degree of saturation. The product \( [R]_{\text{tot}}(Y_i - Y_{i-1}) = [RL]_i \) is the amount of complex formed in the duration of injection \( i \). The calculation of \( Y \) requires knowledge of \( [RL] \). The latter can be obtained by combining the equation defining \( K_A \) with the equations of mass conservation:

\[
K_A = \frac{[RL]}{[R][L]} = \frac{[RL]}{([R]_{\text{tot}} - [RL])[L]_{\text{tot}} - [RL]}
\]  

(2)

After rearrangement, one obtains a quadratic equation:

\[
[RL]^2 - \left( \frac{1}{K_A} + [R]_{\text{tot}} + [L]_{\text{tot}} \right)[RL] + [R]_{\text{tot}}[L]_{\text{tot}} = 0
\]  

(3)

The only physically meaningful root yields \( [RL] \). The combined equations 1–3 can be fit to the experimental data to calculate \( K_A \) and \( \Delta H \).

Once \( K_A \) and \( \Delta H \) are known, all relevant thermodynamic parameters can be calculated. The binding Gibbs free energy change is related to \( K_A \) by \( \Delta G = -RT \ln K_A \). \( (R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1} \) is the gas constant; \( T \) is the absolute temperature.) The binding entropy change is \( \Delta S = (\Delta H - \Delta G)/T \). Modern titration calorimeters facilitate measurements in a broad temperature interval. From experiments performed at different temperatures, the heat capacity change can be calculated by \( \Delta C_p = d\Delta H/dT \). Hence, ITC has the potential to yield a full thermodynamic description of a binding reaction. This objective can easily be achieved in one day (2-3 experiments of 2-3 hours duration). Yet the main advantage of the method is the precision of the \( \Delta H \), \( \Delta S \), and \( \Delta C_p \) determination. With non-calorimetric data, these quantities are calculated by using temperature derivatives of \( K_A \) and \( \Delta G \) (van’t Hoff analysis). The intrinsic difficulties to
measure $K_A$ with good precision render the accuracy of the so-derived parameters low. Moreover, ITC experiments can be designed in principle with molecules of arbitrary size and “spectroscopically silent” compounds; there is no need for derivatization and immobilization. Of course, the method has also limitations. Very high affinity and very low affinity processes cannot be studied by standard protocols (see below). Binding involving very small heat changes cannot be detected. Sometimes, the large amounts of material required for accurate measurements make ITC experiments impractical. Nonetheless, ITC is regarded as the “gold standard” in measuring the energetics of binding, and ITC data are often used to benchmark data obtained by other methods or computer-based predictions of thermodynamic quantities, as we will discuss in the following sections.

In recent years we have faced a true explosion of published studies reporting results of ITC experiments. In biologically relevant context, ITC is mainly used to complement structural data by measuring the affinity of diverse ligands to proteins. Analysis of mutant complexes or measurements with closely related compounds allows the identification of energetically important contacts. Fewer reports attempt an in-depth thermodynamic characterization of binding reactions in an extended temperature range. Typically, such studies search for correlations between the magnitude of binding parameters and the shape and chemistry of the interacting molecular surfaces. Notwithstanding the persisting problems in the field of structural thermodynamics, there is a steady accumulation of high-precision data collected with high-resolution complexes, expanding the empirical foundation of current concepts about the principles of macromolecular recognition. But the use of ITC is by no means limited to biomedical applications. ITC admittedly enriches the arsenal of experimental tools in virtually all branches of the chemical sciences, and in many branches of the material sciences.

We found about 600 publications reporting ITC data in 2007. Since in many cases “ITC” or “titration calorimetry” is not contained in the title or in the list of keywords, we expanded the search to the full text wherever this option was supported by the publisher. Only full papers reporting original data were considered, with the exception of few publications reviewing and analyzing previously collected data [8-12]. Undoubtedly, there are publications which were not identified. To help the interested reader in navigation through the reference list, we defined the following subsections:

(i) General subjects and references cited in the text [1-56].

(ii) Protein-protein. Papers reporting data on association between folded proteins are grouped here. [21, 25, 35-37, 57-144].
Protein-peptide binding of unstructured peptides to proteins is discussed in these papers [28, 54, 145-208].

Protein/peptide-small ligand. The term “small ligand” refers to low-molecular weight, non-peptidic compounds: nucleotides, sugars (mono- or oligosaccharides), co-factors, fatty acids, detergents, drugs, etc. [9-11, 38, 40-42, 50-53, 209-351].

Protein/peptide-metal [352-391].

Protein/peptide-nucleic acid [8, 12, 20, 392-412].

Protein/peptide-lipid [39, 413-428].

Protein/peptide-polymer [429-440].

Nucleic acid-small ligands (drugs) [38, 441-471].

Enzyme activity and kinetics [47, 472-478].

Miscellaneous. These papers cannot be classified in the above categories. They describe studies of various processes involving mainly ions, small inorganic or organic molecules, and polymers. [479-623].

The selection of papers discussed below is limited by space restraints and is the product of our subjective judgment. We have not intended to give a comprehensive picture of all data accumulated in 2007. Rather, we have tried to select papers illustrating the versatility of the technique and its capacity to provide information in different areas of biomedical research. The emphasis is on work providing deeper insights into the particular molecular process and/or describing new methodological developments, in keeping with the tradition of the annual surveys of the literature on biocalorimetry published so far [13-17].

NEW DEVELOPMENTS AND NON-STANDARD APPLICATIONS

Global analysis of ITC data

Formation of ternary and higher-order protein/protein complexes is vital in many biological processes. Allosteric communication between binding sites is a wide-spread phenomenon and provides powerful mechanisms of control and regulation. Cooperativity relationships in protein-protein interactions (or protein-ligand interactions in general) can be characterized by ITC [18, 19]. A good example from 2007 is the paper of Krell et al. [20], which will be discussed in more detail below. Recently, a full analytical treatment of heterotrophic effects in the case of binding of two ligands to a receptor to form a ternary complex was published [18]. However, quantification of cooperativity effects by ITC remains difficult, in part due to insufficient quality of the data. On the other hand, if binding is multivalent and cooperativity relationships in binary and ternary complexes are present, mixing of the
components will result in multi-protein assemblages. It is very difficult to design a priori ITC experiments suitable to detect cooperativity, as well as to determine the underlying thermodynamic parameters. The paper of Houtman et al. [21] reports the development of a strategy aimed at characterization of binary and ternary protein-protein interactions exhibiting cooperativity. They propose a global analysis of a series of ITC experiments performed by variation of the experimental configuration (mixing order and direction). Global analysis of ITC data is not new and has been applied occasionally in different context [22-24] (see also ref. [25] for an instructive example from 2007), but study of Houtman et al. is probably the first application at this level of complexity. The analytical model is conceived in very general terms, taking into account also potential dissociation of complexes that are preformed in the injection syringe upon dilution in the cell. The methodology was checked for consistency on the example of a simple 1:1 reaction between carbonic anhydrase II and 4-carboxybenzenesulfonamide. The presented global analysis provides a further level of understanding of the interactions between LAT, Grb2 and Sos1 promoting formation of large multiprotein complexes with crucial impact for T-cell receptor activation. The model has been implemented as an extension of the public software domain SEDPHAT (http://www.analyticalultracentrifugation.com/sedphat/sedphat.htm), a widely used platform for global analysis of analytical ultracentrifugation and static light scattering data. The authors critically assess the advantages and limitations of the model and provide guidelines for optimization of the experimental design.

**Expanding the boundaries**

One of the well known and much discussed limitations of ITC is that binding affinity too strong or too weak cannot be straightforwardly determined with the currently available calorimeters. In studies of protein-ligand binding, the lowest and highest dissociation constants that can be measured utilizing standard protocols lie approximately in the low nanomolar and middle micromolar range, respectively. Indeed, the problem is based in the properties of the binding isotherm: reliable measurement of $K_d$ (and $K_A = 1/K_d$) is possible only if the concentrations of both bound and unbound ligand are comparable at partial saturation. In the ITC community, the problem is often stated by the so-called “Wiseman $c$-value”, defined as $c = K_A[R]_{tot}$, where $[R]_{tot}$ is the total receptor concentration in the calorimetric cell [1]. Simultaneous determination of $K_A$ and $\Delta H$ with good precision is possible only in the window $10 < c < 200$. In the high affinity limit ($K_A$ approaching $1\times10^9$ M$^{-1}$), $[R]_{tot}$ must be low and, therefore, the heats of reaction can fall below the sensitivity of detection. In cases of low affinity
(K_A lower than, say, 1×10^4 M^{-1}), the experiment fails due to low solubility and/or aggregation, or it simply becomes too expensive. In the high affinity range, thermodynamic linkage (competition) may provide the solution (for details of the theoretical background see refs. [18, 26, 27]). A strong-binding ligand is titrated to the receptor in the presence of weaker-binding competitor ligand. Since the binding equilibria are linked, that is, the strong ligand displaces the weak ligand, depending on the actual concentrations, the observed apparent binding constant may fall in the favorable c-value window. The approach is illustrated in the paper of Tse et al. [28]. The authors measured the binding of three peptides derived from the Ca^{2+}/calmodulin-dependent protein kinase to calmodulin (CaM). Binding of the shortest peptide (S) was amenable to ITC characterization, while the affinity of the intermediary-sized peptide (I) and the longest peptide (L) were too high to be determined by ITC. Titration of I to the CaM/S complex allowed determination of the affinity of I to CaM (K_A ~ 6×10^9 M^{-1}). The affinity of L to CaM was subsequently measured by titration of L to the preformed CaM/I complex (K_A ~ 2×10^{13} M^{-1}). This is probably the most potent binding measured to date using ITC. It should be noted, however, that the success of a displacement experiment crucially depends on the ratio of the equilibrium constants characterizing binding of the competing ligands and on the magnitude of the corresponding molar enthalpies. Furthermore, the paper of Tse et al. emphasizes the importance of maintaining a large excess of the ligand present in the cell (weaker binder) over the available binding sites, such that only a negligible amount of this ligand is bound to the receptor. For full analytical treatment of competition experiments at any arbitrary concentrations the reader is advised to consult ref. [18].

Joel Tellinghuisen explored the limits of the method in the low-c-value region [29]. Of special interest is the situation where the product ΔH×n is a small number, which typically will be the case for c < 1. Working at low c-values requires a large excess of the titrant to be injected, as it has been pointed out also earlier [30, 31]. With the standard approach of performing many, equally-sized injections, the reaction heat will be distributed mainly in the early part of the titration. The new idea is to use a small number of injections, which differ in size substantially. Based on simulations, statistical analysis and real experiments, the author demonstrates that the binding parameters can be obtained with good precision from experiments involving only a few variable-sized injections and presents an algorithm for optimization of the injection volume. The prerequisites for the success of the procedure are (i) knowledge of the stiochiometric model, (ii) precise determination of receptor concentration, and (iii) evolution of specific heats that are sufficiently different from the heats of dilution. Volume optimization might offer additional
advantages in situations where the procedure is not strictly required, since the runtimes could be shortened to 15-20 min, giving a significant throughput advantage.

**Determination of association and dissociation rate constants of reversible bimolecular reactions**

As explained in the Introduction, each ligand injection produces a characteristic peak, which can be integrated over time to obtain the heat associated with occupation of $n$ number of receptor sites. However, equilibrium is not achieved instantaneously. Therefore, the heat flow signal is expected to contain kinetic information, since it monitors the approach to the equilibrium distribution of ligand, receptor, and ligand-receptor complex. Egawa et al. [32] presented the formalism and experimental method to calculate the microscopic rate constants of association, $k_a$, and dissociation, $k_d$, from ITC data. The procedure is based on the concept of relaxation kinetics: addition of a small amount of one reactant disturbs the equilibrium and time is required for the new equilibrium to be re-established. The method involves the following steps. (1) Measure $K_A$ by conventional ITC titration. Determine the equilibrium concentrations of L, R, and LR at each titration step. (2) Determine the value of a special criterion $r$, which is a function of $K_A$, [L], [R] and [LR]. The exact analytical form of $r$ can be found in the original publication [32], but it essentially indicates portions of the binding isotherm, where the concentrations are such that the equilibrium perturbation is small. (3) Based on the value of $r$, select those injections where relaxation kinetics applies. (4) Correct for the response time function of the instrument. (5) Fit a single-exponential function to the heat flow trace to extract the apparent rate constant, $k_{app} = k_a + k_d$. Steps (4) and (5) can be done simultaneously (See Supporting Information to the article for details about step (4) and (5)). (6) Plot $k_{app}$ as function of ([L] + [R]). Calculate $k_a$ and $k_d$ from the slope and y-axis intercept of the resulting line, respectively. Since the extrapolation error is large, better estimates for $k_d$ are obtained from $k_d = k_a / K_A$.

**New calibration procedure**

A new calibration procedure for perfusion type calorimeters was proposed in ref. [33]. It is based on the strong and non-linear dependence of the relative apparent molar enthalpy ($L_o$) of NaCl(aq) on the concentration. From a series of dilution experiments (NaCl solutions injected into water), calibration factors for both the measured heat and the active cell volume can be calculated. For the particular instrument tested (VP-ITC, MicroCal. Ltd.), the heat factor was 0.987. The cell volume factor was 0.93; however, the latter result should be considered with caution, since it strongly relies on the precision of tabulated $L_o$. In the course of this study, the
syringe volume delivery factor was found to be 0.973, which is consistent with a gear ratio error detected for some instruments by the manufacturer. Temperature calibration showed systematic net deviation from the set-temperature of 1 °C between 25 and 45 °C. The author suggests that such temperature “errors” might be responsible for a large proportion of the discrepancies observed between calorimetric and van’t Hoff estimates of ΔH ([34] and references therein).

**New instrumentation**

In 2007 MicroCal Ltd. announced the availability of a new titration calorimeter. The iTC\textsubscript{200} instrument is equipped with cells of 200 µl volume, a seven-fold reduction of the cell volume in comparison to the instruments available to date from the company. Due to the small cell volume, the amount of material required for experiments is reduced; equilibration and power compensation are faster, leading to lowering of the experimental costs. The handling is also more robust. The instrument can be easily up-graded to full automation allowing running of up to ~400 samples in the unattended mode.

**DIVERSE APPLICATIONS UTILIZING STANDARD PROTOCOLS**

**Protein-ligand binding**

One advantage of ITC over other available methods exploring binding energetics is the possibility to detect protonation/deprotonation taking place upon binding, and to quantify the number of protons transferred between the interacting entities and the buffer. If association causes pK\textsubscript{a} shifts of ionizable groups, protons will be released from or will bind to the complex. Necessarily, protons are taken up into or released from the buffer compound, respectively. Since the ionization enthalpies of many commonly used buffers are large, the apparent binding heats in the ITC experiment will contain a contribution from the buffer ionization heat. Due to thermodynamic linkage relationships and the intrinsic temperature dependence of pK\textsubscript{a} shifts, the observed magnitude of all thermodynamic parameters and their apparent temperature dependencies will be influenced by proton transfer. The traditional way to detect proton exchange is to perform a series of experiments at the same pH in solutions buffered with compounds having different ionization enthalpies. Dozens of studies have demonstrated that plots of the observed enthalpy, ΔH\textsubscript{obs}, as function of the buffer ionization heat, ΔH\textsubscript{b}, are linear. Formally, the data can be described by the equation ΔH\textsubscript{obs} = ΔH\textsubscript{b,0} + n\textsubscript{H+}ΔH\textsubscript{b}, where the slope, n\textsubscript{H+}, and the y-axis intercept, ΔH\textsubscript{b,0}, quantify the number of transferred protons and the enthalpy of association in a (hypothetical) buffer with zero ionization enthalpy, respectively. Usually, ΔH\textsubscript{b,0} is interpreted as representing the intrinsic (genuine) binding enthalpy in the absence of
proton transfer effects. Knowledge of the magnitude of intrinsic binding parameters is, indeed, of prime importance in establishing correlations linking experimentally observed energetics with structural features. The paper of Armstrong & Baker [25] illustrates the necessity of performing a global analysis of experiments done by variation of both pH and temperature as a rigorous way to characterize genuine energetic changes. In particular, it is stressed that \( \Delta H_{b,0} \) determined as explained above does not necessarily represent the intrinsic \( \Delta H \) of binding, because it can include contributions from pH and the magnitude of pK\(_a\) shifts. Binding of the \( \alpha \beta \) T-cell receptor A6 to the class I major histocompatibility molecule HLA-A2 presenting a nonapeptide derived from the Tax protein was measured. Altogether 17 titrations performed in 5 different buffers in the pH range 5.4-7.4 and at temperatures between 4 and 37 °C were globally analyzed. The model explicitly separates the intrinsic \( \Delta G \), \( \Delta H \) and \( \Delta C_p \) and the corresponding contributions due to protonation. The globally fitted isotherms are in good agreement with the isotherms locally fitting each data set. The analysis reveals that, inherently, the binding \( \Delta H \) is small, and \( \Delta S \) favors association. Interestingly, the intrinsic negative \( \Delta C_p \) is unexpectedly large. The authors conclude that protonation of the A6-Tax/HLA-A2 complex perhaps leads to creation of an ion binding site. Overall, the thermodynamic signature suggests conformational adjustments within the complex.

A couple of papers describing ITC investigations of peptide binding to PDZ domains appeared in 2007. Saro et al. [35] reported the results of a voluminous and very thorough study of the energetic determinants governing PDZ3 (PSD-95) domain recognition by a series of linear peptides derived from the natural protein ligands CRIP7, neurologin-1 and citron, as well as by mutants of a consensus hexapeptide. By systematic variation of the peptide length (N-terminal truncation) the authors demonstrate that in all cases six residues warrant maximal affinity. Binding is driven by favorable \( \Delta H \), the binding entropy being slightly positive or slightly negative but in most cases entropic effects add up to binding affinity. The heat capacity change is negative and small (−0.5 to −0.7 kJ K\(^{-1}\) mol\(^{-1}\)), as expected for burial of a small number of residues in the interface. Interestingly, the thermodynamic signature differs from the one derived for another class I PDZ domain (PDZ2 of hPTP 1E). Peptide binding in this case is linked to entropic losses and exhibits twice higher \( \Delta C_p \) (−1.5 kJ K\(^{-1}\) mol\(^{-1}\); ref. [36]). Replacement of the class I canonical C-terminal valine by threonine does not fully abolish binding, the loss of affinity being attributed to more unfavorable \( \Delta S \). Further, the authors followed a double mutant strategy to explore the presence of cooperative effects between adjacent peptide sub-sites. No significant coupling was detected, yet the negligible coupling \( \Delta \Delta \Delta G \) was the result of compensating coupling \( \Delta \Delta \Delta H \) and \( \Delta \Delta \Delta S \).
The laboratory of Mark Spaller contributed another paper dealing with the energetics of multivalent binding to the PDZ3(PSD-95) domain [37]. The problem of multivalent interactions has two aspects. First, many proteins contain tandems of PDZ domains and recruitment by a given binding partner may serve as a pivot facilitating other interactions. Therefore, careful characterization of multivalent binding may provide biologically relevant insights. Second, the thermodynamic signature of multivalent binding has been tackled only occasionally by ITC. Klosi et al. [37] synthesized homobivalent ligands of PDZ3(PSD-95) by linking two C-terminal peptides of the CRIP protein via a diacid succinate as the linker. Indeed, ITC experiments revealed the formation of a ternary complex. The observation was confirmed by ESI mass spectrometry. As with monovalent ligands, elongation of the ligand peptide beyond position six hardly changes the binding affinity (see above). The enthalpy and entropy of bivalent binding are essentially the same as those describing formation of binary complexes. The data were tested against several binding models included in the MicroCal ORIGIN (v.5) software, which is supplied by the manufacturer. Using the $\chi^2$ criterion, best fits were obtained with the “sequential-binding” model, as opposed to the “one-set-of-sites” and “two-sets-of-sites” models. The paper leaves open the question whether the thermodynamic parameters acquired for the two binding sites using the best fitting procedure are statistically relevant. Intriguingly, however, $\Delta C_p$ for formation of the ternary complex is positive (+0.3 kJ K$^{-1}$ mol$^{-1}$), in contrast to the negative value measured for monovalent binding (−0.5 to −0.7 kJ K$^{-1}$ mol$^{-1}$). Further work is required to clarify the molecular origin of the discrepancy. However, the heat capacity of hydration of polar groups is known to be negative. Therefore, a plausible hypothesis is that, as the bivalent ligand is short, the two neighboring PDZ3 domains contact each other, and the resulting solvent-inaccessible interface is mainly polar.

**Nucleic acid recognition**

The thermodynamic signature of protein-DNA recognition has been object of studies ever since the appearance of sensitive titration calorimeters. In contrast to protein binding sites, which have arbitrary shapes and are flexible, the DNA duplex exhibits fixed geometry and is much more rigid. Because the spatial position of hydrogen donor/acceptor functions, the negative charges of the phosphate backbone and the overall van der Waals shape can be deduced from sequence information only, understanding the thermodynamic principles governing site-specific DNA recognition by proteins might fruit in new ideas about development of DNA-binding drugs. Peter Privalov and colleagues summarized the results with approximately 20 proteins, which bind either into the major groove or into the minor groove [12]. They stress that credible
structure-oriented comparisons require correction of $\Delta H$ and $\Delta S$ determined by ITC for any contributions from partial refolding and conformational flexibility. Many DNA-binding protein domains are marginally stable and/or flexible. DNA binding induces refolding on local or global scale and restriction of thermal motions. The correction of $\Delta H$ is done by integrating the heat capacity differences between the associated and dissociated state of the system. It should be noted that the corrections are not a matter of “cosmetics”. The temperature dependence of the corrected $\Delta H$ can be dramatically different from the experimentally observed one; sometimes even the sign in a given temperature interval can change. Furthermore, in many cases protein-DNA binding is too strong to be measured with good precision by ITC and reliable $K_A$ and $\Delta G$ data (hence also $\Delta S$ data) must be collected by spectroscopic, mostly fluorescence-based methods.

The data reveal a surprisingly contrasting picture. Binding to the major groove is an enthalpically driven process, whereas recognition in the minor groove is enthalpically unfavorable and is under entropic control. This signature is not affected by the extent of DNA bending. The electrostatic component largely dominates the entropy of formation of complexes in the major groove. In contrast, binding in the minor groove is dominated by a large non-electrostatic contribution. The area-normalized heat capacity changes are invariably larger in major groove binding. The analysis concludes that the qualitative differences in the thermodynamic signatures are the consequence of distinct hydration properties of the major and minor grooves. Minor groove binders recognize principally AT-rich sequences, where water ordering is prevalent. The entropy of releasing of ordered waters is the major driving force for minor groove binding. However, the entropic gains are not in line with the concept of “hydrophobic force”, since the ice-like organization of waters in the minor groove is maintained by the regular arrangement of polar groups.

Krell et al. [20] published an interesting study of cooperative protein binding to DNA. They investigated the interaction between the TtgR protein, a specific transcriptional repressor of the TtgABC efflux pump and the corresponding operator. TtgR forms a dimer in solution. AUC and ITC experiments revealed that two dimers bind to the pseudo-palindromic operator site. Analysis of the obtained “non-trivial” ITC isotherms detected cooperative binding mode: the affinities for the two sites differed by a factor of 20, corresponding to a Hill coefficient of $n_H = 1.6$. Empirical optimization of the palindromicity of the operator site led to an increase of the overall affinity in an interesting way. The lower-affinity binding event was not affected, yet the affinity for the adjacent site was higher by a factor of 80 ($n_H = 1.8$). In the “Material and
Methods” section of the paper, one can find a detailed description of the thermodynamic formalism used in deconvolution of the binding isotherms.

Buurma & Haq [38] reviewed the current state of affairs in analysis of complicated ligand-DNA binding reactions, including multiple (1:n) binding and ligand self-association equilibria coupled to DNA-binding. Although devoted to small molecule binding to DNA, in fact the paper describes experimental procedures and discusses theoretical concepts in general terms, and will help ITC users from different fields in experimental design and data handling.

ITC has not yet found wide application in studies of ligand and protein binding to RNA, but the number of reports has clearly increased. We advise the reader to consult ref. [8] for an overview of recent work on RNA systems. The versatility of the method in studies of small molecule or protein binding to RNA, and in the analysis of RNA folding is demonstrated.

**Lipid/membrane systems**

Of several publications reporting results on peptide/protein interactions with model membranes/liposomes, we would like to mention the paper by Meier & Seelig [39]. They used a synthetic octadecapeptide (3 direct hexameric repeats) containing systematic replacements of adjacent lysine and alanine residues by D-enantiomers as a model for studying the thermodynamics of β-sheet formation and aggregation in membrane environment. Binding of the peptide variants to small unilamellar vesicles was studied by ITC and CD spectroscopy. The overall process is driven by the hydrophobic effect, as the reaction is endothermic, in distinct contrast to membrane binding of α-helix forming peptides. The extent of β-sheet formation was determined by CD spectroscopy. It was possible to separate the energetic contributions of binding to the lipid phase from those intrinsically describing formation of β-sheets. Plots of $\Delta G$, $\Delta H$, and $\Delta S$ versus the number of residues engaged in β-sheets allowed estimation of the per-residue energetic contributions. It turns out that the per-residue free energy is small and negative and is comparable to that measured for α-helix formation. However, the segmental contributions accumulate cooperatively and the interaction with the membrane can confer substantial stabilization of the β-sheet. The per-residue enthalpy change is favorable, yet is three times lower than the values measured for some α-helical peptides. The segmental unfavorable $\Delta S$ change is significantly lower for this β-sheet peptide in comparison to α-helices. Multiple D, D substitutions in peptides with high β-sheet propensity may have the potential to become a general approach in ITC studies of the conformational and thermodynamic properties of amyloid-forming peptides.
ITC AS A TOOL TO BENCHMARK RESULTS OF VAN’T HOFF ANALYSIS AND THEORETICAL CALCULATIONS

As mentioned in the Introduction, ITC enjoys growing popularity as a relatively fast, simple, and reliable method for determination of the thermodynamic parameters of binding in solution. Nonetheless, because of the inherent limitations of the method, high instrumental costs, and practical considerations (solubility, costs of production of macromolecules, etc.) it will remain only one of the large variety of tools available for analysis of binding reactions. Non-calorimetric methods rely on van’t Hoff analysis to extract thermodynamic parameters. In view of the discrepancies between calorimetric and van’t Hoff parameters reported in certain systems (ref. [34] and references therein), it is highly desirable to critically compare data obtained by ITC and other methods. In 2007, the results of the sixth benchmark study using Biacore technology were published [40]. In 22 laboratories, binding of four small ligands to carbonic anhydrase II was measured by Biacore at six temperatures between 5 and 36 °C. The eight complete data sets were highly reproducible. Both $K_d$ measured as the ratio of the association and dissociation rates and $\Delta H$ calculated from van’t Hoff plots, $\ln K_d = f(1/T)$ were in excellent agreement with data measured directly by ITC ($R = 0.9992$ and $R = 0.9998$, respectively). The results of this study are highly encouraging, although it is uncertain whether every system will be unaffected by immobilization. Ref. [25] (discussed above) reports quantitative discrepancy, even considering parameter error at two standard deviations, between $\Delta H$ and $\Delta S$ measured by ITC and calculated from Biacore data.

Rapid increase of computer power and new theoretical developments drive active work in the field of computer-aided free energy calculations. This task is a central challenge in ligand design based on the known structure of a given binding pocket. Clearly, comparison of in-silico results with experimental in-solution data is a crucial feedback mechanism. Two publications in 2007 exemplify the role of ITC as a benchmark in free energy predictions. Mobley et al. [41] performed alchemical calculations in explicit solvent to predict in blind prospective tests the binding free energy of several substituted aromatic rings to a well-defined cavity in T4 lysozyme. Subsequent ITC experiments confirmed the predictions within a RMS error of $\sim 2.5 \text{ kJ mol}^{-1}$. Direct measurements of $\Delta G$ by ITC were also important in discrimination of different algorithms for predicting the binding affinity of four ligands of porcine odorant binding protein possessing an extremely hydrophobic and occluded cavity [42].
ENZYME KINETICS

Apart from its main application in studies of association reactions, ITC can be used to explore enzyme kinetics [43, 44]. Typically, the experiment is performed by titration of substrate to the enzyme placed in the calorimetric cell but the order can be reversed [44, 45]. In either case the change in differential power caused by the heat of substrate transformation monitors the initial velocity of the enzymatic reaction and the data collected at different substrate concentrations can be treated according to the traditional Michaelis-Menten approach to extract $K_m$, $V_{max}$, and $k_{cat}$. It has been shown recently that the sensitivity of the method to measure enzyme activity and kinetics is not impaired in crowded protein solutions [46]. Olsen et al. investigated the effect of osmolytes in combination with macromolecular crowding (simulated by addition of up to 250 mg/ml BSA) on the kinetic parameters of yeast hexokinase action [47]. They found that small organic osmolytes (glycerol, TMAO, betaine), which are generally considered compatible with enzyme function, significantly decrease $k_{cat}/K_m$. The effects of these osmolytes are practically independent on the crowding exerted by BSA. In contrast, the effects of the incompatible osmolyte urea on hexokinase kinetics (increase of $V_{max}$ and $K_m$) differ in diluted and crowded solutions. High protein concentrations counteract the observed perturbation induced by urea. All studied compounds decrease $k_{cat}/K_m$ but have different influence on $V_{max}$ and $K_m$. Interestingly, the perturbing effect of the studied osmolytes on the specificity constant of hexokinase correlates with the degree of exclusion of the compounds from the protein surface. This study underlines the feasibility of ITC experiments performed in complex, non-ideal solutions.

DRUG DESIGN

ITC is widely recognized nowadays as an important tool in the process of lead optimization toward development of high-potency drugs and other biologically-active compounds [48]. Knowledge of the factorization of the binding affinity ($\Delta G$) into $\Delta H$ and $T\Delta S$ components provides the basis for the rational design of chemical changes in a given scaffold as a way to improve binding. In principle, introduction of functions with high “enthalpic potential”, for example groups capable of forming hydrogen bonds to the target, and optimization of the van der Waals complementarity within the binding pocket, as to enhance the hydrophobic effect, are expected to improve binding. However, the picture is much more complex. First, enthalpy/entropy compensation is a ubiquitous phenomenon, which stems from the very nature of non-covalent interactions. For example, the enthalpic gain from a hydrogen bond per se is offset by the dehydration penalty for burying polar chemical functions and entropic losses from
immobilization of the constituent groups. Second, the binding pockets of proteins are flexible and can “respond” in a not-easily predictable way to the incoming ligand. Moreover, mutations within the binding pocket can lead to drug resistance.

A typical example of a drug optimization strategy combining structural and thermodynamic approaches is the development of high potency inhibitors of the HIV-1 protease [49]. Most of the early protease inhibitors have been designed and optimized as binders of the wild type protease and are much less potent against mutants thereof. Clearly, the goal is to develop inhibitors, which preserve affinity to mutant proteins. The study of Muzammil et al. [50] illustrates how new ideas in this direction could be conceived by analyzing thermodynamic data in a structural context. The authors characterized by ITC and crystallography the binding of several medium-to-low picomolar protease inhibitors to wild type protease, active-site mutants, a multi-drug resistance octuple mutant, and an in vitro selected hextuple mutant. Among them, tipranavir (TPV) binds to the wild type enzyme with only slightly favorable \( \Delta H \), the very tight binding (\( K_d \sim 20 \) pM) resulting from the extremely high favorable entropic contribution. Interestingly, the very large negative \( T \Delta S \) does not correlate to the amount of surface burial. Rather, it can be attributed to the fact that less water molecules are trapped at the TPV-protease interface in comparison to other protease/inhibitor complexes. Furthermore, the TPV molecule obtains binding-competent conformation more readily than other compounds in the set. Binding of all inhibitors to mutant protease variants is impaired. TPV stands out in the set. All other mutant protease/inhibitor complexes suffer enthalpic destabilization, which is partly compensated by more favorable (or nearly neutral) entropy. Differently, TPV is capable to compensate for severe entropic losses by gain in binding enthalpy, or else with only modest enthalpic penalty. The reasons for this particular behavior are not exactly known at the moment, but structural analysis reveals that TPV forms an extensive network of hydrogen bonds to backbone atoms and catalytic residues, interactions which cannot be removed by spontaneous mutations resulting in active protease variants. Also, the number of water-mediated hydrogen bonds and the number of immobilized water molecules in mutant protease/TPV complexes is the smallest. The authors conclude that TPV is an example of an “enthalpically restraint inhibitor, i.e. and inhibitor that binds to the WT protease with a barely favorable enthalpy but that contains the potential to enhance its enthalpic interactions when facing protease mutants.”

Two clear cases illustrating the hurdles which enthalpy/entropy compensation poses to binding affinity optimization by rational design were published in 2007. Lafont et al. [51] compared the binding properties of the experimental HIV-1 protease inhibitors KNI-10033 and KNI-10075. The only difference between the two inhibitors is the replacement of the thioether
group of KNI-10033 by a sulfonyl function in KNI-10075, with the idea to engineer a strong hydrogen bond acceptor. Indeed, the crystal structure of the KNI-10075/protease complex reveals the existence of the anticipated hydrogen bond with the amide of Asp 30. The binding enthalpy is improved by almost 4 kcal mol$^{-1}$. However, the gain in enthalpy is completely compensated by unfavorable entropy changes, leading to no increase in binding affinity. Structural analysis suggests that the entropy loss stems partly from desolvation effects and partly from decrease of the conformational entropy.

Gerlach et al. [52] tested one of the basic rules followed by medicinal chemists in the process of systematic optimization of lead compounds, namely that the group contribution of a methylene group to $\Delta G$ of binding is 3-4 kJ mol$^{-1}$. They examined the thermodynamic profiles of two homologous thrombin inhibitors differing only by one methylene group in the cycloalkyl moiety designed to bind in the S3/S4 specificity pocket. Contrary to the expectation that the affinity of the cyclohexyl-containing compound (CH) should be higher than that of the cyclopentyl-containing homologue (CP), both molecules had virtually the same affinity for thrombin. Analysis by ITC revealed surprising differences in the enthalpy/entropy balance of the thrombin/inhibitor complexes. While binding of CP is driven by favorable and approximately equal in magnitude $\Delta H$ and $T\Delta S$ (at 25 °C), CH exhibits a strong entropic advantage, which compensates the significant reduction of enthalpic contributions. The intriguing observation was further analyzed by crystallography and MD simulations. The difference electron density of the cyclopentyl ring of CP in the S3/S4 pocket was well defined, whereas very poorly defined density was observed for the six-membered ring of CH. In MD simulations the cyclopentyl moiety undergoes jump rotation and populates two distinct but geometrically identical states. In contrast, the cyclohexyl group exhibits high mobility, non-concerted motions, and tumbles in and out of the binding pocket. A reasonable interpretation of the combined results is that the pronounced mobility of the cyclohexyl ring is linked to enthalpic loss due to sub-optimal packing interactions, but at the same time renders the entropy change more favorable since less degrees of freedom are lost upon binding. These examples demonstrate that the binding properties of closely related ligands are the result of a complex superposition of structural, dynamic, and energetic factors. Homologues in a congeneric series can bind the target with different enthalpy/entropy signatures, thus destroying simple structure-activity relationships.

Two papers by Steven Homans and colleagues (one of them discussing measurements done earlier) contain interesting results and discussions with potential to be implemented in drug optimization [9, 10]. Binding of hydrophobic compounds (homologous aliphatic alcohols and substituted pyrazine rings) to the mouse major urinary protein was measured by ITC. Because of
the hydrophobic character of the interactions, it was expected that binding would exhibit the typical signature of the “classical” hydrophobic effect, namely large negative $\Delta C_p$ and positive $\Delta S$. Surprisingly, association was driven by favorable $\Delta H$ and opposed by negative $\Delta S$. The analysis reveals that $\Delta C_p$ measured for binding of alcohols can be attributed almost exclusively to dehydration of the alcohol, with quite small contribution from desolvation of the protein binding site. The negative binding entropy of the cyclic compounds tested can also be rationalized in terms of incomplete desolvation of the binding pocket (along with other effects). The conclusion is that in some cases, binding sites, especially hydrophobic pockets, are sub-optimally hydrated. If such areas can be identified, a reasonable way toward gaining affinity would be to optimize the shape complementarity exclusively in these regions, since protein-ligand interactions would not be (partially) compensated by protein-solvent and ligand-solvent interactions.

**STRUCTURE-ENERGY CORRELATIONS**

Since the early nineties, there has been a strong interest in finding and refining correlations between observed changes in thermodynamic quantities characterizing binding and structural features of the complex. The problem is not only central to in-depth understanding of the physical principles of molecular recognition, but echoes the need for developing simplified algorithms to predict the energetic response of structural perturbations in rational design. Several reports in 2007 deal with parameterization of binding parameters in terms of buried surface. A number of different systems were characterized; different parameterization schemes were used. By analyzing the entropy of binding, inhibition of the binding of angiotensin-converting enzyme and allosamidin to a family 18 chitinase was shown to involve (unexpected) conformational changes [53]. Loss of conformational freedom over-compensates the favorable entropy stemming from the hydrophobic effect in the binding of peptide mimotopes to human IgG1 [54]. In these reports (see also ref. [36]), a breakdown of the correlations between buried surface and $\Delta C_p$ was found. In contrast, Casares et al. [55] were able to reproduce the experimental $\Delta C_p$ for binding of a nonapeptide to Abl tyrosine kinase SH3 domain. Yet the predicted $\Delta H$ and $\Delta S$ were completely flawed not only in magnitude but in sign as well. The authors discuss two potential sources of discrepancies. (i) Calculations based on surface burial cannot account for reduced thermal fluctuations in the bound protein, which contribute to the experimentally observed negative enthalpy and entropy. (Ref. [36] discusses discrepancies along similar lines.) (ii) Water molecules at the binding interface were ignored in the calculations of the buried surface. When
the water molecules were explicitly considered, a better qualitative agreement with the experimental $\Delta H$ and $\Delta S$ was achieved. (The correlation with $\Delta C_p$ was lost, however). The importance of including structural waters in surface-based predictions has been highlighted previously [56]. The paper of Lafont et al. [51] (discussed above) documents the important (yet sometimes neglected) prerequisite of using master equations and correlation coefficients, which have been properly calibrated for the nature of the system under study (chemical composition, size, etc.). They were capable to reproduce $\Delta H$ for binding of inhibitor KNI-10033 to HIV-1 protease. Application of the same equation to the closely related inhibitor KNI-10075/HIV-1 protease complex significantly underestimated $\Delta H$. The likely reason is that the sulfonyl group in KNI-10075 has not been part of the training set used to derive the master equation.

The papers of Steve Homans et al. [9, 10] discussed in the previous section might provide rationale in explaining observed discrepancies between measured and calculated thermodynamic quantities in specific cases.

**CONCLUDING REMARKS**

Since the launch of the new generation of sensitive instruments, the interest in using the potential of ITC to answer scientific questions in diverse fields of modern research is steadily growing. The expansion of the ITC community, so obvious form the increasing number of publications reporting ITC results in a specific area of research, is mirrored also in the trend showing a clear diversification of the systems that have been characterized using ITC data. For example, the number of articles classified as treating “miscellaneous subjects” has more than doubled in 2007 in comparison to the preceding year. It appears that both the advantages and limitations of the method are nowadays well anticipated. The principle advantage is the potential to collect a full set of thermodynamic parameters characterizing an association process at “benign” conditions, without uncertainties arising from derivatization and immobilization. The principal limitation is the relatively restricted range of affinities accessible by “standard” experiments. Experimental strategies have been devised facilitating characterization of very high-affinity and low affinity reactions. New theoretical developments provide ways to analyze complicated binding equilibria and heterotropic effects. In the future, the task will be primarily to evaluate critically at the accumulated results. In this respect the creation and maintenance of databases may be very helpful. Examples are the recently advanced databases SCORPIO (http://www.biochem.ucl.ac.uk/scorpio/scorpio.html) and proNIT (http://gibk26.bse.kyutech.ac.jp/jouhou/pronit/pronit.html), both using WWW interfaces. Furthermore, it will be important to collect in a systematic way high-precision data on carefully
selected systems. The challenge is still the discovery of rigorous links between experimental thermodynamics and molecular structure.

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