Applications of calorimetric methods to drug discovery and the study of protein interactions

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Recent studies report the application of isothermal titration calorimetry and differential scanning calorimetry to the study of protein–ligand interactions, allosteric cooperativity and aspects of protein folding. New methods of data analysis compare alternative methods for determining ligand binding enthalpy and analyze potential sources of error in the experimental measurement of other thermodynamic parameters. Several reports examine issues concerning drug design and the correlation of thermodynamic and X-ray structural data. New instruments allow volumetric effects in biochemical systems to be evaluated calorimetrically and to substantially expand the throughput of differential scanning calorimetry measurements in drug discovery and other high-throughput applications.

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Abbreviations

CBS	4-carboxybenzenesulfonamide
DNSA	5-dimethyl-amino-1-naphthalene-sulfonamide
DSC	differential scanning calorimetry
ITC	isothermal titration calorimetry
RalGDS	Ral guanine nucleotide dissociation stimulator

Introduction

A review in this journal last year described the methodology of isothermal titration calorimetry (ITC) and its application to the study of biochemical systems [1]. A second recent review described the molecular events underlying aspects of the thermodynamics of biological systems and the origins of entropy-enthalpy compensation [2••]. This review summarizes recent calorimetric studies of protein–ligand and protein–protein interactions, and describes some new applications of high-throughput calorimetry to drug discovery.

Protein–ligand interactions

Ligand association with proteins typically involves changes in the intramolecular and intermolecular interactions and dynamics of the system components, including the protein, the ligand, water and any additional components that may be present [3]. The changes in bonding interactions or dynamics that occur upon ligand binding are reflected in the reaction enthalpy and entropy, which in turn determine the free energy of ligand association. As shown in Table 1, which is a partial compilation of recent thermodynamic studies of protein–protein and protein–ligand binding interactions, intermolecular associations reflect virtually all possible net favorable combinations of enthalpy and entropy components.

It has proved difficult to predict ligand binding thermodynamics from first principles or even to predict variations in binding affinity when apparently conservative chemical modifications are made to the protein or ligand structures. One significant practical consequence is that structure-based drug design has proved substantially more difficult than originally envisioned [3]. This situation has motivated the expanding use of calorimetry to empirically quantify enthalpic and entropic contributions to protein stabilization and ligand binding affinity.

Recent studies outlined in Table 1 illustrate several examples in which structurally similar ligands interact at common protein binding sites with disparate thermodynamic parameters. For example, recent work reported the interactions of carbonic anhydrase II with the arylsulfonamides 4-carboxybenzenesulfonamide (CBS) and 5-dimethylamino-1-naphthalene-sulfonamide (DNSA) [4•]. These compounds, which could easily represent variations on a common drug pharmacophore, bind with similar free energies ($\Delta G_{CBS} = -8.4$ kcal/mol and $\Delta G_{DNSA} = -8.8$ kcal/mol). However, the binding enthalpies differ by nearly 2.5-fold and the binding of one compound is entropically favored, whereas the other is entropically disfavored (Table 1). This example illustrates how difficult it is to predict binding parameters and supports the notion that, where possible, heats of reaction should be experimentally measured.

A study of lectin–sugar interactions correlated ITC ligand binding data with structures obtained from X-ray crystallography. The mono-, di- and tri-saccharides galactose, lactose and fucosyllactose each bind *Erythrina cristagalli* lectin with similar affinity [5]. Moreover, the conformations of the disaccharide portions of lactose and fucosyllactose are nearly identical in the high-resolution crystal structures. Contrary to expectation, binding enthalpy within the series does not correlate with buried surface area. The authors suggest factors that could contribute to

Table 1

Thermodynamic binding parameters.

System	ΔH (kcal/M)	TΔS (kcal/M)	ΔT_m (°C)	References
Protein–small molecule interactions				
Zn-α_2-glycoprotein + (dansylamino)undecanoic acid	−5.2	9.7	~2	[24]
HIV-1 protease + Amprenavir	−6.9	6.3		[6]
HIV-1 protease + Nelfinavir	2.6	15.7		[7]
HIV-1 protease + Indinavir	2.1	14.8		[7]
HIV-1 protease + Saquinavir	1.9	14.7		[7]
HIV-1 protease + Ritonavir	−3.7	10.7		[7]
HIV-1 protease (I50V) + Amprenavir	−4.2	6.1		[6]
HIV-1 protease (V82F/I84V) + Amprenavir	−3.9	6.6		[6]
HIV-1 protease subtype A + Ritonavir	−2.9	10.3		[7]
HIV-1 protease subtype B + Ritonavir	−3.7	10.7		[7]
HIV-1 protease subtype C + Ritonavir	−3.1	10.3		[7]
Farnesyl protein transferase + inhibitor	−7.1	3		[25]
Acyl-CoA dehydrogenase + octenoyl-CoA	−21.7	−12.9		[26]
Carbonic anhydrase II + CBS	−11.9	−3.5		[4•]
Carbonic anhydrase II + DNSA	−4.8	4.0		[4•]
E. cristagalli lectin + galactose	−5.3	−1.3		[5]
E. cristagalli lectin + lactose	−7.1	−2.4		[5]
E. cristagalli lectin + fucosyllactose	−4.7	0.0		[5]
Glucose transporter GLUT 1 + ATP (pH 4.3)			−12	[21]
Glucose transporter GLUT 1 + ATP (pH 7.4)			−0.5	[21]
Human H-chain ferritin + Fe^{2+}	2.0	9.0		[27]
Protein–protein interactions				
Fungal xylanase + xylanase-inhibiting protein	−11.6	−1.5		[9]
Ubiquitin-fused complement-type repeat domains 5 and 6 + receptor-associated protein domain 1	−11.5	−3.9		[10]
Ubiquitin-fused complement-type repeat domains 5 and 6 + receptor-associated protein domain 3	−4.9	4.1		[10]
Ferri-cytochrome *c* + ferri-cytochrome *c* peroxidase	−2.6	5.5		[11•]
(Ferri-cytochrome *c* + ferri-cytochrome *c* peroxidase) in trisaccharides (site 1)	−2.8	8.5		[12•]
(Ferri-cytochrome *c* + ferri-cytochrome *c* peroxidase) in trisaccharides (site 2)	−0.3	8.4		[12•]
Peripheral subunit-binding domain of dihydrolipoyl acetyltransferase + pyruvate decarboxylase	−8.4	4.5	4.8	[13]
Peripheral subunit-binding domain of dihydrolipoyl acetyltransferase + dihydrolipoyl dehydrogenase	2.2	14.8	6	[13,14]
Ras-binding domain of Raf + Ras	−5.2	4.5		[15]
Ras-binding domain of RalGDS + Ras	−14.5	−6.2		[15]
Ras-binding domain of Raf + Rap	−3.7	4.8		[15]
Ras-binding domain of RalGDS + Rap	−14.5	−4.7		[15]

this effect, which include differences in the number of water molecules that are immobilized at the lectin–carbohydrate interface.

Calorimetry and drug design

Recent papers discuss aspects of HIV-1 protease inhibitor design and related binding thermodynamics. Thermodynamic binding parameters have been reported for several marketed HIV-1 protease inhibitors, including Amprenavir, Indinavir, Nelfinavir, Saquinavir and Ritonavir [6]. The dissociation constants of these molecules are subnanomolar and all show strongly favorable entropy components, despite the fact that inhibitor binding at the protease active site is accompanied by the ordering of two surface loops that are observed to be conformationally flexible in the unliganded enzyme. Only Amprenavir and Ritonavir exhibit favorable binding enthalpies (Table 1).

Studies were also reported for HIV-1 protease inhibitors binding to drug-resistant enzyme variants (I50V and V82F/I84V) in which mutations near the active site catalytic residues alter the size and shape of the substrate-binding site [6,7]. Drug binding to the resistant variants was generally characterized by unfavorable enthalpy changes relative to the native protease, although the change in entropic contribution for Amprenavir was negative and partially off-set the positive change in enthalpic contribution to produce a relatively small decrease in inhibitor affinity. Studies were also performed that

compared drug binding to HIV-1 protease subtypes that primarily differ at sites remote from the enzyme active site, but display different affinities for existing clinical HIV-1 protease inhibitors [7].

Based on thermodynamic analyses of inhibitor binding to wild-type and drug-resistant forms of HIV-1 protease, Freire and co-workers [6] proposed matching inhibitor conformational flexibility (e.g. by incorporating rotatable bonds and asymmetric centers in the drug) with highly mutable protease sites as a strategy for combating the emergence of drug resistance [6]. Other applications of ITC to drug design were recently reviewed by Ladbury [8].

Protein–protein interactions and protein domain organization

Several ITC and differential scanning calorimetry (DSC) studies investigating protein–protein interactions and protein domain organization [9,10,11•,12•,13–15] are summarized in Table 1. The results again defy broad generalizations regarding common thermodynamic driving forces for these associations.

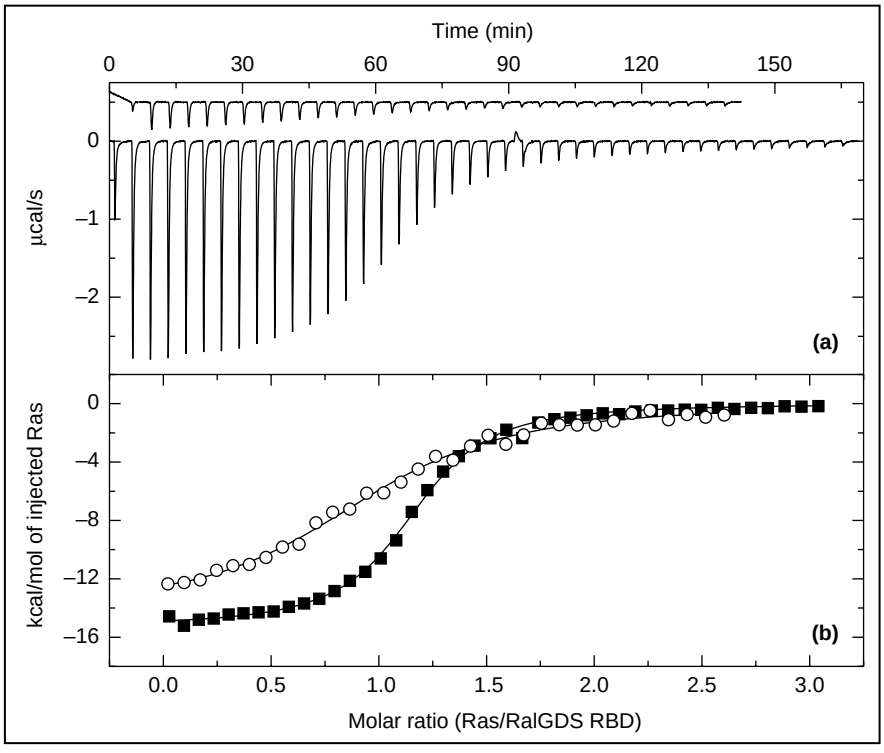
Protein–protein interactions

The association of four Ras family GTPases with four Ras-binding domains from different effector molecules was examined by ITC to correlate structure and interaction specificity in different signaling pathways [15]. The Ras family GTPases interacted similarly with each of the effector domains, although large differences in thermodynamic binding parameters were observed among the respective effector domains (some examples are shown in Table 1). Figure 1 shows ITC data from a concentration study performed to ascertain the potential for protein oligomerization in this system. Some effects correlated well with available three-dimensional structural information on the free and complexed GTPase and effector molecules, although a comprehensive explanation remains equivocal.

Protein domain organization

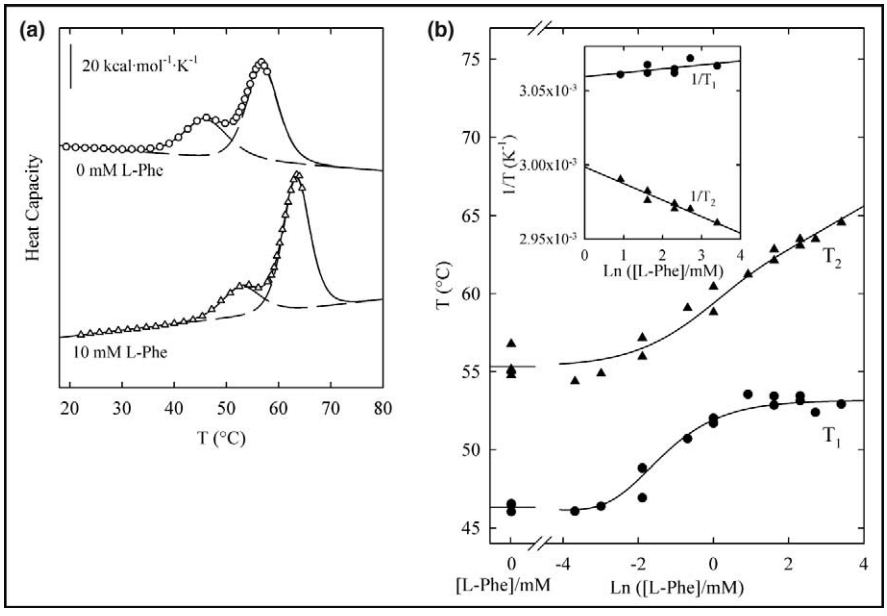
A DSC study described the domain interactions and ligand-induced cooperativity of phenylalanine hydroxylase, a tetrameric enzyme that exhibits a Hill coefficient of 2 when binding its substrate, phenylalanine. Each

Figure 1



Concentration independence of the interaction between Ras and the Ras-binding domain (RBD) of Ral guanine nucleotide dissociation stimulator (RalGDS). **(a)** Calorimetric titration of Ras into 6 μM (upper curve) and 50 μM (lower curve) RalGDS. The baseline was subtracted from the raw data and an off-set of 0.5 μcal/s was added to the upper curve. **(b)** The theoretical curves fitted to the integrated data yield $K_d = 1.2$ μM and $\Delta H^\circ = -15.0$ kcal/mol for RalGDS at 6 μM (open circles), and $K_d = 1.1$ μM and $\Delta H^\circ = -14.8$ kcal/mol for RalGDS at 50 μM (closed squares). Titrations conducted at protein concentrations varying by nearly 90% exhibit similar heats of reaction and dissociation constants. This indicates that, at both concentrations, the proteins possess the same oligomeric form and, in this system, the proteins are monomeric. Reproduced with permission from [15].

Figure 2



DSC profiles of phenylalanine hydroxylase. **(a)** Heat capacity versus temperature profiles for the thermal denaturation of human phenylalanine hydroxylase in the absence of L-phenylalanine (circles) and in the presence of 10 mM L-phenylalanine (triangles). The continuous lines represent the best fits of the sum of two non-two-state transitions to the experimental data; the individual transitions resulting from the fitting are shown with dashed lines. Profiles have been displaced on the y-axis for the sake of clarity. **(b)** Effect of L-phenylalanine concentration on the transition temperature for the two transitions observed in the DSC thermograms of the thermal denaturation of human phenylalanine hydroxylase. The symbols are the values of T_1 and T_2 derived from the experimental thermograms. Inset: plots of $1/T_1$ and $1/T_2$ versus the logarithm of L-phenylalanine concentration. Reproduced with permission from [16].

polypeptide chain of the enzyme incorporates three domains. DSC analysis revealed distinct transitions reflecting the sequential melting of the regulatory and catalytic domains [16]. A general binding-polynomial formalism was developed to analyze ligand effects on transition temperatures. Differences in the dependence of the transitions on phenylalanine concentration (Figure 2), together with DSC studies of truncated forms of the enzyme and comparison of the observed and computed transition enthalpies, support the hypothesis that the regulatory domain influences the cooperativity of the catalytic domain without directly binding phenylalanine. This result contrasts with earlier work suggesting that phenylalanine bound to the enzyme at independent sites as substrate and allosteric effector.

A DSC study of the thermal unfolding of streptokinase under a wide variety of environmental and experimental conditions systematically illustrated how solution pH, protein concentration and thermal scan rate altered the observed DSC curves and the apparent derived thermodynamic parameters [17•]. Many of these effects are related to the rate of irreversible aggregation following heat-induced unfolding.

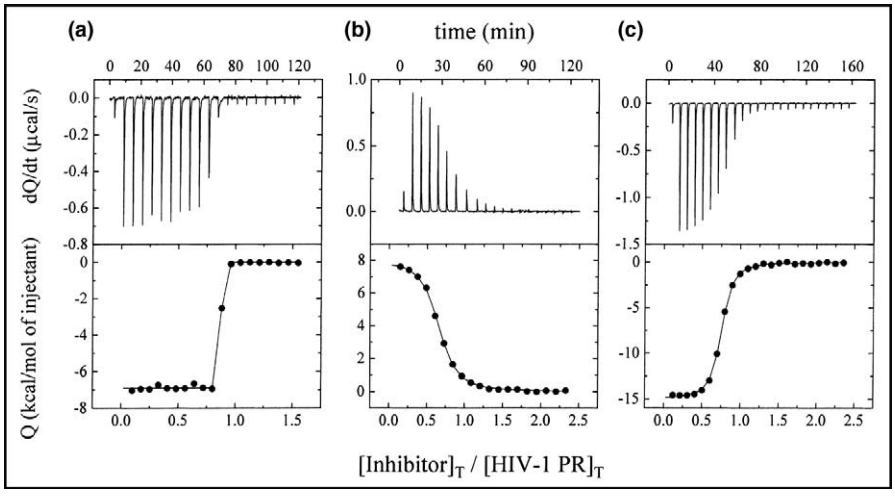
Using a new high-sensitivity Nano-DSC instrument, Dragan and Privalov [18••] studied thermal transitions in a simple leucine zipper structure (a coiled coil of two α helices). Initial unfolding was found to involve two concentration-independent transitions reflecting distinct unimolecular unfolding of helical regions within the zipper dimer. At higher temperatures, cooperative concentration-dependent unfolding and dissociation of the two polypeptide chains occur. The observed behavior contrasts with earlier work that had characterized leucine zipper unfolding as a simple two-state process.

New methods

Several publications have reported new experimental approaches and methods of data treatment.

Reaction enthalpies can be measured directly by calorimetric methods (ΔH_{cal}) or estimated from the temperature dependence of an equilibrium constant (known as the van't Hoff enthalpy, ΔH_{vH}). Day *et al.* [4•] found a good correlation between values of ΔH_{vH} computed from surface plasmon resonance experiments and values of ΔH_{cal} determined from solution ITC experiments. Murphy and co-workers [19••,20••] described sources of error leading to discrepancies between observed and estimated values of ΔH_{cal} and ΔH_{vH}. These can reflect experimental or computational errors, or poor instrument calibration. However, the authors noted that, in nonequilibrium binding situations such as those caused by irreversible protein

Figure 3



Calorimetric titrations of HIV-1 protease with **(a)** Amprenavir, **(b)** acetyl pepstatin and **(c)** Amprenavir injected into the acetyl pepstatin–protease complex solution (displacement titration). In all experiments, the protease was in the calorimeter reaction cell. Reproduced with permission from [6].

aggregation or in 'open' experimental systems in which the proton concentration is held constant while the temperature is varied, genuine differences between ΔH_{cal} and ΔH_{vH} can occur.

ITC measurements to derive accurate binding affinities for very tightly binding ligands pose specific experimental challenges. Freire and co-workers [6] reported an approach to accurately determine the binding affinities of highly potent inhibitors of HIV-1 protease by performing a displacement ITC experiment, in which a concentrated high-affinity inhibitor was injected into a solution of HIV-1 protease complexed with a weak inhibitor exhibiting a positive enthalpy of displacement (Figure 3).

Pressure perturbation calorimetry (PPC) is a recently introduced technique [2••] that applies relatively small pressure pulses to sample and reference cells of a modified DSC instrument. The method senses effects associated with changes in the partial specific coefficients of thermal expansion of the system components in order to measure changes in volumetric properties. The approach has been used to measure volumetric changes associated with protein unfolding, as well as those associated with ligand binding to lysozyme [2••].

Instrumentation for parallel measurements of thermal transitions in proteins

Conventional instruments for ITC or DSC studies generally require ~0.5 mg of purified protein per experiment and must be operated by careful experimentalists to produce reliable data, limiting manual throughput to 5–10 experimental measurements per instrument per day. Although the value of thermodynamic analysis for studying ligand binding has been appreciated for many years, the instrumental limitations have restricted the broader use of ITC and DSC in modern biochemical and drug discovery applications, for which experimental strategies may involve the evaluation of the relative stability of thousands of mutant proteins or the screening of a large library of chemical compounds against a protein drug target. Nevertheless, several approaches have recently emerged that can substantially increase the throughput of DSC or 'DSC-like' measurements relative to conventional instrumentation.

A primary motivation for these developments emerges from the utility of DSC or 'thermal shift' measurements in primary or secondary drug screening. DSC measures the cooperative melting of a folded protein's structure, which occurs at a temperature, T_m, that reflects the protein's stabilization energy. When compounds bind to a protein, the energy of ligand binding adds incrementally to the protein's stabilization energy and the protein's T_m is increased by an amount that is proportional to the ligand binding energy. Thermodynamic analysis of the ΔT_m, or thermal shift, associated with ligand binding can be semi-quantitatively related to ligand binding affinity, subject to reasonable assumptions regarding the ligand binding enthalpies of drug-like compounds. The approach has a broad dynamic range (typically $10\,\mu M > K_a > 1$ nM) and can be applied to a very broad range of drug targets, including both enzymes and cell-surface receptor domains.

Although favorable ligand–protein associations typically stabilize proteins, one study [21] reported destabilizing interactions of ATP with the glucose transporter GLUT 1 (Table 1). In fact, the massively parallel use of thermal

shift measurements in drug screening [22] often reveals compounds that apparently destabilize the protein structure. Usually these act as covalent modifiers of the target protein, as extractors of ions that stabilize the protein structure or as relatively nonspecific detergents.

Recent advances in DSC instrumentation to facilitate its application to drug discovery involve automated integration with a sampling robot to allow unattended throughput of 50 samples per day [23]. The MicroCal VP-Capillary DSC platform system uses ~100 μg of the target protein in a 130 μl volume, which is about one-quarter the amount required using more conventional instrumentation. Such systems are relatively low throughput relative to modern ultra-high-throughput drug screens, but are well suited to secondary screens. In such screens, DSC measurements can confirm that a screening hit in fact corresponds to a reversibly binding, high-affinity ligand for the target, as opposed to an inhibitor that acts through some spurious mechanism.

An approach that emulates a DSC measurement uses environmentally sensitive dyes that selectively fluoresce when bound to the melted state of the protein to determine the protein's T_m [10]. In this application, called ThermoFluor®, an instrument has been developed that uses a high-resolution CCD camera to image a sample plate in which each well contains the target protein of interest, a different test ligand and the reporter dye. As the plate is heated, a fluorescent signal progressively develops as the target protein melts. Analysis of the intensity curve defines the midpoint of the transition or the T_m for each potential protein–ligand complex. In wells in which the test compound binds to the protein, the T_m is shifted to a higher temperature. Typical screening applications use ~0.5 μg of test protein in a 5 μl sample volume and a 384-well plate that can be thermally scanned in 30–90 min. The method also provides a high-throughput approach to the determination of solution conditions that stabilize proteins and the analysis of the thermal stability of mutant proteins.

Conclusions

Establishing a comprehensive physical and structural understanding of the events that give rise to thermodynamic phenomena in biochemical systems is a difficult task that lies beyond currently available computational approaches to the analysis of molecular structure and interaction. Nevertheless, improvements in instrumentation and experimental methodology, together with an expanded appreciation of the utility of thermodynamic approaches to such diverse areas as protein folding and drug discovery, are providing a wealth of new experimental data. The expanded correlation of these data with physical studies of protein structure and dynamics has the potential to greatly expand our understanding of how biological systems actually work.

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