

ITC XXX – Reviews

Ababou A. and Ladbury J. E. (2006) Survey of the year 2004: literature on applications of isothermal titration calorimetry. *J Mol Recognit* **19**, 79-89.

Abstract: The market for commercially available isothermal titration calorimeters continues to grow as new applications and methodologies are developed. Concomitantly the number of users (and abusers) increases dramatically, resulting in a steady increase in the number of publications in which isothermal titration calorimetry (ITC) plays a role. In the present review, we will focus on areas where ITC is making a significant contribution and will highlight some interesting applications of the technique. This overview of papers published in 2004 also discusses current issues of interest in the development of ITC as a tool of choice in the determination of the thermodynamics of molecular recognition and interaction.

Ababou A. and Ladbury J. E. (2007) Survey of the year 2005: literature on applications of isothermal titration calorimetry. *J Mol Recognit* **20**, 4-14.

Abstract: Isothermal titration calorimetry (ITC) can provide a full thermodynamic characterization of an interaction. Its usage does not suffer from constraints of molecular size, shape or chemical constitution. Neither is there any need for chemical modification or attachment to solid support. This ease of use has made it an invaluable instrumental resource and led to its appearance in many laboratories. Despite this, the value of the thermodynamic parameterization has, only quite recently, become widely appreciated. Although our understanding of the correlation between thermodynamic data and structural details continues to be somewhat naive, a large number of publications have begun to improve the situation. In this overview of the literature for 2005, we have attempted to highlight works of interest and novelty. Furthermore, we draw attention to those works which we feel have provided a route to better analysis and increased our ability to understand the meaning of thermodynamic change on binding. Copyright (c) 2006 John Wiley & Sons, Ltd.

Ambrosone L. and Ragone R. (2000) Electrochemical methods of evaluating the van't Hoff enthalpy in reactions involving biological macromolecules. *Int J Biol Macromol* **27**, 241-244.

Abstract: We highlight conditions under which coincidence of van't Hoff and calorimetric enthalpies can be experimentally verified for reactions of biochemical interest. First, we clarify that, often, chemical equations in condensed phase do not explicitly contain information on all processes involved. Second, we underline that the accuracy of electrochemical methods is much higher than that of other non-calorimetric techniques. Electrochemical data on the binding of ethidium ion to DNA are re-examined to verify that the entropy evaluated as the temperature derivative of the free energy agrees in full with the calorimetric one. Third, we point out that unfolding or self-association enthalpies of redox proteins can be reliably obtained by electromotive force measurements, taking advantage of their linkage to redox enthalpies. Thermodynamic cycles coupling biochemical transformations to redox systems are briefly discussed.

Bains G. and Freire E. (1991) Calorimetric determination of cooperative interactions in high affinity binding processes. *Anal Biochem* **192**, 203-206.

Abstract: It is demonstrated that isothermal titration calorimetry can be used to determine cooperative interaction energetics even for extremely tight binding processes in which the binding affinity constants are beyond the limits of experimental determination. The approach is based on the capability of calorimetry to measure the apparent binding enthalpy at any degree of ligand saturation. When calorimetric measurements are performed under conditions of total association at partial saturation, the dependence of the apparent binding enthalpy on the degree of saturation is a function only of the cooperative binding interactions. The method developed in this paper allows an independent estimation of cooperative energetic parameters without the need to simultaneously estimate or precisely know the value of the association constants. Since total ligand association at partial saturation is achieved only at macromolecular concentrations much larger than the dissociation constants, the method is especially suited for high and very high affinity processes. Biological associations in this category include fundamental cellular processes like cell surface receptor binding or protein-DNA interactions.

Baker B. M. and Murphy K. P. (1996) Evaluation of linked protonation effects in protein binding reactions using isothermal titration calorimetry. *Biophys J* **71**, 2049-2055.

Abstract: A theoretical development in the evaluation of proton linkage in protein binding reactions by isothermal titration calorimetry (ITC) is presented. For a system in which binding is linked to protonation of an ionizable group on a protein, we show that by performing experiments as a function of pH in buffers with varying ionization enthalpy, one can determine the pKa's of the group responsible for the proton linkage in the free and the liganded states, the protonation enthalpy for this group in these states, as well as the intrinsic energetics for ligand binding (ΔH_o , ΔS_o , and ΔC_p). Determination of intrinsic energetics in this fashion allows for comparison with energetics calculated empirically from structural information. It is shown that in addition to variation of the ligand binding constant with pH, the observed binding enthalpy and heat capacity change can undergo extreme deviations from their intrinsic values, depending upon pH and buffer conditions.

Baker B. M. and Murphy K. P. (1998) Prediction of binding energetics from structure using empirical parameterization. *Methods Enzymol* **295**, 294-315.

Abstract: We have presented an empirical method that can be used to predict the binding energetics for protein-protein or protein-peptide interactions from three-dimensional structures. The approach differs from other empirical methods in yielding a thermodynamic description of the binding process, including ΔC_p , ΔH degree, and ΔS degree, rather than predicting ΔG degree alone. These thermodynamic terms can provide a wealth of detail about the nature of the interaction, and, if sufficient experimental data are available for comparison, a greater assessment of the accuracy of the calculations. A recurring theme throughout this article is the need for more complete thermodynamic and structural characterizations of protein-ligand interactions. This includes not only characterization of the binding ΔH degree, ΔS degree, and ΔC_p , but a thorough investigation into equilibria linked to binding, such as protonation, ion binding, and conformational changes. Sufficient data will allow parameterization on binding data rather than protein unfolding data. Further inclusion of information obtained from unfolding studies is not likely to generate significant improvement in the accuracy of the calculations. As additional binding data become available, the parameterization can be further extended to include relationships derived from analyses of these data. Not only will this increase accuracy and thus confidence, but allow extension of the method of additional types of interactions.

Bjelic S. and Jelesarov I. (2008) A survey of the year 2007 literature on applications of isothermal titration calorimetry. *J Mol Recognit* **21**, 289-312.

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

Blandamer M.J. (1998) Thermodynamic background to Isothermal Titration Calorimetry, *in* Biocalorimetry: Applications of Calorimetry in the Biological Sciences. Ladbury, J.E., Chowdhry, B.Z., eds., John Wiley & Sons Ltd., Chichester UK, pp. 5-38.

Blandamer M. J., Cullis P. M., and Gleeson P. T. (2003) Three important calorimetric applications of a classic thermodynamic equation. *Chem Soc Rev* **32**, 264-267.

Abstract: The thermodynamic background to three calorimetric techniques is discussed; (i) titration microcalorimetry, (ii) adiabatic calorimetry, and (iii) heat conduction calorimetry. Relevant equations for each technique are derived from a common equation for the enthalpy H of a closed system. General

patterns which emerge in the measured parameters are presented for adiabatic and heat conduction calorimeters linked to applications of these techniques.

Breslauer K. J., Freire E., and Straume M. (1992) Calorimetry: a tool for DNA and ligand-DNA studies. *Methods Enzymol* **211**, 533-567.

Bouchemal K. (2008) New challenges for pharmaceutical formulations and drug delivery systems characterization using isothermal titration calorimetry. *Drug Discov. Today* **13**, 960-972.

Abstract: Long viewed as the 'method of choice' for characterizing thermodynamics and stoichiometry of molecular interactions, with high sensitivity, isothermal titration calorimetry (ITC) has been applied to many areas of pharmaceutical analysis. This review highlights ITC employment to measure binding thermodynamics and their use for pharmaceutical formulations and drug delivery system characterization particularly cyclodextrin-guest interactions, investigation of micellar-based systems, polyelectrolytes, nucleic acid interactions with multivalent cations and the optimization of DNA targeting and delivery. Furthermore, the potential of ITC for the characterization of different functionalities carried by nanoparticles as well as their interaction with living systems was outlined

Bundle D. R. and Sigurskjold B. W. (1994) Determination of accurate thermodynamics of binding by titration microcalorimetry. *Methods Enzymol* **247**, 288-305.

Buurma N. J. and Haq I. (2007) Advances in the analysis of isothermal titration calorimetry data for ligand-DNA interactions. *Methods* **42**, 162-172.

Abstract: Isothermal titration calorimetry (ITC) is a well established technique for the study of biological interactions. The strength of ITC is that it directly measures enthalpy changes associated with interactions. Experiments can also yield binding isotherms allowing quantification of equilibrium binding constants, hence an almost complete thermodynamic profile can be established. Principles and application of ITC have been well documented over recent years, experimentally the technique is simple to use and in ideal scenarios data analysis is trivial. However, ITC experiments can be designed such that previously inaccessible parameters can be evaluated. We outline some of these advances, including (1) exploiting different experimental conditions; (2) low affinity systems; (3) high affinity systems and displacement assays. In addition we ask the question: What if data cannot be fit using the fitting functions incorporated in the data-analysis software that came with your ITC? Examples where such data might be generated include systems following non 1:n binding patterns and systems where binding is coupled to other events such as ligand dissociation. Models dealing with such data are now appearing in literature and we summarise examples relevant for the study of ligand-DNA interactions.

Chaires J. B. (2008) Calorimetry and thermodynamics in drug design. *Annu Rev Biophys* **37**, 135-151.

Abstract: Modern instrumentation for calorimetry permits direct determination of enthalpy values for binding reactions and conformational transitions in biomolecules. Complete thermodynamic profiles consisting of free energy, enthalpy, and entropy may be obtained for reactions of interest in a relatively straightforward manner. Such profiles are of enormous value in drug design because they provide information about the balance of driving forces that cannot be obtained from structural or computational methods alone. This perspective shows several examples of the insight provided by thermodynamic data in drug design

Chen X., Lin Y., and Gilson M. K. (2001) The binding database: overview and user's guide. *Biopolymers* **61**, 127-141.

Abstract: The large and growing body of experimental data on molecular binding is of enormous value in biology, pharmacology, and chemistry. Applications include the assignment of function to biomolecules, drug discovery, molecular modeling, and nanotechnology. However, binding data are difficult to find and access because they are available almost exclusively through scientific journals. BindingDB, a public, web-accessible database of measured binding affinities, is designed to address this problem. BindingDB collects data for natural and modified biomolecules and for synthetic compounds, and provides detailed experimental information. Currently, measurements by isothermal titration calorimetry are fully supported; measurements by enzyme inhibition will soon be included as well. The web site allows data to be searched by a range of criteria, including binding thermodynamics, sequence homology, and chemical structure,

substructure, and similarity. Experimentalists are encouraged to publicize their data by entering it into BindingDB via the online forms. Such data can be updated or revised by the depositor, if necessary, and will remain publicly accessible. User involvement and feedback are welcomed.

Chen X., Liu M., and Gilson M. K. (2001) BindingDB: a web-accessible molecular recognition database. *Comb Chem High Throughput Screen* **4**, 719-725.

Abstract: This paper presents an initial description of the BindingDB, a public web-accessible database of measured binding affinities for various molecular types (<http://www.bindingdb.org>). The BindingDB allows queries based upon a range of criteria, including chemical similarity or substructure, sequence homology, numerical criteria (e.g. $\Delta G_0 < 5$ kcal/mol) and reactant names (e.g. "lysozyme"). Principles of Human-Computer Interactions are being employed in creating the query interface and user-feedback is being solicited. The data specification includes significant experimental detail. A full dictionary has been created for isothermal titration calorimetry data in consultation with experimentalists and data dictionaries for enzyme-inhibition and other measurement techniques are being developed. Currently, the BindingDB contains several data sets of broad interest, such as antigen-antibody binding and cyclodextrin/small molecule binding. However, it is anticipated that online deposition by experimentalists will ultimately contribute to a larger flow of data. We are actively developing software and file specifications to facilitate such deposition.

Chen X., Lin Y., Liu M., and Gilson M. K. (2002) The Binding Database: data management and interface design. *Bioinformatics* **18**, 130-139.

Abstract: MOTIVATION: The large and growing body of experimental data on biomolecular binding is of enormous value in developing a deeper understanding of molecular biology, in developing new therapeutics, and in various molecular design applications. However, most of these data are found only in the published literature and are therefore difficult to access and use. No existing public database has focused on measured binding affinities and has provided query capabilities that include chemical structure and sequence homology searches. METHODS & RESULTS: We have created Binding DataBase (BindingDB), a public, web-accessible database of measured binding affinities. BindingDB is based upon a relational data specification for describing binding measurements via Isothermal Titration Calorimetry (ITC) and enzyme inhibition. A corresponding XML Document Type Definition (DTD) is used to create and parse intermediate files during the on-line deposition process and will also be used for data interchange, including collection of data from other sources. The on-line query interface, which is constructed with Java Servlet technology, supports standard SQL queries as well as searches for molecules by chemical structure and sequence homology. The on-line deposition interface uses Java Server Pages and JavaBean objects to generate dynamic HTML and to store intermediate results. The resulting data resource provides a range of functionality with brisk response-times, and lends itself well to continued development and enhancement.

Chun P. W. (2000) Thermodynamic molecular switch in macromolecular interactions. *Cell Biochem Biophys* **33**, 149-169.

Abstract: It is known that most living systems can live and operate optimally only at a sharply defined temperature, or over a limited temperature range, at best, which implies that many basic biochemical interactions exhibit a well-defined Gibbs free energy minimum as a function of temperature. The Gibbs free energy change, $\Delta G_0(T)$, for biological systems shows a complicated behavior, in which $\Delta G_0(T)$ changes from positive to negative, then reaches a negative value of maximum magnitude (favorable), and finally becomes positive as temperature increases. The critical factor in this complicated thermodynamic behavior is a temperature-dependent heat capacity change ($\Delta C_{p_0}(T)$) of reaction, which is positive at low temperature, but switches to a negative value at a temperature well below the ambient range. Thus, the thermodynamic molecular switch determines the behavior patterns of the Gibbs free energy change, and hence a change in the equilibrium constant, K_{eq} , and/or spontaneity. The subsequent, mathematically predictable changes in $\Delta H_0(T)$, $\Delta S_0(T)$, $\Delta W_0(T)$, and $\Delta G_0(T)$ give rise to the classically observed behavior patterns in biological reactivity, as demonstrated in three interacting protein systems: the acid dimerization reaction of alpha-chymotrypsin at low pH, interaction of chromogranin A with the intraluminal loop peptide of the inositol 1,4,5-triphosphate receptor at pH 5.5, and the binding of L-arabinose and D-galactose to the L-arabinose binding protein of Escherichia coli. In cases of protein unfolding of four mutants of phage T4 lysozyme, no thermodynamic molecular switch is observed.

Cliff M. J., Gutierrez A., and Ladbury J. E. (2004) A survey of the year 2003 literature on applications of isothermal titration calorimetry. *J Mol Recognit* **17**, 513-523.

Abstract: Over the last decade isothermal titration calorimetry (ITC) has developed from a specialist method which was largely restricted in its use to dedicated experts, to a major, commercially available tool in the arsenal directed at understanding molecular interactions. The number of those proficient in this field has multiplied dramatically, as has the range of experiments to which this method has been applied. This has led to an overwhelming amount of new data and novel applications to be assessed. With the increasing number of publications in this field comes a need to highlight works of interest and impact. In this overview of the literature we have attempted to draw attention to papers and issues for which both the experienced calorimetrist and the interested dilettante hopefully will share our enthusiasm.

Cooper A. (2005) Heat capacity effects in protein folding and ligand binding: a re-evaluation of the role of water in biomolecular thermodynamics. *Biophys Chem* **115**, 89-97.

Abstract: Large "anomalous" heat capacity (ΔC_p) effects are a common feature of the thermodynamics of biomolecular interactions in aqueous solution and, as a result of the improved facility for direct calorimetric measurements, there is a growing body of experimental data for such effects in protein folding, protein-protein and protein-ligand interactions. Conventionally such heat capacity effects have been ascribed to hydrophobic interactions, and there are some remarkably convincing demonstrations of the usefulness of this concept. Nonetheless, there is also increasing evidence that hydrophobic interactions are not the only possible source of such effects. Here we re-evaluate the possible contributions of other interactions to the heat capacity changes to be expected for cooperative biomolecular folding and binding processes, with particular reference to the role of hydrogen bonding and solvent water interactions. Simple models based on the hydrogen-bonding propensity of water as a function of temperature give quantitative estimates of ΔC_p that compare well with experimental observations for both protein folding and ligand binding. The thermodynamic contribution of bound waters in protein complexes is also estimated. The prediction from simple lattice models is that trapping of water in a complex should give more exothermic binding ($\Delta\Delta H$ of -6 to -12 kJ mol⁻¹) with lower entropy ($\Delta\Delta S$ of approximately -11 J mol⁻¹ K⁻¹) and more negative ΔC_p (by about -75 J mol⁻¹ K⁻¹) per water molecule. More generally, it is clear that significant ΔC_p effects are to be expected for any macromolecular process involving a multiplicity of cooperative weak interactions of whatever kind.

Cooper A. and Johnson C. M. (1994) Isothermal titration microcalorimetry. *Methods Mol Biol* **22**, 137-150.

Cooper A. and Johnson C. M. (1994) Introduction to microcalorimetry and biomolecular energetics. *Methods Mol Biol* **22**, 109-124.

Cooper A. (1998) Microcalorimetry of protein-protein interactions. *Methods Mol Biol* **88**, 11-22.

Cooper A. (1999) Thermodynamic analysis of biomolecular interactions. *Curr Opin Chem Biol* **3**, 557-563.

Abstract: Direct measurement of the thermodynamics of biomolecular interactions is now relatively easy. Interpretation of these thermodynamics in simple molecular terms is not. Recent work shows how the multiplicity of weak noncovalent interactions, and the inevitable enthalpy/entropy compensation that these interactions engender, lead to difficulties in teasing out the different components.

Cooper A., Johnson C. M., Lakey J. H., and Nollmann M. (2001) Heat does not come in different colours: entropy-enthalpy compensation, free energy windows, quantum confinement, pressure perturbation calorimetry, solvation and the multiple causes of heat capacity effects in biomolecular interactions. *Biophys Chem* **93**, 215-230.

Abstract: Modern techniques in microcalorimetry allow us to measure directly the heat changes and associated thermodynamics for biomolecular processes in aqueous solution at reasonable concentrations. All these processes involve changes in solvation/hydration, and it is natural to assume that the heats for these processes should reflect, in some way, such changes in solvation. However, the interpretation of data is still somewhat ambiguous, since different non-covalent interactions may have similar thermodynamic signatures, and analysis is frustrated by large entropy-enthalpy compensation effects. Changes in heat capacity (ΔC_p) have been related to changes in hydrophobic hydration and non-polar accessible surface areas, but more recent empirical and theoretical work has shown how this need not always be the case.

Entropy-enthalpy compensation is a natural consequence of finite ΔC_p values and, more generally, can arise as a result of quantum confinement effects, multiple weak interactions, and limited free energy windows, giving rise to thermodynamic homeostasis that may be of evolutionary and functional advantage. The new technique of pressure perturbation calorimetry (PPC) has enormous potential here as a means of probing solvation-related volumetric changes in biomolecules at modest pressures, as illustrated with preliminary data for a simple protein-inhibitor complex.

Cooper M. A. (2003) Label-free screening of bio-molecular interactions. *Anal Bioanal Chem* **377**, 834-842. **Abstract:** The majority of techniques currently employed to interrogate a biomolecular interaction require some type of radio- or enzymatic- or fluorescent-labelling to report the binding event. However, there is an increasing awareness of novel techniques that do not require labelling of the ligand or the receptor, and that allow virtually any complex to be screened with minimal assay development. This review focuses on three major label-free screening platforms: surface plasmon resonance biosensors, acoustic biosensors, and calorimetric biosensors. Scientists in both academia and industry are using biosensors in areas that encompass almost all areas drug discovery, diagnostics, and the life sciences. The capabilities and advantages of each technique are compared and key applications involving small molecules, proteins, oligonucleotides, bacteriophage, viruses, bacteria, and cells are reviewed. The role of the interface between the biosensor surface (in the case of SPR and acoustic biosensors) and the chemical or biological systems to be studied is also covered with attention to the covalent and non-covalent coupling chemistries commonly employed.

Cooper M. A. (2004) Advances in membrane receptor screening and analysis. *J Mol Recognit* **17**, 286-315. **Abstract:** During the last decade there has been significant progress in the development of analytical techniques for the screening of ligand binding to membranes and membrane receptors. This review focuses on developments using label-free assays that facilitate ligand-membrane-receptor screening without the need for chemical-, biological- or radiological-labelled reagents. These assays include acoustic, optical surface plasmon resonance biosensing, sedimentation (analytical ultracentrifugation), chromatographic assays, isothermal titration calorimetry and differential scanning calorimetry. The merits and applications of cell-based screening systems and of different model membrane systems, including planar supported lipid layers, bead-supported membranes and lipid micro-arrays, are discussed. Recent advances involving more established techniques including intrinsic fluorescence, FRET spectroscopy, scintillation proximity assays and automated patch clamping are presented along with applications to peripheral membrane proteins, ion channels and G protein-coupled receptors. Novel high-throughput assays for determination of drug- and protein-partitioning in membranes are also highlighted. To aid the experimenter, a brief synopsis of the techniques commonly employed to purify and reconstitute membranes and membrane receptors is included.

Dam T. K. and Brewer C. F. (2004) Multivalent protein-carbohydrate interactions: isothermal titration microcalorimetry studies. *Methods Enzymol* **379**, 107-128.

Doyle M. L., Louie G., Dal Monte P. R., and Sokoloski T. D. (1995) Tight binding affinities determined from thermodynamic linkage to protons by titration calorimetry. *Methods Enzymol* **259**, 183-194. **Abstract:** A general titration calorimetry method is described that can be used to determine the affinity of tight binding interactions with proteins. The method is based on the thermodynamic linkage between ligand binding and coupled protonation reactions. The protons linked to a given ligand-binding reaction are measured by titration calorimetry, and integration of the resulting data set yields the pH dependence of the binding affinity based on thermodynamic relationships developed elsewhere. When the pH dependence of the binding affinity is combined with the absolute affinity determined independently at a pH at which the affinity can be conveniently measured, the absolute binding affinity over the entire pH range is determined. The method is well suited for determining high-affinity binding interactions of protein antigens with antibodies, but is applicable to any macromolecular ligand-binding reaction that is coupled to protonation.

Doyle M. L. (1997) Characterization of binding interactions by isothermal titration calorimetry. *Curr Opin Biotechnol* **8**, 31-35.

Abstract: Isothermal titration calorimetry is a high-accuracy method for measuring binding affinities. Titration calorimetry is a universal method that has broad impact throughout biotechnology. In recent years, microcalorimeters that are capable of characterizing binding interactions of biological

macromolecules have become commercially available. Results from these studies are providing new insight into the molecular nature of macromolecular interactions.

Doyle M. L. and Hensley P. (1998) Tight ligand binding affinities determined from thermodynamic linkage to temperature by titration calorimetry. *Methods Enzymol* **295**, 88-99.

Abstract: A general isothermal titration calorimetry method is described that can be used to determine equilibrium binding constants for high-affinity interactions of ligands with biological macromolecules. The method exploits the thermodynamic linkage between the ligand binding equilibrium constant and temperature. By measuring the binding enthalpy change for an interaction as a function of temperature directly, the change in affinity can be calculated with an integrated form of the van't Hoff equation that is applicable to ligand binding to biological macromolecules. When the temperature dependence of the affinity is combined with the absolute affinity determined independently at a convenient temperature (where the affinity can most accurately or most easily be measured), the absolute binding affinity over the entire temperature range is determined.

Edgcomb S. P. and Murphy K. P. (2000) Structural energetics of protein folding and binding. *Curr Opin Biotechnol* **11**, 62-66.

Abstract: Structural energetics is a method for calculating the energetics of protein folding and binding reactions as a function of temperature. This approach allows measured energetics to be interpreted with regards to the protein structure and the prediction of energetics from known structures. Recent advances include improvements in the parameterization of enthalpy, entropy and heat capacity terms and new applications, especially with regards to understanding dynamic properties of proteins and how these are affected by ligand binding.

Fisher H. F. and Singh N. (1995) Calorimetric methods for interpreting protein-ligand interactions. *Methods Enzymol* **259**, 194-221.

Freire, E., Mayorga, O.L., and Straume, M. (1990) Isothermal Titration Calorimetry. *Anal Chem* **62**, 950A-959A.

Freyer M. W. and Lewis E. A. (2008) Isothermal titration calorimetry: experimental design, data analysis, and probing macromolecule/ligand binding and kinetic interactions. *Methods Cell Biol* **84**, 79-113.

Abstract: Isothermal titration calorimetry (ITC) is now routinely used to directly characterize the thermodynamics of biopolymer binding interactions and the kinetics of enzyme-catalyzed reactions. This is the result of improvements in ITC instrumentation and data analysis software. Modern ITC instruments make it possible to measure heat effects as small as 0.1 microcal (0.4 microJ), allowing the determination of binding constants, K 's, as large as $10(8) - 10(9)M(-1)$. Modern ITC instruments make it possible to measure heat rates as small as 0.1 microcal/sec, allowing for the precise determination of reaction rates in the range of $10(-12) mol/sec$. Values for $K(m)$ and $k(cat)$, in the ranges of $10(-2) - 10(3) microM$ and $0.05 - 500 sec(-1)$, respectively, can be determined by ITC. This chapter reviews the planning of an optimal ITC experiment for either a binding or kinetic study, guides the reader through simulated sample experiments, and reviews analysis of the data and the interpretation of the results

Gohlke H. and Klebe G. (2002) Approaches to the description and prediction of the binding affinity of small-molecule ligands to macromolecular receptors. *Angew Chem Int Ed Engl* **41**, 2644-2676.

Abstract: The influence of a xenobiotic compound on an organism is usually summarized by the expression biological activity. If a controlled, therapeutically relevant, and regulatory action is observed the compound has potential as a drug, otherwise its toxicity on the biological system is of interest. However, what do we understand by the biological activity? In principle, the overall effect on an organism has to be considered. However, because of the complexity of the interrelated processes involved, as a simplification primarily the "main action" on the organism is taken into consideration. On the molecular level, biological activity corresponds to the binding of a (low-molecular weight) compound to a macromolecular receptor, usually a protein. Enzymatic reactions or signal-transduction cascades are thereby influenced with respect to their function for the organism. We regard this binding as a process under equilibrium conditions; thus, binding can be described as an association or dissociation process. Accordingly, biological activity is expressed as the affinity of both partners for each other, as a thermodynamic equilibrium quantity. How

well do we understand these terms and how well are they theoretically predictable today? The holy grail of rational drug design is the prediction of the biological activity of a compound. The processes involving ligand binding are extremely complicated, both ligand and protein are flexible molecules, and the energy inventory between the bound and unbound states must be considered in aqueous solution. How sophisticated and reliable are our experimental approaches to obtaining the necessary insight? The present review summarizes our current understanding of the binding affinity of a small-molecule ligand to a protein. Both theoretical and empirical approaches for predicting binding affinity, starting from the three-dimensional structure of a protein-ligand complex, will be described and compared. Experimental methods, primarily microcalorimetry, will be discussed. As a perspective, our own knowledge-based approach towards affinity prediction and experimental data on factorizing binding contributions to protein-ligand binding will be presented.

Hansen L. D., Russell D. J. and Choma C. T. (2007) From biochemistry to physiology: the calorimetry connection. *Cell Biochem Biophys* **49**, 125-140.

Abstract: This article provides guidelines for selecting optimal calorimetric instrumentation for applications in biochemistry and biophysics. Applications include determining thermodynamics of interactions in non-covalently bonded structures, and determining function through measurements of enzyme kinetics and metabolic rates. Specific examples illustrating current capabilities and methods in biological calorimetry are provided. Commercially available calorimeters are categorized by application and by instrument characteristics (isothermal or temperature-scanning, reaction vessel volume, heat rate detection limit, fixed or removable reaction vessels, etc.). Advantages and limitations of commercially available calorimeters are listed for each application in biochemistry, biophysics, and physiology.

Haq I., Jenkins T. C., Chowdhry B. Z., Ren J., and Chaires J. B. (2000) Parsing free energies of drug-DNA interactions. *Methods Enzymol* **323**, 373-405.

Haq I., Chowdhry B. Z., and Jenkins T. C. (2001) Calorimetric techniques in the study of high-order DNA-drug interactions. *Methods Enzymol* **340**, 109-149.

Holdgate G. A. (2001) Making cool drugs hot: isothermal titration calorimetry as a tool to study binding energetics. *Biotechniques* **31**, 164-6, 168, 170.

Abstract: Characterization of the thermodynamics of binding interactions is important in improving our understanding of bimolecular recognition and forms an essential part of the rational drug design process. Isothermal titration calorimetry (ITC) is rapidly becoming established as the method of choice for undertaking such studies. The power of ITC lies in its unique ability to measure binding reactions by the detection of the heat change during the binding interaction. Since heat changes occur during many physicochemical processes, ITC has a broad application, ranging from chemical and biochemical binding studies to more complex processes involving enthalpy changes, such as enzyme kinetics. Several features of ITC have facilitated its preferential use compared to other techniques that estimate affinity. It is a sensitive, rapid, and direct method with no requirement for chemical modification or immobilization. It is the only technique that directly measures enthalpy of binding and so eliminates the need for van't Hoff analysis, which can be time consuming and prone to uncertainty in parameter values. Although ITC has facilitated the measurement of the thermodynamics governing binding reactions, interpretation of these parameters in structural terms is still a major challenge.

Hopkins H. P., Jr. (1997) Calorimetric techniques for studying drug-DNA interactions. *Methods Mol Biol* **90**, 259-268.

Horn J. R., Russell D., Lewis E. A., and Murphy K. P. (2001) Van't Hoff and calorimetric enthalpies from isothermal titration calorimetry: are there significant discrepancies? *Biochemistry* **40**, 1774-1778.

Abstract: The enthalpy of a reaction is most often determined through one of two means; it can be determined directly using calorimetry or indirectly by measuring the temperature dependence of the equilibrium constant (i.e., the van't Hoff method). Recently, discrepancies have been noted between the enthalpy measured by calorimetry, and the enthalpy determined by the van't Hoff method. This has been suggested to indicate that the binding reaction is more complex than the simple one-to-one binding model used to describe the data. To better understand possible discrepancies between and, we have undertaken

both experimental studies using isothermal titration calorimetry to measure the binding energetics of Ba(2+) binding 18-crown-6 ether and 2'-CMP binding RNase A, along with a simulation of a system involving a molecule in conformational equilibrium coupled with binding. We find that when experimental setup and analysis are correctly performed, no statistically significant discrepancies between and exist even for the linked system.

Horn J. R., Brandts J. F., and Murphy K. P. (2002) van't Hoff and calorimetric enthalpies II: effects of linked equilibria. *Biochemistry* **41**, 7501-7507.

Abstract: The complexity of binding reactions, including the linkage with other equilibria, is becoming increasingly apparent in biological processes such as signal transduction. Understanding these interactions requires obtaining thermodynamic profiles for each of the equilibria that occur in a binding event. Concern has been raised as to whether linked equilibria contribute differently to thermodynamics, such as ΔH degrees and ΔC_p , obtained from calorimetric and van't Hoff methods. We have previously shown that linked equilibria do not contribute differently to the van't Hoff and calorimetrically determined ΔH degrees for processes such as linked folding or hydration. Here, examples of proton and ion linkage are examined. We show that there is no reason to expect the calorimetric and van't Hoff ΔH degrees to be different, even without prior knowledge of the presence or absence of linked equilibria, as long as the system is permitted to equilibrate. However, it is possible to create experimental scenarios that result in and discrepancies. Furthermore, it is found that the presence of linked equilibria in all cases can result in "nonconventional" ΔH degrees and ΔC_p profiles, making data analysis nontrivial.

Indyk L. and Fisher H. F. (1998) Theoretical aspects of isothermal titration calorimetry. *Methods Enzymol* **295**, 350-364.

Jelesarov I. and Bosshard H. R. (1999) Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J Mol Recognit* **12**, 3-18.

Abstract: The principles of isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) are reviewed together with the basic thermodynamic formalism on which the two techniques are based. Although ITC is particularly suitable to follow the energetics of an association reaction between biomolecules, the combination of ITC and DSC provides a more comprehensive description of the thermodynamics of an associating system. The reason is that the parameters ΔG , ΔH , ΔS , and ΔC_p obtained from ITC are global properties of the system under study. They may be composed to varying degrees of contributions from the binding reaction proper, from conformational changes of the component molecules during association, and from changes in molecule/solvent interactions and in the state of protonation.

Koenigbauer M. J. (1994) Pharmaceutical applications of microcalorimetry. *Pharm Res* **11**, 777-783.

Abstract: General principles and applications of microcalorimetry are reviewed. Microcalorimetry is useful in the study of physical, chemical, and biological drug interactions. The sensitivity of the present instrumentation is approximately 0.1 μW . With this high sensitivity, additional applications have been developed, including the interactions of drugs with food, lymphoma cells, microorganisms, blood, excipients, and cyclodextrin. A recent application of microcalorimetry is the measurement of degradation rates of drugs.

Krell, T (2008) Microcalorimetry: a response to challenges in modern biotechnology. *Microbial Biotechnol* **1**, 126-136.

Abstract: Almost any process in life is accompanied by heat changes which can be monitored by isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). Both techniques are now established tools in fundamental research but over the last decade a clear tendency towards more problem driven applications is noted. This review aims at summarizing these problem-oriented applications of microcalorimetry and the solutions both techniques can provide to problems in biotechnology. The biotechnological issues to which microcalorimetry has been successfully applied are as diverse as rational drug design, overcoming drug resistance, optimization of long-term stability of proteins, estimation of the bioavailability of drugs, control of complex pharmaceutical products or the optimization of gene delivery efficiency. The main limitation of microcalorimetry, which is the relatively large amounts of

sample necessary for analysis, is less important in the biotechnology sector which frequently uses large-scale produced bulk products for analysis. The recently developed high-throughput DSC and ITC microcalorimeters will additionally reduce the labour intensity of these techniques. Due to the precision of microcalorimetric analyses and the versatility of processes which can be studied, it is expected that ITC and DSC will soon be key technologies in biotechnological research.

Ladbury J. E. (2004) Application of isothermal titration calorimetry in the biological sciences: things are heating up! *Biotechniques* **37**, 885-887.

Ladbury J. E. (1995) Counting the calories to stay in the groove. *Structure* **3**, 635-639.

Abstract: High-sensitivity microcalorimetry is beginning to make an impact on the determination of thermodynamic parameters associated with protein-DNA interactions and the understanding of the relationship of these data to structural details of complex formation

Ladbury J. E. and Chowdhry B. Z. (1996) Sensing the heat: the application of isothermal titration calorimetry to thermodynamic studies of biomolecular interactions. *Chem Biol* **3**, 791-801.

Abstract: Biomolecular interactions can be defined by combining thermodynamic data on the energetic properties of the interaction with high-resolution structural data. The development of high sensitivity isothermal titration calorimetric equipment provides a dramatic advance in the gathering of thermodynamic data, and the interactions between biological macromolecules can now be described with unprecedented accuracy.

Ladbury J. E. (1996) Just add water! The effect of water on the specificity of protein-ligand binding sites and its potential application to drug design. *Chem Biol* **3**, 973-980.

Abstract: Recent data have highlighted the enigmatic role that water plays in biomolecular complexes. Water at the interface of a complex can increase the promiscuity of an interaction, yet it can also provide increased specificity and affinity. The ability to engineer water-binding sites into the interface between a drug and its target might prove useful in drug design.

Ladbury J. E. and Williams M. A. (2004) The extended interface: measuring non-local effects in biomolecular interactions. *Curr Opin Struct Biol* **14**, 562-569.

Abstract: Improvements in the sensitivity and availability of biophysical techniques for the detection of the formation of complexes in solution are revealing that the effects of binding are not restricted to the direct contacts between the biomolecules or even to a localised site. Rather, information about the binding event is transmitted throughout the biomolecules and the surrounding solution through changes in the hydrogen bonding, hydration and electrostatic field as the complex is formed. Calorimetric, volumetric and NMR methods are beginning to provide a quantitative view of the nature and thermodynamic consequences of this extended interface, and the resulting data pose a major challenge for computational models of binding.

Lakey J. H. and Raggett E. M. (1998) Measuring protein-protein interactions. *Curr Opin Struct Biol* **8**, 119-123.

Abstract: The binding of one protein to another provokes a variety of biophysical changes that can then be used as a measure of the binding reaction. Optical spectroscopy, particularly fluorescence, is the most flexible technique, but surface plasmon resonance biosensors, microcalorimetry and mass spectroscopy have recently shown significant development.

Leavitt S. and Freire E. (2001) Direct measurement of protein binding energetics by isothermal titration calorimetry. *Curr Opin Struct Biol* **11**, 560-566.

Abstract: Of all the techniques that are currently available to measure binding, isothermal titration calorimetry is the only one capable of measuring not only the magnitude of the binding affinity but also the magnitude of the two thermodynamic terms that define the binding affinity: the enthalpy (ΔH) and entropy (ΔS) changes. Recent advances in instrumentation have facilitated the development of experimental designs that permit the direct measurement of arbitrarily high binding affinities, the coupling of binding to protonation/deprotonation processes and the analysis of binding thermodynamics in terms of structural

parameters. Because isothermal titration calorimetry has the capability to measure different energetic contributions to the binding affinity, it provides a unique bridge between computational and experimental analysis. As such, it is increasingly becoming an essential tool in molecular design.

Lewis E. A. and Murphy K. P. (2005) Isothermal titration calorimetry. *Methods Mol Biol* **305**, 1-16.

Abstract: Isothermal titration calorimetry is an ideal technique for measuring biological binding interactions. It does not rely on the presence of chromophores or fluorophores, nor does it require an enzymatic assay. Because the technique relies only on the detection of a heat effect upon binding, it can be used to measure the binding constant, K , the enthalpy of binding, ΔH degrees and the stoichiometry, or number of binding sites, n . This chapter describes instrumentation, experimental design, and the theoretical underpinnings necessary to run and analyze a calorimetric binding experiment.

Li L., Dantzer J. J., Nowacki J., O'Callaghan B. J. and Meroueh S. O. (2008) PDBcal: a comprehensive dataset for receptor-ligand interactions with three-dimensional structures and binding thermodynamics from isothermal titration calorimetry. *Chem Biol Drug Des* **71**, 529-532.

Abstract: Compounds designed solely based on structure often do not result in any improvement of the binding affinity because of entropy-enthalpy compensation. Thermodynamic data along with structure provide an opportunity to gain a deeper understanding of this effect and aid in the refinement of scoring functions used in computational drug design. Here, we scoured the literature and constructed the most comprehensive hand-curated calorimetry dataset to date. It contains thermodynamic and structural data for more than 400 receptor-ligand complexes. The dataset can be accessed through a web interface at <http://www.pdbcal.org>. The thermodynamic data consists of free energy, enthalpy, entropy and heat capacity as measured by isothermal titration calorimetry (ITC). The dataset also contains the experimental conditions that were used to carry out the ITC experiments. The chemical structures of the ligands are also provided. Analysis of the data confirms the existence of enthalpy-entropy compensation effect for the first time using strictly ITC data

Liang Y. (2008) Applications of isothermal titration calorimetry in protein science. *Acta Biochim Biophys Sin. (Shanghai)* **40**, 565-576.

Abstract: During the past decade, isothermal titration calorimetry (ITC) has developed from a specialist method for understanding molecular interactions and other biological processes within cells to a more robust, widely used method. Nowadays, ITC is used to investigate all types of protein interactions, including protein-protein interactions, protein-DNA/RNA interactions, protein-small molecule interactions and enzyme kinetics; it provides a direct route to the complete thermodynamic characterization of protein interactions. This review concentrates on the new applications of ITC in protein folding and misfolding, its traditional application in protein interactions, and an overview of what can be achieved in the field of protein science using this method and what developments are likely to occur in the near future. Also, this review discusses some new developments of ITC method in protein science, such as the reverse titration of ITC and the displacement method of ITC

Lopez M. M. and Makhatadze G. I. (2002) Isothermal titration calorimetry. *Methods Mol Biol* **173**, 121-126.

Lundqvist T. (2005) The devil is still in the details--driving early drug discovery forward with biophysical experimental methods. *Curr Opin Drug Discov Devel* **8**, 513-519.

Abstract: This review comments on some recent trends and insights in the field of lead identification and optimization with a bias toward the increased use of biophysical methods, particularly in combination with three-dimensional structural information. While high-throughput screening, combinatorial chemistry and, most recently, in silico virtual screening techniques have made well-resourced but only partially successful attempts to meet the challenge of identifying new drug candidates by playing 'the large numbers game', another group of technologies are now approaching the same challenge from what might be considered the opposite extreme. The common strategy of these technologies is to focus on a smaller set of low-molecular-weight compounds whose interactions with a target are characterized with the aid of sensitive assays, most often high-quality biophysical techniques such as biosensors, calorimetry, nuclear magnetic resonance spectroscopy and X-ray crystallography. The advantages of such an approach include more optimal and chemically attractive starting points, immediate access to reliable measurements of binding properties, the

mapping of ligand interactions on the atomic level and, most importantly, a greater control of experimental errors at the initial stages of drug discovery where compounds are either discovered or lost. When correctly supported, this more careful approach appears to deliver quality leads, even for the so-called 'difficult' targets. As these techniques are complementary to traditional methods, companies should be less hesitant to invest in them. The biophysical methods that are used to drive this approach have made something of a return to drug discovery after having been discarded for being too slow, too expensive or too old-fashioned by the over-optimistic supporters of high-throughput and statistical/computational in silico methods.

Luque I., Leavitt S. A., and Freire E. (2002) The linkage between protein folding and functional cooperativity: two sides of the same coin? *Annu Rev Biophys Biomol Struct* **31**, 235-256.

Abstract: During the course of their biological function, proteins undergo different types of structural rearrangements ranging from local to large-scale conformational changes. These changes are usually triggered by their interactions with small-molecular-weight ligands or other macromolecules. Because binding interactions occur at specific sites and involve only a small number of residues, a chain of cooperative interactions is necessary for the propagation of binding signals to distal locations within the protein structure. This process requires an uneven structural distribution of protein stability and cooperativity as revealed by NMR-detected hydrogen/deuterium exchange experiments under native conditions. The distribution of stabilizing interactions does not only provide the architectural foundation to the three-dimensional structure of a protein, but it also provides the required framework for functional cooperativity. In this review, the statistical thermodynamic linkage between protein stability, functional cooperativity, and ligand binding is discussed.

Minor D. L., Jr. (2007) The neurobiologist's guide to structural biology: a primer on why macromolecular structure matters and how to evaluate structural data. *Neuron* **54**, 511-533.

Abstract: Structural biology now plays a prominent role in addressing questions central to understanding how excitable cells function. Although interest in the insights gained from the definition and dissection of macromolecular anatomy is high, many neurobiologists remain unfamiliar with the methods employed. This primer aims to help neurobiologists understand approaches for probing macromolecular structure and where the limits and challenges remain. Using examples of macromolecules with neurobiological importance, the review covers X-ray crystallography, electron microscopy (EM), small-angle X-ray scattering (SAXS), and nuclear magnetic resonance (NMR) and biophysical methods with which these approaches are often paired: isothermal titration calorimetry (ITC), equilibrium analytical ultracentrifugation, and molecular dynamics (MD).

Mizoue L. S. and Tellinghuisen J. (2004) Calorimetric vs. van't Hoff binding enthalpies from isothermal titration calorimetry: Ba²⁺-crown ether complexation. *Biophys Chem* **110**, 15-24.

Abstract: The 1:1 complexation reaction between Ba²⁺ and 18-crown-6 ether is re-examined using isothermal titration calorimetry (ITC), with the goal of clarifying previously reported discrepancies between reaction enthalpies estimated directly (calorimetric) and indirectly, from the temperature dependence of the reaction equilibrium constant K (van't Hoff). The ITC thermograms are analyzed using three different non-linear fit models based on different assumptions about the data error: constant, proportional to the heat and proportional but correlated. The statistics of the fitting indicate a preference for the proportional error model, in agreement with expectations for the conditions of the experiment, where uncertainties in the delivered titrant volume should dominate. With attention to proper procedures for propagating statistical error in the van't Hoff analysis, the differences between ΔH_{cal} and ΔH_{vH} are deemed statistically significant. In addition, statistically significant differences are observed for the ΔH_{cal} estimates obtained for two different sources of Ba²⁺, BaCl₂ and Ba(NO₃)₂. The effects are tentatively attributed to deficiencies in the standard procedure in ITC of subtracting a blank obtained for pure titrant from the thermogram obtained for the sample.

Morikis D. and Lambris J. D. (2004) Physical methods for structure, dynamics and binding in immunological research. *Trends Immunol* **25**, 700-707.

Abstract: We present four experimental physical methods--X-ray and neutron diffraction, nuclear magnetic resonance spectroscopy, mass spectrometry and calorimetry--and two computational methods--molecular dynamics simulations and electrostatics calculations--which are general and widely applicable in the study of protein structure, dynamics and binding. These methods are useful tools for biologists that lead

to structure-function, dynamics-function and binding-function correlations, in efforts to understand biomolecular function. Standard and emerging technologies within these methods are discussed and representative examples of applications in immunology are presented, from antigen-antibody, complement and MHC-T-cell receptor research. The examples demonstrate the power of the reviewed methods in immunological studies at the molecular level.

Murphy K. P. and Freire E. (1995) Thermodynamic strategies for rational protein and drug design. *Pharm Biotechnol* **7**, 219-241.

Murphy K. P. (1999) Predicting binding energetics from structure: looking beyond ΔG degrees. *Med Res Rev* **19**, 333-339.

Abstract: Structure-based design of pharmaceuticals requires the ability to predict ligand affinity based on knowledge of structure. The primary term of interest is the binding affinity constant, K , or the free energy of binding, ΔG degrees. It is common to attempt to predict ΔG degrees based on empirically derived terms which represent common contributions such as the hydrophobic effect, hydrogen bonding, and conformational entropy. Although these approaches have met with some success, when they fail it is difficult to know which parameter(s) need refinement. Confidence in these approaches is also limited by the fact that ΔG degrees typically is made up of compensating enthalpic and entropic terms, ΔH degrees and ΔS degrees, so that accurate prediction of a ΔG degrees value may be fortuitous and may not indicate a reasonable understanding of the underlying relationship between structure and affinity. This is further complicated by the fact that both ΔH degrees and ΔS degrees are strongly temperature dependent through the heat capacity change, ΔC_p . In order to avoid these difficulties, we attempt to use structural data to predict ΔH degrees, ΔS degrees, and ΔC_p from which ΔG degrees can be calculated as a function of temperature. The predictions are then compared to experimentally determined values. These calculations have been applied to several systems by ourselves and others. Systems include the binding of angiotensin II to an antibody, the dimerization of interleukin-8, and the binding of inhibitors to aspartic and serine proteases. Overall the calculations are very successful, and suggest that our understanding of the contributions of the hydrophobic effect, hydrogen bonding, and conformational entropy are quite good. Several of these systems show a strong dependence of the binding energetics on pH, indicative of changes in proton affinity of ionizable groups upon binding. It is critical to account for these protonation contributions to the binding energetics in order to assess the reliability of any computational prediction of energetics from structure. Methods have been developed for determining the energetics of proton binding using isothermal titration calorimetry. The availability of these methods provides a means of understanding how protein structure can modify the pKa's of ionizable groups. This information will further add to our understanding of structural energetic relationships and our ability accurately to predict binding affinities.

Naghbi H., Tamura A., and Sturtevant J. M. (1995) Significant discrepancies between van't Hoff and calorimetric enthalpies. *Proc Natl Acad Sci U S A* **92**, 5597-5599.

Abstract: In this paper we show that the usual assumption in studies of the temperature variation of equilibrium constants for equilibria of the form $A+B \rightleftharpoons AB$ that a plot of $\ln K$ vs. $1/T$ (K = equilibrium constant, T = temperature in degrees kelvin) is a straight line with slope equal to $-\Delta H_vH/R$ (ΔH_vH = van't Hoff or apparent enthalpy, R = gas constant) is not valid in many cases. In all the cases considered here, ΔH_vH is temperature dependent and is significantly different from the true or calorimetrically measured enthalpy, and the respective values for ΔC_p are also significantly different.

Navratilova I., Papalia G. A., Rich R. L., Bedinger D., Brophy S., Condon B., Deng T., Emerick A. W., Guan H. W., Hayden T., Heutmekers T., Hoorelbeke B., McCroskey M. C., Murphy M. M., Nakagawa T., Parmeggiani F., Qin X., Rebe S., Tomasevic N., Tsang T., Waddell M. B., Zhang F. F., Leavitt S. and Myszkka D. G. (2007) Thermodynamic benchmark study using Biacore technology. *Anal Biochem* **364**, 67-77.

Abstract: A total of 22 individuals participated in this benchmark study to characterize the thermodynamics of small-molecule inhibitor-enzyme interactions using Biacore instruments. Participants were provided with reagents (the enzyme carbonic anhydrase II, which was immobilized onto the sensor surface, and four sulfonamide-based inhibitors) and were instructed to collect response data from 6 to 36 degrees C. van't Hoff enthalpies and entropies were calculated from the temperature dependence of the binding constants. The equilibrium dissociation and thermodynamic constants determined from the Biacore

analysis matched the values determined using isothermal titration calorimetry. These results demonstrate that immobilization of the enzyme onto the sensor surface did not alter the thermodynamics of these interactions. This benchmark study also provides insights into the opportunities and challenges in carrying out thermodynamic studies using optical biosensors.

O'Brien R., and Haq I. (2004) Applications of Biocalorimetry: Binding, stability and enzyme kinetics *in* Biocalorimetry 2: Applications of Calorimetry in the Biological Sciences. Ladbury, J.E., Doyle, M.L., eds., John Wiley & Sons Ltd., Chichester UK, pp. 3-34.

O'Brien R., Ladbury J.E., and Chowdhry B.Z. (2001) Isothermal titration calorimetry of biomolecules, *in* Protein-Ligand Interactions: Hydrodynamics and Calorimetry. Harding, S.E., Chowdhry, B.Z., eds., Oxford University Press, Oxford UK, pp. 263-286.

Okhrimenko O. and Jelesarov I. (2008) A survey of the year 2006 literature on applications of isothermal titration calorimetry. *J Mol Recognit* **21**, 1-19.

Abstract: Isothermal titration calorimetry (ITC) is a fast and robust method to determine the energetics of association reactions in solution. The changes in enthalpy, entropy and heat capacity that accompany binding provide unique insights into the balance of forces driving association of molecular entities. ITC is used nowadays on a day-to-day basis in hundreds of laboratories. The method aids projects both in basic and practice-oriented research ranging from medicine and biochemistry to physical chemistry and material sciences. Not surprisingly, the range of studies utilizing ITC data is steadily expanding. In this review, we discuss selected results and ideas that have accumulated in the course of the year 2006, the focus being on biologically relevant systems. Theoretical developments, novel applications and studies that provide a deeper level of understanding of the energetic principles of biological function are primarily considered. Following the appearance of a new generation of titration calorimeters, recent papers provide instructive examples of the synergy between energetic and structural approaches in biomedical and biotechnological research

Olsson T. S., Williams M. A., Pitt W. R. and Ladbury J. E. (2008) The thermodynamics of protein-ligand interaction and solvation: insights for ligand design. *J Mol Biol* **384**, 1002-1017.

Abstract: Isothermal titration calorimetry is able to provide accurate information on the thermodynamic contributions of enthalpy and entropy changes to free energies of binding. The Structure/Calorimetry of Reported Protein Interactions Online database of published isothermal titration calorimetry studies and structural information on the interactions between proteins and small-molecule ligands is used here to reveal general thermodynamic properties of protein-ligand interactions and to investigate correlations with changes in solvation. The overwhelming majority of interactions are found to be enthalpically favoured. Synthetic inhibitors and biological ligands form two distinct subpopulations in the data, with the former having greater average affinity due to more favourable entropy changes on binding. The greatest correlation is found between the binding free energy and apolar surface burial upon complex formation. However, the free-energy contribution per unit area buried is only 30-50% of that expected from earlier studies of transfer free energies of small molecules. A simple probability-based estimator for the maximal affinity of a binding site in terms of its apolar surface area is proposed. Polar surface area burial also contributes substantially to affinity but is difficult to express in terms of unit area due to the small variation in the amount of polar surface buried and a tendency for cancellation of its enthalpic and entropic contributions. Conventionally, the contribution of apolar desolvation to affinity is attributed to gain of entropy due to solvent release. Although data presented here are supportive of this notion, because the correlation of entropy change with apolar surface burial is relatively weak, it cannot, on present evidence, be confidently considered to be correct. Further, thermodynamic changes arising from small differences between ligands binding to individual proteins are relatively large and, in general, uncorrelated with changes in solvation, suggesting that trends identified across widely differing proteins are of limited use in explaining or predicting the effects of ligand modifications

Patston P. A., Church F. C., and Olson S. T. (2004) Serpin-ligand interactions. *Methods* **32**, 93-109.

Abstract: One of the more common features of serpins is the ability to bind various ligands. Ligand binding can occur so that the inhibitory properties of the serpin are regulated, so that the serpin can be localized, or to produce or modulate some other biological function of the serpin. Ligands known to affect

serpin biologic activity include glycosaminoglycans such as heparin, heparan sulfate and dermatan sulfate, DNA, extracellular matrix proteins such as vitronectin and collagen, and small organic molecule hormones. Many different biochemical and biophysical techniques in conjunction with molecular biology and cell biology approaches have been used to study the binding of various ligands to serpins and to assess the influence of this binding on activity and structure. We summarize here the different approaches that have been used to identify serpin ligands and the many methods that have been used to characterize the interactions of these ligands with their cognate serpins.

Perozzo R., Folkers G., and Scapozza L. (2004) Thermodynamics of protein-ligand interactions: history, presence, and future aspects. *J Recept Signal Transduct Res* **24**, 1-52.

Abstract: The understanding of molecular recognition processes of small ligands and biological macromolecules requires a complete characterization of the binding energetics and correlation of thermodynamic data with interacting structures involved. A quantitative description of the forces that govern molecular associations requires determination of changes of all thermodynamic parameters, including free energy of binding (ΔG), enthalpy (ΔH), and entropy (ΔS) of binding and the heat capacity change (ΔC_p). A close insight into the binding process is of significant and practical interest, since it provides the fundamental know-how for development of structure-based molecular design-strategies. The only direct method to measure the heat change during complex formation at constant temperature is provided by isothermal titration calorimetry (ITC). With this method one binding partner is titrated into a solution containing the interaction partner, thereby generating or absorbing heat. This heat is the direct observable that can be quantified by the calorimeter. The use of ITC has been limited due to the lack of sensitivity, but recent developments in instrument design permit to measure heat effects generated by nanomol (typically 10-100) amounts of reactants. ITC has emerged as the primary tool for characterizing interactions in terms of thermodynamic parameters. Because heat changes occur in almost all chemical and biochemical processes, ITC can be used for numerous applications, e.g., binding studies of antibody-antigen, protein-peptide, protein-protein, enzyme-inhibitor or enzyme-substrate, carbohydrate-protein, DNA-protein (and many more) interactions as well as enzyme kinetics. Under appropriate conditions data analysis from a single experiment yields ΔH , K_B , the stoichiometry (n), ΔG and ΔS of binding. Moreover, ITC experiments performed at different temperatures yield the heat capacity change (ΔC_p). The informational content of thermodynamic data is large, and it has been shown that it plays an important role in the elucidation of binding mechanisms and, through the link to structural data, also in rational drug design. In this review we will present a comprehensive overview to ITC by giving some historical background to calorimetry, outline some critical experimental and data analysis aspects, discuss the latest developments, and give three recent examples of studies published with respect to macromolecule-ligand interactions that have utilized ITC technology.

Piehler J. (2005) New methodologies for measuring protein interactions in vivo and in vitro. *Curr Opin Struct Biol.* **15**, 4-14.

Abstract: The identification and characterization of protein interactions is a key topic in current life science research; a huge variety of methodologies have been established in recent years to expedite research in this area. Generic methods have been established for monitoring protein interactions in vivo by protein fragment complementation and for screening protein interactions in vitro by highly parallel solid-phase techniques. Substantial progress has been made in identifying and characterizing interactions with and between membrane proteins. Studying protein interactions on the single-molecule level has become an important tool for understanding protein function in vivo and in vitro.

Pierce M. M., Raman C. S., and Nall B. T. (1999) Isothermal titration calorimetry of protein-protein interactions. *Methods* **19**, 213-221.

Abstract: The interaction of biological macromolecules, whether protein-DNA, antibody-antigen, hormone-receptor, etc., illustrates the complexity and diversity of molecular recognition. The importance of such interactions in the immune response, signal transduction cascades, and gene expression cannot be overstated. It is of great interest to determine the nature of the forces that stabilize the interaction. The thermodynamics of association are characterized by the stoichiometry of the interaction (n), the association constant (K_a), the free energy ($\Delta G(b)$), enthalpy ($\Delta H(b)$), entropy ($\Delta S(b)$), and heat capacity of binding (ΔC_p). In combination with structural information, the energetics of binding can provide a complete dissection of the interaction and aid in identifying the most important regions of the interface and the

energetic contributions. Various indirect methods (ELISA, RIA, surface plasmon resonance, etc.) are routinely used to characterize biologically important interactions. Here we describe the use of isothermal titration calorimetry (ITC) in the study of protein-protein interactions. ITC is the most quantitative means available for measuring the thermodynamic properties of a protein-protein interaction. ITC measures the binding equilibrium directly by determining the heat evolved on association of a ligand with its binding partner. In a single experiment, the values of the binding constant (K_a), the stoichiometry (n), and the enthalpy of binding ($\Delta H(b)$) are determined. The free energy and entropy of binding are determined from the association constant. The temperature dependence of the $\Delta H(b)$ parameter, measured by performing the titration at varying temperatures, describes the ΔC_p term. As a practical application of the method, we describe the use of ITC to study the interaction between cytochrome c and two monoclonal antibodies.

Plum G. E. and Breslauer K. J. (1995) Calorimetry of proteins and nucleic acids. *Curr Opin Struct Biol* **5**, 682-690.

Abstract: The availability of sensitive calorimetric instrumentation has led to a considerable increase in thermodynamic studies of proteins, nucleic acids, and their interactions. This article reviews some of the recent contributions of calorimetry to characterizing the thermodynamic origins of protein and nucleic acid stability and conformational preferences, as well as the interactions of proteins with each other, with small molecules, and with nucleic acids.

Privalov G. P. and Privalov P. L. (2000) Problems and prospects in microcalorimetry of biological macromolecules. *Methods Enzymol* **323**, 31-62.

Privalov P. L. (2007) Reflections on the origins of microcalorimetry of biopolymers. *Biophys Chem* **126**, 13-15.

Privalov P. L. and Dragan A. I. (2007) Microcalorimetry of biological macromolecules. *Biophys Chem* **126**, 16-24.

Abstract: The capabilities of contemporary differential scanning and isothermal titration microcalorimetry for studying the thermodynamics of protein unfolding/refolding and their association with partners, particularly target DNA duplexes, are considered. It is shown that the predenaturational changes of proteins must not be ignored in studying the thermodynamics of formation of their native structure and their complexes with partners, particularly their cognate DNA duplexes.

Remmele R. M. (2005) Microcalorimetric approaches to biopharmaceutical development in Analytical techniques for biopharmaceutical development, Rodriguez-Diaz, R., Wehr, T. Tuck, S., eds., Marcel Dekker, New York NY, pp. 327-381.

Roos H., Karlsson R., Nilshans H., and Persson A. (1998) Thermodynamic analysis of protein interactions with biosensor technology. *J Mol Recognit* **11**, 204-210.

Abstract: A methodology using biosensor technology for combined kinetic and thermodynamic analysis of biomolecular interactions is described. Rate and affinity constants are determined with BIAcore. Thermodynamics parameters, changes in free energy, enthalpy and entropy, are evaluated from equilibrium data and by using rate constants and transition state theory. The methodology using van't Hoff theory gives complementary information to microcalorimetry, since only the direct binding is measured with BIAcore whereas microcalorimetry measures all components, including e.g. hydration effects. Furthermore, BIAcore gives possibilities to gain new information by thermodynamic analysis of the rate constants.

Salim N. N. and Feig A. L. (2008) Isothermal titration calorimetry of RNA. *Methods (epublication)*.

Abstract: Isothermal titration calorimetry (ITC) is a fast and robust method to study the physical basis of molecular interactions. A single well-designed experiment can provide complete thermodynamic characterization of a binding reaction, including $K(a)$, ΔG , ΔH , ΔS and reaction stoichiometry (n). Repeating the experiment at different temperatures allows determination of the heat capacity change ($\Delta C(P)$) of the interaction. Modern calorimeters are sensitive enough to probe even weak biological interactions making ITC a very popular method among biochemists. Although ITC has been applied to protein studies for many years, it is becoming widely applicable in RNA biochemistry as well, especially in studies which involve RNA folding and RNA interactions with small molecules, proteins and with other

RNAs. This review focuses on best practices for planning, designing and executing effective ITC experiments when one or more of the reactants is an RNA

Schwarz F. P. (2004) Calorimetric analysis of mutagenic effects on protein-ligand interactions. *Methods Enzymol* **379**, 128-145.

Sigurskjold B. W. (2000) Exact analysis of competition ligand binding by displacement isothermal titration calorimetry. *Anal Biochem* **277**, 260-266.

Abstract: A rigorous method for the least-squares nonlinear regression analysis of displacement isothermal titration calorimetric data is presented. The method can fit the binding isotherm of a ligand which is competitively inhibited in its binding by another bound ligand to a molecule with n identical and independent binding sites. There are no other assumptions for the method and no approximations. Analysis of previously published data of the strong binding of acarbose to glucoamylase is presented as an example. The regression equations have been programmed for the Origin software supplied with the widely used titration calorimeters from Microcal, Inc., and an Origin Function Definition File with instructions is freely available from the author upon e-mail request.

Tellinghuisen J. (2003) A study of statistical error in isothermal titration calorimetry. *Anal Biochem* **321**, 79-88.

Abstract: In isothermal titration calorimetry (ITC), the two main sources of random (statistical) error are associated with the extraction of the heat q from the measured temperature changes and with the delivery of metered volumes of titrant. The former leads to uncertainty that is approximately constant and the latter to uncertainty that is proportional to q . The role of these errors in the analysis of ITC data by nonlinear least squares is examined for the case of 1:1 binding, $M+X \rightleftharpoons MX$. The standard errors in the key parameters—the equilibrium constant K_o and the enthalpy ΔH_o —are assessed from the variance-covariance matrix computed for exactly fitting data. Monte Carlo calculations confirm that these "exact" estimates will normally suffice and show further that neglect of weights in the nonlinear fitting can result in significant loss of efficiency. The effects of the titrant volume error are strongly dependent on assumptions about the nature of this error: If it is random in the integral volume instead of the differential volume, correlated least-squares is required for proper analysis, and the parameter standard errors decrease with increasing number of titration steps rather than increase.

Tellinghuisen J. (2004) Statistical error in isothermal titration calorimetry. *Methods Enzymol* **383**, 245-282.

Tellinghuisen J. (2004) Volume errors in isothermal titration calorimetry. *Anal Biochem* **333**, 405-406.

Turnbull W. B. and Daranas A. H. (2003) On the value of c : can low affinity systems be studied by isothermal titration calorimetry? *J Am Chem Soc* **125**, 14859-14866.

Abstract: Isothermal titration calorimetry (ITC) allows the determination of ΔG degrees, ΔH degrees, and ΔS degrees from a single experiment and is thus widely used for studying binding thermodynamics in both biological and synthetic supramolecular systems. However, it is widely believed that it is not possible to derive accurate thermodynamic information from ITC experiments in which the Wiseman " c " parameter (which is the product of the receptor concentration and the binding constant, K_a) is less than ca. 10, constraining its use to high affinity systems. Herein, experimental titrations and simulated data are used to demonstrate that this dogma is false, especially for low affinity systems, assuming that (1) a sufficient portion of the binding isotherm is used for analysis, (2) the binding stoichiometry is known, (3) the concentrations of both ligand and receptor are known with accuracy, and (4) there is an adequate level of signal-to-noise in the data. This study supports the validity of ITC for determining the value of K_a and, hence, ΔG degrees from experiments conducted under low c conditions but advocates greater caution in the interpretation of values for ΔH degrees. Therefore, isothermal titration calorimetry is a valid and useful technique for studying biologically and synthetically important low affinity systems.

Velazquez-Campoy A. and Freire E. (2005) ITC in the post-genomic era...? Priceless. *Biophys Chem* **115**, 115-124.

Abstract: The information available after decoding the genome of the human species and many others is opening the possibility of new approaches to target thousands of protein interactions critical for a

continuously increasing list of genetic and infectious diseases and pathologies, and to understand complex regulatory pathways and interaction networks describing cell function and interrelation. There is a need for a reliable technique offering the capability of measuring accurately macromolecular interactions (e.g. protein/ligand, protein/protein, protein/nucleic acid) in the laboratory. Compared to other analytical techniques, isothermal titration calorimetry (ITC) exhibits some important advantages for characterizing intermolecular interactions and binding equilibria. ITC is suitable for characterizing both low affinity interactions (e.g. protein network regulation and natural ligands) and high affinity interactions (e.g. rational drug design). Considering the advanced technological level reached as well as the outstanding quality of the information accessible through this technique, ITC is expected to play a very prominent role in the next years in the areas of rational drug design and protein network regulation.

Velazquez-Campoy A., Leavitt S. A., and Freire E. (2004) Characterization of protein-protein interactions by isothermal titration calorimetry. *Methods Mol Biol* **261**, 35-54.

Abstract: Isothermal titration calorimetry (ITC) is a powerful technique to study both protein-ligand and protein-protein interactions. This methods chapter is devoted to describing protein-protein interactions, in particular, the association between two different proteins and the self-association of a protein into homodimers. ITC is the only technique that determines directly the thermodynamic parameters of a given reaction: ΔG , ΔH , ΔS , and ΔCP . Isothermal titration calorimeters have evolved over the years and one of the latest models is the VP-ITC produced by Microcal, Inc. In this chapter we will be describing the general procedure for performing an ITC experiment as well as for the specific cases of porcine pancreatic trypsin binding to soybean trypsin inhibitor and the dissociation of bovine pancreatic alpha-chymotrypsin.

Vickers L. P., Hopkins H. P., Jr., Ali S. Z., and Carey V. (1985) Error analysis in titration microcalorimetry of biochemical systems. *Anal Biochem* **145**, 257-265.

Abstract: A simplified method for titrations of biochemical systems is described as well as extensive error propagation through the data analysis. This work uses a Tronac Model 450 isoperibol titration calorimeter. Sample volumes of 2 ml are used and total heats of less than 5 mcal can be routinely measured. The binding of 3'-CMP to bovine pancreatic ribonuclease A is used to illustrate the methods. The binding enthalpy can be determined with a standard deviation of 1.5% and the free energy with a standard deviation of 2 to 3%.

Waldron T. T., Schrifft G. L., and Murphy K. P. (2005) The salt-dependence of a protein-ligand interaction: ion-protein binding energetics. *J Mol Biol* **346**, 895-905.

Abstract: Using the binding of a nucleotide inhibitor (guanosine-3'-monophosphate) to a ribonuclease (ribonuclease Sa) as a model system, we show that the salt-dependence of the interaction arises due to specific ion binding at the site of nucleotide binding. The presence of specific ion-protein binding is concluded from a combination of differential scanning calorimetry and NMR data. Isothermal titration calorimetry data are then fit to determine the energetic profile (enthalpy, entropy, and heat capacity) for both the ion-protein and nucleotide-protein interactions. The results provide insight into the energetics of charge-charge interactions, and have implications for the interpretation of an observed salt-dependence. Further, the presence of specific ion-binding leads to a system behavior as a function of temperature that is drastically different from that predicted from Poisson-Boltzmann calculations.

Ward W. H. and Holdgate G. A. (2001) Isothermal titration calorimetry in drug discovery. *Prog Med Chem* **38**, 309-376.

Abstract: Isothermal titration calorimetry (ITC) follows the heat change when a test compound binds to a target protein. It allows precise measurement of affinity. The method is direct, making interpretation facile, because there is no requirement for competing molecules. Titration in the presence of other ligands rapidly provides information on the mechanism of action of the test compound, identifying the intermolecular complexes that are relevant for structure-based design. Calorimetry allows measurement of stoichiometry and so evaluation of the proportion of the sample that is functional. ITC can characterize protein fragments and catalytically inactive mutant enzymes. It is the only technique which directly measures the enthalpy of binding (ΔH degree). Interpretation of ΔH degree and its temperature dependence (ΔCp) is usually qualitative, not quantitative. This is because of complicated contributions from linked equilibria and a single change in structure giving modification of several physicochemical properties. Measured ΔH degree values allow characterization of proton movement linked to the association of protein and ligand, giving

information on the ionization of groups involved in binding. Biochemical systems characteristically exhibit enthalpy-entropy compensation where increased bonding is offset by an entropic penalty, reducing the magnitude of change in affinity. This also causes a lack of correlation between the free energy of binding (ΔG degree) and ΔH degree. When characterizing structure-activity relationships (SAR), most groups involved in binding can be detected as contributing to ΔH degree, but not to affinity. Large enthalpy changes may reflect a modified binding mode, or protein conformation changes. Thus, ΔH degree values may highlight a potential discontinuity in SAR, so that experimental structural data are likely to be particularly valuable in molecular design.

Weber P. C. and Salemme F. R. (2003) Applications of calorimetric methods to drug discovery and the study of protein interactions. *Curr Opin Struct Biol* **13**, 115-121.

Abstract: 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.