Review Article

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Biological Applications of Isothermal Titration Calorimetry

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Most of the biological phenomena are influenced by intermolecular recognition and interaction. Thus, understanding the thermodynamics of biomacromolecule ligand interaction is a very interesting area in biochemistry and biotechnology. One of the most powerful techniques to obtain precise information about the energetics of (bio) molecules binding to other biological macromolecules is isothermal titration calorimetry (ITC). In a typical ITC experiment, a macromolecule solution is titrated by a solution containing a reactant at a constant temperature, and exchanged heat of the reaction is measured, allowing determination of thermodynamic parameters (enthalpy change, entropy change, change in Gibbs free energy, binding affinity and stoichiometry) of molecular interactions. In this review article, we describe the ITC approach briefly and review some applications of ITC for studying protein-ligand interactions, protein-protein interactions, self-association, and drug design processes. Furthermore, the application of ITC for determination of kinetic parameters of enzyme catalyzed reactions as well as thermodynamic parameters will be discussed.

Keywords: Isothermal titration calorimetry, Ligand binding, Enthalpy of binding, Drug design, Enzyme kinetics

INTRODUCTION

Heat is perhaps the most common sign of the occurrence of nearly all chemical reactions and physical processes exchange heat. Calorimetry is a very general method that directly measures the heat generated or absorbed. Isothermal titration calorimeters were built in the 1966 to study chemical reactions [1,2]. Improvement of sensitivity in the 1980s introduced biological applications of ITC, such as the study of ligand binding processes [3-5]. In 1989 first commercially available ITC designed for the study of biological systems [6]. Since the beginning of 1990s, the number of published papers related to ITC has increased and nowadays it is a widely used technique in biosciences [7]. ITC directly measures the energy associated with a biochemical reaction or a molecular interaction at constant temperature.

ITC technique is able to accurately measure binding energetics of different ligands to biological macromolecules [8-18], protein unfolding, fibrillation and aggregation [18-24], enzyme inhibition [25-29], enzyme kinetics [30,31] drug discovery [32-34] and quality, safety and shelf-life of materials and material stability [35-43]. Several review articles are available which either review relevant publications within a definite time period [30,44-48] or particular aspects of the field of study of interactions by ITC [49-52]. In this review article, we will discuss several experiences and recent reported results on the applications of ITC to studying protein-ligand interactions, protein-protein interactions, self-association, drug design processes and enzyme kinetics.

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Theory and Experiment

In a typical ITC experiment small aliquots of one reactant (titrant or ligand) are titrated into the sample cell containing the other reactant(s); see Fig. 1. In each injection, some ligand is added to the sample, and the interaction between reactants releases or absorbs a certain amount of heat. The exchanged heat is necessary to maintain the temperature constant and eliminate difference between the sample and reference cells. The amount of heat is proportional to the amount of ligand that binds to the macromolecule and is monitored as a peak of power (microcalori per second) against time for each injection. The exchanged heat of each injection is obtained by calculating the area under each peak and then replotted as a function of the molar ratio of ligand to macromolecule, calculated from dividing total concentration of ligand by macromolecule concentration; see Fig. 2.

Since the amount of free macromolecule available decreases after each successive injection, the magnitude of the peaks becomes gradually smaller until complete saturation is reached. Subsequent injections produce similar peaks after saturation, corresponding to dilutions or mechanical effects that need to be subtracted from each of the injection peaks before analysis. The heat of dilution of the ligand solution is measured in a separate titration of ligand in buffer [53,54].

Advantages and Disadvantages of ITC

Surface plasmon resonance (SPR), nuclear magnetic resonance (NMR), (UV-Vis and fluorescence) spectroscopy and differential scanning calorimetry (DSC) are other techniques which can be used to study biomolecular interactions. Each of these methods has certain advantages and disadvantages. In approaches based on SPR method, one of the interacting components has to be surface-immobilized [55]. NMR provides high resolution information regarding the structure of the formed complex and conformational changes of the molecules upon interaction and binding affinity. However, the main disadvantages of this expensive technique are the complexity of the instrument, limitation of sample molecular weight (it should be less than 40 kDa) as well as the large amount of the sample required [55]. Fluorescence and UV-Vis spectroscopy are easy to use and affordable

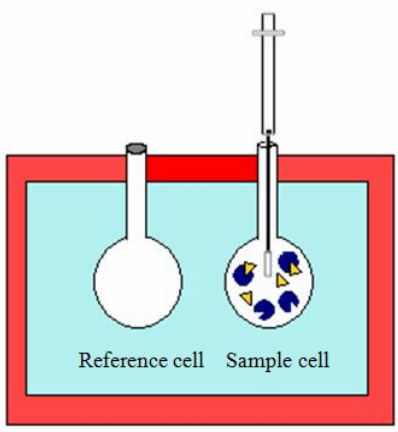

Fig. 1. Configuration of an ITC reaction cell. The sample cell is filled with the protein solution (blue circles). The injection syringe is filled with the ligand solution (yellow triangles).

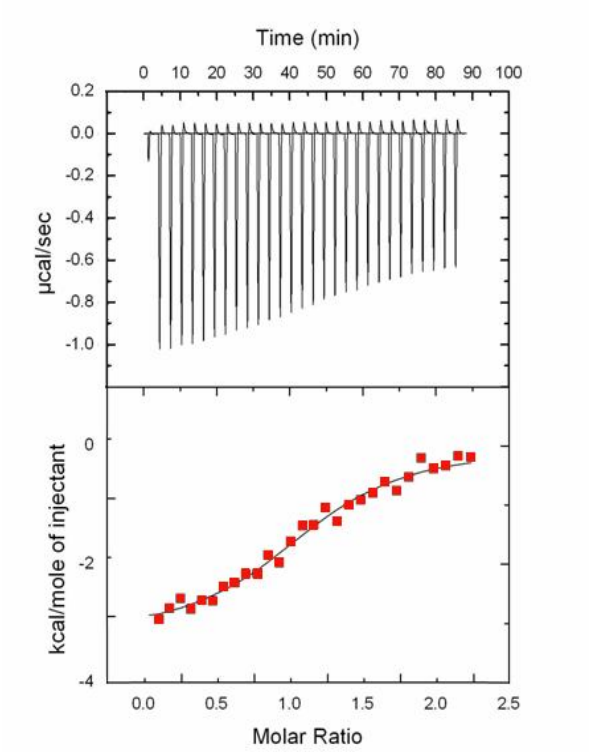

Fig. 2. A typical ITC experiment; The top panel shows the heat measured per injection *vs.* time and the bottom panel shows the integrated enthalpy change measured per mole of the vitamin D_3 injected against the molar ratio of vitamin D_3 to α-La for each injection and the best fit line using one set of binding site model. Reprinted from reference [51].

methods to calculate thermodynamic parameters, using the van't Hoff method. Nevertheless, the main disadvantage of these methods is their low resolution, also they sometimes require labeling [56]. ITC has different advantages in studies of macromolecular interactions; reactants are in solution, it does not need any chemical modification or immobilization of either binding partner, molecular weight of reactants is not limited and it is unaffected by spectroscopic properties of reactants [55,57]. However, ITC method requires expensive equipment, it uses large amounts of the sample and highly purified ligands and macromolecules with accurately determined concentrations [58].

THERMODYNAMIC PROFILE OF MOLECULAR INTERACTIONS

There are several methods for ITC data analysis and obtaining binding thermodynamics [53]. Data analysis method depends on the system of interest. One procedure is data fitting to an appropriate model (*e.g.*, one set of binding sites or n identical sites, multiple independent sites, or sequential binding) using Microcal Origin software provided with the ITC instrument. After subtracting the enthalpy of dilution from the enthalpy of ligand-macromolecule interaction, a nonlinear least-squares algorithm (minimization of χ^2) along with the total concentrations of the ligand and the sample is used to fit the heat flow per injection to an equilibrium binding equation, providing best fit for the values of stoichiometry (the number of binding sites, n), the binding enthalpy change (ΔH), and the binding affinity (K). Enthalpy change is a direct measure of the collective energies of bonds made or broken during complex formation. These include ionic interactions, hydrogen bonds, and van der Waals interactions between macromolecule and ligand, within the macromolecule itself, and between interacting solutes and water. As an example, Fig. 2 Shows a typical ITC experiment corresponds to the titration of α-La (20 μM) with vitamin D3 (200 μM). The experiment was performed in 20 mM Tris buffer, pH 7 at 25 °C. Analysis of the data, using one set of binding site model, yields a binding affinity of 3.66×10^5 M^{-1} and a ΔH of -3.209 kcal mol^{-1} [43].

The change in free energy (ΔG) is a crucial parameter which describes the likelihood of biomolecular interactions occurring. Negative free energy shows that binding event occurs spontaneously and *vice versa*. ΔG and the change in entropy (ΔS) for the binding reaction are calculated by the fundamental thermodynamic equations: $\Delta G = -RT \ln K$; $\Delta S = (\Delta H - \Delta G)/T$, where R is the gas constant and T is the experimental temperature. Furthermore, repeating titration at different temperatures and the temperature dependence of ΔH allows determining the change in heat capacity (ΔC_p) associated with the binding reaction ($\Delta C_p = \delta \Delta H/\delta T$). In order to determine an accurate value for ΔC_p, ITC experiments should be done at different temperatures and just two temperatures is not enough.

ITC gives thermodynamic parameters as well as information about the type of reaction, electrostatic and hydrophobic contributions to the interaction and also cooperativity characterization of binding process by calculating the Hill coefficient. ITC is the only technique that can resolve the enthalpic and entropic components of binding affinity directly. Since the enthalpic and entropic components are related to structural parameters, they can be used as a guide for a molecular design. Contribution of enthalpy and entropy to binding is important when designing high-affinity ligands especially in drug design.

LIGAND-BIOLOGICAL MACROMOLECULE INTERACTION

ITC has become a standard method for investigating the binding of ligands to receptor molecules. ITC is a precise method to study several interactions of macromolecules, *e.g.*, interaction of proteins with a variety of ligands such as small molecules, metal ions, drugs, enzyme inhibitors, nucleotides, polysaccharides and lipids. In spite of some other experimental approaches, ITC is a relatively easy, label-free technique and lack of inaccuracy of turbidity, which can be also successfully applied to a system with highly problematic due to the lack of an optical signal. Although it is not possible to provide a full thermodynamic-structure correlation from an ITC experiment, it is possible to draw sensible conclusions from data by comparing systems where subtle changes have been imposed, for example comparing the thermodynamic parameters for the binding of a ligand with changes at specific atomic sites. It

is essential to recognize the structure-function relationship in proteins and to the structure-based design of pharmaceutical ligands.

Calorimetry is a very general method that directly measures exchanged heat. The generality of calorimetry method is both strength and weakness, because the calorimeter measures all processes occurring simultaneously, significant processes are unlikely to be missed, but the desired signal may be lost in a background of processes. Total heat that ITC measures upon addition of titrant, can include contributions from other phenomena, like enthalpy change due to the protein conformational change or heat exchange resulting from acceptance or release of protons by the protein or the ligand. Some metals displace protons when binding to protein residues, and the deprotonation and subsequent buffer protonation are coupled equilibria that contribute to thermodynamic parameters measured by ITC. If the binding reaction is coupled with protein protonation or deprotonation, the reaction is pH-dependent and the binding enthalpy is dependent on the ionization enthalpy of the buffer. However, ITC measurements in two or more buffers with different heats of protonation can be used to quantify the number of protons whose displacement and transfer to the buffer is coupled with the metal binding event [49,59].

Protein-Metal Ion Interaction

Proteins have crucial roles in uptake, transport, storage and export of metal ions whose interaction with proteins may affect protein stability and/or activity. Milk proteins as natural vehicles, can be potentially utilized for delivery of essential nutrients for human health in food systems. Recently, ITC has been used to quantify the thermodynamics of Zn^{2+} binding to camel alpha-lactalbumin (α-La). α-La is strongly bound to Ca^{2+} in milk and binding of calcium is essential for its native structure and stability [60]. Our results show that the zinc ion binds to two noncooperative sets of binding sites on α-La, which is shown in Fig. 3. Any α-La molecule binds to two Zn^{2+} with the binding affinity of 4.53×10^4 M^{-1} and approximately four Zn^{2+} ions with binding affinity of 963 M^{-1}; binding enthalpy is 6.02 and 41.22 kJ mol^{-1} for the first and second set of binding sites, respectively [61].

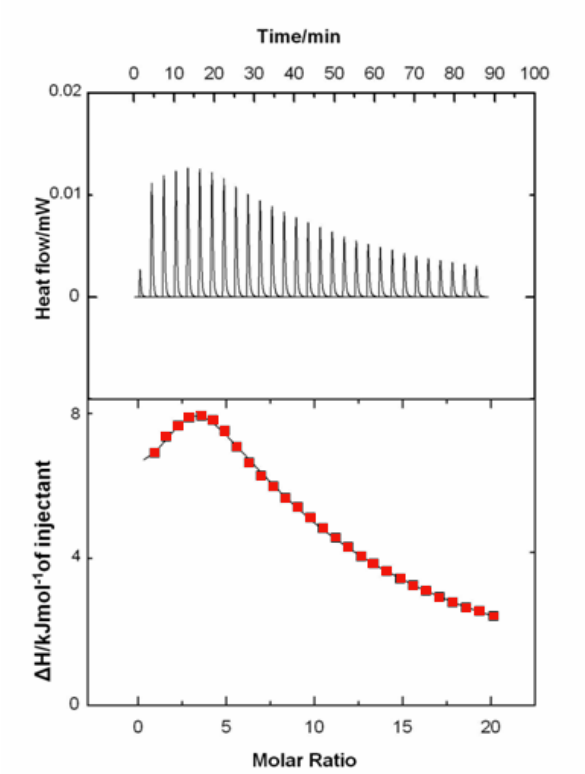

Fig. 3. ITC results for Zn^{2+} titration into camel α-La at 25 °C and pH 7.5 (10 mM Tris buffer containing 2 mM $CaCl_2$). Reprinted from reference [61].

Protein-Nanoparticle Interaction

Proteins bind to the surface of nanoparticles in the physiological environment. The further biological response of the body is influenced by the protein-nanoparticle complex. The stoichiometry and affinity of protein binding depend on the type, size, and surface characterization (*e.g.* hydrophobicity) of nanoparticle. Protein binding affects bioavailability and biodistribution of the nanoparticles throughout the body, furthermore interaction with nanoparticles may disrupt protein native structure. ITC technique has the potential to assess the stoichiometry and binding affinity of proteins to the nanoparticles surface. Understanding the protein-nanoparticle will facilitate a deeper understanding of the molecular basis of cell-nanoparticle interactions and the potential risks associated with nanoparticles [62,63].

Silver nanoparticles have gained much popularity because of their strong antibacterial activity. Most of the

toxic and therapeutic usages of nanoparticles include the introduction of nanoparticles into the blood. Understanding of the nanoparticle interactions with the blood proteins such as hemoglobin is important so that the great antimicrobial properties of these particles may be safely used without threatening human health and disruption of protein structures. Our investigation using ITC and spectroscopic methods showed that there is no significant interaction between citrate-coated silver nanoparticles and bovine hemoglobin at physiological pH. However, polyethylene glycol-coated silver nanoparticles strongly bind to hemoglobin and affect its structure. Our study revealed that citrate-coated Silver nanoparticles do not interact with hemoglobin and this antibacterial agent is a more safe material for biological applications in comparison with polyethylene glycol-coated silver nanoparticles[41].

Carbon nanoparticles continuously generated from industries and vehicles are one of the potent causes of air pollution. Carbon nanoparticles in the polluted air when introduced into the bloodstream of humans through respiration can interact with blood plasma proteins. The interaction of carbon nanoparticles with bovine and human serum albumin has been investigated by ITC. Protein was injected into a nanoparticle solution in the sample cell. Successive injections of bovine or human serum albumin into carbon nanoparticles revealed strong complex formation and showed that each carbon nanoparticle binds 49 and 31 molecules of bovine and human serum albumin, respectively [64].

Protein-Polysaccharide Interaction

Protein-polysaccharide complexes are of increasing interest in research. These natural biopolymers are widely utilized as functional ingredients in the food, pharmaceutical and biomedical industries. ITC has been successfully applied in recent years in a growing number of studies of protein-polysaccharide complexes [65-67].

An interesting research has been carried out to study the interaction between beta-lactoglobulin and sodium alginate (before and after sonication) at 25 °C and pH 4.The injection profiles resulting from the titration of beta-lactoglobulin with intact and sonicated alginate in the sample cell were exothermic due to nonspecific electrostatic nature of the interaction between two biopolymers. Binding stoichiometry (n) for the binding of beta-lactoglobulin molecules to the binding sites along the alginate chains was shown as the weight ratio of the protein to alginate, because exact molecular weight of polysaccharide chain is unknown. ITC measurements indicated that the sonication decreased the interaction strength between alginate and beta-lactoglobulin [68]. Similar results were obtained for the binding of beta-lactoglobulin to kappa-carrageenans which is one of the red algae polysaccharides [69].

PROTEIN-PROTEIN INTERACTION

ITC is the most quantitative means available for measuring the thermodynamic properties of protein (or derived peptide)-protein interaction. Advances in our understanding of these processes will provide useful information about domain-domain interaction, protein recognition, hormone-receptor, antibody-antigen, antibody-receptor binding and membrane fusion.

Antibody binding to its antigen is a fundamental step for the immune responses. Like other protein-protein associations, antigen-antibody complexes arise from noncovalent interactions, including electrostatic and van der Waals forces, hydrogen bonds, and hydrophobic effects. Interaction between cytochrome c and two monoclonal antibodies has been characterized by ITC. In addition to thermodynamic parameters, amount of buried polar and nonpolar surface on formation of the complex for the respective interaction has been estimated using the change in the heat capacity on binding [50]. In another study, interactions of four monoclonal antibodies with a univalent peptide antigen mimetic of Cryptococcus neoformanspolysaccharid are investigated by ITC. It has shown that binding of this peptide to the antibodies is dominated by favorable entropy, with significant differences in thermodynamic binding parameters. These results have established that minor structural differences can affect the interaction of the antibody with an antigen [70].

SELF-ASSOCIATING SYSTEMS

ITC has shown growing use as a technique to follow self-assembly. Micellization parameters of surfactants in aqueous solutions, such as critical micelle concentration

(cmc), micellization enthalpy change (ΔH_{mic}) and aggregation number can be determined from an ITC experiment [71,72]. As an example, the heat effect as a function of total tetradodecylammonium bromide surfactant (TTAB) concentration was measured in an ITC experiment. The concentration at which the numerical derivative of the enthalpy graph is maximum is taken as the cmc value. The difference between the extrapolated values for low concentrations and high concentrations (which follow straight lines) at the cmc is taken as the value for the standard enthalpy of association. The maximum value of the derivative, the cmc value, and the value for the standard enthalpy of association are used to calculate the aggregation number [73].

An advantage of ITC measurement is the direct determination of the micellization enthalpy change. Other thermodynamic parameters of micellization, the standard Gibbs free energy of micellization (ΔG_{mic}), the entropy of micellization (ΔS_{mic}) and the heat capacity of micellization ($\Delta C_{p,mic}$) can also be obtained for surfactant systems [74,75]. This is not applicable only for surfactants but ITC has also been applied to determine thermodynamic parameters and driving force for peptide and protein self-assembly [55]. In an interesting work, the self-association of beta-casein as a natural amphiphilic protein was studied by ITC and cmc and demicellization enthalpy were obtained at different temperatures [76].

DRUG DESIGN

Access to the thermodynamic signature (enthalpy change, entropy change, binding affinity and stoichiometry) of binding is of a special importance for drug development. Computational tools and modeling studies are used widely in drug discovery approaches. Thermodynamics is increasingly common besides longer standing modeling studies. ITC can be effectively used to gain valuable insights into the thermodynamic signatures. Therefore, ITC is essential for understanding molecular interaction between a drug candidate and its binding target. ITC studies will play an important role in designing more specific and more potent drugs with higher affinity and optimized ΔH and ΔS for a wide variety of human diseases. ΔH arises from binding and interactions and ΔS is due to conformational changes of protein and ligand, and the organization and release of water molecules [34,52,57]. Freire and co-workers analyzed the thermodynamics of a series of HIV-1 protease inhibitors. They concluded that extremely high binding affinity requires a favorable binding enthalpy, and developed enthalpy optimization approach as a drug design principle [77]. Excellent reviews [34,52,57,78,79] are available illustrating role of calorimetry in drug design.

ITC can also be applied to improve drug delivery systems. Chitosan is a biomaterial, which has low solubility at physiological pH. To overcome low solubility and improve its use as an absorption enhancer in a neutral environment, chitosan derivatives have been examined. The binding affinity of carboxymethyl chitosan with bovine serum albumin studied by ITC analysis. ITC demonstrated that bovine serum albumin binds to carboxymethyl chitosan with a molar ratio of 4.5:1 and binding is an exothermic interaction [80].

ENZYME KINETICS

ITC is a powerful tool for studying the enzyme kinetics as well as thermodynamic. Its usage for determination of the kinetics of enzyme catalyzed reactions is increasing as a consequence of availability of more sensitive isothermal titration calorimeters. ITC allows a continuous monitoring of enzymatic activity, it does not require addition of any modified substrates or coupled reactions.It also permits a high degree of control of the temperature, and in a single experiment a complete set of kinetic parameters can be obtained [51,81,82]. An example of ITC usefulness is the study of the trypsin-catalyzed hydrolysis of several substrates at different temperatures. These enzymatic reactions could not be measured by absorption or fluorescence spectroscopy because of the spectrophotometrically blind substrates. ITC was carried out in a VP-ITC microcalorimeter (MicroCal) over the temperature range 5-45 °C and the thermal power signal was recorded until reaction completion. Then nonlinear least squares fitting of the data to the Michaelis-Menten equation determined the steady-state kinetic parameters [83]. Generally, multiple-injection method and a single-injection method are two ITC methods for determining enzyme kinetics. In the multiple injection method, the steady-

state heat rate is measured after each injection of substrate into an enzyme solution. The data produced is a plot of heat rate versus concentration of substrate. In this method in addition to the step-wise heat rate measurements, an additional single injection experiment must be done to determine the enthalpy change for the catalyzed reaction. In the single-injection method, a single injection of enzyme is made into a solution of substrate. The data produced consists of measurements of heat rate versus time until the substrate is mostly consumed and the reaction is complete. In comparison to multiple-injection, the single-injection method is significantly faster and requires less enzyme, but its data analysis might be more challenging [31,84]. Oxidation of syringic acid by laccase is another example of process monitored by ITC. This study compared multiple-injection and a single-injection method and determined kinetic parameters using single injection method [82].

CONCLUSIONS

In this review we have presented applications of ITC in studying interaction of protein with different kinds of ligands, self-association, drug design and determination of enzyme kinetic parameters. Furthermore, the procedure and analysis of the thermodynamic parameters derived from the exchanged heat have been described. In combination with structural information, ITC provides a thorough description of the interactions of biological macromolecules, and it is a reliable, sensitive and accurate tool. The information obtained from ITC studies should aid in molecular design in general and drug design in particular as well in the elucidation of factors determining the specificity of macromolecular interactions. Moreover, determination of the enzyme kinetics using ITC is growing as a consequence of improvements in sensitivity of calorimeters.

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