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Review

Isothermal titration calorimetry in membrane protein research

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ABSTRACT

Isothermal titration calorimetry is a versatile method to characterize energetics of intermolecular reactions and in particular interactions between drug molecules and their macromolecular targets. The assay is widely used in medicinal chemistry to quantification and characterization of molecular mechanisms of these interactions. The article reviews applications of the method to study ligand interactions with various classes of embedded membrane proteins. These proteins constitute very important fraction of macromolecular targets for medicinal compounds used in current therapies and recently emerged for detailed structural, functional and biochemical characterization of their interactions with drug molecules.

Contents

1. Introduction

Isothermal titration calorimetry (ITC) is a versatile analytical method that has become a gold standard in characterization of molecular interactions. This label free technique is based on precise measurement of heat absorbed or generated during binding event in a sequential (titrating) manner. During a single experiment it allows simultaneous determination of binding constants (K_a), reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS) change, providing a complete thermodynamic profile of investigated interaction. It is worth to notice that ITC is the only technique able to directly determine enthalpic and entropic contribution to the total energy of binding. Many techniques allow binding constant determination, but to estimate enthalpy or entropy the van't Hoff relationship has to be used. In that case enthalpy is indirectly obtained from temperature dependence of equilibrium constant, however, the enthalpy itself might also be a temperature dependent and it is often necessary to use correction term in the form of heat capacity (ΔC_p). It leads to widely reported discrepancies between calorimetric and van't Hoff derived enthalpies [1–3]. Therefore to determine accurate thermodynamic parameters of investigated interaction, ITC should be the technique of choice. Obtained results facilitate understanding of mechanism and driving forces of studied reaction and in combination with mutagenesis calorimetric data can also indicate residues crucial for binding event.

Since the experimental protocol requires having all molecules of interest in homogeneous solutions, ITC analysis are mostly applied to water soluble systems. However, rapidly developing field of membrane biochemistry and increasing interest in membrane proteins lead to growing demand for adopting this technique also for lipophilic biomolecules. As reported by Comley on basis of HT Stec's recent Label-Free Binding Analysis Trends 2008, membrane proteins were the second most frequently indicated target class of molecules worked with or plan to work with for binding analysis by respondents to the survey (72% investigated) [4]. Incorporation

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into liposomes or detergent micelles or use of recently developed nanodisc technique [5,6] should provide solution to overcome solubility problems and assist the study of membrane associated molecules. However still a limited number of papers reporting use of ITC for characterization of lipophilic proteins interactions were published.

The aim of this review is to show possible application of ITC in investigations of membrane proteins by presenting a collection of available works from that field with focus on three main membrane protein families: G-protein coupled receptors, ion channels and transporters. Due to the fact that calorimetric approaches to study lipid vesicles (liposomes) and biological membranes were previously reviewed by Heerklotz [7], this topic will be not discussed in details.

2. Principles of the ITC assays

ITC is a high-accuracy method that can directly measure heat change of a reaction and can provide information regarding the Gibbs free energy of interaction, the binding enthalpy or entropy change, the stoichiometry and equilibrium constant of a given reaction in a single experiment. High precision of the technique stems from the fact that since the assay is label-free there is no need to use radioactive, chromo- or fluoro-phore tagged reactants or immobilized proteins. Therefore interactions can be measured in conditions resembling physiological with macromolecules in their native forms. Thus, irreproducibility resulting from secondary reactions and protein chemical modifications can be avoided. Due to its universality and simplicity of experimental protocol accompanied by accuracy of obtained results ITC became a routinely used technique to explicit thermodynamics of biopolymer binding interactions and measure kinetics of enzyme-catalyzed reactions. Ladbury has presented a series of annual reviews on ITC summarizing year-by-year applications of ITC, proving versatility of the technique [8–11].

Standard ITC experiment is carried out in a series of successive injections of a titrant solution (e.g. ligand) to a sample cell containing a solution of the second reactant (macromolecule). The reaction that takes place after each injection absorbs or generates a certain amount of heat which is proportional to the amount of ligand binding to the protein and enthalpy (ΔH) characteristic of the interaction. Value of heat released or absorbed is determined by integrating peaks of signal which reflects heat flow after addition of ligand. In majority of ITC instruments heat flow is measured in a power compensation way. A thermoelectric device measures the temperature difference between sample and reference cell (usually filed with water) and keeps it at level near to zero by the addition or removal of heat to the cell containing macromolecule solution (Fig. 1). The amount of power applied by this cell feedback system to maintain $\Delta T = 0$ per unit of time reflects the heat flow. Exothermic reactions will trigger a temporary decrease in the applied power, on the contrary endothermic reactions will produce an increased feedback. During the experiment with each subsequent injection titrant saturates binding sites on macromolecule what is observed as a decrease in corresponding integrated heats. Finally when all binding sites are occupied by ligand molecules only a heat signal resulting from mixing, dilution effects and liquid friction is observed (Fig. 2). Value of these unspecific heats must be further confirmed in control experiments and subtracted. Since the heat of dilution of macromolecule is in most cases negligible only experiment in which ligand is injected to sample cell filed with buffer is performed. Starting molarity of the titrant in calorimeter cell is zero, and it is added as a highly concentrated solution to achieve final solution in the cell that will be several times the protein concentration. This leads to heats of dilution for

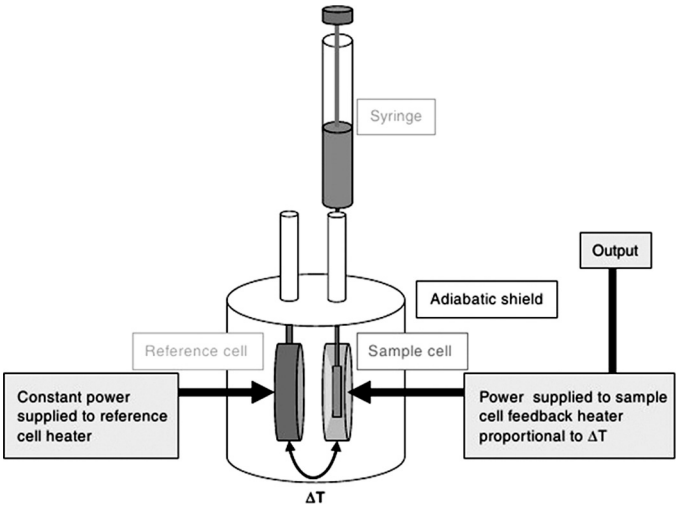

Fig. 1. Illustration of a standard power compensating isothermal titration calorimeter. Two chambers inside are surrounded by adiabatic shield, one is used as a reference cell and the other as reaction cell. A thermoelectric device measures the temperature difference between the two cells. After each injection of a titrant into sample cell filled with an analyte, the two materials interact, and certain amount of heat is generated or absorbed. The temperature difference between the sample and reference cells (ΔT) is kept at a constant value (i.e. baseline) by the addition or removal of heat to the sample cell. The integral of the power required to maintain $\Delta T = 0$ over time is a measure of total heat resulting from the process being studied. To keep the solution homogeneous after addition of titrant spinning syringe with a paddle shape needle tip is used for injecting and subsequent mixing of the reactants [15].

ligands which cannot be neglected. To minimize other unspecific heat, buffers for preparing titrant and protein solution should be exactly matched. To achieve that usually macromolecule solution is extensively dialysed against pure buffer which further is used for preparation of ligand solution. In membrane proteins studies unspecific heats changes may be particularly pronounced due to a mismatch in detergent concentrations between solubilized protein solution and ligand solution. To minimize this heat effects a series of buffer solutions with varying concentration of detergent can be prepared and then titrated one by one into macromolecule solution. For final experiments ligand should be dissolved in buffer, which results in lowest mismatch heats. Additionally ratio of the detergent concentration in the buffer to the protein solution can be established by NMR studies and further used to calculate optimal buffer match [12]. Alternatively the buffer mismatch effect can be reduced by using detergent in concentration below the CMC or the protein can be reconstituted into small unilamellar vesicles [13]. Hayashi et al. [14] demonstrated that different salts in injection solution may lead to different buffer mismatch effects. Therefore, buffer composition should be adjusted for each of them individually. Finally, appropriate mathematical binding model is fitted to a set of corrected heats of injections by nonlinear regression resulting in a titration curve from which values of binding constant, enthalpy of interaction and its stoichiometry can be determined. Other thermodynamic parameters i.e. change in Gibbs free energy (ΔG) and entropy (ΔS) are obtained from relationship described by equation:

$$-RT \ln K_a = \Delta G = \Delta H - T\,\Delta S$$

The shape of the curve depends on both the binding constant, K_a, and the concentration of binding sites, $[M]_T$. ITC is able to define affinity of reactions which value of equilibrium constant is in the range 10^3–$10^9\ M^{-1}$. Relationship between affinity and accuracy of results is summarized by so-called Wiseman parameter "c":

$$c = n \times K_a \times [M]_T$$