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# Isothermal titration calorimetry of ion-coupled membrane transporters

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ABSTRACT

Binding of ligands, ranging from proteins to ions, to membrane proteins is associated with absorption or release of heat that can be detected by isothermal titration calorimetry (ITC). Such measurements not only provide binding affinities but also afford direct access to thermodynamic parameters of binding – enthalpy, entropy and heat capacity. These parameters can be interpreted in a structural context, allow discrimination between different binding mechanisms and guide drug design. In this review, we introduce advantages and limitations of ITC as a methodology to study molecular interactions of membrane proteins. We further describe case studies where ITC was used to analyze thermodynamic linkage between ions and substrates in ion-coupled transporters. Similar type of linkage analysis will likely be applicable to a wide range of transporters, channels, and receptors.



## 1. Introduction

Majority of chemical reactions are associated with absorption or release of energy in the form of heat. Isothermal titration calorimetry (ITC) measures this heat, and thus the energy of a reaction directly as reactants are mixed in the instrument cell. Over the last several decades, ITC has been applied to numerous systems. Majority of these studies focused on molecular interactions, such as between proteins, or between proteins and small ligands, DNA or other macromolecular systems. Other phenomena, including enzyme kinetics have also been probed by ITC. Excellent reviews [1–4] are available covering appropriate methodologies including studies of membrane proteins [5]. Though lagging behind soluble proteins, interactions of membrane proteins with a variety of partners are also being actively interrogated. An incomplete list includes channels binding ions [6–8] and gating ligands [9,10], secondary transporters binding substrates and coupled ions [11–28], and assembly of protein complexes [29,30]. ITC is routinely used to establish functionality and substrate specificity of channels and transporters. However, other questions such as stoichiometry of binding [26] and ion-coupling mechanisms [12,15] have also been probed using this technique. Here, we will discuss applications of ITC to studies of membrane proteins with further focus on the mechanistic studies of ion-coupled transporters.

### 1.1. Advantages of ITC

There are two key advantages of ITC in studies of molecular interactions. First, ITC is performed on native proteins without a need for modifications. By contrast, approaches based on fluorescence spectroscopy require that at least one of the reactants is either intrinsically fluorescent or chemically labeled. Furthermore, reactants in ITC are in solution, as opposed to, for example, surface plasmon resonance spectroscopy, where one of the interacting components has to be surface-immobilized. Hence arguably, binding observed by ITC approximates binding processes in cells most closely among common in vitro methods. Finally, ITC is unaffected by spectroscopic properties of reactants. For example, there are no limitations on their internal fluorescence or optical density. This property allows one to study binding in solutions with variable compositions. In the case of membrane proteins, these include detergent micelles, bicelles or membrane mimetics, such as nanodiscs and proteo-liposomes. Importantly, ITC experiments are technically simple, requiring only basic training, and the instruments are widely available and relatively inexpensive.

Second, ITC provides rich thermodynamic information, including values of enthalpy ($\Delta H$), entropy ($\Delta S$) and heat capacity

*Abbreviations:* ITC, isothermal titration calorimetry; CMC, critical micelle concentration; $K_D$, dissociation constant; $\Delta H$, enthalpy change; $\Delta S$, entropy change; $\Delta G°$, standard free energy change; $\Delta C_p$, heat capacity change; $R$, gas constant; DMSO, dimethyl sulfoxide; SEC, size exclusion chromatography; $Na^+$, sodium; $Cl^-$, chloride; $H^+$, proton; GPCR, G-protein coupled receptor; TRP, transient receptor potential.

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($\Delta C_p$) of binding in addition to standard free energy ($\Delta G^\circ$) and dissociation constants ($K_D$). Moreover, information on a reaction mechanism can often be obtained such as the number of binding sites ($n$), presence or absence of cooperativity, and coupling of ligand binding to protonation/deprotonation events. Access to thermodynamic parameters ($\Delta H$, $\Delta S$ and $\Delta C_p$), which define the thermodynamic signature of a process, is of significant value in data interpretation. Collectively, these parameters determine the values of $\Delta G^\circ$ and $K_D$ and their temperature dependencies.

$$K_D = \exp\left(-\frac{\Delta G^\circ}{RT}\right), \quad (1)$$

$$\Delta G^\circ = \Delta H - T\Delta S, \quad (2)$$

$$\Delta H = \Delta H_{REF} + \Delta C_p(T - T_{REF}), \quad (3)$$

$$\Delta S = \Delta S_{REF} + \Delta C_p \ln\left(\frac{T}{T_{REF}}\right), \quad (4)$$

where $R$ is the gas constant, $T$ is an absolute temperature, $T_{REF}$ is a reference temperature, and $\Delta H_{REF}$ and $\Delta S_{REF}$ are the reaction parameters at $T_{REF}$. Importantly, $\Delta G^\circ$ is the standard free energy defined as the free energy of binding at 1 M concentration of reactants. The relationships of $\Delta H$, $\Delta S$ and $\Delta C_p$ to structures and structural changes of interacting components have been extensively studied and challenges associated with these interpretations recognized [31,32].

In general, $\Delta C_p$ is probably the most information-rich parameter. It reflects the complexity of the underlying reaction inasmuch as the magnitude of $\Delta C_p$ is related to the multiplicity of the formed cooperative weak interactions [33]. Thus, binding of a small ligand to a preformed binding site is expected to produce small $\Delta C_p$. In contrast, reactions involving protein restructuring, rigidification and extensive changes of interactions with water are expected to produce large $\Delta C_p$ [33–35]. We discuss measurements and interpretation of $\Delta C_p$ in Section 4.3.

Access to the thermodynamic signature of binding is of a special importance for drug development [36–39]. $\Delta H$ is a direct measure of the collective energies of bonds that are made and broken during complex formation. These include ionic interactions, hydrogen bonds, and van der Waals interactions between protein and ligand, within the protein itself, and between interacting solutes and water. Hydrogen bonds formed between protein and ligand are the principal contributors to favorable $\Delta H$ of binding and are important for specificity of drug interactions with the target, as well as strength of binding. However, *in silico* modeling and optimization of the bonds is challenging because their strength depends on precise distance and orientation of relevant atoms. Therefore, selecting lead compounds that already show favorable binding $\Delta H$ for further optimization might be a useful strategy [36–39]. $\Delta S$ of binding comprises cratic, solvation, and conformational entropies. While cratic $\Delta S$ is typically unfavorable, reflecting loss of rotational and translational degrees of freedom upon complex formation, solvation and conformational entropies can be both favorable and unfavorable. Solvation entropy is usually favorable and is due to dehydration of the hydrophobic regions of reactants and release of water molecules into the bulk. Conformational entropy is typically, but not always [40], unfavorable due to increased conformational constraints on both protein and ligand. Rational optimization of $\Delta S$ during drug maturation is relatively straightforward: addition of hydrophobic moieties to the drug will increase solvation entropy gains, and rigidifying the drug would decrease the conformational entropy penalty [31,37].

### 1.2. Limitations of ITC

Ideally, protein concentration in ITC experiments should be between 10 and 500 fold over the $K_D$ of the complex. For a simple binding model of $n$ identical independent binding sites, this requirement is usually discussed in terms of a parameter $c$:

$$c = nM_T/K_D, \quad (5)$$

where $M_T$ is the total protein concentration [41]. Complexes that are too tight or too weak cannot be optimally studied (Fig. 1).

To stay within favorable range of $c$ values for tight binding with $K_D$ below 1–10 nM, concentrations of the reactants may become too low to obtain good signal to noise levels. When $c$ values exceed 1000, determination of binding $K_D$ becomes challenging (Fig 1C). However under high-$c$ conditions, an upper bound of $K_D$ can always be obtained, and values of $n$, $\Delta H$ and $\Delta C_p$ are very well determined. Notably, the sensitivity of ITC instruments is continuously improved and therefore with time, tighter complexes will become fully accessible. Improvements in hardware are accompanied by optimization of ITC data analysis procedures that now allow for more accurate baseline determination and error evaluation [42]. Finally, if two competitive ligands are available with one binding too tightly, but the other binding within the suitable range, displacement titrations can yield all thermodynamic parameters for both ligands [43,44]. Typically, protein is first titrated with the moderate affinity ligand and then with the tight ligand, which displaces the weaker competitor. An optimized single titration protocol has been recently been described [45]. From these experiments, the free energy and other parameters for the ligands are calculated based on competitive binding equilibrium equations.

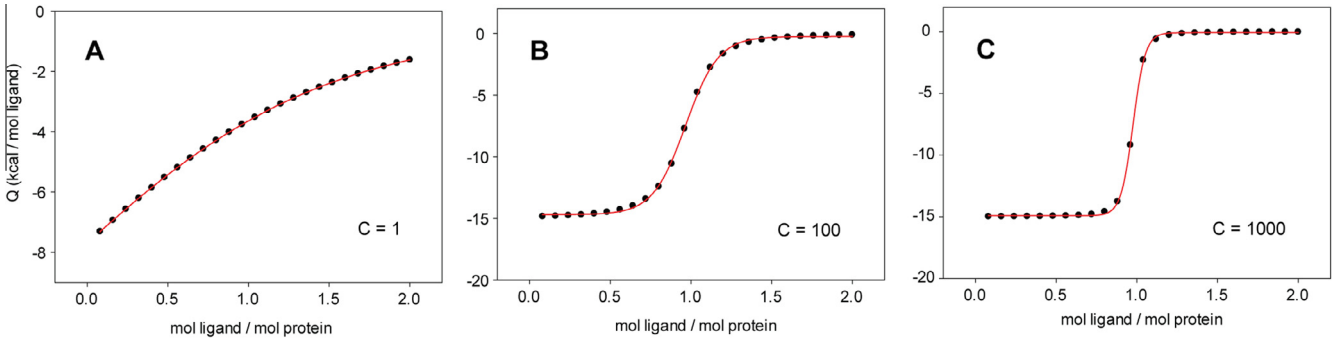


**Fig. 1.** Binding isotherms for low (A) intermediate (B) and high (C) affinities. Integrated injection heats produce binding isotherms, from which $K_D$, $\Delta H$ and $n$ are determined. Simulated data are shown with $\Delta H$ of −15 kcal/mol, $n$ of 1. The solid red lines through the data are fits to the independent binding sites model. Corresponding $c$ values are on the panels.