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Isothermal Titration Calorimetry of Membrane Proteins – Progress and Challenges

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Summary

Integral membrane proteins, including G protein-coupled receptors (GPCR) and ion channels, mediate diverse biological functions that are crucial to all aspects of life. The knowledge of the molecular mechanisms, and in particular, the thermodynamic basis of the binding interactions of the extracellular ligands and intracellular effector proteins is essential to understand the workings of these remarkable nanomachines. In this review, we describe how isothermal titration calorimetry (ITC) can be effectively used to gain valuable insights into the thermodynamic signatures (enthalpy, entropy, affinity, and stoichiometry), which would be most useful for drug discovery studies, considering that more than 30% of the current drugs target membrane proteins.

1. Introduction

Integral membrane proteins play fundamental roles in all aspects of human physiology, and mediate diverse functions from triggering various signaling pathways to ion transport and metabolism. There is no question that their functions, as mediators between the extracellular and intracellular worlds, are exquisitely regulated; not surprisingly, their dysregulation leads to a wide variety of diseases. Considering 30% of the human genome encodes membrane proteins, very little is known regarding the molecular mechanisms underlying their function, and much less is known regarding the thermodynamic basis of their binding interactions. In fact, a PubMed search failed to identify even a single experimental thermodynamic characterization on intact mammalian membrane proteins prior to 2009. This is in sharp contrast to soluble proteins, and can be directly attributed to the major bottleneck of requiring large amounts of the homogeneous, stable, and active preparations needed for structural and biophysical studies. Nevertheless, the publication of two recent reports, one describing ligand binding to a GPCR and another to a ligand-gated ion channel, bodes well for the future [1,2].

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Isothermal titration calorimetry (ITC) is the only experimental technique that can provide thermodynamic parameters (enthalpy, entropy, binding constant, and stoichiometry) from a single titration, and most importantly, the technique is label-free and therefore does not introduce artifacts. ITC is robust, and in general, can measure binding affinities over 5 log units from ~100 μM to 1 nM. ITC's ability to measure high affinity interactions is worth noting, as this is not easily achieved with other label-free methods. For instance, most commonly used technique of competitive binding requires radiolabeled ligands, and similarly, in principle, fluorescence spectroscopy can be used to measure high affinity binding but requires the judicious introduction of highly sensitive fluorescent label at a specific site, which may not be straightforward.

Considering availability of membrane proteins is limiting, it is very important to have extremely sensitive instrumentation. Currently, the leading instruments are the Microcal ITC series and the TA Instruments Nano-ITC. Typical traces obtained from multiple injections (baseline subtracted) are shown in Figure 1A. The area under each peak represents the enthalpy (heat energy) associated with the corresponding injection of ligand. Summing up all the heats from the individual peaks yields the familiar titration curve for high-affinity binding (Figure 1C). The Figure also indicates the thermodynamic properties yielded by different parts of the ITC curve. Each enthalpy point represents an integrated injection peak. The amplitude of the curve is proportional to the enthalpy of the reaction, the inflection point gives the stoichiometry, and the width yields the ratio of the affinity to the protein concentration.

Thermodynamic insights from ITC studies are also of significant interest for drug development. A large fraction of the current drugs targets GPCRs, and it has become quite evident from clinical data that there is an immediate need for drugs that are more specific and more potent in order to minimize side effects that could be quite detrimental. Most drug discovery approaches use computational tools and modeling studies, which fail to capture the nuances of the molecular underpinnings of binding interactions. Structural and dynamic measurements using solution NMR studies of a wide variety of proteins have shown substantial and subtle structural and dynamic changes between the free and bound forms. Even such detailed knowledge may not be sufficient to tease out the enthalpic and entropic components that arise from packing and electrostatic interactions, structural and dynamic changes, and the organization and release of water [3-5]. Therefore, experimental thermodynamic data from ITC studies are ideally suited to provide this information, and will therefore play an integral role in designing next-generation high-affinity and high-specificity drugs.

The major challenges and limitations for studying membrane proteins are two-fold. In addition to the requirement for relatively large amounts of protein, there is also a need to keep the protein folded and functional in native membrane lipid mimics (detergents) over a period of days. A tremendous amount of work has gone into obtaining large amounts of proteins mainly by optimizing heterologous expression in insect, yeast, mammalian, and bacterial systems, and in parallel, developing and using detergents that are mild and at the same time efficient in solubilizing membranes [6,7]. Until recently, the major goals of these efforts have been focused on structure determination, and indeed, a recent flurry of papers on structures of various GPCRs bodes well that protein expression and obtaining sufficient amounts of proteins will not be limiting [8-10], and will become less cumbersome, setting the stage for future ITC studies of this highly challenging but important class of proteins.

We initially discuss various criteria that must be taken into consideration for ITC, and in particular, focus on factors that are unique to membrane proteins. We then discuss various ITC studies on membrane proteins and what has been learned. The two sections are not

mutually exclusive, and whenever possible we have used currently available published data to describe and highlight the experimental criteria. Several comprehensive review articles are available for more general details on technical aspects, experimental design, and applications of ITC [11-13].

2. How to set up an ITC experiment for membrane proteins?

ITC is extremely sensitive; it is therefore essential to measure enthalpy changes only from binding interactions, and eliminate or significantly minimize enthalpy changes from other processes such as buffer mismatch due to differences in pH or ionic strength, ligand dilution, and in the case of membrane proteins, mismatch in detergent concentrations. Further, ITC measurements, compared to other biophysical techniques, require relatively large amounts of protein. This becomes all the more challenging for membrane proteins, which are more difficult to purify in large amounts. As much as several milligrams may be needed for a series of experiments, depending on the specific system under investigation. However, substantially less material may be needed for the investigation of very high affinity interactions combined with very large enthalpies of binding. Taking these factors into consideration, we discuss various factors that should be considered before setting up the experiment, and having done the experiment, discuss how to maximally interpret and get the most out of the data. For simplicity, we consider only the case of independent and equal binding sites.

In general, prior knowledge from the literature on the binding affinities and/or stoichiometry is useful. For most receptors, functional data generally exist, in which case it is very likely that EC_{50} values also exist. EC_{50} is defined as the ligand concentration at which 50% of the maximal response is observed, and is determined by measuring the functional response over many log units. This knowledge is most useful as the binding affinities often correlate with EC_{50}. EC_{50} values, in contrast to binding constants, are also more easily determined. In the context of ITC experiments, it must also be remembered that there is no correlation between enthalpy change and binding affinity; if the binding is predominantly entropically driven, the signal could be weak even for high-affinity interactions, and easily misinterpreted as lack of binding.

2.1 Initial choice of protein and ligand concentrations

The 30, 30, 30 approach—Initial ITC measurements generally work best, when (i) the concentration of protein is about 30 times higher than the expected dissociation constant, (ii) the ligand concentration in the syringe is about 30 times higher than the protein concentration in the cell, and (iii) about 30 injections of 5 μL are performed. In this section, we explain the rationale, and in the following sections also consider alternate approaches in which the 30, 30, 30 approach is not practical.

Impact of protein and ligand concentrations on curve shape—It is important to choose a set of conditions under which the ITC curve has a meaningful shape that allows the extraction of affinity, stoichiometry, and enthalpy in a reliable manner. A peculiar property of ITC curves is that the shape primarily depends on the ratio of the protein concentration to the dissociation constant. This ratio is defined as the c-value, where $c = PN/K_D$ (where P is the protein concentration in the cell, N is the number of binding sites per protein molecule, and K_D is the dissociation constant). Note that when $N \geq 2$, there are multiple equal and independent binding sites on each protein, and the protein concentration has to be multiplied by the number of sites (N) to obtain the correct c-value. Figure 2 shows how the shape of an ITC curve depends on the c-value. Note that the shape depends only on the c-value, not on the specific protein. Only amplitudes or noise-levels will vary between proteins; but the shape will be exactly as shown in Figure 2 for a protein with one binding site. The shape is

also preserved for multiple equal and independent sites, and only the numbers on the abscissa will change.

Why do c-values matter? To answer this question, we first consider extremely high c-values. At a c-value of 3000 or more (filled triangle in Figure 2), the titration curve allows the extraction of just two parameters: the stoichiometry is obtained from the molar ratio at which the signal jumps, and the binding enthalpy from the difference in signal levels before and after the jump. At intermediate c-values (around 30; filled square in Figure 2), more information is obtained. Namely, the progression of the actual transition is seen, revealing the affinity of the interaction. At low c-values (below 3; filled circle in Figure 2), not much information can be obtained unless there is prior knowledge of some aspect of the binding reaction, e.g. the stoichiometry [14]. If such knowledge exists, then the known parameter can be fixed in the curve fit allowing the extraction of the other parameters. This latter approach of fixing the stoichiometry has been applied in the case of membrane proteins; ITC was used to characterize the binding of NAD(H) and NADP(H) to *E. coli* transhydrogenase, and due to the low c-value, the authors assumed a 1:1 stoichiometry and were able to determine the enthalpy and entropy of binding [15]. High protein concentrations (>mM) would have been needed in this case to achieve c-values >10, which is not practical due to non-availability or solubility, highlighting the challenges of characterizing low-affinity ligands.

2.2. Final choice of protein and ligand concentrations

The 30, 30, 30 approach is a good place to start if there is no *a priori* knowledge about the affinities, or if the signal is sufficiently large to yield good parameter estimates without fine-tuning the experimental conditions. If the signal is weak but large enough to extract approximate parameters, further optimization is necessary. One such approach includes optimizing the concentrations of protein and ligand [16]. It is recommended to (i) use as high a protein concentration as possible while maintaining the c-value <1000, (ii) make only 10 injections to maximize the signal per injection, and (iii) set the ligand concentration such that the ligand-to-protein concentration ratio after the final injection is $R_m=6.4*c^{-0.2}+13/c$, but not smaller than 1.1 [16]. This translates to a syringe concentration of $R_m/(-1+(1+R_v)^{11})$, where R_v is the ratio between the volume in each injection and the cell volume, and c is the c-value. While 10 injections are sufficient to sample the transition within this approach, we recommend doing a few more than 10 injections to establish the signal level after the transition.

Sometimes, it may be difficult to balance out the magnitude of the signal against the shape of the curve. For example, in the case of the membrane protein arginine-agmatine exchange transporter [17], signals of good magnitude were achieved using relatively large (20 μL) injection volumes. Applying the principle from the last paragraph, we can calculate that an optimal ligand concentration would have been about double of what the authors used. The calculation is as follows: a c-value of 1.6 results from a protein concentration of 0.15 mM and a K_d of 0.094 mM. The injection volume is 20 μL, and the cell volume is likely 1400 μL (the exact instrument type was not reported), thus the ratio of volumes, Rv, is about 0.014. Inserting these values gives $(6.4*1.6^{-0.2}+13/1.6)/(-1+(1+0.014)^{11})=82.5$ for the ratio between ligand syringe concentration and cell protein concentration, i.e. 82.5*0.15=12 mM arginine concentration in the syringe. What was actually used was two-fold lower, viz. 6 mM. Note that any of the parameters, protein concentration, ligand concentration, or injection volume could have been modified. But the ligand concentration is the most convenient in the given case. For agmatine, however, the used concentration of 6 mM was optimal, as can be easily calculated with the same equation (use 0.023 mM for the affinity). Of course, much better results could be obtained with c-values >1.6, if sufficient protein were available.

In the case of the membrane protein transferrin receptor [18], some of the titrations show sharp transitions and lack sufficient data points in the transition phase to accurately determine binding affinity. In this case, to get a curve shape that gives a better accuracy for the affinities, smaller injection volumes or lower injection concentrations could have been used at the expense of signal strength.

The choice of buffer can be another way to enhance or inadvertently reduce the signal [19]. Many binding reactions are accompanied by a change in protonation state of either protein or ligand. In that case, the buffer will respond by taking up or releasing protons, resulting in buffer-dependent enthalpy change that must be either added or subtracted from the experimentally observed heat release. The relationship is given by the following equation, $\Delta H_{ITC} = \Delta H_{binding} + n\Delta H_{ionization}$, where ΔH_{ITC} is the experimental observed enthalpy, $\Delta H_{binding}$ is the buffer-independent binding enthalpy, $\Delta H_{ionization}$ is the ionization enthalpy of the buffer, and n is the number of protons transferred during binding. Thus it can be helpful to use alternative buffers with large protonation enthalpies of opposite sign. Buffer ionization enthalpies are available in the literature [20].

2.3. Effect of detergents

Detergent-related problems in the ITC signal can be identified based on two major symptoms, viz. unusually large amplitudes and unusual peak shapes. Detergents in presence of membrane proteins exist in equilibrium between three forms – as a monomer; as a micelle, which depends on the CMC that is known; and a mixed micelle with the membrane protein where the CMC is generally not known. Protein-detergent interactions will contribute to the overall thermodynamics of the binding process. For instance, the release of bound detergents and/or changes in micellar properties during the titration can influence the heat released (enthalpy) to the extent that they obscure the heat release directly related to the binding event. For example, in the case of the human glycine receptor solubilized in DDM, extensive buffer mismatch experiments using different detergent concentrations were carried out to obviate large dilution heats [2]. This was all the more important as the authors used individual protein fractions of different concentrations for their ITC experiments, and so each fraction also contains different amounts of detergents. A similar problem, though to a much lesser degree, seems to have also occurred in ITC studies of photosystem II solubilized in DDM [21]; this issue was not mentioned in the publication, but evident in at least one of the titrations. Though such heats of dilution is normally a mere nuisance, the possibility should be kept in mind that coupling between ligand and detergent binding could affect the results beyond an additive contribution.

For soluble proteins, the conventional approach is to dialyze the protein against the same solution that is used for the ligand. However, this is complicated for solutions containing detergents for reasons outlined above. Only a fraction of the detergent is present in a monomeric form that can freely cross the dialysis membrane, and the micelles may be too large to pass which is also dependent on the pore size of the dialysis membrane used. Slow diffusion of detergents across the membrane may not allow equilibrium to be reached in a reasonable amount of time, making dialysis essentially impractical for biophysical studies of membrane proteins. This is why a trial and error approach to finding the right detergent concentration, as carried out for the glycine receptor, may be superior compared to dialysis. However, dialysis could be a viable option if protein levels are not limiting and are also highly stable. For example, *E. coli* AcrAB-TolC membrane protein was extensively dialyzed for ITC experiments, though the details of the extensiveness and the stability and activity of the proteins were not described by the authors [22].

Unusual peak shapes may point to undesired processes in the sample cell, such as release or uptake of detergent upon ligand binding. Figure 3 shows the typical shape of an ITC peak

(black points and grey line). This shape results from the fact that heat transfer from the molecules in the cell to the temperature sensor is not instant. If it were infinitely fast, then we would get the "input signal" shown as a dashed line in Figure 3. In this case, it has a breadth of 15 seconds, corresponding to a 15 second injection. The area is equal to the heat energy (enthalpy) change caused by the injection. The distorted "observed signal" has still the same area, but reflects the instrument response (~13s time constant for our instrument), and to a lesser degree on the kinetics of stirring. Under normal circumstances, the peaks should always have the same shape for any given ITC: an approximately exponential signal change in one direction, followed an approximately exponential change in the other direction – with a time constant that is reproducible across all titrations on the respective instrument.

Further distortions of ITC peaks can occur if the binding of the ligand is slow compared to the instrument response [23, 24], or if the critical micelle concentration (CMC) slowly readjusts. It is thus worthwhile to monitor peaks for deviations from normal behavior. Such deviations could be broadened peaks (slower exponential decay) or double peaks. For example, SDS-mediated α-synuclein aggregation results in very unusual peak shapes that could be attributed to the kinetic process of both protein aggregation and micelle dilution [25]. Another example is the broadened peaks that were observed in the case of transferrin receptor [18]. Some of the injection peaks include a slower exponential decay or even double peaks. With the given information it is not easy to tell whether the binding reaction itself is slow or there was a detergent rearrangement such as association or dissociation. It may be sometimes desirable to attempt to reconstruct the "input signal" in order to understand unexpected peak shapes. Relatively simple mathematical procedures are available for such purposes [26, 27]. Normally, it is sufficient to use the longest time constant (the instrument response; e.g. ~13s for our instrument, as shown in Figure 3) and do a first order deconvolution, as described in the cited chapters.

In general, it seems that the binding of ligands to membrane proteins is relatively slow. In many examples, injection peaks broaden as the titration approaches 50% completion, and subsequently sharpen again. This is the typical behavior expected for kinetic processes such as ligand binding, and that they become visible in the experiment is indicative of their slow speed compared to the response time of the ITC [24]. The origin of double peaks is not clear *a priori* and could occur for various reasons. The time frame of the peaks may give a clue to their origin. Very sharp additional peaks may for example be due to a mechanically generated heat of injection, caused by altered hydrodynamic properties of lipid-containing solutions. Unusually broad secondary peaks are likely due to kinetic processes that are triggered by the presence of the injected ligand, such as slow fusion of vesicles.

2.4. Optimizing experimental parameters

Choice of temperature—If the amplitude of the peaks is too small, a change in experimental temperature may improve the situation. This is because binding enthalpies often strongly depend on temperature. If the experimental temperature happens to be just around the point where ΔH passes through zero, not much of a heat signal will be observed.

Injection time and peak spacing—Protein samples may not be stable for a long time, and this can be a factor towards shortening experiments. Such a decrease in time could be achieved by using very short times between injections [28,29]. The disadvantage is that the injection peaks may overlap resulting in reduced enthalpy values. An alternative approach for very fast ITC measurements is the use of the single injection method [30]. This method consists of one single prolonged injection. Its shape is fit to the applicable binding model to yield the thermodynamic parameters. The disadvantage is that the single injection method

requires a very stable baseline and will therefore usually result in increased parameter uncertainty.

2.5. Choice of ITC instruments

To our knowledge there are two major brands of high sensitivity ITC instruments that are useful for membrane proteins -- the Microcal (General Electric) VP-ITC and iTC_{200}, with 1.4 and 0.2 ml cell volumes, respectively, and the TA-instrument Nano-ITC which is available with 1.0 and 0.2 ml cell volumes [31]. We will focus on the Microcal instruments for which more information is available, but the same principles should apply to the TA-instruments as well. Considering protein amounts and achieving high concentrations are limiting for membrane proteins, we discuss the advantages and disadvantages of VP-ITC (large volume) and iTC_{200} (small volume) instruments.

In general, about three times less total protein is required for iTC_{200} compared to the VP-ITC. Because a smaller volume is required, the concentration of the protein needs to be two-fold higher. Formally, their sensitivity is equal, but a smaller volume leads to a faster response time, resulting in sharper, better-defined peaks. Therefore, the iTC_{200} has about three-fold sharper peaks compared to the VP-ITC, because the response time is three times shorter. However, its volume is seven fold lower (1.4 vs. 0.2 ml). To obtain comparable signals, one needs then a three-fold lower concentration based on the response time, but a seven-fold higher concentration based on the seven-fold smaller volume, resulting in a factor of about two (7/3) in concentration. Because the volume is seven times smaller, the total protein required is three times less (7/3/7=1/3).

So, the advantage of a small cell volume is that less material is required and that the measurement is faster, leading to moderately high throughput capabilities (up to about 50 samples per day). The tradeoff is the higher protein concentration, leading to higher c-values. Therefore, instruments with larger cell volumes are better suited for very high affinity measurements. If measurement time is a problem due to stability issues, then iTC_{200} is better because of shorter measurement time. If the elevated protein concentration is a problem, due to protein aggregation, then VP-ITC is preferable.

3. ITC of membrane proteins

3.1 ITC studies of mammalian membrane proteins

ITC studies of a G protein-coupled receptor (GPCR) – human chemokine receptor CCR5—GPCRs bind a diverse array of ligands from a photon to small molecules and proteins, and play crucial roles from developmental biology and sensory processes to regulating host immune response. A number of current drugs actually target GPCR, and therefore knowledge of the structural basis and molecular mechanisms underlying binding interactions is of significant interest and being actively pursued in both academia and industry. Despite their importance, there is only one study in the literature reporting an ITC study of a GPCR. Nisius *et al.* [1] have used ITC to characterize the functional characteristics of a recombinant purified CCR5 receptor by measuring binding to its native ligand RANTES. The authors were successful in obtaining relatively high amounts of purified receptor (~1 mg/liter of culture) from baculovirus-infected Sf9 insect cells. Screening a panel of detergents resulted in FosCholine-12 showing the best results for solubility. Both circular dichroism (CD) and ^{1}H NMR spectra showed a folded receptor and characteristic spectral signatures of a helical protein, and a conformation-specific antibody also recognized the detergent-solubilized receptor. ITC studies of RANTES binding to the receptor showed that the binding is exothermic; however, the measured apparent binding

affinity of ~1 μM was substantially lower than the nanomolar binding affinities observed for the native receptor.

Large differences in binding and functional properties between purified and membrane-bound proteins are not uncommon. These differences could be a true reflection of the differences in membrane environment and/or that the biophysical and functional methods are limiting and do not capture the actual activity of the protein. Therefore in the case of CCR5, did the purified receptors in detergent micelles lack some of the native interactions, and/or the ITC studies not carried out under optimal conditions? Ligand binding involves two sites on the receptor – the N-terminal domain (Site-I) and the extracellular loops (Site-II) (Figure 4), and it is also well established that a GPCR exists as an ensemble. It is possible that with the purification scheme and/or under the experimental conditions used, the receptor exists in and/or adopts a low affinity conformation, and binding at Site-I does not trigger the conformational change required for binding at Site-II. Various biophysical studies have indicated that CCR5 in detergent micelles exists as both monomers and dimers. RANTES oligomerizes at physiological pH, and so the authors use a mutant (E66S) that has a lower tendency to aggregate. This is important, because previous binding studies have shown that only the monomer, and not even the dimer can bind the receptor. However, the binding and functional characteristics of the monomeric vs. dimeric forms of the receptor are not known. Therefore, it is possible that the lower affinities could be due to differences in the binding characteristics of monomeric vs. dimeric/oligomeric forms of the ligand to the monomeric vs. dimeric forms of the receptor.

From a technical point of view, these titrations are challenging due to the low c-value (~5) that could be achieved with the available receptor amounts. Further, only a factor of 10 between GPCR and ligand concentration was achieved, whereas a factor of 30 would be desirable (30, 30, 30 criteria); therefore, using higher RANTES concentrations could have resulted in a stronger signal. However, this may not be also practical due to the tendency of the ligand to oligomerize. The authors doubled the injection volume from the recommended 5 μL to 10 μL; this partially compensated for the limitations of low c-value and low protein/ligand ratio. Considering 30-30-30 criteria were not feasible, with other parameters being constant, we propose the use of ~ 17 μL injection volumes according to the criteria discussed in Section 2.2 [16]. However, for such large volumes, it is important to verify first in a simple water-to-water reference measurement whether the solution in the syringe has sufficient time to reach the sample temperature before being injected, as a temperature mismatch would result in large peaks.

This study provides compelling proof-of-principle that ITC studies of GPCRs are feasible and realistic, and at the same time highlights some of the challenges. Future studies of other GPCRs, and also of CCR5 using a monomeric RANTES, a receptor preparation containing only monomers or dimers, data collection under more optimal conditions, and receptors solubilized using different detergents are necessary to provide the framework for formulating the experimental principles for studying thermodynamics of GPCRs.

ITC studies of an ion channel – the human glycine receptor—The glycine receptor belongs to the class of pentameric ligand-gated ion channels that rapidly respond to changes in extracellular effectors in the neural signaling network. Recently, Wohri *et al.* have succeeded in expressing high receptor levels in yeast and obtaining high quality ITC data comparable to those for soluble proteins [2]. The authors have succeeded in preparing a well folded, highly stable (over a week), homogeneous, and active receptor using DDM as the detergent in high yields (up to 5 mgs/liter of culture). However, the authors had to overcome multiple hurdles at every step along the way from optimizing expression vectors to minimizing experimental artifacts during ITC data collection. The study highlights how a

systematic approach, and the integration of the expertise from molecular biology, heterologous protein expression, and detergents to ITC data collection are essential to be successful. The design strategy involved the initial optimization of conditions for protein homogeneity including cloning and characterizing a series of mutants, optimizing conditions for stability including use of endogenous ligands, and finally optimizing conditions for collecting ITC data.

The authors report the binding of strychnine an antagonist and glycine an agonist. The chemical structures of glycine and strychnine are quite different, and these ligands bind with differing affinities to distinct but overlapping sites. Interestingly, the calorimetry data showed that the binding of glycine is enthalpically driven and that of strychnine is entropically driven. The data could be fitted to a simple model, with both ligands binding with a stoichiometry of one ligand to a single pentameric receptor, and the affinities were similar to those observed from radioligand binding studies. These data together suggest that the purification scheme and the choice of detergent captured the native fold and function of the receptor. A rigorous analysis of the data also suggests that strychnine has additional low affinity binding sites.

One of the challenges of ITC studies of membrane proteins is the buffer mismatch that arises due to variations in detergent composition of the membrane proteins between fractions and between preparations, and the difficulties encountered in quantifying the exact amount of detergent present. DDM shows fairly high buffer mismatch but is also one of the best detergents for membrane protein studies, as was the case for the glycine receptor. Therefore, researchers carried out a series of titrations with different detergent concentrations to minimize buffer mismatch, and observed negligible mismatch only over a narrow window. It was also evident from these experiments that minimizing buffer mismatch may not be possible at high total DDM concentrations, and the authors propose that using protein fractions containing high protein to low detergent ratio will minimize buffer mismatch-related issues and yield the best and most reliable data.

A comparison between glycine and strychnine binding highlights the importance of optimal c-values. The c-values were >100 for strychnine and ~1 for glycine. Thus, strychnine titrations yield recognizable titration curves, whereas glycine titrations result in curves that are more difficult to interpret. Error limits for K_d are not provided, but they should be quite high for glycine binding, likely above 100% in the linear scale, which means that only the order of magnitude, and not the actual value, of the K_d can be assessed. A more reliable direct estimation of the binding affinity requires a higher receptor concentration, but will result in a higher detergent concentration resulting in significant buffer mismatch and thereby rendering the data difficult to interpret. On the other hand, more reliable competition titrations resulted in significantly lower affinity estimates than direct glycine titrations. Such competition experiments are an excellent alternative for titrations with ligands whose affinity is out of range (high or low). The idea is that the presence of a weaker affinity ligand increases the K_d of the stronger affinity ligand $K_{d,app,strong}=K_{d,strong}(1+C_{weak}K_{d,weak})$, where $K_{d,app,strong}$ is the apparent affinity of the strong binder in the presence of the weak binder, $K_{d,strong}$ and $K_{d,weak}$ are the intrinsic affinities of the ligands, and C_{weak} the concentration of the weak binder. In the case of glycine receptor, $K_{d,weak}$ was determined by first measuring $K_{d,strong}$ and then $K_{d,app,strong}$ at one value of C_{weak} (a better approach would be to use a range of concentrations). This approach will also work the other way around for very strong affinities: determining $K_{d,strong}$ from measurements of $K_{d,weak}$ and $K_{d,app,strong}$ [32].

Independent of c-value limitations, these studies were able to validate the currently proposed mechanism and stoichiometry of ligand transport. This study also provides compelling

proof-of-principle that ITC studies of ion channels, and by extension of other membrane proteins, can yield thermodynamic data comparable to those obtained for soluble proteins.

3.2 Bacterial membrane proteins

In contrast to mammalian membrane proteins, ITC studies have been reported for various bacterial and a few archaeal membrane proteins [summarized in Table 1; refs. 15, 17, 18, 21, 28, 29, 33-40]. These include transporters and pumps of metal ions, peptides, and proteins. Studying bacterial membrane proteins, in general, does not require heterologous expression, they tend to be more stable, and therefore obtaining relatively large amounts of stable and folded proteins (>10 mgs/liter of culture) is more straightforward.

Measuring the enthalpy, entropy, affinity, and stoichiometry of binding for a number of these proteins have enabled the authors to propose the structural basis of the binding (for proteins where structures are available), and in other cases, the mechanisms of binding and models for receptor assembly and cellular transport. Whereas some of the studies exclusively used ITC alone in describing the binding interactions, others used a combination of biophysical and functional techniques where calorimetry data complemented other structural/functional data towards describing the mechanism of binding and function. Whereas some of the membrane proteins function as monomers, quite a few exist as homooligomers and heterodimers. Despite better protein expression, the challenges of studying these complex systems and of having to use detergents are obvious in some of the studies, some of which have poor signal-to-noise ratios, whereas others could have been performed under more optimal conditions. We have discussed some of these studies in Section 2.

Higher stability of these proteins allows studies under conditions not feasible for mammalian membrane proteins. For instance, in the case of archaeal rhodopsins, the ITC studies of the WT were carried out at 45°C and of the mutants at 35°C and in the presence of high NaCl concentrations (400 mM) [38]. Krell *et al.* studied the binding of the two individual subunits and the entire complex of the transferrin receptor, and in addition, also studied the binding to various domains of the individual subunits [18]. Further, transferrin exists in both apo and holo (iron-bound) forms, and by characterizing the binding of both forms, the authors were able to provide a thermodynamic basis of the mechanism of transferrin receptor function.

Bacteria have multiple drug-resistance mechanisms, including the expression of membrane transporters that efficiently clear drugs. *E. coli* encodes two such transporters called SugE and EnrE. Sikora and Turner have used ITC to study the binding of a family of quaternary ammonium compounds (ethidium, methyl viologen, proflavin, TPP, and CTPC) to these proteins in two different detergents, SDS and DDM, and in small unilamellar vesicles (SUV) made from *E. coli* polar lipids [36,37]. These transport proteins can withstand harsh treatments including exposure to organic solvents, and so could be purified in large quantities in a straightforward manner. The authors initially carried out a series of experiments to obtain sufficient signal by varying protein concentrations in the cell, ligand concentration in the syringe, and eliminating buffer constituents that decreased the signal-to-noise ratio. Such experiments allowed the detailed thermodynamic characterization of the binding of multiple ligands to two transporters in three different systems, including in SUVs. DDM is a neutral detergent, SDS is negatively charged, and the lipids used in the SUV preparation are neutral, and the properties of the DDM and SDS micelles and the SUV vesicles are different in terms of size and shape, among other parameters. The ITC data show that the binding affinities, stoichiometries, enthalpies, and entropies of the binding of various drugs to the two transporters, but for rare exceptions, did not vary among membrane mimetics. To our knowledge, these are the only ITC studies of membrane proteins

reconstituted in SUVs. Essentially similar thermodynamic parameters between SUVs and micelles are striking, considering that SUVs are more similar to cells in shape and size. These observations suggest that the presence of the membrane environment is essential for a functional transporter and is not strictly dependent on the type of membrane mimetic used. Considering that these drugs are lipophilic, control experiments of binding to the micelles and vesicles showed weak binding (K_d ~mM) compared to the micromolar binding to the transporters. These experiments suggest that the drug efflux is mediated by transporters and is not due to passive diffusion across the membrane. Structural studies have shown that EmrE forms oligomers, but ITC studies show that all of the drugs bound both transporters with a 1:1 stoichiometry suggesting that oligomerization is not essential for binding. These studies show how ITC can be effectively used to describe the structural and thermodynamic basis and the molecular mechanism underlying ligand-membrane protein interactions.

3.3. Calorimetry of ligand binding to functional domains of GPCR class of receptors

Considering the various challenges that face calorimetric studies of intact receptors, some authors including us have taken a ‘divide and conquer’ approach of characterizing binding interactions to the receptor extracellular domains. These studies are all the more relevant if such interactions could provide insights into the thermodynamic signatures that are otherwise hard to come by. Examples in the literature include ITC studies of ligand binding to chemokine, C5a, and parathyroid receptor N-terminal domains (summarized in Table 1, refs. 41-48). In addition to providing stoichiometry, these studies have also provided functional insights into ligand and receptor specificity and mechanistic insights into ligand-receptor interactions.

Chemokines, a large family of protein ligands (MW ~8–10 kDa), are atypical agonists for class-A GPCRs, as most agonists tend to be small molecules. Receptor activation involves interactions between the chemokine N-loop and receptor N-terminal residues (Site-I) and between the chemokine N-terminal and receptor extracellular loop/transmembrane residues (Site-II) (Figure 4). Chemokines bind their receptors with nanomolar affinity, but how the two sites contribute to the overall affinity, and the thermodynamic basis of binding, such as whether the binding to two sites are independent or coupled, and the role of enthalpy and entropy, are not known [49]. Further, chemokines exist as both monomers and dimers, and the mode of binding, receptor selectivity, and affinity vary among different chemokine-receptor pairs [50, 51].

Chemokine CXCL8 exists as monomers and dimers, and binds two receptors, CXCR1 and CXCR2. Whereas CXCR1 binds only the monomer with high affinity, CXCR2 binds both the monomer and the dimer with high affinity. ITC studies of the binding of both monomer and dimer to the CXCR1 receptor N-domain peptide have shown that the dimer dissociates on binding, providing a structural basis for the lower receptor affinity of the dimer [41], and that the binding of the monomer is enthalpically driven [42]. A recent crystal structure of CXCR1 has shown that the N-terminal domain is unstructured [52]. Therefore, studies using isolated N-domain peptides most likely mimic binding to the intact receptor, indicating that ligand binding to the N-domain of the intact receptor is also enthalpically driven. Structural studies of the CXCL8-receptor N-domain complex have shown that a combination of packing/hydrophobic and electrostatic interactions mediate the binding process [53]. The thermodynamic signatures are interesting, because the entropic component of the binding is relatively small. Structures and dynamic measurements reveal that both the receptor and ligand, though structured, continue to be dynamic in the bound form, suggesting that entropy does play a role; further, it is possible that entropic contributions from the release/reorganization of bound water on binding also play a role. Recently, ITC was used to characterize IL-8 monomer binding to a series of CXCR1 and CXCR2 receptor constructs that consisted of N-domain (Site-I binding site) and/or an extracellular loop domain (Site-II

binding site) on a protein scaffold to understand whether Site-II influences the thermodynamics of Site-I binding [43]. This study showed that Site-II influences Site-I binding in the case of CXCR1 and not CXCR2, demonstrating how studying soluble functional domains could provide valuable knowledge, that is otherwise intractable, of the intact membrane-bound receptors. HIV infection of the host cells involves binding of the viral envelope protein gp120 to the CD4 receptor and chemokine CCR5 receptor. CCR5 N-terminal domain contains sulfated tyrosines, which are known to be critical for binding to gp120. Brower *et al.* have characterized the binding of gp120 to the CCR5 N-terminal sulfated and unsulfated peptides using ITC in presence and absence of CD4, and show that the gp120 binds to the sulfated CCR5 N-domain peptide only in the presence of CD4, that the binding is mediated by both favorable enthalpic and entropic interactions, and that the unsulfated peptide is inactive [44]. In addition to providing molecular insights, these studies also indicate that compounds containing sulfated aromatic compounds could act as potential inhibitors of HIV entry.

The parathyroid (PTH) receptor is a class-B GPCR receptor, and in contrast to class-A receptors, the N-domains of class-B receptors are folded and structured and could be easily visualized to function as independent structural and functional units. The structure of the ligand-bound PTH receptor N-domain has been solved by X-ray crystallography [45], providing insights into the binding specificity and how some of the conserved residues unique to PTH receptors mediate the binding process. In an independent study, ITC was extensively used to characterize the binding affinities of PTH peptides of various lengths and single substitution mutants of residues known to be important for binding, with the overall goal of designing high affinity drugs [46]. This study was able to identify and confirm various residues that function as hot spots, and also showed that increasing hydrophobic interactions could result in higher binding affinities, paving the way for the future structure-based design of therapeutics.

The C5a receptor, like chemokine receptors, is a class-A receptor, and is characterized by N-terminal tyrosine sulfation that is essential for function. Its endogenous ligand C5a is generated during complement activation, and plays an important role in combating infection by activating the C5a receptor. At the same time, the C5a receptor is also an attractive drug target, as a dysregulation in its response also results in collateral tissue damage and disease. Interestingly, *S. aureus* secretes a protein called CHIPS, which binds to the C5a receptor and prevents its activation, allowing the bacterium to evade the host immune response. CHIPS binds to the sulfated receptor N-domain, and ITC studies have shown that CHIPS bind to various singly and doubly sulfated N-domain peptides with nM affinities [47]. ITC measurements of the unsulfated peptide showed reduced binding affinities whereas phosphorylated peptides showed similar nM binding affinities, indicating that negative charge, and not sulfation in itself, is critical for the binding process [48].

These studies using isolated receptor domains indicate that membrane proteins can be classified into those which contain a soluble ligand binding domain and those which do not, that thermodynamic insights for the former class can be captured by measuring binding to recombinant soluble ligand binding domains with out any requirement for detergents, and that these measurements most likely reflect binding to these domains in the full-length receptor. However, for the latter class, binding measurements can be carried out only using detergent solubilized membrane proteins.

Conclusions

In this review, we have discussed the various studies on the thermodynamic characterization of ligand-membrane proteins interactions using ITC; we have highlighted the knowledge

gained and the challenges that are multifactorial from purifying membrane proteins in large quantities to experimental setup and data analysis. Recent advances in protein expression and purification, detergents, instrumentation and data analysis together have contributed to considerable progress, considering that all of the published studies were carried out in the past decade, and that the only two studies on mammalian membrane proteins were reported in the last few years. We conclude that the recent successes are a harbinger of what we can expect in the coming decades, and ITC studies will undoubtedly provide valuable and unique knowledge on the molecular mechanisms and to the drug discovery process for a wide variety of human diseases.

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Highlights

- This review describes how ITC can be used to study membrane protein thermodynamics.
- We survey and summarize the literature on ITC studies of membrane proteins.
- Recent successes in membrane protein ITC bode well for similar future analyses.
- Such studies could provide unique insights into the drug discovery process.

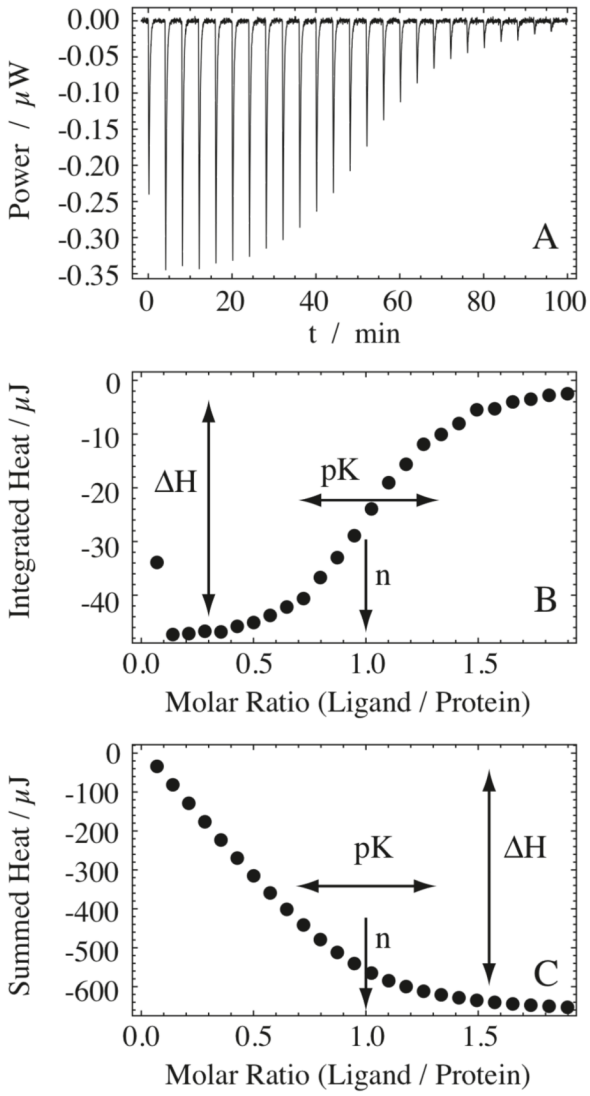

Figure 1.

A representative ITC dataset. (A) In the power vs. time titration curve, each peak represents an injection of ligand into the sample cell that contains protein. (B) Typical representation of ITC data. (C) Atypical representation of ITC data in the form of regular high-affinity binding curves.

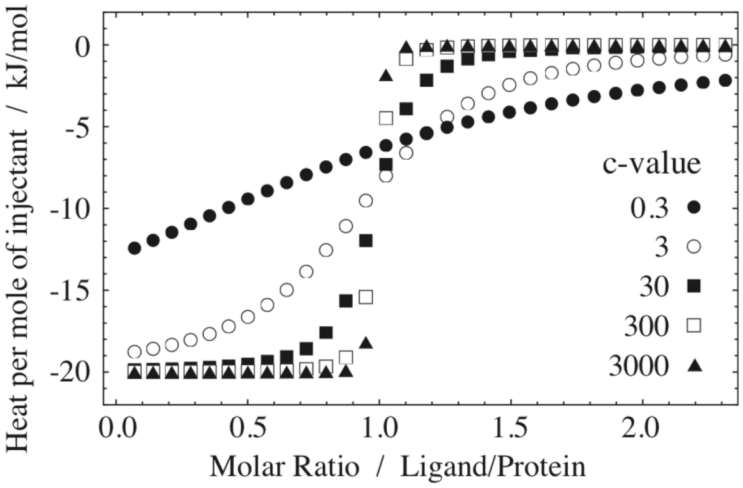

Figure 2.
Dependence of the shape of ITC curves on the c-value (the ratio between protein concentration and dissociation constant). The preferred range for the c-value is between about 3 and 100, with the optimum around 30.

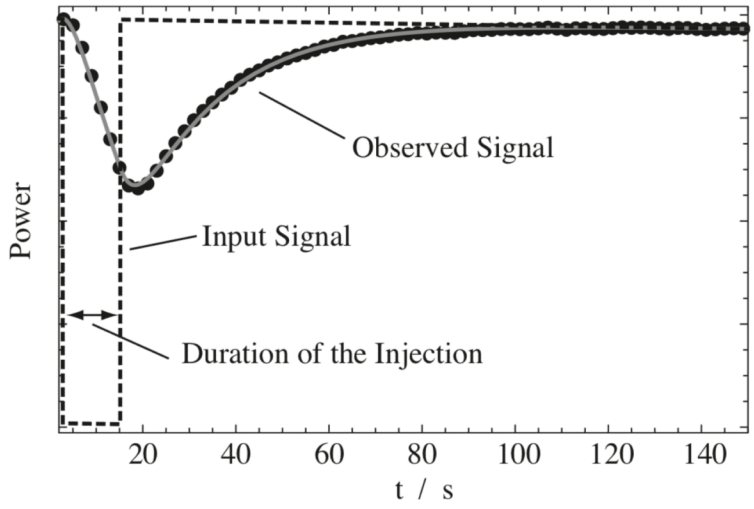

Figure 3.
Typical shape of an ITC peak (“observed signal”) caused by a 15-second injection. The ideal signal (“input signal”) would be obtained if mixing, binding reaction, and instrument response were infinitely fast.

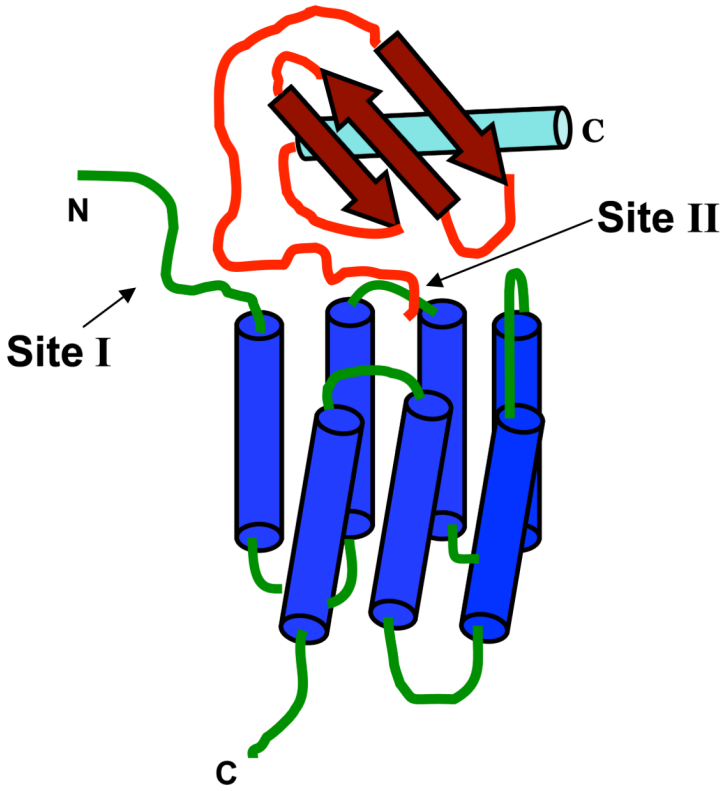

Figure 4.
A schematic of the two-site interaction of chemokine ligand binding to its receptor. Chemokine N-loop residues bind to the receptor N-terminal domain (defined as Site-I) and chemokine N-terminal residues bind to the receptor extracellular/transmembrane residues (defined as Site-II). In the case of RANTES, receptor binding and dimer interface residues overlap and so dimeric RANTES cannot bind the receptor. In the case of IL-8, dimer interface residues are located away from the receptor binding site, and so both the monomer and dimer can bind the receptor.

Table 1
ITC studies of membrane proteins and receptor extracellular domains

Membrane protein/ligand	Detergent	ref.
A) Mammalian membrane proteins		
i) chemokine receptor CCR5/RANTES	0.5% FosCholine-12	ref. 1
ii) Glycine receptor/ glycine and strychnine	~0.04 to 0.1 % DDM	ref. 2
B) Bacterial and other non-mammalian membrane proteins		
i) *E. coli* transhydrogenase/NADH	0.1% Brij-35	ref. 15
ii) *E. coli* AdiC transporter/Arginine	5 mM DM	ref. 17
iii) *N. meningitides* transferrin receptor/transferrin	0.05% DDM	ref. 18
iv) *T. elongates* PS II/herbicides	0.03% DDM	ref. 21
v) *E. coli* AcrAB-TolC multidrug efflux system	0.02% DDM	ref. 22
vi) *E. coli* Iron transporter FhuA/ microcin J25	1% OG	ref. 28
vii) *P. denitrificans* Surf1/ heme	0.02% DDM	ref. 29
viii) *E. coli* zinc transporter YiiP/Zn2+, Cd2+, and Hg2+	0.05% DDM	ref. 34
ix) *E. coli* vitamin B12 receptor/colicins	1% β-OG	ref. 35
x) *E. coli* MDR EmrE/drugs	8 % SDS/2% DDM	ref. 36
xi) *E. coli* MDR EmrE and SugE/drugs	8 % SDS/2% DDM	ref. 37
xii) Archaeal rhodopsin/transducer protein	0.05% DDM	ref. 38
xiii) *A. aeolicus* twin-arginine translocation protein/ signal peptide	0.02% LMNG	ref. 39
xiv) *E. coli* NhaA Na+/H+ antiporter/ Li2+	0.04% DDM	ref. 40
C) GPCR Receptor domains		
i) CXCR1 N-domain/IL-8 dimer and monomer		ref. 41
ii) CXCR1 N-domain/IL-8 monomer		ref. 42
iii) CXCR1 extracellular domains/IL-8 monomer		ref. 43
iv) CCR5 N-domain/CD4/HIV gp120		ref. 44
v) PTH receptor N-domain/PTH		ref. 45
vi) PTH receptor N-domain/PTH variants		ref. 46
vii) C5a receptor N-domain variants/CHIP		ref. 47
viii) C5a receptor N-domain variants/CHIP		ref. 48

Abbreviations – LMNG, lauryl maltose neopentyl glycol; DDM, dodecyl maltoside; OG – octyl glucoside; DM – decyl maltoside; SDS –sodium dodecyl sulfate