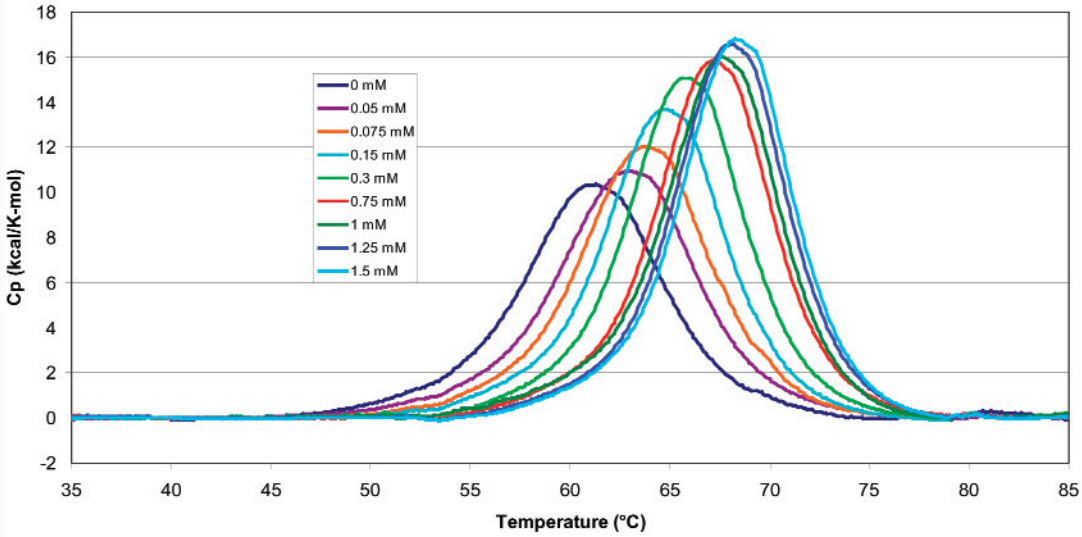
Characterizing Membrane Protein/Ligand Binding by DSC

Christin T. Choma
TA Instruments, 109 Lukens Drive, New Castle, DE 19720, USA



As the concentration of ligand bound to a protein receptor increases, the thermal stability of the protein/ligand complex increases.

Like ITC, DSC is a valuable approach for studying binding between a biological macromolecule and a ligand such as another biopolymer or a drug. Unlike ITC, DSC allows the thermodynamics that drive binding to be correlated, at least to a degree, with conformational changes in the macromolecule caused by the binding reaction. DSC is particularly useful for characterizing very tight or slow binding interactions. DSC also allows characterization of binding reactions that are incompatible with the organic solvent requirements of some ITC experiments (i.e., where ligand solubility for an ITC experiment requires concentrations of organic solvent not tolerated by the protein).

All proteins are capable of recognizing and binding specific molecules such as other proteins, cofactors, prosthetic groups or drugs. Efforts to understand the mechanisms controlling selective binding were initially prompted by the realization that recognition and binding are universal features of all biochemical processes. These efforts have intensified with the awareness that knowledge-based drug design requires not only high-quality structural data on both the protein and the drug candidate, but also a quantitative understanding of the thermodynamics driving binding. A ligand will bind to a protein (or other macromolecule) only if the resulting complex is more stable than the original, non-liganded protein. Binding can occur to the native, folded protein (stabilizing the native state), or it can bind preferentially to the denatured protein, in which case the ligand will destabilize the native protein. In either case, binding triggers changes in intramolecular and intermolecular interactions, and in the dynamics of both the protein and the ligand. Since the degree of stabilization or destabilization of the native protein depends on the magnitude of the binding energy, comparison of the stability of the complex with the stability of the ligand-free protein allows the binding energy to be estimated.

Differential scanning calorimetry (DSC) is particularly suited to studying the thermodynamics controlling conformational transitions in macromolecules such as proteins. As explained in CSC's overview note entitled 'Life Science Applications of DSC', DSC is generally used to measure the partial molar heat capacity of a protein over a temperature range of approximately 80 °C. If a ligand binds preferentially to the native state of the protein, the temperature at which the protein-ligand complex denatures will be higher compared to the temperature at which the free protein unfolds. DSC thus provides a direct measure of whether ligand binding to a protein is stabilizing or destabilizing, and so can complement studies of binding equilibria obtained by isothermal titration calorimetry (ITC). For a discussion of the utility of ITC in binding studies, please see CSC's application note entitled 'Characterizing protein/ligand binding by ITC'.

Assessing ligand binding by DSC

Proteins are large and flexible, and constantly sample the continuum of conformational states from partially folded to fully native. The native state itself is not one conformation, but a rapidly-changing ensemble of closely related structures (Ringe and Petsko, 1985; Palmer 1997). The binding between a flexible protein and a small ligand therefore produces a complex energy profile involving many favorable and unfavorable contributions. The thermodynamic assessment of a binding event is further complicated by the fact that ligands such as drug molecules often contain hydrophobic moieties which interact with hydrophobic patches on the surface (or in the binding crevice) of the protein. Water is more ordered adjacent to hydrophobic surfaces (Shinoda, 1977); when the protein and ligand bind and hydrophobic surfaces interact, bound water at these surfaces is transferred into the bulk solvent. Since the free energy of the entire system must decrease if binding is to occur, these solvent effects must be accounted for in addition to the energy changes resulting from direct noncovalent interactions between the protein and ligand (Jelesarov and Bosshard, 1999).

ITC is the most direct approach for assessing binding interactions: several rapid incremental or continuous ITC experiments can provide a direct determination of the binding constant, the stoichiometry of binding, the enthalpy and entropy of the reaction, and the change in heat capacity due to binding. In contrast, DSC indirectly estimates the binding constant from measurements of the equilibrium between the folded and unfolded protein. However, characterizing binding by DSC is distinctly advantageous if a) the binding constant is extremely high, or binding is extremely slow and cannot be measured by ITC or b) if the ligand is only soluble in organic solvents such as dimethyl sulfoxide (DMSO) or dimethylformamide (DMF). Organic solvents are problematic in ITC experiments in two circumstances. The first is if formation of the ligand/protein complex produces only a small heat of binding. This heat can be masked by the high heat of dilution of the organic during an ITC experiment, although careful matching of organic concentrations in the blank and sample titrations can often (but not always) overcome this obstacle. For example, Fig. 1 shows the heat of dilution of a 5% DMSO solution titrated into actetate buffer, plotted above the heat released when 2'-CMP binds to RNase A. The 2'-CMP/RNase system yields heats of binding substantially higher than that obtained from many protein/ligand systems, but even so its heats are dwarfed

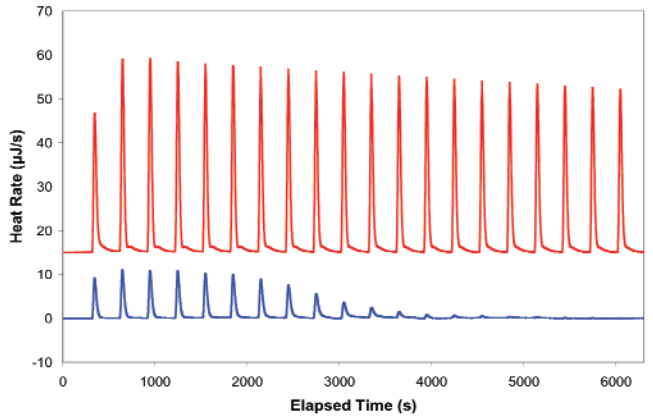


Fig. 1. ***Red:*** *DMSO (5% v/v in 15 mM acetate buffer, pH 5.5) titrated into acetate buffer in 20, 5 µL increments at 25 °C.* ***Blue:*** *RNase A (80 µM in acetate buffer) titrated with 20, 5 µL aliquots of 2'-CMP (1.58 mM in acetate buffer) at 25 °C. Scans are offset for clarity.*

by the heat of dilution of the DMSO solution. Clearly, data from protein/ligand systems producing smaller heats of binding could not provide meaningful results if there was any mismatch between the concentration of DMSO in the sample cell and in the syringe.

The second potential problem with organics arises if the concentration of solvent required to solubilize sufficient ligand for an ITC binding study exceeds the protein's tolerance for the solvent. 'Batch' DSC studies may require lower concentrations of organic solvent since some ligand will immediately bind to the protein and be removed from solution, necessitating less solvent to solubilize the remaining (unbound) ligand.

a) DSC measurements of very tight or very slow binding:

As noted in the CSC ITC application notes 'Characterizing Binding Interactions by ITC' and 'Characterizing Binding Using Continuous Isothermal Titration Calorimetry', the most accurate ITC binding constant measurements are obtained for reactions with binding constants in the range 10^2 to 10^9 M^{-1}. This range of accessible binding constants can be extended by using competitive binding conditions, but extremely tight binding (picomolar or higher) may be difficult to assess. Since DSC compares the extent to which the ligand/protein complex is stabilized towards thermal denaturation compared to the free protein, DSC allows estimation of binding energies for extremely tightly associated complexes, and also of complexes that equilibrate very slowly (hours to days) and thus are not compatible with the ITC timeframe (seconds to minutes) (Kroe *et al.*, 2003). In addition, by conducting several DSC binding experiments at various concentrations of reactants, the population of free and bound species can be calculated as a function of temperature and concentration (Holdgate and Ward, 2005).

If a ligand (L) binds to the native conformation of a protein (P) with high affinity, the resulting protein/ligand complex (PL) will have a higher thermal stability than either of the two free components (Jelesarov and Bosshard, 1999). Figure 2 shows an idealized example where the stabilized complex has a significantly higher melting temperature than the protein or ligand alone. The symmetry of this PL peak suggests that the dissociation and thermal denaturation of the complex are tightly coupled. In practice, the shape of the protein/ligand endotherm can be quite complex due to the presence of intermediates and/or denatured protein, the sensitivity of the complex to pH and ionic strength, or to the ligand preferentially binding to and stabilizing only part of the protein (*e.g.*, one domain) (Brandts and Lin, 1990; Jelesarov and Bosshard, 1999; Luque *et al.*, 2002). For many applications, only an approximation of the T_m of the complex (the temperature at which half the protein/ligand complex molecules are folded and half are unfolded) *vs.* the T_m of the free protein is required; this information can generally be obtained by visual inspection of the endotherms. However, since the free energy of complex formation results from a delicate

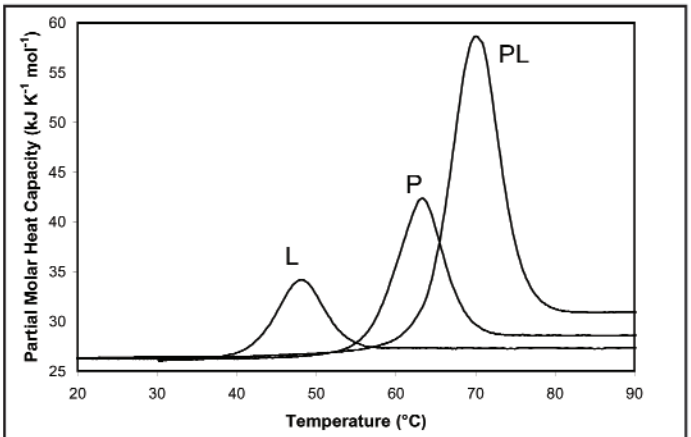


Fig. 2. Simulated DSC data showing the molar heat capacity of free ligand (L), free protein (P) and the protein-ligand complex (PL), where the complex is a tightly-associating system.

balance between large favorable and unfavorable entropic and enthalpic contributions, precautions must be taken not to over-interpret the data. For example, binding driven by hydrophobic interactions (an entropic effect) tend to give rise to larger shifts in T_m than enthalpically-driven binding (*e.g.*, changes in solvation). Therefore, a large observed T_m shift is not necessarily an indication of high affinity binding, since a range of different affinities, with different entropic and enthalpic contributions, could result in the same T_m (Holdgate and Ward, 2005).

If actual estimation of the binding constant is required, the equations derived by Brandts and Lin (1990) (reviewed by Holdgate and Ward (2005) and Bruylants *et al*. (2005)) can be applied. The equations make the following assumptions: 1) the ligand concentration is much larger than K_a; 2) unfolding is reversible and two-state; 3) ligand does not bind to the unfolded protein; 4) all measurements are made at equilibrium. The association constant for a single ligand molecule binding per protein molecule at the melting temperature of the complex is given by:

$$K_a^{Tm} = \frac{\exp\{\frac{-\Delta H_{D-N}}{R}(\frac{1}{T_m}-\frac{1}{T_0})+\frac{\Delta C_{pD-N}}{R}(\ln(\frac{T_m}{T_0})+\frac{T_0}{T_m}-1)\}-1}{[L]}$$

where $\Delta H_{D\text{-}N}$ is the enthalpy change for the unfolding of the uncomplexed protein (native (N); denatured (D)) obtained from the area under the transition peak, R is the universal gas constant, T_0 is the melting temperature of the free protein, T_m is the melting temperature of the protein/ligand complex, $\Delta C_{pD\text{-}N}$ is the change in heat capacity of the free protein upon unfolding measured from the shift in baseline before and after the unfolding transition, and [L] is the concentration of the free ligand at T_m.

In order to compare the binding constants of various ligands, each producing a protein/ligand complex with a different stability, each K_a^{Tm} must be extrapolated to the same reference temperature (typically 25 °C):

$$K_a^{Tref} = \frac{K_a^{Tm}}{\exp\{\frac{\Delta H_L^{Tref}}{R}(\frac{1}{T_{ref}}-\frac{1}{T_m})-\frac{\Delta C_{pL}}{R}(\ln(\frac{T_{ref}}{T_m})+1-\frac{T_{ref}}{T_m})\}}$$

where ΔH_L^{Tref} is the enthalpy of ligand binding at T_{ref}, ΔC_{pL} is the heat capacity change of ligand binding, and T_{ref} is the reference temperature. Because the extrapolation to the reference temperature may be quite long, there is potential for significant error in K_a^{Tref}. However, for very slow binding (Kroe *et al*., 2003), for extremely tight binding affinities (K_a of 10^{12} M^{-1} or higher (Brandts and Lin, 1990; Bruylants *et al.,* 2005)), or for the binding of ligands soluble only in organic solvents (see below), DSC allows estimation of binding constants not accessible by other calorimetric approaches. In addition, changes in the shape of the endotherm upon ligand association can provide information on conformational changes triggered by binding. The deconvolution of multicomponent endotherms and the analysis of complex binding systems are described in Jelesarov and Bosshard (1999), Cooper *et al.* (2001), and references therein.

b) DSC measurements of ligand binding in the presence of organic solvents:

Hydrophobic ligands are often only soluble in the presence of organic solvents such as DMSO or DMF. Determining the binding characteristics of these ligands by ITC is problematic because, as discussed above, the heat of dilution of the organic solvent (as the ligand solution is titrated into the aqueous protein solution) can be substantially more than the heat of binding, making binding difficult to measure. This problem is addressed by matching as closely as possible the concentration of organic solvent titrated into both the sample and the blank, although any errors made in precisely matching the organic concentration will invalidate the experiment. A more insurmountable problem in ITC is if the final concentration of organic required to deliver the ligand exceeds the protein's tolerance for the solvent. This problem can potentially be overcome using DSC, since during the preparation of the 'batch' samples, some ligand will immediately bind to the protein and be removed from solution, leaving less ligand to be solubilized and resulting in an overall lower concentration of solvent being used for the experiment.

The RNase/2'-CMP system was used to determine if DSC measurements in an aqueous/organic solvent mixture can provide reasonable estimates of the binding constant. A series of RNase A samples (RNase A concentration 46 μM) containing increasing concentrations of 2'-CMP (0 to 1.5 mM) in the presence or absence of 5% (v/v) DMSO were scanned from 20 – 95 °C at 1 °C/min using an N-DSC III. Data were analyzed for T_m and calorimetric enthalpy.

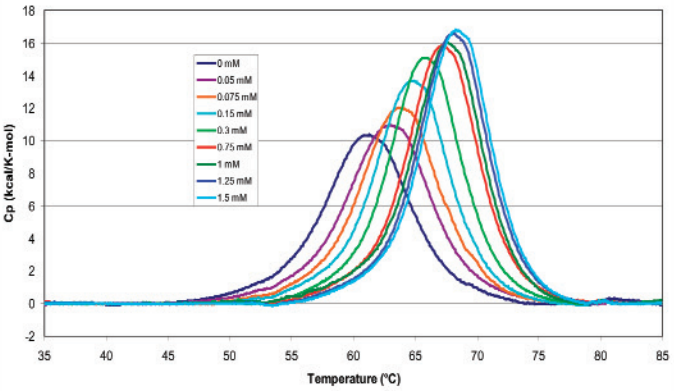


Fig. 3. Increasing concentrations of 2′-CMP (0 – 1.5 mM) added to 46 μM RNase A in 15 mM acetate buffer, pH 5.5. Samples were scanned at 1 °C/min. Comparable data were obtained in the presence of 5% DMSO.

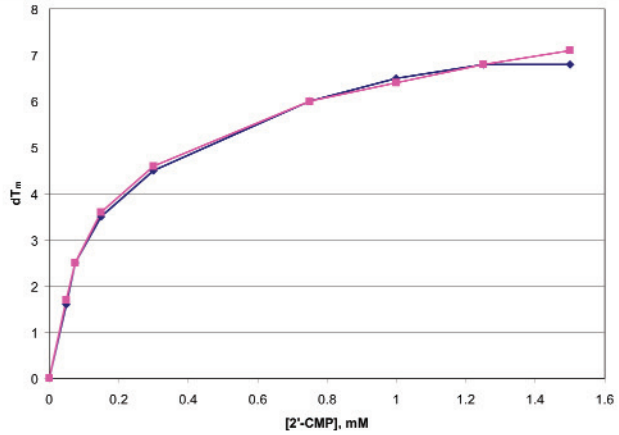


Fig. 4. The change in T_m (dT_m) with increasing concentration of 2′-CMP. Blue: without DMSO; magenta: 5% DMSO in the sample.

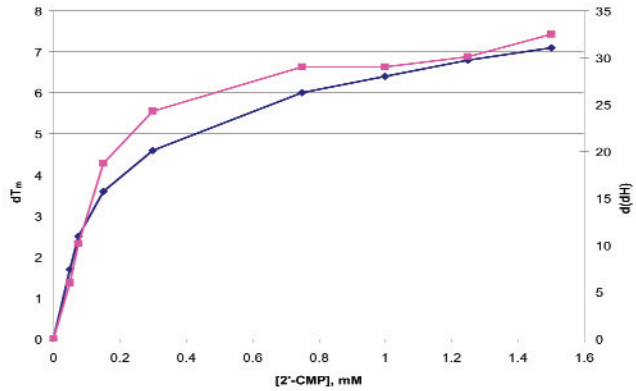


Fig. 5. The change in calorimetric enthalpy (d(dH), magenta) with increasing 2′-CMP concentration in the absence of organic solvent compared to the corresponding increase in T_m (blue). Comparable results were obtained in the presence of 5% DMSO.

In both the presence and absence of DMSO, T_m increased with increasing concentration of 2′-CMP (Fig. 3). Samples in 5% DMSO consistently unfolded approximately 1 °C lower for a given concentration of 2′-CMP than in the absence of organic solvent, but the incremental increase in T_m with increasing ligand concentration was the same for both data sets (Fig. 4). In addition, the change in calorimetric enthalpy with increasing 2′-CMP concentration followed the same general trend as the change in T_m (Fig. 5) whether DMSO was present or not.

The above results show that the temperature of unfolding is more accurately determined than the enthalpy of unfolding, as the former is a direct measurement while the latter is affected by the accuracy of the baseline fit under the transition peak. The change in T_m with increasing ligand concentration was therefore used to estimate the binding constant of 2′-CMP both in the presence and absence of DMSO. This approach required two simplifications: first, that the shift in T_m is due solely to the change in concentration of 2′-CMP and second, that K_a remains constant despite the increasing T_m. Fitting the data provided a K_a of 5900 M^{-1} in the range 61-68 °C in the absence of organic solvent, and a K_a of 6900 M^{-1} in the presence of 5% DMSO. The validity of these estimates was tested by ITC. 2′-CMP was titrated into 46 μM RNase A at 50 °C (just below the start of the RNase unfolding transition in the absence of 2′-CMP); low salt concentrations (15 mM acetate buffer, pH 5.5) identical with the DSC experiments were used, and no DMSO was added. A K_a of 76,500 M^{-1} was calculated. This is consistent with values reported in the literature for this system (Wiseman *et al.*, 1989; Horn *et al.*, 2001) given that i) a low salt buffer was used (salt increases T_m; Murphy *et al.*, 2004) and ii) the ITC experiment was conducted at 50 °C, 10 – 18 °C lower than the DSC data (increasing temperature substantially decreases K_a; Horn *et al.*, 2001). These results support the validity of using DSC to estimate binding constants under conditions incompatible with ITC measurements.

Summary:

Although ITC remains the method of choice for determining binding constants, DSC can provide estimates of K_a where binding occurs very slowly, is too tight to be measured by ITC, or where high concentrations of organic solvent are required. In addition, when combined with structural and ITC data, DSC provides an independent approach to elucidating the free energy changes accompanying the formation of protein/ligand complexes, which in turn can shed light on the mechanism of the association reaction. Used together, ITC and DSC provide the most direct, comprehensive approach for monitoring and interpreting binding interactions involving biological macromolecules.

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(Preference has been given to current references. Citation does not imply that a paper is necessarily the original reference to a study.)

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