Characterizing non-covalent nucleic acid interactions with small molecules and proteins by calorimetry

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The expression or replication of genes is affected by the binding of small molecule ligands and proteins to nucleic acid sequences. Such binding events are critical for the physiological integrity of organisms and therefore are of fundamental interest to life scientists. Recently, the thermodynamics driving these interactions have also become important to pharmaceutical scientists investigating the anticancer, antibacterial and antiviral potential of nucleic acid/ligand interactions. In addition, as the number of diseases identified as being due to a malfunction of cellular control processes increases, the possibility of treating disorders by manipulating gene expression is further focusing attention on the thermodynamics underlying nucleic acid binding affinity and specificity.

Calorimetry is the most accurate and rapid approach for obtaining direct thermodynamic information which, in combination with high resolution structural data and mechanistic studies, provides the most complete picture possible of the factors involved in the recognition and binding of nucleic acids to ligands. This application note examines the utility of differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) for characterizing the energetics governing nucleic acid/ligand complexes. For general descriptions of the principles behind DSC and ITC, please see TA's overview notes entitled *Life Science Applications of DSC* and *Life Science Applications of ITC*.

Non-covalent nucleic acid/small molecule binding interactions

Since the impetus for understanding the rules governing recognition and binding to nucleic acids largely arises from interest in designing drugs capable of sequence- and structure-specific nucleic acid recognition, most thermodynamic binding studies on nucleic acids have focused on small molecule, drug-like ligands binding to DNA. The enthalpic and entropic contributions to nucleic acid binding are the same as those driving protein/ligand interactions: increased number of hydrogen bonds, more favorable van der Waals interactions, and electrostatic and polar interactions (all enthalpic factors), and conformational changes and the release of bound solvent (entropic factors). The physical basis and relative importance of these contributions are discussed in Gohlke and Klebe (2002). However, although these interactions are the same as those driving protein/ligand binding, the models used for ligand binding to proteins cannot be used for ligand binding to nucleic acids. This is primarily because the three dimensional structures of nucleic acids generally lack the intricacy of protein structures, requiring that nucleic acids be modeled as a lattice. Thus, the statistics governing ligand binding to proteins and nucleic acids are different, a distinction that is not always appreciated in the published literature.

Small molecules usually bind non-covalently to double-stranded DNA either by intercalation or minor groove binding (Haq, 2002). Intercalation generally occurs when the ligand contains flat heteroaromatic rings that can insert between two adjacent base pairs

and be stabilized by π–π interactions. Intercalation structurally perturbs the DNA, causing it to unwind and lengthen slightly. In contrast, ligands which bind in the minor groove often contain several aromatic groups connected by rotationally-flexible linkers, allowing the ligand to insert and follow the curve of the groove without disturbing packing. The binding of these ligands is stabilized by van der Waals interactions, hydrogen bonds and hydrophobic interactions, in particular between the ligand and A-T base pairs (Haq *et al.*, 2000).

General thermodynamic considerations:

The energetics involved in reversible reactions can be most directly determined by calorimetry because calorimetry alone directly measures the enthalpy of a reaction; all other techniques require that enthalpy be calculated from measurements, which introduces a degree of inaccuracy. The enthalpy, entropy, free energy and binding constant of the reaction are all related and defined at equilibrium by the standard thermodynamic relationship:

$$\Delta G^o = -RT \ln K_a = \Delta H^o - T\Delta S^o \qquad (1)$$

where ΔG^o, ΔH^o and ΔS^o are the changes in the equilibrium free energy, standard enthalpy and standard entropy, respectively, R is the gas constant, T is the absolute temperature and K_a is the equilibrium concentration of the complex divided by the concentrations of the free reactants. The effect of temperature on the free energy is described by:

$$\Delta G(T) = \Delta H(T_{ref}) + \int_{T_{ref}}^{T} \Delta C_p dT - T\Delta S(T_{ref}) - T\int_{T_{ref}}^{T} \Delta C_p d\ln T \qquad (2)$$

where ΔC_p is the change in heat capacity and T_{ref} is the reference temperature. At constant pressure, the heat capacity can be defined in terms of the change in enthalpy or entropy with temperature:

$$\Delta C_p = \left(\frac{\partial \Delta H}{\partial T}\right)_p = \left(T\frac{\partial \Delta H}{\partial T}\right)_p \qquad (3)$$

The binding constant and the stoichiometry of a reaction can be determined from a single ITC experiment, but probing the temperature dependence of the equilibrium, which is reflected in the enthalpy change for the binding, requires titrations at at least two (and preferably several) temperatures. Measuring ΔH as a function of temperature by ITC allows, in principle, the change in the heat capacity for the binding reaction to be determined, as discussed below. Guidelines for designing DNA-drug ITC binding experiments are outlined in detail in the review by Haq *et al*. (2001), while more general considerations for experiment design are presented in the TA application note *Characterizing binding interactions by ITC*, and in O'Brien *et al*. (2001). A review of current approaches for analyzing complex DNA/ligand ITC binding data is provided by Buurma and Haq, 2007.

Heat capacity

The heat capacity of a compound or complex arises from vibrations and rotations of all the molecules in the reaction, including the solvent. The heat capacity of a biopolymer clearly will change as it changes conformation upon ligand binding, and this change can be measured as the temperature dependence of the enthalpy or entropy. As equation 3 implies, ΔH and ΔS for a given transition change together, so transitions with large changes in heat capacity could exhibit a degree of enthalpy/entropy compensation. However, care must be taken not to over-interpret the data: since the enthalpy and entropy values are derived from the same data, apparent compensation could merely be an artifact of statistical coupling

between the calculated values.

The relative contributions of different physical processes to heat capacity changes in proteins have been studied extensively (Cooper, 2005), but there have been fewer comparable studies on nucleic acids. Given the highly ordered localized, repetitive structure of nucleic acids, and their very ionic nature (resulting in a large number of associated counterions), the relative importance of each factor to the heat capacity of nucleic acids is likely different from what it is for proteins (Mikulecky and Feig, 2006). However, whether heat capacity effects in nucleic acids are due primarily to differential solvation of polar and nonpolar surfaces, or are the general result of order-disorder transitions in a system stabilized by many weak interactions (Cooper, 1999), the heat capacity of nucleic acids is observed to change non-linearly with temperature, and increases during the binding reaction. Therefore, binding studies conducted over even a relatively narrow temperature range need to take into account the non-linear change in heat capacity of the nucleic acid as well as potential temperature-dependant changes in structure (Mikulecky and Feig, 2006). Inherent difficulties and necessary precautions when performing calorimetric studies on various types of nucleic acids are comprehensively summarized by Mikulecky and Feig (2006).

Traditionally, ΔC_p is determined by measuring ΔH as a function of temperature and taking the slope of ΔH vs. T. In practice, this provides only an estimate of ΔC_p due to the magnification of any errors in ΔH on taking the derivative. In addition, the heat of association measured by ITC includes heats associated with conformational rearrangement of the reactants, which should be subtracted from the heat of binding. The change in heat capacity is most accurately determined from DSC scans conducted on an instrument with an ultra-stable and reproducible baseline, such as the TA Nano-DSC (Privalov and Dragan, 2007). The change in molar heat capacity of each reactant due to conformational changes and hydration effects is measured by DSC, then the individual molar heat capacities of the reactants are subtracted from the molar heat capacity of the complex to provide the change in heat capacity for the binding reaction. These measurements are direct, do not involve taking derivatives, and allow the heat of solute and solvent rearrangement to be separated from the heat of binding. An excellent review of the importance of heat capacity in protein thermodynamic studies is provided by Privalov (2007).

Estimating the enthalpic and entropic contributions of DNA-ligand binding

Two ligands can bind to a nucleic acid target with similar affinity and yet, due to different enthalpic and entropic contributions, the energetics driving the reaction can be quite different. In general, an interaction dominated by enthalpic contributions (arising from an increased number of hydrogen bonds, favorable van der Waals interactions and polar effects) would give rise to more specific binding than an equally tight association dominated by entropic contributions (conformational changes and release of bound water as hydrophobic groups interact). Complete dissection of all the enthalpic and entropic contributions to a binding reaction is not presently possible, but the problem can be partially deconvoluted using calorimetry together with computational approaches.

Calculations of binding parameters from an ITC experiment generally assume that binding only involves the target molecules in the syringe and calorimeter cell, whereas in fact there are also interactions between the reactants and ions, solvent and protons. The binding constant is therefore dependant on salt concentration and pH, as well as on temperature and pressure. Since nucleic acids are polyanions, the binding of a positively charged ligand to its target sequence will displace cations clustered around the binding site phosphate groups. Nucleic acid binding reactions are therefore generally highly sensitive to salt concentration. The free energy of binding is comprised of two terms: an electrostatic component (generally favorable and largely due to the ionic nature of the nucleic acid,

which can be quantified from the dependence of the equilibrium constant on salt concentration; Chaires, 1996), and a non-electrolytic component. The non-electrolytic component is comprised of free energy contributions from conformational changes in the nucleic acid and ligand upon binding, losses in rotational and translational freedom upon complex formation, hydrophobic transfer of the ligand from solution to the binding site, and from non-covalent ligand-nucleic acid interactions (Chaires, 1998).

Conformational changes in DNA upon ligand binding, and the loss of rotational and conformational degrees of freedom, present free energy barriers to binding that must be overcome by favorable contributions from hydrophobic, non-covalent and electrostatic components. The change in free energy due to groove binding has been reported to be close to zero (Haq, 2002), which could suggest (but does not prove) that groove binding demands little conformational change in either the nucleic acid or the ligand. In contrast, DNA melting studies clearly show that intercalation carries a penalty of between 5 to 10 kcal/mol for formation of the intercalation cavity (Chairs, 1998). For both intercalators and groove binders, the rotational and translational cost of ligand binding to DNA is approximately 15 kcal/mol (Spolar and Record, 1994).

The remaining non-electrolytic components, together with the (generally favorable) electrolytic contribution, must overcome unfavorable entropy due to losses in conformational, rotational and translational freedom. For both intercalating and groove binding ligands, the major favorable contribution is due to hydrophobic effects arising from the transfer of the ligand from aqueous solution to the interior of the DNA molecule or to its minor groove. Changes in the solvent accessible surface area have been correlated with heat capacity changes, allowing the free energy contribution from the hydrophobic interactions to be estimated using heat capacity measurements from ITC or DSC (Chaires, 1998). Evaluation of the last contribution to the free energy of binding, non-covalent interactions such as hydrogen bonds and van der Waals interactions, is best addressed by systematically altering specific ligand functional groups followed by ITC binding studies. Although this process is laborious, the hope is that, once a database of binding free energies correlated with specific interactions is established, this data base will provide a training set against which computational approaches can be tested, eventually eliminating the need to experimentally determine this complex component of the free energy of binding (Chaires, 1998; Haq and Ladbury, 2000).

Practical considerations regarding heat capacity and other calorimetric measurements of DNA/ligand interactions are presented in Haq *et al*. (2000). Detailed calorimetric, structural and theoretical studies on a number of ligands have focused on identifying specific differences in the binding modes of intercalators and groove binders, and the targeting of ligands to duplex, triplex and tetraplex nucleic acid structures.

Thermodynamics driving minor groove binding and intercalation, studied by ITC

The best studied minor groove binder is the dye Hoechst 33258, which binds to AT-rich domains of double-stranded DNA. ITC studies in conjunction with other techniques (Haq *et al.,* 1997; Han *et al.,* 2005) have shown that at physiologically-relevant temperatures, the binding of Hoechst 33258 is entropically driven, with a free energy change of -11.7 kcal mol^{-1} at 25 oC, and a large negative change in heat capacity (-330 cal mol^{-1} K^{-1}). Hydration provides a large contribution to the binding entropy, about 71 kcal mol-1 K-1, consistent with each bound Hoechst 33258 displacing about 55 water molecules from the minor groove of the DNA (Han *et al.,* 2005). Configurational, rotational and translational contributions to entropy from both the dye and the DNA were determined to be insignificant, and the binding of the ligand is accompanied by the displacement of only a single cation (typically Na^{+}) from the minor groove (Han *et al.,* 2005). These analyses show that

the binding of Hoechst 33258 to DNA is overwhelmingly driven by hydrophobic effects, with new hydrogen bonds between the ligand and the DNA replacing hydrogen bonds from the displaced water molecules. This conclusion was somewhat unexpected, as NMR and crystallographic structures indicated a fairly optimized network of hydrogen bonds and van der Waals interactions between the ligand and DNA. In the event, these interactions were actually shown to be slightly unfavorable.

Much of the impetus behind studies such as those on Hoechst 33258 is the desire to understand binding thermodynamics sufficiently so that therapeutic agents can be designed to bind tightly to specific stretches of DNA. The results from the Hoechst 33258 studies serve to highlight the difficulty of designing sequence-specific nucleic acid ligands, since affinity may often be driven primarily by (relatively non-specific) hydrophobic interactions. In addition, small molecules can only interact with a few base pairs, which is insufficient to direct them to a unique binding site on the human genome (which would require on the order of 15 base pairs). Studies comparable to those conducted on Hoechst 33258 have been reported for four other minor groove binding ligands (propamidine, netropsin, distamycin and berenil; Haq, 2002) and show that in all cases, binding is accompanied by a large negative change in heat capacity, consistent with changes in solvent accessible surface. However, whereas the binding of Hoechst 33258 is entopically driven, of these four ligands all but berenil are enthalpically driven, suggesting that it may not yet be possible to generalize about the binding mechanism of small minor groove ligands.

Although the binding of intercalators is also accompanied by a negative change in heat capacity, ITC experiments with five intercalating ligands indicated that this change is in general smaller than that for minor groove binding ligands (Ren *et al.,* 2000). The change in free energy due to hydration effects was large, showing that the transfer of the ligand from solvent to the intercalation site was a significant factor driving the binding process. However, in contrast to minor groove binding, which is largely driven by hydrophobic interactions, analysis of all the components contributing to the overall free energy change accompanying intercalator binding showed that non-covalent interactions, and in particular hydrogen bonding, contribute as much as hydrophobic interactions to binding (Haq, 2002). There is indeed some evidence that intercalators can be tailored to target DNA sequence-specifically more readily than minor groove binding ligands (Ren *et al.,* 2000). However, caution is required when designing and interpreting ITC experiments with intercalators, as at high concentrations, intercalators can stack and form self complexes.

DNA sequences designed to fold into triplexes which recognize gene-specific stretches of DNA, or which interact with transcription factors that bind DNA, have been characterized thermodynamically (Jenkins, 2000), as have G-rich single-stranded DNA molecules that fold into tetraplex conformations and target telomerase, an enzyme over-expressed in tumor cells (Olsen *et al.,* 2006). To date, however, too few thermodynamic studies have been completed to allow general conclusions regarding the energetics driving these higher-order structure interactions to be drawn.

DSC studies of DNA-ligand binding.

Although ITC is generally the most direct approach for measuring binding constants, there are situations when estimating binding constants by DSC is preferable: namely, when the binding constant is very high, or when the ligand is too poorly soluble to prepare a solution to titrate into a DNA sample. The principle and experimental approach behind estimating binding constants from DSC scans of ligand/receptor complexes is described in detail in the TA application note entitled *Characterizing protein/ligand binding by DSC*. Revisiting these principles in the context of nucleic acids, if a small molecule such as a groove binder or intercalator binds preferentially to duplex DNA, it will stabilize the

duplex structure and elevate its melting temperature (alternatively, if the ligand binds preferentially to single-stranded DNA, the melting temperature of the sample will decrease as the equilibrium shifts towards the single-stranded structure). If the double-stranded DNA is saturated with ligand, if the ligand does not bind to single-stranded DNA, and if the enthalpy of the melting of the DNA is known, the shift in the midpoint of the thermal unfolding can be used to estimate the binding constant of the ligand:

$$\frac{1}{T_m^0} - \frac{1}{T_m} = \frac{R}{\Delta H_{DNA}} \ln[(1+K_h L)^{1/n}]$$

where T_m^0 and T^m are the melting temperature in Kelvin of the DNA and the DNA-ligand complex, respectively, R is the gas constant, ΔH_{DNA} is the enthalpy of melting a DNA base pair, K_h is the binding constant for the ligand at T_m, L is the ligand concentration and n is the size of the binding site expressed in base pairs (Crothers, 1971; McGee, 1976; Spink and Wellman, 2001). ΔH_{DNA} is determined from the number of base pairs and the enthalpy of denaturation of the DNA alone, while n is determined either by ITC or from fitting melting curves at various non-saturating ligand concentrations (Chaires 1998). At non-saturating concentrations, complex DSC thermograms are obtained arising from the dissociation of ligand from a thermally-unfolded portion of the DNA molecule, followed by transient binding to an intact portion of the sequence (Crothers, 1971; McGee, 1976; Spink and Chaires, 1997; Leng *et al.,* 1998). Obtaining thermograms over a range of DNA-ligand concentrations provides detailed thermodynamic information on the binding process, as described in Spink and Wellman (2001).

Although the DSC approach is less direct than ITC, DSC can measure extremely high binding constants. This is because DSC does not depend on a signal from the ligand, and so the ligand can be present at an extremely low concentration. In addition, since samples can be prepared by equilibrating DNA solutions with solid ligand, the binding of very sparingly soluble compounds can be studied. However, a drawback of the DSC approach is that the binding constant can only be estimated at the temperature of unfolding of the complex, which is unlikely to be the temperature of interest (often 37 oC). Extrapolation from the unfolding temperature to the temperature of interest requires knowledge of the change in heat capacity for binding over that temperature range, or making the assumption (usually incorrect) that the heat capacity does not change over that temperature range.

Nucleic acid/protein binding interactions

In 1994, Spolar and Record published a study of the forces driving DNA site-specific recognition and binding by proteins. Prior to this it was known that structural changes occurred to double-stranded DNA when it bound to a protein, ranging from relatively minor deformations to sharp bending of the helical axis and disruption of base-pair stacking. Spolar and Record showed that site-specific protein-DNA recognition is accompanied by a large negative change in heat capacity, resulting from the removal of a large amount of water from non-polar surfaces during the binding event and a significant increase in the order of the protein at the binding site. Numerous studies since have demonstrated the generality of these conclusions, as reviewed in Jayaram and Jain (2004) and von Hippel (2007), amongst others.

Arguably the most detailed analyses of the thermodynamics driving DNA/protein binding have been by Privalov and coworkers. From a large body of publications, two are particularly noteworthy. In the first paper (Dragan *et al.,* 2004), DSC was used to study the thermodynamics driving DNA binding to two versions of a high-affinity DNA binding domain called the HMG box: sequence-specific HMG boxes, which show a strong preference

for a specific DNA sequence, and non-sequence specific HMG boxes, which bind a wide range of DNA sequences. Both categories of protein are partially unfolded in the absence of DNA, and become fully folded when DNA is bound. DSC showed that DNA binding to sequence-specific HMG boxes is accompanied by essentially no change in enthalpy and a negative change in heat capacity, while binding to non-sequence-specific HMG boxes is accompanied by a positive enthalpy change and a positive change in heat capacity. The authors conclude that the formation of DNA/sequence-specific complexes is specified by increased packing between non-polar groups, whereas the formation of non-sequence-specific complexes is driven mainly by electrostatics.

The second noteworthy paper (Privalov and Dragan, 2007) is a review of the thermodynamics driving protein binding to either the major or minor groove of DNA. Since the proteins become more ordered after binding DNA, this needs to be taken into account when

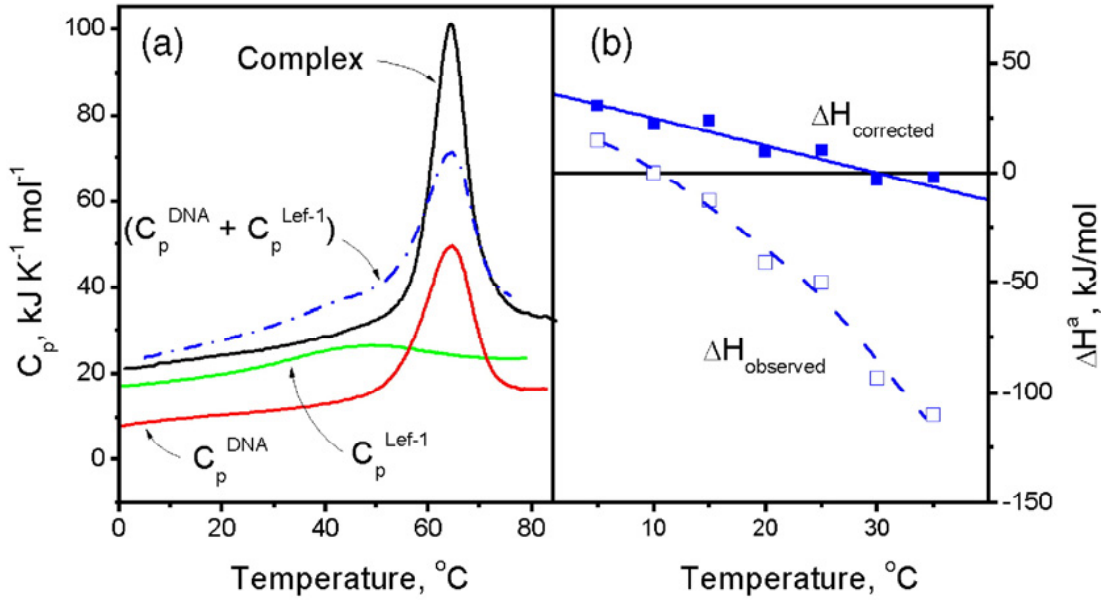

Fig. 1. (a) The heat capacity functions of a DNA binding domain (the HMG box from the protein LRF-1), the domain's target DNA duplex, and the DNA-domain complex. The uncomplexed protein slowly unfolds over a broad, low temperature range, whereas upon complexation with its DNA target, the protein folds and forms a stable complex that dissociates and unfolds cooperatively at approximately 62ºC. The heat of protein unfolding at any temperature can be determined by integrating the difference between the sum of the heat capacity of the free protein and the free DNA (dot-dashed line) and the heat capacity of the complex (black solid line). (b) The enthalpy of binding of the LRF-1 HMG box with its target DNA was measured by ITC at various temperatures, then the values were corrected for the heat of protein folding upon binding DNA. The corrected curve corresponds to the enthalpy of binding of the fully folded DNA binding domain with its target DNA sequence. Solutions for all experiments were extensively dialyzed against 10 mM potassium phosphate (pH 6.0), 100 mM KCl. Macromolecule concentrations for the DSC experiment were 0.2 mM. Samples were scanned on a TA Nano-DSC at 1 ºC/min. ITC experiments were conducted on a TA Nano-ITC. Protein (0.2 mM) in the syringe was titrated into DNA (16 µM) in the sample cell in 5 µL increments until the protein was saturated with DNA and no further binding occurred. Data from Privalov et al., 2007; used with permission.

analyzing temperature-dependant ITC data, as the observed titration heats are comprised of both the heat of binding and the heat of refolding. Following this correction (essentially accounting for the change in heat capacity of the protein), the enthalpies measured from ITC data at different temperatures followed a straight line when plotted vs. temperature, rather than a curved function (Fig. 1). The results from over 20 DNA binding proteins show, without exception, that the enthalpy change for protein binding to the minor groove is always positive, while the enthalpy change for binding to the major groove is always negative. However, the free energy of binding is similar for both categories of protein, indicating that the enthalpic differences are balanced by entropic factors. In fact, the entropic contribution of minor groove binding is larger than that of major groove binding. The different energy profiles for these two classes of protein are apparently due to differences in hydration of the major and minor grooves, with water being highly ordered in the minor groove, and substantially less so in the major groove. Thus, the removal of water from the minor groove upon protein binding is a major driving force for minor groove binding, while major groove binding appears to be driven by increased hydrogen bonding and van der Waals interactions.

CONCLUSIONS

The non-covalent interactions between nucleic acids and their small molecule and macromolecule ligands are governed by the same physical-chemical factors as those governing protein-small molecule and protein-protein interactions. However, due to the highly ionic nature of nucleic acids, and the partially unfolded structures of nucleic acid binding proteins in the absence of their ligand, studying these systems can be challenging. Accordingly, the models used for more 'typical' proteins cannot be applied indiscriminately to nucleic acid systems. Used correctly, however, calorimetry provides the most sensitive and reliable approach available for understanding the thermodynamics controlling the expression and regulation of genes.

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