

**Differential scanning calorimetry in life science: Thermodynamics, stability, molecular recognition and application in drug design**

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## **Differential scanning calorimetry in life science: Thermodynamics, stability, molecular recognition and application in drug design**

G. Bruylants<sup>1</sup>, J. Wouters<sup>2</sup> and C. Michaux<sup>2\*</sup>

All biological phenomena depend on molecular recognition, which is either intermolecular like in ligand binding to a macromolecule or intramolecular like in protein folding. As a result, understanding the relationship between the structure of proteins and the energetics of their stability and binding with others (bio)molecules is a very interesting point in biochemistry and biotechnology. It is essential to the engineering of stable proteins and to the structure-based design of pharmaceutical ligands. The parameter generally used to characterize the stability of a system (the folded and unfolded state of the protein for example) is the equilibrium constant (K) or the free energy ( $\Delta G^\circ$ ), which is the sum of enthalpic ( $\Delta H^\circ$ ) and entropic ( $\Delta S^\circ$ ) terms. These parameters are temperature dependent through the heat capacity change ( $\Delta C_p$ ). The thermodynamic parameters  $\Delta H^\circ$  and  $\Delta C_p$  can be derived from spectroscopic experiments, using the van't Hoff method, or measured directly using calorimetry. Along with isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC) is a powerful method, less described than ITC, for measuring directly the thermodynamic parameters which characterize biomolecules.

In this article, we summarize the principal thermodynamics parameters, describe the DSC approach and review some systems to which it has been applied. DSC is much used for the study of the stability and the folding of biomolecules, but it can also be applied in order to understand biomolecular interactions and can thus be an interesting technique in the process of drug design.

**Keywords:** calorimetry, DSC, thermodynamics,  $\Delta H^\circ$ ,  $\Delta C_p$ , binding, stability

## 1. Thermodynamic parameters <sup>[1]</sup>

The stability of biological macromolecules, and of biomolecular associations, is quantified by the standard free energy  $\Delta G^\circ$  or the difference in Gibbs energy between different states (the native and denaturated structures in the case of protein and nucleic acid stability). At equilibrium (when  $\Delta G=0$ ),  $\Delta G^\circ$  is related to the equilibrium constant (K) between the two states by:

$$\Delta G^\circ(T) = -RT \ln K(T)$$

where R is the universal gas constant and T the absolute temperature in Kelvin.  $\Delta G^\circ$  is the sum of two contributions:

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

where  $\Delta H^\circ$  and  $\Delta S^\circ$  are the enthalpy and entropy changes at the temperature at which  $\Delta G^\circ$  is being evaluated. When values of K can be determined experimentally as a function of temperature (this can be achieved by various spectroscopic methods) the data can be fitted to yield values for  $\Delta H^\circ$  (called van't Hoff enthalpy to distinguish it from a calorimetrically determined enthalpy, see below).

$$\ln K(T) = \left(\frac{-\Delta H^\circ(T)}{R}\right)\left(\frac{1}{T}\right) + \left(\frac{\Delta S^\circ(T)}{R}\right)$$

$$\frac{d \ln K(T)}{d(1/T)} = \frac{-\Delta H^\circ(T)}{R}$$

If  $\Delta H^\circ$  is independent of the temperature ( $\Delta C_p = 0$ ), a plot of  $\ln K$  in function of  $1/T$  will give a straight line with an angular coefficient of  $-\Delta H^\circ/R$ . This case is however very unusual,  $\Delta H^\circ$  being generally temperature-dependant. In this case, it is necessary to make a hypothesis on this dependence in order to be able to determine  $\Delta H^\circ$ . It has also to be noted that the fit assumes a simple two-state equilibrium, without for example stable folding intermediates in

the case of protein folding. This non-calorimetric and indirect approach to obtain thermodynamic parameters is called the van't Hoff analysis.

When a macromolecule changes its thermodynamic state (*e.g.*, unfolds), a heat capacity change ( $\Delta C_p$ ) is observed. This change is due to the fact that the heat required to raise the temperature of a solution of unfolded protein is greater than that required for a solution of folded protein. Heat capacity changes are primarily due to restructuring of the solvent molecules around the non polar sidechains exposed to the solvent during the unfolding process.<sup>[2]</sup> Assuming a constant temperature-independent  $\Delta C_p$ ,  $\Delta G^\circ$  is described by:

$$\Delta G^\circ(T) = [\Delta H^\circ(T_R) + \Delta C_p(T - T_R)] - T[\Delta S^\circ(T_R) + \Delta C_p \ln(\frac{T}{T_R})]$$

$$\Delta G^\circ(T) = \Delta H^\circ(T_R) - T\Delta S^\circ(T_R) + \Delta C_p[(T - T_R) - T \ln(\frac{T}{T_R})]$$

where  $T_R$  is the reference temperature and  $T$  the temperature of interest.

The unfolding transition occurs at a characteristic temperature called transition midpoint,  $T_m$ . Assuming a two-state transition,  $\Delta G^\circ$  is equal to zero if  $T_R$  is equal to  $T_m$  (i.e  $K=1$ ). As a result,  $\Delta S^\circ(T_m)$  is just  $\Delta H^\circ(T_m)/T_m$ .  $\Delta G^\circ(T)$  thus becomes:

$$\Delta G^\circ(T) = \Delta H^\circ_m * (1 - \frac{T}{T_m}) + \Delta C_p[(T - T_m) - T \ln(\frac{T}{T_m})]$$

where  $\Delta H^\circ_m$  is the value of  $\Delta H^\circ$  at  $T_m$  ( $\Delta H^\circ(T_m)$ ). This equation is often called “the modified Gibbs-Helmoltz equation”.<sup>[3]</sup>

To characterize the thermodynamics of an unfolding-folding process means to determine  $\Delta G^\circ$ ,  $\Delta H^\circ$  and  $\Delta S^\circ$  at a given temperature and to obtain  $\Delta C_p$  to predict the change of these three parameters with temperature. The denaturation enthalpy and entropy at temperature  $T$  can be calculated according to the following relations (Kirchhoff's law):

$$\Delta H^\circ(T) = \Delta H^\circ_m + \Delta C_p(T - T_m)$$

$$\Delta S^\circ(T) = \Delta S^\circ_m + \Delta C_p \ln\left(\frac{T}{T_m}\right)$$

where  $\Delta S^\circ_m$  is  $\Delta H^\circ_m/T_m$ , the denaturation entropy at  $T_m$ .

## 2. Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a technique able to study thermally induced transitions and particularly, the conformational transitions of biological macromolecules (for example between the folded and the unfolded structure of a protein or between single and double stranded DNA). Reviews on this technique are available.<sup>[1,4-10]</sup> DSC measures the excess heat capacity of a solution ( $C_p$ ) of the molecule of interest as a function of temperature (Fig.1). The transition is recognized as a sharp endothermic peak centred at  $T_m$  and the maximum in  $C_p$  occurs directly at  $T_m$ . Integration of the  $C_p$  versus  $T$  curve yields the transition enthalpy ( $\Delta H^\circ_m$ ) and the shift in the baseline,  $\Delta C_p$ . DSC is the only method for the direct determination of  $\Delta H^\circ_m$ .

The value  $\Delta H^\circ_m$ , calculated from the area under the transition peak, is correlated with the content of ordered secondary structure of a protein.<sup>[11]</sup> The  $\Delta H^\circ_m$  value is actually a net value from a combination of endothermic contributions, such as the disruption of hydrogen bonds,<sup>[12]</sup> and exothermic ones, such as the break-up of hydrophobic interactions.<sup>[13]</sup> The sharpness of the transition peak can be measured as the width at half-peak height, and is an index of the cooperative nature (two-state or multistate process) of the transition from native to denatured state. If denaturation occurs within a narrow temperature range, the transition is considered highly cooperative.<sup>[14]</sup>

DSC curve can be integrated incrementally to give a progress curve, i.e. the proportion of the total heat absorbed as a function of temperature, which indicates the progress of the

reaction, just like a change in spectroscopic parameters (see above). Fitting of the progress curve will yield a van't Hoff enthalpy ( $\Delta H^{\text{vH}}$ ) which can be different from the calorimetric enthalpy,  $\Delta H^{\circ\text{m}}$ . The calorimetric enthalpy is the total enthalpy change including the contributions from all processes and determined independently of any model while the corresponding  $\Delta H^{\text{vH}}$  assumes a simple two-state transition.<sup>[15]</sup> Comparison of  $\Delta H^{\circ\text{m}}$  with  $\Delta H^{\text{vH}}$  therefore allows one to assess whether the transition occurs as a two-state or multistate process.<sup>[16]</sup> If  $\Delta H^{\text{vH}} = \Delta H^{\circ\text{m}}$  then the denaturation can be considered to be well approximated by a two state process. If  $\Delta H^{\text{vH}} < \Delta H^{\circ\text{m}}$  then most likely there is an unfolding intermediate (i.e. non-2 state process). If  $\Delta H^{\text{vH}} > \Delta H^{\circ\text{m}}$  the molecule may be a dimer or multimer and the  $K$ , which is used in the calculation of  $\Delta H^{\text{vH}}$ , will exhibit a much sharper transition due to the larger effective concentration of the denatured species during the unfolding transition.

From  $\Delta H^{\circ\text{m}}$  and  $\Delta C_p$ ,  $\Delta G^{\circ}(T)$  and  $\Delta S^{\circ}(T)$  can be determined. As a result, DSC is able to provide all the thermodynamic parameters of a conformational transition. The technique is however limited by the requirements for high concentrations of protein (at least 1mg/ml).<sup>[1]</sup> This may lead to difficulties arising from aggregation of the denatured protein or, possibly, self-association of the native state. Accurate DSC studies thus require an assessment of the concentration dependence of the thermodynamics.

It is essential to determine the reversibility of the unfolding before extracting thermodynamic properties from the data. The usual test for reversibility is to perform two DSC scans and check that the second scan gives all of the endotherm observed in the first scan.

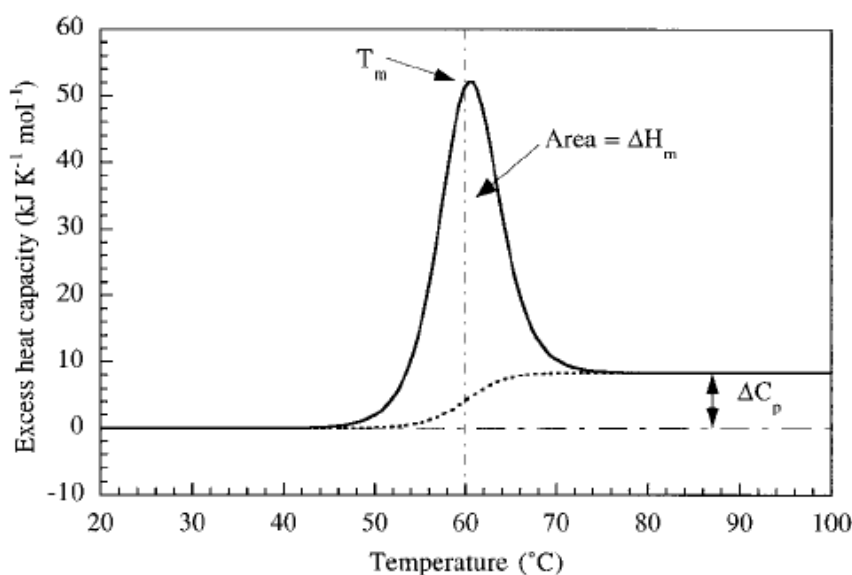


Figure 1: DSC experiment for the two-state unfolding of a globular protein (Adapted from Fig.1 of ref<sup>[1]</sup>)

In this review, we focus on DSC and present recent calorimetric studies using this technique.<sup>[17,18]</sup> The ITC method that has been extensively reviewed<sup>[19-26]</sup> will not be covered. DSC is much used for the study of the stability of biological macromolecules and the folding of proteins, but it can also be applied in order to understand biomolecular interactions and can thus be an interesting technique in the process of drug design.<sup>[27]</sup>

### 3. DSC and protein folding/stability

One goal of protein engineering and biopharmaceutical formulation (galenic formulation) is the development, production and storage of stable proteins with full functionality. It is therefore essential to understand how proteins fold into their biological states and how these active states are stabilized. A denatured protein has a higher heat capacity than the native one. As a result, the  $\Delta C_p$  for protein unfolding is almost always positive. The variation of heat capacity associated with protein unfolding is primarily due to changes in hydration of side-chains that were buried in the native state, which become exposed to the solvent in the denatured state. Many factors are responsible for the folding and

stability of native proteins, including hydrophobic interactions, hydrogen bonding, conformational entropy.<sup>[28,29]</sup> Moreover,  $T_m$  is an indicator of thermostability and generally, the higher is the  $T_m$ , the more thermodynamically stable is the protein. Proteins with higher  $T_m$  are less susceptible to unfolding and denaturation at lower temperatures. Here are described some examples where DSC is used to assess the stability of proteins and the effect of experimental conditions and/or the presence of ligands on their stability.

Evaluation of the effect of some formulation factors as 2-hydroxypropyl- $\beta$ -cyclodextrin (HP $\beta$ CD), sucrose concentrations and pH on the thermal stability of **lysozyme** was performed using DSC thermograms.<sup>[30]</sup> From these studies, it was observed that pH plays an important role in the conditions at the start of the experiment as well as in the irreversible degradation step. The maximal thermal stability would be reached at pH 5. Sucrose does not affect the initial equilibrium between the folded and the unfolded moieties but there is evidence of a stabilizing role of sucrose on the folded protein. No influence of HP $\beta$ CD on the stability of lysozyme was observed. Another study shows that thermal stability of lysozyme and bovine serum albumin is mostly unaffected by the presence of poly(ethylene oxide) and its copolymers with poly(propylene oxide).<sup>[31]</sup> Furthermore, thermodynamic analysis reveals that the stability of the folded conformation of lysozyme in glycerol is similar to that in water at 20-80°C but exceeds it at lower and higher temperature.<sup>[32]</sup> Conformation of lysozyme in glycerol is native-like and upon heating it cooperatively unfolds not into a random coil (as in water) but apparently into a highly ordered molten globule which can be further unfolded cooperatively at a much higher temperature.

Other stability studies were performed with lysozyme.<sup>[33-37]</sup>

The  **$\beta$ -1,4-endoxylanase A** from the mesophylic actinomycete *Streptomyces lividans* is a member class of polysaccharidases being investigated by the pulp and paper industries for their potential use in the biobleaching of wood pulp and for other applications in textile,



biomass energy and chemical industries.<sup>[38]</sup> Calorimetric studies show that the thermal denaturation of the enzyme is a complex process consisting of two endothermic events centred near 57 and 64°C, attributing to the thermal unfolding of the xylan binding and catalytic domains, respectively, and an exothermic event centred near 75°C, due to the aggregation and precipitation of the denatured catalytic domain (Fig.2).

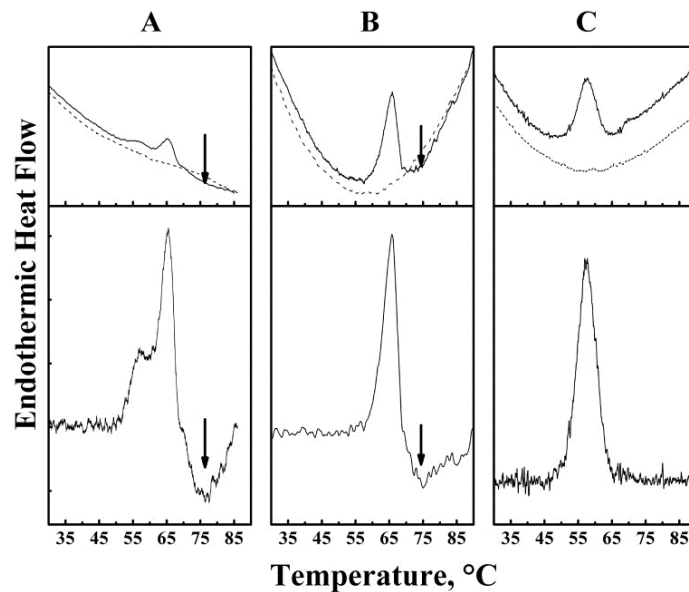


Figure 2: DSC thermograms observed during the thermal unfolding of *S. lividans* xylanase A. The data are representative of the intact enzyme (A), the catalytic domain (B), and the xylan binding domain (C). The top panels show thermograms observed during the unfolding of the protein (solid lines) and those obtained in subsequent heating scans after complete suppression of all protein-related thermotropic events (broken line). The bottom panels show the unfolding thermograms after baseline subtraction. (Adapted from Fig.1 of ref<sup>[38]</sup>)

The thermostability of other xylanases, like Xyn10A from thermophilic bacterium *Rhodothermus marinus*,<sup>[39]</sup> a cold-adapted family 8 xylanase,<sup>[40,41]</sup> a family 10 xylanase from *Cryptococcus adeliae* was also studied by DSC.<sup>[42]</sup>

Recombinant human **interferon  $\alpha 2b$**  was expressed intracellularly in *E. coli* using a new expression vector. Its conformational stability as a function of pH and temperature was studied by DSC and circular dichroism.<sup>[43]</sup> Thermal unfolding as a function of pH showed a

single endothermic transition at a temperature higher than 50°C, and was reversible at pH 2-3.7 and irreversible at pH 4-10. At pH 7.0, the most stabilizing conditions, the conformational stability depends on protein concentration, ionic strength (showing that electrostatic interactions play a significant role in protein stability) and scan-rate (showing a thermal denaturation kinetically controlled). The fact that  $T_m$  depends on protein concentration indicates the existence of intermolecular cooperativity. The  $\Delta H^{\circ}_m/\Delta H^{\text{VH}}$  ratio of 0.53 corresponds to a dimeric protein undergoing a single coupled transition on unfolding.

**Arginine kinase** (AK), catalyzing the reversible phosphorylation of arginine by ATP, plays a key role in the interconnection of energy production and utilization. Thermal stability of wild-type AK and two Trp mutants was determined by DSC.<sup>[44]</sup>  $\Delta C_p$  profiles revealed that an endothermic reaction took place just before the exothermic reaction in AK. Both reactions began at low temperatures in the order W218A<W208A<Wild-type AK, which suggested that W218A was the most sensitive to the thermal changes in the environment and Trp218 takes part in stabilizing the conformational structure of AK.

A mutant of dimeric rabbit muscle **creatine kinase** (CK), in which six residues (2-7) at the N-terminal (CKND<sub>6</sub>) were removed, was studied to assess the role of these residues in dimer cohesion and to determine the structural stability of the protein.<sup>[45,46]</sup> The DSC profiles of wild-type CK exhibited maximum endothermic and exothermic temperatures at 57.3 and 62.5°C, while the CKND<sub>6</sub> mutant showed peaks at 66 and 69.5°C, respectively. The deletion of the six residues results in more stable secondary, tertiary and quaternary structures, indicating that no interaction between the two monomers was mediated by residues 2-7 on the N-terminal of CK.

The **cold-active** and heat-labile  **$\alpha$ -amylase** from the antarctic bacterium *Pseudoalteromonas haloplanktis* (AHA) was thermodynamically studied in order to understand the relationship between activity, stability and flexibility.<sup>[47-50]</sup> This psychrophilic

enzyme shows a low thermal stability compared to mesophilic (PPA) or thermophilic (BBA) homologous enzymes and unfolds reversibly according to a two-state transition (Fig.3). The heat-labile psychrophilic enzyme is therefore also cold-labile, contrary to what is intuitively expected. In environmental conditions, its structure is stabilized entropically, whereas the enthalpic contribution becomes a destabilizing factor. It is thought that hydration of polar and nonpolar groups is responsible for the decrease of stability at low temperatures, leading to cold unfolding, and accordingly, this factor should contribute to the required mobility for function at low temperatures.

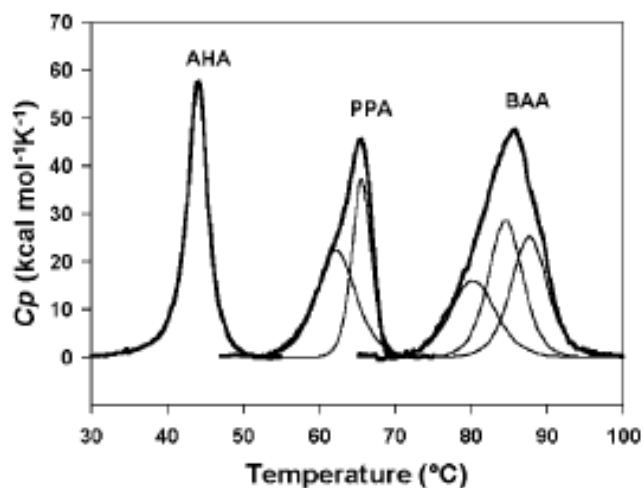


Figure 3: Thermal unfolding of  $\alpha$ -amylase recorded by DSC. AHA: psychrophilic  $\alpha$ -amylase from *P. haloplanktis*; PPA: pig pancreatic  $\alpha$ -amylase; BBA: *Bacillus amyloliquefaciens*  $\alpha$ -amylase. Deconvolution shows two and three calorimetric domains for PPA and BAA respectively (Adapted from Fig.1 of ref<sup>[47]</sup>)

The thermostability and thermal denaturation of other enzymatic systems, like PsbQ protein of higher plant photosystem II,<sup>[51]</sup> ervatamin C,<sup>[52]</sup> progenipoietin,<sup>[53]</sup> the tryptophan synthase beta2 subunit (Pfbeta2),<sup>[54]</sup> beta-lactoglobulins A and B,<sup>[55]</sup> HIV-1 gp41 and gp160,<sup>[56]</sup> NAD(+) synthetase,<sup>[57]</sup> DNA ligase,<sup>[58]</sup> streptokinase from *Streptococcus equisimilis* were also studied by DSC.<sup>[59,60]</sup>

#### 4. DSC and molecular recognition <sup>[61]</sup>

Ligand or protein association with proteins, or drug-DNA interaction involves changes in the intra and intermolecular interactions and in the dynamics of all the components present, as for example the protein/DNA, the ligand and the surrounding water.<sup>[62]</sup> These changes are reflected in the binding  $\Delta H_B^\circ$  and  $\Delta S_B^\circ$ , governing the  $\Delta G_B^\circ$  of association. For example, when a ligand binds preferentially to the native state of a protein, this one will be stabilized. The new  $T_m$  will occur at a higher temperature than the midpoint in the absence of ligand ( $T_{m0}$ ). On the other hand, binding to the unfolded state will decrease the  $T_m$  relative to  $T_{m0}$ .

The DSC method for measuring binding constant is indirect, compared for example to ITC, because the estimate binding  $K_B$  is made from measurements on the equilibrium between folded and unfolded protein rather than that between bound and unbound forms. Assuming a two-state reversible transition, the binding constant  $K_B(T_m)$  at  $T_m$  can then be estimated from  $T_{m0}$ ,  $T_m$ ,  $n$  (number of identical and independent binding sites on the protein), the concentration of unbound ligand at  $T_m$  ( $[L]$ ),  $\Delta H^\circ_m$  and  $\Delta C_p$ , the enthalpy and heat capacity change at  $T_{m0}$ .<sup>[8,63-65]</sup>

$$K_B(T_m) = \frac{\exp\left\{\frac{-\Delta H^\circ_m}{nR}\left(\frac{1}{T_m} - \frac{1}{T_{m0}}\right) + \frac{\Delta C_p}{nR}\left(\ln \frac{T_m}{T_{m0}} + \frac{T_{m0}}{T_m} - 1\right)\right\} - 1}{[L]}$$

The value of free ligand concentration at  $T_m$  can be calculated from  $L_{tot}$  and  $P_{tot}$ , the total concentration of protein and ligand in the solution:<sup>[63]</sup>

$$[L] = L_{tot} - \frac{nP_{tot}}{2} \quad (L_{tot} \geq nP_{tot})$$

This equation assumes total saturation of the binding sites on the native protein.

This treatment assumes only binding to the native form and no binding to the unfolded form. DSC can therefore be used to empirically quantify the enthalpic ( $\Delta H_B^\circ$ ) and entropic contributions ( $\Delta S_B^\circ$ ) to a ligand-protein binding.  $\Delta H_B^\circ$  can be obtained from:

$$n\Delta H_B^\circ = \Delta H^\circ(T_m) - [\Delta H^\circ_m + \Delta C_p(T_m - T_m0)]$$

where  $\Delta H^\circ(T_m)$  is the experimental enthalpy at  $T_m$  in the presence of ligand. The second term on the right-hand is the expected enthalpy at  $T_m$  with no bound ligand and the first term is the actual enthalpy at the same temperature in the presence of ligand.  $\Delta S_B^\circ$  can then be deduced from:

$$\Delta G_B^\circ = -RT \ln K_B = \Delta H_B^\circ - T\Delta S_B^\circ$$

This method can estimate binding constants up to  $10^{20} \text{ M}^{-1}$ , so it is used for ultratight binding that cannot be measured by other methods. It can also be used to characterize protein-protein or drug-DNA interactions. It has to be noted that the relationship between binding affinity and the magnitude of the observed temperature shift is not a simple correlation and is complicated by interaction with the denatured state.<sup>[66]</sup>

#### 4.1 DSC and protein-ligand interaction<sup>[67]</sup>

**Interactions of ATP with native Heat Shock Protein 90 (hsp90)** and its recombinant N-terminal and C-terminal domains were studied by DSC and other techniques (ITC, fluorescence spectroscopy).<sup>[68]</sup> The hsp90 melting curve consisted of two transitions. The experiment performed in presence of ATP showed a shift of the two transition peaks (+1.9°C and +2.9°C, respectively), indicating that the nucleotide stabilized both the N- and C-terminal domain of hsp90. Furthermore, the analyses show that hsp90 possesses a second ATP-binding site located on the C-terminal and suggest allosteric interactions between N- and C-terminal domains.

**Tetracycline repressor (TetR)**, constituting the most common mechanism of bacterial resistance to an antibiotic, is a homodimeric protein. Binding of **tetracycline (Tet)** in the protein pocket is accompanied by conformational changes in TetR, which abolish the specific interaction between the protein and DNA. DSC results show that, in the absence of

Tet, the thermally induced transitions of TetR can be described as an irreversible process, strongly dependent on scan rate and indicating that the protein denaturation is under kinetic control.<sup>[69]</sup> On the other hand, in the presence of Tet, the thermal unfolding of the protein can be described by a two-state model. In this model, TetR with Tet undergoes cooperative unfolding characterized by a  $\Delta H^{\circ}_m$  and a  $\Delta S^{\circ}_m$  of 1067 kJ.mol<sup>-1</sup> and 3.1 kJ.mol<sup>-1</sup>, respectively.

The glucose transporter **GLUT-1** is an integral membrane protein facilitating the passive glucose transport. Results from DSC studies indicate that the thermal denaturation temperature of GLUT-1 is significantly lower in presence of **ATP**, showing that ATP destabilizes the native structure (Fig.4).<sup>[70]</sup> The lowering of this transition temperature is dependent on pH. At more acidic pH, ATP has greater effect of lowering T<sub>m</sub> of the protein. However, a change in pH alone without ATP has little effect on T<sub>m</sub>. Both glucose and salt (suggesting that the interaction between GLUT-1 and ATP must partly be electrostatic) partially reverse the lowering of T<sub>m</sub> caused by ATP.

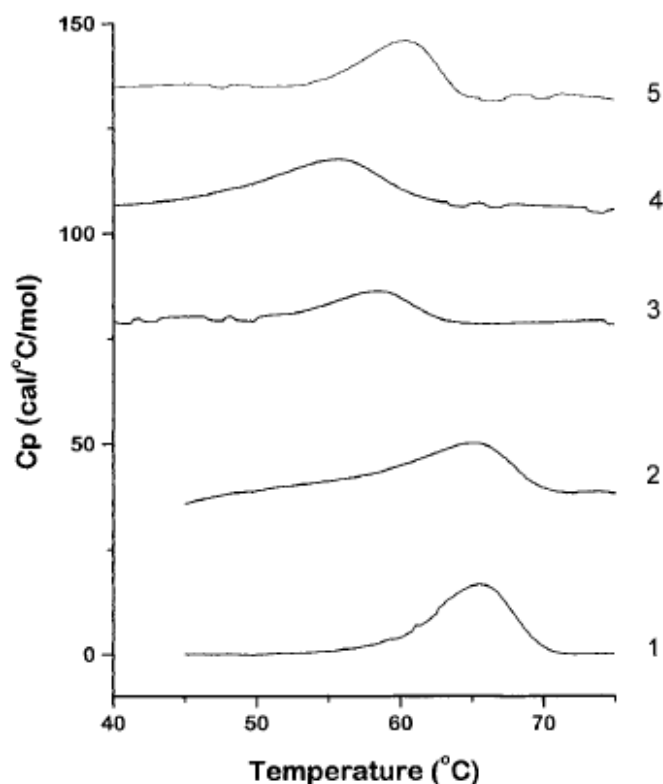


Figure 4: DSC heating curves for the thermal denaturation of GLUT 1. Curve 1, No addition; curve 2, in the presence of 0.4 M NaCl; curve 3, in the presence of 4.8 mM ATP and 0.4 M NaCl; curve 4, in the presence of 4.8 mM ATP; curve 5, in the presence of 4.8mM ATP and 500mM D-glucose (Adapted from Fig.3 of ref<sup>[70]</sup>)

**Bovine serum albumin (BSA)** and **anilinonaphtalene sulphonate (ANS)** derivatives were used to study the correlation between changes in protein thermal stability and conformational dynamics induced by ligand binding.<sup>[71]</sup> In the absence of ligands, BSA shows a typical two-state thermally induced unfolding with a  $T_m$  centred at 59°C and a  $\Delta H^{\circ}_m$  of 134 kcal.mol<sup>-1</sup>. The effect of ligand binding on protein stability depends on the total ligand:protein mole ratio and the type of ANS derivative. The binding of either 1,8-ANS or 2,6-ANS increases the thermal stability of BSA and the thermostability is gradually augmented as the ligand-protein mole ration is raised. On the other hand, bis-ANS has a dual effect on  $T_m$  and  $\Delta H^{\circ}_m$  depending on the ligand concentration. At low ratio ( $\leq 5:1$ ), bis-ANS produces a similar effect than that observed for the other compounds, but at higher proportions, a gradual

decrease in protein stability is observed. This dual effect is compatible with the presence of high affinity sites in the folded conformation and multiple low affinity binding sites in the non-native conformation.

**Zn- $\alpha$ 2-glycoprotein (ZAG)**, member of the major histocompatibility complex (MHC) class I family of proteins, was shown to bind fatty acids like **DAUDA**, a saturated fatty acid with a dansyl fluorophore attached at its  $\omega$ -methyl terminus.<sup>[72]</sup> DSC experiments showed that ZAG proteins undergo a typical endothermic cooperative reversible unfolding transition in solution with a  $T_m$  at around 65-70°C under physiological conditions (Fig.5). Recombinant ZAG samples had the slightly lower thermal stability ( $T_m$  65°C) compared with serum-derived material ( $T_m$  70°C) under the same conditions, possibly because the recombinant proteins lack the ligand or *N*-linked carbohydrates. Addition of increasing concentrations of DAUDA raised the  $T_m$  progressively for both the natural and recombinant ZAG by up to 5°C with 200  $\mu$ M added ligand.

Other DSC studies were also performed to observe conformational changes of apolipoprotein-A induced by lysine analog epsilon-aminocaproic acid<sup>[73]</sup> or to understand effects of L-phenylalanine binding on the stability of human phenylalanine hydroxylase<sup>[74]</sup> or to see different effects of individual ligands on the structure of the water-soluble receptor of the bacterial histidine permease.<sup>[75]</sup>



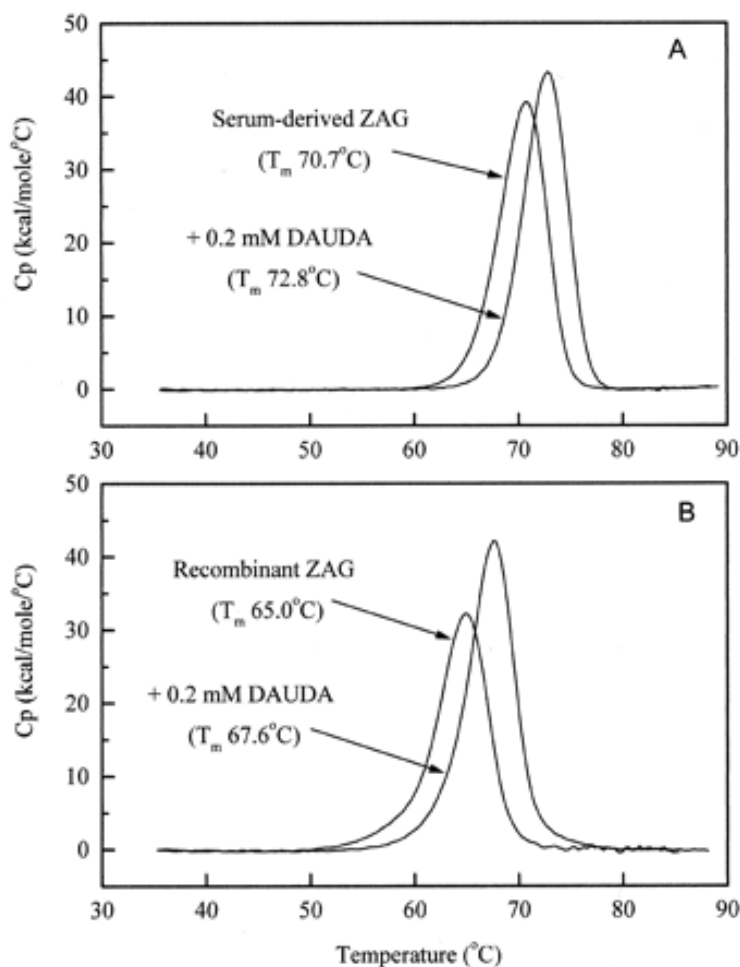


Figure 5: Increased thermal stability of ZAG upon ligand binding. Normalized DSC data, corrected for buffer base line showing cooperative endothermic unfolding of wild-type (A) and recombinant ZAG (B) in the presence and absence of 0.2 mM DAUDA. (Adapted from Fig.4 of ref<sup>[72]</sup>)

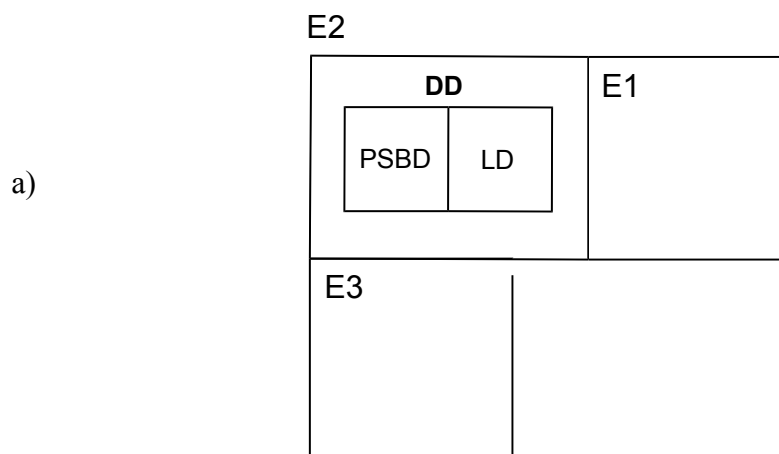
#### 4.2 DSC and protein-protein/lipid or drug-lipid interaction

Proteins often bind very tightly and specifically to another protein to form a complex, in order to regulate a biological process and DSC assay can be useful to understand it.

**Barnase** and **binase** are microbial ribonucleases synthesized by *Bacillus amyloliquefaciens* and *Bacillus intermedius*, respectively. These proteins bind **barstar** and **barstarA** (the Cys40,82Ala double mutant of barstar), one of their inhibitors. It is postulated that this association would be due to electrostatic interactions between positive charges in barnase/binase and negative ones in barstar. DSC studies show that mutations (leading to a

loss in negative charge of the active site of barstar) in bartsarA decrease the affinity of the inhibitors for barnase and binase and increase the thermal stability of the barstar mutants.<sup>[76]</sup> Indeed these mutations reduce the electrostatic repulsion among the negatively charged groups at the binding site of barstar, leading to an increased stability and, reduce favourable interactions between the ribonucleases and the mutants.

The peripheral subunit-binding domain (PSBD) of the **dihydrolipoyl acetyltransferase** (E2) binds tightly but mutually exclusively to **dihydrolipoyl dehydrogenase** (E3) and **pyruvate decarboxylase** (E1) in the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus* (Fig.6a). Two distinct transitions were observed in a DSC experiment with DD (a recombinant di-domain representing the lipoyl domain, LD, and PSBD of E2) alone, implying that the LD and PSBD are unfolding independently (Fig.6b).<sup>[77]</sup> E1 and E3 were found to unfold in single cooperative transitions, with a T<sub>m</sub> of 65.2 and 91°C, respectively. Both T<sub>m</sub> values were shifted higher (to 70 and 97.1°C, respectively) in the presence of DD, representing the enhanced thermal stability that accompanies tight complex formation.



b)

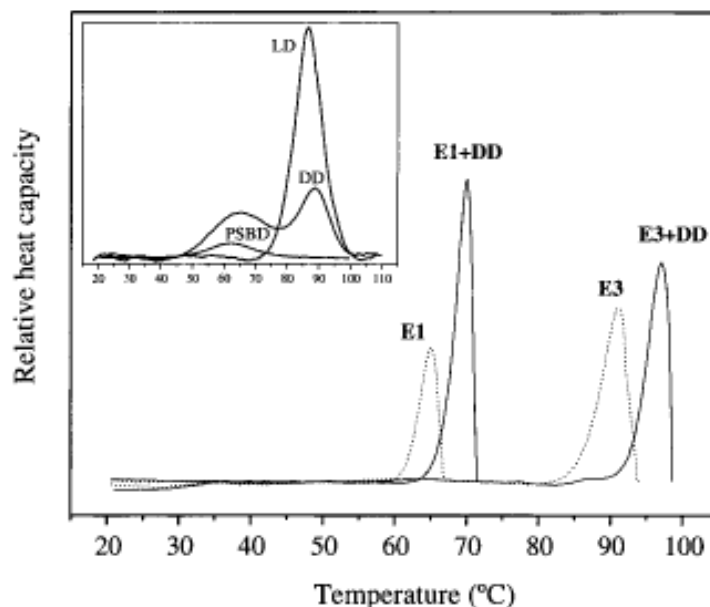


Figure 6: a) Schematic arrangement of subunits of the pyruvate dehydrogenase multienzyme complex: E1=pyruvate decarboxylase, E2=dihydrolipoyl acetyltransferase, E3=dihydrolipoyl dehydrogenase, PSBD=peripheral subunit-binding domain, LD=lipoyl domain, DD= recombinant di-domain representing the LD and PSBD of E2 ;

b) DSC trace of the thermal denaturation of E3 and E1 in the absence (dotted line) and presence (solid line) of PSBD (Adapted from Fig.5 of ref<sup>[77]</sup>)

DSC and ITC in conjunction with ultrasonic velocimetry, high-precision densimetry, and fluorescence spectroscopy were used to characterize the binding of **turkey ovomucoid third domain (OMTKY3)** to  **$\alpha$ -chymotrypsin**.<sup>[78]</sup> At each temperature studied, OMTKY3 association with  $\alpha$ -chymotrypsin is entropy driven with a large, unfavourable enthalpy contribution. The observed entropy of the binding reflects interplay between two very large favourable and unfavourable terms. The favourable term reflects an increase in the hydrational entropy resulting from release to the bulk of 454 water molecules. The unfavourable term is related to a decrease in the entropy and, consequently, a decrease in the conformational dynamics of the two proteins.

Extensive DSC investigations with membranes have demonstrated this technique is valuable for the analysis of materials that stabilize or destabilize membranes and lipids.

Physicochemical methods were used to study the thermal and dynamic changes caused by **losartan**, an antagonist of AT1 receptor, in the membrane bilayers.<sup>[79]</sup> Losartan causes progressive changes in the thermotropic properties of **DPPC bilayers** (Fig.7). At losartan:phospholipids 1:99 molar ratio, the presence of the drug causes broadening of the pretransition while at 5:95 and 10:90 molar ratio, losartan abolishes the pretransition observed in DPPC lipids and causes lowering and broadening of its main phase transition. At higher concentration of 20 mol%, the broadening of the transition is increased and a shoulder at the high temperature side of the main transition is observed. Such structural transitions would arise from the enhanced membrane elasticity accompanying the lipid state fluctuations on chain melting and solvent-associated interactions that favour a change in membrane curvature.

The structural and functional consequences of disrupting a previously identified interaction between a molecule of the **diacidic lipid cardiolipin** and the **purple bacterial reaction** centre were examined by DSC.<sup>[80]</sup> In the case of the wild-type and HM145F (His M145 to Phe) reaction centres, the DSC measurements revealed two overlapping endothermic transitions. In contrast, the purified RM267L (Arg M267 to Leu) reaction centre showed only a single transition. The HM145F, which has no discernable effect on the spectroscopic or biochemical properties of the reaction centre, does not affect the affinity of cardiolipin for the binding site on the reaction centre, and the two-component DSC profile is maintained, albeit with some small variations in T<sub>m</sub> values. However, the RM267L mutation weakens cardiolipin binding to such an extent that the lipid is no longer bound to the purified form of the complex and the RM267L reaction centre exhibits only the lower of the two melting temperatures.

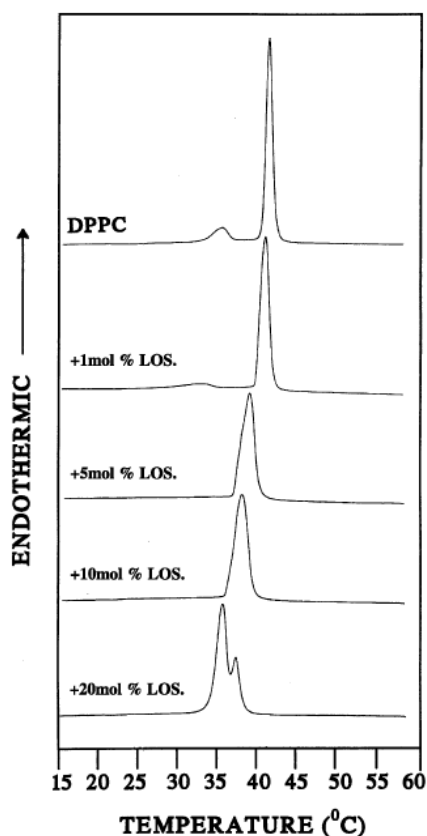


Figure 7: Normalized DSC scans of DPPC bilayers containing losartan at different concentrations (Adapted from Fig.2 of ref<sup>[79]</sup>)

Interactions between lipids and the assembly factor P17 from bacteriophage PRD1<sup>[81]</sup> were also studied by DSC. Effect of variations in the structure of a polyleucine-based  $\alpha$ -helical transmembrane peptide on its interaction with phosphatidylcholine bilayers was investigated.<sup>[82]</sup> DNA-lipid interactions are also studied by DSC.<sup>[83]</sup>

#### 4.3 DSC and drug/protein-DNA interaction

There are number of drugs, including antibiotics and chemotherapeutic agents that bind directly to DNA or RNA. Information about structure, function and thermodynamics of drug-nucleic acid interactions is essential for the design and optimization of new drugs. DSC is used to understand the molecular mechanism and energetics of drug-DNA interactions. Statistical mechanical theories were developed by Crothers and McGhee allowing

interpretation and calculation of DNA melting curves in the presence of ligands or proteins.<sup>[84-</sup>

<sup>86]</sup> The McGhee's model shows that:

$$\frac{1}{Tm0} - \frac{1}{Tm} = \frac{R}{\Delta H^\circ} \ln[(1 + K_B[L])^{\frac{1}{n}}]$$

where  $Tm0$  and  $Tm$  are the melting temperature without or with drug;  $R$  is the gas constant;  $\Delta H^\circ$  is the enthalpy for melting a DNA base pair;  $K_B$  is the binding constant for drug-DNA interaction at  $Tm$ ;  $[L]$  is the free drug concentration;  $n$  is the binding site size expressed in base pairs. Such equation is similar to that of ligand-protein or protein-protein interactions.

From this equation,  $K_B$  can be calculated from the experimental shift in melting temperatures and the  $\Delta H^\circ$  determined from denaturation of DNA alone. The  $n$  value can be obtained either by ITC or optical methods, or by fits to melting curves at intermediate drug concentrations.

The combination of DSC and temperature dependent UV melting methods allowed the first direct determination of the binding enthalpy for **echinomycin**, a **bisintercalator** that is difficult to study because of its poor solubility.<sup>[87]</sup> For the association of echinomycin with DNA, we found  $\Delta G_B^\circ = -7.6$  kcal/mol,  $\Delta H_B^\circ = +3.8$  kcal/mol and  $\Delta S_B^\circ = +38.9$  cal/mol.K at 20°C. The binding reaction is clearly entropically driven, a hallmark of a process that is predominantly stabilized by hydrophobic interactions, though a deeper analysis of the free energy contributions suggests that direct molecular recognition between echinomycin and DNA, mediated by hydrogen bonding and van der Waals contacts, also plays an important role in stabilizing the complex. The echinomycin DNA binding constant  $K_B$  was found to be  $4.5 \times 10^5 \text{ M}^{-1}$ .

Among various anti-tumor antibiotics, **distamycin** belongs to a class of DNA groove binding ligands, and specifically binds to sites consisting of at least four successive A-T base pairs. DSC was applied to understand interaction of distamycin with ColE1 DNA, a well-characterized plasmid.<sup>[88]</sup> The results show that with increasing the  $[\text{distamycin}]/[\text{base pair}]$

ratio, nine peaks observed in the absence of distamycin converged at near 110°C and gave rise to two endothermic peaks (Fig.8). Because the temperature of 110°C is close to the parameter given for melting of a G-C base pair, it indicates that distamycin molecules bind to almost all AT tracts found in the colE1 sequence. It is also shown that the affinity of distamycin to DNA, at low distamycin concentration, depends highly on the DNA sequence, and preferential binding occurs to the sites of four to six successive A-T pairs having two or more successive G-C pairs on both ends.

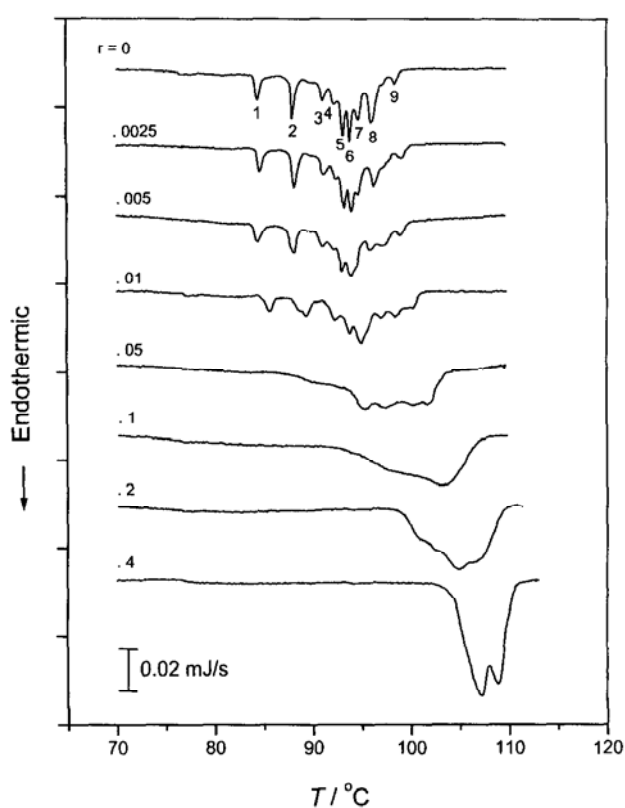


Figure 8: DSC curves of ColE1 plasmid DNA in solutions of various distamycin concentrations. Distamycin concentration was indicated as r, the molar ratio of distamycin to base pairs of DNA (Adapted from Fig.3 of ref<sup>[88]</sup>)

The energetics of the specific interaction of a protein fragment (**zfl-3**) containing the three N-terminal zinc fingers of the *Xenopus laevis* **transcription factor TFIIIA** with its cognate DNA sequence, contained in a 15 bp DNA duplex were studied using ITC and DSC.<sup>[89]</sup> In the temperature range from 13°C to 45°C (where all the binding reaction

components are folded), formation of the complex is enthalpically driven with a negative heat capacity effect. Comparison of the experimental values of  $\Delta H_B^\circ$  and  $\Delta C_p$  with expected values of these parameters (calculated from the burial of polar and nonpolar molecular surfaces) indicates that the polar groups at the protein/DNA interface are not completely dehydrated upon formation of the complex.

**Chartreusin** is an antitumor antibiotic that contains the chromophore chartarin and an uncharged disaccharide moiety. Binding to DNA is believed to be central to the mechanism by which chartreusin exerts its antitumoral activity. It can be used as a model to study multivalent intercalation, which also involves interaction through the minor groove of DNA. DSC, ITC and UV melting experiments have been used to analyze the multivalent binding of this compound to DNA.<sup>[90,91]</sup> For example, DSC scans show that  $T_m$  of DNA was 74.4°C while in the presence of saturating concentrations of chartreusin the melting temperature rose to 78.0°C. The enthalpy of the DNA melting was 8.8(0.2) kcal/mol bp. Such value was used to calculate the binding constant  $K_B$  ( $4.9 \cdot 10^4 \text{ M}^{-1}$  with 18mM [Na<sup>+</sup>]) to DNA at the DNA melting temperature,  $T_m$ .

DSC measurements were conducted to characterize the thermally induced denaturation of the 20-bp duplex with the specific goal of elucidating the thermal and thermodynamic consequences of modifying and constraining DNA via a single, site-specific interstrand cross-link of antitumor **cisplatin** or its clinically ineffective *trans* isomer (Fig.9).<sup>[92]</sup> The structural perturbation resulting from the interstrand cross-link of cisplatin increases entropy of the duplex and in this way entropically stabilizes the duplex. This entropic cross-link-induced stabilization of the duplex is partially but not completely compensated by the enthalpic destabilization of the duplex. By contrast, the cross-link formed by transplatin does not considerably alter the enthalpic stability of the duplex, whereas entropically destabilizing the host duplex. It has been proposed that these results support the view that the impact of the



interstrand cross-links of cisplatin and transplatin on DNA is different for each and might also be associated with the distinctly different antitumor effects of these platinum compounds.

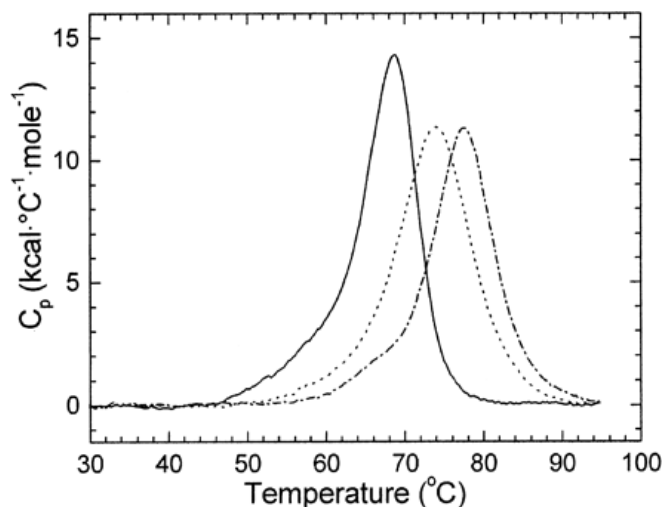


Figure 9: DSC thermograms for the d(TGCT)·d(AGCA) unplatinated duplex (*solid line*) containing a single, site-specific interstrand cross-link of cisplatin (*dot-dash line*) or transplatin (*dashed line*). (Adapted from Fig.2 of ref<sup>[92]</sup>)

The interaction of other drugs, like chlorobenzylidin,<sup>[93]</sup> phenoxazone drug<sup>[94]</sup> or CP-31398,<sup>[95]</sup> an anticancer drug, with DNA has been also investigated by DSC.

## 5. Conclusion

Differential scanning calorimetry is a powerful technique for studying thermal transitions in biological systems. It is possible using DSC to determine not only the melting temperatures of these systems (described as the temperature at which 50% of the molecules are denatured) but also to obtain the thermodynamic parameters associated with these changes. It is also able to yield, albeit indirectly, information on the stability of (bio)molecular associations. This is the only technique that allows direct determination of the enthalpy and heat capacity changes of the systems of interest in a single experiment. Because enthalpy changes are a universal property of all chemical processes, calorimetry is applicable to a wide variety of systems.

In this article, we review some of the recent contributions of DSC to characterize the thermodynamics of protein and nucleic acid stability, as well as the interactions of proteins with each other, with small molecules, and with nucleic acids.

Such data are useful to understand how biological systems work and to facilitate drug discovery, for example, in galenic formulation, in molecular mechanism and energetics of drug-DNA interactions, in lipid phase transitions or in order to estimate binding constants of two molecules.

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