



Differential Scanning Calorimetry: Theory and Practice


Application Note

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Ultrasensitive Calorimetry for the Life Sciences™



Calorimetry is the only technique for directly measuring the thermodynamics of the interactions that stabilise the structures of biological macromolecules and govern how they interact with one another. All other methods measuring thermodynamics are indirect since they require assumptions about the mechanism for these processes. Differential Scanning Calorimetry (DSC) measures the thermodynamics of interactions by inducing changes in the macromolecules with increasing or decreasing temperature. This technique was historically the domain of a few specialised academic groups, but current instrumentation is simple to use and requires only modest amounts of material for accurate and precise measurement and DSC is now a routine part of most biophysics labs.

The thermodynamics of biological systems in solution are not easy to interpret in isolation. They are always the net effect of interactions between groups in the molecule and interactions that these same groups could make with the solvent. The energies stabilising biological systems are thus the difference between the many favourable as well

as potentially unfavourable interactions. This fact comes as a revelation to many researchers and DSC measurements make the point very clearly. All is not lost, however, and a careful, methodical approach can begin to dissect out the complexity of the system. DSC also finds many other uses, based in part on this ability to see the totality of the thermodynamics associated with changes in the system and the absence of any optical components. It can measure how simple effects related to changes in protonation of the macromolecules affect the thermodynamics and stability. This approach can be extended to include any non-covalently bound ligand, giving a new and almost universal method of screening for binding. Under favourable circumstances, this approach can be used to determine binding constants. Here we discuss the thermodynamic background to DSC measurement and its application in studying the stability of proteins and their interactions with ligands. However, the techniques are equally transferable to other biological macromolecules such as nucleic acids, lipids etc.

Basic equilibrium thermodynamics

Proteins undergo a transition, or 'melting', between a structured, native and biologically active conformation (N) and an unstructured, denatured and inactive conformation (D) when temperature is increased in a physiologically relevant range. They share this behaviour with many other biological macromolecules (DNA, lipids etc) and with organic polymers in general. If we follow some property (signal) from the protein that reports on this conformational transition, then we will see a sigmoidal trace such as below:

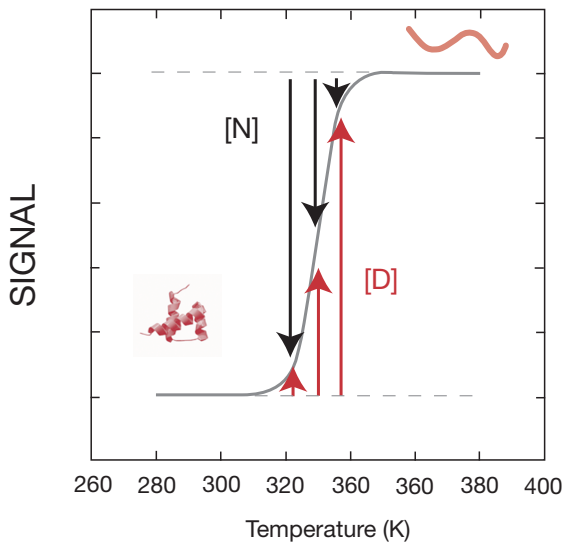


Figure 1: Typical sigmoidal transition for the denaturation of a 100 amino acid protein. Below 300 K the protein is essentially native, above 360 K the protein is denatured. Between these temperatures, the relative occupancy of each state is indicated by the length of the black and red arrows respectively.

When the structure of the protein is 'melted' in this way there are no changes to the covalent nature of the molecule. It is only non-covalent interactions that are perturbed and, in many cases, if the protein is cooled down again these interactions will reform spontaneously, yielding the active native conformation. Thus N and D are in reversible equilibrium with temperature as an intensive variable:



In Figure 1 we can see the proportions of N and D change as this equilibrium is driven toward D with increasing

temperature. At any one temperature we can define an equilibrium position, the equilibrium constant (K_{eq}), which merely reflects the relative concentrations of N and D. In a logarithmic scale this equilibrium constant is expressed in the Gibbs free energy (ΔG):

$$K_{eq} = \frac{[D]}{[N]} \quad (2)$$

$$\Delta G = -RT \ln K_{eq} \quad (3)$$

with R , the gas constant, and T , the temperature in degree Kelvin. The temperature at which the concentrations of D and N are equal is defined as the midpoint of the transition or melting temperature T_m . At this temperature K_{eq} is equal to 1 and ΔG is 0. The T_m is an important parameter for any protein since it indicates its thermal stability. Below this temperature the concentration of native protein is higher than that of denatured, while above the T_m , more of the protein is denatured.

The reason that proteins undergo this melting behaviour is because their native structures are stabilised by numerous interactions that have temperature dependence themselves. Stabilisation by enthalpy (ΔH) requires interactions involving bond making, structuring and reduction in internal energy, while stabilisation by entropy (ΔS) reflects disordering interactions and increasing the number of ways the system can be organised with same energy. These terms are related to ΔG in the familiar equation:

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Combining equations (3) and (4) and rearranging gives the van't Hoff equation (5) from which the variation in equilibrium constant with temperature can be plotted to yield the enthalpy and entropy of the thermal denaturation in a simple linear relationship ($\ln K_{eq}$ versus $1/T$):

$$\ln K_{eq} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (5)$$

We can use the data in Figure 1 to determine K_{eq} at temperatures in the transition region and from this evaluate ΔH and ΔS for the process. The units of enthalpy determined from the slope of the van't Hoff plot are in calories or Joules *per mol* (1 calorie = 4.184 Joule). But in this case the 'mol' term is derived from R, the gas constant, raising the questions as to *per mol* of what? The answer to this is *per mol* of the species in the native to denatured equilibrium, often termed 'the cooperative unit'. This value is, therefore, indirectly determined and model dependent. Obviously this is far from ideal and a direct method of measurement is preferable.

Direct measurement of thermodynamics using calorimetry

Not surprisingly, calorimetry, from the Latin, *calor*, meaning heat, and *metrium*, to measure, is the only way to determine directly the enthalpy for denaturation of a protein. Ultra sensitive calorimeters, such as the MicroCal VP-DSC, suitable for accurately measuring enthalpies from fractions of mg of material are available. These instruments are simple to use, accurate and reliable, making calorimetric measurements of this type a routine part of any biophysical lab. They work by measuring the heat capacity (C_p) of a sample of protein solution while scanning up or down in temperature. C_p is simply the amount of energy required to raise the sample temperature some amount, normally 1 deg K and is related to enthalpy in Kirchoff's law:

$$\Delta H = \int_{T_n}^{T_d} C_p \cdot \delta T \quad (6)$$

The excess (differential) heat capacity of the protein is measured relative to a carefully matched solvent reference cell during the scanning and hence these types of instruments are known as differential scanning calorimeters (DSC).

The DSC measurement is identical to Figure 1 except that now the property of the protein followed during the denaturation is its heat capacity. A conformational transition such as in Figure 2 will be observed.

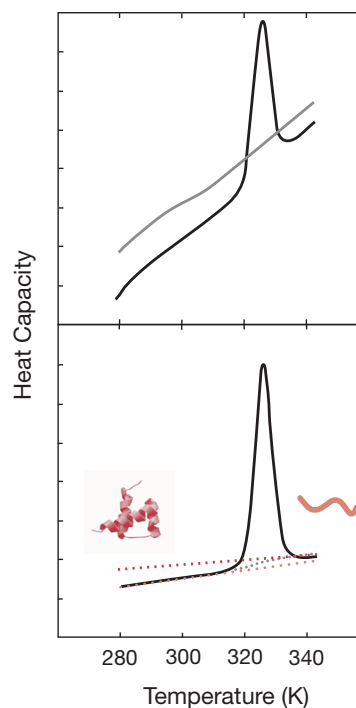


Figure 2: DSC data for the denaturation of the 118 amino acid protein barnase measured at 20 μ M (0.25 mg/ml) at 60 K/hr in a VP-DSC instrument. The instrumental baseline recorded with buffer is indicated in grey. The lower panel shows the data after 'buffer' subtraction and illustrates the process of baseline generation prior to integration of the peak.

From Eq 6 it is clear that to obtain the enthalpy we must simply integrate the excess heat capacity function. Before we can do this we have to remove the instrumental baseline which is observed when both the sample and the reference cells contain solvent only. For technical reasons the instrumental baseline does not give a zero excess heat capacity between the cells (Grey line, Figure 2). Having removed this instrumental contribution, we have to extrapolate the linear regions on either side of the transition peak, which represent the heat capacity of the native and denatured states of the protein, into the transition and then merge them in relation to the progression through the transition. This is done with software routines. Finally, we can integrate the area under the resulting peak to give us the excess energy that the DSC requires to denature the protein in the sample cell. Providing we know the concentration of the protein solution and the operational volume of the calorimeter cell, we can convert this energy to ΔH in calories or Joules *per mol* of protein. Normally we use ΔH_{cal} to indicate that this is a directly measured calorimetric enthalpy.

Experimental Tip: The DSC is a very sensitive instrument capable of measuring the very small changes in heat capacity associated with protein denaturation in a dilute solution. The heat capacity of the solvent exceeds that of the protein by many orders of magnitude. Thus for the most accurate and reliable measurement we must eliminate this background heat capacity carefully by ensuring that the solvent for the protein sample and reference solutions are exactly matched in composition. Dialysis or chromatography is the best method with the final dialysate or column flow through used as the reference solution.

Two-state and non two-state equilibria

The data in Figure 2 can also be analysed in the same way as any other property of the protein that undergoes a change on denaturation as discussed above. This gives us the T_m and the model dependant van't Hoff enthalpy; now ΔH_{vH} to distinguish it from ΔH_{cal} . Comparison of these two measurements of enthalpy, which are obtained in the same experiment, provides a powerful test for our model of protein denaturation. ΔH_{vH} gives the energy *per* mol of the cooperative unit in our equilibrium while ΔH_{cal} gives the energy *per* mol of protein. If these energies are the same then the protein and the cooperative unit are the same, or can be envisaged as having identical molecular weight, thus confirming the two state assumption of our equilibrium.

In alternative scenarios, ΔH_{vH} is smaller than ΔH_{cal} suggesting that the average molecular weight of the equilibrium species is lower than that of the protein and that a denaturation scheme involving intermediates may be more appropriate:



In extreme cases of intermediates occurring during denaturation, such as when proteins have independently folding domains of differing thermal stability, two distinct transitions may be observed. Here, ΔH_{vH} of each transition will reflect the molecular weight of each domain while ΔH_{cal} , based on the molecular weight of the entire protein, will reflect the total energy to denature the system, i.e., the total area under the two transitions.

There can also be cases where ΔH_{vH} is larger than ΔH_{cal} suggesting that the size or molecular weight of the species in our equilibrium is larger than that of the protein. This would be the case where the protein forms dimer, tetramer or higher order

'aggregates' with the ratio of ΔH_{vH} to ΔH_{cal} reflecting, at least in principle, the order (n) of association:



Unlike Eq 7, this is still a two state equilibrium since only N and D are included. The discrepancy in the ratio of ΔH_{vH} to ΔH_{cal} reflects the fact that the concentration *per* mol term in our calculation was based on the subunit rather than the oligomeric protein.

This also raises the important point that the value of ΔH_{cal} is totally dependent on the sample concentration. Any errors in the concentration will be transferred directly into our estimate of enthalpy. These include errors in the physical measurement of concentration, usually by absorbance spectroscopy, errors in the extinction coefficient of the protein, as well as errors introduced by less than 100% pure material (contaminant proteins contribute to absorbance but have different T_m 's which may not be seen in DSC) or less than 100% folded native material (denatured protein contributes to absorbance but is already unfolded). Thus some care must be taken in interpreting smaller discrepancies in the ratio of ΔH_{vH} to ΔH_{cal} .

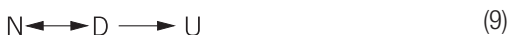
Experimental Tip: The accurate quantification of sample purity and concentration is the most important element of experimental design and data interpretation in DSC.

If the ratio of ΔH_{vH} to ΔH_{cal} suggests an oligomeric denaturation scheme (Eq 8) it is easy to confirm this by checking the concentration dependence of the DSC measurement. Since the equilibrium involves a change in concentration of say, the monomeric form, then the concentration of monomer will, by mass action or Le Chatelier's principle, affect the equilibrium position. Thus we expect the thermal stability of an oligomeric system to increase with increasing concentration as is seen in practice (Neet and Timm, 1994; Johnson *et al.*, 1995).

Experimental Tip: Checking for sample concentration dependence in DSC measurement is a key test of the mechanism and is always worth doing, if sample availability permits, even when the calculated ratio of ΔH_{vH} to ΔH_{cal} is close to 1.

Irreversible and non-equilibrium denaturation

Having completed a DSC measurement one of the simplest and potentially informative experiments one can make is to simply rescan the same sample again. If we observe an identical endotherm during the rescan we can conclude the system is fully repeatable with the native and denatured states in equilibrium. But in a significant number of cases there is no transition or one of reduced magnitude on rescanning the protein. This indicates that there is an irreversible step in the denaturation mechanism:



In this unimolecular scheme processes such as deamidation, proline isomerisation etc., can lead to irreversible modification and prevent refolding. Often, repeatability can be improved by scanning to temperatures just above the transition region, rather than to the highest temperature the DSC will reach, because there is a kinetic element (rate) to the irreversible step. This rate will most likely be faster at higher temperatures. Thus the higher the temperature accessed and the longer the time spent denatured, the more the irreversible step proceeds.

The irreversible step may also result from association or aggregation of the denatured state:



This will manifest itself in a concentration dependence of the initial scan parameters. At higher sample concentrations the concentration of D will be increased so the irreversible step is accelerated and the protein will have a lower T_m . In cases of significant and rapid aggregation it may even be possible to distort and ‘truncate’ the denaturation transition with the exothermic aggregation event and observe atypically noisy data above the transition region, such as below:

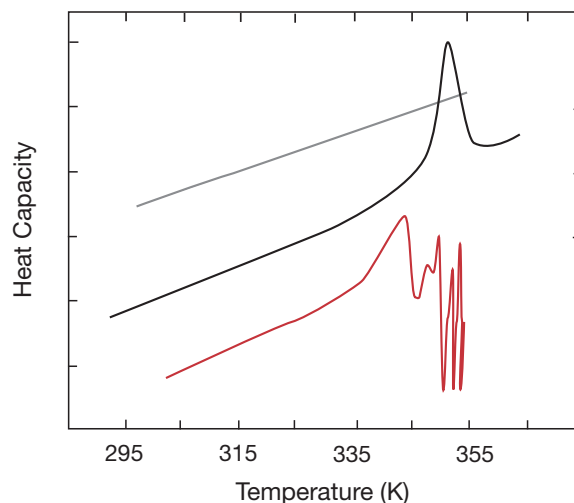


Figure 3: Raw DSC data for the denaturation of an aggregating protein (red). Data for the buffer baseline (grey) and the same protein (black) under different conditions where aggregation is almost completely eliminated are shown for comparison.

Calorimetry is not a forgiving technique in this respect and it is often DSC that flags up aggregation problems that were not obvious in spectroscopic-based thermal denaturation experiments done at similar sample concentrations. In both cases of irreversibility it is informative to examine the sample concentration and scan rate dependence of the DSC measurements. Indeed, in some cases such information can be used to extract meaningful parameters from the initial equilibrium event and even estimate values of the irreversible rate (Freire *et al.*, 1990; Lepock *et al.*, 1992).

Experimental Tip: Checking for scan rate dependence in DSC measurement is a key test of the mechanism and is always worth doing, if sample availability permits, even when the system is highly repeatable and at equilibrium. Even in the simplest unimolecular equilibrium (Eq 1) we must confirm that the equilibrium concentrations of N and D reflect the stability of the system under those conditions. These concentrations change at a rate equal to the sum of the forward (unfolding) and reverse (folding) reactions in the equilibrium. Thus if we increase temperature faster than the system can change we will distort the position and shape of the DSC endotherm. This will give incorrect T_m 's and anomalous ΔH_{vH} to ΔH_{cal} ratios. As a rule of thumb, 60 K/hr is a good starting point for most proteins.

Thermal stability; interpreting the T_m

DSC is the only technique that gives an indication of the potential complexity of protein denaturation with a single measurement. As well as this comparative use of the enthalpies we have an accurate determination of the enthalpy itself and the T_m . As already noted, the T_m is a reflection of the thermal stability of the protein. This should not be directly equated with 'stability' in the sense of longevity of activity in the sample or its 'shelf life' since this can have an additional kinetic dimension introduced by an irreversible step. In our simplest equilibrium (Eq 1) we have a system with perfect reversibility, giving the protein, in theory, infinite shelf life. Obviously, if we go to a temperature above the T_m then the active native state is less populated and our level of activity is correspondingly reduced. However, if we go below the T_m the system will potentially regain the active native structure. In the simplest case of irreversible denaturation (Eq 9), if we are above the T_m , the rate of non-equilibrium step, which is in competition with the rate of folding from D to N, will determine how long the protein will retain the opportunity of populating the active native state. Even well below the T_m , where the equilibrium constant means that most of the protein is in the native state, the fact that D to U is irreversible means that once converted to U the molecules are no longer in the equilibrium. To maintain the appropriate [D]/[N] ratio, as dictated by K_{eq} , native protein will become denatured. Once again, it is the kinetic rate that determines how long native and active protein will remain. This said, it is clearly judicious to store a protein well below its T_m and common sense dictates that T_m and shelf life will be correlated. All rates of reaction will be slowed at lower temperature including the D to U step. In addition, if, as is often the case, the irreversible step involves aggregation of two or more denatured proteins (Eq 10), then the lower the concentration of D the slower will be the rate for this reaction.

It is also misleading to equate thermal stability with the equilibrium stability of a protein at temperatures far from its T_m . Extrapolation of the equilibrium constant, and thus ΔG , away from the T_m is complex and depends on a number of parameters that are considered below. This extrapolation is markedly nonlinear so that protein with a high T_m will not necessarily have a larger ΔG at lower temperatures than other proteins with lower T_m 's.

Thermal stability; interpreting the thermodynamics

The enthalpies that we determine in a DSC experiment apply to the T_m of measurement, since this is the midpoint of their determination. At this temperature we know that the ΔG is 0, so from Eq 4 we can see that $\Delta S = \Delta H / T_m$. In other words we get the ΔS by elimination, but not by measurement, and all errors in determination of ΔH and T_m will propagate into ΔS .

It would be nice to think that these fundamental thermodynamic quantities would give us a profound insight into the forces stabilising protein structures. After all, proteins are very much like biological 'lego'. For the toy, there are only a few types of brick and they only join in a few ways, yet the diversity of structure and function that can be built up within these limitations is enormous. In biology there are only a limited number of chemical groups (bricks) in a protein polypeptide that can interact; groups from the backbone carboxyl and amide and from the amino acid side chains. These groups use an equally small set of interactions to join themselves together: Van der Waals forces, hydrogen bonding, electrostatic charge-charge attractions and hydrophobic effects. Such interactions, by their non-covalent nature, are systems at equilibrium between two extremes, interacting and non-interacting and so can be described by the thermodynamics described above. Thus the ΔH and ΔS that we determine for the denaturation of a protein must tell us something about the nature and strength of these interactions.

Regrettably, these are information rich quantities. In an average protein there will be many hundreds of interactions and these will sum over the whole molecule to determine its thermodynamics. It is not possible to disturb one interaction in isolation. Typically, the protein shows a high level of 'cooperativity' in its structure so that either all the interactions are made and the protein is native, or all the interactions are broken and the protein is denatured.

Furthermore, each of the chemical groups in the protein can also interact intermolecularly with the solvent water and dissolved salts as well as intramolecularly with each other. The thermodynamics of each interaction will thus reflect the net difference between the groups coming together and when they are separately solvated by water molecules, making the situation even more complex.

Extrapolation and comparisons of stability

Despite our inability to interpret values of ΔH and ΔS in isolation, there is still a great deal we can do with these parameters. They are determined at T_m where ΔG is 0 so if we were to know their temperature dependence then we could calculate ΔG at all other temperatures. To do this we need to know the change in heat capacity (ΔC_p) for denaturation of the protein. Since the DSC measures heat capacity we can potentially determine the change on denaturation directly from our data (Figure 2). Indeed, we had to apply the progress baseline function prior to integration of the peak area because of this very difference.

ΔC_p for proteins is, as seen in Figure 2, large and positive. Its value is available from each DSC experiment but it can also be determined just as accurately simply from the size of the protein (Myers *et al.*, 1995). The larger the protein the larger is ΔC_p reflecting the fact that its value correlates very well with the total non polar surface buried by the protein when it folds to the native state.

The variation of ΔH and ΔS with temperature are another way of expressing the Kirchoff relation:

$$\Delta H_T = \Delta H_{T_m} + \Delta C_p (T - T_m) \quad (11)$$

$$\Delta S_T = \Delta S_{T_m} + \Delta C_p \ln \left[\frac{T}{T_m} \right]$$

leading to a temperature dependence of ΔG calculated according to:

$$\Delta G_T = \Delta H_{T_m} + \Delta C_p (T - T_m) - T \left[\Delta S_{T_m} + \Delta C_p \ln \left(\frac{T}{T_m} \right) \right] \quad (12)$$

Eq 11 also provides another method of determining ΔC_p . If we measure ΔH for a protein under differing conditions of stability, then the variation of ΔH , $\delta\Delta H/\delta T$, will give us the ΔC_p . Providing these experiments use appropriate buffers (as

discussed below) then this is probably the most accurate estimate of ΔC_p since it is based on a number of independent measurements of ΔH .

With Eq 12 we can calculate ΔG , ΔH and ΔS at any other temperature of interest. Such estimates will have an error reflecting the original error in the measured T_m and ΔH as well as the length of temperature extrapolation away from the T_m . With longer extrapolations in temperature the compounded errors can become significant and some estimation of their size should be made when they are quoted. Should we wish to compare ΔG , ΔH and ΔS between systems or under different solvent conditions then it is obviously essential that we extrapolate the measured data to a common temperature of comparison.

It is quite meaningless to compare the experimentally determined ΔH of denaturation for two proteins since these are measured at their respective T_m 's. Such a comparison requires extrapolation of the ΔH data to a common temperature using the ΔC_p of each protein. This temperature is preferably the mid point between the two T_m 's ($(T_{m1} + T_{m2})/2$) to minimise the extrapolation errors.

On a qualitative level we can make comparisons of this type and they may flag up some gross change in our system. But, as already noted, the complexity of the denaturation process makes any detailed interpretation of data, or changes in its values, extremely difficult. Nevertheless, there are certain experimental strategies that may eventually begin to shed some light on the contributions of specific interactions to stabilising protein structures. Site directed mutagenesis changes specific side chains in a protein and can be used in a very subtle way, e.g. deleting a single methyl moiety buried in the core of the protein.

If the thermodynamics of these mutations can be augmented with high-resolution structural information on the parent protein and the mutant we at least stand some chance of interpreting the changes. Such an approach can be extended to double or triple mutant cycles to confirm the validity of interaction energies or to include changes in solvent (H_2O vs D_2O) to probe hydrogen bonding (Connelly, P.R., *et al.*, 1994).

A unique way of measuring protonation changes

Thus far we have seen that the complexity and information content of thermodynamic data is problematic. However, there are ways we can take advantage of this that gives DSC some unique uses. For example, the amino acid side chains in proteins that have protonatable groups may undergo a shift in pK_a during denaturation because in the native state they are interacting with other charged groups while in the denatured molecule they are interacting with the solvent. The pK_a is simply a representation of the equilibrium constant for the group between its protonated and unprotonated forms and if this equilibrium changes during denaturation, then protons will be released or taken up by the protein. Since we invariably include a buffer in our studies, which acts to maintain pH (proton concentration), the protons involved in the protein denaturation equilibrium will be taken up or provided by the buffer, a process that will also have an associated enthalpy. This enthalpy is different for each buffer depending on its chemistry. So if we measure ΔH for protein denaturation in different buffers at the same pH we will observe different values reflecting the sum of ΔH for the protein event and ΔH for the buffer ionisation. Plotting out our observed values versus the ΔH of ionisation for the buffers will give us a linear function whose slope (positive or negative) reflects the number of protons (sum of all pK_a shifts) associated with the denaturation of the protein:

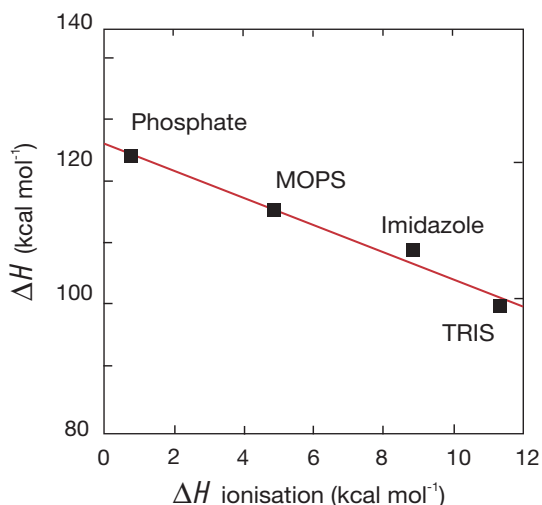


Figure 4: ΔH of denaturation of a 150 amino acid protein at pH 7.5 versus ΔH of buffer ionisation. ΔH ionisation is for the process $HA \rightarrow H^+ + A^-$. The negative slope (-2) indicates 2 protons are released by the protein upon denaturation at this pH.

This utility in measuring what would be a technically challenging measurement by other means highlights one of the important factors in designing any DSC measurement, i.e., the choice of buffer. For routine work a buffer with a small or zero enthalpy of ionisation is preferable since this eliminates any ionisation contributions in the measurement. Buffers such as formate, acetate and phosphate are ideal since they have negligible heats of ionisation, are available in high purity and are cheap. These have the added advantage that their pH remains constant with changes in temperature; obviously an important factor for DSC measurement. This is again a consequence of their small heats of ionisation.

Why thermal stability varies with pH

Another consequence of a protein having protonatable groups whose pK_a is different in the native and denatured state is that its K_{eq} , and thus ΔG , will be dependant on solution proton concentration, i.e., pH. This follows from the law of mass action or Le Chatelier's principle. If the denaturation K_{eq} affects the pH of the solution by releasing or taking up protons then it holds that the solution pH will affect K_{eq} of the protein. This effect can be quantified in:

$$\frac{\delta \Delta G}{\delta pH} = 2.303 RT \Delta \nu \quad (13)$$

Where $\Delta \nu$ is the change in protonation. The larger the change in protonation upon denaturation the larger the change in stability for each pH unit. The effect is only manifest where there is a potential difference in protonation states between the pK_a of the group in 'solution', exposed as it is in the denatured state, and the pK_a of the group in the native state where it may interact with oppositely charged groups, lowering its pK_a or with like-charged groups which act to raise its pK_a . Typical pK_a shifts in proteins are up to 2 units. Proteins normally have a bell shaped stability pH profile and this reflects exactly this effect. The pK_a of acidic groups in proteins is around 4 and that of basic groups around 10 so the major changes in stability occur between 2 and 6 and between 8 and 12. At neutral pH, the only pK_a is that of Histidine and this may or may not modulate stability in this region.

DSC can be used to determine the thermal stability of a protein at virtually any pH of interest. The T_m and ΔH can be used to

calculate ΔG at a common temperature of comparison and the pH stability profile can be analysed using Eq 13 to obtain the protonation change of denaturation at any pH. The effects of specific mutation of charged residues in the protein on these profiles can indicate which groups are involved in the protonation behaviour.

DSC to detect and quantify ligand binding

It could be convenient to think of protons as simple non-covalently attached ligands that have different 'affinities' (pK_a) for the native and denatured states. With this analogy in mind, we can immediately see that the effects of mass action must apply to any ligand that has different binding affinity to the native and denatured states. Most, if not all biologically relevant ligands bind tightly and specifically to the native states of their cognate proteins and have no affinity for the corresponding denatured states. Therefore, when they denature they release ligand into solution affecting the concentration. The mass action effect is that when the concentration of ligand in solution is changed it will affect the native to denatured equilibrium, i.e., the stability of the protein.

This approach can be used in two ways. Firstly as a crude screening tool for ligands that bind to a protein. The thermal stability of a protein is measured on its own and then in the presence of the ligands to be screened. These are added at some level of excess concentration over the protein to ensure that ligands binding with reasonable affinities will saturate the binding sites on the protein. In cases where the thermal stability is increased there is evidence that at the temperature of denaturation there was stabilisation of the protein through binding to the native state. The opposite holds in that a decrease in thermal stability suggests a ligand binding to the denatured but not the native state. Some caution must be attached if the changes in thermal stability are small since the ligands may also act indirectly to change ionic strength or pH, which could then modulate stability. Suitable controls can resolve these possibilities.

A second more quantitative use of this method involves measuring thermal stability as a function of increasing ligand concentration. Data such as in Figure 5 result and this can be used to obtain an estimate of the binding constant between the ligand and protein (Brandts and Lin, 1990):

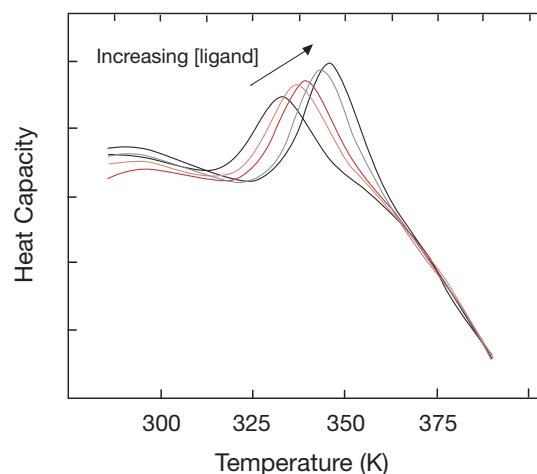


Figure 5: Raw DSC data for the denaturation of a 60 amino acid protein in the presence of its cognate peptide ligand. The protein concentration was 200 μM (1 mg/ml) and the ligand concentration increased from 0 – 250 μM .

There are several advantages to this approach over other more conventional methods. The method may be used to determine very tight binding constants that cannot be measured in conventional equilibrium methods. The signal measured is ΔH , which is a universal feature of protein denaturation, so there is no need to develop specific methods for each protein studied. There is also no reliance on an optical probe, so high concentrations of ligands that can be problematic in spectroscopic methods can be used. Indeed, there is no reason why mixtures of ligands cannot be used, screening for competitive binding for a known ligand, or in elaborate combinatorial screening protocols.

The future of DSC in biophysical studies

Recent advances in DSC instrumentation and automation technology have allowed the production of an automated 'high' throughput DSC; MicroCal's Cap DSC (Plotnikov *et al.*, 2002). As well as using automatic robotic sample loading, these instruments scan at faster rates making the whole measurement more rapid. Prior to using higher scan rates it is wise to check the dependence of the T_m and enthalpies on this parameter to make sure the system is at equilibrium as discussed above. Once this is established the Cap DSC can acquire up to 25 scans or so in a 24 hr period and can be loaded

with samples, held in a refrigerated compartment, that will allow the instrument to run for a week or so:

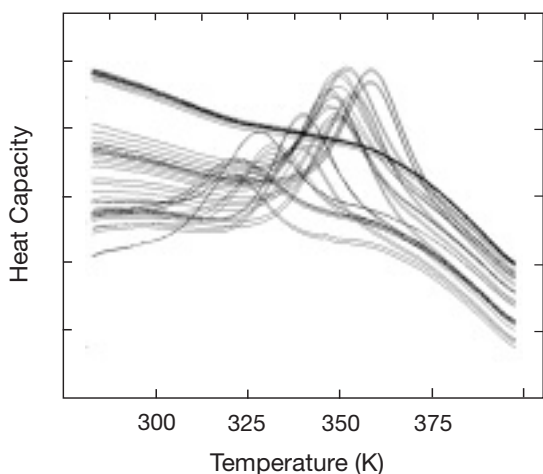


Figure 6: Raw DSC data for the denaturation of 20 different point mutants of a 60 amino acid protein recorded on a Cap DSC. Protein and baseline scans were collected at 125 K/hr over a weekend.

This automated approach to DSC presents new opportunities to use the technique as a screening tool. Solution conditions (pH, ionic strength, additives) that affect the thermal stability of a protein can be rapidly assessed. This information might be of use indicating optimal conditions for shelf life studies or crystallisation trials. Similarly, ligands that bind to a protein, and thereby increase its thermal stability, could be screened for, or evaluated in detail following their identification in a higher throughput primary screen.

References

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Text citations

Plotnikov, V.V., *et al.* (1997) A new ultrasensitive scanning calorimeter. *Anal. Biochem.* 250, 237-244.

Neet, K.E. and Timm, D.E. (1994) Conformational stability of dimeric proteins: Quantitative studies by equilibrium denaturation. *Prot. Sci.* 3, 2167-2174.

Johnson, C.R., *et al.* (1995) Thermodynamic analysis of the structural stability of the tetrameric oligomerization domain of p53 tumour suppressor. *Biochemistry* 34, 5309-5316.

Freire, E., *et al.*, (1990) Calorimetrically determined dynamics of complex unfolding transitions in proteins. *Ann. Rev. Biophys. Chem.* 19, 159-188.

Lepock, J.R., *et al.* (1992) Influence of transition rates and scan rate on kinetic simulations of differential scanning calorimetry profiles of reversible and irreversible protein denaturation. *Biochemistry* 31, 12706-12712.

Myers, J.K., *et al.*, (1995) Denaturant m values and heat capacity changes: relation to changes in accessible surface areas of protein unfolding. *Prot. Sci.* 10, 2138-2148

Connelly, P.R., *et al.*, (1994) Enthalpy of hydrogen bond formation in a protein ligand binding reaction. *Proc. Natl. Acad. Sci.*, 91, 1964-1968.

Brandts, J.F. and Lin, L.N. (1990). Study of strong to ultratight protein interactions using differential scanning calorimetry. *Biochemistry* 29, 6927-6940.

Plotnikov, V.V., *et al.* (2002) An autosampling differential scanning calorimeter instrument for studying molecular interactions. *Assay Drug Dev. Technol.* 1, 83-90.

Further reading

Cooper, A. *et al.*, (2000) Differential scanning microcalorimetry. in S. E. Harding and B. Z. Chowdhry (Eds.), *Protein-Ligand Interactions: hydrodynamics and calorimetry.* Oxford University Press. p 287-318

Freire, E. (1995) Differential Scanning Calorimetry. *Methods Mol. Biol.* 40, 191-218.

Privalov, P.L. and Potekhin, S.A. (1986). Scanning microcalorimetry in studying temperature-induced changes in proteins. *Methods Enzymol.* 131, 4-51.

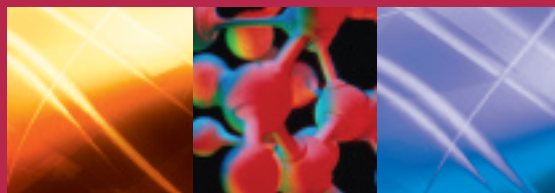
Plum, G.E. and Breslauer, K.J. (1995). Calorimetry of proteins and nucleic acids. *Cur. Opin. Struct. Biol.*, 5, 682-690.

Clas, S.D., *et al.*, (1999). Differential scanning calorimetry : applications in drug development. *Pharm. Sci. Technol. Today* 8, 311-320

Clausse, D., *et al.*, (2005). Morphology characterization of emulsions by differential scanning calorimetry. *Adv. Colloid Interface Sci.* 117,59-74.

Beezer, A.E., *et al.*, (1999). Pharmaceutical microcalorimetry: applications to long-term stability studies. *Int. J. Pharm.* 179, 159-165.

Jelesarov, I. and Bosshard, H.R. (1999). Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J. Mol. Recognit.*, 12, 3-18.



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