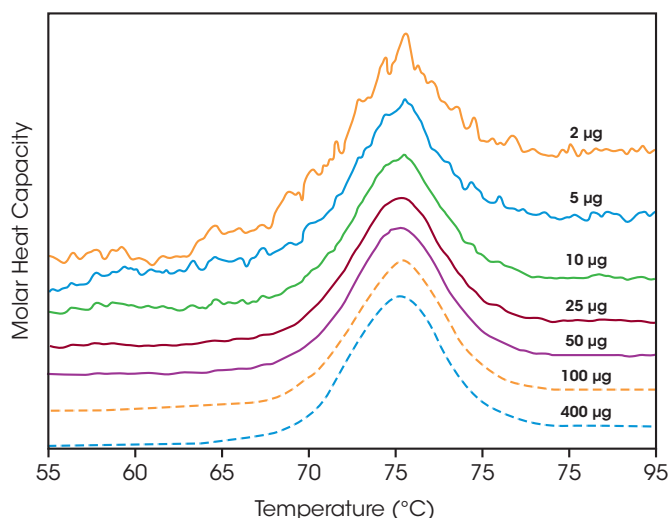


Keywords: Proteins, amino acids, DSC

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Fifty years ago, the elegant experiments of Christian Anfinsen showed unequivocally that the linear sequence of amino acids of a protein (its primary structure) encodes all the information for its three-dimensional structure (Sela *et al.*, 1957; Anfinsen, 1973). It is now clear that the sequence directs the interactions both between component amino acids, and between the protein and the solvent, and that these interactions are governed by thermodynamics. What is not clear is how the sequence encodes the complex structure of a protein.



Just µg of lysozyme allows accurate determination of enthalpy and entropy of unfolding.

DSC is the most direct and sensitive approach for characterizing the thermodynamic parameters controlling noncovalent bond formation (and therefore stability) in proteins and other macromolecules. In an experiment requiring only a few micrograms of material, the protein is thermally unfolded, allowing the relationship between enthalpy and entropy of the denaturation process to be established in about one hour. Correlating thermodynamic properties to stability is necessary for the rational design of engineered proteins and protein therapeutics.

The thermodynamic balance in proteins is delicate: the folded structure of a small protein is stabilized by approximately 80-120 kJ/mol (20-30 kcal/mol) compared to its unfolded structure, and many proteins thermally unfold by 70 °C. If most proteins can be denatured by relatively small increases in temperature, and if the balance between folded and unfolded states is held by the equivalent of 5 – 8 hydrogen bonds, how are thermophilic proteins stabilized, allowing organisms to thrive at elevated pressures and at temperatures above 100 °C (Perl and Schmid, 2002)? In the absence of thermal changes, what thermodynamic events trigger proteins that cause amyloid and prion diseases to switch from one stable conformation to another, with fatal consequences

(Mastrianni, 2004)? Would a thorough comprehension of the interactions controlling conformation permit the full complement of three-dimensional protein structures to be accurately predicted from an organism's genome (Webster 2000)? Achieving a fundamental understanding of the thermodynamics directing the formation of macromolecular noncovalent bonds would answer these questions, and critically impact such diverse endeavors as the self-assembly of protein inspired supramolecular structures (Yeates and Padilla, 2002), the composition of shape-memory biocompatible polymers (Langer and Tirrell, 2004), the rational design of protein-specific small molecule drugs (Waldrón and Murphy, 2003) and the formulation of stable protein therapeutics (Remmele and Bombotz, 2000).

The thermodynamic parameters that control noncovalent bond formation in proteins and other macromolecules can be directly studied and quantified by differential scanning calorimetry (DSC). This Application Note examines the utility of DSC for characterizing protein stability.

ANALYZING PROTEIN THERMAL STABILITY

As described in the DSC Overview Note, analyzing the stability of a protein in dilute solution involves determining changes in the partial molar heat capacity of the protein at constant pressure (ΔC_p). The change in heat capacity of a compound reflects its ability to absorb heat and experience a defined increase in temperature. Because of its extensive hydrogen bond network, water has a substantially higher heat capacity than organic compounds such as proteins. In addition, water is more ordered and tightly packed adjacent to hydrophobic patches on the protein's surface: since water cannot make hydrogen bonds to nonpolar moieties, hydrogen bonding between water molecules is maximized (clathrate formation) at the protein-solvent interface (Shinoda, 1977). As the temperature is raised, the ordered water shell around the protein starts to disorder and become more like bulk water. This 'melting' produces the anomalously large heat capacity of protein aqueous solutions. The contribution of the protein to the calorimetrically measured heat capacity (its partial C_p) is determined by subtracting a scan of a buffer blank from the sample data prior to analysis. The partial C_p is not just the heat capacity of the protein, but also includes any effects the protein had on the solvent, such as promoting clathrate formation (Freire, 1995). Heating the protein sample initially produces a slightly increasing baseline but as heating progresses, heat is absorbed by the protein and causes it to thermally unfold over a temperature range characteristic for that protein, giving rise to an endothermic peak (Fig. 1). During the unfolding process, water molecules around the protein reorganize and restructure as more non-polar sidechains are exposed. Once unfolding is complete, heat absorption decreases, and a new baseline is established.

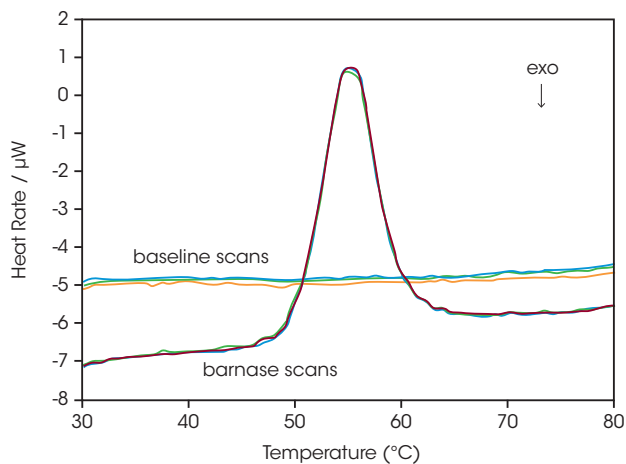


Fig. 1. Three baseline temperature scans, and three scans of 60 μg of barnase in 0.3 mL 20 mM phosphate buffer, pH 5.5 obtained at a scan rate of 1 $^{\circ}\text{C}/\text{min}$ using DSC.

After blank subtraction, the data can be analyzed to provide a complete thermodynamic characterization of the unfolding process. Integration of the heat capacity of the sample vs. temperature yields the enthalpy (ΔH)

$$\Delta H = \int_{T_1}^{T_2} C_p dT$$

of the unfolding process, which is due to endothermic events such as the breaking of hydrogen bonds, and exothermic processes such as the disruption of hydrophobic interactions (Bruylants *et al.*, 2005). The shift in baseline before and after the transition represents the change in heat capacity (ΔC_p of the protein (and associated water) caused by unfolding. The transition midpoint (T_m , or the melting temperature) is the temperature at which half the protein molecules are folded and half are unfolded. The entropy (ΔS) is obtained from the area under the curve of C_p/T vs. T .

$$\Delta S = \int_{T_1}^{T_2} (C_p / T) dT$$

Establishing the reversibility of the unfolding reaction is not necessary if only the determination of T_m is of interest. However, a full thermodynamic characterization of the unfolding event requires that the reversibility of the unfolding transition be verified (Marky and Breslauer, 1987). This is accomplished by rescanning the sample under identical conditions and obtaining a second endotherm that is superimposable on the first. Irreversibility, if observed, may be due to a second (irreversible) unfolding event occurring after the first (reversible) unfolding transition at T_m . The capillary cells in the DSC delay the onset of irreversible protein aggregation and precipitation, allowing a complete, interpretable scan to be obtained on essentially any sample. For example, 100 μM bovine serum albumin (in 0.1 M phosphate buffer, pH 7.2, scanned from 20 to 90 $^{\circ}\text{C}$ at 1 $^{\circ}\text{C}/\text{min}$) precipitated after a single scan, whereas under identical conditions precipitate was not observed below 300 μM in the DSC (capillary cells).

The sharpness of the transition peak is indicative of the cooperative nature of the unfolding process. If unfolding produces a narrow,

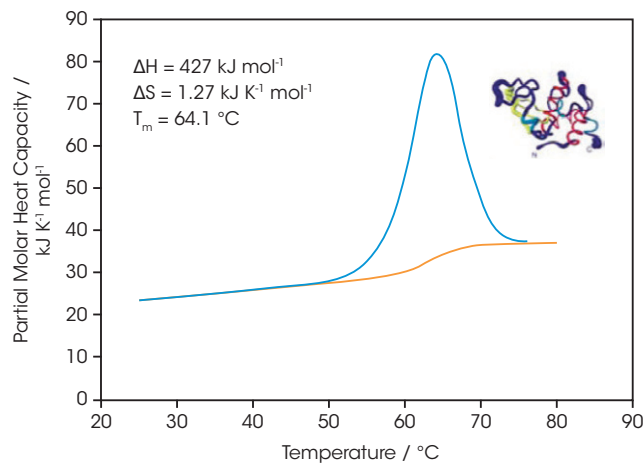


Fig. 2. Temperature scan, converted to partial molar heat capacity, and fitted baseline for 1 mg mL^{-1} hen egg white lysozyme in 0.2 M glycine buffer, pH 2.7. Data obtained at a scan rate of 1 $^{\circ}\text{C}/\text{min}$ using DSC.

relatively symmetric peak such as in Fig. 2, the transition is likely to be two-state, reversible and highly cooperative (Bruylants *et al.*, 2005). This is often the situation for small, single domain proteins; the more complex behavior of multidomain proteins is considered in detail in a separate application note. Two-state behavior can be verified by fitting the curve to the two-state van't Hoff equation; this calculation is performed by software supplied with DSC. The van't Hoff enthalpy obtained (ΔH_{vH}) can be different from the calorimetric enthalpy since the latter includes all contributions from the sample, including solvent rearrangement. If the van't Hoff and calorimetric enthalpies are the same, the denaturation process may be accurately approximated by the two-state model. If the van't Hoff enthalpy is smaller than the calorimetric enthalpy, an unfolding intermediate is likely formed, while if it is larger than the calorimetric enthalpy, the protein is likely multimeric (Marky and Breslauer, 1987).

CORRELATING THERMODYNAMIC PROPERTIES TO STABILITY

The stability of a protein (and hence its T_m) depends on environmental conditions. For example, a protein will be optimally stable at a characteristic pH and will be destabilized at pH values deviating from that optimum (Privalov and Khechinashvili, 1974). The conformational stability of a protein may be affected by its concentration (showing the importance of protein-protein interactions), ionic strength of the solution (showing the role of electrostatic interactions) and scan rate (showing that thermal denaturation has a kinetic component). A complete thermodynamic characterization of a simple protein such as lysozyme illustrates the complex interplay of stabilizing and destabilizing interactions and shows that lysozyme is unusually stable for a small protein (Pfeil and Privalov, 1976; Griko *et al.*, 1995). The different heat capacities of the folded and denatured form of the protein show that the enthalpies and entropies of both states are strongly temperature dependent, but they largely compensate each other so that the net stability of a typical small protein is approximately 80–120 kJ/mol (20–30 kcal/mol). The large ΔC_p of unfolding also indicates that a protein will have maximum stability at a given temperature (depending on its sequence, pH, etc.), and that its net stability will decrease above and below that temperature. Thus, proteins can not only be denatured by heating but can also be denatured by cooling.

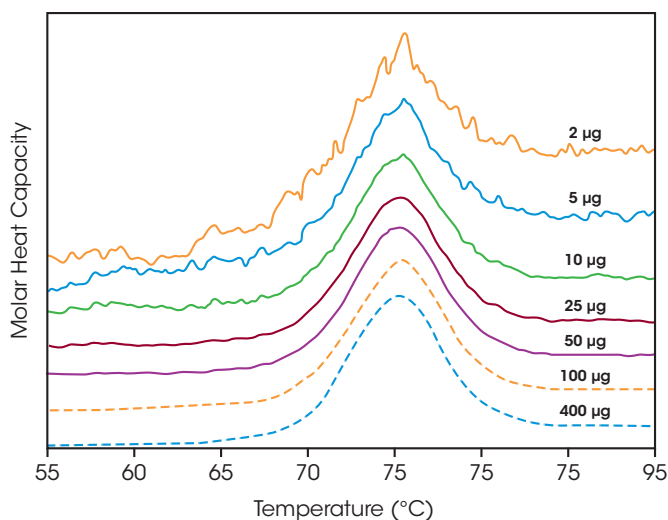


Fig. 3. Temperature scan, converted to molar heat capacity, for hen egg white lysozyme solution in 0.2 M glycine buffer, pH 4.0. Lysozyme concentrations ranged from 400 μg to 2 μg in the 300 μl sample cell. Curves are offset to aid presentation. Data were obtained using a scan rate of 2 $^{\circ}\text{C}/\text{min}$ on a DSC.

The amount of protein available for characterization can be very limited, which in the past restricted the application of biophysical techniques such as DSC. Recent advances in instrument sensitivity have alleviated this constraint by significantly decreasing the amount of sample required for DSC analyses. Fig. 3 shows raw data for lysozyme scanned on a DSC. Protein amounts ranged from 400 μg to 2 μg in the 300 μl sample cell. Thermodynamic parameters obtained at all concentrations are consistent (Table 1), showing that even 2 μg of protein can be sufficient to allow accurate thermodynamic characterization of a protein.

Can the thermodynamic properties of a protein, analyzed by DSC, be correlated to structure and stability? As more proteins are carefully characterized both experimentally and computationally, correlations between thermodynamics and structure are being revealed, albeit not yet at a reliably predictive level. A discussion of the stabilizing and destabilizing interactions outlined above comprises a vast body of literature; much of this literature has been rigorously and comprehensively reviewed by Robertson and Murphy (1997).

RATIONALIZING THE STABILITIES OF FOLDED CONFORMATIONS: MUTANT VS. WILD-TYPE PROTEINS

Hydrophobicity scales of the amino acids, and propensity scales for amino acids in various secondary structures (Koehl and Levitt, 1999; Acevedo and Lareo, 2005), are used in conjunction with molecular modeling to predict the effect of specific mutations. Systematic mutation of proteins with known three-dimensional structures often aims to quantify the resultant stability changes in terms of the energetics of unfolding. A single site-specific mutation can give rise to a large increase or decrease in stability, but many mutations produce only small effects. The ability of proteins to make subtle solvation and conformational changes in order to compensate for the enthalpic and entropic disturbances caused by a mutation can result in only small free energy changes being observed upon thermal denaturation of the protein. Nonetheless, the growing database of proteins thoroughly characterized by DSC is aiding comprehension of mutational effects on the stability of proteins, although care must be exercised in the analyses (Sturtevant, 1994; Plum and Breslauer, 1995; Robertson and Murphy, 1997; Brylants *et al.*, 2005). The recent redesign of proteins for increased stability (Fowler *et al.*, 2005; Lee *et al.*, 2005), and in particular the rational redesign of enzymes for both enhanced entropic stability and catalytic function (O’Fagain, 2003; Eijsink *et al.*, 2004), illustrate that as long as the mechanisms controlling the stability and thermal unfolding of a protein are sufficiently understood, it is possible to logically and predictably alter the properties of proteins of known structure.

SUMMARY

Understanding the thermodynamics controlling the formation of macromolecular noncovalent bonds is necessary if the promise of diverse endeavors such as the design of supramolecular assemblies and therapeutic proteins are to be realized. Many factors are responsible for the stability of a protein, including hydrogen bonds to the solvent and intramolecular interactions. Rigorous characterization of the thermodynamic basis of a protein’s stability is prerequisite for enhancing its thermal and functional characteristics by sequence alteration. DSC is the most direct approach for determining the intrinsic stability of a protein in dilute solution, allowing the relationship between enthalpy and entropy of the denaturation process to be quickly established. The superior sensitivity of the CSC N-DSC II or N-DSC III allows accurate thermodynamic parameters to be obtained from just a few micrograms of protein.

Table 1. Thermodynamic parameters obtained from the lysozyme scans presented in Figure 3.

Lysozyme in cell (μg)	Calorimetric		van’t Hoff	
	ΔH (kJ mol^{-1})	ΔS ($\text{kJ K}^{-1} \text{mol}^{-1}$)	T_m ($^{\circ}\text{C}$)	ΔH (kJ mol^{-1})
400	512	1.46	78.0	515
100	512	1.46	78.0	509
50	517	1.47	77.9	513
25	513	1.46	77.8	513
10	515	1.47	78.0	515
5	490	1.40	78.1	510
2	503	1.43	77.8	499

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