# Characterizing Protein Structure by DSC

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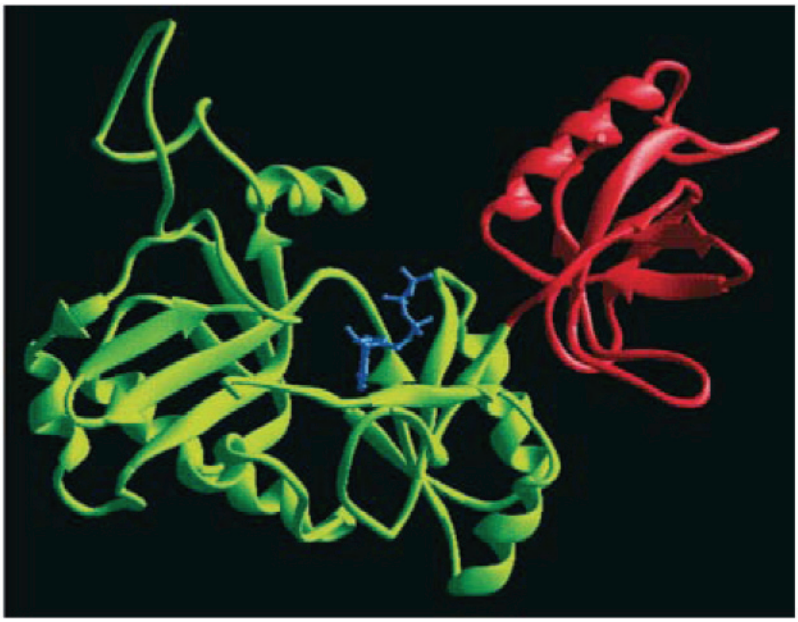

*DSC is a valuable approach for characterizing the domain and quaternary structures of proteins.*

*Various techniques yield either structural or functional information about a protein, but DSC uniquely provides insights into both the three-dimensional arrangement and the functional properties of a protein's structural components.*

The non-covalent interactions dictating the three dimensional structures of proteins can be segregated into two major categories: those resulting from interactions between component amino acids in the sequence, and those resulting from interactions between the protein and the solvent. These interactions, which also control the stability of the protein, are governed by thermodynamics. Controlled temperature changes have been used extensively to perturb protein structures in an effort to learn how heat affects these inter- and intramolecular interactions, but it is only in the past 10 years or so that calorimeters with the sensitivity and baseline stability required for studying very dilute protein solutions have become commercially available. This note examines the application of differential scanning calorimetry (DSC) for monitoring tertiary and quaternary structure changes in solubilized proteins, and forms part of a series of application notes dealing with the biophysical characterization of proteins by DSC. In particular, the Calorimetry Sciences Corporation (CSC) application note entitled *Characterizing Protein Stability by DSC* provides a concise description of the thermodynamic principles underlying protein stability, and the application note entitled *Characterizing Protein/Ligand Binding by DSC* describes the interactions controlling protein/ligand interactions and their effects on protein stability. For a general description of the principles behind DSC, how an experiment is conducted and interpreted, and a summary of the types of questions that can be addressed by this technique, please refer to CSC's Overview Note entitled *Life science applications of DSC*.

### *Thermally-induced Structural Changes*

When globular proteins unfold, significant heat is absorbed over a temperature range that is characteristic for that protein in those specific solvent conditions (pH, protein concentration, buffer salt concentration, *etc.*). As the temperature of a protein solution is slowly raised (but prior to the protein unfolding), the baseline observed on the thermogram increases slightly, then as heating continues, the protein thermally unfolds and gives rise to an endothermic peak. During the unfolding process, water molecules around the protein reorganize and restructure as hydrophobic residues from inside the protein are exposed; this rearrangement of water molecules, coupled with their increasing disorder as the temperature continues to rise, produces the anomalously large heat capacities of protein aqueous solutions. Once unfolding is complete, heat absorption decreases and a new, higher baseline is established. The contribution of the protein to the calorimetrically measured heat capacity (its partial heat capacity) is determined by subtracting a scan of a buffer blank from the sample data. The data can then be analyzed to provide a complete thermodynamic characterization of the unfolding process. The area under the endotherm represents the calorimetric enthalpy of the unfolding process, the shift in the baseline before and after the transition represents the change in heat capacity of the protein caused by unfolding, and the sharpness of the transition peak is indicative of the cooperative nature of the unfolding process. A narrow, relatively symmetric peak indicates that the transition is probably reversible and highly cooperative. For small single-domain globular proteins, the endotherm can often be accurately represented by a two-state transition, indicating that the protein unfolds without populating any intermediate states. The appropriateness of the two-state model for a given protein can be verified by fitting the curve to the two-state van't Hoff equation; this calculation is performed by the software supplied with CSC DSCs. 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, whereas if it is larger, the protein likely associates and forms oligomers (Marky and Breslauer, 1987). The thermodynamic analysis of single domain proteins is described in detail in the CSC application note *Characterizing Protein Stability by DSC*.

A narrow, symmetric endotherm is not necessarily indicative of a single-domain protein: a multi-

domain protein can give rise to an endotherm that mimics a two-state transition if all the domains have very similar thermal stabilities and thus unfold essentially simultaneously (Privalov and Privalov, 2000). More commonly, however, multi-domain proteins produce endotherms that are broad and have a complex shape due to the independent unfolding of the constituent domains, each over its own characteristic temperature range (Privalov and Potekhin, 1986; Luque *et al.*, 2002). Therefore, the shape of the endotherm encodes thermodynamic information on the states populated by the protein throughout the unfolding process. After blank subtraction, deconvolution analysis of the endotherm shows that thermal unfolding proceeds through several cooperative stages, with each stage generally (but not always) corresponding to the unfolding of an independent structural domain or subunit (Freire, 1995; Privalov and Dragan, 2006). Although tertiary and quaternary structure can be inferred from other types of experimental information (*e.g.*, homology modeling, limited proteolysis or sequence data), calorimetric data alone provides a direct measure of the thermodynamic parameters specifying the stabilities of each structural unit, in addition to information on all the states populated during the unfolding process.

### *Ligand-induced Structural Changes*

DSC can also be used to characterize both the specific binding of a ligand (for example, a drug to a receptor binding site), or nonspecific binding (for example, detergents binding to hydrophobic patches on a protein surface). In some instances ligand binding, even if to a specific receptor site, results in long-range protein structural rearrangements that stabilize the entire complex. For example, the binding of $Zn^{2+}$ to specific sites on insulin triggers the monomeric protein (which unfolds at 68 °C) to oligomerize to a hexameric state that unfolds at 87 °C (Huus *et al.*, 2005). DSC can also provide a framework for understanding the thermodynamic basis driving preferential ligand binding: rubredoxin has been shown to bind metals in the order $Fe^{2+} << Fe^{3+} < Zn^{2+} < Cd^{2+}$, with a corresponding increase in the thermal stability of the entire protein that can be correlated to specific structural changes occurring at the metal-binding site (Bonomi *et al.*, 2000).

The use of DSC for studying tertiary structure changes triggered by ligand binding to a protein is illustrated by the binding of $Zn^{2+}$ to bovine α-lactalbumin. α-Lactalbumin is comprised of two domains: an α-helical domain (residues 1-34 and 86-123) and a mixed β-sheet domain (residues 35-85; Chrysina *et al.*, 2000). The two domains are linked by a flexible loop, with the single $Ca^{2+}$ being bound by two residues on the loop and three residues from the two domains ($K_a = 2.9 \times 10^8\ M^{-1}$; Hendrix *et al.*, 2000). There is also a single strong $Zn^{2+}$ binding site ($K_a = 5 \times 10^5\ M^{-1}$; Permyakov *et al.*, 1991) comprised of three residues at the N-terminus, and several putative weaker $Zn^{2+}$ binding sites. *Figure 1* shows DSC scans of $Ca^{2+}$-saturated bovine α-lactalbumin at various protein:$Zn^{2+}$ ratios. The midpoint of the thermal unfolding of the protein decreases from 65 °C in the absence of $Zn^{2+}$ to 35 °C at a protein:$Zn^{2+}$ ratio of 1:70; the protein precipitates at higher $Zn^{2+}$ concentrations. The enthalpy of unfolding is also decreased substantially by high $Zn^{2+}$ concentrations. At low concentrations of $Zn^{2+}$, the thermal unfolding of α-lactalbumin is reversible, so the thermograms were deconvoluted with a two-state reversible model. In the absence of or at low concentrations of $Zn^{2+}$, the thermogram could be closely approximated by two transitions. For example, in the absence of $Zn^{2+}$, one transition, with an enthalpy of 200 kJ/mol, has a midpoint at 63.5 °C, and the second, with an enthalpy of 347 kJ/mol, has a midpoint at 66.3 °C. This is indicative of the independent thermal unfolding of two domains of different sizes and stabilities, consistent with the crystal structure of the protein (Chrysina *et al.*, 2000). At higher $Zn^{2+}$ concentrations (above 1:5 protein:$Zn^{2+}$ molar ratio), the thermogram becomes more asymmetric and fitting the curve requires the introduction of a third transition. For example, at a protein:$Zn^{2+}$ ratio of 1:35, the thermogram can be best fit with transitions centered at 38.5, 41.3 and 44.9 °C, with enthalpies of 38.9, 54.4 and 16.3 kJ/mol, respectively.

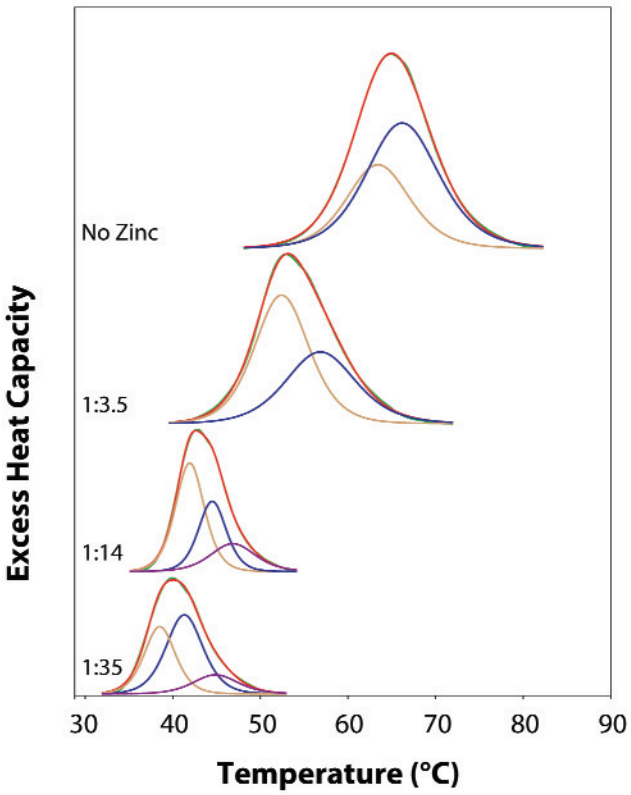


*Figure 1. Thermograms for bovine α-lactalbumin in the presence of increasing concentrations of $Zn^{2+}$. $Ca^{2+}$-loaded α-lactalbumin (purchased from Sigma-Aldrich) was dialyzed against 10 mM Tris, 2 mM $CaCl_2$ (pH 7.5) at 4 °C overnight. The protein (0.67 mg/mL) in the presence of different concentration of $ZnCl_2$ was scanned at 1 °C/min on a 6300 N-DSC III. Thermograms were fit as described in the text. Scans are offset for clarity.*

As explained in the CSC application notes entitled *Characterizing Binding Interactions by ITC* and *Characterizing Protein/Ligand Binding by DSC*, the binding of a ligand to a protein stabilizes the protein. Since $Zn^{2+}$ decreases the stability of α-lactalbumin and, above a 70-fold molar excess, precipitates the protein, $Zn^{2+}$ apparently binds more strongly to unfolded α-lactalbumin than to the native structure, driving the equilibrium of the population further towards the unfolded state with increasing $Zn^{2+}$ concentration. Supporting experimental data from limited proteolysis or mutation/stability studies would be required before drawing more substantive conclusions from these DSC results, but the above example clearly demonstrates the utility of DSC for following tertiary structure changes in proteins.

In contrast to the above example, in other ligand/protein systems only select domains or subunits are stabilized or destabilized. DSC studies on such water-soluble or membrane-soluble protein complexes can provide functional and structural information about the interactions driving complex formation that cannot be derived from crystallographic or NMR structural data. For example, DSC studies showed that L-phenylalanine binds to the active sites of tetrameric phenylalanine hydroxylase rather than to the regulatory domain as previously thought, and that the regulatory domain exerts its control through interactions with the catalytic domains and with the tetramerization motif (Thorolfsson *et al.*, 2002). In another study it was shown that phosphoglycerate kinase unfolds in two distinct stages thought to correspond to the sequential unfolding of the N- and C-terminal structural domains. However, DSC characterization of both the intact protein and fragments corresponding to the structural domains showed that the stability domains in fact do not correspond to the structural domains: only a portion of the C-terminal domain is thermally stable, while another portion of the C-terminal domain interacts with the N-terminal domain to form a thermally unstable unit (Zecchinon *et al.*, 2005). In a third example, the interaction of the $Ca^{2+}$ channel blocker D-600 (Ortego *et al.*, 2000) with sarcoplasmic reticulum ATPase, an ion-transporting membrane protein, showed that in the presence of $Ca^{2+}$ the blocker binds to and stabilizes the transmembrane domain, and not the catalytic cytoplasmic domain as previously believed.

It is clear that even for complex or insoluble proteins, equilibrium thermodynamics analysis in conjunction with functional data provides information regarding domain organization and interdomain interactions that cannot be obtained from structural data alone. In addition, if binding stabilizes a specific domain, the resulting change in heat capacity of the domain

reflects changes in the hydration of the protein, and the binding-induced alterations in the ratio of exposed polar and nonpolar groups provide information on the polarity and size of the binding interface (Spolar and Record, 1994).

### *Summary*

Differential scanning calorimetry provides the most direct approach for monitoring the effect of environment (*e.g.*, temperature, ligands) on the tertiary and quaternary structures of proteins, and allows quantification of the thermodynamic parameters that specify the stabilities of a protein's components. Because of the direct nature of the technique, DSC can provide structural and mechanistic insights into protein function that are difficult or impossible to obtain using other approaches.

### *References*

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


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