3.014 Materials Laboratory
October 24-28, 2005

Lab Week 2 – Module α_2

AFM/DSC Study of Protein Denaturation

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Objectives

- Understand the theory and instrumentation in Differential Scanning Calorimetry (DSC) and Atomic Force Spectroscopy
- Observe the denaturation of Bovine Serum Albumin (BSA) via DSC and force spectroscopy
- Quantify the energy required to denature the protein by each method

Summary of tasks

(1) Obtain the DSC thermogram of BSA using the TA Instruments Q10 DSC

Lesson to be learned: Understand how thermal energy can be used to denature proteins.

(2) Prepare a sample of BSA adsorbed to gold

Lesson to be learned: Why proteins readily adsorb to surfaces such as gold

(3) Stretch individual proteins using the Molecular Force Probe (MFP-3D, Asylum Research)

Lesson to be learned: How AFM can be used to study the structural properties of a material and the energy required to denature supramolecular architecture

Introduction

As was discussed in 3.012, a great deal about the thermodynamic properties of a material can be learned by studying its behavior in response to heat and various forms of work. In this laboratory experience, we will study the energetics of denaturation (unfolding) of a protein in response to heat and mechanical force. By doing so, we will be able to quantify the enthalpy associated with the phase transition of the protein from the folded to the unfolded state.

The protein we will use is bovine serum albumin (BSA), a protein found in cow blood. Albumins are present in the blood of many mammals and typically are among the most abundant proteins in the bloodstream. BSA exists in its native folded state at room temperature under "biological" conditions; that is, in water of a fairly neutral pH. To mimic this environment, we will dissolve BSA in a buffer solution (phosphate buffered saline, PBS) with ionic strength 1.0 M and pH 7.4.

Thermal denaturation of BSA will occur in a differential scanning calorimeter (DSC). We will obtain a thermogram that depicts the heat flow into or out of the sample as the temperature is increased at a set rate. Above some given temperature, the bonds holding BSA together will be broken and the protein will denature. We will observe this as a peak on the thermogram, and from this data we will determine the enthalpy of denaturation.

We also will deposit a layer of BSA onto a gold surface and attempt to mechanically denature the protein by physically pulling it apart. Using an atomic force microscope (AFM) as a molecular force probe (MFP), we will "grab" onto individual proteins and extend them. When the protein reaches a certain extension, it will no longer be able to elastically deform, and the secondary bonds giving it its structure will have to break. By analyzing the force required to unfold BSA in this way, we will obtain another (hopefully similar!) value for the energy of denaturation.

Experimental techniques

Differential scanning calorimetry (DSC)

DSC is a technique to measure the heat flow into or out of a sample (relative to a reference sample) as a function of temperature. By measuring the amount of heat a sample absorbs or releases (ΔH) as we change the temperature (T) at a constant rate, we can directly determine thermodynamic quantities such as:[1,2]

- The enthalpy of melting, ΔH_m (and that of other phase transitions)
- The temperatures of various phase transitions (e.g. solid→liquid, liquid→vapor, and vice versa), as well as the crystallization and glass transition temperatures (T_c and T_g, respectively) of polymers
- The thermal degradation temperature

Why is ΔH the relevant thermodynamic variable in this situation? Consider the definition of ΔH:

$$dH = TdS + VdP\,.$$

At constant pressure, we find that

$$dH = TdS = dq$$

$$\Delta H = \int dH = q\,.$$

Thus, the enthalpy change is directly related to the heat flow in a system, which is what we measure with DSC.

Most of the thermodynamic quantities accessible by DSC are associated with phase transitions, such as melting. Recall from class that the change in enthalpy upon melting is known as the enthalpy of melting, ΔH_m, and that there is a discontinuity in the enthalpy at the solid-liquid phase transition. More practically, consider what happens to ice when it is heated at a constant rate. At first, its temperature rises as it is heated. When it

reaches its melting temperature, T_m, the ice begins to melt and become liquid water. However, until the entire sample has melted, the temperature does not rise above T_m even though heat is still being added to the system. Only once the entire sample has become liquid does the temperature rise again. This is because heat is required to overcome the energetic barrier between the two phases and drive the solid→liquid phase transition.

In DSC, the temperature of a sample is increased (or decreased) at a constant rate, and the heat flow into (or out of) the sample in order to maintain that rate is measured. In our above example, the heat flow is constant until we reach T_m. Then, a large amount of heat must be added in order to melt all the ice and maintain the imposed rate of temperature increase. Thus, the DSC will detect an endothermic peak (energy being put into the system) in the heat flow at T_m, from which we can calculate the enthalpy of melting.

A typical DSC curve of a crystalline polymeric material such as high density polyethylene (HDPE), for example, would look like:

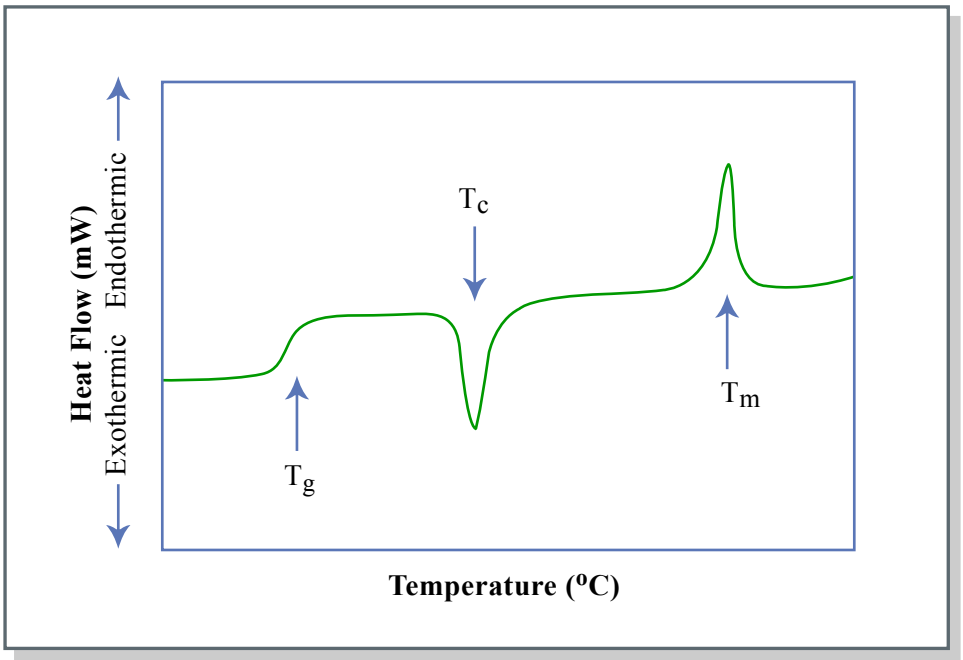


Figure by MIT OCW.

where T_m is the melting temperature, T_c is the crystallization temperature, and T_g is the glass transition temperature. Exothermic peaks, such as that of crystallization, point down, indicating that energy is being released from the sample. Endothermic peaks, such as those associated with melting, are positive because energy is being put into the sample.

Interpreting the DSC Curve[3]

Zero Line: the curve obtained by running through the heating cycle with the instrument empty

Baseline: The line constructed in such a way that it connects the measured curve before and after a peak

Peak: Deviation from the baseline as a result of disruption of steady state by some production or consumption of heat by the sample

Initial Peak Temperature, T_i: The T at which the curve begins to deviate from the baseline

Extrapolated Peak Onset Temperature, T_e: The T at which a line drawn through the almost linear section of the ascending peak slope intersects the baseline

Extrapolated Peak Completion Temperature, T_c: The T at which a line drawn through the almost linear section of the descending peak slope intersects the baseline

Final Peak Temperature: The T at which the curve returns to the baseline

General notes about data interpretation:

- Positive peaks are endothermic (a positive amount of energy is being put into the sample), and negative peaks are exothermic.
- Endothermic peaks (melting, solid-solid transitions, e.g.) are often reversible on cooling; exothermic peaks (crystallization, decomposition) are not.
- The enthalpy of a phase transition (ΔH) can be determined by integrating the peak.
- Crystallization is not instantaneous; parts of the sample begin to crystallize before others, and so the magnitude of the exothermic peak of crystallization is rate-dependent. Often it take an asymmetric shape, tailing off to the baseline as crystallization is completed.

To calculate the heat capacity C_p and the entropy change ΔS, you must use the initial and final peak temperatures and the area under the peak:

$$C_p: \begin{cases} \Delta H = \int_{T_1}^{T_2} C_p dT \\ \Delta H = C_p (T_2 - T_1) \\ C_p = \dfrac{\Delta H}{(T_2 - T_1)} \end{cases}$$

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

To calculate ΔG of transition, you must use the peak temperature, the ΔH from the thermogram, and the ΔS from calculations as given above:

$$\Delta G = \Delta H - T\Delta S$$

Atomic Force Spectroscopy

The atomic force microscope (AFM) is used for nanometer-scale topographic imaging of surfaces. In AFM, a microfabricated cantilever (Figure 2) is scanned across a surface, bending in response to nanometer-scale changes in surface topography. A focused laser beam is reflected off the backside of the cantilever and directed into a position-sensitive photodetector (PSPD). As the cantilever deflects, the laser beam strikes different points on the PSPD. The vertical sensor output difference, s (V), of the top minus bottom quadrants of the PSPD is used to measure cantilever deflection, δ (nm). δ is then converted into height data, producing a topographic profile, or "contour map", of the surface.

Figure 2 *An SEM micrograph of a typical AFM cantilever. (Veeco Instruments, http://www.veecoprobes.com)*

Atomic force spectroscopy measures atomic-level forces between a fine probe tip and an underlying substrate, as a function of tip-sample separation distance, D (nm). In this case, the cantilever is driven vertically by a piezoelectric crystal, which expands or contracts in response to an applied voltage. Deflection data is collected as the cantilever approaches and retracts from the surface. In this case, δ is converted into the force, F (nN), that is exerted on the sample by the cantilever or vice versa. The expansion and contraction of the piezo crystal, z (nm), is converted into the tip-sample separation D. The result is a plot of the tip-sample interaction force versus the tip-sample distance. This procedure is known as high-resolution force spectroscopy (HRFS).[4]

A typical HRFS experiment on a hard substrate is depicted in Figure 3. The raw data is seen on the left; it consists of two curves, one for the approach and one for the retraction of the tip. At large separations, there are no interactions between tip and sample, the cantilever does not deflect, and there is no measured PSPD output. When the tip nears the sample, it begins to bend and a signal is detected on the PSPD. As the tip is retracted, often there are adhesive forces between the tip and the sample that will cause it to bend

the other way and stay in contact with the surface for longer. This gives rise to the region of negative PSPD output. At some point, the piezo crystal has raised the tip so far that the attractive forces are not enough to keep it in contact with the sample, and it returns to its undisturbed, undeflected state. Conversion of this data produces the plot seen on the right (see below for data conversion procedure). From this force vs. distance plot, tip-sample interaction forces and system energetics can be determined.

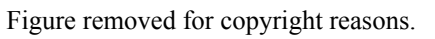


Figure 3 *Raw and converted data from a force pull depicted at right.*

It should be noted that in all HRFS experiments, the spring constant of the cantilever, *k*, is much less than the stiffness of the sample. Hence, little or no deformation of the substrate occurs, leading to the D = 0 vertical region of apparent infinite slope in the high-force, constant compliance regime.

To take a thermodynamic approach to this experiment, consider that we are performing mechanical work on the system. Our intensive/extensive pair of coupled variables is force and displacement, giving us incremental work $dW = F \cdot dl$. This framework now enables us to write and determine relevant thermodynamic expressions.

Procedure for data conversion

To convert the y-axis from PSPD output (s (V)) to Force (nN):

1. $\delta(nm) = \dfrac{-s(V)}{m(V/nm)}$

   where *m* is the slope of the raw data curve in the constant compliance regime (e.g. when the tip and sample are in contact)
2. By Hooke's law, $F(nN) = k(nN/nm) \cdot \delta(nm)$

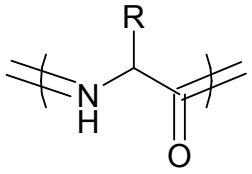
where k, the spring constant of the cantilever, is an experimentally measured quantity.

To convert the x-axis from z-piezo deflection (z (nm)) to tip-sample separation distance (D (nm)) is simpler: $D = z + \delta$ where δ is calculated in step 1 above.

To determine the energy supplied to the system, calculate the area enclosed by the "adhesion triangle" of the force-distance plot (e.g., integrate that portion of the curve).

Protein structure

Proteins are random copolymers of amino acids, all of which possess the basic structure:

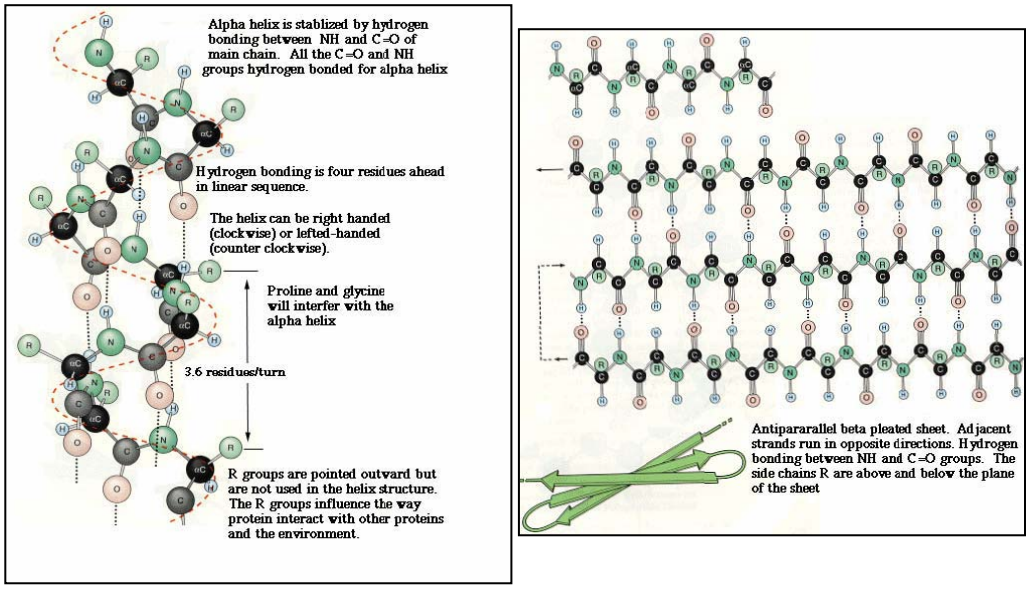


where "R" is a chemical group such as -CH_3, -COOH, etc. There are typically hundreds to thousands of amino acids per protein. Every function in the living cell depends on proteins, including but not limited to:

- Motion and locomotion of cells and organisms depends on contractile proteins.
- The catalysis of all biochemical reactions is done by enzymes, which contain protein.
- The structure of cells, and the extracellular matrix in which they are embedded, is largely made of protein. (Plants and many microbes depend more on carbohydrates, e.g., cellulose, for support, but these are synthesized by enzymes.)
- The transport of materials in body fluids depends on proteins.
- The receptors for hormones and other signaling molecules are proteins.
- The transcription factors that turn genes on and off to guide the differentiation of the cell and its later responsiveness to signals reaching it are proteins.

The primary structure of proteins consists of a series of amino acids; each protein has a particular composition and structure, but different proteins consist of varying numbers of amino acids in diverse conformations. In the "native" state, proteins fold into specific three-dimensional structures that are determined by the amino acid sequence; examples of common structures are twisted alpha-helices (Figure 4, left) or crimped beta-sheets (Figure 4, right). The folded structures are held together by secondary bonds, such as hydrophobic interactions, hydrogen bonding, or electrostatic interactions.

Why do proteins fold in this way? The amino acids that compose proteins may be either hydrophilic (energetically favorable interaction with water) or hydrophobic (energetically unfavorable interaction with water). Recall that in investigating an energetically driven phenomenon, we always want to minimize the Gibb's free energy. In this case, there is an entropic penalty of folding into tight, ordered structures. However, this is overcome by the enthalpic gains of folding in such a way that the hydrophobic residues are not exposed to aqueous biological environments. However, the secondary bonds keeping the three-dimensional structure of the protein are easily broken by the application of external forces. This is called denaturation. For example, thermal energy can overcome weak intramolecular interactions, causing protein unfolding. Similarly, mechanical force can physically "pull apart" a protein and unravel its secondary structure.



Courtesy of Natural Toxins Research Center. Used with permission.

Figure 4 *Left: the structure of a polypeptide α-helix. Right: the structure of a polypeptide β-sheet (Adapted from reference 5).*

Some proteins possess rather unique, modular supramolecular structures. For example, the giant muscle protein titin possesses some ten "domains" of tightly ordered structure that are linked together by amino acid "strings" of random structure, much like pearls on a necklace. Mechanically, in tension, these domains behave like springs in series, so if the protein is extended, the weakest spring (or domain) will unfold (denature) first,

followed by the next weakest, and so on.[6] If we had nano-sized fingers and could pull on titin, we would feel the tension in it suddenly release every time we unfolded another domain. Atomic force spectroscopy does just that by "grabbing" individual proteins with an AFM cantilever and extending them. Such an experiment on titin is shown in Figure 5. At first, as the cantilever retracts, we see only elastic stretching of the protein. However, because this is a modular protein, at some given force (that is characteristic of the protein being studied), the protein unfolds, relaxing the stretching force and generating a negative peak in the force plot. Multiple unfolding events generate a sawtooth pattern.[8]

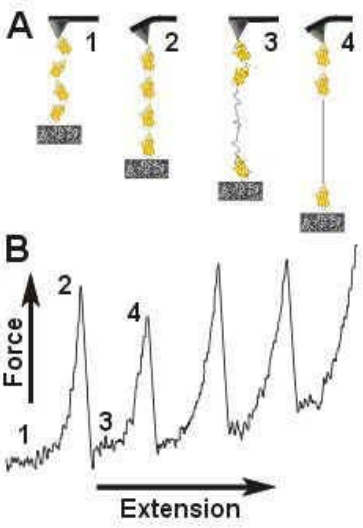


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Figure 5 *(a) Schematic of the modular unfolding of the protein titin. (b) Force-extension curve of titin shows a peaks corresponding to each successive unfolding event. (Adapted from reference 7)*

In addition, proteins will denature with heat. As thermal energy increases, the magnitude of molecular vibrations and the range of bond rotations both increase, vibrations allowing a greater number of molecular conformations to be sampled by the protein. At some point, the thermal energy overcomes the enthalpic driving force maintaining the protein in its complicated supramolecular structure, and the protein "melts". Using DSC, we can measure the ΔH per gram of this phase transition, and convert it into the energy of denaturation per molecule or per mole. (A similar conversion can be done on the energy obtained from integrating the force spectroscopy data, thus enabling us to compare the denaturation energies obtained by each method.)

Protein adsorption:[9]

Proteins will spontaneously adsorb to most surfaces, including biomedical devices and implants (even if they have been made "protein-resistant"). The adsorption process is fast: adsorption begins within seconds of the immersion of a substrate into a protein solution, and a monolayer of proteins is often formed after only a few minutes. Although there are no chemical bonds formed, the process is practically irreversible; adsorbed

proteins do not easily come off of a surface with rinsing, unless there is the addition of a denaturing agent (e.g. surfactant) or a change in pH or temperature. The adsorption process can be thought of as a balancing of repulsive and attractive nonspecific interactions, such as electrostatic forces, steric repulsion, hydrophobic interactions, van der Waals forces, and hydrogen bonding. The same protein can adsorb onto both hydrophobic and hydrophilic surfaces; it contains both hydrophobic and hydrophilic amino acids, and it can adopt a conformation relative to the surface that maximizes favorable interactions.

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