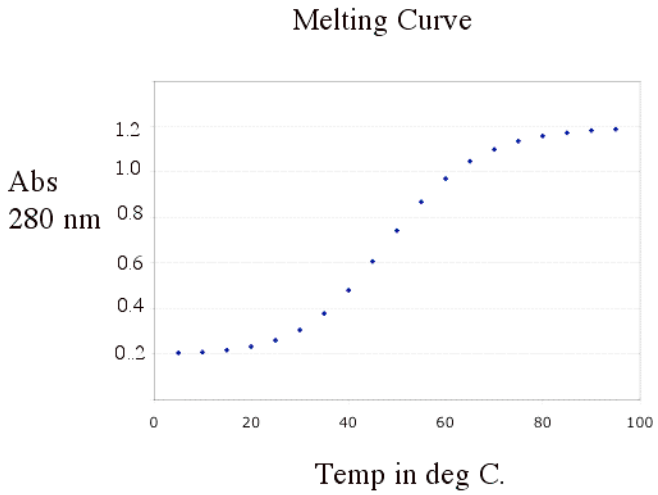
Protein Melting Curves

In previous classes we talked a lot about what aspects of a protein structure stabilize a protein and what aspects destabilize it. But how would one actually test such predictions? In other words we would like to know the enthalpic and entropic changes associated with the folding and unfolding of a protein.

The easiest way to get the entropy and enthalpy of protein folding is by recording protein melting/renaturation curves like this one.



To get such a curve we take a protein solution and heat it up until the protein is completely unfolded. While doing this we record a protein property that changes as a result of unfolding.

We will usually follow this unfolding process by some spectroscopic method. For example we could be looking at the fluorescence from a tryptophan residue or at a CD spectrum. Either way we will get a graph in which some measured quantity will vary as a function of temperature. The beauty of this approach is that we do not care about the physical mechanism underlying the spectroscopic change, all we care about is that the signal depends linearly on the concentration of the folded state and the unfolded state.

The shape of the curve then contains all the information we need to determine the entropy and the enthalpy of the folding reaction. But how do we get this information out.

Lets look at this in a more formal way.

Here is our reaction

F -> U

This is about as simple a reaction as one can find.

We know that

$$\Delta G = -RT \ln K_{eq}$$

and

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

In this equations K_{eq} is the one quantity that is easy to get a handle on experimentally and once we have K_{eq} as a function of temperature, we can then determine ΔS and ΔH. Note, as long as we are dealing with a monomeric protein, we have a unimolecular reaction so K_{eq} and ΔG are independent of the concentration of the reactant. So we can do our experiments at any concentration that is convenient for our measurements and still get ΔG^0 directly without any further calculation.

But how do we convert our experimentally observed melting curve (in the above example in absorbance units) into a curve that relates T to K_{eq}.

Due to the concentration independence K_{eq} is simply given by the ratio of the concentration of the molecules that are folded and unfolded. So we do not have to know the actual concentrations, but we just need the relative concentrations e.g. the fraction of all molecules that are folded (where f varies between 0 and 1)

$$K_{eq(folded \rightarrow unfolded)} = \frac{f_{unfolded}}{f_{folded}}$$

and

$$f_{folded} + f_{unfolded} = 1$$

$$f_{folded} = 1 - f_{unfolded}$$

so

$$K_{eq} = \frac{f_{unfolded}}{1 - f_{unfolded}}$$

So how do we go from our spectroscopically determined signal to f?
The observed spectroscopic signal A_{obs} is a linear combination of the signal A_{folded} observed for this sample in the fully folded state and the signal $A_{unfolded}$ seen in the fully unfolded state (in the curve shown above A_{folded} is 0.2 and $A_{unfolded}$ is 1.2).

$$A_{obs.} = f_{unfold} \cdot A_{unfold} + f_{fold} \cdot A_{fold}$$

$$= f_{unfold} \cdot A_{unfold} + (1 - f_{unfold}) \cdot A_{fold}$$

$$= f_{unfold} \cdot A_{unfold} + A_{fold} - f_{unfold} \cdot A_{fold}$$

$$A_{obs.} - A_{fold} = f_{unfold}(A_{unfold} - A_{fold})$$

$$f_{unfold} = \frac{A_{obs.} - A_{fold}}{A_{unfold} - A_{fold}}$$

Now we have a plot of f versus temperature, which allows us to calculate K_{eq} for every temperature and thereby ΔG for every temperature. There are now many different ways to read of the value for ΔH and ΔS from these plots.

The most traditional way to do this is to do a Van't Hoff plot.

Lets start with our famous equations for ΔG again.

$$\Delta G = \Delta RT \ln K_{eq} = \Delta H \Delta T \Delta S$$

so

$$\ln K_{eq} = \Delta \frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

$$\ln K_{eq} = \frac{1}{T} \left(\Delta \frac{\Delta H}{R} \right) + \frac{\Delta S}{R}$$

The last equation should look to you like the equation for a straight line

$$\ln K_{eq} = \frac{1}{T} \left(\Delta \frac{\Delta H}{R} \right) + \frac{\Delta S}{R}$$

$$y = xa + b$$

with

$$y = \ln K_{eq}$$

$$x = 1/T$$

$$a = \left(\Delta \frac{\Delta H}{R} \right)$$

$$b = \frac{\Delta S}{R}$$

So what we need to do is to plot ln K_{eq} vs. 1/T. This is called the Van't Hoff plot. The slope of the line times –R is ΔH and the y-axis times R is ΔS.
When you make these plots make sure that you are using absolute temperatures (deg C +273). Otherwise your plots will come out all wrong.

Warning !!!!!!
If I would show the above calculation to theoreticians working on thermodynamics, their hair would be standing up and they might kick me out of their office. In deriving the Van't Hoff plot in this way we do some serious cutting of corners. In other words we are making some very gutsy assumptions. In particular we are assuming that both ΔH and ΔS are not temperature dependent themselves. This means two things:

a) we have to be dealing with a true two-state mechanism. This means that the folded state of the protein at room temp (where we want to know the ΔG) is exactly the same as in the temperature range where it is undergoing unfolding. In other words there can be no continuous process in which individual protein molecules go from fully folded through a molten globule state to a partially unfolded state etc. For this approach to work individual protein molecules have to be in one of two states: folded or unfolded.
b) Our assumption that ΔH and ΔS are independent of temperature. In the case of ΔH this implies that the heat capacity of the folded and the unfolded state are identical. This is to say that H of the folded and the unfolded state (which are both temperature dependent; remember $C_p=dH/dT$) vary with temperature in exactly the same way. While this second assumption appears to be rather unreasonable (as a matter of fact it is flat out wrong based on first principles), it turns out that in practice this assumption typically works well enough that the errors introduced by this assumption are smaller than the measurement errors.

There are many different ways to skin this cat

There are many different ways to plot our data to get ΔG, ΔH and ΔS. The above warning equally applies to all of them. But some are more convenient than the standard Van't Hoff plot.

One particularly simple approach is:

Plot ΔG as a function of temperature, the slope of this plot then gives us ΔS
And the point where $\Delta G = 0$ is our melting temperature.

At T_m -> $\Delta G = 0$

so

$\Delta H = T_m \Delta S$

so

$T_m = \Delta H / \Delta S$

Combining protein melting curves with site-directed mutagenesis

Now we have our values for ΔH and ΔS, but how usefull are they really.
The values for ΔH and ΔS of the folding reaction are the aggregate of all the coulombic, hydrophobic, conformational entropy etc. energies that contribute to the folding reaction. Since all these contributions are glommed together into two numbers it is very hard to interpret those numbers in terms of a molecular picture. So while protein-melting curves are easy to record, we do not get all that much meaningful information out of them and

that was the state of the field until the late 80's and early 90's. That is the time when site-directed mutagenesis and bacterial protein expression became routine techniques.

With these techniques in hand it now became possible to compare the melting curves of two proteins, in which only a particular sidechain was changed.
Then one could determine the ΔH and ΔS for the folding of the two proteins and from that determine the $\Delta\Delta H$ and $\Delta\Delta S$ values for that particular change in the protein.
Where one of the first Δ symbolizes the difference between the folded and the unfolded protein and the second Δ symbolizes difference between wild-type and mutant protein.
One man pioneered and then went all the way with this approach.
His name is Brian Matthews and he picked T4 lysozyme as a model system and mutated the hell out of it. His work is probably provides the most useful data for the understanding the structural basis of the energetics of protein folding.