Overview of Stability & Folding Block of Laboratories

This module addresses issues related to protein folding and stability followed using the spectroscopic techniques fluorescence and circular dichroism. In the exploration module, you investigated the spectral properties of the "aromatic" amino acids, tyrosine and tryptophan, and the effects of local environment [i.e. solvent polarity] on them, as well as the absorption properties of the peptide bond. Since changes in solvent polarity to a certain extent mimic the changes of these amino acid side chains that occur in the protein folding process, understanding the effects of solvent polarity on the absorbance and fluorescent properties of these side chains yields insight into changes that might be expected as a protein folds or unfolds. Furthermore, a thorough understanding of local environment effects can help in identifying the basis of UV or fluorescence difference effects that might occur when a ligand binds to a protein. A detailed description of the absorbance or fluorescence spectra of tyrosine and tryptophan can be of use in solvent perturbation experiments to probe overall conformation.

In addition to using the native chromophores of a protein, extrinsically added chromophores such as 1-Anilinonaphthalene-8-Sulfonic Acid (ANS) or Sypro Orange can also be used in a variety of ways to study protein conformation and changes in conformation that accompany unfolding.

In this module, you will explore the effects of temperature and two chemical denaturants, guanidine hydrochloride (GudHCl) and urea, on the spectral properties of a protein. As the temperature or concentration of the chemical denaturant is increased, you should expect to find changes in either the UV or the fluorescence

spectra of the protein that are related to local environment changes in the contributing tyrosine and tryptophan residues in the protein. Since the concentration of the chemical denaturant that "unfolds" the protein is related to the overall stability of the protein [the higher the concentration of denaturant required to "unfold" the protein, the more stable the protein], it is possible to assess the effects of ligands on the stability of the protein by determining the "denaturation" profile in the presence or absence of an additional ligand. With such thermal protein unfolding experiments, you can also follow a probe of "secondary" structure such as circular dichroism or follow the quenching of a fluorescent probe that preferentially associates with unfolded, but not aggregated proteins.

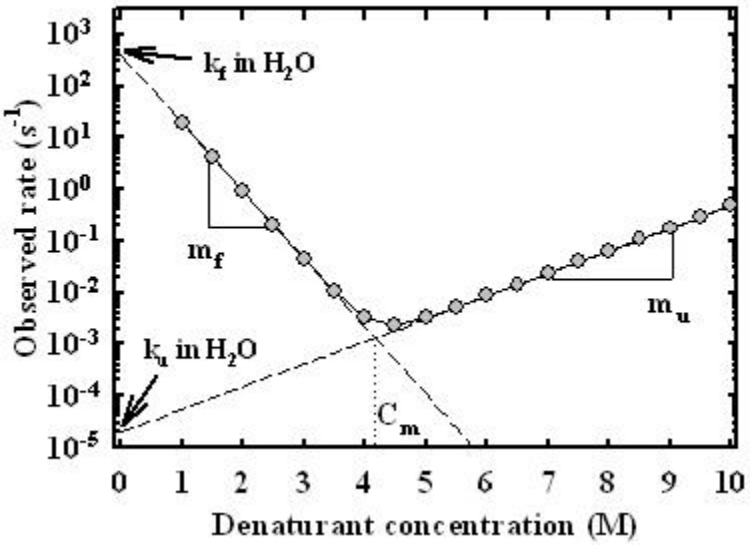


Figure 2. A typical chevron plot observed in protein folding experiments (Credit: Wikipedia).

All of the above approaches can be used to follow the equilibrium position between folded (F) and unfolded (U) protein and hence used to determine thermodynamic parameters for the F-U transition. They can also be used to determine rates of unfolding or folding allowing so called 'Chevron" analysis of the F-U transition.

Whenever you unfold a protein a critical question that always arises is whether or not the process is "reversible." Using the various spectral changes that you will find, you should investigate whether the effects are reversible. For example, if you dilute out or otherwise remove the chemical denaturant or cool the protein mixture, do you get the exact same spectral properties that the original native protein had? In addition to potential "regain" of spectral properties on removal of the denaturant or cooling, you can also design an experiment to determine whether or not activity is recoverable from the "refolding" process.

UV and Fluorescence Spectral Studies of Proteins

The average protein contains 3.5% phenylalanine, 3.5% tyrosine and 1.1% tryptophan: these three amino acids contribute the majority of the protein's absorbance and fluorescence properties in the 250-300nm wavelength

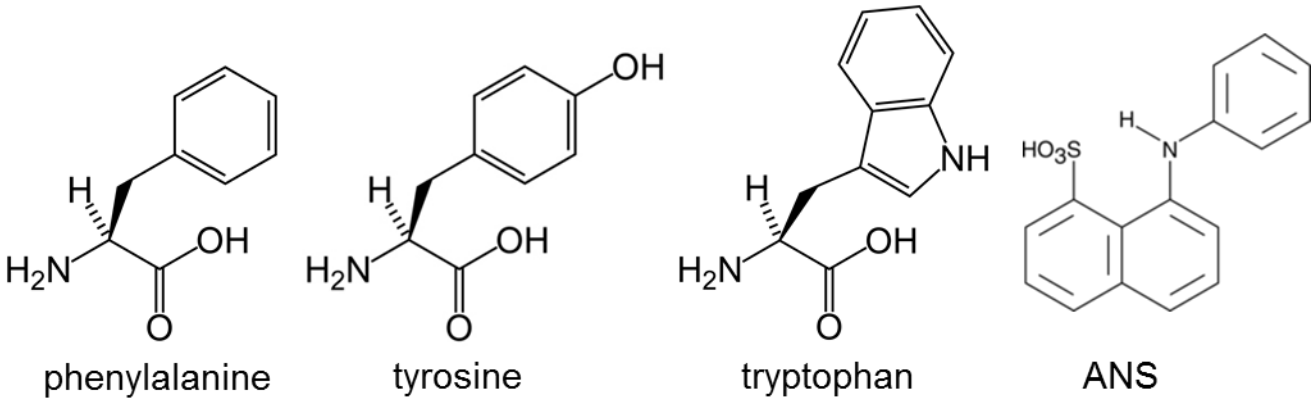


Figure 1. Natural and chemical chromophores.

range. Although, if the protein contains significant numbers of disulfide bonds, these, too, will contribute to the absorption properties in the wavelength range 250-280nm. The peptide bond and other amino acids contribute to absorbance properties in the 210-220nm region. In theory, the protein absorption properties in the range 250-300nm could be calculated from the absorption properties of the individual amino acids if the content of the particular protein was known. Alternatively, the amino acid composition of these three amino acids could be calculated from the protein absorption spectrum if the properties of the individual amino acids are known. This presumes that each of the three amino acids contributes to the absorption properties of the protein to the same extent as they do individually in solution. *Is this true?*

What aspects of the local environment may change the absorption properties of a given amino acid? Polarity is the major contributing factor. During the absorption transition, an electron must go from a ground state to an excited state with a consequent charge separation and hence the local polarity will affect the energy difference [i.e. absorption wavelength] between the ground and excited states. $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions will behave somewhat differently in this respect, but both are subject to wavelength shifts as the polarity of the local environment is changed. The influence of nonpolar solvent addition is generally attributed to the differential polarity of the ground and excited state orbitals. The relative polarities of n, π, and π* orbitals are $\pi < \pi^* < n$. Based upon this, n orbitals interact the most strongly with polar solvents, followed by π *, then by π. Using these considerations, $\pi \rightarrow \pi^*$ transitions should exhibit a blue shift with addition of a nonpolar solvent while $n \rightarrow \pi^*$ transitions should exhibit a red shift. Although other considerations, such as hydrogen bonding with the ground state, may affect these predictions. Another consideration is the overall pH environment of the chromophore: are the absorption properties of the individual amino acids subject to pH induced changes in properties?

The peptide bond can undergo both $n \rightarrow \pi^*$, and $\pi \rightarrow \pi^*$ transitions, with the $\pi \rightarrow \pi^*$ transition having an intense absorption around 190nm (ε_{max} = ~7000), while the $n \rightarrow \pi^*$ transition occurs at 210-220nm with a much weaker ε_{max} (~100). Asp, Glu, Asn, Gln, Arg, and His sidechains also have absorption in this region (190 - 230 nm), but it is very weak compared to peptide bond $\pi \rightarrow \pi^*$ transition. Absorption in the 190-230 nm region can be and is used to quantitate protein/peptide concentrations, but this is complicated by many compounds used in buffers which also absorb at these wavelengths.

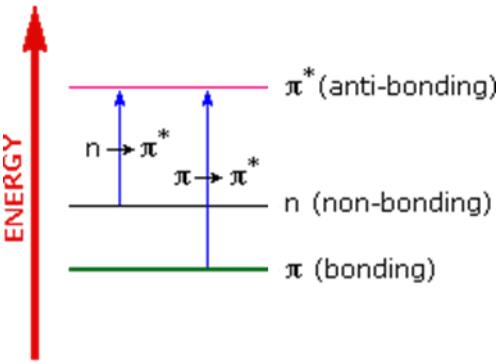


Figure 3. Diagram showing the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic excitation transitions that may occur in organic molecules.

The local environment [and hence contribution to the overall absorption properties of the protein] of an individual amino acid in a protein is governed by the native conformation of the protein: what would happen to the absorption properties of the individual amino acids if the native conformation was destroyed by a chemical denaturant such as GudHCl or Urea?

In the various experiments in this laboratory, you will explore the fluorescence properties of the aromatic amino acids, phenylalanine, tyrosine & tryptophan, and the effects of local environment within the context of a protein. In this way, you will learn how the spectral properties of a protein can be used to investigate the conformation of a protein and conformational changes in proteins. Depending upon the

level of detail that can be obtained about the contribution of individual amino acids to the spectral properties of a protein [these can be investigated using site directed mutagenesis of individual side chains or chemical modification of individual side chains], spectral changes in the protein may be able to give quite detailed information about conformation or conformational changes.

EXPERIMENT 1

Rate Constants and Equilibrium Constants for the Unfolding Process

Introduction:

The experiments used to explore UV, fluorescence & circular dichroism established that for a number of different spectroscopic parameters there is a large difference between the fully folded and fully unfolded protein which we can refer to as ΔObs.

ΔObs can be followed as a function of time to give a rate constant for the process, k_{OBS}. If the process has reached equilibrium, the magnitude of the parameter being followed at any given concentration of the perturbant relative to the magnitude of the parameter being followed for the complete unfolding represents the fraction of the protein that is in the unfolded state.

Determining the Unfolding and Overall Stability of a Protein

You are provided with following solutions:
0.5 M phosphate buffer @ pH 6 or 7 or 8
8 M stock solution of Guanidine hydrochloride (buffered @ pH 8.5)
2 mg/ml glutamate dehydrogenase, subunit MW 56, 000 daltons
10 mM ADP in water

And equipment:
Fluorometer
Quartz fluorometer cuvettes

Experiment 1.1

Determine the unfolding and overall stability of glutamate dehydrogenase using GudHCl as the chemical denaturant

Green group: Complete study at pH 6
Yellow group: Complete study at pH 7
Red group: Complete study at pH 8
Blue group: Complete study at pH 8 + ADP

Using Fluorescence determine the effects of increasing concentrations of guanidine hydrochloride on the spectral properties of the protein. You need to use 15 concentrations of guanidine hydrochloride between 0 and 6M. Make up tubes containing the appropriate guanidine hydrochloride concentration in the presence and absence of protein. ***Incubate reactions for 30 minutes at room temperature prior to collecting spectra.*** You will use the appropriate solution in the absence of protein as the reference or blank when you measure the spectrum of the protein containing solution.

Note: Be sure to check that your final concentration of protein is within range of the fluorometer intensity scale.

Fluormeter setup:
You will be collecting emission spectra.
Given that you are working with a protein that contains phenylalanine, tyrosine, and tryptophan, what wavelength should you excite your sample? You can choose several wavelengths based upon this information. Justify/explain why you have selected a particular wavelength.

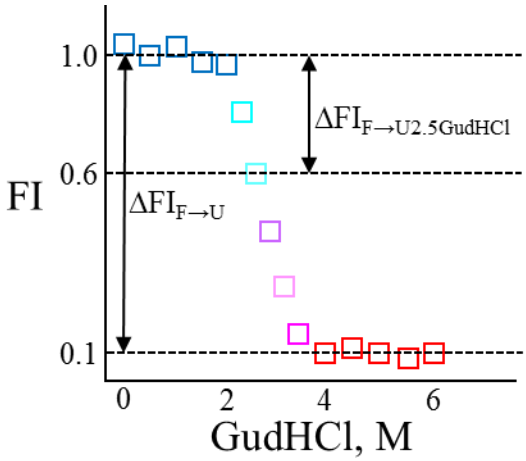
When you excite the aromatic amino acids, where is the emission maxima? You should collect approximately 100 nm in your emission spectrum. Note your emission spectrum should start at least 2 x your bandwidth of the fluorometer slits. (The PTI machine has its slits set at 2 nm.)

From one or more of the higher guanidine hydrochloride concentrations, design and conduct an experiment to determine whether or not the effects produced by the chemical denaturant are "reversible" in terms of the observed spectral effects. Be sure to consider and use the appropriate controls.

Experimental Set Up Worksheet 1.1

Tube	Phosphate buffer stock	GDH	ADP	GudHCl	Conc. GudHCl	H_2O
#	*mL*	*mL*	*mL*	*mL*	*M*	*mL*
1	0.2	0.045			0	
2	0.2	0.045			0.5	
3	0.2	0.045			1	
4	0.2	0.045			1.5	
5	0.2	0.045			2	
6	0.2	0.045			2.25	
7	0.2	0.045			2.5	
8	0.2	0.045			2.75	
9	0.2	0.045			3	
10	0.2	0.045			3.5	
11	0.2	0.045			4	
12	0.2	0.045			4.5	
13	0.2	0.045			5	
14	0.2	0.045			5.5	
15	0.2	0.045			6	

Analyzing your data:
The goal of your analyses is to calculate the $\Delta G°$ describing the standard free energy difference between the folded and unfolded states of the protein. To do this: Plot your fluorescence intensity (FI) either at a single wavelength or as a function of λ_{max} versus the guanidine hydrochloride concentration. What is the FI of the folded state? What is the FI of the unfolded state? What is the change in FI between the folded and unfolded state (ΔFI)? In the transition between folded and unfolded (see cyan, purple and pink markers in graph within Figure 5), calculate the percentage of protein in the folded or unfolded state. Use this information to calculate



$$\% \text{ unfolded} = \Delta FI_{F\rightarrow U@2.5GudHCl}/\Delta FI_{F\rightarrow U}$$
$$= 0.3/0.9$$
$$= 33\%$$
$$K_{eq@GudHCl2.5} = \frac{U}{F} = \frac{33\%}{67\%} = 0.49$$

Figure 5. Schematic of protein denaturation curve and derivation of K_{eq} at a defined guanidine hydrochloride concentration.

the equilibrium constant at each guanidine hydrochloride concentration that falls within the transition of the protein from folded to unfolded state. Plot your K_{eq} values versus their corresponding guanidine hydrochloride concentrations – should be a linear relationship. Extrapolate to GudHCl equal to zero to derive the K_{eq} of the folded state. Using this constant, calculate the ΔG° describing the standard free energy difference between the folded and unfolded states of the protein. What does this number tell you? Is this a valid calculation?

Quantitative Analysis of the Data you can Obtain from such Experiments

The unfolding transition of a protein can be represented by:

$$F \underset{k_F}{\overset{k_U}{\rightleftarrows}} U$$

Where k_U is the rate constant for unfolding and k_F is the rate constant for folding. The rate constant of "denaturation," followed by stopped flow kinetics, (k_{OBS}) is the sum of both:

$$k_{OBS} = k_F + k_U$$

and can be measured as a function of the concentration of a denaturant in a rapid mixing experiment. The obtained data is usually represented as a "chevron" plot:

EXPERIMENT 2

Determining the Unfolding and Overall Stability of a Protein

You are provided with following solutions:
0.5 M phosphate buffer @ pH 6 or 7 or 8
8 M stock solution of urea in H_2O
2 mg/ml glutamate dehydrogenase, subunit MW 56, 000 daltons
10 mM ADP in water

And equipment:
Fluorometer
Quartz fluorometer cuvettes

Experiment 1.1

Determine the unfolding and overall stability of glutamate dehydrogenase using urea as the chemical denaturant

Green group: Complete study at pH 7
Yellow group: Complete study at pH 8
Red group: Complete study at pH 8 + ADP
Blue group: Complete study at pH 6

Using Fluorescence determine the effects of increasing concentrations of urea on the spectral properties of the protein. You need to use 15 concentrations of urea between 0 and 6M. Make up tubes containing the appropriate urea concentration in the presence and absence of protein. ***Incubate reactions for 30 minutes at room temperature prior to collecting spectra.*** You will use the appropriate solution in the absence of protein as the reference or blank when you measure the spectrum of the protein containing solution.

Note: Be sure to check that your final concentration of protein is within range of the fluorometer intensity scale.

Fluormeter setup:
You will be collecting emission spectra.
Given that you are working with a protein that contains phenylalanine, tyrosine, and tryptophan, what wavelength should you excite your sample? You can choose several wavelengths based upon this information. Justify/explain why you have selected a particular wavelength.

When you excite the aromatic amino acids, where is the emission maxima? You should collect approximately 100 nm in your emission spectrum. ***Note***, your emission spectrum should start at least 2 x your bandwidth of the fluorometer slits. (The PTI machine has its slits set at 2 nm.)

From one or more of the higher urea concentrations, design and conduct an experiment to determine whether or not the effects produced by the chemical denaturant are "reversible" in terms of the observed spectral effects. Be sure to consider and use the appropriate controls.

Experimental Set Up Worksheet 2.1

Tube	Phosphate buffer stock	GDH	ADP	urea	Conc. urea	H_2O
#	*mL*	*mL*	*mL*	*mL*	*M*	*mL*
1	0.2	0.045			0	
2	0.2	0.045			0.5	
3	0.2	0.045			1	
4	0.2	0.045			1.5	
5	0.2	0.045			2	
6	0.2	0.045			2.25	
7	0.2	0.045			2.5	
8	0.2	0.045			2.75	
9	0.2	0.045			3	
10	0.2	0.045			3.5	
11	0.2	0.045			4	
12	0.2	0.045			4.5	
13	0.2	0.045			5	
14	0.2	0.045			5.5	
15	0.2	0.045			6	

Analyzing your data:

The goal of your analyses is to calculate the $\Delta G°$ describing the standard free energy difference between the folded and unfolded states of the protein. To do this: Plot your fluorescence intensity (FI) either at a single wavelength or as a function of λ_{max} versus the urea concentration. What is the FI of the folded state? What is the FI of the unfolded state? What is the change in FI between the folded and unfolded state (ΔFI)? In the transition between folded and unfolded (see cyan, purple and pink markers in graph within Figure 5), calculate the percentage of protein in the folded or unfolded state. Use this information to calculate the equilibrium constant at each urea concentration that falls within the transition of the protein from folded to unfolded state. Plot your K_{eq} values versus their corresponding urea concentrations – should be a linear relationship. Extrapolate to urea equal to zero to derive the K_{eq} of the folded state. Using this constant, calculate the $\Delta G°$ describing the standard free energy difference between the folded and unfolded states of the protein. What does this number tell you? Is this a valid calculation?

EXPERIMENT 3
CD Spectra of Proteins

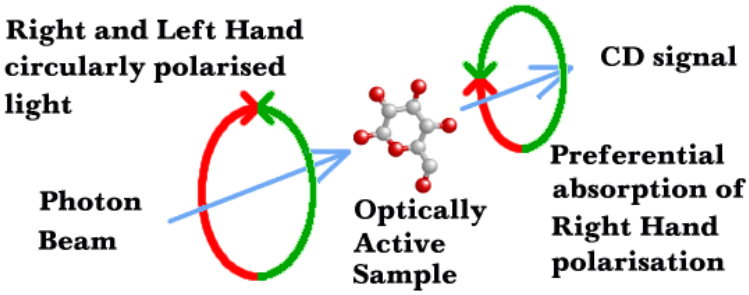


Figure 3.1. Schematic of left & right handed circularly polarized light unequally absorbed by a chiral molecule.

"Dichroism is the phenomenon in which light absorption differs for different directions of polarization. In circularly polarized light, the magnitude is constant and the direction is modulated. The wavelength is defined by the repeat in space, and the frequency by the repeat in time. The electric vector of circularly polarized light describes a helix, and circularly polarized light may describe either a right-handed helix, or a left-handed helix. Circular dichroism investigates the absorption of the two different kinds of circularly polarized light. The circular dichroism spectrum of a biopolymer is exquisitely sensitive to its secondary structure.

Circular dichroism is a special kind of absorption spectroscopy, and occurs only at energies where normal absorption occurs. The instruments needed are similar to normal absorption instruments, although optical elements are added to produce the polarized light. As a practical matter, it is important to investigate the normal absorption first to ensure that the absorbance of the solution is not more than 1.0, so that at least 10% of the light is transmitted for the instrument to use in determining the dichroism.

The major chromophore in proteins is the amide group that forms when two amino acids are joined. The super asymmetric secondary structures formed by polypeptides and proteins have distinctive CD spectra. Typical of the α helix is a negative band at about 222 nm due to the $n \rightarrow \pi^*$ transition, and a negative and positive couplet at about 208 and 190 nm due to the parallel and perpendicular components of the $\pi \rightarrow \pi^*$ transition, respectively. The magnitude of the negative 222 nm band is a good measure of α helix content in a peptide or protein. There is a fairly linear relationship with $\Delta\varepsilon = 0$ corresponding to 0% α helix and $\Delta\varepsilon = -10$ corresponding to 100% α helix. The CD for a β strand has a negative band at about 215 nm and a positive band at about 198 nm, but the positions and intensities of these two bands vary considerably with the sample. The CD spectra of polypeptides that form a series of type II β turns or α helices, or β strand or random coil is shown in Figure 3.2. Since protein secondary structures have very different CD spectra, we would expect the CD of a native protein to depend on the fractions of its component secondary structures."[1]

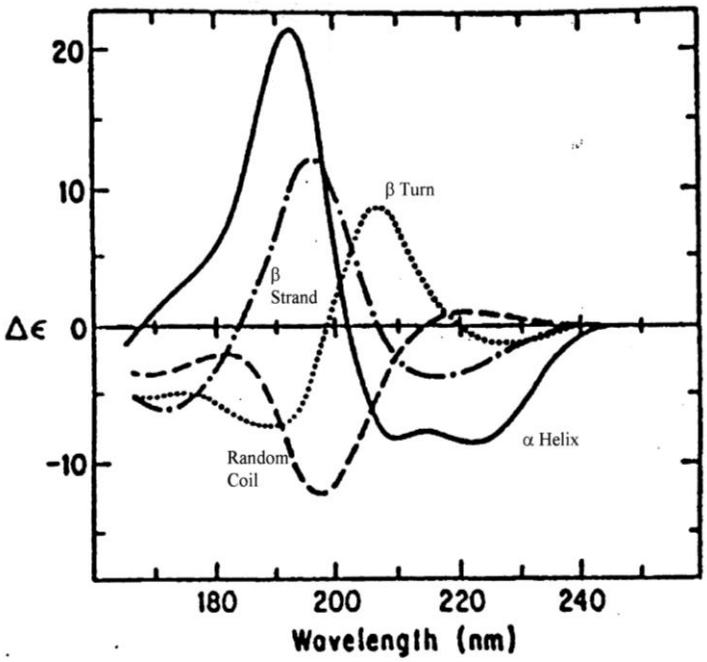
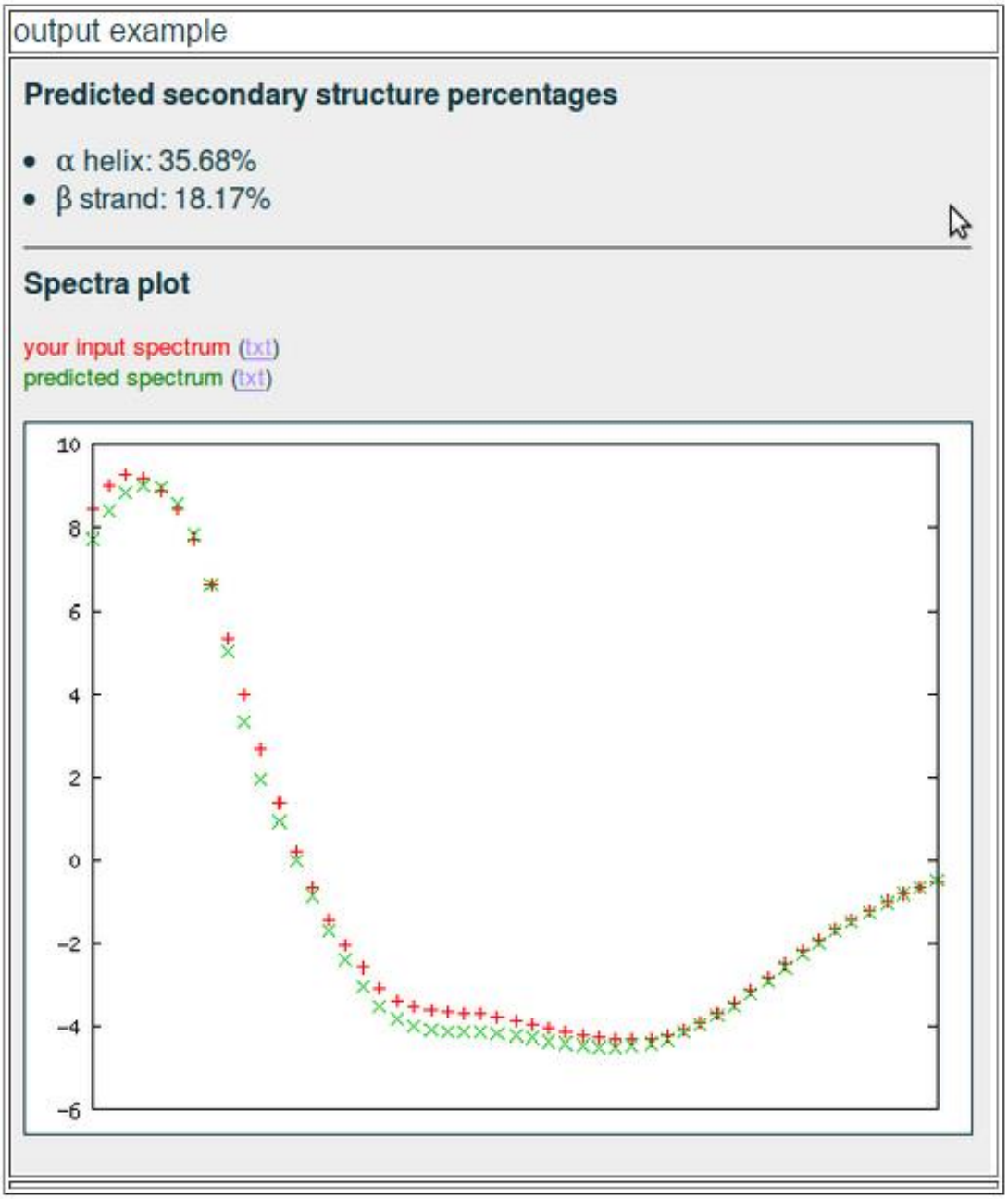


Figure 3.2. CD spectra of various types of secondary structure. Calculated CD spectra for α-helix, β strand, β turn and random coil.

Proteins, depending upon their secondary structure content, will have a CD spectrum in the region of 200-240nm that allows estimation of the overall secondary structure content. While there are a number of web based resources for interpreting CD spectra of proteins and calculating estimates of secondary structure, one

Figure 4.3. Sample data output from K2D3.



of the simplest to use is the web-based resource, K2D3[2]. http://k2d3.ogic.ca/

Using K2D3, one simply inputs the wavelength range used (usually 200-240nm), the CD units used (either in deciliter mol^{-1} cm^{-1} (difference in molar extinction coefficients, Δε, also called molar circular dichroism) or in deg cm^2 $dmol^{-1}$ per residue (mean residue ellipticity units, MRE), and the protein size (either chain length or molecular weight), as well as entering the CD data as a column of numbers (cut and pasted from the instrument output table). The program returns the % alpha helix and beta strand as well as the predicted spectrum overlaying the actual measured spectrum, see Figure 4.3 for an example output.

Proteins, depending upon their secondary structure content, will have a CD spectrum in the region of 200-240nm that allows estimation of the overall secondary structure content. If the secondary structure is perturbed as would occur in unfolding, the CD spectrum is a convenient way to follow phenomena such as denaturation. In particular CD at 222nm is often used in conjunction with thermal unfolding to follow the transition from folded to unfolded and obtain a "melting" temperature for the protein which reflects the global stability of the protein.

If the thermal unfolding of a macromolecule such as a protein is reversible, thermodynamic parameters can be obtained from such "thermal melts." When the CD of a protein, polypeptide or other asymmetric polymer such as a polynucleotide or polysaccharide changes as a function of temperature, the change can be utilized to analyze the thermodynamics of unfolding or folding. In the simplest case, a molecule undergoes an unfolding transition between two states: folded, F, and unfolded, U:

$$F \rightarrow U$$

We know that

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

and

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

In this equations K_{eq} is the quantity that is easily determined experimentally. With K_{eq} values as a function of temperature, ΔS and ΔH can be determined. Note, as long as the protein is monomeric, it is a unimolecular reaction, and K_{eq} and ΔG are independent of the concentration of the reactant. Therefore, experiments can be completed at any concentration that is convenient for measurements and still obtain $\Delta G°$ directly. But how does one convert an experimentally observed melting curve 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. The actual concentrations are not needed, but just need the relative concentrations e.g. the fraction of all molecules that are folded (where f varies between 0 and 1).

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

Where f is fraction of species and

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

so

$$K_{eq(folded \rightarrow unfolded)} = \frac{f_{unfolded}}{1 - f_{unfolded}}$$

How does one go from the 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.

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

$$= f_{unfold} \times A_{unfold} + (1 - f_{unfold}) \times A_{fold}$$
$$= f_{unfold} \times A_{unfold} + A_{fold} - f_{unfold} \times A_{fold}$$
$$A_{obs.} - A_{fold} = f_{unfold} (A_{unfold} - A_{fold})$$

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

Now, one can plot f versus temperature, which allows calculation of Keq for every temperature and thereby ΔG for every temperature. There are now many different ways to read the value for ΔH and ΔS from these plots. The most traditional way to do this is from a van't Hoff plot.

$$\Delta G = -RT \ln Keq = \Delta H - T\Delta S$$

So

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

And can be rearranged

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

From this equation, one can see that plotting $ln K_{eq}$ versus 1/T yields a linear relationship – the Van't Hoff plot. Fitting the data to the equation for a straight line will yield a slope of –ΔH/R and an intercept of ΔS/R.

Note: This derivation assumes that both ΔH and ΔS are not temperature dependent. For this assumption to be true: 1) The system under study undergoes a true two-state mechanism. That is, the protein does not undergo a continuous process as it unfolds (i.e. folded to molten globule state to unfolded state). 2) For ΔH to be T independent, C_p (heat capacity) is identical in the folded and unfolded state. This means that H of the folded and unfolded state vary with temperature in exactly the same way. Not a reasonable assumption, <u>*but*</u> errors introduced by this assumption are smaller than the measurement errors.

Experiment 3.1
Thermal denaturation of a protein
You will be provided with the follow reagents:
1mg/ml bovine glutamate dehydrogenase (GDH) in 5 mM Na/H phosphate, pH 8, ε = 0.93 $(mg/ml)^{-1}cm^{-1}$
5 mM Na/H phosphate buffer 5 mM, pH 6 or pH 7 or pH 8
10 mM ADP in H_2O

And equipment:
CD spectrometer
CD cuvettes, 2 mm

Green group: Complete study at pH 8
Yellow group: Complete study at pH 8 + ADP
Red group: Complete study at pH 6
Blue group: Complete study at pH 7

Collect a CD spectrum (190 – 240 nm) of your initial sample. Then, collect a thermal melt for GDH sample from 15 to 85°C monitoring the ellipiticity at 222nm. Following your thermal melt, return the sample to 15°C and collect a final spectrum from 190-240 nm. Compare your initial and final spectrum. What can you conclude about this process? As stated in the background reading, be sure that the sample concentration allows at least 10% transmittance in the range of 200-240 nm. ***Remember*** that when you measure your UV absorbance at 220 nm, you are using a 1 cm cuvette. When you are collecting CD data, you are using a 2 mm cuvette. **THINK** about the impact of this on your calculations for preparing your CD sample: $A = \varepsilon cl$. *When A = 0.8 as measured in a 1 cm cuvette, you will need 5x the concentration in the CD cuvette to have an equivalent absorbance.*

From your data, calculate a minimum of 5 K_{eq} values at temperatures within the transition from folded to unfolded. Use the van't Hoff plot to derive thermodynamic parameters for this process. Are these calculations valid?

References

1. Van Holde KE, Johnson WC, Ho, PS *Principles of Physical Biochemistry* Prentice Hall New Jersey 2nd ed.
2. Louis-Jeune C, Andrade-Navarro MA, Perez-Iratxeta C "Prediction of protein secondary structure from circular dichroism using theoretically derived spectra" Proteins: Structure, Function, and Bioinformatics 2012 80(2) 374–381.
3. Greenfield N, Fasman GD "Computed circular dichroism spectra for the evaluatioon of protein conformation." Biochemistry 1969 8:4108-4116.

EXPERIMENT 4
Fluorescence-based thermal shift assay (FTS) assay to assess protein stability

As proteins unfold, the hydrophobic residues typically buried within the interior become exposed to the aqueous environment. To monitor the exposure of these hydrophobic residues, the binding of a hydrophobic fluorescent dye (SYPRO Orange) is quantitated as a function of the protein unfolding. The SYPRO Orange dye is highly fluorescent upon binding to hydrophobic sites on unfolded proteins. Typically in a Fluor-thermo assay, the thermal denaturation of a protein is marked by a change (increase) in fluorescence intensity as the protein unfolds, generating a sigmoidal curve that can be described by a two-state transition.

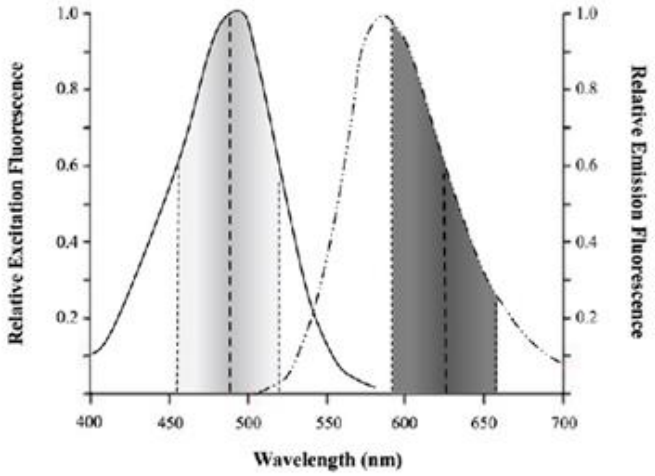


Figure 1. Excitation (solid) and emission (dashed) spectra of SYPRO orange fluorescent dye in BSA. Shaded areas represent the relative range of light transmitted through the BioRad 485 ± 30 nm excitation and 625 ± 30 nm emission filters used in this experiment. This figure is modified from Invitrogen Life Technologies product information (Cat. No. S-6650) and copied from Biotechniques (2012) 53:231-238.

Experiment 4.1
Fluorescent thermal shift assay to assess glutamate dehydrogenase stability

You are provided with the following solutions:
1 mg/ml Glutamate dehydrogenase in 5 mM phosphate buffer, pH 8 (subunit MW of 56,000)
0.5 M phosphate buffer, pH 6 or pH 7 or pH 8
80% Glycerol
8 M guanidine hydrochloride (buffered at pH 8.5)
8 M urea in H_2O
5 M NaCl
200X stock of SYPRO Orange

And equipment:
BioRad thermocycler with CFX96 Reaction Module
96 well plate or 8-tube strips with caps

You will examine the denaturation of glutamate dehydrogenase by fluorescence-based thermal shift (FTS) assay under several buffer conditions using SYPRO Orange fluorescence as your readout. You will set up your reactions in a 96 well plate or 8-tube strips on ice. Your final enzyme concentration in the reaction will be 4 μM. Each reaction will occur in a total volume of 25 μl. *You should complete each reaction in triplicate.* Fill out the experimental setup worksheet below to aid in mapping out your reaction setup. For these reactions you will use p2 and p10 pipettes as well as the pipettes in your lab drawer. Setup your reactions such that you do not pipette any volumes less than 1 μl.

Experimental Setup Worksheet 4.1

Reaction	Tube	GDH	SYPRO	Buffer	NaCl	GudHCl	Urea	Glycerol	H_2O
	#	4 μM, μl	20X	100 mM, μl	0.5 M, μl	1 M, μl	1M, μl	1% or 5%, μl	μl
Ctl. No GDH	1								

Ctl. GDH std. conditions	**2**								
pH 6	**3**								
pH 7	**4**								
pH 8	**5**								
1M GudHCl	**6**								
1M urea	**7**								
0.5 M NaCl	**8**								
1% glycerol	**9**								
5% glycerol	**10**								

The thermal scans will be performed from 10 to 95°C at 1.5°C increase/minute and fluorescence scans collected every 30 s. Return the temperature to 10°C and record a final fluorescence intensity. Calculate average of triplicates. Subtract average "No GDH" FI control from the average of your samples. Make plots of the average, corrected fluorescence intensity versus temperature. From these plots, calculate the Tm. Compare the Tm for your different samples. What conclusions can you draw with respect to protein stability and the different conditions used in each reaction? Using a minimum of 5 points within the transition between the folded and unfolded state, calculate the K_{eq} for the folded to unfolded process. Create a van't Hoff plot from this analysis and derive the thermodynamic parameters. Are these analyses valid?