Protein Unfolding of metMyoglobin Monitored by Spectroscopic Techniques

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Abstract: In this experiment the unfolding of the protein, myoglobin, will be monitored using both fluorescence and UV–vis absorption spectroscopy. Changes in the absorbance at 409.5 nm, the absorption maximum of the native state, will be monitored in order to probe changes in the protein conformation after initiation of unfolding by addition of a chemical denaturant. Protein unfolding will also be monitored after exciting the sample at 280 nm and following protein fluorescence emission at 345 nm, the fluorescence maximum for the unfolded state. The absorption run follows the time-dependent decrease in the concentration of the native-state species, whereas the fluorescence experiment monitors the increase in the concentration of the unfolded state. Kinetic rate constants obtained using the two techniques will be compared.

Introduction

I. Protein Folding. An outstanding problem in biophysical chemistry that is currently attracting considerable research activity is the determination of the factors governing the transformation of a nascent polypeptide chain into a functional three-dimensional protein structure (see Figure 1). Objectives of research focusing on the "protein-folding problem" include:

- Predicting protein conformations directly from DNA-sequencing information.
- Controlling the folding process via knowledge of folding kinetics.
- Designing novel proteins.
- Modifying protein activity via specific structural mutations.

Researchers hope to ultimately be able to both computationally model and experimentally control the folding process. Currently, computational chemistry cannot simultaneously and unequivocally predict the complete secondary and tertiary structure of a protein given its amino acid sequence. The ability to predict protein structure will alleviate the reliance on experimental methods of protein structural determination using X-ray crystallography or multidimensional NMR spectroscopy. Advantages of structural determination via computational methods include: (1) a relatively rapid determination of the structure (i.e., compared to the time scale of sample preparation or crystal growth, data collection, and data analysis), (2) the ability to obtain structural information for proteins for which crystals cannot be grown or that can only be isolated in such minute quantities as to preclude experimental structural analysis, (3) ease of structurally mutating the protein and predicting the new conformation, and (4) ease of designing novel proteins.

A goal of protein folding research is to control experimental conditions in order to increase yields of genetically expressed recombinant proteins. Aggregation and other processes currently compete with folding to reduce yields both in vitro and in vivo. For example, biotechnological methods for synthesizing proteins such as insulin exist, but they are hindered by lowered yields compared to in vivo synthesis because proteins are produced that are incorrectly folded and, therefore, not viable.

Recent evidence also suggests that misfolded and aggregated proteins may be involved in pathological conditions such as Altzheimer's, cystic fibrosis, and in the neurodegenerative diseases scrapie, bovine spongiform encephalopathy, and Creutzfeldt–Jakob disease.

Kinetics of Protein Folding

Kinetics experiments can be used to study the time scales and mechanisms associated with the folding and unfolding reactions of proteins. Figure 2 shows the simplest possible model (i.e., a two-state model with no intermediates) for the interconversion of a protein between its native or folded conformation and its unfolded conformation. Conventional methods for initiating the folding/unfolding reaction include changing the sample temperature, adding or changing the concentration a chemical denaturant, or changing the solution pH. Chemical denaturants such as urea or guanidine hydrochloride (GuHCl) disrupt hydrogen-bonding interactions, which are important in the secondary structure formation of proteins.

II. Protein Structure and Function. *Protein Function.*Proteins perform a variety of functions including:

- Transport and storage (binding of other molecules). *Example*: myoglobin and hemoglobin bind and store oxygen.
- Mechanical support (proteins bind one another). *Example*: contractile fibers in muscle, fibrin.
- Immune protection (binding of foreign molecules). *Example*: antibody proteins.
- Control of cellular growth. *Example*: cyclins and kinases

Protein Structure. Proteins themselves are comprised of a set of 20 amino acids with the general structure shown in the upper-left-hand corner of Figure 3. These 20 amino acids differ in their size, shape, polarity, chemical reactivity, and ability to hydrogen bond. Different proteins are comprised of different numbers and different sequences (i.e., primary structure) of amino acids. Amino acids link together into

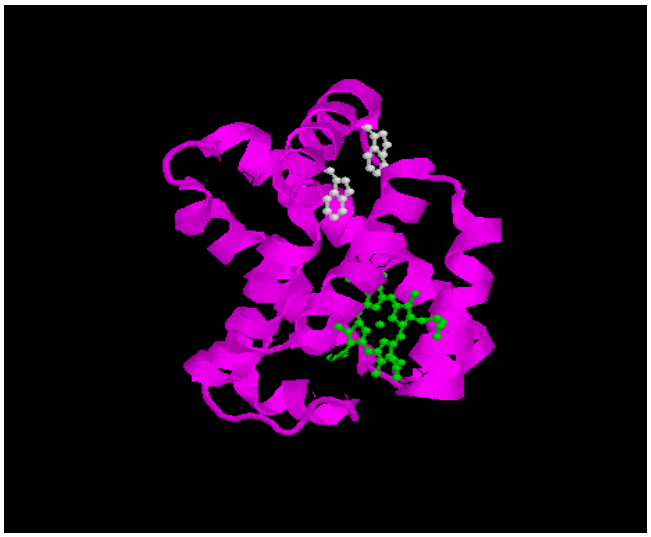


Figure 1. Three-dimensional structure of the protein Horse-heart metmyoglobin (1 yMB.PDB). The Heme group is shown in green and the tryptophan residues are shown in white. (Image was created using Weblab ViewerLite 3.10 by Molecular simulations, Inc.)

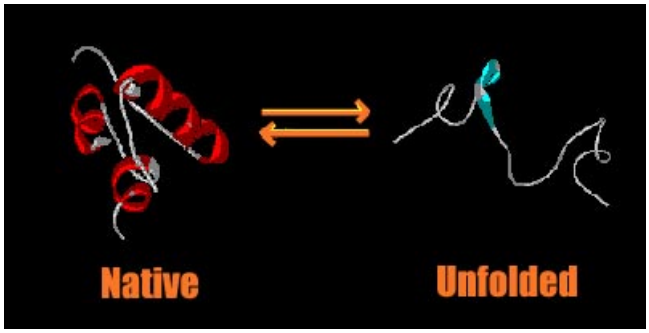


Figure 2. Unfolding/folding reaction of a Protein. n = native state. u = unfolded state. (Image was created using Weblab ViewerLite 3.10 by Molecular simulations, Inc.)

polypeptide chains by forming peptide bonds between adjacent residues. By convention, the residue at the amino end marks the chain beginning and is written first in the sequence. A carboxyl group terminates the chain.

Proteins are not simple one-dimensional strings of amino acids. Although the peptide unit is planar, there is rotational motion about the C–C bonds, imparting flexibility to the chain. This flexibility allows the polypeptide chain to assume a three-dimensional structure, which is intimately related the protein's ability to function. Disulfide bond formation can link different chains or parts of the same chain. Hydrogen-bonding interactions result in two forms of secondary structure, namely, α-helix and β-pleated sheet structure. The α-helix is a coiled chain stabilized by hydrogen-bonding interactions between an N–H group and a C=O group on another amino acid four residues away. Alpha helices can entwine into coiled coils; this structure imparts added mechanical stability to the chain and is found, for example, in fibrin in blood clots.

The β-pleated sheet is an extended structure formed via hydrogen bondings between different polypeptide chains. The polypeptide chains can run in a parallel (i.e., amino chain ends aligned) or antiparallel direction (i.e., amino end of one chain adjacent to the carboxyl end of the other chain). Proteins can have regions of both periodic forms of secondary structure.

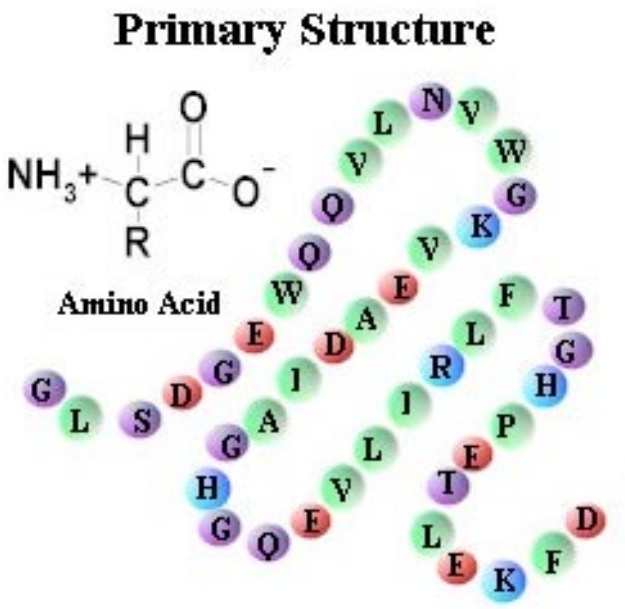


Figure 3. Primary Structure of METMb**.** First 44 of the 153 total residues of horse-heart metMb. Key for the amino acids: green = nonpolar residues; purple = polar; blue = basic; red = acidic. The letters represent the standard one-letter symbols for the amino acids.

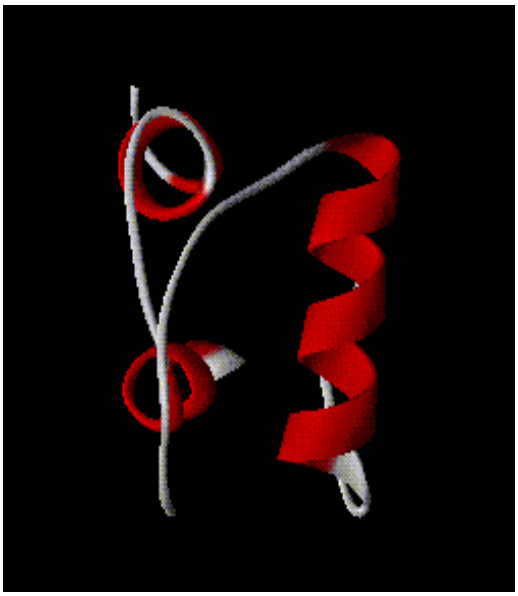
Chains routinely reverse their directions via the formation of an α-turn. The overall global arrangement of the polypeptide chain comprises the protein's tertiary structure. For proteins with more than one polypeptide subunit, the relative packing of these subunits is termed the quaternary structure.

Forces controlling the three-dimensional shape of a protein include: electrostatic forces (i.e., charge–charge interactions), hydrogen bonding interactions, and van der Waals forces (i.e., intermolecular attractive forces between neutral molecules). It is the three-dimensional shape that determines the ability of a protein to function.

III. Fundamentals of Fluorescence Spectroscopy. Fluorescence spectroscopy has proven its utility for the study of biomolecules. The number of applications with regard to protein, nucleic acid, and immunochemical research continues to grow. One of the main advantages of fluorescence spectroscopy is its sensitivity. Single-molecule detection has been achieved through the combination of state-of-the-art separation techniques such as capillary zone electrophoresis with fluorescence detection methods [1].

What is Fluorescence Spectroscopy?

When an atom or molecule is excited by the absorption of electromagnetic radiation, relaxation back to the ground state can occur, either by the release of thermal energy (heat) or by the emission of a photon (radiative energy). Fluorescence is an emission process in which a molecule or atom relaxes back to the ground electronic state by releasing its excess energy as a photon. The energy level diagram in Figure 5 summarizes various relaxation processes for an excited singlet electronic state. Nonradiative relaxation processes, which include vibrational relaxation and internal conversion, occur on timescales of $< 10^{-11}$ s. Vibrational relaxation occurs within the vibrational manifold of an excited electronic state and



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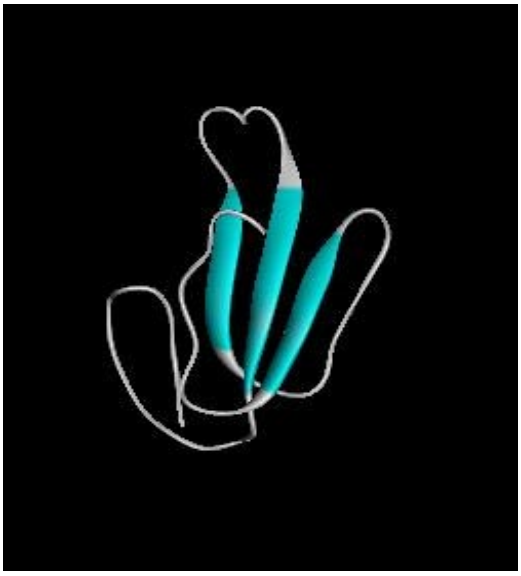

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Figure 4. Secondary Structure Types**.** The protein in panel a (Relaxin; 6RLX.PDB) exhibits α-helical secondary structure. The protein (Cobratoxin; 1CTX.PDB) in panel b contains β-pleated sheet structure. (Images were created using WebLab ViewerLite 3.10 by Molecular Simulations, Inc.)

involves either the transfer of vibrational energy via collisions between the excited molecule and the solvent or dissipation of energy through internal vibrational motions. If energy is transferred to the solvent, the temperature of the medium increases. Internal conversion is a nonradiative relaxation process occurring between the lowest vibrational level of an excited electronic state and an upper vibrational level of another electronic state. These mechanisms of dissipation of energy compete directly with radiative relaxation processes. Fluorescence emission typically occurs on time scales of $> 10^{-8}$ s. When much faster nonradiative processes occur, fluorescent photons are typically less energetic than the photon originally absorbed (due to the energy lost in the nonradiative relaxation processes). Also, as a consequence of these faster rates, fluorescence typically occurs from the lowest vibrational

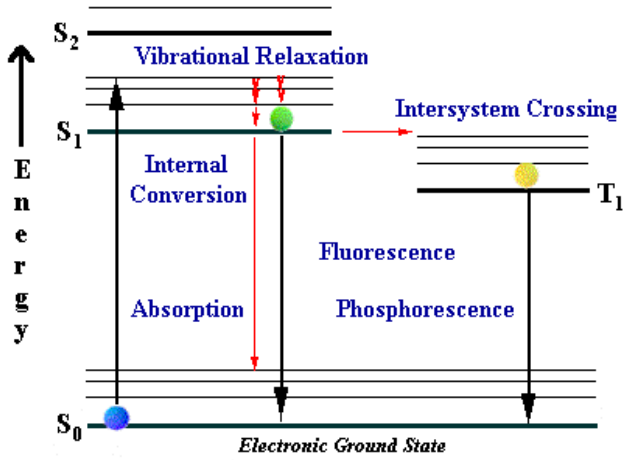


Figure 5. Jablonski Diagram. S_N indicates the singlet state; T_N indicates the triplet state. The heavy solid horizontal line indicates the electronic level and the light solid horizontal line the vibrational level. Absorption into an upper vibrational level of the first excited electronic state is represented by the blue photon. The short vertical red lines represent radiationless vibrational relaxation to the ground vibrational level of the first excited electronic state. Radiationless internal conversion between electronic states is indicated by the long vertical red line, radiative emission (fluorescence) to the ground electronic state by the green photon, intersystem crossing to the triplet state by the horizontal red line, and radiative emission (phosphorescence) from the triplet state by the yellow photon.

level of the excited electronic state. Excitation into the higher vibrational levels of an excited electronic state is normally followed by rapid vibrational relaxation to the ground vibrational level of that electronic state. Most molecules do not fluoresce because of the greater efficiencies and short time scales of the nonradiative pathways. Another process, phosphorescence, involves photon emission from a triplet electronic state. In order for this competing, longer-lived ($> 10^{-4}$ s) radiative process to occur, there must first be nonradiative coupling, called intersystem crossing, between states of different multiplicity. In Figure 5 the radiationless intersystem crossing process occurs between the first excited singlet electronic state, S_1, and the first excited triplet electronic state, T_1.

Species that Fluoresce

Compounds that contain aromatic rings or multiply conjugated double bonds are possible candidates for fluorophores (i.e., species that fluoresce). The aromatic amino acids (tryptophan, tyrosine, and phenylalanine) contribute to fluorescence in proteins. For those compounds that don't fluorescence, a fluorescent probe can be covalently attached. For example, the nucleotide bases of nucleic acids do not fluoresce, but studies on DNA and RNA are routinely conducted via derivitization.

Environmental Effects on Fluorescence Intensities

Temperature: Increasing temperature typically decreases the quantum yield of fluorescent emission due to the increased collisional frequency at higher temperatures.

Solvent Polarity: Polar solvents often strongly quench fluorescence. Nonpolar solvents or rigid environments often enhance fluorescence intensities.

Solvent Viscosity: Increasing the solvent viscosity typically leads to increased fluorescence quantum yields.

Resonance-Energy Transfer: The presence of other chromophores with absorption energies that overlap the emission energies of the fluorophore can quench fluorescence emission intensities if those chromophores are < 50 Å away. Essentially, these chromophores absorb the emitted radiation of the fluorophore.

Relationship Between Fluorescence Intensity and Concentration

Fluorescence intensity (F) varies linearly with concentration (c) at low concentrations (i.e., where $\varepsilon bc < 0.05$) and follows the relationship:

$$F = 2.3 I_0 \cdot \theta_f \varepsilon bcg(l)k(\theta)$$

where $k(\theta)$ is a geometrical factor that accounts for the solid angle of fluorescence radiation subtended by the detector, ε is the sample absorptivity, b is the path length of the cuvette, $g(l)$ is the wavelength-dependent detector efficiency, I_0 is the incident intensity, and ϕ_f is the fluorescence quantum yield. At higher concentrations selfquenching and selfabsorption mechanisms can lead to nonlinearities. The fluorescent intensity is usually measured relative to either a fluorescence standard such as Rhodamine B or a photodiode in order to correct the data for wavelength-dependent changes of the source intensity.

Protein Fluorescence

Of the 20 amino acids, only the three aromatic amino acid residues (i.e., tryptophan, tyrosine, and phenylalanine) fluoresce. These residues absorb at ca. 280 nm. Excitation in this region results in fluorescent emission at wavelengths between approximately 300 and 400 nm. Selective excitation of these three residues is possible by judicious choice of excitation frequency because exact positions of the absorption maxima for these amino acids are slightly shifted from one another. Free tryptophan in solution exhibits a fluorescence maximum at ca. 350 nm that is sensitive to solvent polarity. The fluorescence spectra of tryptophans located on the protein's surface exhibit similar maxima; however, tryptophans buried in the hydrophobic protein interior typically fluoresce with maxima near ~330 nm. Surface and buried tyrosine residues, on the other hand, exhibit no such environmental sensitivity. The intensities of tyrosine fluorescence signals are influenced by neighboring residues. Tryptophan is the strongest fluorophore of the three residues and phenylalanine the weakest. Due to the low molar absorptivity of the phenylalanine absorption band along with its low fluorescence quantum yield, phenylalanine fluorescence is typically not observed. The slightly larger fluorescence quantum yield and molar absorptivity values for tryptophan compared to tyrosine result in a protein fluorescence that is typically dominated by tryptophan fluorescence for those proteins containing both types of residues. Furthermore, if the tryptophan and tyrosine residues are located in close proximity to one another, tryptophan quenches the tyrosine fluorescence via a Förster energy-transfer mechanism. The polypeptide backbone also somewhat quenches the fluorescence of the aromatic amino acids.

Fluorescence of Native and Unfolded Myoglobin

Tryptophan fluorescence dominates protein fluorescence in solution. As mentioned above, if the unfolding of a protein exposes buried tryptophan residues to a polar solvent, the environmental sensitivity of the tryptophan emission will more closely approximate that of free tryptophan in solution. In the native protein the emission will occur at ca. 330 nm, but will shift to ca. 350 nm as the protein denatures and residues come into contact with solvent molecules. Horse-heart myoglobin contains two tryptophan (see Figure 1), two tyrosine, and seven phenylalanine residues. In the native state, the very weak fluorescence occurs at ca. 330 nm. In the presence of 2 M guanidine hydrochloride, a chemical denaturant, the fluorescence emission red-shifts to ca. 345 nm and significantly increases in intensity. This intensity increase is due primarily to the loss of Förster energy-transfer processes upon unfolding. These resonance-energy processes are distance-dependent. The heme group (see Figure 1) quenches the fluorescence intensity of the tryptophan residues in the native state where these groups are in close proximity. Similarly, due to Förster energy transfer, tyrosine fluorescence is completely quenched by the tryptophan residues in the native state. As the protein unfolds, the physical separation between the heme and tryptophan groups increases, thus decreasing the quenching. The observed fluorescence intensities of metMb are, therefore, greater in the unfolded state than in the native protein conformation.

Experimental

Equipment.

- Abbe refractometer (thermostated)
- Spectrofluorometer (an SLM-AMINCO Model 8100 spectrofluorometer was used to measure the data shown in this paper)
- Thermostated bath
- Hamilton syringes or autopipet
- Scanning UV–vis absorption spectrometer (a Shimadzu Model UV2101PC Ultraviolet–visible spectrophotometer was to acquire the experimental data shown here)
- Fused-silica fluorescence and absorption cuvettes (1-cm pathlength)
- Volumetric glassware

Supplies.

- Horse-heart myoglobin (Sigma)
- Monobasic and dibasic phosphate
- Sequanal-grade GuHCl (Pierce)
- Deionized or distilled water

Software.

- Nonlinear-least-squares-fitting program (the data in this paper were analyzed using Matlab, The Mathworks, Inc.)

Sample Preparation. *Preparation of ~7 M Guanidine Hydrochloride Denaturant in 0.10 M Phosphate Buffer*. Weigh approximately 33.44 ± 0.01 g of sequanal-grade guanidine hydrochloride (GuHCl) and quantitatively transfer to a 50-mL volumetric flask. (Note: Other grades of GuHCl contain UV-absorbing impurities that will interfere with this experiment.)

Add 0.27 g of monobasic phosphate and 0.52 g of dibasic phosphate to the flask.

Record the measured weights of the solids. The final solution concentration should be based on these measured weights, not on the estimated weights above.

Carefully add deionized water. The dissolution of guanidine hydrochloride is endothermic. The solution will, therefore, feel cold to the touch. Be careful not to exceed the 50-mL line on the volumetric flask. The solids were added to the flask first because the solution is approximately 7 M. For such concentrated solutions, the solute itself occupies a considerable fraction of the solution volume.

The actual concentration of guanidine hydrochloride must be determined via a refractive index measurement. Guanidine hydrochloride is hygroscopic, so the measured weights are not accurate monitors of the sample weight. Purified guanidine hydrochloride does not absorb in the near UV–vis wavelength region, so a simple absorption measurement to relate measured absorbance to concentration is not possible.

1. If the refractometer is thermostated, set the temperature to 25 °C and allow the system to equilibrate.
2. Place a drop of water on the prism of the refractometer and measure its refractive index. The refractive index of water should be 1.3333 at 25 °C; if your value is not close to this, consult the instructor.
3. Gently dry the prism surface.
4. Place a drop of the guanidine hydrochloride solution on the prism and record the refractive index of the solution.
5. The concentration of the guanidine hydrochloride can be obtained using the measured refractive index using the following equation: concentration (M) = 60.836 × (refractive index) – 81.106 (@ 25 °C; [2].
6. The calculated concentration should be close to 7 M; if not, consult the instructor.
7. Cap the volumetric flask with a glass stopper and label the contents.

1.0 M Monobasic and 1.0 M Dibasic Phosphate Stock Solutions. Weigh approximately 34.03 ± 0.01 g of monobasic phosphate (or 43.55 ± 0.01 g of the dibasic phosphate). Place into a 250-mL volumetric flask and dilute to the mark with deionized water. Mix to homogenize. Label the contents of the flask.

0.10 M Phosphate Buffer Solution at pH 7.0. Pipet 20 mL of 1.0 M monobasic phosphate and 30 mL of 1.0 M dibasic phosphate into a 500-mL volumetric flask and dilute to the mark with deionized water. Thoroughly mix the solution. Measure the pH of the solution and its temperature and record. Label the volumetric flask with both the contents and pH.

Preparation of metMyoglobin (Stock Protein) in 0.10 M Phosphate Buffer . Weigh approximately 0.01 g of horse-heart myoglobin. Quantitatively transfer the sample into a vial. Note: 0.01 g of a protein (a relatively high molar mass species) is a very small amount of material!

Dissolve the sample in a minimal amount of 0.10 M phosphate buffer. Cap the vial and mix by gently inverting a few times. Do not vigorously stir the protein solution. Strong agitation will denature the protein.

Determine the concentration of the stock protein solution as follows.

Using a scanning UV-visible absorption spectrophotometer, set the following parameters: Wavelength range: 600–240 nm, Bandwidth: 0.5 nm, Scan rate: medium, and, Temperature: 10.0 °C.

Zero the instrument. Place 0.10 M phosphate buffer in both the sample and reference cuvettes and perform a base-line run to compensate for cell and solvent absorbance over the wavelength range of interest.

If the same cuvette is used for the protein, be sure to thoroughly dry the cell. Residual droplets of liquid adhering to the inside surface of the cuvette may significantly change the volume of the sample due to the small volumes being used. Deliver a 100-µl aliquot of the native protein sample into the cell using a Hamilton microliter syringe or autopipet. (Note: If a Hamilton syringe is used, avoid bubbles, which will lead to large errors in the delivered volume.) Deliver 3000 µl of the 0.10 M phosphate buffer into the same cell. Rinse out the remaining protein from the Hamilton syringe by drawing the protein solution into the syringe a few times. Seal the top of the cell with a cuvette cap or a piece of parafilm and invert gently a few times to mix.

Measure the absorption spectrum over the specified wavelength range. Save the absorption spectrum. Plot the absorbance versus the wavelength (nm) as shown in Figure 6 and save the spectrum.

1. Determine the wavelength of maximum absorbance. This wavelength should occur at 409.5 nm for the native protein (blue curve). If distinct maxima are not observed at 409.5 nm and 280 nm, consult the instructor.
2. Convert the measured absorbance at 409.5 nm into a sample concentration using Beer's law (the molar absorptivity of horse-heart metMyoglobin at 409.5 nm is 188,000 L $mol^{-1} \cdot cm^{-1}$) at 25 °C and pH of 6.4. Remember to include the dilution factor of (3100/100) because the stock protein solution was diluted with buffer in order to perform the absorbance measurement. A stock concentration between 100 and 200 µM is a reasonable working concentration.

Preparation of the metMyoglobin Sample for the Kinetics Runs. You will prepare a total of 4000 µL of protein (stock + denaturant + 0.10 M phosphate buffer). The final concentration of protein for the kinetics run should be about 5 µM and that of the denaturant about 2 M. This concentration of denaturant is adequate to ensure complete denaturation. The measured volumes for the three solution components (i.e., protein stock, denaturant, and buffer) depend critically on the measured concentrations for the protein and denaturant stock solutions.

Example calculation #1:

Given that the stock GuHCl concentration is 7.0 M and the stock protein concentration is 100 µM, the sample should be prepared as follows (see Table 1):

Calculation of volume of protein stock:

$$(100\ \mu M)(x\ \mu L) = (5\ \mu M)(4000\ \mu L);\ x = 200\ \mu L$$

Calculation of the volume of guanidine hydrochloride stock:

$$(7.0\ M)(x\ \mu L) = (2.1\ M)(4000\ \mu L);\ x = 1200\ \mu L$$

Calculation of Volume of Phosphate Buffer:

$$4000\ \mu L - 1200\ \mu L - 200\ \mu L = 2600\ \mu L$$

Using these volumes gives a final protein concentration of 5.0 µM and a GuHCl concentration of 2.1 M.

Example calculation #2:

Given that the stock stock GuHCl concentration is 8.0 M, that the protein concentration is 200 µM, and that the desired concentrations for the sample are ~5 µM protein and ~2.0 M GuHCl (see Table 2):

Table 1. Summary of Calculations for Example #1

Total Volume (μL)	Volume Protein Stock (μL)	Volume GuHCl Stock (μL)	Vol. 0.10 M Phosphate Buffer (μL)
4000	200	1200	2600

Table 2. Summary of Calculations for Example #2

Total Volume (μL)	Volume Protein Stock (μL)	Volume GuHCl Stock (μL)	Vol. 0.10 M Phosphate Buffer (μL)
4000	100	1000	2900

Table 3. Typical Parameters for the Kinetics Runs

Experimental Parameter	Absorption Run	Fluorescence Run
Excitation wavelength	409.5 nm	280 nm
Emission wavelength	NA	345 nm
Temperature	10 °C	10 °C
Acquisition type	Time Trace	Time Trace
Lower limit	0 min	0 min
Upper limit	25 min	25 min
Resolution	0.5 min	0.5 min
Number of data points	49*	49*
Repetitions	1	1
Integration time	NA	0.01 min

*Select a resolution to obtain an adequate number of early time points.

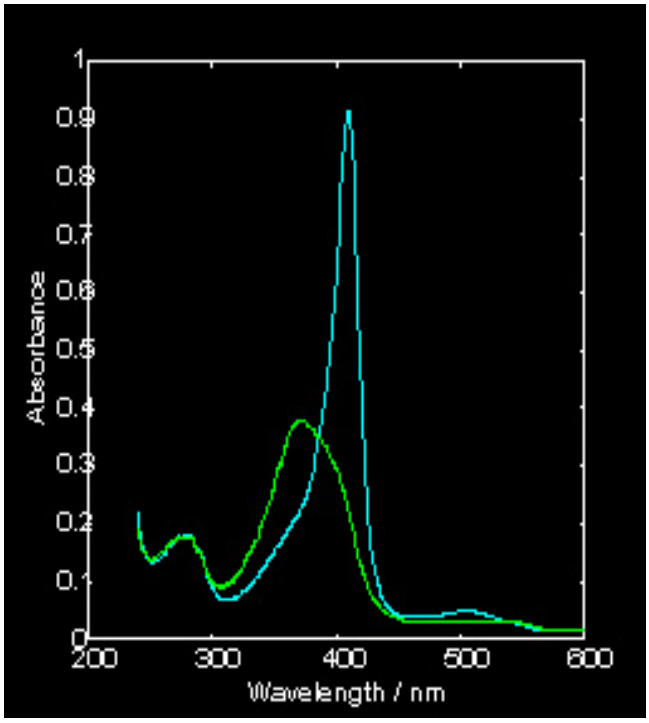


Figure 6. Absorption Spectra. Absorption spectra of native (blue) and denatured (green; in ~5 M GUHCl) METMb in 0.10 M phosphate buffer. The protein concentration is ~5 μM.

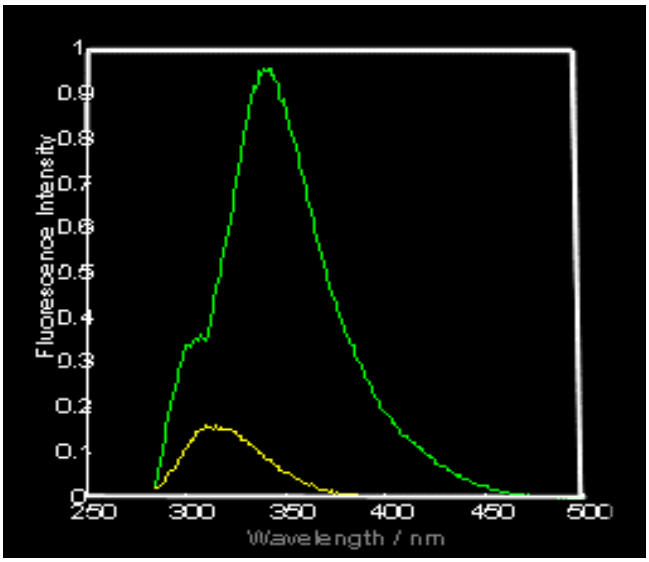


Figure 7. Fluorescence Spectra**.** Fluorescence spectra of native (yellow) and denatured metMb (green; in ~5 M GUHCl) in 0.10 M phosphate buffer. The protein concentration is ~5 μM. The feature at ~300 NM in the denatured protein derives from tyrosine fluorescence.

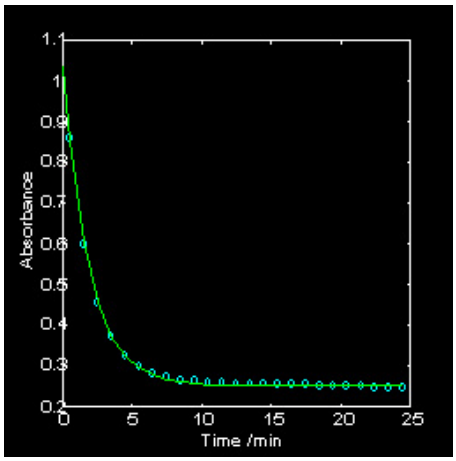
Using these volumes gives a final protein concentration of 5.0 μM and a GuHCl concentration of 2.0 *M*.

Following Example 1 above, add 1200 μL of the stock GuHCl solution with 2600 μL of phosphate buffer to a quartz cuvette. The buffer and GuHCl should be equilibrated to 10 °C. The protein (200 μL) should only be added to the GuHCl/buffer solution immediately before insertion into the spectrophotometer and should also be equilibrated to 10 °C before mixing. Denaturation begins upon contact of the protein and GuHCl solutions. If there is too long of a delay between addition of the GuHCl and initiation of the scan, significant absorbance decreases occur outside of the observation time frame. The spectrometer should have all parameters set and the only step remaining should be insertion of the cuvette into the sampling chamber and initiation of the scan. After addition of the protein, the cuvette should be quickly covered with a cuvette cap or a piece of parafilm and then gently inverted a few times to ensure sample homogeneity prior to data acquisition. A magnetic-stirring flea should also be inserted into the sample to be used for the fluorescence run prior to injection of the protein. The fairly viscous GuHCl will not be mixed by the agitation from the stirring bar alone.

An identical solution (i.e., the same concentrations of protein and denaturant and the same total volume) should be prepared for both the absorbance and fluorescence kinetic runs.

Experimental Details for the Fluorescence Run. The sample is susceptible to photo-induced effects, such as photobleaching, if too intense a beam irradiates the sample. Before the kinetics run, establish that a reproducible signal can be measured and that the beam intensity is not extreme.

The fluorescence spectra for the native and denatured protein are shown in Figure 7. The native protein fluoresces very weakly and exhibits a maximum in intensity at ca. 330 nm. The denatured protein fluorescence, which is dominated tryptophan fluorescence, increases dramatically in intensity compared to the fluorescence of the native state and shifts to 345 nm. In the kinetics run the fluorescence intensity will be measured at 345 nm to monitor the unfolding reaction via the observation of the formation of the unfolded state. Because the signal intensity increases in time, the voltage and gain of the spectrofluorometer should be adjusted using a fully denatured sample



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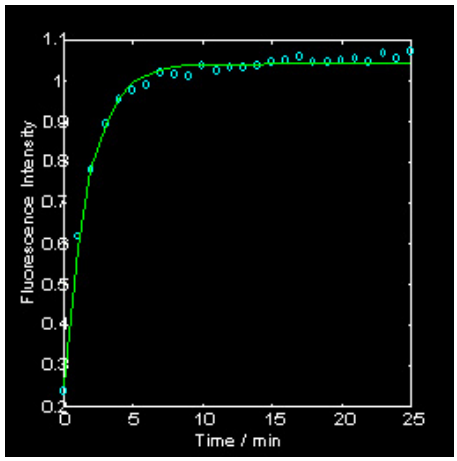

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Figure 8. A.) Kinetic Run: Absorption**.** Absorbance of metMb (at 409.5 nm) in 2.1 M GUHCl and 0.10 M phosphate buffer as a function of time (blue circles). The green line is a fit to the data using the function presented below. Fit parameters are summarized in Table 4. For clarity, only half of the fitted data were plotted in the Figure. B.) Kinetic Run: Fluorescence. Fluorescence intensity of metMb (at 345 nm) in 2.1 M GUHCl and 0.10 M phosphate buffer as a function of time (blue circles). the green line is a fit to the data using the function presented below. Fit parameters are summarized in Table 4. For clarity, only half of the fitted data were plotted.

Table 4. Fit Parameters for the Kinetics Experiments

Fit Parameter	Absorbance Data	Fluorescence Data
Amplitude	0.78	-0.78
Rate constant	0.53 min^{-1}	0.55 min^{-1}
Baseline	0.25	1.0
Sum of squared residuals	8.4×10^{-4}	1.1×10^{-2}

prepared at the same protein and GuHCl concentration as for the sample to be used in the kinetics run.

Data Analysis

Time-Dependent Data. Plots of the absorbance (at 409.5 nm) and the fluorescence intensity (at 345 nm) as a function of time are shown in Figures 8a and 8b. Note the difference in curvature between the two plots. Recall that in the absorption run 409.5 nm corresponds to the maximum of the absorption peak of the *native* protein. Upon addition of the denaturant, the protein unfolds. Decreases in the intensity of the absorption signal attributed to the native-state species are observed as its concentration decreases. The fluorescence intensity, on the other hand, increases with time because 345 nm, the observation wavelength, corresponds to the emission maximum of the *unfolded* state. Recall from Figure 7 that the native protein fluoresces only weakly at all wavelengths scanned in this experiment. As the protein unfolds, the measured fluorescence intensity increases significantly due to the contributions from the fluorescence of the unfolded protein. In terms of a simple two-state model:

$$N \rightarrow U$$

Nonlinear-Least-Squares Fit of the Data. Given that a simple two-state model following first-order reaction kinetics adequately describes the experimental data, the concentration of the reactant (the native state) should exponentially decrease with time, whereas the product concentration (the unfolded state) should exponentially increase with time. The following equation is expected to model the absorption data:

$$N_t = N_0 e^{-kt}$$

where N_t is the concentration of the native-state species at time = t, N_0 is the initial concentration of the native-state species, and k is the rate constant. The experimental data consisted of time-dependent absorbance values (Figure 8a); however, note that these absorbance values did not decay to zero amplitude at long times. The following function can be used to fit the absorption data and to accommodate the nonzero base-line amplitude:

$$f(t) = p_1 e^{-p_2 t} + p_3$$

In this equation p_n are fit parameters (p_1 is the amplitude, p_2 is the rate constant, and p_3 is the base-line amplitude). The fluorescence data will also be fitted using three parameters. Table 4 summarizes the results of the nonlinear-least-squares fits to the absorbance and fluorescence data:

When fitting data with exponentially, keep the following points in mind:

- Choose initial parameters that are expected to be close to those of the fitted solution.
- Try fitting the data with several sets of initial parameters to avoid false minima.
- Examine the values for the statistical parameters (e.g., the sum of squared residuals) to assess the quality and reliability of a fit. As the sum of squared residuals approaches zero, the fit generated by the given model closely approximates the experimental data.

A single exponential adequately fits both the absorbance and fluorescence data. (Note: If a single exponential does not adequately fit the data, more complex kinetics may be observed due to photobleaching, selfabsorption, or other effects. Fluorescence intensities should scale linearly with the incident intensity.) A two-state model with first-order kinetics appears to best represent the data under the current experimental conditions. Note that the rate constants, 0.53 min^{-1} for the absorption data and 0.55 min^{-1} for the fluorescence data, agree within experimental error. The data show that by either observing the decrease in the concentration of the reactant or the increase in the concentration of the product for a first-order

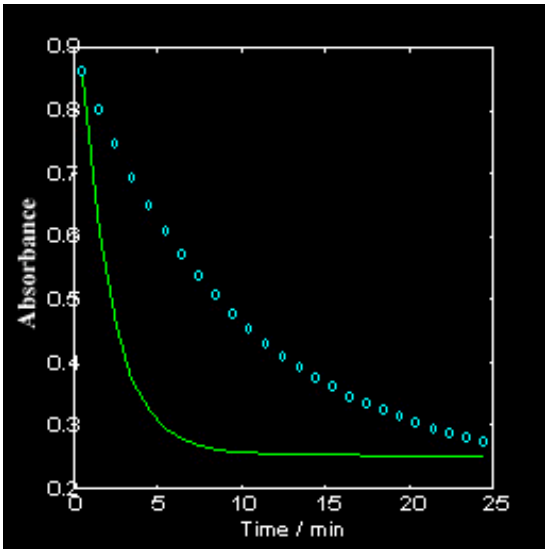

Figure 9. Simulated Kinetics Run: Absorption. Time-dependent absorption of METMb at 409.5 nm. Data = blue circles. Green line = single-exponential fit using the following fit parameters: amplitude = 0.78; rate constant = 0.53 min^{-1}; base-line amplitude = 0.25.

process in a two-state system, essentially the same rate constant is observed. Note the change in sign of the amplitude parameter for the two data sets. Recall that the kinetics trace for the absorption data exponentially decays, whereas the fluorescence data exponentially increases in time.

Discussion Questions

Extensions of the Experiment: Experimental. Repeat the fluorescence kinetics run using an excitation wavelength of 280 nm,but with an observation wavelength of 375 nm. It was assumed that the contribution from the fluorescence of the native state was negligible given an excitation wavelength of 345 nm. The assumption was taken to be valid, not only due to the low fluorescence quantum yield of the native state at 345 nm, but also due to its very low concentration under the experimental conditions. Test the validity of this assumption.

Extensions of the Experiment: Data Analysis. Verify that a single-exponential fit with a parameter accounting for the baseline is the best description of the data. Repeat the nonlinear-least-squares fit using the following equations:

Single-exponential fit without the base-line fitting parameter

$$\text{Fit Function: } f(t) = p_1 e^{-p_2 t}$$

Two-exponential fit with the base-line fitting parameter

$$\text{Fit Function: } f(t) = p_1 e^{-p_2 t} + p_3 e^{-p_4 t} + p_5$$

Questions.

1) Given the plot shown in Figure 9, explain the adequacy of the single-exponential fit (green line) in modeling the experimental data (blue circles). How might you attempt to fit the data? Is 25 min an adequate time to sample the kinetics?
2) The fluorescence kinetic data did not start at zero amplitude at the first time point (assigned to t = 0) in the data set. How would you conduct an experiment to observe the complete kinetics of formation of the unfolded species?
3) In this experiment the unfolding kinetics of metMb were monitored. What changes would have to be made to the experimental procedure in order to monitor the reverse reaction (i.e., the folding kinetics)?
4) The data were fitted using a *nonlinear*-least-squares fitting algorithm. How could you use a *linear*-least-squares fitting algorithm to analyze the data?
5) Would an observation wavelength of 280 nm be appropriate for a kinetics experiment in which it was desired to monitor the changes in absorbance of the *native-state species*? Why? What other wavelengths in addition to 409.5 nm are suitable for monitoring the unfolding kinetics of metMb?

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