# Determination of Myoglobin Stability by Visible Spectroscopy

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Proteins are biopolymers that fold spontaneously into a well-defined three-dimensional structure (*1–5*). The stability of these intricate structures in solution is usually measured by their resistance to denaturation in the presence of either heat or chemical denaturants. These demonstrations of protein stability are rarely encountered in the biochemistry teaching laboratory despite publications in this *Journal* of denaturation experiments involving chymotrypsin (*6*) and ribonuclease (*7*). Both of those studies used as a denaturant the guanidinium ion, which increases the solubility of both polar and nonpolar amino acid side chains and thereby reduces the hydrophobic effect on protein stability (*8–10*). The chymotrypsin experiment is noteworthy in that both protein conformation and enzyme activity are monitored simultaneously, directly illustrating the structure–function relationship. However, the protein conformation is monitored by intrinsic fluorescence, a technique that may be difficult for a modestly equipped teaching laboratory to utilize. The ribonuclease unfolding experiment is monitored in the ultraviolet (287 nm) where the total absorbance change upon denaturation is very small and difficult to measure accurately. The simplest procedure would be to utilize a visible protein that undergoes a significant change in color intensity upon denaturation. This would allow the denaturation to be followed with an inexpensive spectrophotometer or possibly even a colorimeter. In our opinion, the best candidate for such a protein is myoglobin.

Myoglobin ($M_r$ 16,700) is a simple monomeric oxygen-binding protein found within muscle cells. This protein's crystal structure was the first elucidated and its reversible unfolding has been well studied (*11, 12*). Because of their obvious historical and biological significance, myoglobin and hemoglobin are discussed extensively in most biochemistry texts. Myoglobin contains one polypeptide chain of 153 residues and a heme prosthetic group. The heme group is buried in a hydrophobic pocket within the protein's interior. This interaction of the heme with a structural feature of the protein results in the Soret band, a strong absorbance peak in the visible spectrum at 409 nm for myoglobin. Upon denaturation of myoglobin, a decrease in absorbance at 409 nm occurs owing to the exposure of heme to the polar aqueous solvent (*11–13*). This denaturation is easily and reproducibly measured in the teaching laboratory either by fluorescence spectroscopy, as described in a recent review of protein folding by Jones (*13*), or by visible spectroscopy as described below.

## Experimental Procedure

A stock solution of 2 mg/mL myoglobin (from horse skeletal muscle, 95–100%, Sigma #M-0630) was prepared using sodium phosphate buffer, 0.05 M, pH 7.0, as the solvent. A similarly buffered denaturant stock solution of 6 M guanidine-HCl (99+%, Sigma #G-3272) was also prepared at pH 7.0.

For each reaction tube, the final concentration of myoglobin was held constant at 0.2 mg/mL and the concentration of guanidinium hydrochloride (GuHCl) was varied from 0 to 3 M in 0.2 M increments, with smaller increments in the transition region between 1.0 and 2.0 M. All samples were prepared in duplicate. Appropriate volumes of buffer and denaturant were added to each tube, and then the contents of the tubes were mixed. Myoglobin was added last, and the gentle mixing was repeated. The samples were incubated at room temperature (25 °C) for 30 minutes before measurement of absorbance at 409 nm.

## Results and Discussion

The time necessary to reach equilibrium for GuHCl denaturation appears to be less than 5 min (Fig. 1), and so an incubation time of 30 min was used to insure complete equilibration. The absorbance at 409 nm of each incubated sample was plotted against denaturant concentration to generate an unfolding profile with a denaturation midpoint of 1.5 M GuHCl, as illustrated in Figure 2. The equilibrium constant ($K_{eq}$) for unfolding at each denaturant concentration in the transition region was determined from the ratio of unfolded to folded forms using the equation

$$K_{eq} = (A_f - A)/(A - A_u)$$

where $A_f$ is the absorbance of folded native myoglobin, $A_u$ is the absorbance of the unfolded denatured myoglobin, and $A$ is the absorbance obtained at the specified denaturant concentration. The free energy of stabilization at each specific denaturant values can subsequently be obtained from $K_{eq}$ using the equation $\Delta G = -RT \ln K_{eq}$. This is followed by
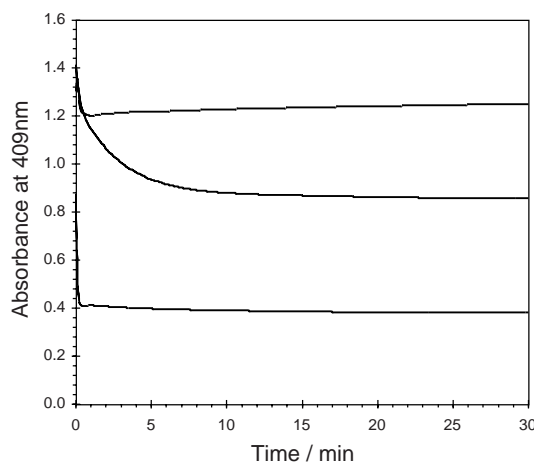


Figure 1. Time course of myoglobin denaturation. Guanidium hydrochloride concentrations are, from top to bottom, 0.8, 1.5, and 2.4 M. Myoglobin concentration was 0.2 mg/mL.

extrapolation to zero denaturant (Fig. 2 insert) to derive the stability of the folded protein under nondenaturing, and presumably physiological, conditions (*14*). The experimentally determined myoglobin free energy of stabilization is highly reproducible (45.7 ± 0.7 kJ/mol, *n* = 5) and well within the range of published values for horse heart myoglobin of 39 to 50 kJ/mol (*11–13*). The differences in published values are probably due to the use of a variety of buffers, salts, equilibration times, and protein concentrations.

Several variations on the above procedure can be imagined. Changing the pH or raising the temperature of the denaturation will shift the unfolding transition to lower denaturant concentrations and consequentially lower $\Delta G_{(H_2O)}$. This shows how dependent the stability of the folded protein is on environmental conditions. Obtaining $K_{eq}$ at multiple temperatures can allow the dissection of the free energy of stabilization into the entropic and enthalpic contributions. A further biophysical extension would be the illustration of both equilibrium and rate constant derivation by incorporating the benzene-enhanced myoglobin unfolding kinetics experiment of Schuh (*15*) with the experiment described here. If a fluorometer is available, a comparison of the measurement of myoglobin stability by the method of Jones (*13*) with that presented here may be of interest. Using urea instead of guanidinium hydrochloride as a denaturant is also a possibility, although it is experimentally more difficult because the transition region is at a higher denaturant concentration (*11, 12*) and the unfolding rate is considerably slower. It was not as reproducible in our hands, probably because of these technical difficulties.

In addition to absorbance in the visible range, another advantage of myoglobin in stability experiments is the variety of similar myoglobins readily available. Contributions to stabilization energy could be assigned to particular amino acids or groups of amino acids by using "natural mutagenesis"— that is, measuring the stability of commercially available muscle myoglobins from various species of mammals (sheep, pig, dog, etc). The sequences of these myoglobins are readily available through the NCBI databases at *http://www.ncbi.nlm.nih.gov*. Numerous structures of myoglobins are also available from the Protein Data Bank (*http://www.rcsb.org/pdb/* and can be viewed from most personal computers using either Rasmol (*http://www.umass.edu/microbio/rasmol/*) or Kinemage (*http://kinemage.biochem.duke.edu).*

In summary, myoglobin is an easily observed system for examining the stability of a typical folded protein's tertiary structure. The wealth of information on myoglobin and related heme proteins in the primary literature and general
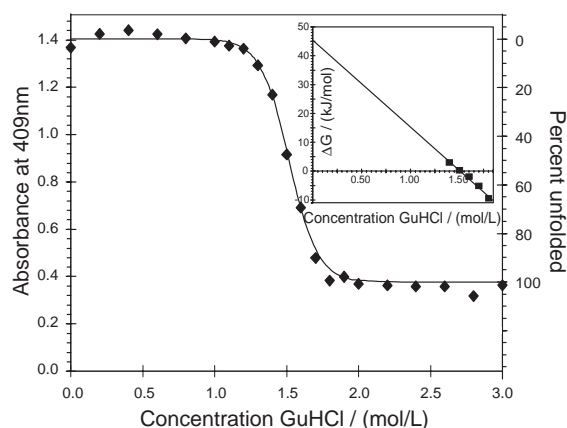


Figure 2. Denaturation profile of myoglobin in the presence of increasing concentrations of guanidium hydrochloride. Absorbance was measured after a 30-min incubation. Points are averages of duplicates. The error of each point is less than 10% (CV). The insert is a replot of the free energies derived from the transition region and is extrapolated to zero denaturant.

biochemistry texts provides ample background material for the inquiring student, and the simplicity of the experimental design enables this experiment to be performed in any teaching laboratory equipped with the most inexpensive visible spectrophotometers.

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