# Folding Myoglobin within a Sol-Gel Glass: Protein Folding Constrained to a Small Volume

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ABSTRACT The unfolding and refolding reaction of myoglobin was examined in solution and within a porous silica sol-gel glass. The sol-gel pores constrain the protein to a volume that is the same size and shape as the folded native state accompanied by a few layers of water solvation. Denaturants such as low pH buffers can be diffused through the gel pores to the protein to initiate unfolding and refolding. Acid-induced unfolding was hindered by the steric constraints imposed by the gel pores such that more denaturing conditions were required within the gel than in solution to create the unfolded state. No new folding intermediates were observed. Refolding of myoglobin was not complete in millimolar pH 7 buffer alone. Addition of 25% glycerol to the pH 7 buffer resulted in nearly complete refolding, and the use of 1 M phosphate buffer resulted in complete refolding. The role of this cosolvent and salt in disrupting the ordered water surrounding the protein within the gel is discussed in light of the Hofmeister series and entropic trapping via a diminished hydrophobic effect within the gel. These results are consistent with the premises of folding models in which secondary and tertiary structures are considered to form within a compact conformation of the protein backbone.

## INTRODUCTION

A current view of protein folding describes the reaction as occurring via an initial rapid collapse of the unfolded, extended protein backbone into a compact, low-volume conformation. Some secondary structure may form during this process. Within the compact state, most of the helices, turns, and sheets then assume their final fold and dock with each other to create the tertiary structure of the functional protein. If this description of folding is correct, then a protein should be able to unfold and refold within a small volume. The question, then, is how much volume is typically required for these processes, and what would be the effect on the folding pathway if the volume available to the protein were limited? These questions become particularly germane if one considers that the cytoplasm of a cell is much more congested than the buffer solutions used in most protein studies. We present a novel approach to address these issues by confining a folding protein within the pores of a sol-gel glass to ascertain if volume constraints can prohibit it from accessing the same states as in solution. We chose Mb for our study because its folding mechanism in solution is well characterized.

The folding reactions of apoglobin and holomyoglobin have been studied extensively as model systems. The folded state of Mb is composed of 8 alpha helices that make up ~85% of the protein sequence (1). The heme group sits within a hydrophobic pocket bordered above and below by the E and F helices and on the sides by the G helix and solvent (2). One covalent bond between the proximal histidine in the F helix and the iron atom connects the heme to the protein. (Proximal denotes the side of the heme bound to this His.) In solution, the unfolding of holoMb typically is not reversible due to aggregation of the heme group upon its release from the unfolded protein and, depending on reaction conditions, one or more intermediate states exist on the folding pathway. Understanding how and when the heme (un)binds to the globin during (un)folding has been a primary focus of many studies conducted on holoMb. A common theme in the literature is that the initial steps in the acid-induced unfolding of Mb in solution involve loosening or partial unfolding of the helices, thereby admitting water to the heme pocket. Unfolding, in part, is driven by the protonation of 6 histidine side chains throughout the protein (3). Sage et al. (4) and Tang et al. (5) found that an increase in solution ionic strength results in a shift of the folding equilibria toward the unfolded states due to increased shielding of these additional positive charges.

Various studies have noted that heme binding/loss is modulated by the strength of the proximal Fe-His bond (2, 6, 7, and references therein). Generally, it has been accepted that heme loss from ferric Mb occurs upon protonation of the proximal His and cleavage of the Fe-His bond within a

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*Abbreviations used*: Mb or holoMb, myoglobin with heme; apoMb or globin, myoglobin without heme; ferric or metMb, Mb with oxidized heme iron, $Fe^{+3}$; deoxyMb, Mb with a 5-coordinate heme containing a reduced iron, $Fe^{+2}$; COMb, Mb with a 6-coordinate heme containing a reduced iron ligated with CO; Fe-His bond, the covalent bond between the proximal His in the Mb F-helix and the heme; UV/Vis, ultraviolet and visible; TMOS, tetramethylorthosilicate.

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largely intact pocket; whereas heme loss in ferrous Mb is more difficult and also requires hydration of the pocket. Sage and colleagues (4) used Raman, visible absorption, and circular dichroism (CD) spectroscopies to determine that ferric Mb loses the proximal histidine heme ligand below pH 4, resulting in a 5-coordinate heme. (One water molecule remains bound on the distal side, which is the side of the heme where small molecules such as oxygen and carbon monoxide bind.) Sage et al. (4) also demonstrated that this transition is accompanied by a 50% loss of alpha helix content. Chi and Asher (8) used UV Raman, infrared absorption, and UV/Vis absorption spectroscopies to further substantiate the high correlation between changes in the heme environment and loss of helicity in the protein. Specifically, they observed that the A through F helices unfold in a concerted fashion in a sharp transition between pH 3 and 4, and that this transition is accompanied by a significant shift in the heme spectrum assigned to loss of the heme. Tang et al. (5) and Palaniappan and Bocian (9) reported similar results for metMb and that deoxyMb can form a partially folded intermediate containing heme. Hargrove et al. (2), Tang et al. (6), and Liong et al. (10) identified the role of water in heme stability through mutation of amino acids on either side of the heme. These researchers demonstrated that changes to smaller or more polar side chains dramatically increase both cleavage of the Fe-His bond and the heme dissociation rate in deoxyMb as a result of increased water access to the pocket (2,6,10).

Coincident with the studies of heme loss, these researchers and others found that heme association is governed by nonspecific binding to hydrophobic areas on the protein, which is primarily the empty but partially folded heme pocket for apoMb (2,7,10,11). Once the heme is within the pocket, large-scale folding occurs in the globin to create the final conformation, including binding of the proximal histidine to the heme iron. These events were well fit as a first-order process with a rate of $\sim 500\ s^{-1}$. A second pathway involves nonspecific binding of the heme to the protein outside the heme pocket, from where it must dissociate and then enter the pocket before the final folding steps can proceed. In solution, this second pathway requires tens of seconds.

The loss of helical content that accompanies pocket opening and subsequent dissociation of the heme must result in an increase in the protein's volume. Our study characterizes the (un)folding of ferric metMb within a sol-gel glass to effectively limit the volume available to the protein. A sol-gel glass is a porous $SiO_2$ matrix that can be formed around individual protein molecules (12). Steric constraints within the gel pores hinder or prevent protein motions involving substantial changes in shape or volume, but smaller internal motions still occur as in bulk solution. For example, encapsulated hemoglobin can be locked into either the T or the R quaternary conformation regardless of whether oxygen is bound, but many of the internal tertiary changes within the heme pocket and between subunits that normally accompany ligand binding still occur ((13,14); E. S. Peterson and J. M. Friedman, unpublished data). Similarly, rotational diffusion of Mb is slowed dramatically within the sol-gel pores (15). The hemoglobin studies and other studies on Mb (16) also demonstrate that the gel significantly dampens—but does not prevent—fluctuations that open the heme pocket to CO or $O_2$. These results indicate that the gel pores are nearly the same size and shape as the protein. If the pores were much larger, the observed gel-induced inhibitions on protein motion would not occur.

An alternative scenario is that the proteins have adsorbed onto the gel walls within large pores, which could explain the slowing of the ligand binding and rotational dynamics described above and the folding reactions presented below. However, because adsorbates are known to be quite mobile at room temperature, we do not think that adsorption alone would slow any of these dynamics to the degree observed (slowed from $10^{-7}$–$10^{-3}$ s in solution to seconds–months in the gels). Diffusion on the surface still would allow rotation because the pore walls are curved. The R–T transition in hemoglobin and the escape of CO from either Mb or Hb should occur on a surface with rates comparable to those in solution. Sufficient immobilization of the protein to prevent these processes would require that the gel wall contact the protein on more than one side; nonspecific adsorption, with only a few points of the protein touching the wall, would be insufficient. In other words, one must assume the equivalent of the tight pores that we describe and that are assumed in the cited references.

Protein folding in the gel is expected to be hindered at points along the reaction pathway that require large-scale fluctuations of the protein backbone or large motions of one part of the protein relative to another. Yet, smaller internal rearrangements should occur in a facile manner.

Proteins in the gel are solvated by a thin layer of water, which can be inferred from successful encapsulation of many proteins within their native states. Removal of surface waters dramatically destabilizes proteins causing them to unfold. Small, solute molecules can be diffused from a bathing solution through the glass to the encapsulated protein. This diffusive connectivity between the bulk solvent and the gel interior is also consistent with a solvation layer.

Previous studies have shown that sol-gel encapsulation influences the acid-induced folding and refolding of Mb. Ellerby et al. (12) and Lan et al. (17) were among the first to demonstrate that Mb can be encapsulated in a functional state, as determined by changes in the absorption spectrum upon ligand binding. Edmiston et al. (18) encapsulated metMb and found that the encapsulation process used in their study resulted in perturbations of the Mb structure, as evidenced in an altered absorption spectrum. Das et al. (19) demonstrated that cleavage of the Fe-His bond is slowed dramatically in encapsulated deoxyMb. Samuni et al. (20) reported that unfolding of COMb in the gel below pH 2 involves disruption of the tertiary structure, partial opening of the heme pocket, and alteration of the Fe-His linkage. Sustained exposure to

these low pH conditions over many hours resulted in loss of the Fe-His bond and ligation of the heme iron by a water molecule. Complete refolding of Mb within their gels was unsuccessful (20).

Eggers and Valentine (21) used CD to ascertain that apoMb loses significant helical structure upon encapsulation within a sol-gel, and that this loss persists from pH 4 to pH 10. As for Edmiston et al. (18), the sol-gel protocols used in their study were found to partially denature met holoMb in some preparations. They also found that the unfolded apoMb helical content is invariant upon addition of 2 M KCl, but that the addition of 1 M $KH_2PO_4$ increases helical content. Alternatively, addition of a few percent by volume of hexafluoroisopropanol increases the helical content of apoMb to 81% in the gel, a value greater than that which occurs in solution. In a second publication, Eggers and Valentine (22) reported that, relative to a control buffer, $Mg^{+2}$ and $Na^{+1}$ slightly decrease helicity of apoMb in the gels, whereas the addition of $K^{+1}$ or glycerol results in a minor increase in helicity; this increase is not nearly as much of an increase as that induced by 1 M phosphate. No cation was found that restored the helicity measured for apoMb in solution. The authors proposed that the salt and cosolvent data are evidence of an altered water structure within the gel pores that changes the strength of the hydrophobic effect involved in protein folding.

The conclusions of our study significantly extend what is known about Mb folding and have implications for interpreting the reaction using folding models that invoke compact states, such as molten globules. In particular, we address the following issues. If ferric Mb is restricted to a small volume, can the heme escape from the pocket and is protonation of the proximal histidine still sufficient to cause this loss? Are new metMb folding intermediates sterically trapped within the gel? What are the effects of cosolvents and osmolytes on Mb folding? And, finally, can conditions be found such that Mb will reversibly refold within the gel?

## MATERIALS AND METHODS

### Mb solution and sol-gel preparation

Mb solution and sol-gel samples were prepared using modified versions of previously described methods (E. S. Peterson and R. D. Salisbury, unpublished data; (12,16)). In brief, ~0.5 mM stock solutions of horse skeletal Mb (Sigma, Saint Louis, MO) were prepared by dissolving lyophilized protein in 50 mM pH 7.0 potassium phosphate buffer. Solution experiments were always performed as a point of comparison for the sol-gel experiments. Sol-gels were prepared by first mixing 500 $\mu$L of TMOS with 1000 $\mu$L of 2 mM HCl to form $Si(OH)_4$ via a hydrolysis reaction. Nitrogen gas was bubbled through this mixture to mix it and remove > 95% of the methanol byproduct. Next, 25 $\mu$L of $Si(OH)_4$ was combined with 25 $\mu$L of buffered Mb stock solution to form the sol-gel. This solution was briefly vortexed and allowed to harden on the walls of a 10-mm glass sample tube (Fisher Scientific, Pittsburgh, PA) to a thickness of a few hundred microns. The sol-gels were allowed to finish polymerizing overnight while soaking in phosphate buffer at pH 7.0 before folding reactions were conducted. The gels were homogenous and completely transparent, which is ideal for optical studies.

In all cases, the Mb started in its native folded state in 50 mM potassium phosphate buffer at pH 7.0. Unfolding at pH 2.0 was done in 50 mM phosphate, and unfolding near ~pH 4 was in 50 mM potassium acetate/HCl. Unfolding was allowed to proceed from 15 min to several days. Unless otherwise specified, refolding was initiated with 50 mM phosphate ± 25% glycerol (v/v) at pH 7.0. Refolding also was done with 1–3 M KCl or $MgCl_2$ added to the 50 mM phosphate buffer at pH 7 and in 1 M phosphate (no other added salts) at pH 7. Proton diffusion through our gels was complete within minutes, as determined by encapsulation of pH-sensitive dyes.

### UV/Vis absorption spectra

UV/Vis absorption spectra were measured using a spectrophotometer (Varian/Cary 400; Varian Instruments, Walnut Creek, CA) from 325 to 700 nm using 1-nm steps, 0.5 s per step. An air reference was used because the thickness of the gel varied with each sample, as did the optical quality of the round culture tube. These factors were enough to alter the light path within the instrument such that an empty culture tube or one filled with buffer did not result in a baseline value of 0. Most spectra were baselined by subtracting the absorption at 700 nm, typically ~0.2. In some cases, the spectra also were normalized by dividing the baselined spectrum by the maximum intensity at the peak of the Soret band.

### Basis set fitting of Mb absorption spectra

The UV/Vis spectra of Mb at several points along the unfolding and refolding pathways within the sol-gels were fit to a two-state model. The spectra of Mb at pH 7 and pH 2 in solution were used as the basis functions for the native and completely unfolded states, respectively. Based on the close similarity between the heme (see below) and Mb spectra at pH 2, most of the heme at pH 2 must be dissociated from the protein; however, there is certainly a small population that also is bound nonspecifically to the globin (11). The basis spectrum at pH 2 contains contributions from both of these populations. The sol-gel absorption spectra were fit to a weighted sum of the basis spectra as follows:

$$\mathrm{F}(\lambda) = [\mathrm{c}\,\mathrm{N}(\lambda) + (1\text{-}\mathrm{c})\,\mathrm{U}(\lambda)] * \mathrm{f}_{\mathrm{heme}}.$$

Here F(λ) is the fitting function, N(λ) and U(λ) are the native and unfolded state basis spectra, respectively, and $c$ is the fraction of native state in the sample. Free heme can leach out of the gel into the bathing solutions (see below), which reduces the absorption of the gel. The factor $f_{\mathrm{heme}}$ represents the fraction of heme remaining in the gel. Both $c$ and $f_{\mathrm{heme}}$ varied between 1 and 0.

It was necessary to account for absorbance differences between the basis spectra and spectra of Mb in other solutions and gels due to variations in pathlength and concentration. All spectra being fit were normalized by expressing the absorbance values in terms of molar extinction coefficients. For solutions of the same concentration and pathlength, the extinction coefficients at each wavelength were obtained by using the measured absorption values to scale the known extinction coefficient of the Soret band at pH 7 (23) as follows:

$$\varepsilon(\lambda) = \varepsilon(Soret)\frac{Abs(\lambda)}{Abs(Soret)\,\mathrm{at\,pH\,7}}.$$

For each gel sample, the Soret extinction coefficient was used to determine the product of pathlength and concentration for the folded protein at pH 7 as follows:

$$lc = \frac{Abs(Soret)\,\mathrm{at\,pH\,7}}{\varepsilon(Soret)}.$$

This factor was then used to determine the extinction coefficient for Mb under any subsequent condition in that gel from the measured absorption values as follows:

$$\varepsilon(\lambda) = \frac{Abs(\lambda)}{lc} = Abs(\lambda)\frac{\varepsilon(Soret)}{Abs(Soret)\,\mathrm{at\,pH\,7}}.$$

### Free heme in solution and in sol-gels

Prolonged exposure of Mb in the gel to denaturing conditions resulted in release of the heme into the gel and eventually into the buffer solution bathing the gel. To assist in spectral assignments, UV/Vis absorption spectra were acquired for free heme both in solution and within the sol-gel pores. These spectra then were compared to spectra taken separately of the Mb gels and the bathing buffers. Hemin chloride (metheme; Porphyrin Products, Logan, UT) was dissolved to saturation in methanol. This solution was centrifuged, and the supernatant was diluted 10:1 (v/v) with phosphate buffer at pH 7 to prepare an aqueous met stock solution. Sol-gel samples containing heme were prepared as described above.

## RESULTS

The unfolding/refolding reaction of ferric Mb was characterized in solution and within the sol-gel pores by using UV/Vis absorption spectra to follow changes in the heme environment. Table 1 summarizes the observed positions of the Soret band for equilibrium solution samples at pH 7 and pH 2. The Soret band is a strong absorption near 400 nm that is assigned to an allowed $\pi \rightarrow \pi^*$ heme transition. Most of the ensuing discussion focuses on changes in this band that occur when Mb undergoes conformational changes. This band shifts dramatically upon loss of the Fe-His bond, hydration of the heme pocket, and ligation of water to the heme, all of which are steps involved in Mb folding. Several weaker, spin-forbidden $\pi \rightarrow \pi^*$ transitions result in the $\alpha$- and $\beta$-bands between 500 and 600 nm, and weak charge transfer bands appear above ~600 nm.

### Unfolding in solution versus sol-gel pores

To compare unfolding within the gel to unfolding in solution, it is important to establish that the same native structure is the reaction starting point. A previous study encountered difficulties with Mb partially unfolding during encapsulation (18). Examination of the protocol used in that study reveals two issues that also have given us difficulties (E. S. Peterson, R. D. Salisbury, and J. M. Friedman, unpublished data). First, not enough water was initially added to the TMOS to allow for complete hydrolysis of the TMOS methoxy groups. Second, the methanol formed in the hydrolysis was not removed from the solution; methanol can denature Mb if present in sufficient quantities. In our protocols, both of these issues were eliminated. The absorption spectrum of Mb encapsulated within our sol-gels at pH 7 is identical to the spectrum of a solution sample, indicating that our encapsulation process does not perturb the Mb conformation.

**TABLE 1 Soret band absorption maxima for Mb and free heme solutions**

| Species | pH 7 buffer | pH 2 buffer | pH 2 buffer + 25% glycerol |
|---|---|---|---|
| Mb | 408 nm | 365–370 nm | 370 nm + 395 nm |
| Ferric heme | 365 nm + 390 nm | 370 nm | 365 nm + 395 nm |

Spectra of Mb in solution between pH 7 and pH 2 display clear isosbestic points, indicative of a conformational transition that is well characterized as a two-state system (Table 1 and Fig. 1 *a*, spectra not shown for every pH value). When the pH is decreased below 4, dramatic changes occur within a few minutes, as the Soret band broadens and shifts from 408 nm to 365 nm, the $\beta$-band shifts from 502 nm to 525 nm, and the charge-transfer band shifts from 630 nm to 650 nm. No further evolution of the spectra was observed over longer times.

The most likely cause of heme loss within the gels at low pH values is unfolding of the helices. Stronger denaturing conditions are required to unfold Mb within the gel pores, that is, to induce the same spectral changes that are observed in solution. At pH 3.8, encapsulated Mb exhibits very little spectral change from the native state (Fig. 1 *b*). (The overall intensity decrease from pH 7 to pH 3.8 is due to desorption of Mb from the gel surface, not unfolding (see below).) In the gel at pH 2, Mb exhibits the same spectral changes as in solution at pH ~4, but they take place over a much longer timescale (Fig. 1 *c*). Within 15 min, the Soret band at 408 nm decreases substantially in intensity (~60%), and a concomitant increase in absorption occurs at 365 nm. Smaller magnitude changes continue over the course of 2–4 more hours (~5–10% additional change in Soret intensity). This very slow phase is likely due to a minority Mb population within pores that have either a volume or a shape that is not conducive to unfolding.

### Refolding Mb in solution versus within sol-gel pores

Refolding of Mb within the sol-gel upon return to millimolar phosphate buffer at pH 7 is not complete for samples unfolded at either pH 2 or pH 3.8 (Fig. 1, *b* and *c*). Half the population refolds over ~1 h. The progress of this reaction is evident as an increase in the native state (408-nm peak) accompanied by a decrease in the unfolded state (365-nm peak). Heme loss from the gel accounts for 5–20% of the nonfolding population, increasing with longer times at acidic pH (Supplementary Material, Data S1).

Several modifications were made in the refolding procedure to increase the Mb refolding yield within the gels. Previously, addition of glycerol was shown to increase protein stability and refolding (E. S. Peterson and J. M. Friedman, unpublished data; (20)). A total of 25% (v/v) glycerol added to the refolding buffer at pH 7 resulted in substantial, but not complete, refolding. The maximum reproducible refolding yield with glycerol was 80% (data identical to Figs. 1 *d* (1 M at pH 7 phosphate overnight) and 2 *d*).

The surface of an $SiO_2$ glass is negatively charged at neutral pH due to $SiO^{-1}$ groups. It is possible that either the protein or the heme becomes adsorbed on the walls of the gel pores via electrostatic interactions and is therefore unable to participate in refolding. KCl or $MgCl_2$ were added up to 3 M

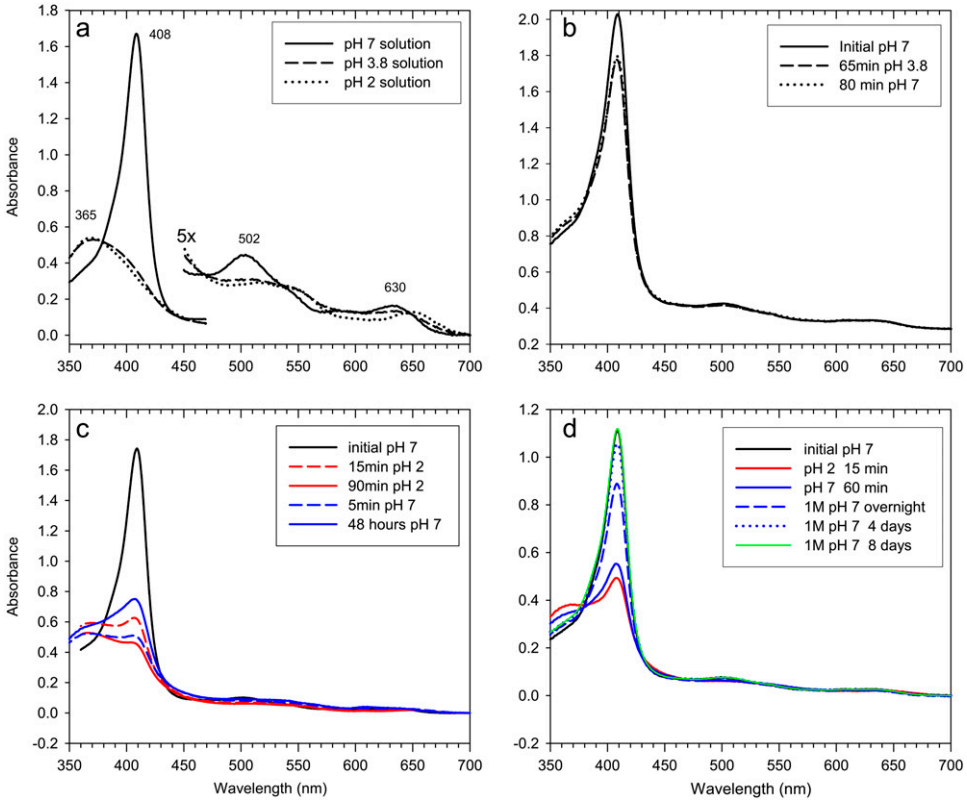


FIGURE 1 Absorption spectra of Mb in solution and sol-gels. The Soret band of the native state appears at 408 nm, and the unfolded species appears at 365 nm. (*a*) Solutions of folded Mb at pH 7 and unfolded Mb at pH 3.8 and pH 2. (*b*) A Mb sol-gel sequentially placed in buffers at pH 7.0, pH 3.8, and returned to pH 7 to unfold and then refold the Mb. (*c*) A Mb sol-gel sequentially placed in pH 7, pH 2, and pH 7 buffers. (*d*) Sol-gel–encapsulated Mb unfolded at pH 2 and refolded at pH 7 using two concentrations of potassium phosphate buffer, first 0.05 M and then 1 M.

to provide counter-ions to screen charged groups on the gel and protein surfaces and thereby free the Mb and/or the heme. Addition of these salts had no effect on either Mb in solution or on the refolding kinetics and yield in the gel samples.

Complete refolding was achieved in the gel when a sample unfolded at pH 2 was placed in a 1.0 M potassium phosphate buffer at pH 7 rather than the 0.050 M buffer used in our typical protocol. Over the course of several days, the native-state Soret band at 408 nm and the bands at 630 and 502 nm all recovered to their original intensity, which was present before the Mb was unfolded (Fig. 1 *d*). The spectrum of the refolded sample had a slightly elevated baseline at 370 nm compared to the original pH 7 spectrum, but otherwise these two spectra overlaid exactly. A total of 1.0 M phosphate at pH 7 was the only condition that consistently resulted in the complete refolding of Mb within the gel; 1 M phosphate had no effect on the folded state in solution as evidenced by no change in the absorption spectrum.

## Two-state modeling of Mb folding

Previous work on ferric Mb in solution has not resulted in evidence for equilibrium intermediate conformational states that contain heme along the folding pathway. To ascertain whether the constrained environment within the sol-gel results in kinetic trapping of a Mb folding intermediate, solution spectra at pH 7 and pH 2 were used as a basis set to fit spectra of Mb in the sol-gel. Representative fits for an unfolding/refolding series (data displayed in Fig. 1 *d*) are shown in Fig. 2. We found that this two-state model could successfully fit all encapsulated Mb spectra at pH 7, pH 3.8, pH 2, and at several time points during the refolding process upon return to pH 7 (Data S1). Thus, there was no evidence that the gel pores had trapped a third, partially folded intermediate state containing heme. Furthermore, when the time at pH 2 was sufficiently short, little heme escaped, as evidenced in the $f_{\text{heme}}$ factor being $>0.9$. This implies that nearly all of the heme was bound in folded native Mb, nonspecifically bound to Mb, or present as aggregated free heme. As described below, the absorption spectra of these latter two heme states are very similar, presumably because both effectively sequester the heme from the polar solvent. It is possible that the Mb that remains in its apoform or that contains nonspecifically bound heme adopts more than one conformation.

## Heme is mobile within the sol-gel matrix

A heme-bearing species in the bathing solution was observed for Mb gels stored overnight in buffer at pH 2, but a heme species was never observed in a bathing solution at pH 7. Spectra of solutions used to soak and rinse Mb gels were compared to spectra of Mb and free heme to identify the source of this acidic bathing solution heme. Encapsulation of heme in a sol-gel did not significantly perturb its spectrum (Fig. 3). The Soret band of ferric heme at neutral pH dis-

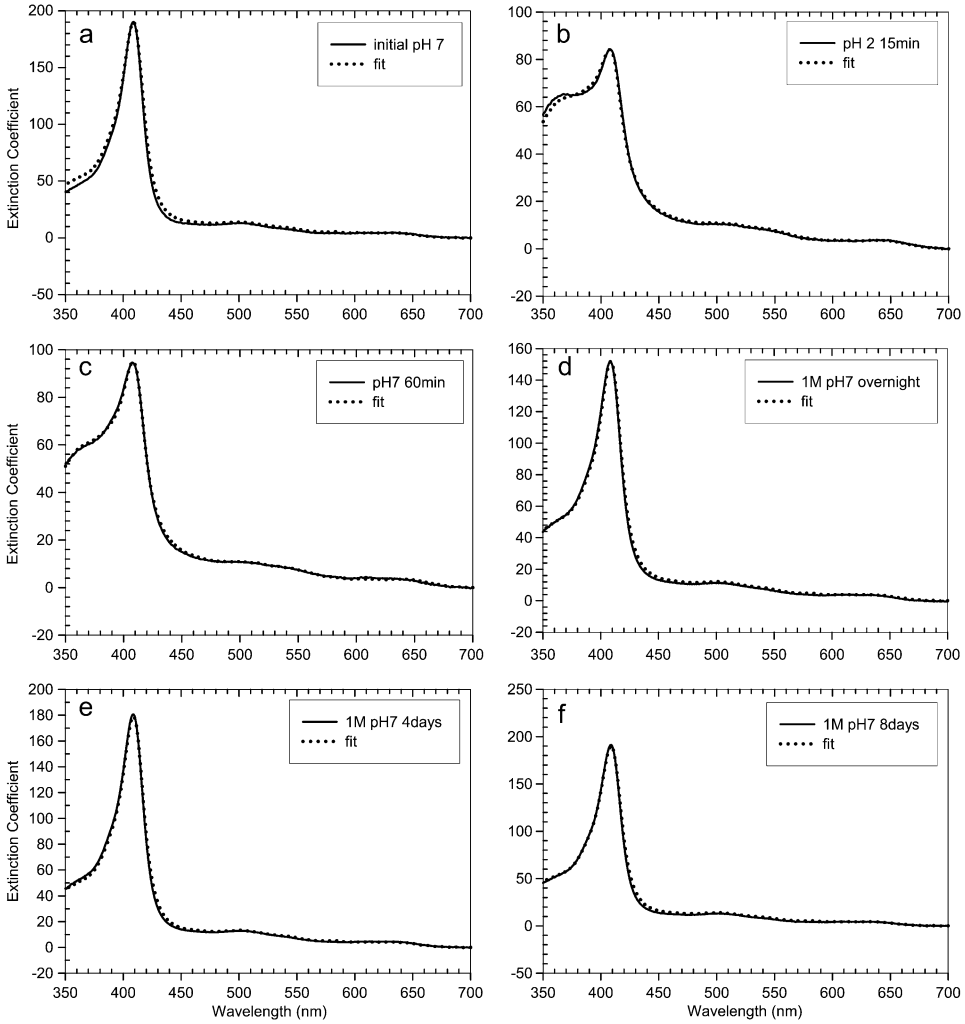


FIGURE 2 Two-state spectral fit of the Mb sol-gel spectra for the unfolding/refolding sequence in Fig. 1 *d*. Solution spectra of Mb at pH 7 and pH 2 comprise the basis set. The protein status and percent native state in each panel are as follows: (*a*) folded, 100%; (*b*) low pH unfolded, 30%; (*c*) refolding in 50 mM phosphate, 40%; (*d–f*) refolding in 1 M phosphate, 85%, 99%, 100%, respectively.

played two distinct components at 365 and 390 nm that correspond to aggregated and free heme, respectively. At pH 2, the two components merged together into a single broad feature at 370 nm, very similar to Mb under the same conditions.

The $SiO_2$ glass is quite stable at low pH, and the gel pores are too small for Mb diffusion within the gel; encapsulated Mb, therefore, cannot escape the gel. As discussed below, the species in the acidic bathing solutions came from two sources: heme that had diffused out of the gel and a small amount of Mb that had been adsorbed on the outer gel surface and only dissociates at low pH.

The solutions at pH 2 used to rinse the gels for 1 min contained heme, and three to four rinses were sufficient to remove this heme source from the gels (spectra of subsequent solutions contained no heme peaks). If these initial rinsing solutions at pH 2 were returned to pH 7, their spectra had the 408-nm Soret band of holoMb, indicating that adsorbed protein was rinsed off the gel surfaces. These data also indicate that the proteins inside the gel cannot be encapsulated within large pores; if the Mb were simply adsorbed onto the walls of such pores, it should unfold within seconds as does the Mb adsorbed on the outside surface of the gels.

If rinsed gels were then soaked in buffer at pH 2 for several hours, the soaking solution was again found to contain substantial heme. When these solutions were returned to pH 7, however, their Soret band remained at 370 nm. This indicates that Mb cannot escape the gel and that these solutions contained heme that had diffused out of the matrix into the bathing solution. Heme migration out of the gel can be minimized by shortening the duration of the unfolding step at pH 2.

## DISCUSSION

Our results provide several new insights into the folding mechanism of Mb. Mb can unfold within the gel pore, but it only refolds completely after the addition of cosolvents or certain salts. In both solution and the gel, heme loss from ferric Mb requires sufficient opening of the heme pocket to admit water rather than just a simple protonation of the proximal His imidazole. We discuss these observations in terms of the

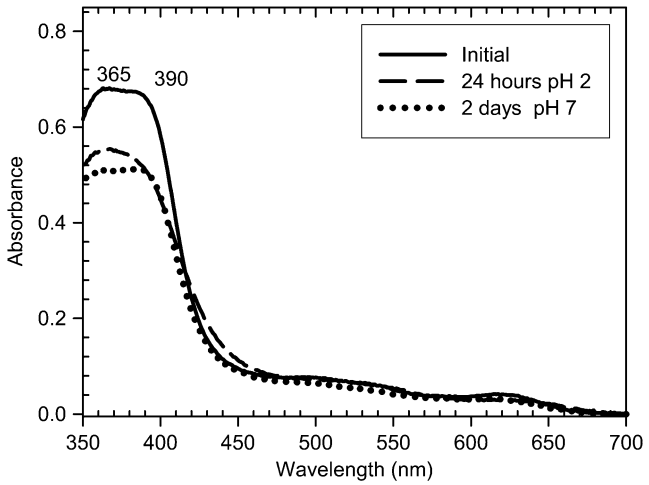


FIGURE 3 Absorption spectra of ferric heme encapsulated in a sol-gel at pH 7. The gel was then soaked in buffer at pH 2 and subsequently returned to pH 7. The intensity difference between the two pH 7 spectra is due to loss of heme from the gel into the bathing solutions.

volume required for (un)folding, migration of the heme within the gel, and entropic trapping of the unfolded protein due to a diminished hydrophobic effect inside the gel pores.

## Heme loss in Mb requires opening of the pocket to admit water

Mb unfolding occurs within sol-gel pores, albeit more slowly and requiring more denaturing conditions than unfolding in aqueous solution. In solution, complete unfolding occurs within minutes below ~pH 4 (Fig. 1 *a*), a pH very close to the $pK_a$ of the proximal His imidazole ring. In contrast, very little unfolding occurs in the gel at pH 3.8 (Fig. 1 *b*), whereas unfolding proceeds over tens of minutes at pH 2 to the same endpoint observed in solution (Fig. 1 *c*). These results and the arguments given below indicate that the sol-gel pores inhibit Mb unfolding and that heme loss from ferric Mb in both solution and the gels involves at least some opening of the heme pocket, not simply protonation of the proximal His, as has been suggested previously (2,6).

It has been well established that the heme pocket in sol-gel–encapsulated Mb and sol-gel–encapsulated Hb can undergo fluctuations large enough to admit small molecules, such as CO, $O_2$, dithionite, and $H_2O$, but that these motions are significantly damped within the gel ((13,14,16); E. S. Peterson and J. M. Friedman, unpublished data). Given this, protons also must be able to enter the heme pocket of encapsulated Mb. Protonation of the imidazole occurs at $N_\varepsilon$, the same nitrogen bound to the heme iron because the other nitrogen is protonated already. Thus, protonation requires cleavage of the Fe-His bond. This, in turn, results in a clear shift of the Soret band. We did not observe a Soret band shift for Mb in the gel until the pH had been decreased to ~2, well below the His $pK_a$.

One possible heme loss mechanism in the gel is heme dissociation after His protonation and Fe-His bond cleavage, but with helices that remain folded. Previous work (2,6,10) indicates that water must enter the pocket before the heme can dissociate. Because the folded heme pocket is very hydrophobic and the gel significantly dampens motions that open this pocket, one would not expect enough water access to dislodge the heme in this scenario. We therefore suggest His protonation and heme loss cannot occur within a heme pocket that remains largely intact, as must be the case in the gel at pH 4.

A more likely mechanism for opening the heme pocket to water within the gel is unfolding of the helices. Our results are consistent with this model as are conclusions in the literature. Chi and Asher (8) demonstrated that heme loss and the unfolding of helices A through F exhibit the same $pK_a$, a strong indication that the heme cannot exit the pocket until substantial loss of helicity has occurred. Eggers and Valentine (21) used CD to demonstrate that loss and reformation of helical structure in several small proteins, including apoMb, is possible within sol-gels to the same extent as in solution. In addition, Samuni and coworkers (20) have reported evidence of helix disruption in COMb, as described above. Thus, we suggest that heme loss from ferric Mb in both solution and gels can occur only after sufficient unfolding allows water to enter the pocket, resulting in weakening of the Fe-His bond and disruption of the hydrophobic interactions between the heme and pocket residues. In solution, heme loss is observed at pH 3.8, because significant loss of helicity admits water under these conditions (4,5,8,9). For Mb in the gels, however, sufficient pocket hydration requires a lower pH, near ~2, because the gel walls increase the unfolding barrier.

## The holoMb folding reaction proceeds through a compact state

The template that establishes the size and shape of the gel pore is Mb in its solvated native state. The mobility of the solvation layer in the pore affords the protein slightly more volume than its fully folded conformation. However, protein reactions requiring more volume than this when they occur in solution will be unable to proceed within the gel, as described earlier for the quaternary change in hemoglobin. We observed that the dynamics of both unfolding and refolding were slowed by the gel, but they are not wholly prevented. These observations indicate that helix formation in Mb in solution must occur within a very compact conformation that is slightly larger than the fully folded structure, such as a molten globule state. This conclusion is supported by the literature. Palaniappan and Bocian (9) equated the Mb molten globule with the apoMb ''I'' conformation, a partially unfolded intermediate with only the A, G, and H helices intact (~60% of the apo N state helicity) (24,25).

### Heme binding in ferric Mb folding is well-described as a two-state process

Previous studies of ferric Mb unfolding did not find evidence of a stable, partially folded, intermediate species containing bound heme analogous to the I′ state observed for deoxyMb (4,5,9). Our study reexamines the unfolding of ferric Mb within the sol-gel, where the steric constraints provide a real possibility of trapping an intermediate species in which the heme remains within the pocket after loss of the proximal Fe-His bond. Our spectral evidence suggests that, even within the gel, the unfolding of metMb proceeds as far as losing the heme without forming an intermediate population and thus remains well-described by a two-state model. As unfolding proceeds, the native-state Soret band at 408 nm merely decreases in intensity and does not shift slightly in wavelength or broaden, as is observed for the deoxyMb and COMb adducts when a stable heme containing intermediate population is created ((4,5,9); E. S. Peterson, J. A. Foulke, and E. F. Leonard, unpublished data). As a further indication of a two-state system, both solution and gel spectra contain isosbestic points.

As a quantitative test of whether a two-state model can account for the spectral changes observed when Mb unfolds within a gel pore, spectra of encapsulated Mb were fit to a linear combination of the native- and unfolded-state solution spectra. The solution-unfolded spectrum was used because this should also correspond to the endpoint in the gel if comparable unfolding has occurred. For all reaction conditions, excellent fits were achieved over the entire spectrum including the Soret, $\alpha$-, $\beta$-, and charge-transfer bands, as shown in Fig. 2 for a complete unfolding and refolding sequence. Based on these fits, we conclude that the steric constraints found within the sol-gel pores do not trap a significant population of a third species with a partially folded heme-containing species with a well-defined pocket, which is further evidence that the mechanism of unfolding in the gel in terms of heme dissociation/binding is the same as in solution. As described in the Results section, however, it is possible that more than one backbone conformation is present in the population that does not contain heme.

### Cosolvents or salts are required for complete Mb refolding within the sol-gel

We found that Mb refolding and rebinding of the heme proceeds over the course of many days within the gel, a rate that is notably slower than unfolding and much slower than the two mechanisms described by others (2,7,10,11). Furthermore, we observed that complete refolding does not occur without the addition of cosolvents or particular salts, suggesting that the unfolded state is somehow stabilized after it is formed within the sol-gel. This difficulty in refolding is counterintuitive because the expected result is that the spatial limitations imposed by the gel walls should limit the conformational search necessary to find the native state. The resulting loss of entropy in the unfolded state should provide a thermodynamic driving force for refolding. Three possible explanations exist for why Mb will not refold in 50 mM phosphate buffer. 1), The gel pores are too tight to allow refolding; 2,) the unfolded protein electrostatically adheres to the gel wall and becomes trapped; and 3), some other influence within the gel traps the unfolded protein. Option one can be dismissed based on microscopic reversibility; if the protein can unfold within the pore then, considering only the sterics are involved, it should also be able to move sufficiently to refold if given enough time. Options two and three are explored below through the addition of cosolvents and salts. We note that the lack of complete refolding is not due to heme loss, as this can be prevented by shortening the time at pH 2. The fitting results account for lost heme and confirm that a population exists that has not refolded.

During the course of developing different sol-gel protocols, it was noted that, in some cases, the addition of glycerol resulted in clearer gels with proteins closer to their native conformational folds (E. S. Peterson and J. M. Friedman, unpublished data). With this in mind, glycerol was added to the buffers at pH 7 that were used to initiate refolding. The primary effect of glycerol is to reduce the dielectric constant such that heme solubility and mobility increase. (This explanation was readily confirmed at pH 2 because the addition of glycerol greatly increased heme diffusion out of the gel into the bathing solution.) When glycerol was added at pH 7, the increased heme mobility led to a larger fraction of dissociated heme that could rebind to the refolded apoMb. Approximately 80% refolding was obtained reproducibly for Mb in 25% glycerol buffer at pH 7. Our interpretation that glycerol helps the heme diffuse back to the protein rather than helping the globin itself to fold is supported by the results of Eggers and Valentine (22), who demonstrated that adding glycerol to encapsulated apoMb only slightly increased helicity. Samuni et al. (20) also found that glycerol aided in refolding Mb. Our findings with glycerol have important implications for heme-based sensor design (17). Use of glycerol during both unfolding and folding may provide a simple and facile way to exchange the heme group for other porphyrins to impart different substrate sensitivities to a gel containing a heme protein.

The $pK_a$ of the SiOH groups within the gel pores is likely between 2 and 4, and so the interior of the gels is negatively charged at a neutral pH given the presence of surface $SiO^{-1}$ groups. Because acid unfolding of Mb is driven, in part, by the protonation of His and other residues, creating increased positive charge throughout the protein (3), one might expect that the charged interior of the gel could stabilize unfolded intermediates through salt bridges or hydrogen bonds between the protein surface and the gel wall. Addition of 3 M KCl or $MgCl_2$ had no effect on the refolding yields. Such ion concentrations should be more than adequate to screen the wall charges from the protein. This result strongly suggests

that electrostatic interactions are not what is preventing reversible folding. Eggers and Valentine (21,22) attempted to refold apoMb in a sol-gel using 1–2 M KCl, NaCl, and $MgCl_2$ and noted that these salts had minimal effects. However, they found that addition of 1 M $KH_2PO_4$ resulted in increased helix formation in apoMb. We also found that phosphate concentration had a strong influence on the refolding yield. Within our gels, Mb only refolded ~40% in 50 mM $KH_2PO_4$. When placed in 1 M phosphate, however, ~85% of the Mb refolded within 24 h, 99% within 4 days, and 100% within 8 days (see Figs. 1 *d* and 2 and Data S1). Overlaying the original and final spectra, as well as fitting of the spectra, indicated that the original Soret peak at 408 nm and the smaller bands were fully recovered and that no heme had been lost from the gel. This is the first quantitative evidence that complete refolding of holoMb can be accomplished within a sol-gel.

It is interesting that we observed the same salt dependence as Eggers and Valentine (21,22) because our samples contain heme, which significantly stabilizes the Mb fold. If the globin could fold in 50 mM phosphate to form a heme pocket that excluded water, then heme binding would occur and could shift the equilibrium in the gel toward the folded state; however, this is not the case.

The sensitivity of the folding yield on the type of ions present suggests that the influence of the gel walls on the protein is not simply electrostatic in nature. An explanation for these observations lies in the Hofmeister series, a ranking of ions based on their interactions with water (26). Depending on how tightly these ions bind to water, they can act to salt out the polar peptide bonds and/or salt in nonpolar groups and vice versa. Thus, these ions can influence secondary structure formation by modulating the strength of the hydrophobic effect as a driving force in folding.

The mechanism by which Hofmeister salts might promote folding is becoming clearer. Navati et al. (27) have conducted studies on the solvation environment inside sol-gel pores using the vibrational sidebands present in the emission spectrum of $Gd^{+3}$, a direct probe of the hydrogen bonds within the ion's solvation shells. In a newly formed gel, the water molecules are involved in stronger hydrogen bonds than occur in the bulk solvent, which is a result of increased ordering of the water around the ion as expected in the early stages of gel polymerization because fewer waters are available. The $Gd^{+3}$ study by Navati et al. (27) offers insight as to why the addition of phosphate increases the refolding yields for Mb within the sol-gels. In a cured gel, the protein environment is not that of bulk water. The solvating water resides in a limited space bounded on one side by the polar glass wall and on the other side by the surface of the protein. It is known that a silicon dioxide surface can induce order in water (28). This water ordering is influenced by ions and modulates the strength of the hydrophobic effect experienced by the unfolded protein in the gel pore, as discussed below.

### Ordered water solvating a protein within a sol-gel reduces the hydrophobic effect in protein folding

The origin of the hydrophobic effect can be understood by considering the entropy of hydration for nonpolar molecules (29 and references therein). Water forms a clathrate-like cage around nonpolar solutes in an effort to maintain favorable water-water interactions and avoid the less favorable interactions with the solute. Water in these structures is more ordered than bulk water due to stronger hydrogen bonds and is therefore entropically unfavorable. This entropic barrier is the source of the hydrophobic effect in protein folding, and it would be diminished if the water around the protein was already ordered by some other influence, such as through interactions with the gel wall and confinement in a thin layer as described above. As a result, proteins unfolded within a gel pore can be stabilized and partially trapped in this conformation. The addition of high concentrations of Hofmeister salts, such as phosphate ion, presumably disrupts the gel-induced order in the water when the water reorganizes to solvate these ions. This in turn restores enough of the hydrophobic driving force to allow full folding of holoMb within the gel. Our results with holoMb complement the results with apoMb and other small proteins as reported by Eggers and Valentine (21,22), who first described the ordered water in a sol-gel affecting protein behavior. Further experiments using more salts and proteins without the dissociating heme group are warranted to solidify this interpretation.

Recent simulations by Sorin and Pande (30) and Sorin et al. (31) on peptides trapped within carbon nanotubes also demonstrate that water order has a strong influence on protein folding . They found that decreasing the tube diameter results in thinning and ordering of the water layer solvating the helix and that the loss of entropy in this water correlates exactly with the fraction of helicity lost in the peptide. These results are particularly interesting in the context of our work, because the nanotubes are nonpolar whereas the gel pore walls are polar. Pande's conclusions and our results suggest the intriguing possibility that the water in both of our systems becomes ordered primarily in response to the confinement and that the nature of the wall causing this confinement is not a defining factor. In both cases, the final result is that the peptide is surrounded by an ordered, polar water surface and responds by unfolding. Pande has coined the term ''hydrophilic destabilization'' to describe this particular example of a decreased hydrophobic effect.

## CONCLUSION

Ferric Mb was encapsulated within the pores of a sol-gel glass to confine the protein to a volume slightly larger in size and shape than the folded native state. Within the framework of the folding funnel (32), the volume limitation imposed by the gel pore effectively steepens the funnel walls to constrain protein motion to conformations near the bottom of the

funnel. This is the same region spanned by a protein that is folding in solution after it has undergone the initial collapse from an extended, random coil. Acid-induced unfolding and refolding of Mb was possible within the gel, although the reaction was slowed significantly and conditions that were more acidic by 1–2 pH units were required to produce the same unfolded state observed in solution. No new partially folded heme-bound intermediates were trapped as a result of the steric constraints within the gel. These results represent the first study in which complete refolding of a protein within a sol-gel glass pore was quantitatively confirmed. They also indicate that the basic Mb folding mechanism is the same in the gel as in solution. Thus, helix formation and heme binding in Mb occur within a low-volume, compact state for both sol-gel and solution samples. This conclusion is consistent with the use of molten globule folding models to describe the folding mechanism of Mb in solution.

Our results also demonstrate that cleavage of the proximal Fe-His bond and loss of heme in ferric Mb requires hydration of the heme pocket, previously only thought necessary in ferrous Mb.

Lastly, our results demonstrate the utility of sol-gels as a new environment in which to study protein function and point to the importance of surface waters in affecting the dynamics of protein conformational change. The ordering of the water layer solvating the silica wall and protein within the gel results in a diminished hydrophobic effect relative to what occurs in bulk solution. This change in the protein free-energy surface causes the unfolded protein to be partially stabilized in the gel. The addition of high concentrations of salts that interact strongly with water, such as phosphate, disrupts the ordered water layer and reestablishes the hydrophobic effect, which in turn allows the protein to refold. The decreased water activity present in the gel pores is more reminiscent of the crowded solution conditions within a cell than are dilute buffers (33). Studies such as this, therefore, are significant in understanding how cellular protein function may be altered from what we typically see in the lab. These results may also have ramifications in understanding how chaperonins, such as GroEL/GroES, function.

## SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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