Preservation of the Native Structure in Myoglobin at Low pH by Sol−Gel Encapsulation

Tapan Kanti Das, Imran Khan, Denis L. Rousseau, and Joel M. Friedman*

Department of Physiology and Biophysics
Albert Einstein College of Medicine
1300 Morris Park Avenue, Bronx, New York 10461

Received June 18, 1998

Silica-based porous sol−gels[1] have been shown to be relatively inert while allowing encapsulated proteins to retain their structure and reactivity.[2−3] Encapsulated proteins typically exhibit enhanced stability at elevated temperatures, suggesting that the gel matrix limits large amplitude conformational fluctuations. The possibility that conformational degrees of freedom are reduced for encapsulated proteins raises the prospect of using such materials for studying nonequilibrium protein structures. In the present study, resonance Raman spectroscopy is used to show that sol−gel encapsulation of deoxymyoglobin (deoxyMb) dramatically slows the low pH induced cleavage of the iron proximal histidine (Fe−His) bond at pH values as low as 2.

Acid unfolding of deoxyMb in solution[4−9] has been shown to proceed through two intermediates both of which exhibit a loss of the Fe−His bond. Between pH 4.5 and 3.5, intermediate I′ is formed. In this intermediate, the heme remains five-coordinate, but water replaces the proximal histidine as the fifth ligand. Between pH 3.5 and 2.5, a second spectroscopically distinguishable intermediate, U′, is formed. In this intermediate, the heme becomes four-coordinate but appears to remain within the heme pocket. At the low end of this pH regime, further unfolding and associated heme loss become the dominant processes. In the present work, we examine the extent to which sol−gel-imposed stability influences the acid-induced formation of the deoxyMb unfolding intermediates.

In resonance Raman studies of various myoglobins (Mb) and hemoglobins (Hb), the vibrational stretching frequency of the Fe−His bond (ν_{Fe-His}) is seen in the 200−230 cm^{-1} region.[10−12] Figure 1 compares the resonance Raman spectra[13] obtained with 441.6 nm excitation in the low-frequency region of deoxyMb encapsulated in a tetramethyl orthosilicate (TMOS) derived wet sol−gel matrix,[14] at pH 7 and 2.6. The ν_{Fe-His} mode of deoxyMb in sol−gel at pH 7 is seen at 220 cm^{-1} (spectrum a in Figure 1) which is very similar to that of deoxyMb in solution at neutral pH.[15] The intensities and frequencies of other Raman bands are also nearly identical to those observed in solution, showing that the protein, encapsulated in the gel, retains its structure and spectroscopic properties. This finding confirms that deoxyMb encapsulated in a wet gel at neutral pH is stabilized in its native form.[2]

When the pH is jumped from 7 to 2.6, the frequencies in the spectra (b−c) remain nearly unchanged for more than 30 min; however, the overall intensity gradually diminishes. Similar observations were made with an excitation wavelength of 413.1 nm in pH-jump experiments (Figure 2, spectra a−c). The spectrum taken 100 minutes after the pH jump (Figure 2, spectrum d), shows the loss of ν_{Fe-His}. These measurements demonstrate that deoxyMb loses the Fe−His bond very slowly compared to solution, where the acid induced cleavage of the Fe−His bond is several orders of magnitude faster even at substantially higher pH values.

Since 441.6 nm excitation is in resonance with the native deoxy species, the intensity of the spectrum at low pH can decrease with time (See Figure 1) as intermediates, with blue-shifted Soret transition, are formed. However, with a bluer excitation (413.1 nm), the spectrum (Figure 2, spectrum d) of the low pH intermediate could be measured. This spectrum differs from that of the deoxy form in that the ν_{Fe-His} mode at 220 cm^{-1} is completely lost, indicating full cleavage of the Fe−His bond. The bands assigned to the heme propionate and vinyl groups at 370 and 404 cm^{-1}, respectively, are shifted to 375 and 408 cm^{-1}, respectively, indicating that the heme environment is different. The heme spectral changes are similar to those seen in solution[5,6] for the formation of the I′ intermediate, upon lowering the pH from 6.9 to 3.9.

Unlike the low frequency spectra that showed clear changes with time, the high-frequency region of the spectra (Figure 3) remains essentially unchanged even after 2 h of acidification of the protein in the gel. The high-frequency region is sensitive to spin, coordination, and oxidation state of the heme iron.[16] The frequencies of the marker bands, particularly ν_2, ν_3, and ν_4 (1563, 1472, and 1355 cm^{-1}, respectively) are unchanged although some are slightly broadened. The spectrum is consistent with that of the I′ intermediate species in which the Fe−His bond has been ruptured, but the ferrous heme remains five-coordinate high-spin, with water as the fifth ligand. The spectrum after 20 h (not shown) is basically unchanged except for the appearance of a

* Corresponding author. Tel: (718) 430-3591. Fax: (718) 430-8819. E-mail: jfriedma@aecom.yu.edu.

(1) Brinker, C. J.; Scherer, G. W. In *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*; Academic Press: Boston, 1990.

(2) (a) Dave, B. C.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Anal. Chem.* **1994**, *66*, 1120A−1127A and references therein. (b) Ellerby, L. M.; Nishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Science* **1992**, *255*, 1113−1115. (c) Ji, Q.; Lloyd, C. R.; Ellis, Jr., W. R.; Eyring, E. M. *J. Am. Chem. Soc.* **1998**, *120*, 221−222. (d) Shen, C.; Kostic, N. M. *J. Am. Chem. Soc.* **1997**, *119*, 1304−1312. (e) Shibayama, N.; Saigo, S. *J. Mol. Biol.* **1995**, *251*, 203−209. (f) Bettati, S.; Mozzarelli, A. *J. Biol. Chem.* **1997**, *272*, 32050−32055.

(3) (a) Miller, J. M.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Non-Cryst. Solids* **1996**, *202*, 279−289. (b) Chen, Q.; Kenausis, G. L.; Heller, A. *J. Non-Cryst. Solids* **1996**, *202*, 4582−4585. (c) Williams, A. K.; Hupp, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 4366−4371.

(4) Sage, J. T.; Morikis, D.; Champion, P. M. *Biochemistry* **1991**, *30*, 1227−1237.

(5) Han, S.; Rousseau, D. L.; Giacometti, G.; Brunori, M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 205−209.

(6) Palaniappan, V.; Bocian, D. F. *Biochemistry* **1994**, *33*, 14264−14274.

(7) Tang, Q.; Kalsbeck, W. A.; Olson, J. S.; Bocian, D. F. *Biochemistry* **1998**, *37*, 7047−7056.

(8) Tang, Q.; Kalsbeck, W. A.; Bocian, D. F. *Biospectroscopy* **1997**, *3*, 17−29.

(9) (a) Postnikova, G. B.; Komarov, Y. E.; Yumakova, E. M. *Eur. J. Biochem.* **1991**, *198*, 233−239. (b) Giacometti, G. M.; Traylor, T. G.; Ascenzi, P.; Brunori, M.; Antonini, E. *J. Biol. Chem.* **1977**, *252*, 7447−7448.

(10) Rousseau, D. L.; Friedman, J. M. In *Biological Application of Raman Spectroscopy*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1988; Vol. 3, pp 133−215 and references therein.

(11) Kitagawa, T. In *Biological Application of Raman Spectroscopy*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1988; Vol. 3, pp 97−131 and references therein.

(12) Friedman, J. M. *Methods Enzymol.* **1994**, *232*, 205−231.

(13) The resonance Raman experiments were carried out at room temperature with laser excitation at 413.1 nm from a Kr-ion laser (Spectra Physics, CA) and 441.6 nm from a He-Cd laser (Liconix, CA). The laser power used for the measurements was ~10 mW. The NMR tubes containing the sol−gel encapsulated protein samples were spun to avoid local heating. The instrumental details have been described elsewhere.[16]

(14) The wet porous sol−gels were prepared by reported methods[2,3] with some modifications. For the preparation of the sol−gel, 10 μL of fresh TMOS and 10 μL of 50 mM potassium phosphate buffer (pH 7) were injected in a spinning glass NMR tube. When the mixture becomes homogeneous, 10 μL of buffered Mb (400 μM) was added and mixed thoroughly. The spinning is continued for ~20 min to allow a transparent sol−gel to form. The sol−gel samples were washed twice with the same buffer and finally 100 μL of buffer was poured on the top of sol−gel. The samples were incubated overnight at 4 °C before experiments.

(15) Hu, S.; Smith, K. M.; Spiro, T. G. *J. Am. Chem. Soc.* **1996**, *118*, 12638−12646.

(16) Wang, J.; Caughey, W. S.; Rousseau, D. L. *Methods in nitric oxide research*; Feelisch, M., Stamler, J. S., Eds.; John Wiley & Sons Ltd.: New York, 1996; pp 427−454.

S0002-7863(98)02127-1 CCC: $15.00 © 1998 American Chemical Society
Published on Web 09/17/1998

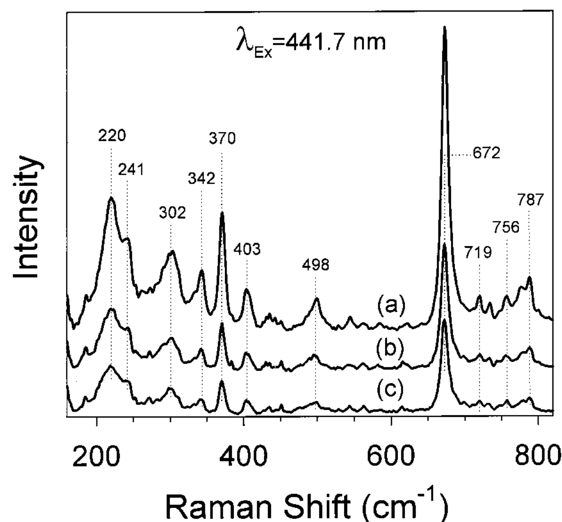

Figure 1. Low-frequency resonance Raman spectra of sol−gel encapsulated deoxyMb. The spectra shown are (a) pH 7.0, (b) 10 min after jump of pH from 7.0 to 2.6, and (c) 30 min after pH jump. Data collection time and other experimental conditions are the same for each of these measurements, and the intensities as obtained are shown in the three spectra. For the sake of clarity, the spectra a and b are vertically displaced.

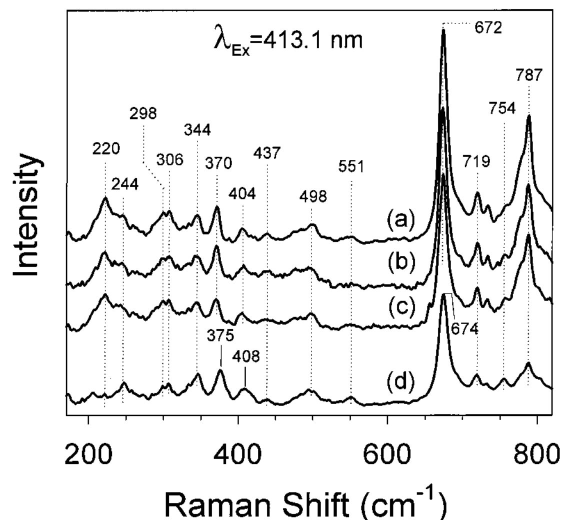

Figure 2. Low-frequency resonance Raman spectra of sol−gel encapsulated deoxyMb. The top spectrum (a) is at pH 7.0. After the jump of pH from 7.0 to 2.6, spectra were taken at (b) 5 min, (c) 15 min, and (d) 100 min. Data collection time was 30 s each for spectra a, b, and c and 15 min for spectrum d. For better clarity, the intensity of spectrum a is scaled by one-half and the spectra a−c are vertically displaced. The intensity of spectrum d is normalized to the same collection time (30 s).

weak shoulder at ∼1375 cm^{-1} which could be due the formation of the four-coordinate U′ intermediate or oxidized heme (Fe^{3+}). Nonetheless, it is clear, even after 20 h at pH 2.6, that the sample retains a predominantly five-coordinate ferrous high-spin heme.

The effect of other final pH values on the resonance Raman spectra of encapsulated deoxyMb were also examined. At pH ∼2, deoxyMb still retains its Fe−His linkage for several minutes, and a complete loss occurs only after tens of minutes (data not shown). At pH ∼4, the bond is stable for tens of hours.

The slow response of the protein to the change in pH at all three pH values, cannot be attributed to a retarded diffusion of

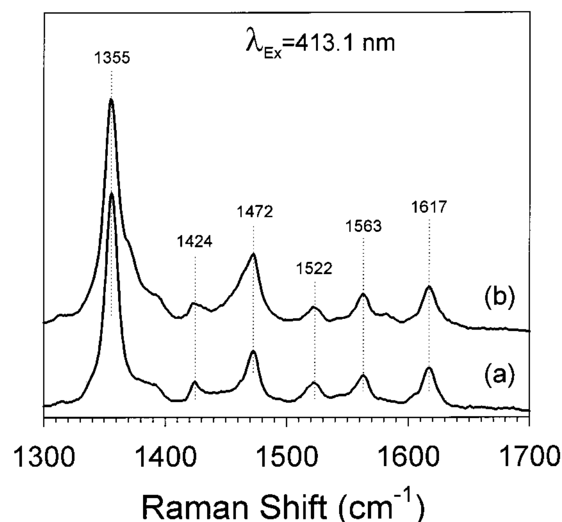

Figure 3. High-frequency resonance Raman spectra of sol−gel encapsulated deoxyMb. The bottom spectrum (a) is at pH 7.0. The top spectrum (b) was taken 2 h after the pH jump from 7.0 to 2.6.

the protons to the protein. We have seen that heme proteins encapsulated in thin gels respond very rapidly and completely (within seconds) to added substrates such as D_2O, sodium dithionite, oxygen, and carbon monoxide. Other groups also have reported that encapsulated rhodamine 6G has substantial rotational mobility[17] and that small ions diffuse in sol−gels almost as rapidly as in solution.[18]

Despite the fact that protons are accessible to the protein in these low pH experiments, the proton-induced rupture of the Fe−His bond within the sol−gel occurs at a markedly slowed rate, and the formation of the four-coordinate intermediate occurs at an even slower rate. A consistent picture that emerges from this study is that large amplitude conformational fluctuations are necessary for the formation of the unfolding intermediates and that the sol−gel inhibits the amplitude of these fluctuations thereby inhibiting the steps leading to protein unfolding.

In conclusion, it appears that the conformational fluctuations that are necessary to open the heme pocket to allow the Fe−His bond to break and the histidine to become protonated are severely damped by incorporation of the protein in a sol−gel matrix. Thus, conformational opening of the heme pocket in deoxyMb facilitates the disruption of the Fe−His linkage and the subsequent loss of the heme and unfolding of the globin, a result consistent with the solution phase observations of Tang et al.[7] It is likely that protein encapsulation within porous sol−gels can be used as a general technique both to trap unfolding intermediates and to stabilize active forms under conditions (high temperature, extremes in pH) that enhance reactivity but would normally denature the protein. The latter property augers well for the use of sol−gel encapsulated proteins either as probes or as industrial catalysts that can operate under rate-enhancing conditions that normally could not be applied to a protein-based solution process.

Acknowledgment. This work was supported by the NIH grants GM54806 and GM54812 to D.L.R., PO1 HL51084 and RO1 HL58247 to J.M.F.

JA982127I

(17) Narang, U.; Wang, R.; Prasad, P. N.; Bright, F. V. *J. Phys. Chem.* **1994**, *98*, 17−22.
(18) McKiernan, J.; Simoni, E.; Dunn, B.; Zink, J. I. *J. Phys. Chem.* **1994**, *98*, 1006−1009.