Communications to the Editor

Kinetics of the Allosteric Transition in Hemoglobin within Silicate Sol−Gels

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Received October 19, 1998

Structural changes are essential to the functions of proteins. In multisubunit proteins, the rearrangement of the packing of the subunits, the quaternary conformational change, is responsible for the cooperative behavior of the proteins.[1] For several allosteric proteins, X-ray crystallography has provided atomic resolution structures of the end states of allosteric transitions, but very little is known about the molecular mechanisms of the allosteric transitions. Beginning with the work of Sawicki and Gibson,[2] the allosteric transition in hemoglobin (Hb) has been studied by kinetic methods with the use of photodissociation of the CO complex, COHb, to generate unliganded Hb, which then evolves from the R quaternary structure toward the equilibrium T quaternary structure before it fully recombines with the CO. Despite significant progress in these studies,[3] the results are difficult to interpret because the deoxyheme spectral changes resulting from the change in quaternary structure are much smaller than, and often totally obscured by, the spectral changes that result from the ligand rebinding. Another problem inherent in these studies using nanosecond laser is that about 40% of the dissociated ligands geminately rebind to the hemes prior to the quaternary structural change. Here, we report a new method for studying the allosteric transition in Hb without interference from ligand rebinding by using sol−gel encapsulation. This work is based on our recent discoveries that oxy- and deoxyHbs encapsulated in the water-filled pores of a sol−gel-derived transparent silica gel maintain their original quaternary structures during the oxygen equilibrium measurements and that they are solvated and retain their intact properties in solution.[4] This gel matrix provides a means of slowing large-scale protein motions[4,5] while allowing relatively small molecules to diffuse in the gel as rapidly as in bulk solutions.[6,7] Thus, we have explored the possibility of observing the allosteric transition in deoxyHb on a much slower time scale after rapid deoxygenation of oxyHb in the wet sol−gel by dithionite.

The encapsulation of human adult Hb was carried out under CO atmosphere, by mixing 0.033 mL of TMOS sol[6] with 0.05 mL of 4.1% COHb in 0.05 M potassium phosphate buffer, pH 7.18, in a rotating glass tube (1 cm diameter). Gelation occurred within 3 min at 0 °C. The resultant COHb-doped thin film (i.e., 0.02−0.05 mm thickness), which adhered to the inner surface of the glass tube, was soaked in CO-saturated 0.1 M potassium phosphate buffer, pH 7.0, and was allowed to stand at 4 °C for 12 h. Then, the encapsulated COHb was converted to oxyHb by irradiation of light at 0 °C for 30 min with O_2 bubbling through the external solution. Because of the porosity of the silica gels,[6] immersing the oxyHb-doped thin film in a solution of sodium dithionite (i.e., 9.5 mM dithionite in N_2-saturated 0.1 M potassium phosphate buffer, pH 7.0) resulted in formation of R-state deoxyHb (R_0) within 2 min at 20 °C.[8] In this study, therefore, the spectrum after 2 min of the reduction period was used as the R_0 spectrum. Note that the thin films have faster response times with external reagents, such as dithionite, compared to monolithic silica gels.

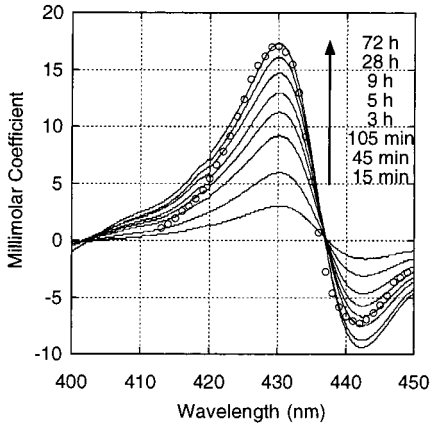

Figure 1. Absorption spectra of deoxyHb in sol−gel in the presence of 0.1 M potassium phosphate buffer, pH 7.0, at 20 °C following reduction of oxyHb with sodium dithionite. The spectrum measured after 2 min of the reduction period was used as reference (defined as $t = 0$) in order to eliminate the initial lag due to the diffusion of dithionite into the gel. The transient difference spectra of deoxyHb measured at 15 min, 45 min, 105 min, 3 h, 5h, 9h, 28 h, and 72 h, were shown as difference spectra. The absorbance was converted to its millimolar coefficient by assuming that the coefficient at 430 nm for deoxyHb in the gel at 72 h is equivalent to the value of equilibrium deoxyHb in solution (i.e., $\epsilon = 133$ mM^{-1} cm^{-1} at pH 7.0, 20 °C).[9] The circles indicate the published T_0-minus-R_0 difference spectrum for deoxyHb in solution (see text).[9]

The kinetics of the allosteric transition in deoxyHb from the R-state (R_0) to the equilibrium T-state (T_0) within the wet sol−gel was measured by monitoring the change in the spectra of the deoxyheme.[3,9] Figure 1 shows the transient Soret difference spectra (T_0-minus-R_0 spectra) of deoxyHb within the gel containing 0.1 M potassium phosphate buffer, pH 7.0, at 20 °C. The spectral change is very slow, but the spectral shape and amplitude are similar to those of the published T_0-minus-R_0 difference spectrum between deoxygenated, isolated α and β chains in equimolar amounts (R_0) and their reactive product (T_0) in 0.1 M

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(8) The completion of the deoxygenation within 2 min is evident from the existence of a characteristic isosbestic point at 437 nm in the transient Soret difference spectra between the spectrum measured after 2 min of the reduction period and the spectra at later times (Figure 1). We also confirmed that the time required for the diffusion of dithionite into a film, 0.05 mm thick, is less than 2 min, by measuring the time course of the reduction of ferric cytochrome *c* in the film by dithionite.

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10.1021/ja983652k CCC: $18.00
Published on Web 12/29/1998

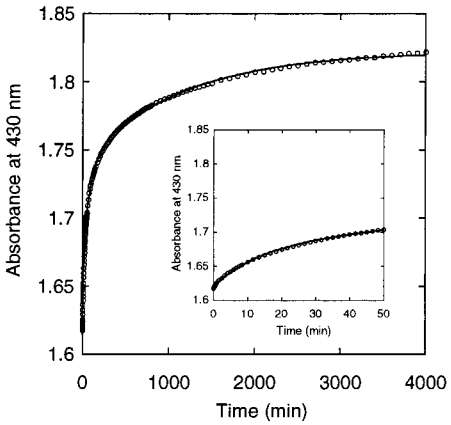

Figure 2. Time course of the T_0-to-R_0 transition in deoxyHb in sol−gel observed at 430 nm following reduction of oxyHb. The experimental conditions are the same as in Figure 1. The solid line shows the fit of the data to three exponentials with time constants of 13 min (relative amplitude, 25%), 1.5 h (35%), and 19 h (40%). The inset shows the same time course during the initial 50 min.

phosphate buffer, pH 7.0, at 20 °C.[9] Figure 2 shows the time course of the R_0-to-T_0 transition in the gel by plotting the Soret peak absorbance of deoxyHb at 430 nm as a function of time. Clearly, it does not follow a single-exponential time course. The time course can be fitted well to three exponentials of nearly equal amplitudes, with time constants of 13 min, 1.5 h, and 19 h. The presence of the fastest phase is more clearly seen in the inset of Figure 2, which shows the same time course during the initial 50 min.

Previous time-resolved optical studies by Eaton and co-workers[3] and recent time-resolved resonance Raman studies by Spiro and colleagues[10] have shown that five relaxations occur following full photolysis of COHb in a solution at neutral pH at room temperature. These relaxations have been interpreted as rebinding of CO from inside the protein matrix (i.e., geminate rebinding) at about 50 ns (called relaxation I), some kind of conformational relaxation which starts from nanoseconds and extends out to microseconds (relaxation II),[11] establishment of the T quaternary structure at about 10 μs (relaxation III), and overall bimolecular rebinding of CO from the solvent to the R and T quaternary structures at a later time (relaxations IV and V). Since the deoxyheme spectral changes in relaxations II and III are similar in shape,[3] the deoxyheme spectrum evolves continuously over three decades in time, in agreement with our observation in the gel (Figures 1 and 2). Therefore, it is reasonable to assume that the slowest phase in the gel ($k = 1.4 \times 10^{-5}$ s^{-1}) corresponds to relaxations III in solution ($k = 1.0 \times 10^{5}$ s^{-1}),[3d] and the other faster phases in the gel ($k = 1.9 \times 10^{-4}$, 1.3×10^{-3} s^{-1}) correspond largely to relaxation II ($k = 1.2 \times 10^{6}$, 1.1×10^{7}, 5.0×10^{7} s^{-1}).[11] Overall, the kinetics would be approximately 10 orders of magnitude slower in the gel than in solution.

Another possible explanation for the multiphasic kinetics in the gels is that Hb molecules exist in different environments in the pores of the silica gels. In fact, Shen and Kostic[12] recently demonstrated that photoinduced electron-transfer reactions from the triplet state of zinc cytochrome *c* to several charged electron acceptors, such as $[Fe(CN)_6]^{3-}$, are monophasic in bulk solutions but multiphasic in the water-filled pores of the wet sol−gels, suggesting heterogeneity of the pore environments in terms of electrostatic interaction between the pore walls and charged small molecules. Such microscopic heterogeneity can be expected since the pore walls contain several kinds of functional groups, such as silanol (Si−OH), siloxide (Si−O$^-$), siloxane (Si−O−Si), and silaketone (Si═O), that may exist in variable proportions and individually or in clusters of various sizes. However, our independent study using metal-substituted hybrid Hbs[13] has shown that the time course of the T_2-to-R_2 transition in a doubly liganded hybrid trapped in sol−gels is very slow and approximately monophasic.[14] This finding implies that microscopic heterogeneity of the gel interior, if any, does not significantly complicate the kinetics of the allosteric transition in Hb and does not alter the main conclusions drawn from our present study.

In summary, the present sol−gel trapping technique affords a unique opportunity to investigate the kinetics of the allosteric transition in Hb on a much (approximately 10 orders of magnitude) slower time scale without interference from ligand rebinding. This finding opens up a completely new area of investigation on transient species along the allosteric pathway. For example, it will become feasible to measure the oxygen equilibrium curves of the transient species trapped in the sol−gels. In addition, kinetics studies can be conducted on the T_4-to-R_4 transition in COHb following CO binding to deoxyHb within the sol−gel films. These studies are currently in progress in this laboratory.

Acknowledgment. This work was supported in part by a grant (10780413) from the Ministry of Education, Science, Sports, and Culture, Japan, to N.S.

JA983652K

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(11) The extended, continuous nature of relaxation II was apparent from the data of Jones et al.,[3d] which showed that the description of relaxation II required three exponentials with time constants of 20, 90, and 820 ns ($k = 5.0 \times 10^{7}$, 1.1×10^{7}, and 1.2×10^{6} s^{-1}) at 20 °C. At present, however, the molecular event that occurs in relaxation II is still a matter of controversy. Relaxation II was previously assigned as a *pure* tertiary change on the basis of its independence of the degree of photolysis,[3] while the recent time-resolved resonance Raman studies using ultraviolet excitation showed that a T-like $\alpha 1\beta 2$ interface begins to form in relaxation II.[10]

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