Nitrite reductase activity of deoxyhemoglobin (HbA) in the red blood cell has been proposed as a non-nitric-oxide synthase source of deliverable nitric oxide (NO) within the vasculature. An essential element in this scheme is the dependence of this reaction on the quaternary/tertiary structure of HbA. In the present work sol-gel encapsulation is used to trap and stabilize deoxy-HbA in either the T or R quaternary state, thus allowing for the clear-cut monitoring of nitrite reductase activity as a function of quaternary state with and without effectors. The results indicate that reaction is not only R-T-dependent but also heterotropic effector-dependent within a given quaternary state. The use of the maximum entropy method to analyze carbon monoxide (CO) recombination kinetics from fully and partially liganded sol-gel-encapsulated T-state species provides a framework for understanding effector modulation of T-state reactivity by influencing the distribution of high and low reactivity T-state conformations.

The physiological role of the nitrite ion is currently attracting considerable research interest arising primarily from observations that indicate the anion can function as a non-nitric-oxide synthase source of nitric oxide (NO) (1–6). It has also been suggested that hemoglobin (Hb)5 within red blood cells may be a source of deliverable nitrite-derived NO, generated from Hb nitrite reductase activity (deoxy–Hb + nitrite → met-Hb + NO) (6–10). Such a mechanism also has clear implications for the vasoactivity of acellular hemoglobin-based blood substitutes. Although there are studies that support the physiological role of a hemoglobin-based source of nitrite-derived NO, there are still fundamental mechanistic questions that remain unanswered including the key issue of how NO, once bound to a ferrous heme, can be efficiently delivered (11, 12).

Studies show that there is a relationship between the hemoglobin P50 and nitrite reductase activity (2, 3, 13, 14) as well as S-nitrosothiol synthase function (14). This result has led to the hypotheses that red blood cell/Hb enzymatic pathways can modulate blood flow and play a role in regulating hypoxic vasodilation (3, 14). The results also imply a relationship between the conformational properties of Hb and nitrite reductase activity (2, 5, 6, 15) and S-nitrosothiol synthase function (14). Direct measurements of the nitrite reductase activity of deoxy-Hb as a function of added allosteric effectors and mutagenic/chemical modifications support the concept of conformational control of Hb nitrite reductase reactivity with the T-state conformation having reduced reactivity compared with R-state conformations of Hb (5, 6). Two limitations of these studies are the inability to stabilize and compare the T and R-state forms of deoxy-HbA (human adult hemoglobin) without mutagenic or chemical modification and the complexity of the reductase reaction in solution due to autoacceleration arising from the allosteric nature of Hb reactivity.

The present study directly addresses the question of how the conformational state of HbA affects the interaction with nitrite by isolating T and R-state forms of the deoxy derivatives of HbA using sol-gel encapsulation to “lock in” global conformational populations. Several sol-gel encapsulation protocols have been developed that limit the conformational dynamics of the protein without significantly limiting diffusion of small solute and solvent molecules from the surrounding medium (16–21). In the case of HbA, the sol-gel matrix greatly slows the interconversion of R and T structures to time scales that greatly exceed those of standard kinetic measurements. As a result encapsulated deoxy-HbA retains the T quaternary structure for days to weeks subsequent to binding ligands. Similarly, deoxygenation of encapsulated R-state oxy HbA samples yields deoxy-HbA samples that can remain in the R-state for periods that greatly exceed the time scale of typical kinetic reactions such as those associated with nitrite reductase measurements (16, 22). As a result we are able to prepare T and R-state forms of deoxy-HbA and directly compare nitrite reductase activity. This approach also allows a direct evaluation of the influence of pH and added allosteric effectors inositol hexaphosphate (IHP) and L35 (23) on the nitrite reductase activity within a given quaternary state of HbA.

In the present study nitrite reductase activity of different sol-gel-encapsulated deoxy-HbA samples are compared by monitoring optical changes in the spectrum that follow the disappearance of deoxy heme and appearance of reaction products...
Nitrite Reductase Activity of Sol-Gel-encapsulated Hemoglobin

such as NOHbA and metHbA. The results show that the nitrite reductase activity of HbA is a function not only of quaternary structure but also of both pH and added allosteric effectors for a given quaternary state of Hb. Kinetic patterns for the recombination of CO to a variety of stable and transient forms of encapsulated T-state CO Hbs are also shown. These results show that effectors and the degree of ligation all influence the distribution of functionally distinct T-state tertiary conformations, a finding that provides a biophysical basis for modulation of T-state enzymatic activity of HbA.

EXPERIMENTAL PROCEDURES

Sample Preparation

Source of Hb—OxyHbA, provided by Drs. Manjula and Acharya, was prepared according to previously described protocols (24, 25). All other chemicals were reagent grade (Sigma). The buffer used in this experiment was 0.05 mM Bis-Tris acetate either at pH 6.5 or at pH 7.5. Purified solutions of iron-metal symmetric hybrid forms of HbA were generously provided by Dr. R. Noble.

Sol-Gel-encapsulated Deoxy-HbA—Sol-gel-encapsulated deoxy-HbA samples were prepared using two different protocols. In one case the protocol referred to as the T-state protocol is designed to trap and maintain the T-state of HbA. The second protocol referred to as the R-state protocol is designed to trap and maintain the R-state of HbA.

T-state protocol—Oxy-HbA (2 mM in heme) in 0.05 mM Bis-Tris acetate, pH 6.5, was first purged with nitrogen and then treated with dithionite (2 times the heme concentration) to generate deoxy-HbA. The T-state deoxy-HbA peak positions of the Q-band (555 nm), band III (758 nm), and the Soret band (430 nm) were verified by visible absorption spectroscopy for each sample of deoxy-HbA before its use. A 100-μl aliquot of this deoxy-Hb solution was used to generate a thin (<1 mm) layer of an encapsulated deoxy-HbA sample on the inside of a 1-cm-diameter quartz NMR tube (Wilmad Glass, Buena, NJ) configured for either the 1-cm NMR tubes or standard cuvettes. The effector-containing samples were prepared by adding IHP and L35 (1:1:tetramer:IHP:L35) to this initial deoxy-HbA sample before making the sol-gel. IHP and L35 were also included at 0.125 mM (the approximate concentration of the tetramer in the sol-gel) in the buffer bathing the HbA-containing gel.

R-state protocol—These samples were prepared by first encapsulating HbA as the oxy derivative in the sol-gel. The ligation status of the oxy samples was verified by optical absorption. The encapsulated fully oxy HbA samples were then aged for the same period of time as those prepared using the T-state protocol. The aging of the sol-gel samples enhances the locking in of the initial conformational population. After the aging process, the oxygenation status was confirmed by optical spectra. The samples were then converted to the five coordinate high spin ferrous derivative (also known as deoxy) by first purging the sample extensively with nitrogen gas and then adding a slight excess of dithionite that was then flushed out subsequent to full deoxygenation. Deoxygenation and the R-state status of the resulting deoxy sample were confirmed by optical spectra as discussed below.

Sol-Gel Preparation—All protein samples were encapsulated using a sol-gel protocol demonstrated to favor tight locking of the initial protein conformational distribution (19, 26, 27). Basically, buffer 0.05 mM Bis-Tris acetate, pH 6.5, containing 25% glycerol was added to ultrapure tetramethylorthosilicate (Sigma) followed by the addition of protein in 0.05 mM Bis-Tris acetate at pH 6.5. The resulting solution was a 1:1:1 mix of each component, and the final concentration of protein was 0.5 mM in heme. The resulting solution was vortexed slightly and then spun in a 1-cm-diameter NMR tube (Wilmad Glass, Buena, NJ) under nitrogen (for the deoxy samples) until gelation was complete. Deoxy samples were sealed in an oxygen-free hood with a septum. Once the sample gelled, an excess of nitrogen-purged buffer (0.05 mM Bis-Tris acetate, 25% glycerol, pH 6.5) was added to the tube. All samples were aged for the same time (1–2 weeks) before the addition of nitrite. Oxy samples were prepared similarly except using oxygen-purged buffers for preparation and for storage.

Sample Characterization

Absorption Spectra—The absorption spectra in the visible region were recorded using a Lambda 2 (PerkinElmer Life Science) absorption spectrometer with a sample compartment configured for either the 1-cm NMR tubes or standard cuvettes. The visible absorption spectrum was used to evaluate the ligation and oxidation status of the various solution phase and encapsulated HbA samples. In addition the peak positions of the Q-band at ~555 nm and the near IR band (band III) at ~760 nm were recorded for all preparations of deoxy samples. The peak positions of these two absorption peaks are known to be responsive to quaternary structure (28).

Geminate and Solvent Phase/Bimolecular Recombination—The visible and near infrared absorption spectra peaks for deoxy-HbA are adequate to assess the R-T status of the initial deoxy-HbA samples prepared using the two different encapsulation protocols. Less straightforward is how to monitor the extent to which the protocols maintain T and R-state status subsequent to ligand binding and the extent to which the sol-gel allows for ligation induced evolution of functionally distinct tertiary structures within a given quaternary state. As will be discussed subsequently, these issues are essential for sorting out both the role of effectors in modulating nitrite reductase activity and the possibility of autocatalytic behavior within the T or R states. We have previously shown (21) that CO recombination and maximum entropy method (MEM) analysis of the different kinetic phases together provide a means of monitoring not only the R-T status of encapsulated HbA samples but also the evolution of functionally distinct T-state populations with time. The CO recombination kinetics were generated after extensively flushing both T-state protocol and R-state protocol deoxy samples with CO. The conversion of the samples to the CO derivative was confirmed by optical spectra. The geminate yield and the solvent phase recombination rates (under the pseudo first order conditions of having a large excess of CO) for sol-gel-encapsulated COHb samples are highly responsive to both the quaternary and tertiary status of the sample (19–21, 26, 29, 30). The CO recombination
traces of samples of sol-gel-encapsulated carbonmonoxy Hbs were generated at 3.5 and 25 °C using a previously described protocol in which a 7-ns 532-nm excitation pulse (10 Hz) was used to photodissociate the CO from the heme and the attenuated 442 nm CW output from a HeCd laser was used as the probe of the recombination over several decades in time (19, 27, 29). The evolution of T-state protocol samples was followed over a period of weeks to monitor the evolution of the kinetic traces as a function of added effectors. The evolution was followed for two categories of samples. In one case the bathing buffer contained 25% by volume glycerol, and in the second case the bathing buffer contained 75% glycerol as a means of further slowing conformation relaxation and, thus, better exposing the role of effectors in modulating relaxation within the T-state.

The role of partial ligation with and without added effectors within the T-state was monitored using encapsulated Fe²⁺/Zn and Fe²⁺/Cr³⁺ symmetric hybrids of HbA. The Fe²⁺/Zn²⁺ hybrids are known both to bind oxygen non-cooperatively with very low affinity (31) and to remain in the T-state when liganded under a variety of solution conditions (20). The Zn²⁺ substitution mimics the behavior of the five coordinate ferrous irons in deoxy-HbA. The Fe²⁺/Cr³⁺ hybrids bind oxygen cooperatively with an affinity consistent with either enhanced stabilization of the R-state or destabilization of the T-state due to the heme-in plane nature of the Cr³⁺-heme unit (thus, mimicking ligation of the Fe²⁺-heme) (32).

Maximum Entropy Method—Kinetic traces were analyzed using the MEM as a means of distinguishing kinetic phases and following their evolution as a function of time as previously described (21, 33). The MEM analysis was performed using an algorithm described previously (34–36) that is now part of a commercially available package contained within the analysis module in Felix 3.2 software (Photon Technology International, Lawrenceville, NJ).

Nitrite Reductase Activity

The reaction was initiated with the addition of an excess of nitrite containing buffer; however, before the addition of nitrite, the bathing buffer of each gel sample was repetitively exchanged to remove glycerol and any remaining dithionite. The initial bathing buffer was replaced with 0.05 M Bis-Tris acetate adjusted to either pH 6.5 or 7.5. Samples were allowed to equilibrate 30–60 min with new bathing buffer before initiating the reaction with nitrite.

Reaction Conditions

Solutions of nitrite were prepared by adding the solid sodium salt to degassed buffer (0.05 M Bis-Tris acetate) and adjusting the pH to either 6.5 or 7.5. An aliquot of this stock was added to the bathing buffer of each T-state protocol and R-state protocol deoxy sample using a gas-tight syringe in an oxygen-free environment. The final concentration of nitrite in the bathing buffer was 15 mM for most measurements, creating a situation where nitrite was in large excess relative to the heme concentration. In one instance the concentration was reduced to 0.5 mM to determine whether the lower concentration of nitrite reduced the rate of reaction and, thus, might permit the appearance of intermediates not detected when the higher concentration of nitrite is used. All reactions were conducted in the absence of oxygen.

After the addition of nitrite to the buffer bathing the sol-gel, absorption spectra were scanned at regular intervals (starting at one spectrum every 30 s and progressing to 1-min intervals as the reaction proceeded and slowed). Kinetic curves as a function of time were constructed from these data. In this manner the progress of the reaction could be traced at different wavelengths reflecting both starting species and reaction products (see Table 1). The original deoxy spectrum of each sample was subtracted from each of the intermediate and end point absorption spectra taken at timed intervals. Fractions of aquo-met-Hb were calculated and subtracted from the final absorption spectrum obtained in each kinetic run to generate the final spectrum of the product(s).

RESULTS

Quaternary State of the Encapsulated Deoxy-HbA Sample Absorption Spectra—Fig. 1 shows that the Q-band from a representative R-state protocol sample is red-shifted by ∼3 nm from that of a T-state protocol sample at 555 nm. The encapsulated deoxy-HbA sample (T-state protocol) yields a Q-band peak position that is identical to that of deoxy-HbA solution phase samples, whereas the red-shifted peak from the R-state protocol sample is characteristic of solution phase deoxy samples from mutants or chemically modified samples that are known to be R-state species (28, 37). The peak position of R-T-sensitive band III in the near IR at ∼760 nm (28, 37) is also red-shifted for the deoxy-encapsulated derivative prepared using the R-state protocol relative to the T-state protocol sample, which yields a spectrum identical to that of deoxy-HbA in solution (spectra not shown, see Table 1).
Quaternary/Tertiary Structure Status of Encapsulated Samples upon Ligation CO/Recombination—Fig. 2 shows as a function of time, subsequent to the addition of an excess of CO, the evolution of the CO recombination traces of the CO-saturated derivative generated from encapsulated T-state protocol deoxy-HbA samples with added effectors (designated as [deoxy-HbA + IHP + L35] + CO). The corresponding trace for a sample that is directly encapsulated as a COHbA derivative (designated as [COHbA]) is also shown. This trace is included as the R-state reference. The two kinetic phases shown in the figure have been well characterized (19, 26, 27, 30). The faster phase is geminate recombination (38–42), and the second slower phase is the ligand concentration-dependent solvent phase recombination. It can be seen that over the time course of the sample evolution, the [deoxy-HbA + IHP + L35] + CO sample consistently has a lower geminate yield and slower bimolecular rate compared with the R-state [COHbA] reference. Similar time courses were obtained for the other T-state protocol samples with preparation-dependent differences in the evolution, as will be discussed in the following sections. The kinetic traces from CO-exposed R-state protocol deoxy samples were identical to that of the COHbA sample. Preliminary and still ongoing studies are being conducted on [oxyHbA] and [cyanomet-HbA] samples that are converted first to deoxy derivatives and then, after varying aging periods as a deoxy sample, are converted to the CO derivative. That study shows that it takes many days to weeks before the liganded-R converted to deoxy-R samples begin to manifest T-state signatures in either the spectra (Raman) or the kinetics of CO recombination.

The MEM-derived lifetimes for the contributing phases to the CO recombination are shown beneath the complex kinetic traces. These plots show the distribution of lifetimes (1/k in seconds) for the different recombination phases contained within the kinetic trace. It can be seen that as the sample evolves over the 52-day period, the lifetime values of the MEM populations get progressively faster, and the geminate yield of the faster geminate phase gets progressively larger. A summary of the MEM analyses of the solvent phase from several kinetic traces is given in Table 2. The MEM peaks associated with the solvent phase are assigned as high affinity T (HT), low affinity T (LT), and R based on both the lifetime value of the midpoints of each peak, as previously published (21), and on the previous accounts of high and low affinity forms of the T-state (26, 43, 45). The LT populations are further subdivided, albeit somewhat arbitrarily, based on the appearance and behavior of the different distinct LT peaks. Table 2 displays MEM results in five groupings arranged to highlight specific concepts.

The top grouping in Table 2 shows the MEM populations for T-state protocol samples in 25% glycerol buffer at days 1 and 52 as a function of added effectors. Also shown are the corresponding MEM populations for a half-liganded T-state protocol sample (day 1) derived from an Fe2+/Zn2+ hybrid in the presence of effectors. It can be seen that the MEM populations for the half-liganded species is heavily biased toward the lowest affinity LT populations (LT1 and LT2). A comparison of the corresponding day 1 populations for the fully liganded samples

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TABLE 1

| Sample | Q-band | Band III |
|--------|--------|----------|
| Deoxy-Hb (T solution) | 555 nm | 757 nm |
| Deoxy-Hb (R solution) | 558 nm | 760 nm |
| T-state protocol deoxy pH 6.5 | 555 nm | 756 nm |
| T-state protocol deoxy pH 7.5 | 555 nm | 757 nm |
| T-state protocol deoxy pH 6.5 + IHP + L35 | 555 nm | 757 nm |
| R-state protocol deoxy pH 6.5 | 558 nm | 760 nm |
| R-state protocol deoxy pH 7.5 | 559 nm | 762 nm |
| NOFe(II) Hb | 544 nm, 572 nm |
| NOFe(III) Hb | 534 nm, 564 nm |
| Aquo-metHb | 500 nm |

*From Ref. 37. The 631-nm band was observed but has been omitted from the table.
*From Ref. 37. High background precluded an accurate peak position measurement.
*From Ref. 55.

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FIGURE 2. A, kinetic trace showing the recombination of CO to HbA under pseudo first order conditions at 3. 5 °C subsequent to nanosecond photodissociation at pH 6.5 for sol-gel-encapsulated COHbA samples. The top three traces show the evolution of the kinetics as a function of time (red, day 1; blue, day 7; green, day 52) subsequent to the addition of CO to an initially prepared T-state protocol sample plus added IHP and L35. The bottom trace is derived from an encapsulated COHbA sample. N.A., normalized absorbance. B, kinetic phases derived from the above complex traces using the maximum entropy method. The distinct phases are presented as peaks with well defined recombination lifetimes. The populations are grouped into those originating from geminate recombination (GR) and those from the solvent phase recombination. The color code is the same as in A. The solvent phase peaks are labeled to indicate distinct populations based on correlations with samples, and proteins of known reactivity that have been designated as HT (high affinity T) and LT (low affinity T) species (see “Results” for details).
with and without effectors shows that the sample with effectors is skewed toward the lower affinity populations with no HT present but not skewed as far as the corresponding Fe$^{2+}$/Zn$^{2+}$ sample. At day 52, both fully liganded T-state protocol samples have sizable contributions from the HT population as noted. There are as yet no MEM peaks corresponding to the R-state population that are shown for R-state protocol samples in the second from top grouping. We have found that for a given temperature and solvent, there is very little if any variation in this MEM peak position for a wide variety of mutant and chemically modified R-state Hbs.\(^3\)

The third grouping shows the MEM populations for evolving T-state protocol samples with and without effectors bathed in 75% glycerol buffer. In contrast to the corresponding samples in the lower viscosity bathing buffer (top grouping), these samples do not manifest any large population difference at day 1. Instead they appear to evolve at a different rate as seen in the day 20 comparison. At day 20 the effector containing sample is less evolved toward the HT populations as reflected in a longer lifetime HT population and an LT population skewed toward LT$_1$ and LT$_2$. The MEM distributions at day 20 for these higher viscosity samples closely resemble those of the corresponding lower viscosity samples at day 1.

The fourth grouping shows how for the encapsulated half-ligated FeCO/Zn$^{2+}$ hybrid, the presence of effectors shifts the equilibrium distribution of T-state conformational populations toward the lower affinity end of the distribution. Because the sol-gel greatly slows even tertiary relaxation on the time scale of the kinetic measurements, the resulting distributions represent the unrelaxed populations associated with the starting unphotolyzed liganded species.

The bottom grouping shows that for two different encapsulated half-ligated Fe$^{2+}$/metal hybrids in the presence of the same effector, the nature of the metal impacts the distribution of T-state conformations. The FeCO/Cr$^{3+}$ hybrid is fully R (data not shown), whereas the deoxy derivative is T-state. The results shown in the figure indicate that for a FeCO/Cr$^{3+}$ T-state protocol sample turned CO, the distribution of LT and HT at early times are biased much more toward the high affinity end of the T-state distribution when compared with an analogous FeCO/Zn$^{2+}$ sample that is encapsulated from the start as the CO derivative.

Several clear results emerge from the kinetics and MEM analysis. The first is that the T-state protocol samples continue to manifest T-state population without the appearance of any R-state over an extended period of days to weeks. The second is that the R-state protocol samples remain fully R despite having been cycled from oxy to deoxy to CO on the same time scale as samples being prepared for the nitrite reaction. Third is that T-state distribution of functionally distinct tertiary conformations is highly responsive to the ligand binding, the extent of ligand binding, and the presence of allosteric effectors. It was also observed in both this and earlier studies (21, 26) that the progression of the kinetic traces and MEM populations are very similar for the different preparative protocols but with the time scale and end points for the evolution being highly dependent on Hb type, added heterotropic effectors, the gelation protocol, and added solutes (e.g. glycerol).

Nitrification Reduction Reaction Kinetics—Fig. 3 shows the absorption spectrum of a T-state protocol deoxy-HbA sample as a function of time subsequent to the addition of a large excess of nitrite. The Q-band features characteristic of the deoxy heme disappear (a prominent band at 555 nm) as new features appear as a function of time subsequent to the addition of the nitrite.

3 C. J. Roche, D. Dantsker, U. Samuni, and J. M. Friedman, unpublished observation.

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**TABLE 2**

MEM generated solvent phase CO recombination lifetimes for sol-gel-encapsulated HbAs

The brackets represent sol-gel encapsulation. Species listed within brackets are those that are encapsulated as part of the initial encapsulation process, whereas those listed outside the brackets are added after the sample had gelled and aged. Unless otherwise noted, all kinetic measurements were conducted at 3.5 °C. Increasing the temperature to 25 °C slightly shortened the lifetimes of the phases but had relatively minor effects on the rates of evolution of the traces beyond the initial day. Not shown are the more extensively evolved traces that contain R state populations. These traces required much longer evolution times. dx stands for deoxy, and the Fe is all cases ferrous.

| Sample                                      | Day | R HT LT$_1$ LT$_2$ LT$_3$ LT$_4$ |
|---------------------------------------------|-----|---------------------------------|
| [dxHbA] + CO$^a$                            | 1   | 781 7 27 11                     |
| [dxHbA] + IHP + L35 + CO$^a$                | 52  | 477 4 38                        |
| [dxFe]/β(Zn) + IHP + L35 + CO              | 52  | 417 15 33                       |
| [COHbA]$^a$                                | 280 | 176                             |
| [oxyHbA] + dithionite + CO (25 °C)$^a$     | 750 | 11                              |
| [dxHbA] + CO$^b$                            | 20  | 438 10.7 40                     |
| [dxHbA] + IHP + L35 + CO$^b$               | 20  | 877 10 50                       |
| [α(COFe)/β(Zn)]$^a$                        | 750 | 11                              |
| [α(COFe)/β(Zn)] + IHP + L35$^a$            | 7.5 | 12                              |
| [α(COFe)/dxHbA] + CO$^a$                   | 54  | 356.9 10.6                      |
| [α(COFe)/dxHbA] + CO$^b$                   | 1   | 10 10                            |

$^a$ In 0.05 M Bis-Tris acetate, pH 6.5, +25% glycerol; effectors are as noted.

$^b$ In 0.05 M Bis-Tris acetate, pH 6.5, +75% glycerol; effectors are as noted.

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**FIGURE 3.** Absorption spectra as a function of time for the reaction mixture between encapsulated deoxy-HbA (T-state protocol) in 0.05 M Bis-Tris buffer, pH 6.5, with 15 mM nitrite. The arrow represents increasing time (t) after the addition of the nitrite.
time. A band at 631 nm, consistent with the formation of aquo-met-HbA, appears as well as a band at 539–540 nm and another at 569–570 nm. These second two bands approach the values of the ferrous NOHbA derivative (46) (see values in Table 1). The appearance of the final spectrum was similar to that which was reported for the solution phase studies (47).

The spectral decomposition of the final absorption trace in the time series for the reaction of encapsulated deoxy-HbA with 15 mM nitrite was evaluated by subtracting out the contribution from the absorption spectrum of aquo-met-HbA. The result of this subtraction is the absorption spectrum shown in Fig. 4. The major peak positions of this spectrum in Fig. 4 are consistent with spectra of the ferrous NO derivative. This same treatment was applied to the final spectrum for each of the nitrite reactions using either T- or R-state protocols as well as the T-state protocol sample exposed to the lower concentration of nitrite.

The use of 0.5 mM nitrite instead of 15 mM nitrite resulted in a substantial slowing of the reaction; nevertheless, the spectra obtained throughout the run and the final end point spectrum showed very little if any difference when compared with those derived from the faster reacting samples. No spectroscopic signatures were observed for any substantial build up (transient or otherwise) of the NO metHbA derivative in any of the resulting spectra using either the low or high concentration of nitrite.

Nitrite Reductase Activity as a Function of Preparative Protocol—Fig. 5 shows a plot of the absorption at 631 nm as a function of time subsequent to the addition of nitrite to T-state protocol/H11006 effectors and R-state protocol samples at pH 6.5. It can be seen that the sample prepared as R-state manifests the faster rate. The same ordering of rates was also observed for the two protocols at pH 7.5 (data not shown). T-state protocol samples containing IHP and L35 at pH 6.5 with 15 mM added nitrite show a further rate reduction relative to the effector-free T-state protocol samples. The figure illustrates the results obtained from one of five such runs, which all yielded comparable curves with the same ordering of rates.

Pseudo first order rates for the reaction of nitrite with the T-state protocol samples with and without effectors under the high nitrite concentration limit were generated using the decrease in the deoxy band (555 nm) as a function of time for five separate samples. The data from the effector-free samples were best fit to a biexponential decay, yielding two lifetimes that differ by a factor of between 3 and 5 within the sample group. The shorter lifetime is comparable with kinetics reported in solution that yield a single rate constant in the range of 0.098 s$^{-1}$ in the absence of the effectors IHP and L35. In the presence of both effectors the data are best fit to a single exponential yielding a lifetime comparable with the longer of the two lifetimes observed in the absence of added effectors. The R-state lifetime proved difficult to extract from the spectroscopic changes but does appear to be almost a factor of two faster than the faster T-state lifetime.

Effect of pH—Fig. 6 shows the pH sensitivity of the reaction rate for a T-state protocol sample. It can be seen that overall
the rate is faster at pH 6.5 than at pH 7.5. A similar ordering of the rates with pH is seen for the R-state protocol samples (data not shown). The reduction in the nitrite reaction rate for both T and R populations with an increase in pH is likely to arise from a direct pH influence on the occupancy of nitrite within the distal heme pocket due to changes in protonation of the nitrite.

**DISCUSSION**

*Assignment of Quaternary State*—The spectroscopic and kinetic data support the anticipated assignments for the quaternary states of T-state protocol and R-state protocol samples. Based on the previously reported capacity of the sol-gel to lock in the conformational distribution of the initially encapsulated population, (16, 21, 26) it is not unexpected that the samples encapsulated as deoxy-HbA (T-state protocol) and oxyHbA (R-state protocol) would manifest T- and R-state properties, respectively. The absorption spectra of the deoxy T- and R-state protocol samples exhibit peak positions for the Q-band and band III that are reflective of T and R, respectively (see Table 1). Similarly, the CO rebinding traces from CO-saturated samples prepared using the protocols described in this study, respectively, resemble the previously described T- and R-state rebinding traces derived from functionally and spectroscopically well-characterized samples. Significantly, the kinetic traces and MEM peaks clearly indicate that on the time scale of the nitrite reductase measurements, these samples do not contain mixtures of quaternary states but are instead purely T or R respectively (see Table 1). Together, the absorption and kinetic data support not only the R-T assignments for the starting samples but also indicate that the R-T status is maintained over a time scale comparable with that associated with the nitrite reductase activity measurements (less than 2 h).

*Effect of Quaternary State on Nitrite Reductase Activity*—The sample-specific differences in the rate at which nitrite is reduced by encapsulated deoxy derivatives of HbA observed in this study strongly confirm the pattern inferred for solution phase samples (47, 48). In the sol-gel study, in contrast to the solution phase study, the R-T status of the samples is relatively unambiguous and remains unchanged during the nitrite reaction. In both cases, samples having properties attributed to the R-state samples react more rapidly. It is anticipated that with the refinement of these techniques and improved quality of the kinetic traces, it will be possible to apply MEM analysis to the nitrite reductase reaction. Under those circumstances, it should be possible to unambiguously identify the presence of small contributing populations of R occurring in the presence of a primarily T-state distribution as might occur for mutant or chemically modified Hbs.

The Influence of Heterotropic Effectors—The sol-gel technique permits evaluation of the nitrite reaction as a function of added effectors within the constraints of a given quaternary state. For solution phase samples it is difficult to isolate modulation by the effectors from that by the quaternary state since effectors significantly alter the autocatalytic process by stabilizing one quaternary state over the other. In the sol-gel, where the quaternary state is locked in the impact of an autocatalytic process arising from a quaternary structure, the switch was removed.

The addition of IHP and L35 to the T-state deoxy sample slows the rate of the nitrite reaction. This slowdown could arise either from a direct influence on either reox properties of the heme pocket or properties relating to conformation and dynamic that impact substrate binding to the heme groups directly. Spectroelectrochemical studies (49) have allowed for a separation of the influence of heterotropic effectors on reox and oxygenation properties of HbA. That study indicates that IHP is not likely to have a direct influence on the actual electron flow component of reox reactions within the deoxy T-state. That assessment implies that effector modulation of heme reactivity occurs largely through either substrate accessibility or direct heme-substrate stabilization (transition state or product).

Heterotropic effector-induced decreases in T-state reactions or processes have been reported. These decreases include the reactivity of the β93 cysteines (50), the on-rates for CO binding (51, 52), T-state oxygen affinity (17, 31, 43, 45, 51–54, 56), and the rate of T-state tertiary relaxation upon ligand binding (26). These results have been interpreted in terms of effector-induced decreases in the amplitudes of those conformational dynamics that (i) transiently expose the sulfhydryl groups of Cys-β93, (ii) transiently enhance ligand access to the heme iron (57), and (iii) facilitate ligand binding induced conformational relaxation (21). These earlier studies in combination with the present MEM results provide a basis for evaluating variations in reactivity within the T-state.

The present CO rebinding results directly support several emerging concepts (20, 21, 26, 58–60) that directly relate to T-state reactivity. (i) There is a distribution of functionally distinct tertiary conformations (reflected in the HT/LT distributions) accessible within the T-state family, (ii) for deoxy-HbA, the distribution is heavily skewed toward the low reactivity T-state conformations (LT), and (iii) ligand binding within the T-state shifts the distribution toward the higher reactivity T-state conformations (HT, LT₃) with the extent of the shift dependent on the degree of ligation. There are three potential roles for effectors within this picture. For the deoxy T-state, effectors do not change the equilibrium distribution of conformations (21, 61). They do, however, decrease the amplitude of thermally driven equilibrium fluctuations that allow for interconversion among the members of the equilibrium distribution of conformations. Upon ligand binding to the T-state hemes, the initial distribution of very low reactivity LT conformations begins to evolve toward higher reactivity conformations. The presence of effectors can slow the rate of relaxation toward the high reactivity conformations by decreasing the amplitude of those conformational fluctuations that drive the nonequilibrium relaxation (fluctuation-dissipation theorem). And finally, for stable or quasi-stable populations of partially or fully liganded T-state Hb, the presence of effectors will shift the conformational distribution back toward the lower reactivity conformations.

A structural basis for the linkage between heme reactivity and both effector and ligation-dependent distributions of
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HT and LT within the T-state can be seen from previous spectroscopic earlier works (20, 21, 26, 62, 63). These UV and visible resonance Raman studies on T-state populations with different HT/LT composition show that the highly stable T-state \( \alpha_1 \beta_2 \) interface associated with deoxy-HbA undergoes progressive loosening in going from the extreme LT to HT populations. Concomitant with that loosening is the tightly linked progressive decrease in proximal strain that impacts ligand reactivity. X-ray crystallography (60) shows the same progression in a series of T-state species. That x-ray study as well as functional and spectroscopic results (64) obtained on semi-Hbs (dimeric \( \alpha_1 \beta_2 \) derivatives composed of one apo and one holo globin chain) suggest that intra-T-state communication occurring during this loosening process is likely to be facilitated through a bending of the \( \alpha \beta \) dimers. Thermodynamic studies have directly provided indications of intra dimer communication within the T-state (65, 66).

The MEM results in Table 2 and the above Hb model provide a framework for explaining the effector induced slow-down in the T-state nitrite reductase reaction. The very initial rate at which nitrite reacts with a heme in a fully deoxy T-state species under a given set of solution conditions is dominated by the high proximal strain and steric hindrance limiting both reactivity with and access to the iron. Modulation of this pattern through effectors occurs through damping of motions that transiently decrease proximal strain and increase accessibility but not by changing the actual equilibrium distribution of LT species within the deoxy-HbA population (21, 61). Once a given heme site undergoes ligand binding (as either aquo-met, met-NO, or ferrous-NO), the overall ternary conformation begins to evolve in a manner that enhances reactivity at the remaining sites (probably within the same \( \alpha \beta \) dimer) (64 – 66). This point is illustrated by the comparison of FeCO/Cr\(^{3+}\) and FeCO/Zn\(^{2+}\) data showing that within the T-state, ligand binding (as mimicked by the Cr\(^{3+}\) ion) biases the HT/LT distribution toward HT with respect to the remaining deoxy sites. The Raman results show that in shifting toward the HT population, proximal strain decreases, which is associated with enhanced ligand reactivity (20, 21, 27, 62). In addition we anticipate that it is also likely that access to the \( \beta \) heme is enhanced due to either a shifting of the Val E11 side chain or to an interface-associated loosening that enhances the amplitude of side chain fluctuations that transiently increase access to the \( \beta \) heme. However, the rate and extent of this evolution is effector-dependent. For a partially oxygenated T-state species we also have the situation where the addition of effectors will bias the HT/LT distribution toward the lower reactivity populations. Overall the addition of effectors can exert influence on the overall reaction by damping conformational fluctuations and stabilizing the lower affinity T-state tertiary conformations. This picture implies that there could well be a degree of autocatalytic behavior even within the T-state for the nitrite reductase reaction.

Reactivity at Half-saturation—These results also help account for the maximizing of nitrite reductase activity at half-saturation (6). In addition they account for the special role recently attributed to partially oxygenated HbA in generating bioactive nitric oxide from nitrite due to the combination of nitrite reductase activity within the T-state and the onset of S-nitrosothiol synthase activity upon switching to the R-state (14). As discussed above, partial ligation within the T-state results in a build up of the higher reactivity T-state conformations with enhanced reactivity for the remaining available open heme sites. In earlier work we provided evidence that the HT population with its loosened \( \alpha_1 \beta_2 \) interface and enhanced heme reactivity has features that give it transition state properties with respect to the T to R transition. Thus, conditions such as partial oxygenation create species that have both enhanced nitrite reductase activity (for either T or R) and the low energetic threshold for T-R interconversion.

Summary—Recent studies on the allosteric mechanism in HbA that utilize sol-gel encapsulation (20, 21, 26, 59) or crystal matrices (60, 67, 68) to modulate various tertiary and quaternary relaxation rates show that there is a range of accessible tertiary conformations within both the T and R family of quaternary structures. Functional and spectroscopic studies are consistent with equilibrium populations of these different functionally distinct conformations being modulated by added heterotropic effectors (54, 64), with the degree and symmetry of partial ligand binding (65, 66, 69), and with the nature of the surrounding solvent/environment (44, 70–72). The present study shows that nitrite reductase activity is also responsive to this tuning of conformational distributions and, thus, provides a biophysical framework for understanding the interplay between hemoglobin enzymatic reactivity and the complex in vivo physiological milieu.

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