Oxygen and CO binding to Triply NO and Asymmetric NO/CO Hemoglobin Hybrids

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ABSTRACT The bimolecular and geminate CO recombination kinetics have been measured for hemoglobin (Hb) with over 90% of the ligand binding sites occupied by NO. Since $Hb(NO)_4$ with inositol hexaphosphate (IHP) at pH below 7 is thought to take on the low affinity (deoxy) conformation, the goal of the experiments was to determine whether the species $^{IHP}Hb(NO)_3(CO)$ also exists in this quaternary structure, which would allow ligand binding studies to tetramers in the deoxy conformation. For samples at pH 6.6 in the presence of IHP, the bimolecular kinetics show only a slow phase with rate 7×10^4 M^{-1} s^{-1}, characteristic of CO binding to deoxy Hb, indicating that the triply NO tetramers are in the deoxy conformation. Unlike $Hb(CO)_4$, the fraction recombination occurring during the geminate phase is low (<1%) in aqueous solutions, suggesting that the $^{IHP}Hb(NO)_3(CO)$ hybrid is also essentially in the deoxy conformation.

By mixing stock solutions of HbCO and HbNO, the initial exchange of dimers produces asymmetric ($\alpha^{NO}\beta^{NO}/\alpha^{CO}\beta^{CO}$) hybrids. At low pH in the presence of IHP, this hybrid also displays a high bimolecular quantum yield and a large fraction of slow (deoxy-like) CO recombination; the slow bimolecular kinetics show components of equal amplitude with rates 7 and 20 $\times 10^4$ M^{-1} s^{-1}, probably reflecting the differences in the α and β chains.

Samples of symmetric hybrids ($\alpha_2^{NO}\beta_2^{CO}$ or $\alpha_2^{CO}\beta_2^{NO}$) showed a lower (R-like) bimolecular yield and less slow phase for the CO bimolecular recombination, relative to the asymmetric hybrid or the triply NO species. The slower (T state) bimolecular rate of 7×10^4 M^{-1} s^{-1} was observed for CO rebinding to a β chain.

While oxygen equilibrium studies with $^{IHP}Hb(NO)_3$ were hampered by a high oxidation rate, it was possible to perform experiments with samples equilibrated with a mixed CO/oxygen atmosphere. Photodissociation of CO allows a temporary exposure of the binding sites to oxygen. The results confirm that $^{IHP}Hb(NO)_3$ has a low oxygen affinity.

INTRODUCTION

Deoxy hemoglobin (Hb) tetramers possess a low oxygen affinity (T state), and upon oxygenation the tetramers switch to a high affinity (R state) structure. Although the R and T affinities are generally associated with the oxy and deoxy forms, the ligation level cannot be used as a definition of these states. It has been shown that deoxy R state tetramers can be produced by photodissociation (Gibson, 1956), although this form relaxes in about 100 μs to the T state conformation (Sawicki and Gibson, 1976). Mutant hemoglobins, such as Hb Chesapeake (Charache et al., 1966) may have a greatly shifted allosteric equilibrium, so that the forms with zero or one ligand may show an appreciable amount of R state (Edelstein, 1975; Shulman et al, 1975). If the mechanism for the heme-heme interaction is lost, the resulting Hb may behave as dimers or isolated chains, which have R-like properties.

At the other extreme, there are cases where the liganded form may adopt the T state conformation at low pH, such as certain fish Hb exhibiting a Root effect (Root, 1931; Brittain, 1987), or Hb Kansas in the presence of the strong effector IHP (Bonaventura and Riggs, 1968). Use of multiple effectors may also shift the allosteric equilibrium so that HbA with three ligands bound remains in the T state (Marden et al., 1988). When constrained by the crystal lattice, Hb has also been reported to show a single low oxygen affinity for all four sites (Mozarelli et al., 1991).

Another example of liganded Hb in the T state is HbNO in the presence of IHP under acid conditions. Upon addition of IHP to HbNO, there is a change in the absorption spectrum (Cassoly, 1974) and a decreased β93 cysteine reactivity (Cassoly, 1975), which were interpreted as a change from the R to T state. This hypothesis was supported by the observation that sickle cell HbS-NO with IHP will participate in polymer formation as does deoxy HbS, whereas no polymer occurs for the oxy form (Briehl and Salhany, 1975). Further characterization of HbNO has been made by IR absorption studies (Maxwell and Caughey, 1976; Friedman et al., 1983), circular dichroism (Perutz et al., 1976), EPR spectra (Henry and Cassoly, 1973; Nagai et al., 1978), and variation of the physical-chemical parameters such as pH, NO saturation level, temperature (Hille et al., 1979; Wajnberg et al., 1992), and conditions of dehydration (Sanches, 1988).

These examples of greatly shifted allosteric equilibrium indicate that a better working definition of the Hb state is required. In the present ligand binding studies we make use of the large differences in the oxygen affinity and the CO association rate to probe the allosteric state of Hb partially saturated with NO.

NO, a biologically important effector (Ignarro, 1989) and participant in redox reactions (Kosaka, 1989), exhibits many intrinsic differences compared to other ligands (CO and O_2) of ferrous Hb. While CO strengthens the bond to the proximal histidine, NO weakens this bond; the specific electronic structure of NO with an unpaired electron involves disruption

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or a weakening of the fifth iron bound with the proximal base (Traylor and Sharma, 1992). It was reported that relative to the ligands CO and O_2, samples with NO will show slightly more T state behavior (Moore and Gibson, 1976). EPR studies of HbNO indicated that disruption of the iron-histidine bond occurs at low pH; addition of IHP enhances the fraction with a rupture of the fifth coordinate bond (Hille et al., 1979). From the point of view of the iron atom, the subunit is liganded, but the proximal histidine no longer feels the pull of the liganded iron atom and adopts a deoxy-like local structure. Without the iron-histidine bond there is apparently a breakdown in the transmission of the signal necessary for the heme-heme interaction.

While the change in ligand affinity between the allosteric forms is mainly due to differences in the association rates for CO, the difference is predominantly in the dissociation rates for NO and oxygen (Szabo, 1978; Sharma and Ranney, 1978). The kinetics of NO dissociation have been measured in the presence of IHP (Moore and Gibson, 1976; Sharma and Ranney, 1978): a rapid phase accounted for 40% of the kinetics, indicating that loss of the first two NO ligands occurs in the T state conformation. This result suggests that $^{IHP}Hb(NO)_3(CO)$ may also be in the T state conformation. We confirm this hypothesis by flash photolysis studies of samples which are 90% NO and the remaining sites as CO bound. The present results for the bimolecular rate and yield indicate that the tetra-liganded species $^{IHP}Hb(NO)_3(CO)$ at pH < 7 is predominantly in the low affinity conformation.

MATERIALS AND METHODS

Purified stripped Hb A was prepared from bank blood and stored in the oxy form in liquid nitrogen. Lyophilized Horse MetMb Type III (batch M-1882; Sigma Chemical Co., St. Louis, MO) was equilibrated under CO and then reduced by a buffered dithionite solution. Protein concentrations were determined by spectrophotometry using extinction coefficients for HbCO of 14.3 mM^{-1} cm^{-1} at 540 nm (Zijlstra et al., 1991) and 15.4 mM^{-1} cm^{-1} at 540 nm for MbCO (Antonini and Brunori, 1971).

Freshly prepared stock of 1 atm NO gas (Air Liquide Co., Paris, France) previously washed with aqueous sodium hydroxide was stored in a sealed 10-ml tube containing 1 ml of concentrated NaOH solution. In all steps, great care was taken to exclude contact with oxygen to avoid the production of nitrite.

CO/NO hybrids

Method 1: $^{IHP}Hb(NO)_3(CO)$

A Hb solution (about 800 μM in heme) at pH 6.6, 50 mM in Bis-Tris buffer, 100 mM NaCl, and 0.5 mM IHP (Sigma) was deoxygenated thoroughly under argon in a tonometer attached to an optical cuvette (0.2 cm). No dithionite was added. Then with a gas-tight syringe flushed with argon, a slight excess of NO gas relative to the heme concentration was introduced in the tonometer. Because of the slow equilibrium of the stirred gas/liquid mixture, the increase in percent NO bound could be followed spectrophotometrically. The induced spectral changes (Salhany et al., 1974; Moore and Gibson, 1976) were examined in a range of wavelength between 500 and 600 nm. The reaction was stopped between 90 and 100% NO bound (after about 10 min) by flushing the tonometer with argon to remove the excess NO gas, and the sample was kept on ice until the measurements. The partially NO Hb stock solution (over 90% NO bound) was then diluted in a buffered solution previously equilibrated with 0.1 or 1 atm CO.

Method 2: $Hb(NO)_3(CO)$

Samples of Hb (without IHP) fully saturated with NO were prepared and then equilibrated with argon to the excess NO. This stock was then diluted in a buffered solution previously equilibrated with CO. The desired fraction of sites with NO bound could be obtained by replacement of NO by CO. For samples equilibrated with 1 atm CO, there is a slow displacement of NO by CO, but at 0.1 atm CO the percent NO remained stable for hours. A small amount of sodium dithionite solution may be used to remove the bound NO more efficiently (Moore and Gibson, 1976).

Method 3: $\alpha^{NO}\beta^{NO}/\alpha^{CO}\beta^{CO}$

Asymmetric hybrid samples were prepared (without IHP) by mixing stock solutions of HbCO and HbNO (without excess NO). The exchange of dimers leads to the formation of (dimer-NO/dimer-CO) asymmetric hybrids (Ackers et al., 1992). Mixtures with a large excess of HbNO were used to lower the relative contribution of $Hb(CO)_4$. IHP was added just before the measurements. Note that if IHP is added before mixing, the exchange of dimers is not as efficient due to the increased stability of the (T state) $^{IHP}Hb(NO)_4$ tetramer.

Method 4: $\alpha_2{}^{NO}\beta_2{}^{CO}$ or $\alpha_2{}^{CO}\beta_2{}^{NO}$

CO bound α_{PMB} and β_{PMB} chains were prepared from normal hemolysate. The regeneration of -SH groups was performed by reaction with β-mercaptoethanol (Bucci and Fronticelli, 1965), and the purity of the preparation was controlled by IEF. The symmetric hybrids were prepared by exposing one type of chain to NO, flushing with argon to remove the excess NO, and adding a slight (10%) excess of the NO-bound chain to a solution containing the other (CO bound) chain.

Photodissociation studies

Flash photolysis measurements were made using 10-ns laser pulses at 532 nm (Quantel); kinetic curves were an average of at least three (32,000 point) traces recorded on a LeCroy 9400 oscilloscope (Marden et al., 1988). Since the CO recombination to the low affinity form of Hb may be slow, a low intensity light source (50-watt quartz halogen lamp filtered at 436 nm) was used to avoid partial photolysis by the detection beam. Care was also taken to use a sufficiently low repetition rate to permit full ligand recombination between photolysis pulses. Samples with a total heme concentration of typically 60–100 μM were prepared in cuvettes of 0.1-, 0.2-, or 0.4-cm optical pathlength.

No signal was observed for 100% NO samples; the geminate rebinding of NO occurs on a picosecond time scale, and the bimolecular yield is very low (0.003; Saffran and Gibson (1977)). On time scales greater than nanoseconds, the kinetic signal for the hybrid CO/NO samples should therefore be essentially due to CO rebinding. For hybrid samples, the fraction CO bound could be controlled by the amplitude of the photodissociation signal.

A large fraction NO bound is necessary to isolate the signal for CO binding to the triply NO species. For samples with 95% of the sites occupied by NO, use of the binomial distribution would imply that 86% of the kinetic signal is due to CO rebinding to triply NO tetramers (assuming no signal for the NO bound sites); a difference in the α and β chains in their affinity for NO would change the distribution and increase the contribution of the triply NO form (to 90% for a large difference).

For $Hb(CO)_4$ samples the fraction dissociated was normalized relative to static difference spectra. In the spectrophotomer or the flash detection system, we observed a static absorbance difference (Hb minus HbCO) of 0.8 OD at 436 nm for 100 μM samples in 1-mm cuvettes. The actual bimolecular signal depends on the concentration of CO bound hemes, the optical pathlength, the fraction dissociated, and the bimolecular yield (50–60% for R state Hb). Typical bimolecular signals for studies of the triply liganded form ranged from 0.024 OD corresponding to 5% dissociation of 60 μM HbCO samples in 1-mm cuvettes, to 0.13 OD for 80 μM ^{IHP}Hb samples with 95% NO bound in 4-mm cuvettes.

Buffer/glycerol mixtures were used for the measurements at low temperature; the kinetics at the lowest temperatures were measured first to minimize oxidation or other changes in the sample. Since the partially NO samples may change more quickly at higher temperatures, we made only one measurement above 0°C per sample by preheating the sample holder and making a single transition from 0°C to the final temperature, passing the cuvette first in a liquid bath for 5 min to facilitate the temperature equilibration. Since the bimolecular yield increases with temperature, the absorption change at the highest temperature was used to normalize the entire data set.

CO/oxygen replacement reaction

The partially NO $^{\mathrm{IHP}}$Hb stock (method 1) was diluted in a solution previously equilibrated with the mixed CO/oxygen atmosphere. Since Hb has a higher affinity for CO relative to oxygen, the Hb is essentially NO/CO bound before photolysis.

RESULTS

Overall the CO recombination kinetics to Hb require three levels of analysis. First there is a major distinction between the nanosecond geminate phase and the millisecond bimolecular phase. There is a further splitting in two for the bimolecular phase, since the R and T bimolecular rates differ by over a factor of 30; for Hb(CO)$_4$, the fraction of slow (T state) bimolecular kinetics increases for higher dissociation levels, lower [CO] (to permit completion of the R → T transition) and other conditions which favor the deoxy conformation (lower pH, strong allosteric effectors, etc). Finally within the T state, the α and β chains may differ in rate.

Bimolecular kinetics

Recombination kinetics after photodissociation of NO/CO Hb hybrids with 95% NO bound are shown in Fig. 1. The reference curves for Hb(CO)$_4$ were measured with a low (5%) photodissociation level; without IHP the kinetics exhibit mainly the rapid (R state) bimolecular phase, corresponding to the rebinding between substates R$_3$ and R$_4$; addition of IHP to Hb(CO)$_4$ increases the fraction slow. Samples of Hb (without IHP) with 95% of the hemes bound with NO, denoted Hb(NO)$_3$(CO) as the predominant preflash species which provides a CO rebinding signal, show mainly R-like CO rebinding kinetics.

As shown in Fig. 1, the CO rebinding kinetics occur (to within a few percent) as a single slow process for $^{\mathrm{IHP}}$Hb(NO)$_3$(CO) samples (prepared by method 1), corresponding to recombination to T state tetramers. The fraction slow was independent of the CO concentration and the photolysis level. The association rate is 7×10^4 M^{-1} s^{-1}, the same as that reported for the symmetric valency hybrid α(met-CN)-β(CO) in the presence of IHP (Cassoly and Gibson, 1972); they observed a rate 2.5 times higher for α(CO)β(met-CN) with IHP.

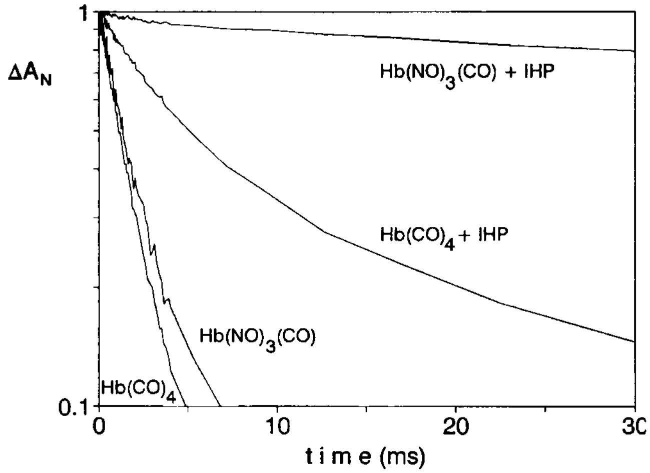

FIGURE 1 Bimolecular recombination kinetics for NO/CO Hb hybrids with 95% of the hemes occupied by NO. Conditions: 50 mM bis-Tris at pH 6.6, 100 mM NaCl, 0.1 mM IHP, equilibration with 0.1 atm CO, 25°C. Since the NO recombination provides no signal on this time scale, the observed kinetics are due to CO recombination. The reference curves for Hb(CO)$_4$ samples, at low (5%) dissociation, represent CO recombination to triply CO tetramers, and show mainly the fast (R-like) component. The partially NO samples prepared with IHP (method 1) show only the slow phase (to within a few percent), characteristic of rebinding to deoxy (T state) Hb. The absorption changes ranged from 0.024 OD (for HbCO, 60 μM on a heme basis, 1-mm cuvette) to 0.13 OD for Hb(NO)$_3$(CO) with IHP, 80 μM, 4-mm cuvette. All curves were normalized (at 4 μs), and are displayed on a log-linear plot.

Asymmetric NO/CO hybrids

By mixing solutions of HbCO and HbNO (method 3), the exchange of dimers (Ackers et al., 1992) forms tetrameric species with 0, 2, and 4 molecules of CO bound. This reaction occurs within seconds for R state tetramers (without IHP). Since we add a large excess of HbNO, the dominant form showing CO kinetics should be the asymmetric hybrid ($\alpha^{NO}\beta^{NO}/\alpha^{CO}\beta^{CO}$). The bimolecular kinetics for these samples without IHP showed mainly the rapid R-like bimolecular phase (data not shown). Upon addition of IHP, the amplitude of the bimolecular signal nearly doubled and we observed over 90% slow (T state) recombination; the fraction slow was independent of the fraction photodissociated. The asymmetric hybrids show two slow phases of equal amplitude (Fig. 2), as expected if both α and β chains participate in CO rebinding; the rates are 7 and 20×10^4 M^{-1} s^{-1}.

Symmetric hybrids

Unlike the triply NO and the asymmetric hybrid samples, the symmetric hybrids ($\alpha_2^{NO}\beta_2^{CO}$ or $\alpha_2^{CO}\beta_2^{NO}$) did not show a large increase in the amplitude of the bimolecular phase upon addition of IHP. They showed less slow phase for the bimolecular kinetics, and the fraction slow was dependent on the fraction of hemes photodissociated, although only weakly for CO rebinding to a β chain. At maximum dissociation, the samples show about 50% slow phase; the slow rates were 20 (for CO rebinding to an α chain) and 7×10^4 M^{-1} s^{-1}, the same rates as for the valency hybrids (Cassoly and Gibson, 1972). The difference in rates can be seen in Fig. 2; note that the kinetics were normalized at 4 ms to better compare the slow (T-like) kinetics. This normalization rep-

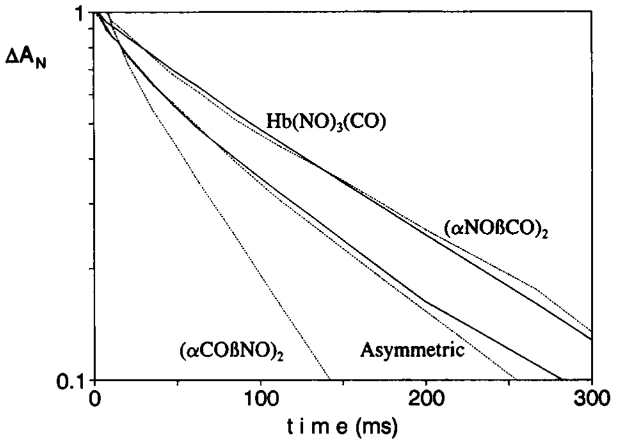

FIGURE 2 Bimolecular kinetics for NO/CO hybrids (with IHP) with two (*dotted lines*) or three (*solid lines*) NO molecules/tetramer; conditions as for Fig. 1. The curves were normalized at 4 ms to better compare the rate of the slow (T-like) bimolecular phase. The fastest rate was observed for the symmetric hybrid $^{IHP}\alpha^{CO}\beta^{NO}$. The slowest rate was for the symmetric hybrid $^{IHP}\alpha^{NO}\beta^{CO}$ and ^{IHP}Hb(NO)$_3$(CO) (method 1, same curve as in Fig. 1). The asymmetric hybrid $^{IHP}(\alpha^{NO}\beta^{NO}/\alpha^{CO}\beta^{CO})$, a mixture of 20 μM HbCO with 120 μM HbNO, shows an equal mixture of the two rates. For samples of triply NO Hb (prepared initially without IHP by method 2, 95% NO bound), the kinetics just (20 s) after addition of IHP also show a mixture of the two rates. The triply NO, and the asymmetric hybrid samples showed predominantly the slow (T-like) CO recombination (see Table 1).

resented only a few percent correction for the triply NO and asymmetric hybrid, but was about a factor of 2 for the symmetric hybrids.

These results indicate that the triply NO samples involve CO rebinding to β chains, which would imply that nearly all the α chains are NO-bound. This is compatible with EPR results indicating that the α chains have a higher affinity for NO (relative to the β chains) in these conditions of pH 6.6, with IHP (Henry and Cassoly, 1973; Hille et al., 1979). It was reported that without IHP the α and β chains have a similar affinity for NO, and that the IHP-induced difference requires several minutes (Hille et al., 1979). To test for such an effect with Hb(NO)$_3$(CO), we first measured the kinetics for samples without IHP (prepared by method 2). We then made kinetic measurements within 20 s after addition of IHP (Fig. 2), which provokes an increase in the amplitude of the bimolecular phase, and shifts the allosteric equilibrium resulting in over 90% slow (T-like) bimolecular phase; the kinetics were now a nearly equal mixture of the two rates observed for the symmetric hybrids, suggesting that both α and β chains participate in the CO rebinding, as for the asymmetric hybrid.

From the bimolecular kinetics alone, it is difficult to determine if the Hb sample is in the T conformation (before photodissociation) or whether there is an R to T transition after the photolysis pulse. The fact that the kinetics are T-like for samples equilibrated under 1 atm CO indicates only that such a transition is much faster than the CO rebinding to the R state. Another test is the direct observation of the allosteric transition at an isosbestic point (near 426 nm) for CO dissociation (Sawicki and Gibson, 1976; Marden et al., 1986). We found no evidence for an R to T transition for the triply NO samples at this wavelength. The increase in the amplitude of the bimolecular phase upon addition of IHP does suggest a change in the preflash conformation.

Geminate kinetics

Measurement of the nanosecond geminate phase provides a better indication of the quaternary structure of Hb before photodissociation, since the protein has less time to relax to a new conformation. The fraction that escapes and rebinds via the millisecond bimolecular phase depends on the competition between direct rebinding and migration away from the iron atom; the fraction bimolecular decreases at low temperature and high viscosity (Beece et al., 1980). Since the CO (bimolecular) rebinding rate is over 30 times slower for the T state relative to the R state, one would expect a larger bimolecular yield for a T state tetramer, assuming similar escape rates for the two conformations. This analysis appears to be valid when comparing HbCO (about 50% bimolecular yield at 25°C) to MbCO (98% bimolecular yield) which has a slower CO association rate. As mentioned above, the rapid (picosecond) rebinding explains the very low bimolecular yield after photodissociation of HbNO. Previous results using techniques of double flash and flow-flash (Marden et al., 1987), or strong effectors (Marden et al., 1988), or (α-iron)-(β-cobalt) symmetric hybrids in combination with strong effectors (Murray et al., 1988) have indicated a greatly diminished fraction of geminate phase for conditions favoring the deoxy conformation.

As seen in Fig. 3, the triply NO form shows a much larger bimolecular yield; high viscosity conditions were used (80% glycerol at 256 K) to increase the fraction geminate. The final

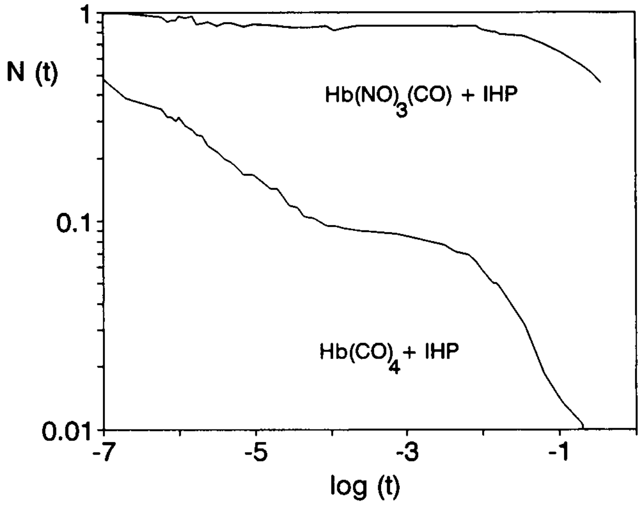

FIGURE 3 Recombination kinetics (log-log plot) after photodissociation of ^{IHP}Hb(CO)$_4$ and ^{IHP}Hb(NO)$_3$(CO) at 256 K in 80% (w/w) glycerol/buffer (50 mM Bis-Tris at pH 6.6), 100 mM NaCl, 0.1 mM IHP, equilibration with 0.1 atm CO. *N*(*t*) is the fraction of deoxy sites, normalized to the signal at high temperature. A much higher bimolecular yield, and a slower bimolecular rebinding can be seen for the triply NO sample (over 90% of the hemes with NO).

(bimolecular) phase is much slower for the triply NO sample relative to HbCO, corresponding to the difference between the T and R bimolecular rates.

The fraction bimolecular versus temperature for samples in 80% glycerol (w/w) are shown in Fig. 4. In general, the slower the bimolecular rebinding rate, the larger the bimolecular yield (Table 1).

CO/oxygen replacement reaction

Similar studies using oxygen instead of CO were difficult due to an elevated oxidation rate. This would indicate that the T state tetramer oxidizes much more quickly than the fully oxy (R state) form, consistent with studies that showed an increased rate of autoxidation for samples at reduced oxygen partial pressure or in the presence of IHP.

It was possible to perform flash photolysis studies with samples equilibrated with a mixed O_2/CO atmosphere, while maintaining the sample with 90% NO bound. This method makes use of the fact that, while CO has a higher affinity than O_2 (by a factor of 200), the overall association (on) rate is higher for oxygen (Antonini and Brunori, 1971).

After photodissociation of CO, one observes a rapid oxygen binding phase followed by the replacement of O_2 by CO (Fig. 5). This technique provides a method of measuring the association (k') and dissociation (k) rates of O_2 (Antonini and Brunori, 1971). Oxidation of the hemes is decreased, because the binding sites are exposed to oxygen for less than 1 s. The standard method involves a series of measurements of different ratios of oxygen and CO (all in saturating conditions). In this study we use nonsaturating levels of oxygen to directly observe the fractional saturation versus PO_2.

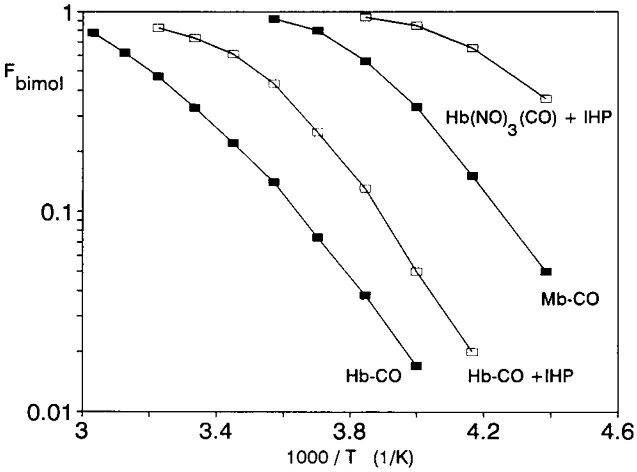

FIGURE 4 The fraction bimolecular phase after photodissociation of the systems indicated. Conditions: 80% (w/w) glycerol/buffer (50 mM Bis-Tris buffer at pH 6.6), 100 mM NaCl, 0.1 mM IHP, 100 μM on a heme basis, equilibration with 0.1 atm CO. The fraction bimolecular is correlated with the bimolecular on-rate (Table 1): the slowest recombination rate is for the triply NO form, while the fastest is observed with HbCO in the absence of effectors. The amplitudes versus temperature were normalized to the maximum absorbance change, which occurs at the highest temperature.

Consider as an example the reference curve (*lowest trace*) in Fig. 5, for a Hb sample (without NO, without IHP) equilibrated with 0.5 atm CO and 0.5 atm oxygen. After photodissociation of CO, oxygen binds in less than 1 ms. CO binding would require over 10 ms even for R state Hb; for T state Hb, the ratio of the on-rates (k'/l') is nearly a factor of 100. From reference static spectra of Hb, HbCO, and oxyHb, the level for pure oxyHb (indicated in Fig. 5) would correspond to 91% of the signal for detection at 436 nm.

For nonsaturating conditions, the amplitude of the (rapid) oxygen binding phase depends on the oxygen partial pressure; it corresponds to the equilibrium oxygen saturation of the exposed binding sites. For $^{IHP}Hb(NO)_3$ at 25°C, even at the maximum oxygen partial pressure used (0.6 atm), the oxygen binding represented only 55% of the total signal with detection at 436 nm (*middle curve* in Fig. 5). From the amplitude of the rapid phase versus PO_2, one can estimate the P_{50} for oxygen binding. A value of P_{50} = 300 mmHg was obtained for measurements of $^{IHP}Hb(NO)_3$ at 25°C; this is a lower oxygen affinity than for ^{IHP}Hb (P_{50} = 80, K_T = 150 mmHg) or Hb crystals ($P_{50} = K_T$ = 270 mmHg, Mozarelli et al. (1991)), and the lowest affinity previously observed for multiple effectors IHP + L345 (K_T = 240 mmHg) (Lalezari et al., 1990). This confirms that the $Hb(NO)_3$ tetramer is in the low oxygen affinity state, although it is clear that systems with effectors represent a T′-state with a lower oxygen affinity than Hb without effectors.

The kinetic data were simulated using a two-state framework which considered a triply liganded form $Hb(NO)_3$ with allosteric equilibrium L3 = T3/R3 and two tetra-liganded forms $Hb(NO)_3(CO)$ and $Hb(NO)_3O_2$ both with L4. The R and T state on-rates for CO were not free parameters, since they can be directly measured (in the absence of oxygen). Similarly the oxygen on-rates are constrained to scale with the oxygen concentration. Simulations were not sensitive to the CO off-rates; they were set to a low value (<1/s), which effectively treats the CO rebinding as irreversible. The oxygen off-rate was varied as well as L3 and L4; good fits could be obtained by setting both L3 and L4 to 1000 (nearly pure T state). For $^{IHP}Hb(NO)_3$ at 25°C, we obtained $^{T}k' = 3 \times 10^6\ M^{-1}\ s^{-1}$ and ^{T}k = 1800/s (as opposed to ^{R}k = 15/s for dissociation of oxygen from oxyHb); at 4°C, the observed oxygen affinity was 60 mmHg and ^{T}k = 50/s. These values confirm than the difference in oxygen affinity for the allosteric states is due mainly to a change in the off-rate k.

A stopped flow method has also been proposed which reduces the oxygen exposure time to about 5 s before observation (Astatke et al., 1992). Similarly the mixed CO/oxygen kinetic method reduces the exposure time to less than 1 s. Since HbCO is one of the more stable forms, this flash method allows measurements under extreme conditions of temperature or pH. In addition, since no mixing is required, low temperature and high viscosity solvents could be used. However, the stopped flow method which starts from deoxy Hb would be preferable in some cases to avoid a dimer contribution.

TABLE 1 Kinetic parameters for CO rebinding to Hb in aqueous solutions (50 mM Bis-Tris at pH 6.6), or in 80% glycerol-buffer (w/w) solutions

	% Bimolecular at			CO on-rate (l') at 298 K (aqueous)		
	298 K, aqueous	300 K	240 K*	R	T	(%slow)
		80% glycerol				
		%		$10^6\ M^{-1}\ s^{-1}$		
$Hb(CO)_4$	55	33	0.8	6.0	0.30	(Variable)
$^{IHP}Hb(CO)_4$	64	73	2	1.5	0.17	(Variable)
MbCO	98	79	15	0.45		
$^{IHP}Hb(NO)_3(CO)$	99	99	66		0.07	(95%)
$^{IHP}\alpha_2(NO)\beta_2(CO)$	65			3.5	0.07	(50–60%)
$^{IHP}\alpha_2(CO)\beta_2(NO)$	65			3.5	0.2	(25–40%)
$^{IHP}(\alpha^{NO}\beta^{NO}/\alpha^{CO}\beta^{CO})$	95				0.2 and 0.07	(95%)

* Temperature.

The percent bimolecular was calculated from the observed absorption change relative to the maximum absorption change observed at high temperature. The bimolecular phase can be further separated into rapid and slow phases; the last column shows the percent slow; this percent varies with the fraction dissociated for $Hb(CO)_4$ samples, and varied less for the symmetric hybrids. The asymmetric hybrid (last row) shows two slow bimolecular rates, correlated with the rates for the symmetric hybrids.

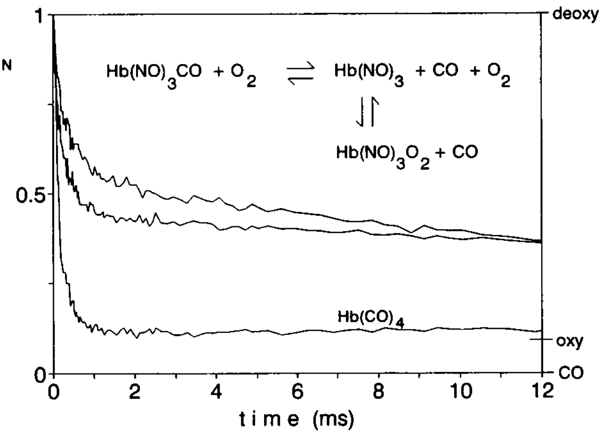

FIGURE 5 Kinetics after photolysis of $^{IHP}Hb(NO)_3(CO)$ samples equilibrated with a mixed CO/O_2 atmosphere, at 25°C, pH 6.6, 0.5 mM IHP, 80 μM on a heme basis. Top curve, 0.38 atm oxygen; middle curve 0.6 atm oxygen. After photolysis of CO, the rapid oxygen association is observed, followed by a (100 ms) replacement reaction to the more stable CO form. From the observed partial saturation level, the oxygen affinity can be estimated. Absorbance changes were typically 0.12 for measurements in 4-mm cuvettes. The reference curve (*bottom trace*, 0.5 atm oxygen) for $^{IHP}Hb(CO)_4$ represents the high oxygen affinity state. Full equilibration to the oxy form corresponds to about 91% of the signal amplitude at this detection wavelength (436 nm).

DISCUSSION

We have examined ligand binding to Hb samples with 95% of the hemes bound to NO. For samples at pH 6.6 in the presence of IHP, the CO and oxygen binding show predominantly T state behavior. The CO bimolecular kinetics display over 95% slow phase, characteristic of CO binding to deoxy Hb. The CO bimolecular yield is greatly increased relative to $Hb(CO)_4$ samples, consistent with a lower rebinding rate. These results indicate that the triply NO species is in the T conformation, with or without the fourth ligand.

Distribution of substates

For hybrid systems, there is always a question as to whether the distribution of ligands is random. For the triply NO samples (above 90% NO bound), we can rule out a contribution of tetramers having three or more CO molecules, since their recombination kinetics at decreased laser energy are known to show rapid (R state) bimolecular kinetics. Since we observe less than 5% rapid kinetics, independent of the photolysis level, the signal is mainly due to CO rebinding to triply NO (IHP bound) tetramers. The same argument holds for the asymmetric hybrids; we did not observe an R state contribution characteristic of $Hb(CO)_4$, indicating that the dimer exchange took place as expected.

Chain type

IR and EPR studies indicate that the α chains possess a higher affinity for NO than the β chains (Henry and Cassoly, 1973; Hille et al., 1979). Addition of IHP enhances the chain difference with a new equilibrium distribution occurring within several minutes (Hille et al., 1979). A higher affinity of the α chains for NO, in the presence of IHP, is consistent with the present results for $^{IHP}Hb(NO)_3(CO)$ which show the same CO rebinding rate as for symmetric hybrid ($\alpha_2^{NO}\beta_2^{CO}$).

The present results with the asymmetric NO/CO hybrids also support this conclusion. The slow (T-like) bimolecular kinetics in this case require a decomposition as a sum of two exponentials of equal amplitude which differ by a factor of 2.5, the slower rate being the same as that for the $^{IHP}Hb(NO)_3(CO)$ samples. Similar biphasic kinetics were obtained by flashing the triply NO samples just after addition of IHP (Fig. 2); this confirms that the chain difference is small in the absence of IHP, and also shows that the large fraction of T-like kinetics is obtained whether the CO bound chain is α or β. Note that, while the effector IHP changes the ligand affinity, the rates and affinities do not depend on the actual hybrid used (valency versus NO/CO, symmetric versus asymmetric). The difference for the α and β chains is apparently not dependent on IHP; a similar difference in the T state on-rates for oxygen binding to Hb (without IHP) was previously reported (Sawicki and Gibson, 1977); they arbitrarily assigned the rapid component to the β chains.

Asymmetric hybrids

It has previously been shown that biliganded symmetric valency hybrids (with IHP) bind CO with the T state rate (Cassoly and Gibson, 1972). Thus in the presence of IHP, both the symmetric and asymmetric biliganded hybrids show T-like properties for addition of the third ligand. However, we observe a difference for the two types of hybrids; surprisingly, the asymmetric (dimer-CO/dimer-NO) hybrids showed only T state behavior after photolysis, which is not the case for the symmetric hybrids. The asymmetric hybrid (in the presence of IHP at pH 6.6) shows little geminate recombination, indicating that even the tetra-liganded Hb hybrid is in the T state. Preliminary results with asymmetric valency hybrids also show this enhancement of T state properties, relative to symmetric valency hybrids. This is a clear departure from the two-state model where the allosteric equilibrium depends on the number of ligands bound, but not their distribution. Based on dimer-tetramer equilibria, Ackers et al. (1992) have previously concluded that the biliganded asymmetric hybrids display more T state behavior that the biliganded symmetric hybrids.

In another study, Cassoly (1978) mixed equal amounts of oxyHb and HbNO and then added dithionite to remove the oxygen; these asymmetric hybrids showed CO binding kinetics (by stopped flow) identical to those for deoxy (T state) Hb. This would imply T-like properties for the asymmetric hybrid, assuming no change in the distribution of substates (due to dimer or ligand exchange). However, such a redistribution has been reported (Perrella et al., 1990): after mixing HbNO and metHb, followed by reduction by dithionite, the intermediate products were then trapped by cryo-techniques; the loss of the asymmetric form occurred within minutes accompanied by an increase in the singly and triply NO forms, suggesting a mechanism due to ligand (rather than dimer) exchange. This is expected if the biliganded asymmetric hybrid is in the T-conformation, since the NO dissociation requires only a few minutes.

Geminate yield

Without using hybrids it is difficult to obtain a high percentage of liganded subunits in the T state. The allosteric nature of the ligand binding tends to cause an all (fully oxygenated) or nothing (deoxy) reaction, which greatly decreases the population of the intermediates, relative to a random binomial distribution. Double flash experiments can provide a better distribution (Marden et al., 1987); however, the starting point is the fully liganded form and thus has some contribution of (R-like) dimers. Lower geminate yields (estimated as lower than 3%) were observed for flow-flash experiments, where the starting material is deoxy tetramers.

For aqueous solutions of $^{IHP}Hb(NO)_3(CO)$, the CO geminate yield was too small to detect. We can only make the same conclusion as Murray et al. (1988) and place the yield as less than 1%; note that in our case, we are probably detecting CO rebinding to a β-chain, while their α(Fe)/β(Co) hybrids (with IHP and bezafibrate) involve CO binding to α-chains. For both cases there is a large decrease of the geminate yield for T state Hb relative to the R state, corresponding roughly to the decrease in bimolecular association rate (Table 1).

Relative to HbCO without effectors, HbCO with IHP has a lower R state (and T state) association rate (Gray and Gibson, 1971); the higher bimolecular yield for HbCO samples with IHP can be explained by the lower R′-state rate rather than being partially T state.

The present results are quite in contrast to those involving symmetric hybrids in the presence of IHP (Bandyopadhyay et al., 1992). Using double mixing techniques, symmetric valency hybrids were first reduced using dithionite and then mixed with a solution equilibrated with CO. They observed slow (T state) bimolecular kinetics for CO binding, yet the flow-flash experiments for the same hybrid did not show a decrease in the geminate yield. Since the (T state) bimolecular kinetics were for the (just reduced) deoxy chains, while the geminate phase necessarily refers to liganded subunits, the authors concluded that the same tetramer might posses two "r" liganded subunits yet the other two deoxy chains are in a "t" conformation.

The nonexponential geminate phase provides information on the energetic barriers between the binding site and the surface of the protein (Austin et al., 1975). The end of the geminate phase can be interpreted as the time necessary for the ligand to migrate to the solvent, if the migration occurs in one dimension; this escape time (Marden, 1982; Marden et al., 1986) is observed to increase with the solvent viscosity. The difference in the fraction geminate between the R and T states is so large that it is difficult to find suitable conditions to compare the escape times. Also, at low viscosity a small (5%) R state contribution in the preflash conditions could mask the T state geminate phase. In order to have an appreciable amplitude for the T state samples, we used conditions of high viscosity (80–90% glycerol and temperatures below 260 K). From the data where a substantial fraction geminate occurs, the escape time for $^{IHP}Hb(NO)_3$ (T state) is only slightly shorter (within a factor of 2) than that for the HbCO (R state) samples. This would suggest that the pathway for escape is not significantly different between the two conformations.

CONCLUSIONS

Triply-NO Hb with IHP is stabilized in the T state conformation without or with CO or O_2 as the fourth ligand. This hybrid species allows ligand binding studies to Hb in the T state. The criteria for the allosteric equilibrium does not seem to be whether the subunit is actually liganded, but whether the other subunits are informed; rupture of the iron histidine bond apparently results in the loss of this transmission of information.

We observe two T state rates for samples where both α and β chains bind CO (asymmetric hybrids and triply NO samples just after addition of IHP). However, triply NO samples prepared initially with IHP show only the slower rate, characteristic of CO rebinding to a β chain.

Based on the amplitude and rate of the bimolecular kinetics, the following species are in the T state in the presence of IHP at pH < 7: $Hb(NO)_3$, $Hb(NO)_3(CO)$, $Hb(NO)_3(O_2)$, where oxygen or CO may be bound to an α or β chain, and the asymmetric hybrid ($\alpha^{NO}\beta^{NO}/\alpha^{CO}\beta^{CO}$) and its photoproducts.

Two-state model: The present work shows evidence for T state species with four ligands bound. This offers no conflict with the two-state model, but shows the switchover point to be widely variable. The small differences (factor of 2 to 3) between the α and β chains are technically not allowed by a simple two-state model, but they represent minor perturbations compared to the factor of 100 shift in allosteric equilibrium for each ligand. The use of strong effectors which change K_T by over an order of magnitude clearly indicate that there are not unique R and T states, but rather a family of R and T states. For given conditions, one can still employ a two-state framework. The results for the asymmetric hybrid are perhaps the greatest departure from a two-state model, implying that in addition to the number of ligands bound, the actual distribution of sites is critical.

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