A Comparison of the Geminate Recombination Kinetics of Several Monomeric Heme Proteins*

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The geminate rate constants for CO, O₂, NO, methyl, ethyl, n-propyl, and n-butyl isocyanide rebinding to soybean leghemoglobin and monomeric component II of Glycera dibranchiata hemoglobin were measured at pH 7, 20 °C using a dye laser with a 30-ns square-wave pulse. The results were compared to the corresponding parameters for sperm whale myoglobin and the isolated α and β subunits of human hemoglobin (Olson, J. S., Rohlfst, R. J., and Gibson, Q. H. (1987) J. Biol. Chem., 262, 12930-12938). The rate-limiting step for O₂, NO, and isonitrile binding to all five proteins is ligand migration up to the initial geminate state, and the rate of this process determines the overall bimolecular association rate constant for these ligands. In contrast, iron-ligand bond formation limits the overall bimolecular rate for CO binding. The distal pockets in leghemoglobin and in Glycera HbII are ~10 times more accessible kinetically to diatomic ligands than that in sperm whale myoglobin. This difference accounts for the much larger association rate constants (1-2 x 10⁶ M⁻¹ s⁻¹) that are observed for O₂ and NO binding to leghemoglobin and Glycera HbII. The rates of isonitrile migration through leghemoglobin are also very large and indicate a very fluid or open distal structure near the sixth coordination position. In contrast, there is a marked decrease in the rate of migration up to and away from the sixth coordination position in Glycera HbII with increasing ligand size.

These results were also used to interpret previously published rate constants and quantum yields for the high (R) and low (T) affinity states of human hemoglobin. In contrast to the differences between the monomeric proteins, the differences between the CO-, O₂-, and NO-binding parameters for R and T state hemoglobin appear to be due to a decrease in the geminate reactivity of the heme iron atom, with little or no change in the accessibility of the distal pocket.

Soybean leghemoglobin and monomeric component II from the coelomic red cells of Glycera dibranchiata are among the best characterized non-mammalian hemoglobins. X-ray crystallographic structures of both proteins have been published (Ollis et al., 1983; Padlan and Love, 1974), and a variety of biophysical techniques have been used to examine their functional and spectral properties (for reviews see Appleby, 1984 and Carson et al., 1986). One key difference between leghemoglobin and sperm whale myoglobin is the flexibility of His E7. A hydrogen bond is formed between bound oxygen and this residue in both proteins. However, in sperm whale myoglobin the distal histidine appears to be rigidly fixed above the sixth coordination position and sterically hinders the binding of ligands containing more than two atoms. In contrast, the distal histidine in leghemoglobin appears to be mobile, and the active site of this protein can be considered open or flexible since most of the straight chain alkyl isocyanides are bound extremely tightly, with little or no steric hindrance (Mims et al., 1983; Appleby, 1984). In Glycera HbII,1 the E7 residue is leucine; no hydrogen bonding to bound oxygen occurs; and as a result, the O₂ affinity of this protein is unusually low (Parkhurst et al., 1980).

Both leghemoglobin and Glycera HbII exhibit extremely large association rate constants for CO, O₂, and NO binding: 1.3-2.7 x 10⁷, 1.2-1.9 x 10⁶, and 1.2-1.5 x 10⁶ M⁻¹ s⁻¹, respectively (see Table II; Mims et al., 1983; Stetzkowski et al., 1985; Parkhurst et al., 1980). These overall rate parameters are 50- to 10-fold greater than the corresponding values for sperm whale myoglobin and roughly equal to the rate constants for gas binding to chelated protoporphyrine and unhindered "picket fence" model compounds in organic solvents (Collman et al., 1983). This increase in reactivity could be due either to favorable proximal effects on the heme iron atom, favorable distal effects which allow ligand molecules to enter the active site more rapidly, or a combination of these effects. The relative importance of these factors can be determined experimentally by measuring geminate recombination time courses using laser photolysis techniques.

The three-step mechanism first proposed by Frauenfelder and co-workers (Austin et al., 1975; Stetzkowski et al., 1985; Ansari et al., 1986) is sufficient to describe ligand rebinding to most monomeric heme proteins during and after flash photolysis at room temperature:

\[ P + X \overset{k_x}{\rightleftharpoons} C \overset{k_b}{\rightarrow} B \overset{k_c}{\rightleftharpoons} A \text{ or } PX \]

where A or PX is the final (equilibrium) bound state, C and B are transient or geminate states, and \( P + X \) represents a

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1 The abbreviation Glycera HbII or Gly is used to indicate the monomeric component II from the red cells of Glycera dibranchiata. Lb is used to denote soybean leghemoglobin.
state where ligand is in the solvent phase (for a more detailed description of this mechanism see Olson et al., 1987; Gibson et al., 1986; or Ansari et al., 1986). Assignment of values to the geminate recombination parameters, \(k_3\) through \(k_6\), allows a quantitative assessment of the rate-limiting steps in the overall association and dissociation reactions. Another key parameter is the overall quantum yield which, in terms of Equation 1, is given by:

\[
Q = \frac{k_3k_5}{k_1k_4 + k_2k_5 + k_6k_4}
\]

(2)

\(Q\) is the probability of a ligand escaping to the solvent phase from the first geminate state and can be measured by conventional photolysis techniques (Gibson et al., 1986). This allows an independent assessment of the rate-limiting step in ligand binding (see “Discussion” and Olson et al., 1987).

Although CO and O\(_2\) rebinding to soybean leghemoglobin has been examined at low temperatures in 75% glycerol (Stetzkowski, 1985), a complete set of parameters for rebinding at room temperature and ordinary aqueous buffers has not been reported. Little or no data for geminate ligand recombination to Glycera HbII has been published. Consequently, we carried out a complete set of nanosecond laser photolysis experiments with the CO, O\(_2\), NO, methyl, ethyl, n-propyl, and n-butyl isocyanide complexes of soybean leghemoglobin and Glycera HbII and, when possible, measured independent values of the overall quantum yields. The conditions and analyses were identical to those used previously for sperm whale myoglobin (Gibson et al., 1986) and the subunits of human hemoglobin (Olson et al., 1987). The results have allowed us to define mechanistically, in terms of Equation 1, why leghemoglobin and Glycera HbII exhibit larger association rate constants than mammalian myoglobins and hemoglobins.

**MATERIALS AND METHODS**

Marine bloodworms, *C. dibranchnia*, were purchased from Woods Hole Marine Biology Laboratory. The monomeric component HbII was isolated by the method of Parkhurst et al. (1980, see also Mims et al., 1983). Purified soybean leghemoglobin was a gift from Dr. C. A. Appleby (Appleby, 1984). The results listed for sperm whale myoglobin (Gibson et al., 1986) and the complex of soybean leg

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n\text{-propyl,  and  n-butyl  isocyanide  complexes  of  soybean leg-}
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**RESULTS**

**Assignment of Geminate Recombination Rate Parameters—** A typical nanosecond photolysis experiment is shown in Fig. 1. Oxygenated leghemoglobin was photolysed with a 30-ns pulse which had been attenuated to one-eighth of the maximum laser light intensity using neutral density filters. The **solid squares** represent the observed absorbance trace, and as shown, very little geminate recombination occurs on the nanosecond time scale after the excitation pulse. The information content of these data resides primarily in the rate and extent of photolysis during excitation and the dependence of these phenomena on laser light intensity. This is shown in Fig. 2 where a complete analysis of the geminate recombination kinetics of oxyhemoglobin is presented.

A detailed description of our data analysis has been presented in two previous publications (Gibson et al., 1986; Olson et al., 1987). As shown in Fig. 2A, sets of three to five absorbance traces were recorded during and after the laser flash and as a function of the relative excitation light intensity. These data were fitted to Equation 1 using a nonlinear, iterative least square procedure which assigns optimum values to rate constants \(k_3\) through \(k_6\). The value of \(k_5\) was determined for each set of experiments by using CO-myoglobin as a standard with \(Q = 1.0\). The ratio \(k_2/k_3\) was determined from the absorbance of MbCO and that of the leghemoglobin or Glycera HbII complex at 77 am, the wavelength of emission of the laser dye.

Since little or no geminate recombination occurs after the laser pulse for oxyhemoglobin, these data were fitted to a two-step scheme with only a single intermediate state, B in Equation 1. The **solid lines** in Fig. 2, panel A represent fitted time courses with \(k_3 = 1260 \mu s^{-1}\) and \(k_5 = 139 \mu s^{-1}\). These parameters define an overall quantum yield of 0.10 which agrees well with that observed by conventional photolysis techniques (last two columns, Table I). Panels B–D in Fig. 2 demonstrate graphically the reliability of the fitted parameters. If \(k_3\) is fixed at 139 \(\mu\)s\(^{-1}\), then \(k_5\) is determined by the extent of photolysis during the excitation pulse (Fig. 2B). Similarly, if \(k_5\) is fixed at 1260 \(\mu\)s\(^{-1}\), \(k_5\) is also well defined. As shown in Fig. 2C, the absolute value of \(k_5\) affects not only the extent of photolysis but also the shape of the time course. If \(k_5\) becomes very large relative to \(k_3\), complete photolysis occurs every time a quantum is absorbed, and the time course becomes identical to that observed for the CO-myoglobin standard. If \(k_5\) becomes very small, the ligand cannot escape to the solvent phase, and complete geminate recombination occurs from state B. In this case, the absorbance trace will follow the excitation pulse and exhibit a “square-wave” shape (bottom curve, Fig. 2C).

In Fig. 2D, the ratio \(k_3/k_5\) was fixed (\(Q = k_3/(k_2 + k_5) = 0.10\)), and the absolute values of \(k_2\) and \(k_5\) were varied. Lowering these parameters significantly below the fitted values changes markedly the shape of the observed time course.
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More net photolysis occurs early during the excitation pulse since $k_2$ is reduced relative to $k_3 I(t)$. In addition, more geminate recombination from state B is observed as the laser flash decays since less escape to the solvent phase occurs due to the decrease in $k_3$. The net result is a square-wave pattern for the absorbance trace which is clearly different from the experimental data ($k_2/4$ curve, Fig. 2D). Increasing both $k_2$ and $k_3$ produces noticeable but less dramatic changes in the absorbance time courses ($k_3 \times 4$ curve, Fig. 2D). In this case, less photolysis occurs at the beginning of the excitation pulse since $k_2$ is larger. In addition, most of the photolyzed ligands have escaped to the solvent phase so that no geminate recombination is observed after the laser pulse ends.

The situation with high values of $k_3$ and $k_3$ points out the limitation of our method. In all cases, the ratio of $k_2/k_3$ can be defined relative to that of MbCO by the extent of photolysis observed at a given laser light intensity. The shape of the absorbance time courses determines the absolute values of these parameters, particularly the value of $k_3$ as shown in Fig. 2C. However, if $k_3 \geq 300 \mu s^{-1}$ or $k_3/(k_2 + k_3) = 1.0$, the individual values of $k_2$ and $k_3$ become undefined, and only the ratio $k_2/k_3$ can be determined with certainty ($k_2 \times 4$ curve, Fig. 2D).

This situation applies at room temperature to most of the CO hemoglobin and myoglobin derivatives that we have examined (Tables I and II).

$O_2$ and NO Geminate Recombination Kinetics—Time courses for $O_2$, NO, methyl, ethyl, n-propyl, and n-butyl isocyanide rebinding to leghemoglobin and Glycera HbII are shown in Figs. 3 and 4. Traces for the CO derivatives of these proteins are virtually indistinguishable from those of the whale MbCO control since the overall quantum yields of these hemoglobins are ~0.9.

The overall photosensitivities of the $O_2$ and NO derivatives of leghemoglobin are fairly similar to those of the corresponding complexes of Glycera HbII. The time courses for both proteins can be fitted to a simple two-step, consecutive reaction scheme (i.e. $A \rightleftharpoons B \rightleftharpoons P + X$); however, the detailed features of the recombination time courses are quite different (Fig. 3). For the nitrosyl leghemoglobin derivative, there is very little state B formation at the beginning of the excitation pulse since the geminate recombination rate is much greater than the rate of photolysis (i.e. $k_3 = 16.2 \, \text{ns}^{-1}$ whereas $k_3 = 1.3 \, \text{ns}^{-1}$, Table I, Fig. 3A). Under these conditions, states A and B in Equation 1 are in equilibrium,
and the fraction of unliganded leghemoglobin is $k_{3}J(t)/(k_{3}J(t) + k_{2})$, which for the LbNO sample in Fig. 3A is roughly 0.1. The further increase in the fraction of deoxy-leghemoglobin during the excitation pulse is due to ligand migration from the first geminate state to the solvent phase. Since states A and B are in rapid equilibrium, the rate of ligand escape is given by $k_{3}h_{2}J(t)/(h_{2}J(t) + k_{2})$ which determines the extent of any further photolysis during the laser flash. For leghemoglobin, $k_{2}$ is fairly large for both O$_{2}$ and NO, $\sim$140 $\mu$s$^{-1}$ (Table I), and this accounts for the upward rise (“ramping”) in the time courses for these derivatives during the laser pulse (Fig. 3A). Since very little B is ever formed and since most of the observed photolysis is due to escape of the ligand to the solvent, little geminate recombination occurs after the flash.

The situation for the O$_{2}$ and NO complexes of Glyceria HbII is somewhat different. For these derivatives both $k_{2}$ and $k_{3}$ are 2.5-5.0-fold smaller than the corresponding parameters for O$_{2}$ and NO leghemoglobin (Table I). As a result, the observed absorbance traces exhibit a square wave appearance (Fig. 3B). The initial increase in deoxyhemoglobin is greater since $k_{3}$ is lower (i.e. $k_{3}J(t)/(k_{3}J(t) + k_{2}) \approx 0.25$ and 0.85 for Glyceria HbNO and HbO$_{2}$, respectively); the rapid equilibrium condition for states A and B is not strictly valid; and some ligand molecules are left in state B after the flash since $k_{2}$ is smaller, $\sim$40 $\mu$s$^{-1}$ (Table I).

Alkyl Isocyanide Rebinding—The quantum yields for the isonitrile complexes of leghemoglobin are uniformly low ($Q < 0.1$) due to extremely large values of $k_{2}$ for all of the compounds examined (Table I). The traces for methyl isocyanide- leghemoglobin are quite similar to those for the O$_{2}$ derivative; in both cases, $k_{2} = 1-2000$ $\mu$s$^{-1}$, $k_{3} \approx 150$ $\mu$s$^{-1}$, and little or no slow geminate recombination is observed after the laser pulse. Small but measurable nanosecond recombination phases are observed for the ethyl, n-propyl, and n-butyl isocyanide derivatives of leghemoglobin (Fig. 4). However, even in these cases,
the overall observed photoinsensitivity is due primarily to the high ratio of $k_3/k_5$ since the fractional escape from state C to the solvent phase is high for all the isonitrile complexes (i.e. $k_5/(k_4 + k_5) \gg 0.6$, Table I).

As with the gases, the values of $k_4$ for the isonitrile-Glycera HbII complexes are much smaller than those observed for the corresponding leghemoglobin derivatives (Table I). The $k_4$ values are roughly the same or greater, and as a result, larger absorbance changes are observed for Glycera HbII at a given laser light intensity (Fig. 4). For the longer isonitriles the observed traces are dominated by large, relatively slow phases representing ligand recombination from state C (Fig. 4). In contrast to leghemoglobin, the fractional escape from the second geminate state in Glycera HbII decreases from 0.8 to 0.01 in going from methyl to n-butyl isonicyanide, and this accounts for the dramatic decrease in $Q$ with side chain elongation (Table I).

**DISCUSSION**

**Rate-limiting Step for Ligand Association**—As described previously (Olson et al., 1987), the overall association rate constant can be defined by the individual rate parameters in Equations 1 and 2:

$$k' = \frac{k_1 k_2}{k_1 k_3 + k_2 k_4 + k_3 k_5} = k_{X=A}(1 - Q)$$

where $k_{X=A}$ is the steady-state rate constant for the formation
of state B starting from ligand in the solvent phase (i.e., $d(C)/dt = 0$; $k_{X-B} = k_{B0}((k_4 + k_5)$) and $K_{X-B}$ is the equilibrium constant for state B formation ($K_{X-B} = k_{B0}/k_3k_5$). The expressions on the right-hand side of Equation 3 can be used to describe the two extreme rate-limiting conditions (see Olson et al., 1987). If $Q$ is small (<0.1), then $k' = k_{X-B}$ and the association reaction is limited by the rate of ligand migration up to the heme iron atom. If $Q$ approaches 1, then $k' = k_{B0}K_{X-B}$, ligand in the solvent is in equilibrium with state B; and iron-ligand bond formation limits the overall association reaction.

A comparison of the key ligand-binding parameters for sperm whale myoglobin, leghemoglobin, and Glycera HbII is presented in Table 5 and Table II (the latter also contains parameters for the isolated $\alpha$ and $\beta$ chains of human hemoglobin). As shown in panels A and B in Fig. 5 and columns 2 and 3 in Table II, there is no direct correlation between $k_0$ and $k'$ for O$_2$, NO, and alkylisocyanide binding to the five monomeric heme proteins that have been examined. For example, the fitted values of $k_0$ for O$_2$ rebinding to leghemoglobin, Glycera HbII, sperm whale myoglobin, and human hemoglobin subunits are 1300, 230, 490, and 1350 ms$^{-1}$, respectively, whereas the corresponding overall association rate constants are 120, 190, 14, and $55 \times 10^6$ M$^{-1}$ s$^{-1}$. This lack of correlation occurs because the rate-limiting step for the binding of these ligands is migration from the solvent to state B. This is shown in Fig. 5C; for all of the ligands except CO, the probability of ligand rebinding from state B, $(1 - Q)$, is $\geq 0.8$ and $k' = k_{X-B}$ (Figs. 5, A and D).

The opposite situation occurs for CO rebinding. For leghemoglobin, Glycera HbII, and sperm whale myoglobin, $Q$ approaches 1.0, and the bimolecular association rate constant becomes proportional to $k_0$ (i.e., $k' = k_{B0}K_{X-B}$). However, since $k_0 \gg k_5$, little or no geminate recombination can be observed in laser photolysis experiments at room temperature and ordinary solvents, and it is very difficult to define the absolute value of $k_0$. Frauenfelder and co-workers (Austin et al., 1975; Ansari et al., 1986) have addressed this problem by carefully measuring the rate of $B \rightarrow A$ transition in high viscosity solvents and at low temperatures where the ligand is “frozen” in the distal pocket. Results obtained under these conditions are then extrapolated to room temperature. Their values of $k_0$ for the CO complexes of sperm whale myoglobin and soybean leghemoglobin (Ansari et al., 1986) in 75% glycerol and phosphate buffer at pH 7 are shown in Table II. Unfortunately, similar data for CO Glycera HbII have not been published. For purposes of illustration in Fig. 5, $k_0$ for CO-Glycera HbII was set equal to that for sperm whale MbCO since the corresponding values for the O$_2$ derivatives are similar.

As shown in Table II, the values of $k_{X-B}$ for the CO derivatives of leghemoglobin, Glycera HbII, and myoglobin are undefined. The relative errors of these parameters are greater than ±100% since iron-ligand bond formation is rate limiting (i.e., $(1 - Q) \approx 0$). The values for $\alpha$ and $\beta$ chains are much better defined since $(1 - Q)$ for the CO derivatives of these proteins are 0.35 ± 0.04 and 0.44 ± 0.14, respectively (Olson et al., 1987). In principle, $K_{X-B}$ for CO binding can be computed from $k'/k_0Q$ (Equation 1); however, only in the case of human HbCO has $k_0$ been measured directly at room temperature in ordinary buffers. As a result, it is difficult to estimate the relative errors of the calculated $K_{X-B}$ values for CO binding.

**Comparisons between Myoglobin, Leghemoglobin, and Glycera HbII**—The 10-fold greater bimolecular rates for NO and O$_2$ binding to leghemoglobin and Glycera HbII compared to sperm whale myoglobin are due solely to greater rates of ligand migration from the solvent phase to the first geminate state ($k_{X-B}$ values, Table II). In the case of leghemoglobin, the reactivity of the heme iron atom toward oxygen is also increased 2–3-fold. These observations are in good qualitative agreement with the earlier low temperature work of Stetzkowski et al. (1985) who also observed that O$_2$ migration through leghemoglobin is roughly 10 times faster than in sperm whale myoglobin and that $k_0$ is greater in the plant protein.\(^5\)

\(^5\)Although our work and that of Stetzkowski et al. (1985) agree qualitatively, there are substantial quantitative and interpretive differences. First, Stetzkowski et al. (1985) reported only a 2-fold difference between the $k'$ values for O$_2$ binding to sperm whale myoglobin and soybean leghemoglobin in 75% glycerol, whereas in 0.1 M phosphate alone there is a 10-fold difference (Table II). Second, in their analysis of O$_2$ rebinding data, Stetzkowski et al. (1985) assumed that the intrinsic photochemical yield of the first geminate state is quite low, 0.1–0.2 for both leghemoglobin and myoglobin, whereas in our analysis the intrinsic photochemical yield has either been assumed or shown to be 1.0 for all ligands (our arguments are presented in Olson et al., 1987). Their assumption led them to propose that iron-ligand bond formation is limiting both O$_2$ and CO binding and that $k'$ is
Greater accessibility of the distal pocket is also observed for isonitrile binding to leghemoglobin which exhibits $k_{x\rightarrow y}$ values 40 times greater than those for sperm whale myoglobin. This observation is readily interpreted in terms of the x-ray crystallographic structure of the soybean protein. The sixth coordination position in leghemoglobin appears to be unhindered and easily accessible to the solvent phase (Ollis et al., 1983; Appleby, 1984). The distal pocket in Glycera HbII is also readily available to diatomic ligands, but a marked decrease in $k_2$ occurs in going from methyl to n-butyl isocyanide (Fig. 5D). As a result, the bimolecular rate constants for the larger isonitriles are roughly the same for sperm whale myoglobin and Glycera HbII (Table II). A structural interpretation of this result is not apparent from the published low resolution crystallographic structure of the Glycera protein (Padlan and Love, 1974).

The uncertainties in $k_x$, $k_{x\rightarrow y}$, and $K_{X-a}$ for CO binding at room temperature make it difficult to determine the cause of the larger overall association rate constants for CO binding to leghemoglobin and Glycera HbII compared to that observed for sperm whale myoglobin (Table II). All three proteins exhibit high quantum yields. Thus, geminate iron-ligand bond formation is rate limiting and $k' = k_x K_{X-a}$, as was suggested originally by Frauenfelder and co-workers (Ansari et al., 1986 and references therein). The data of Stetzkowski et al. (1985) suggest that $k_x$ for CO rebinding to leghemoglobin is ~40 times greater than that for sperm whale myoglobin, whereas the $K_{X-a}$ values are roughly equal (Table II). This result suggests that favorable proximal effects are the major cause of the increased reactivity of leghemoglobin toward carbon monoxide. The situation for Glycera HbII is unknown since...
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FIG. 5. Dependence of the overall association rate constant \( (k') \), the initial geminate recombination rate constant \( (k_2) \), the fraction of rebinding from state B \( (1 - Q) \), and the rate of ligand migration from the solvent to state B \( (k_{X\rightarrow B}) \) on ligand composition and size. Symbols: Lb, open squares, soybean leghemoglobin; Gly, closed triangles, Glyceria HbII and SWMb; closed squares, sperm whale myoglobin. The abbreviations used are MNC, methyl isocyanide; ENC, ethyl isocyanide; nPNC, n-propyl isocyanide; nBNC, n-butyl isocyanide.

\( k_2 \) has not been measured for this protein. The \( O_2 \) and NO-binding data suggest that \( K_{2\rightarrow 0} \) for the diatomic ligands is 6–20 times greater for Glyceria HbII than for sperm whale myoglobin and that \( k_2 \) is similar for these two proteins (Table II). This result would suggest that favorable distal effects are the primary cause of the increased reactivity of Glyceria HbII for CO since \( k' \approx k_0K_{X\rightarrow D} \). Unfortunately, these conclusions for CO binding are tentative, in contrast to those for \( O_2 \) and NO binding where most of the geminate recombination parameters are well defined.

A summary of the photolysis characteristics of leghemoglobin, Glyceria HbII, and sperm whale myoglobin is shown in Fig. 6. The fractional escape from states B to C, \( k_{B\rightarrow C} / (k_B + k_3) \), the fractional escape from C to solvent, \( k_{C\rightarrow 0} / (k_C + k_3) \), and the overall quantum yield, \( Q \), are plotted versus ligand size and composition. For the gases, state C is never populated to any significant extent, and the overall quantum yield for all the CO, \( O_2 \) and NO complexes of these monomeric proteins is determined almost exclusively by \( k_{B\rightarrow C} / (k_B + k_3) \). The key parameter appears to be \( k_3 \) which increases over a hundred-fold in going from CO to \( O_2 \) to NO derivatives. Much smaller variations in \( k_3 \) are observed (Table I).

A similar situation occurs for the photolysis of isonitriles from leghemoglobin and sperm whale myoglobin: \( Q \) is determined primarily by \( k_{C\rightarrow 0} / (k_C + k_3) \). In contrast, the photolysis characteristics of Glyceria HbII isonitrile complexes are dominated by rebinding from state C as the ligand becomes larger. As shown in Fig. 6B, the overall quantum yield for Glyceria HbII decreases 260-fold in going from methyl to n-butyl isocyanide, and this decrease is due primarily to a marked decrease in the fractional escape to the solvent from C (open squares, Fig. 6B). In effect, the larger isonitriles are “stuck” kinetically in the protein matrix of Glyceria HbII, and movement back to state B and rebinding to the iron atom is much more rapid than migration to the solvent. This effect is also seen in the overall dissociation rate constants for the isonitrile complexes of Glyceria HbII (Table III).

A summary of the thermal dissociation behavior of liganded leghemoglobin and Glyceria HbII is presented in Table III. As discussed previously (Gibson et al., 1986; Olson et al., 1987), the rate constant for thermal bond dissociation, \( k_t \), in Equation 1, can be computed from \( k/Q \), where \( k \) is the overall dissociation rate constant measured by conventional rapid mixing techniques. The equilibrium constant for the \( B \rightarrow A \) transition is given in the last column of Table I.

Three major conclusions can be derived from the data for thermal bond dissociation. First, the \( k_t \) values for the CO complexes of the five monomeric proteins are relatively in-
of hydrogen bonding with bound oxygen. Since thermal displacement by a leucine residue, and an analysis of the sequence with His E7, those proteins with the strongest bonds are both expected and observed to exhibit the lowest values of bond, \(-1\) kcal/mol, is formed in the isolated hemoglobin bond between bound 0, and His E7 in leghemoglobin and globin subunits. In \textit{Glycera} HBII, the normal His E7 has been replaced by a leucine residue, and an analysis of the sequence and x-ray crystallographic data suggests that there is no residue near the sixth coordination position which is capable of hydrogen bonding with bound oxygen. Since thermal dissociation of Fe-O2 requires breakage of the hydrogen bond with His E7, those proteins with the strongest bonds are both expected and observed to exhibit the lowest values of \(k_i\) and vice versa. In the case of \textit{Glycera} HBII, \(k_i\) for O2 dissociation is equivalent to the corresponding values observed for unhindered model heme compounds in organic solvents where no hydrogen bonding is possible (Collman et al., 1983).

Third, the thermal bond dissociation rates for the isonitrile complexes of leghemoglobin and \textit{Glycera} HBII change very little with increasing ligand size and are similar to the values observed for chelated protoheme (\(-0.6-2\) s\(^{-1}\), Olson et al., 1983). In contrast, the \(k_i\) values for the methyl isocyanide complexes of whole myoglobin and isolated hemoglobin subunits are significantly larger (25-70 s\(^{-1}\)). We previously interpreted these high thermal bond dissociation rates for methyl isocyanide as due to steric hindrance between the His E7 and the methyl group of the bound isonitrile. This strain is alleviated when the Fe-ligand bond is broken and rotation of the ligand molecule is allowed (Mims et al., 1983; Gibson et al., 1986; Olson et al., 1987). This interaction either does not occur or is not energetically significant in leghemoglobin and \textit{Glycera} HBII.

\textbf{Relevance to the High-to-Low Affinity Transitions in Tetrameric Hemoglobin—}A major question is whether changes in heme reactivity or accessibility of the distal pocket account for the decrease in the association rate constant of tetrameric hemoglobin which is observed in going from the high (R) to the low affinity (T) quaternary conformation. As yet, a complete set of conventional and geminate kinetic parameters has not been obtained for the first (Hb4 X \(\rightarrow\) Hb4X) and last (HbX, \(\rightarrow\) X \(\rightarrow\) HbX,4) steps in ligand binding to human hemoglobin. However, the overall association rate constants and quantum yields of \(\beta\) subunits in the R and T states are reasonably well established (Table IV). These data indicate that the geminate reactivity of the heme iron atom decreases markedly in going from the R to T state hemoglobin, with little or no change in the accessibility of the distal pocket.

The results in Table IV can be simulated qualitatively by assuming a uniform 60-fold decrease in \(k_i\) for all three gaseous ligands in going from \(R\) to \(T\) state \(\beta\) chains, without any other changes in \(k_i\) through \(k_6\). This would account for the increase in the overall quantum yields. As expected, the largest changes would be observed for the O2 and NO derivatives where \(Q\) in the \(R\) state is low and approximately proportional to \(k_i/k_6\). In the case of CO binding, \(Q\) is large in both states and \(k' = k_6K_{X-N}\). As a result, the CO association rate constant is both observed and predicted to decrease markedly in going from the \(R\) to \(T\) state. In the case of O2 association, a smaller change in \(k^i\) is observed. In the \(R\) state, \(Q\) for the O2 complex is small, and the association rate constant is roughly independent of \(k_i\). In the \(T\) state, \(Q\) for \(\Delta O_2\) has increased to 0.5 so that both iron-ligand bond formation (\(k_6\)) and ligand migration through the solvent (\(k_{X-N}\)) are limiting the association reaction. Since in our hypothesis \(k_{X-N}\) is assumed to be the same for the \(R\) and \(T\) states, the decrease in \(k'\) for O2 binding is expected to be much smaller than that observed for CO binding. In the case of NO binding, \(k_6\) and \(k_6\) are equal. For this ligand, \(Q < 0.1\) under all conditions, and ligand migration through the protein is rate limiting in both quaternary conformations.

Cassoly and Gibson's (1975) observation of equal association rate constants for the Hb4 + NO and Hb4(NO3)+ + NO reactions provides the strongest experimental support for the idea that the \(R\) to \(T\) transition does not alter the accessibility of the distal pocket of hemoglobin for diatomic ligands. Mims et al. (1983) came to a similar conclusion based on equilibrium measurements. They observed that the pattern of steric hindrance observed for the binding of CO and a series of 10 alkyl isocyanides was the same for the \(R\) and \(T\) states of human hemoglobin and that there was a uniform 3-4 kcal/mol dif-
**Table III**

Interpretation of dissociation rate constants

Overall dissociation rate constants, $k$, were taken from Mims et al. (1983), Gibson et al. (1986), Olson et al. (1987), or remeasured by us. The errors in $k$ were determined as the standard deviation from the mean or assumed to be ±20%, the mean relative error for the dissociation rate constants of these ligands from sperm whale myoglobin. Again, the errors in $Q_{obs}$ were assumed to be equal to those observed experimentally for $Q_{obs}$. The relative errors in $k$ for NO dissociation are not shown for clarity (~±20%); the errors in $Q_{obs}$ for the NO derivatives were assumed to be ±20%. The uncertainties in $k$, and $K_{S\rightarrow a}$ were calculated using propagation of error theory and the formula: $k_{f} = k_{o}Q_{f}K_{a\rightarrow S}$.

The parameters were rounded off to two significant figures after all calculations had been made. The data for myoglobin were taken from Gibson et al. (1986); the data for $\alpha$ and $\beta$ chains, from Olson et al. (1987). Abbreviations are given in Table I.

<table>
<thead>
<tr>
<th>Ligand and protein</th>
<th>$k$ (s$^{-1}$)</th>
<th>$Q_{obs}$</th>
<th>$k_{f}$</th>
<th>$K_{S\rightarrow a}$ ($\times 10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LbCO</td>
<td>0.016 ± 0.003</td>
<td>0.96</td>
<td>0.016 ± 0.003</td>
<td>(7,500.0)*</td>
</tr>
<tr>
<td>GlyCO</td>
<td>0.042 ± 0.005</td>
<td>0.88</td>
<td>0.048 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>MbCO</td>
<td>0.019 ± 0.005</td>
<td>0.97</td>
<td>0.020 ± 0.006</td>
<td>(500.0)*</td>
</tr>
<tr>
<td>$\alpha$CO</td>
<td>0.013 ± 0.002</td>
<td>0.65</td>
<td>0.020 ± 0.003</td>
<td>5,000.0*</td>
</tr>
<tr>
<td>$\beta$CO</td>
<td>0.008 ± 0.001</td>
<td>0.56</td>
<td>0.014 ± 0.004</td>
<td>7,000.0*</td>
</tr>
<tr>
<td>GlyNO</td>
<td>0.000024</td>
<td>0.0089</td>
<td>0.00028 ± 0.00008</td>
<td>5,800,000.0 ± 2,200,000.0</td>
</tr>
<tr>
<td>MbO$_2$</td>
<td>1.800 ± 200</td>
<td>0.18</td>
<td>10.00 ± 1.200.0</td>
<td>0.023 ± 0.009</td>
</tr>
<tr>
<td>MbO$_2$</td>
<td>11.9 ± 1.8</td>
<td>0.13</td>
<td>92 ± 4.40</td>
<td>5.3 ± 2.7</td>
</tr>
<tr>
<td>$\alpha$O$_2$</td>
<td>28 ± 2.7</td>
<td>0.058</td>
<td>400 ± 180.0</td>
<td>3.1 ± 1.5</td>
</tr>
<tr>
<td>$\beta$O$_2$</td>
<td>15 ± 2</td>
<td>0.058</td>
<td>310 ± 76.0</td>
<td>4.2 ± 2.0</td>
</tr>
<tr>
<td>LbNO</td>
<td>0.000004</td>
<td>0.0028</td>
<td>0.0008 ± 0.0003</td>
<td>28.6 ± 7.5</td>
</tr>
<tr>
<td>GlyNO</td>
<td>0.00015</td>
<td>0.0088</td>
<td>0.0027 ± 0.0003</td>
<td>240,000.0 ± 150,000.0</td>
</tr>
<tr>
<td>MbNO</td>
<td>0.00012</td>
<td>0.0015</td>
<td>0.0027 ± 0.0003</td>
<td>190,000.0 ± 59,000.0</td>
</tr>
<tr>
<td>$\alpha$NO</td>
<td>0.000046</td>
<td>0.00092</td>
<td>0.0056 ± 0.0003</td>
<td>1,400,000.0 ± 250,000.0</td>
</tr>
<tr>
<td>$\beta$NO</td>
<td>0.000022</td>
<td>0.00072</td>
<td>0.0091 ± 0.0004</td>
<td>1,700,000.0 ± 250,000.0</td>
</tr>
<tr>
<td><strong>Isomerytries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LbMNC</td>
<td>0.95 ± 0.07</td>
<td>0.060</td>
<td>1.6 ± 0.3</td>
<td>15,000.0 ± 590</td>
</tr>
<tr>
<td>GlyMNC</td>
<td>0.65 ± 0.07</td>
<td>0.42</td>
<td>1.6 ± 0.6</td>
<td>74 ± 3.8</td>
</tr>
<tr>
<td>MbMNC</td>
<td>4.3 ± 0.3</td>
<td>0.17</td>
<td>20.2 ± 4</td>
<td>0.96 ± 0.20</td>
</tr>
<tr>
<td>$\alpha$MNC</td>
<td>3.9 ± 0.4</td>
<td>0.058</td>
<td>67 ± 28</td>
<td>3.6 ± 1.5</td>
</tr>
<tr>
<td>$\beta$MNC</td>
<td>7.0 ± 0.8</td>
<td>0.14</td>
<td>50 ± 21</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>LbENC</td>
<td>0.026 ± 0.003</td>
<td>0.037</td>
<td>0.70 ± 0.16</td>
<td>2,700.0 ± 1,100</td>
</tr>
<tr>
<td>GlyENC</td>
<td>0.035 ± 0.004</td>
<td>0.092</td>
<td>0.38 ± 0.28</td>
<td>1,000.0 ± 830</td>
</tr>
<tr>
<td>MbENC</td>
<td>0.30 ± 0.02</td>
<td>0.065</td>
<td>4.6 ± 0.5</td>
<td>21 ± 7</td>
</tr>
<tr>
<td>$\alpha$ENC</td>
<td>0.17 ± 0.02</td>
<td>0.052</td>
<td>3.3 ± 2.1</td>
<td>130 ± 90</td>
</tr>
<tr>
<td>$\beta$ENC</td>
<td>0.80 ± 0.09</td>
<td>0.14</td>
<td>5.7 ± 1.8</td>
<td>26 ± 8</td>
</tr>
<tr>
<td>Lb(nPNC)</td>
<td>0.015 ± 0.002</td>
<td>0.023</td>
<td>0.65 ± 0.11</td>
<td>2,500.0 ± 1,300</td>
</tr>
<tr>
<td>Gly(nPNC)</td>
<td>0.012 ± 0.001</td>
<td>0.0150</td>
<td>0.81 ± 0.19</td>
<td>434 ± 160</td>
</tr>
<tr>
<td>Mb(nPNC)</td>
<td>0.39 ± 0.02</td>
<td>0.140</td>
<td>2.9 ± 0.2</td>
<td>16 ± 5</td>
</tr>
<tr>
<td>$\alpha$(nPNC)</td>
<td>0.14 ± 0.02</td>
<td>0.046</td>
<td>3.0 ± 1.0</td>
<td>322 ± 108</td>
</tr>
<tr>
<td>$\beta$(nPNC)</td>
<td>0.54 ± 0.06</td>
<td>0.12</td>
<td>4.5 ± 1.7</td>
<td>110 ± 43</td>
</tr>
<tr>
<td>Lb(nBNC)</td>
<td>0.024 ± 0.003</td>
<td>0.023</td>
<td>1.0 ± 0.2</td>
<td>1,600 ± 790</td>
</tr>
<tr>
<td>Gly(nBNC)</td>
<td>0.0063 ± 0.0004</td>
<td>0.0016</td>
<td>2.1 ± 0.8</td>
<td>240 ± 98</td>
</tr>
<tr>
<td>Mb(nBNC)</td>
<td>0.69 ± 0.05</td>
<td>0.35</td>
<td>2.0 ± 0.5</td>
<td>8 ± 6</td>
</tr>
<tr>
<td>$\alpha$(nBNC)</td>
<td>0.31 ± 0.04</td>
<td>0.059</td>
<td>5.3 ± 0.9</td>
<td>170 ± 130</td>
</tr>
<tr>
<td>$\beta$(nBNC)</td>
<td>1.2 ± 0.14</td>
<td>0.093</td>
<td>13.0 ± 7.0</td>
<td>27 ± 6</td>
</tr>
</tbody>
</table>

* Calculated using $k_{2}$ values taken from Ansari et al. (1986).

† Assumed $k_{z} = 100$ μs$^{-1}$ based on Friedman et al.’s (1985) data (see Olson et al., 1987).

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**Table IV**

Ligand association parameters for the $\beta$ subunits of human hemoglobin in the high (R) and low (T) affinity quaternary states

The bimolecular association rate constants for the first and last steps in CO, O$_{2}$, and NO binding to tetrameric hemoglobin were taken from Sawicki and Gibson (1978b), Sawicki and Gibson (1977), and Cassoly and Gibson (1975), respectively. The overall quantum yields for T-state CO and O$_{2}$ were taken from Sawicki and Gibson (1979) and Morris and Gibson (1984). The quantum yield for T-state NO human hemoglobin has not been published but is estimated to be similar to the value measured for blue-fin tuna hemoglobin at pH 6 (Morris and Gibson, 1980). The values of $k_{S\rightarrow a}$ were calculated from $k'/(1-$Q$)$ (Equation 3).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$k'_{S}$ ($\times 10^{-5}$ M$^{-1}$s$^{-1}$)</th>
<th>$k'_{T}$ ($\times 10^{-6}$ M$^{-1}$s$^{-1}$)</th>
<th>$Q_{S}$</th>
<th>$Q_{T}$</th>
<th>$k_{S\rightarrow a}$ ($\times 10^{-5}$ M$^{-1}$s$^{-1}$)</th>
<th>$k_{T\rightarrow a}$ ($\times 10^{-6}$ M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>6.0</td>
<td>0.1</td>
<td>0.5</td>
<td>~1.0</td>
<td>12</td>
<td>~20</td>
</tr>
<tr>
<td>O$_{2}$</td>
<td>60.0</td>
<td>10.0</td>
<td>0.05</td>
<td>~0.5</td>
<td>63</td>
<td>~20</td>
</tr>
<tr>
<td>NO</td>
<td>30.0</td>
<td>30.0</td>
<td>0.0098</td>
<td>0.035</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
ference between the free energy of binding to the R and T states for all 11 ligands. This also suggests a large change in iron reactivity due to proximal effects and little change in the shape or stereochemistry of the distal pocket in going from the high to the low affinity quaternary conformation. Thus, the available data indicate that R to T differences, unlike those between the monomeric heme proteins, depend primarily on changes in the initial geminate recombination rate constant.

REFERENCES


