The Kinetics of the Reactions of *Parasponia andersonii* Hemoglobin with Oxygen, Carbon Monoxide, and Nitric Oxide*

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Hemoglobin I was isolated from nodules formed on the roots of *Parasponia andersonii* inoculated with *Rhizobium* strain CP 283. The rate of oxygen dissociation from *Parasponia* hemoglobin increases about 12-fold between pH 4 and 7, with apparent pK 6.4, to reach a limiting value of 1.48 × 10^4 s⁻¹. The optical spectrum of oxyhemoglobin in the visible region is also dependent on pH with pK near 6.4. The rate constant for oxygen combination with *Parasponia* hemoglobin increases about 7-8-fold between pH 4 and 7, with apparent pK 5.37, to reach a value of 1.67 × 10^4 s⁻¹ at pH 7. The optical spectrum of deoxyhemoglobin in the visible region and the rate constant for carbon monoxide combination are also dependent on pH with apparent pK 5.65 and 5.75, respectively. The rate constant for carbon monoxide dissociation is independent of pH. The oxygen affinity of *Parasponia* hemoglobin, P50 = 0.049 torr at 20°C, calculated from the kinetic constants at pH 7, is very great. At alkaline pH there is a prominent genimate reaction with oxygen and nitric oxide, with both subnanosecond and tens of nanosecond components. These reactions disappear at acid pH, with pK 6.4, and the effective quantum yield is reduced. In general, the reactions of *Parasponia* hemoglobin with oxygen and carbon monoxide resemble those of soybean leghemoglobin. In each, great oxygen affinity is achieved by unusually rapid oxygen combination together with a moderate rate of oxygen dissociation.

We suggest that protonation of a heme-linked group with pK near 6.4 controls many properties of *Parasponia* oxyhemoglobin, and protonation of a group with pK near 5.5 controls many properties of *Parasponia* deoxyhemoglobin.

Plant hemoglobins (1) are found in nitrogen-fixing nodules formed by symbiotic associations between *Rhizobium* (a bacterium) and legumes (1), between *Rhizobium* and the nonlegume *Parasponia* (2, 3), and between *Frankia* (an actinomycete) and diverse woody dicots (4–6). Legume hemoglobins have been studied extensively; the structures of soybean (7) and lupin (8) leghemoglobins have been determined. In each a histidine residue is the proximal ligand to the heme iron atom, and another histidine residue, located distal to the heme plane, enjoys unusual mobility (9–13) and may form a hydrogen bond to the bound oxygen molecule of oxyhemoglobin (13, 14) or may ligate the iron atom of ferric leghemoglobin (9). The very great oxygen affinity of soybean leghemoglobin is achieved by extraordinarily rapid combination with oxygen together with a moderate rate of oxygen dissociation (13, 15, 16).

*Parasponia*, a medium size (up to 20 m) tropical tree native to the Malay Archipelago region and belonging to the Ulmaceae or elm family, forms the only known nitrogen-fixing, symbiotic association between a non-legume and the bacterium *Rhizobium* (2, 17). *Parasponia* is nodulated by strains of *Rhizobium* that also nodulate certain members of the Leguminosae (17). The hemoglobin found in *Parasponia* nodules is a plant gene product (3, 18). *Parasponia andersonii* hemoglobin I is a dimer of two identical polypeptide chains of 161 amino acids, corresponding to a monomer molecular weight of 18,684 (including the heme group) (3, 19). The amino acid sequence (19) shows extensive homology with lupin and soybean leghemoglobins (19). The secondary structure predicted from the amino acid sequence is similar to those of lupin and soybean leghemoglobins (19). *Parasponia* hemoglobin has 3 histidine residues, in contrast to the soybean leghemoglobins which have 2 and lupin leghemoglobin II which has 5. His-104 is considered to be the proximal ligand to the heme iron atom; His-69 may ligate the heme plane; an additional histidine, His-113, analogous to histidine G3 in lupin leghemoglobin (8), is a candidate for one of the titratable groups to be described.

We report that *Parasponia* hemoglobin shares the high oxygen affinity of soybean leghemoglobin, in each instance achieved by very rapid combination with oxygen together with a moderate rate of dissociation. *Parasponia* hemoglobin has heme-linked protonatable groups controlling, respectively, the

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rates of ligand combination (pK 5.4-5.8) and the rates of oxygen dissociation and geminate recombination (pK 6.4). Only a single heme-linked titratable group, with pK 5.5, controlling oxygen dissociation, is found in soybean Lb* a (13).

**EXPERIMENTAL PROCEDURES**

**Parasponia Hemoglobin**—The major hemoglobin, hemoglobin I, the only component studied in this work, was isolated from fresh nodules of *P. andersonii* Planch infected with *Rhizobium strain CP283 and purified to homogeneity as described (3). Stock solutions of HbO₂ (2 mM) in a buffer containing 100 mM potassium chloride, 1 mM EDTA, and 2 mM potassium phosphate buffer, pH 7.4, were stored frozen under liquid nitrogen. After thawing, the hemoglobin remained entirely oxygenated; no ferric protein was detected in optical spectra.

**Buffer**—A succinic acid/potassium phosphate system (13) was chosen because it buffers adequately over the desired pH range, neither constituent appears to react with *Parasponia* Hb, and all constituents are available as pure, fully deuterated substances for use in parallel experiments using NMR. The mixture contained 100 mM succinic acid (Fluka, purissima grade), 100 mM potassium dihydrogen phosphate (J. T. Baker Chemical Co., ultrax grade), and 1 mM EDTA (Fluka, purissima grade) adjusted to pH 7.40 or other desired pH with sodium hydroxide (J. T. Baker Chemical Co., ultrax grade).

**Molecular Size of Parasponia Hemoglobin**—A model of the molecular size for *Parasponia* Hb was obtained by chromatography on a 4.7-cm column of Sepharcl S-200, 1.6 by 60 cm, developed with air-saturated 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.2, for HbO₂ and ferric Hb or developed with argon-equilibrated buffer containing 1 mM sodium dithionite for ferrous Hb. The sample volume was 2% of the column volume. Fractions 500 µM samples, as loaded, peak effluent concentration was about 200 µM; from 5 µM samples, as loaded, peak effluent concentration was about 1 µM. Good separation in the range of molecular weights from 13,000 to 150,000 was achieved; Kᵣ for the calibrating proteins ribonuclease (13,700), chymotrypsinogen (25,000), ovalbumin (43,000), bovine serum albumin (67,000), aldolase (158,000) were 0.55, 0.49, 0.32, 0.24, and 0.14, respectively.

**pH-dependent Change in Optical Spectrum**—Optical titrations were performed at 20 °C as described by Appleby et al. (13) in a Hitachi Perkin-Elmer model 557 spectrophotometer interfaced to a PDP 11/03 computer and Hewlett-Packard model 7221B plotter.

**Ligand Reaction Rates Using Stopped-Flow**—A Gibson-Milnes (20) stopped-flow apparatus with a 2-cm light path in the observation cell and equipped with a log ratio amplifier was used to measure ligand dissociation rates and the rates of carbon monoxide combination.

**Molecular Size of Parasponia Hemoglobin**—A model of the molecular size for *Parasponia* Hb was obtained by chromatography on a 4.7-cm column of Sepharcl S-200, 1.6 by 60 cm, developed with air-saturated 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.2, for HbO₂ and ferric Hb or developed with argon-equilibrated buffer containing 1 mM sodium dithionite for ferrous Hb. The sample volume was 2% of the column volume. Fractions 500 µM samples, as loaded, peak effluent concentration was about 200 µM; from 5 µM samples, as loaded, peak effluent concentration was about 1 µM. Good separation in the range of molecular weights from 13,000 to 150,000 was achieved; Kᵣ for the calibrating proteins ribonuclease (13,700), chymotrypsinogen (25,000), ovalbumin (43,000), bovine serum albumin (67,000), aldolase (158,000) were 0.55, 0.49, 0.32, 0.24, and 0.14, respectively.

**Oxygen Dissociation Rate**—Solutions of Parasponia HbO₂ (5 mM HbO₂, 2.5 mM free oxygen in buffer) were mixed rapidly with solutions of CO containing 2.5 to 20 mM in buffer containing 125 mM dithionite, and the reaction was followed at 417 nm. The reaction course was first order and homogeneous to more than 90% completion. The rate measured at 430 nm, a minimum in the difference spectrum Parasponia HbCO minus deoxy-Hb, was the same as that measured at 417 nm.

**Ligand Rebinding Rates Following Flash Photolysis**—Ligand recombination following flash photolysis was measured spectrophotometrically as described by Sawicki and Gibson (21, 22). Most measurements were made at 20 °C at a heme concentration near 70 µM in 1-mm cells, using a 300-ns flash. Data were collected with several levels of photolysis light centered at 575 nm, the highest level being below the photochemistry at which artifactual changes set in. The effective rate of work, based on a quantum yield of 1, was about 5 × 10⁹ s⁻¹. Data collection used a Biomation 805 transient recorder coupled to a PDP 8F computer. The observing light was provided by a 75-watt xenon arc, screened with a blue glass filter before reaching the sample.

To measure CO binding, about 1 ml of buffer was placed in a 100-ml tonometer with a 1-mm cell fused to the bottom. The tonometer was flushed for some minutes with purified nitrogen and then with CO. A suitable volume of Parasponia HbO₂ was then introduced, and the fluid equilibrated with the gas phase. Most measurements were made between 50 and 90% saturation, a window near the absorbance change in deoxy-Hb. An excellent isosbestic was found at 422 nm. The time course of the absorbance excursion was accurately first order and there was no slow absorbance change at 419 nm, where the difference between HbO₂ and HbCO is large. This indicates that there was no interference by residual oxygen.

A comparison between the combination of carbon monoxide with *Parasponia* Hb as measured by stopped-flow and by flash photolysis on the same solution was carried out using the flow-flash apparatus described by Ludwig and Gibson (23). Deoxy-Hb and carbon monoxide were first mixed in a stopped-flow mode and the reaction followed using a Biomation 805 recorder. Then, carbon monoxide was dissociated from the same portion of solution which remained at rest in the observation tube by a laser flash using Rhodamine 575. The laser light was excluded from the photomultiplier by two thicknesses of Corning blue glass 7-57. The recombination of CO with the hemoglobin was then followed as before. The second order rate constants calculated for the two procedures agreed within 2%.

Measurements of oxygen binding were made at 436 nm, using air-equilibrated solutions. The kinetic difference at pH 5.1 and 7.0, using the most intense photolysis light, was 89% of the static difference HbO₂ minus deoxy-Hb (dithionite added). An excellent isosbestic was found at 417 nm, suggesting that only two absorbing species were present throughout the experiment. An example of one kinetic run at pH 9.01 is presented as Fig. 1 which shows three data sets collected with maximal light and one set with the light attenuated 32-fold. The data are nearly first order, although theoretically the points should lie on a gentle curve as the oxygen concentration of the sample changes. The difference in slope of the two lines is largely accounted for by the larger amount of oxygen released into solution by the brighter flash, with second order rates of 2.04 and 1.90 × 10⁻⁹ M⁻¹ s⁻¹ for the examples shown.

**The Oxygen Rebinding Rate**—The geminate reactions were followed by flash photolysis using a 25-ns pulse centered at 575 nm, with an energy of 50 mJ. In the optical system used, this was sufficient to cause dissociation of oxymyoglobin at a rate of 5 ns⁻¹. The reaction was followed using a 75-watt Xe arc lamp, pulsed for 0.1 ms to 20 mJ, and passed through a 2 cm light path in the observation cell. The chromat or was monitored using a 1P28 photomultiplier working into 50 ohms. The signals were measured with a Hewlett-Packard 3440A digital multimeter (pK 5.4-5.8) and the rates of oxygen dissociation and geminate recombination (pK 6.4). Only a single heme-linked titratable group, with pK 5.5, controlling oxygen dissociation, is found in soybean Lb* a (13).
Parasponia Hemoglobin Is Dimeric—Appleby et al. (3), on the basis of limited experiments, suggested that Parasponia Hb I was probably a dimer. Chromatography of dilute (initially 5 μM) and concentrated (initially 500 μM) Parasponia Hb on a Sephacryl S-200 column establishes that Parasponia ferric Hb, deoxy-Hb, and HbO₂ exist as concentration-independent dimers (Table I).

Rates of Ligand Combination and Dissociation—Rates of these reactions at the alkaline limits (pH 7–9) of the pH-dependent change of their principal titratable component are presented in Table II, where they are compared to the rates of reaction of soybean leghemoglobin a. The rate constant for oxygen binding does not change from 5 to 250 μM Hb. The rate of oxygen dissociation does not change from 1 to 31 μM HbO₂.

Temperature Dependence of Reaction Rates—The temperature dependence of ligand combination is small (Fig. 2); that of ligand dissociation is about 5-fold greater (Fig. 3). The heats of activation are 3.7, 3.7, 23, and 18 kcal/mol for O₂ combination, CO combination, O₂ dissociation, and CO dissociation, respectively.

pH Dependence of Reaction Rates—The pH dependence of the oxygen combination rate constant between pH 4 and 7 is best represented by assuming a single ionizing group of pK 5.37, with theoretical limiting values of 0.133 and 1.65 × 10⁶ M⁻¹ s⁻¹ for the acid and alkaline forms, respectively (Fig. 4). There is an unexplained discontinuity with an increase in rate at pH values greater than pH 7.

The pH dependence of the oxygen dissociation rate fits the relation for a single ionization of pK 6.41 with limiting values of 1.2 and 14.8 s⁻¹ for the acid and alkaline forms respectively (Fig. 4).

The pH dependence of carbon monoxide combination fits the relation for a single ionizing group of pK 5.75 with limiting values of 2.0 and 13.7 × 10⁶ M⁻¹ s⁻¹ for the acid and alkaline forms (Fig. 5). Carbon monoxide dissociation is independent of pH from pH 5.0 to 8.5.

Nitric oxide combines somewhat more rapidly than oxygen, k₈ = 240 × 10⁶ M⁻¹ s⁻¹ at pH 9 (Table II). Change to acid pH produced a marked drop in the effective quantum yield; recombination could not be measured accurately.

The apparent pK values of these heme-linked ionizations are reported in Table III.

pH-dependent Change of Optical Spectra—A pH-dependent change observed in the visible region of the optical spectrum of Parasponia HbO₂ resembles that described for soybean Lbo₂ (13, 24). It fits the relation for a single ionization, with pK 6.23 (Fig. 6). A smaller, pH-dependent change involving the absorbance maximum of Parasponia deoxy-Hb in the visible region was also observed. It fits the relation for a single ionization with pK 5.65 (Fig. 6).

Geminate Combination of Ligands to Parasponia Hemoglobin—The results with Parasponia hemoglobin are unusual,
Reactions of Parasponia Hemoglobin

TABLE I
Kinetic and equilibrium constants for the reactions of Parasponia Hb with ligands, compared to those of soybean Lb a

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Parasponia Hb</th>
<th>Soybean Lb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k'_m$ s⁻¹</td>
<td>$165 \times 10^6$</td>
<td>$116 \times 10^6$</td>
</tr>
<tr>
<td>$k_n$ s⁻¹</td>
<td>14.8</td>
<td>5.55</td>
</tr>
<tr>
<td>$K$ (mM)</td>
<td>89</td>
<td>48</td>
</tr>
<tr>
<td>$P_{50}$ (nM)</td>
<td>0.049</td>
<td>0.026</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$l'_m$ s⁻¹</td>
<td>$13.7 \times 10^6$</td>
<td>$12.7 \times 10^6$</td>
</tr>
<tr>
<td>$l_n$ s⁻¹</td>
<td>0.0187</td>
<td>0.0078</td>
</tr>
<tr>
<td>$L'$ (nM)</td>
<td>1.4</td>
<td>0.62</td>
</tr>
<tr>
<td>$P_{50}$</td>
<td>$1.0 \times 10^{-3}$</td>
<td>$0.45 \times 10^{-3}$</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k'_m$ s⁻¹</td>
<td>$50$</td>
<td>58</td>
</tr>
<tr>
<td>$M'$ (M⁻¹torr⁻¹)</td>
<td>$240 \times 10^6$</td>
<td></td>
</tr>
</tbody>
</table>

* These numbers are calculated in terms of the gaseous pressures; alternatively, one could define $M'$ in terms of the ratio of the two equilibrium constants $L/K$, in which case $M' = 1.34 M$ at 20 °C.

Values are taken at the alkaline limit of pH-dependent change: pH 9 for Parasponia Hb except for oxygen combination, where the value at pH 7 is taken, and pH 7.0 for soybean Lb. Equilibrium constants are estimated from the kinetic constants. All data were obtained at 20 °C. Data for soybean Lb a are from Appleby et al. (13).

Fig. 4. Kinetic constants for the reactions of oxygen with Parasponia deoxy-Hb as functions of pH at 20 °C. Solid circles, second order rate constant, $k'$, combination; open circles, $k_n$, dissociation. The lines are computer-assisted best fits to the relation for single ionizations. The midpoints are: $pK = 5.4$ for combination and $pK = 6.4$ for dissociation.

Fig. 5. Second order rate constant, $l'$, for the combination of carbon monoxide with Parasponia Hb as a function of pH at 20 °C. $pK = 5.75$. Carbon monoxide dissociation is independent of pH.

Table II
Apparent PK of heme-linked proton-dependent change in Parasponia Hb compared to those of soybean leghemoglobin a

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Apparent PK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyhemoglobin</td>
<td></td>
</tr>
<tr>
<td>Optical spectrum</td>
<td>5.65</td>
</tr>
<tr>
<td>$O_2$ combination</td>
<td>5.37</td>
</tr>
<tr>
<td>CO combination</td>
<td>5.75</td>
</tr>
<tr>
<td>NMR</td>
<td>Invariant</td>
</tr>
<tr>
<td>Oxyhemoglobin</td>
<td></td>
</tr>
<tr>
<td>Optical spectrum</td>
<td>6.23</td>
</tr>
<tr>
<td>$O_2$ dissociation</td>
<td>6.41</td>
</tr>
<tr>
<td>Geminate $O_2$ recombination</td>
<td>6.5</td>
</tr>
<tr>
<td>NMR</td>
<td>5.5</td>
</tr>
<tr>
<td>Carbon monoxide hemoglobin</td>
<td></td>
</tr>
<tr>
<td>Optical spectrum</td>
<td>Invariant</td>
</tr>
<tr>
<td>CO dissociation</td>
<td>Invariant</td>
</tr>
</tbody>
</table>

* Fuchsman and Appleby (24).
* Appleby et al. (13).
* Complex changes are observed above pH 8 and below pH 5.
* Ikeda-Saito et al. (14).
* NMR is of meso heme proton resonances (13) and of the distal histidine (25, 26).
* Almost invariant above pH 5. Change occurs at more acid pH.

Fig. 6. Change in optical spectra of Parasponia HbO₂ and deoxy-Hb as functions of pH at 20 °C. Solid circles, deoxy-Hb ($A_{576nm}$-$A_{662nm}$); open circles, HbO₂ ($A_{585nm}$-$A_{662nm}$). The midpoints are $pK = 5.65$ and $pK = 6.23$ for deoxy-Hb and HbO₂, respectively.

1.6 × 10⁷ s⁻¹. No geminate reaction was observed at pH 7, 20 °C, nor at pH 4 at any temperature.

With oxygen as ligand, a well-marked geminate reaction is observed, accounting for 25% of the absorbance excursion at 20 °C and pH 7, with a half-time of 15 ns. On cooling to 2 °C, the proportion increased to 41%, but the rate was little changed. Change in pH, however, produced a marked effect...
with progressive disappearance of geminate combination as the solutions were made more acid. A sample of oscilloscope records illustrating this effect is shown in Fig. 7. In addition, the effective quantum yield at the end of the flash is only about half as great at pH 4 as at pH 9. This is not due to denaturation of the protein in the acid solutions as a prolonged (300 ns) flash gave the same absorbance change at 432 nm at either pH (lower panels of Fig. 7). The drop in effective quantum yield is also expressed in the obvious difference in the slopes of the reaction records. There is a good correlation between amplitude of geminate reaction and apparent quantum yield expressed in the curves of Fig. 8. The two upper curves show the absorbance excursion at the end of the photolysis pulse expressed in arbitrary units for two different levels of light intensity. The lowest curve is the amplitude of the geminate reaction, measured as the difference between the peak height and the level reached by the trace after 200 ns, as in the upper right panel of Fig. 7. All three curves have half-effect at pH 6.5.

**DISCUSSION**

*Parasponia* Hb is a dimer formed of two identical subunits each bearing one heme. The dimer does not dissociate even in very dilute solution (Table I). The rate constants for oxygen combination and dissociation are each independent of protein concentration, and the progress of the reactions with excess oxygen and carbon monoxide are closely first order throughout most of their course. This indicates that each heme group reacts independently without intra- or intermolecular heme-heme interaction.

The kinetics of the overall reactions of *Parasponia* and soybean hemoglobins with oxygen are strikingly similar (Table II). Oxygen combination is very rapid and, at the alkaline limit, approaches within a decade the expected diffusion-limited rate. The rates of oxygen dissociation are moderate. The resulting oxygen affinities, $P_50$ is 0.049 and 0.026 torr for *Parasponia* and soybean hemoglobin, respectively, are extraordinarily great.

**FIG. 7. Geminate combination of oxygen with *Parasponia* Hb.** The records were obtained with a 25-ns pulse (top panels) and with a 300-ns pulse (lower panels) with energies of 50 and 300 mJ, respectively. The time scale was 50 ns/division throughout. The multiple traces in each panel show the results obtained when the laser flash was either unscreened or attenuated by a factor of 2, 4, or 8 by passage through a neutral density filter. In each panel the largest trace was obtained with the unscreened flash. The absorbance at 432 nm is always greater immediately after the flash. In the two lower panels the top of the grid corresponds to zero light. A back-off voltage was employed for the two upper panels. The solutions contained 75 $\mu$M heme, were equilibrated with air, and were examined in a 1-mm cell at 20 °C.

**FIG. 8. Effect of pH on geminate combination of oxygen with *Parasponia* Hb.** A solution of *Parasponia* HbO, equilibrated with room air, 75 $\mu$M in heme in a 1-mm cell, was photolyzed at 20 °C with a 25-ns pulse. The reaction was followed at 432 nm at each of the pH values shown. The uppermost curve shows the excursion when the laser pulse was attenuated by $\frac{1}{2}$, the middle curve by $\frac{1}{4}$. The measurement of the excursion was made 200 ns after the flash. The maximum excursion shown corresponds to the removal of 45% of the total oxygen. The lowest line is the difference between the peak in deoxyhemoglobin at the end of the flash and the level 200 ns later for the $\frac{1}{2}$ attenuation level and is a measure of geminate recombination. The hemoglobin concentration varied slightly between pH values and the recorded values have been adjusted for this; thus the arbitrary scale of the ordinate has the same relative value for all lines.

The reactions of *Parasponia* Hb with carbon monoxide are 10 to 1000 times slower but in general parallel those with oxygen. The rates of reaction with carbon monoxide are very similar to those of soybean Lb (Table II). This is reflected in the partitions of *Parasponia* and soybean Hb between oxygen and carbon monoxide ($M$ of Table II), which are nearly the same.

Although it has long been recognized that the combination and dissociation of ligands with hemoproteins must be a complex process, it has only recently become possible to demonstrate and measure some of the substeps involved in these reactions (27-33). The reactions are of interest in themselves, but, as the structures of several leghemoglobins have been determined with high resolution, it seems particularly desirable to examine *Parasponia* hemoglobin in this respect.

The reactions with oxygen have been analyzed further by assuming the minimal scheme,

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D$$

where $A$ represents liganded *Parasponia* hemoglobin, and $D$ is free deoxy protein in equilibrium with ligand in solution. The species $B$ and $C$ are intermediates in which a ligand molecule remains associated with the protein but is not bound to the heme. The rate $k_1$ is the rate of breaking the Fe-ligand bond in the light; a quantum yield of unity is assumed. The first step in the reaction with ligand free in solution, $D$ to $C$, is not taken into account as it is too slow, even with *Parasponia* hemoglobin, to make a significant contribution to the absorbance changes observed. The remaining rates have been assigned by fitting the family of absorbance changes obtained with several flash intensities to the integrated form of the rate equations derived from the scheme, as described by Gibson et al. (33). The four undefined parameters can often be determined satisfactorily by such data, depending on the actual values in a given case. In general, the reaction $B$ to $A$, defined by $k_2$, is rapid, and the population of $B$ is roughly $k_1 \times A/(k_1 + k_2)$, giving a starting value for the curve-fitting
procedure. The geminate reaction consists of two parts, a very rapid one as the flash shuts off and A is repopulated from B with rate constant $k_5$ and a slower reaction as species C repopulates A through B. The approximate rate constant for this reaction is $k_4 + k_6$. The amplitudes of the two components are determined by $k_5$ which largely governs the population of C during the photoflash with small values giving a large rapid reaction, and larger values a greater proportion of the slower one.

Values of the parameters derived from curve fitting are given in Table IV. They show, first, that the rate of rebinding of ligand, expressed by $k_3$, is satisfactorily represented with quite similar values of $k_3$ and $k_6$, suggesting that these parameters describe a diffusion process as proposed by Marden (27). Two examples of the results of curve fitting are shown in Fig. 9, A and B.

When the reaction with nitric oxide was studied in the same way, analogous results were obtained, the chief difference at alkaline pH being that a much larger value of $k_6$ was required than with oxygen. At acid pH so little ligand was dissociated at any stage of the reaction, corresponding at the peak to a change of 2-3% in saturation, that the assignment of rates could only be done by analogy with oxygen. The entries in Table IV have been enclosed in parentheses to stress this.

With carbon monoxide as ligand, the problem is just the opposite. So much ligand is dissociated and so little returns to the heme that independent determination of all the parameters is impossible. Again, an estimate of $k_3$ has been given for purposes of comparison. It is much smaller than the corresponding values for oxygen and nitric oxide, suggesting that the difference between ligands derives chiefly from this source, i.e. from a difference in rate of reaction at the heme.

The values in Table IV can be examined for consistency with the overall values given in Table II since the product of the binding constants for each stage of the A $\rightleftharpoons$ B $\rightleftharpoons$ C $\rightleftharpoons$ D scheme should be equal to the overall constant, i.e. $k' = k_3k_4k_5/(k_3k_4 + k_3k_5 + k_3k_6)$. To make the comparison, a value is required for $k_3$, now the dark dissociation velocity constant. This is equal to the product of the observed dissociation rate and the apparent quantum yield for transfer of ligand to solution. An approximate value can be obtained from the data of Fig. 7 using the amplitude after geminate recombination is complete together with the estimated numbers of quanta delivered to the sample as measured by the dissociation of carbon monoxide myoglobin. It is 0.05. The rate of dissociation of oxygen from the heme is then 300 s$^{-1}$ at 20°C. The resulting value for the product of the parameters is $1.2 \times 10^7$ M$^{-1}$ s$^{-1}$ derived from Table II. In making the calculation, it has been assumed that there is no change in activity of ligand in passing from D to C in the scheme. At pH 4.6 and 20°C, the value of $k_3$ is poorly defined, but the calculated value of $1.7 \times 10^7$ M$^{-1}$ s$^{-1}$ does not differ unreasonably from the value derived from Table II and the data on Fig. 4 which is $2.2 \times 10^7$. This agreement, incidentally, strongly supports the assumption that the quantum yield for the step A $\rightarrow$ B is 1.

The temperature dependence of the parameters in Table IV is notably slight, and in some cases ($k_3$) negative. In this specific case, the rate constant is really described two processes, recombination, and diffusion away from the immediate region of the heme. On cooling, there is little change in the recombination velocity, but it is able to operate longer on the newly dissociated ligand which diffuses away less readily at lower temperatures. The compound parameter $k_3$ appears to increase as the temperature is reduced. The slight temperature dependence of the rates is generally consistent with the suggestion that they arise from diffusion, but the large steps in

![Figure 9](image_url)

**Figure 9. Example of curve fitting procedure used to estimate parameter values in the text scheme A $\rightleftharpoons$ B $\rightleftharpoons$ C $\rightleftharpoons$ D.** The observations were made using 85 μM *Parasponia* HbO$_2$ equilibrated with room air at 20°C, in buffers of pH 7 and 4.6 for A and B, respectively. Observation was of a 1-mm path at 422 nm. The laser pulse was used directly and attenuated to ½, ¼, and ½ in A, and to ⅛ and ¼ in B. The points are observed, the lines calculated giving equal weights to all points.

**Table IV**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Temperature</th>
<th>pH</th>
<th>$k_2$</th>
<th>$k_3$</th>
<th>$k_4$</th>
<th>$k_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$</td>
<td>20</td>
<td>7.0</td>
<td>2.2</td>
<td>0.18</td>
<td>0.027</td>
<td>0.030</td>
</tr>
<tr>
<td>O$_2$</td>
<td>2</td>
<td>7.0</td>
<td>1.8</td>
<td>0.22</td>
<td>0.042</td>
<td>0.020</td>
</tr>
<tr>
<td>O$_2$</td>
<td>20</td>
<td>4.6</td>
<td>7.1</td>
<td>0.17</td>
<td>0.002</td>
<td>0.015</td>
</tr>
<tr>
<td>O$_2$</td>
<td>2</td>
<td>4.6</td>
<td>10.7</td>
<td>0.22</td>
<td>0.006</td>
<td>0.018</td>
</tr>
<tr>
<td>NO</td>
<td>20</td>
<td>7.0</td>
<td>6.1</td>
<td>0.17</td>
<td>0.059</td>
<td>0.029</td>
</tr>
<tr>
<td>NO</td>
<td>2</td>
<td>7.0</td>
<td>12.0</td>
<td>0.20</td>
<td>0.048</td>
<td>0.021</td>
</tr>
<tr>
<td>NO</td>
<td>20</td>
<td>4.6</td>
<td>165</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>2</td>
<td>7.0</td>
<td>0.0(2)</td>
<td>0.2</td>
<td>0.04(2)</td>
<td></td>
</tr>
</tbody>
</table>

This is very close to the overall value of $1.1 \times 10^7$ M$^{-1}$ s$^{-1}$ from Table II. Using the amplitude after geminate recombination is complete together with the estimated numbers of quanta delivered to the sample as measured by the dissociation of carbon monoxide myoglobin. It is 0.05. The rate of dissociation of oxygen from the heme is then 300 s$^{-1}$ at 20°C. The resulting value for the product of the parameters is $1.2 \times 10^7$ M$^{-1}$ s$^{-1}$ derived from Table II. In making the calculation, it has been assumed that there is no change in activity of ligand in passing from D to C in the scheme. At pH 4.6 and 20°C, the value of $k_3$ is poorly defined, but the calculated value of $1.7 \times 10^7$ M$^{-1}$ s$^{-1}$ does not differ unreasonably from the value derived from Table II and the data on Fig. 4 which is $2.2 \times 10^7$. This agreement, incidentally, strongly supports the assumption that the quantum yield for the step A $\rightarrow$ B is 1.

The temperature dependence of the parameters in Table IV is notably slight, and in some cases ($k_3$) negative. In this specific case, the rate constant is really described two processes, recombination, and diffusion away from the immediate region of the heme. On cooling, there is little change in the recombination velocity, but it is able to operate longer on the newly dissociated ligand which diffuses away less readily at lower temperatures. The compound parameter $k_3$ appears to increase as the temperature is reduced. The slight temperature dependence of the rates is generally consistent with the suggestion that they arise from diffusion, but the large steps in
the rates as one goes from $k_5$ to $k_6$ and $k_7$ to $k_8$ are not consistent with diffusion in a geometrically simple volume.

Comparison of the values for nitric oxide and oxygen agree with the interpretation suggested for the oxygen data since the main difference is in the values of $k_8$, the chemical barrier step. The geminate reaction with carbon monoxide is small to permit assured analysis, but again the results are consistent with a description in which $k_8$ is primarily affected.

One unusual feature of the geminate reactions of Parasponia Hb with oxygen and nitric oxide is the considerable effect of pH shown in Figs. 7 and 8 and in Table IV. There have been few studies of other hemoproteins with these ligands, and no pH effect has previously been reported. There is, however, a striking parallel between the results with oxygen at acid and alkaline pH and the differences between human hemoglobin subunits observed by Morris et al. (34). When Fe-Co hybrid hemoglobins react with oxygen, $\alpha$ Fe subunits show little geminate recombinations, while with $\beta$ Fe subunits geminate recombination dominates the nanosecond kinetics. With Parasponia Hb the reaction at pH 7 resembles that of $\beta$ subunits, while at pH 4 it resembles that of the $\alpha$ subunits. All of the experiments with the hybrid human hemoglobins were performed at pH 7, however, so the meaning of the parallel is not clear.

The analysis of the geminate reactions summarized in Table IV shows the effect of pH to derive from changes in the reaction B to A ($k_8$) and in the reaction C to B ($k_4$). The first may be more generally associated with the heme and its immediate environs and perhaps with protonation of His-69, but the second change, which is at least as great, affects a diffusion step in the transition between heme and the protein internal environment and, so, presumably reflects a pH-linked change in conformation.

Three properties of Parasponia deoxymyoglobin change with similar pK (Table III). These are the rate of combination with oxygen, the rate of combination with carbon monoxide, and the electronic configuration of the heme as reported by the optical spectrum. For the moment we consider that the three apparent pK values reflect protonation of a common single group. The effect of pH clearly is being exerted at the heme rather than by opening and closing of a hypothetical barrier to diffusion which, if rate determining with oxygen, would have little effect on carbon monoxide, which combines 10 times more slowly. In fact the ratio of rates at the acid and alkaline limits (7–8-fold increase) is the same for oxygen and carbon monoxide.

Three properties of Parasponia oxyhemoglobin change with nearly the same pK. These are the rate of oxygen dissociation, the electronic structure of the heme as reported by the optical spectrum, and the amplitude of geminate recombination of oxygen following photolysis by a very brief flash. The coincidence of the apparent pK values suggests that protonation of the same group may govern all of these properties. It seems reasonable to consider geminate recombination together with the properties of the liganded protein, because the starting point is the oxygenated protein, just as it is for oxygen dissociation, and very little time is available for change in conformation in the few nanoseconds between photolysis and recombination.

We propose, as a working model, that Parasponia hemoglobin has two different protonatable groups, one affecting predominantly the properties of deoxymyoglobin, the other the properties of oxyhemoglobin. An alternative model with a single heme-linked protonatable group with different pK in the deoxygenated and oxygenated states would also fit the facts but we consider it less probable because the protonation governing the properties of soybean oxylegemoglobin is without discernible effect on the deoxy protein (13, 14, 25, 26).

The field of distinguishable pK values for proton effects on deoxy and oxy Parasponia hemoglobin, respectively, implies that the two protonations exert their effects on the heme in different ways. One hypothesis, without present experimental support, is that effects on deoxymyoglobin are exerted through the heme periphery or the proximal ligand to the heme iron, and that effects on oxyhemoglobin, as in soybean legemoglobin (13, 14, 25, 26), are consequences of protonation of the distal histidine.

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REFERENCES

Reactions of Parasponia Hemoglobin
