We report the optical and resonance Raman spectral characterization of ferrous recombinant *Chlamydomonas* LI637 hemoglobin. We show that it is present in three pH-dependent equilibrium forms including a 4-coordinate species at acid pH, a 5-coordinate high spin species at neutral pH, and a 6-coordinate low spin species at alkaline pH. The proximal ligand to the heme is the imidazole group of a histidine. Kinetics of the reactions with ligands were determined by stopped-flow spectroscopy. At alkaline pH, combination with oxygen, nitric oxide, and carbon monoxide displays a kinetic behavior that is interpreted as being rate-limited by conversion of the 6-coordinate form to a reactive 5-coordinate form. At neutral pH, combination rates of the 5-coordinate form with oxygen and carbon monoxide were much faster (\(>10^7 \text{ M}^{-1} \text{s}^{-1}\)). The dissociation rate constant measured for oxygen is among the slowest in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Three hemoglobin genes have been detected in the genome of the green unicellular alga *Chlamydomonas eugametos*. Two of these, *LI637* and *LI410*, have been cloned (1). Both are expressed at the onset of the light period in cells grown under light/dark regimes. Photosynthesis is required for full expression of ferric *Slr2097* with those of several hemoglobins. *Chlamydomonas* hemoglobins are induced by light to a final concentration of approximately 130 nM within the chloroplast. As the internal pH of the stroma of the chloroplast is known to range from about pH 6.9 in the dark to more than pH 8.0 in the light (3), in this investigation we examine acid, neutral, and alkaline forms of *Chlamydomonas* chloroplast hemoglobin.

Phylogenetic analysis based on primary amino acid sequences suggests that *Chlamydomonas* hemoglobin shares a small gene family with the hemoglobins of the cyanobacteria *Nostoc commune* (4) and *Synechocystis* sp. (PCC 6803) (5) and the ciliated protozoa *Paramecium caudatum* (6) and *Tetrahymena* (7). The three-dimensional structure has not been solved for any of them. Multiple sequence alignment and comparison with myoglobin suggests that these hemoglobins have a universally conserved proximal histidine and a phenylalanine at position CD1 (1) (Fig. 1). All hemoglobins of this family are predicted to have glutamine in the distal position instead of the more usual histidine (1). Most are predicted to have tyrosine at position B10 instead of the usual leucine (1). *N. commune* with histidine at position B10 is an exception. Optical spectra of ferric *Chlamydomonas* (8) and *Nostoc* (9) hemoglobins at neutral pH indicate the presence of a low spin 6-coordinate complex, whereas those of *Paramecium* (10) and *Tetrahymena* (7) hemoglobins are consistent with a high spin 6-coordinate conformation reminiscent of the aquo ferric species of myoglobin and hemoglobin.

*Chlamydomonas* LI637 hemoglobin, when expressed in *Escherichia coli* from the cloned gene, contains a non-covalently bound protoheme and forms stable complexes with oxygen and carbon monoxide (8). The ferric protein forms complexes with cyanide and azide and also with the less usual ligands dithiothreitol and \(\beta\)-mercaptoethanol (8). Optical spectra of the 6-coordinate ferrous and ferric forms of the protein suggest structures of the distal ligand different from those usually encountered in hemoglobins. In this study, we use optical, EPR, and resonance Raman spectroscopy to probe the nature of the ligands to the ferrous heme iron of the expressed protein and of single amino acid substitution mutants. We use stopped-flow spectroscopy to establish the kinetics of the reactions with oxygen, carbon monoxide, and nitric oxide. We present evidence that the bound oxygen molecule forms multiple hydrogen bonds with the putative distal glutamine and tyrosine 63 (B10) residues that stabilize the oxygenated structure.

EXPERIMENTAL PROCEDURES

*Chlamydomonas Hemoglobin*—In this investigation, we use the monomeric recombinant protein H21 whose size reflects that of the mature
FIG. 1. Multiple sequence alignment of Chlamydomonas amino acid sequence with those of other hemoglobins and myoglobins.

The alignment was made with using a progressive alignment method (1). Helix position refers to that in sperm whale myoglobin. The position of the CD1 (*) phenylalanine, the E7 ( ), B10 ( ) and F8 ( ) residues and the C2 (−) proline is also indicated. Sperm whale myoglobin (MBSW); human hemoglobin α-chain (HAHU); lamprey hemoglobin (Life Technologies, Inc.); Aplysia kurodai hemoglobin (MAK); earthworm hemoglobin Tytorrhynchus (HECA); Lupinus luteus hemoglobin (HELY); Casuarina glauca hemoglobin (HECY).

Chlamydomonas hemoglobin (16 kDa). This was created by removing the first 24 amino acids of the LI637 protein and by substituting a lysine residue for the unique cysteine residue found at position 41 of the parent protein (8). The first residue of this recombinant protein is thus Thr-25 of the wild type hemoglobin. The H21 protein, which we refer to as Chlamydomonas wild type recombinant hemoglobin and Chlamydomonas hemoglobin in this paper, was purified as described previously (8) with the following modifications. The overall yield of purified hemoglobin in this paper, was purified as described previously (8). The presumed distal glutamine was replaced by a glycine residue with the primer 5'-CGACATGACGGCGCGGTG-3' to create the mutant hemoglobin Gly-84 → Gln; tyrosine 63, presumed to be the B10 residue, was replaced by a leucine residue with the primer 5'-CGACTCGTTTGAGAAGTCC-3' to create the mutant hemoglobin Tyr-63 → Leu; the lysine 87 was replaced by an alanine residue with the primer 5'-GGACATTGGAGGTAGGCTAG-3' to create the mutant hemoglobin Lys-87 → Ala mutant; the methionine 81 was replaced by an alanine residue with the primer 5'-GGACATGACGGCGCGGTG-3' to create the Ala mutant; the methionine 81 was replaced by an alanine residue with the primer 5'-GGACATGACGGCGCGGTG-3' to create the Ala mutant, and the proximal histidine was replaced by a glycine residue with the primer 5'-CGACATGACGGCGCGGTG-3' to create the His-111 → Gly mutant. The ratios of soluble to insoluble reduction of the oxy or ferric protein with sodium dithionite. Optimal Spectra—A Cary model 3E spectrophotometer (Varian) equipped with a thermostatically controlled multicell holder or a modified Cary model 17 recording spectrophotometer (Aviv Associates, Lakewood, NJ), equipped with an Aviv data acquisition and analysis system were used to acquire optical spectra.

The excitation source in the Raman studies was the 413.1-nm line of a krypton ion laser (Spectra Physics, Mountain View, CA). The laser power was maintained in the 0.5–2 mW range to minimize carbon monoxide (or oxygen) dissociation. The sample cell was spun at 3000 rpm to avoid local heating. The Raman scattered light was dispersed by a grating spectrometer (Aviv Associates, Lakewood, NJ), equipped with an Aviv data acquisition and analysis system were used to acquire optical spectra.

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HbO₂ was prepared by reduction of the ferric protein with a ferrodoxin-based enzymatic reducing system (11). Ferrous Hb was formed by reduction of the oxygen or ferric protein with sodium dithionite. Optical Spectra—A Cary model 3E spectrophotometer (Varian) equipped with a thermostatically controlled multicell holder or a modified Cary model 17 recording spectrophotometer (Aviv Associates, Lakewood, NJ), equipped with an Aviv data acquisition and analysis system were used to acquire optical spectra.

The excitation source in the Raman studies was the 413.1-nm line of a krypton ion laser (Spectra Physics, Mountain View, CA). The laser power was maintained in the 0.5–2 mW range to minimize carbon monoxide (or oxygen) dissociation. The sample cell was spun at 3000 rpm to avoid local heating. The Raman scattered light was dispersed by a grating spectrometer (Aviv Associates, Lakewood, NJ), equipped with an Aviv data acquisition and analysis system were used to acquire optical spectra.
through a polychromator (Spx, Metuchen, NJ) equipped with a 1200 grooves/mm grating and detected by a liquid nitrogen-cooled CCD camera (Princeton Instruments, Princeton, NJ). A holographic notch filter (Kaiser, Ann Arbor, MI) was used to remove the laser scattering. Typically, several 10-s (or 1 min) spectra were recorded and averaged after removing cosmic ray spikes by a standard software routine (CCD spectrometric multichannel analysis, Princeton Instruments, NJ). Frequency shifts in the Raman spectra were calibrated using indene as the reference. The accuracy of the Raman shifts was about ±1 cm⁻¹ for absolute shifts and less than ±0.25 cm⁻¹ for relative shifts.

**Electron Paramagnetic Resonance Spectra**—EPR spectra were obtained at liquid nitrogen and liquid helium temperatures using a Varian E112 spectrometer equipped with a Systron-Donner frequency counter and a PC-based data acquisition program. The low spin ferricyanide complex was prepared in 20 mM CHES buffer, pH 9.3, containing 10 mM cyanide, and the spectrum was collected at 9 K. The ferrous nitric oxide complex was prepared in 20 mM CHES buffer, pH 9.3, and the spectrum was collected at 77 and 11 K.

**Tryptophan Fluorescence**—Tryptophan fluorescence was measured using an SLM-Aminco 8000 fluorimeter with an excitation wavelength of 280 nm. Emission spectra were recorded from 290 to 450 nm.

**Buffers**—Different buffers were used to cover different pH ranges as follows: sodium citrate, pH 4.8; sodium acetate, pH 5–5.5; MES-NaOH, pH 5.7–6.5; MOPS-NaOH, pH 6.7–7.5; TAPS-NaOH, pH 7.7–8.5; CHES-NaOH, pH 8.7–9.5; CAPS-NaOH, pH 9.7–11; and pyrophosphate, pH 5.7–6.5; MOPS-NaOH, pH 6.7–7.5; TAPS-NaOH, pH 7.7–8.5; CHES-NaOH, pH 8.7–9.5; CAPS-NaOH, pH 9.7–11; and pyrophosphate, pH 10.0. All buffer concentrations were 50 mM and the solutions contained 50 μM EDTA. As except noted, reaction kinetics and equilibria were determined at pH 9.5 and at a temperature of 20°C.

**pH Dependence of Optical Spectra**—A separate buffer solution at each designated pH was used to prepare the samples for optical measurements. Two concentrated protein stock solutions in 10 mM MES-NaOH, pH 5.8, and CAPS-NaOH, pH 10, were used to demonstrate the reversibility of optical titrations. Each individual diluted solution was reduced anaerobically with a 3-fold molar excess of dithionite to obtain the ferrous proteins. Spectra, recorded at a temperature of 20°C, were monitored at 3-min intervals and were acquired when they showed no further change after 15 min. The midpoints of the titration curves, pKₐ values, and the optical spectra of individual components of mixtures containing multiple chemical species were calculated using Specfit software (Spectrum Software Associates, Chapel Hill, NC).

**Ligand Reaction Rates**—Reaction rates were measured using a Hi-Tech model 61 (Salisbury, UK) stopped-flow apparatus interfaced to an OLIS Data Acquisition/Computation System (On Line Instruments Systems, Bogart, GA). Rates were computed using the OLIS system.

**Carbon Monoxide Combination Rate**—Solutions of ferrous Chlamydomonas hemoglobin (5 μM heme in buffer containing a 3-fold molar excess of dithionite) were mixed rapidly with solutions of oxygen (250–1300 μM in buffer), and the reaction was followed at 426 and 408 nm, a minimum and a maximum in the HbO₂ minus ferrous hemoglobin difference spectrum, respectively.

**Nitric Oxide Combination Rate**—A solution of ferrous Chlamydomonas hemoglobin (5 μM heme in buffer containing a 3-fold molar excess of dithionite) was mixed rapidly with solutions of nitrogen (1300 μM in buffer), and the reaction was followed at 420 nm, a maximum in the HbO₂ minus ferrous hemoglobin difference spectrum.

**Oxygen Dissociation Rate**—Solutions of Chlamydomonas HbO₂ (5 μM HbO₂) were mixed rapidly with solutions of carbon monoxide (25–1000 μM in buffer), and the reaction was followed at 476 nm, a minimum in the HbNO minus ferrous Hb difference spectrum.

**Oxygen Combination Rate**—Solutions of Chlamydomonas hemoglobin (5.0 μM heme in buffer containing a 10-fold molar excess of dithionite) were mixed rapidly with solutions of nitric oxide (30–2000 μM in buffer), and the reaction was followed at 427 nm, a minimum in the HbNO minus ferrous Hb difference spectrum.

**Partition of Chlamydomonas Hemoglobin between Oxygen and Carbon Monoxide**—A solution of Chlamydomonas HbO₂ (2.9 mM heme in buffer, pH 9.5) was equilibrated at 1 atm total pressure with wet gas mixtures containing varying proportions of oxygen and carbon monoxide. Autodissociation was minimal under these conditions where the sum of the gas partial pressures was kept large. After equilibration at each gas composition was complete, optical spectra were acquired from 500 to 380 nm. At equilibrium, HbCO and HbO₂ were the only forms present at significant concentration. Calculations were made from the sum of changes at 420 and 500 nm, wavelengths of the maximum and minimum change in the HbCO minus HbO₂ difference spectrum, respectively.

**RESULTS**

**pH Dependence of the Ferrous Optical Spectrum**—The properties of the acidic, neutral, and alkaline species may be deduced from their optical and resonance Raman spectra. Fig. 2 shows that the optical spectra of ferrous Chlamydomonas hemoglobin show significant changes over the pH range 5 to 9. When the protein is reduced at pH 5.0, there is a time-dependent conversion of an initial 5-coordinate species (λ₅max 428 and 556 nm, with a minor contribution near 520 nm, Fig. 3) to a stable 4-coordinate species (see below) (λ₅max 424, 521, and 580 nm, Figs. 2, 3, and 5a). The optical spectrum at neutral pH (λ₅max 423, 529 shoulder, and 556 nm, Figs. 2 and 5a) is primarily that of a 5-coordinate species. The optical spectrum at alkaline pH (λ₅max 424, 528, and 557 nm, Figs. 2 and 5a) is that of a 6-coordinate low spin species.

In order to obtain the pKₐ values of the spectral transitions and the pure spectra of the individual species giving rise to these spectral changes, spectra, determined over the pH range 5 to 10.5, were deconvoluted using Specfit software (Figs. 4 and 5). The best fit was obtained using a model comprising three different spectral entities and two pKₐ values. Trials using a single pKₐ gave a poor fit to the data, and adding a third pKₐ did not improve the fit. Fig. 4 shows the fit of the experimental
points to the theoretical relation at three different wavelengths. Two inflections are evident in the data taken at 424 nm (Fig. 4a) and at 557 nm (Fig. 4c). The inflection at pH 8.5 is well resolved at these two wavelengths. Both inflections are clearly resolved at 435 nm (Fig. 4b). The midpoints were calculated as $p_{K_a} = 8.5$ and $p_{K_a} = 6.4$. Because the two $p_{K_a}$ values are well separated, optical spectra obtained by deconvolution of composite spectra recorded during the course of the titration corresponded closely to those taken at pH 5.0, 7.5, and 9.5 (Figs. 2 and 5a). The calculated proportions of the three ferrous species as a function of pH are given in Fig. 5b.

We considered the possibility that the protein could be denatured at the pH extremes of our measurements. To test this possibility, we measured the tryptophan fluorescence and observed no significant changes over the pH range studied in this work. This indicated that there was no significant loss of heme from the protein and no significant unfolding of the secondary structure. In addition, we found that the changes in optical absorption spectra of the ferrous protein were completely reversible on cycling the protein over the pH range studied. Furthermore, at pH 7.5, the optical spectrum showed the same mixture of predominantly 5-coordinate species (85% with the balance 6-coordinate and 4-coordinate species), in all buffers used (Hepes, Tris, or phosphate), demonstrating that the buffers have no effect on the heme coordination. This was confirmed at high pH where the same 6-coordinate low spin spectrum was obtained with CAPS, TAPS, CHES, and pyrophosphate buffers.

High Frequency Resonance Raman Spectra of Ferrous Chlamydomonas Hemoglobin—The high frequency region (1200–1800 cm$^{-1}$) of the resonance Raman spectra of hemoproteins is comprised of porphyrin in-plane vibrational modes that are sensitive to the electron density in the porphyrin macrocycle and also to the coordination and spin state of the central iron atom (12, 13). The resonance Raman spectra of the ferrous protein in the high frequency region was recorded as a function of pH (Fig. 6 and Table 1). In the alkaline form (pH 10.5), the frequency for the electron density marker line, $\nu_4$, is 1361 cm$^{-1}$ and that of $\nu_3$, which is coordination-sensitive, is 1492 cm$^{-1}$. These frequencies are typical of the Fe(II) oxidation state in the low spin form of hemoproteins in which the sixth coordination position is occupied by ligands other than oxygen and carbon monoxide, which are electron-withdrawing ligands. The neutral form (pH 7.5) shows a downshift in frequency of $\nu_4$ to 1355 cm$^{-1}$, typical of a deoxy form with no sixth ligand. The frequency of $\nu_3$ is now 1468 cm$^{-1}$, also characteristic of a 5-coordinate high spin heme. In the acidic species (pH 5.0), the frequencies of $\nu_4$ and $\nu_3$ are 1373 and 1499 cm$^{-1}$, respectively. The high frequency spectrum of the acidic species is similar to that reported for 4-coordinate ferrous model compounds (14–16) and for ferric 6-coordinate low spin species (17). For both coordination states, the iron is expected to reside in a planar heme as there are no non-bonded interactions from axial ligands in the 4-coordinate case, and they are balanced in the 6-coordinate case. Consequently, for an intermediate ($S = 1$) or low spin planar configuration of a 4-coordinate species with an unoccupied $d_{x^2-y^2}$ orbital, the frequency of $\nu_3$ would be similar to that of a 6-coordinate low spin heme complex. Raman spectra

![Fig. 3. Optical spectrum of ferrous Chlamydomonas hemoglobin at pH 5 as a function of time. Spectra were recorded immediately (dashed line) and 6 min (solid line) following reduction with a 5-fold molar excess of sodium dithionite.](image)

![Fig. 4. Titration curve of ferrous Chlamydomonas hemoglobin. The titration curve was followed at 424 nm (a), 435 nm (b), and 557 nm (c) as a function of pH. The solid curve is calculated for two ionizations with $p_{K_a}$ at 6.4 and 8.5, respectively. The data residuals are displayed above each titration curve.](image)
of model 4-coordinate ferrous intermediate spin heme complexes (14–16) are, in fact, very similar to those observed for ferric 6-coordinate low spin complexes. To determine the oxidation state of the heme, imidazole was added to the sample, and the resonance Raman spectrum was measured. A spectrum was obtained that was characteristic of a ferrous 6-coordinate low spin heme indicating that prior to the addition of the imidazole the heme was in its ferrous oxidation state. Thus, we assign the acidic form of *Chlamydomonas* hemoglobin as a ferrous 4-coordinate form.

**Low Frequency Resonance Raman Spectra of Ferrous, Oxy, and Carbonmonoxy Chlamydomonas Hemoglobin**—The low frequency region of the resonance Raman spectrum of hemeproteins is comprised of several in-plane and out-of-plane vibrational modes of the heme including heme propionate modes, vinyl modes, and ligand vibrational modes (13). The axial ligand vibrational modes arise from electronic coupling of the ligand to the metal electronic orbitals. The assignment of a ligand vibrational mode is extremely useful as it directly identifies a particular ligand and the nature of its interactions with amino acid residues in the heme pocket. In particular, the Fe-His (proximal) stretching mode \( v_{\text{Fe-His}} \) can be identified in the 5-coordinate deoxy forms of hemoglobins in the 200–250 cm\(^{-1}\) region. The \( v_{\text{Fe-His}} \) mode of deoxy *Chlamydomonas* hemoglobin at pH 7.5 is assigned to the line at 232 cm\(^{-1}\) (Fig. 7). This mode is not seen in 6-coordinate ferrous and ferric forms of hemeproteins. The observation of the Fe-His stretching frequency at neutral pH indicates the presence of histidine as the proximal ligand of a 5-coordinate heme. The absence of this line in the resonance Raman spectrum of ferrous *Chlamydomonas* hemoglobin at high pH (Fig. 8) is consistent with hexa-coordination of the heme.

The low frequency regions of the resonance Raman spectra of the alkaline form of ferrous *Chlamydomonas* hemoglobin in H\(_2\)O and in D\(_2\)O are presented in Fig. 8. The two spectra are very similar except for the band at 335 cm\(^{-1}\). Although this particular mode cannot be assigned to a specific vibration, it

\[ \text{The weak line at 228 cm}^{-1} \text{ in the high pH spectrum (Fig. 8) is assigned as a porphyrin mode rather than a weak Fe-His stretching mode based on the high frequency spectrum (Fig. 6b) which indicates a 6-coordinate low spin heme.} \]
may be a porphyrin internal mode or a mode from an amino acid residue closely coupled to the heme. In D₂O, the peak position of this line undergoes a 6-cm⁻¹ downshift in frequency as well as a drastic loss in intensity. It appears that the band at 335 cm⁻¹ experiences resonance enhancement due to coupling with heme vibrations, but the mode becomes uncoupled in D₂O due to the frequency shift, and it thereby loses its acquired intensity. Intensity enhancement and a frequency shift of Raman bands due to vibrational coupling of the heme porphyrin macrocycle with adjacent chemical species indeed have been shown in the past (18). We invoke a similar vibrational coupling mechanism in Chlamydomonas hemoglobin.

To investigate further the nature of the heme pocket of Chlamydomonas hemoglobin, the carbonmonoxy derivative was also studied. The Fe—CO stretching frequency is sensitive to the nature of distal interactions with carbon monoxide and is also dependent on the nature of the proximal ligand. An added advantage of studying the carbonmonoxy derivative is that the ligand can be photolyzed to yield a transient population of 5-coordinate deoxy species. Fig. 9 shows the resonance Raman spectrum of the CO adduct in which the line at 491 cm⁻¹ is assigned as the Fe—CO stretching mode and the line at 572 cm⁻¹ is assigned as the Fe—C=O bending mode. Each of these assignments was confirmed by isotope (¹³C¹⁸O) substitution experiments (491/482 and 572/559 cm⁻¹, respectively). The C=O stretching mode, also determined from isotope substitution experiments, is assigned at 1957 cm⁻¹ (1868 cm⁻¹ for ¹³C¹⁸O, data not shown). Partial photodissociation of bound carbon monoxide from Chlamydomonas hemoglobin was achieved with high laser power (400 mW). It was observed that the intensity of the line at 232 cm⁻¹ increases with increasing laser power. Thus, this line is seen in the deoxy form of the protein produced either during photodissociation or upon chemical reduction. This ob-
observation is consistent with our assignment of the 232 cm\(^{-1}\) line as the Fe-His stretching mode of the 5-coordinate deoxy heme. In contrast to the observed photolability of the carbon monoxide adduct of Chlamydomonas hemoglobin and the photolability of most oxy complexes of hemoglobins, we find that in the presence of high incident beam powers (\(\sim 600\) mW of power at 413.1 nm), the spectrum of the high frequency region (\(n > 3\)) of the oxy derivative of Chlamydomonas hemoglobin shows no formation of a photodissociated (5-coordinate, deoxy) species. We conclude that this oxyhemoglobin is not photolabile under our experimental conditions.

Electron Paramagnetic Resonance—At alkaline pH and in the presence of 10 mM cyanide (\(\sim 0.8\) mM protein), a signal was observed at \(g = 3.19\), consistent with that of a ferric heme cyanide complex with a trans-histidyl ligand. This signal is likely the \(g_{\text{max}}\) for the cyanide complex and indicates a low spin form with anisotropy similar to that of myoglobin cyanide (19) but greater than that for the cyanide complexes of horseradish or cytochrome \(c\) peroxidases (20). This suggests that the endogenous axial ligand of ferric Chlamydomonas hemoglobin is likely to be a neutral imidazole as in the hemoglobins, rather than one with imidazolate character as in the peroxidases (21).

Upon binding of NO to the ferrous protein at alkaline pH, a rhombic spectrum typical for a 6-coordinate heme-NO complex was obtained (Fig. 10, upper), showing a triplet splitting pattern, characteristics of hyperfine interaction with \(^{14}\)N (\(I = 1\)), near \(g = 2\). A derivative (22) of this spectrum (Fig. 10, lower) shows that the hyperfine pattern is a triplet (21–22 G) of triplets (6–7 G). This hyperfine pattern is indicative of interaction of the unpaired electron with two \(I = 1\) nuclei and strongly suggests the presence of two axial \(^{14}\)N ligands to the ferrous iron of the NO complex of Chlamydomonas hemoglobin. The magnitudes of the two sets of \(^{14}\)N hyperfine splittings are comparable to those found for NO-bound ferrous hemoproteins with axial histidine (23, 24). The larger hyperfine coupling (21–22 G) arises from the \(^{14}\)NO nitrogen, whereas the smaller coupling (6–7 G) is from the proximal histidyl imidazole nitrogen. The EPR of Chlamydomonas HbNO is therefore consistent with histidine as the proximal ligand as suggested by resonance Raman studies of the deoxy ferrous and FeCO proteins and by the EPR study of the ferric cyanide protein.

Oxygen Dissociation—A single homogeneous kinetic event, independent of the observation wavelength or of the presence or absence of dithionite, was recorded upon replacement of bound oxygen by carbon monoxide. The rate was the same in 10 mM Tris-HCl buffer, pH 7.5 (0.0141 s\(^{-1}\)); in 50 mM sodium phosphate buffer, pH 7.5 (0.0133 s\(^{-1}\)); and in 50 mM CAPS-NaOH buffer, pH 10.5 (0.0140 s\(^{-1}\)). The same rate was observed by trapping dissociated oxygen with dithionite alone. These data show that dithionite does not react with the oxygen adduct, but does remove dissociated oxygen. The rates measured in the presence of dithionite reflect oxygen dissociation per se.

Carbon Monoxide Dissociation—Inhomogeneous apparent rate constants could be resolved into a major rate (about 90% of the total), \(k_{\text{off}} = 2.14 \times 10^{-3} \text{ s}^{-1}\), and a faster minority rate. The major rate is similar to those of soybean leghemoglobin and sperm whale myoglobin (Table II).
ferrous Chlamydomonas Hemoglobin

TABLE II

Kinetics and equilibrium constants for the reactions of ferrous Chlamydomonas hemoglobin with oxygen and carbon monoxide compared with those of other proteins

| Protein                | \( k_{on} \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1} \) | \( k_{off} \text{ s}^{-1} \) | \( K_D = (k_{on}/k_{off}) \) | \( t_{on} \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1} \) | \( t_{off} \text{ s}^{-1} \) | \( L_D (=1:1) \) | \( M' \) |
|------------------------|--------------------------------------------------------|------------------------------|-------------------------------|--------------------------------------------------------|------------------------------|-----------------|----------------|
| Chlamydomonas Hb       | 0.141                                                   | 95                           | 1.34                          | 0.0022                                                  | 20                           | 5.0             |                |
| Barley Hb\(^{b}\)      | 0.0272                                                 | 3.82                         |                               | 0.0011                                                 | 1.93                         | 2.0             |                |
| Rice Hb\(^{c}\)        | 0.038                                                   | 0.56                         |                               | 0.001                                                   | 0.14                         | 4.0             |                |
| Arabidopsis Hb\(^{d}\) | 0.12                                                   | 1.6                          |                               |                                                        |                              | 2.0             |                |
| Arabidopsis Hb\(^{e}\) | 0.14                                                   | 130                          |                               |                                                        |                              | 26              |                |
| Lactua HBII\(^{f}\)    | 0.39                                                   | 282                          |                               | 0.0071                                                 | 370                          | 0.76            |                |
| Ascaris HB\(^{g}\)     | 0.0041                                                 | 2.7                          |                               | 0.018                                                   | 1.1                          | 2.5             |                |
| Soybean Lha\(^{h}\)    | 120                                                    | 48                           |                               | 0.0076                                                  | 0.62                         | 75              |                |
| Sperm whale Mb\(^{i}\) | 0.018                                                  | 37                           |                               | 0.015                                                   | 37                           | 23              |                |

\(^{a}\) Combination rates, \( k_2 \) of 5C Chlamydomonas Hb, with ligands are rate-limited by the conversion of a 6C species to a 5C species prior to ligand binding (see Scheme 1 under “Discussion”). The overall ligand binding reactions are best described by \( k_1 \) and the ratio \( k_2/\Delta k_1 \) (see Equation 1 under “Discussion”). The values of \( k_1 \) are 192, 165, and 169 s\(^{-1}\) for oxygen, carbon monoxide, and nitric oxide, respectively. Those of the ratio \( k_2/\Delta k_1 \) are 113 \( \mu \text{M} \) for carbon monoxide and 101 \( \mu \text{M} \) for nitric oxide, respectively. The ratio \( k_2/\Delta k_1 \) for oxygen, calculated from the partition coefficient, \( M' \), the value of \( k_1/\Delta k_1 \), for carbon monoxide, and the dissociation rate constants, \( k_{off} \) and \( t_{off} \) for oxygen and carbon monoxide, is 145 \( \mu \text{M} \).

\(^{b}\) Data of Duff et al. (53). Values of \( k_{on} \) and \( t_{on} \) are valid at low concentration of ligand. At high ligand concentration, the combination rate with ligand is limited by the rate of conversion of a 6C to a 5C species. From the analysis of the CO combination data of Duff et al. (53) according to Equation 1, values of 47 s\(^{-1}\) and 113 \( \mu \text{M} \) were obtained for \( k_1 \) and for the ratio of \( k_2/\Delta k_1 \), respectively.

\(^{c}\) Data of Arredondo-Peter et al. (52).

\(^{d}\) Data of Treviski et al. (37). Data at pH 7.0.

\(^{e}\) Data of Kraus and Wittenberg (32).

\(^{f}\) Data of Gibson and Smith (61).

\(^{g}\) Data of Gibson et al. (62).

\(^{h}\) Data of Springer et al. (41).

Oxygen, Carbon Monoxide, and Nitric Oxide Combination—Single rates following apparent first order kinetics, independent of wavelength, were observed at pH 9.5 at high oxygen concentrations (>200 \( \mu \text{M} \), Fig. 11) and were also observed in the combination of nitric oxide and carbon monoxide with ferrous Chlamydomonas hemoglobin at all concentrations examined. Oxygen binding measurements at low \( p_\text{O2} \) were complicated by competing oxidation of the ferrous protein to the ferric form. As shown in Fig. 11, the rates of combination of oxygen, carbon monoxide, and nitric oxide with alkaline ferrous Chlamydomonas hemoglobin tend toward a limiting value, ~160 s\(^{-1}\), at high ligand concentration. We propose that the rate of conversion of the 6C alkaline species to a 5C species prior to ligand binding limits the rate of combination with ligands (see “Discussion”). The rates of combination with oxygen and carbon monoxide at pH 7.5 were too fast to measure by stopped-flow spectrophotometry (>10\(^3\) M\(^{-1}\) s\(^{-1}\)) in the range of ligand concentration where pseudo-first order kinetics could be achieved.

Partition of Ferrous Chlamydomonas Hemoglobin between Oxygen and Carbon Monoxide—The partition coefficient expresses the relative affinity of the protein for carbon monoxide and oxygen. A plot of the ratio [HbCO]/[HbO\(_2\)] against the ratio \( p\text{CO}p_\text{O2} \) was linear over the range examined (Fig. 12). The partition coefficient, \( M' \), given by the slope of this relation, is \( M' = 3.70 \). This value is expressed in terms of the gaseous pressures. A value, \( M' \), corrected for the solubility of the gases and expressed in molar terms, is related to \( M \) by \( M' = 1.34 \times M = 5.0 \) (Table II).

Chlamydomonas Hemoglobin Mutants—In order to identify the sixth ligand of the alkaline form of ferrous Chlamydomonas hemoglobin, four single amino acid substitutions were studied: Tyr-63 → Leu (B10), Met-81 → Ala (E4), Gln-84 → Gly (E7), and Lys-87 → Ala (E10). A multiple sequence alignment, Fig. 1, indicates how the positions of these residues were assigned. Optical and resonance Raman spectra were used to investigate the role of these residues. Although none of these substitutions allowed us to identify the sixth ligand, they nevertheless revealed distinctive features of the heme pocket structure (Table III).

First, the 4-coordinate form observed at acidic pH depends on the presence of these four residues. Indeed, in contrast to the wild type recombinant protein, none of the mutants showed the 4-coordinate form. Second, the sixth ligand of the alkaline species is stabilized by both tyrosine 63 and lysine 87. As shown in Table III, mutagenesis of these residues has a major disruptive effect on the heme pocket. The Tyr-63 → Leu mutant remains 6-coordinate, low spin from pH 5.0 to 9.5, whereas the Lys-87 → Ala mutant is always a mixture of a majority 6-coordinate low spin species and a minority 5-coordinate, high spin species over the whole pH range studied (pH 5.0–9.5). In contrast, mutation of the glutamine 84 or methionine 81 residues has only minor effects on the pK\(_a\) and ligand coordination. Observations of 6-coordinate species in all of the mutants at alkaline pH indicate that the sixth ligand in the alkaline wild type form is not necessarily the same as that present in the various mutants.

Oxygen Dissociation Rates and Autoxidation Rates of Chlamydomonas Hb Mutants—The oxygen dissociation rates measured for the putative distal glutamine mutant, Gln-84 → Gly, and the B10 tyrosine mutant, Tyr-63 → Leu, increased by 30- and 70-fold (Table IV), and the autoxidation rates increased by 90- and 25-fold, respectively, as compared with the wild type protein (Table IV). In contrast, another heme pocket mutant, Lys-87 → Ala, shows little difference in the oxygen dissociation and the autoxidation rates compared with those of the wild type protein (Table IV).

DISCUSSION

C. eugametos chloroplast hemoglobin (encoded by the gene \( Li637 \)) is expressed in response to light and requires photosynthesis for its full expression (1, 2). Within the chloroplast of the wild type cells, the hemoglobin concentration does not exceed 130 nM, far too little to store a metabolically significant amount of oxygen or to facilitate oxygen movement, except possibly within very limited structural domains. Within the cell, deliv-
ery of oxygen to possible oxygen-consuming enzymes will be limited by the rate of oxygen dissociation. It would appear that oxygen dissociation from *Chlamydomonas* hemoglobin, among the slowest known, $t_{1/2} = 49$ s, is far too slow to support a metabolic function requiring dissociation of hemoglobin-bound oxygen. The function of *Chlamydomonas* hemoglobin remains to be determined.

Sequence alignment, and comparison with myoglobin, suggests that the proximal heme residue of *Chlamydomonas* hemoglobin is histidine; the distal residue is glutamine; and tyrosine occupies position B10 (1) (Fig. 1). EPR and resonance Raman spectra reported here confirm the assignment of histidine as the proximal residue. Site-directed mutagenesis of the putative proximal histidine residue, His-111, leads to expression of an apoprotein, which does not bind heme (data not shown). Several hemoglobins, many characterized by very high oxygen affinity, share with *Chlamydomonas* hemoglobin the simultaneous presence of a distal glutamine and a tyrosine residue in position B10. Among these are the hemoglobins of numerous nematodes (25) including *Caenorhabditis elegans* (25–27) and *Ascaris* (28–31) and, in addition, a hemoglobin of the clam *Lucina*, HbII (31–33). Trematode hemoglobins which have very high oxygen affinities have a tyrosine residue in position B10, in addition to tyrosine in the distal E7 position (34, 35). Tyrosine B10 alone, in the absence of a distal glutamine residue, as in the symbiotic and nonsymbiotic plant hemoglobins (36, 37), plays a poorly understood role in defining oxygen affinity.

**TABLE III**

| Heme coordination of wild type and single amino acid mutants of *Chlamydomonas* hemoglobin at acid, neutral, and alkaline pH |
|---|
| Acid species (pH 5) | Neutral species (pH 7.5) | Alkaline species (pH 10.5) |
| Wild type | 4-Coordinate | 5-Coordinate | 6-Coordinate |
| Gln-84 → Gly | 5-Coordinate | 5-Coordinate | 6-Coordinate |
| Me-81 → Ala | 5-Coordinate | 5/6-Coordinate<sup>a</sup> | 6-Coordinate |
| Tyr-63 → Leu | 6-Coordinate | 6-Coordinate | 6-Coordinate<sup>b</sup> |
| Lys-87 → Ala | 5/6-Coordinate<sup>a</sup> | 5/6-Coordinate<sup>a</sup> | 5/6-Coordinate<sup>a</sup> |

<sup>a</sup> Mixture of 5- and 6-coordinate species.

<sup>b</sup> Spectra recorded at pH 9.5.

**TABLE IV**

| Oxygen dissociation rates and autoxidation rates of ferrous wild type and single amino acid mutants of *Chlamydomonas* hemoglobin |
|---|
| Oxygen dissociation rate $k$ (s$^{-1}$) | Autoxidation rate $t_{1/2}$ (h) |
| *Chlamydomonas* Hb | | |
| Wild type | 0.0141 | 169<sup>a</sup> |
| Lys-87 → Ala | 0.0222 | 172 |
| Gln-84 → Gly | 0.444 | 1.9 |
| Tyr-63 → Leu | 0.948 | 7.1 |
| Horse heart myoglobin | 10.0<sup>b</sup> | 115<sup>b</sup> |

<sup>a</sup> Data of Couture and Guertin (8).

<sup>b</sup> Data of Antonini and Brunori (63).

The rates of ligand combination approach a limiting value of $>160$ s$^{-1}$ at the highest ligand concentration attainable.
due, Gln-84 → Gly, or of tyrosine B10, Tyr-63 → Leu, increases the rate of oxygen dissociation 30- and 70-fold and increases the rate of autoxidation 90- and 20-fold, respectively, as summarized in Table IV. We suggest that these two residues may form hydrogen bonds stabilizing the bound oxygen molecule. The rate of oxygen dissociation from wild type oxy Chlamydomonas hemoglobin is the same from pH 7.5 to pH 10.5. This suggests that the structure of the oxygen complex does not depend on protonatable groups titrating within this range.

Instances in which the distal residue forms stabilizing hydrogen bonds to the hemoglobin-ligated oxygen molecule are many (38, 39). Imidazole from histidine plays this role in the majority of vertebrate hemoglobins and myoglobins but may be replaced by glutamine, as in elephant myoglobin (40) or in mutant versions of sperm whale myoglobin (41). When glutamine replaces histidine, hydrogen bond formation from the distal glutamine to the bound oxygen molecule becomes a common theme among invertebrate hemoglobins and myoglobins (38). Among these, Ascaris hemoglobin offers a strong analogy to Chlamydomonas hemoglobin. In Ascaris hemoglobin, tyrosine B10, together with the distal glutamine residue, contributes to a network of hydrogen bonds believed to stabilize the bound oxygen molecule, see Table II (28–31). A still more elaborate network of hydrogen bonds involving the distal glutamine, tyrosine B10, and, in addition, a water molecule, accounts for the slow dissociation of oxygen from Lucina HbII (31–33), see Table II.

Acidic Ferrous Chlamydomonas Hemoglobin—The optical spectra of ferrous Chlamydomonas hemoglobin are pH-dependent (Figs. 2, 4, and 5a). This dependence is best fit by a model involving two pKₐ values (8.5 and 6.4) and three different optical entities. In the following sections we discuss the properties of these species.

At pH 5.0, ferrous Chlamydomonas hemoglobin exists as a stable 4-coordinate species (Figs. 2, 3, and 5a and Table I). Observation of a 4-coordinate heme in Chlamydomonas hemoglobin at acidic pH indicates that the proximal histidine-iron bond is broken. This is unusual because mammalian ferrous myoglobins and hemoglobins retain their proximal ligand in a similar pH range. Hence, the heme pocket structure and strain on the proximal ligand bond must be different from other hemoglobins and myoglobins. Breaking of the proximal histidine ligation in Chlamydomonas hemoglobin is not caused by protein unfolding, indicated by the absence of any significant increase in the tryptophan fluorescence in the pH range 5–9.5. In addition, the heme does not fall out of the heme pocket, which would also be expected to result in a large increase in fluorescence. The optical absorption spectrum is also consistent with the above suggestion as the Soret band of the 4-coordinate species of Chlamydomonas hemoglobin is located at 421 nm in comparison to highly blue-shifted Soret band (∼380 nm) in acid-denatured deoxymyoglobin (42) and model heme complexes in aqueous solutions. Thus, in Chlamydomonas hemoglobin in the absence of its axial ligation, the heme is stabilized in the pocket by non-bonded interactions. This is consistent with reports of stable 5-coordinate, hydroxide-bound heme groups in mutants of myoglobin and cytochrome c peroxidase in which the proximal histidine has been replaced by glycine (43, 44). The protein structures of these mutants do not unfold. On the other hand, in sperm whale deoxymyoglobin at low pH (pH < 4), unfolding has been proposed to be a prerequisite for cleavage of the proximal histidine bond (42). Reversible formation of a 4-coordinate species at acidic pH has been observed only in wild-type recombinant Chlamydomonas hemoglobin. None of the mutants examined formed this species, suggesting that each of the mutated heme pocket residues contributes toward stabilizing this unique structure.

Neutral Ferrous Chlamydomonas Hemoglobin—The optical spectrum of neutral ferrous Chlamydomonas hemoglobin (Fig. 5 and Table I) is consistent with a high spin 5-coordinate heme as found in other globins at neutral pH. Furthermore, the resonance Raman spectra supply strong evidence that the fifth ligand is histidine. A line at 232 cm⁻¹ (Fig. 7) is assigned to the Fe-His stretching mode of this 5-coordinate species (45). Our assignment of this mode is based on the following lines of evidence. First, the resonance Raman spectrum in the high frequency region demonstrates that the ferrous protein is 5-coordinate. It is only under such conditions that the Fe-His mode appears in the resonance Raman spectrum. Second, EPR of the CN complex and of the NO complex establish that histidine is coordinated to the proximal position of both the ferric and ferrous hemes, respectively. Third, on photodissociation of the CO-bound species, the 232 cm⁻¹ line, which was absent in the presence of CO, reappeared in the spectrum. The frequency of 232 cm⁻¹ is very high for a hemoglobin under equilibrium conditions (46). It is the same as in photodissociated hemoglobins prior to structural relaxation in which there is no strain on the proximal histidine to iron bond (47), and it is the same in peroxidases when the proton on the proximal histidine is either absent or strongly hydrogen-bonded to amino acid residues (48). To distinguish between these two possibilities, we turn to the EPR spectra. The EPR spectrum of the ferric cyanide complex is consistent with a neutral imidazole such as that found in myoglobin and is not consistent with the deprotonated imidazole group. We note that the (first derivative) EPR spectrum of the FeNO protein (Fig. 10, upper) has poorly resolved hyperfine splittings commonly observed for NO-bound globins, whereas for the Fe-NO complexes of peroxidases, it is possible to resolve hyperfine splittings for both axial nitrogens without the use of second derivative analysis such as that shown in the lower part of Fig. 10 (23). Based on the comparison of these two spectroscopic studies, we conclude that the histidine adopts a perpendicular orientation to the heme, and there is no strain induced on the proximal histidine to iron bond in ferrous Chlamydomonas hemoglobin at neutral pH. This should contribute to a high ligand affinity since the iron can be pulled into the heme plane without the restraints imposed to it by the protein as occurs in most other hemoglobins.

The spectra of the CO-bound form provide additional evidence in support of the assignment of a neutral imidazole as the proximal ligand. It is widely accepted that the Fe–CO bond order. The Fe–CO stretching mode, detected at 1941 cm⁻¹ (Fig. 9), and the C–O stretching line at 1957 cm⁻¹, are characteristic of a hemoglobin or myoglobin with an open heme pocket with no positive groups interacting directly with the bound CO. Most myoglobin mutants lacking a distal histidine display similar frequency of the Fe–CO stretching mode (50). The Fe–CO and C–O frequencies of Chlamydomonas hemoglobin fall on the correlation line (data not shown) which is characteristic for hemeproteins that contain histidine as the proximal ligand. The part of the Fe–CO versus C–O frequency correlation line with the higher Fe–CO frequencies and the lower C–O frequencies is generally occupied by per-
oxidases that have a proximal imidazolate, and the part of the line with the lower Fe—CO frequencies and the higher C—O frequencies is occupied by heme proteins that have a neutral proximal imidazole (51). The fact that *Chlamydomonas* hemoglobin falls on the part of the correlation line with the lower Fe—CO frequencies and the higher C—O frequencies is consistent with a neutral imidazolate character of its proximal ligand.

In marked contrast to the 6-coordinate alkaline ferrous form which does not react directly with oxygen, the 5-coordinate ferrous *Chlamydomonas* hemoglobin observed at neutral pH combines very rapidly, >10^7 μM⁻¹ s⁻¹, with oxygen and carbon monoxide. These combination rates are comparable to those of sperm whale myoglobin, see Table II. The behavior of *Chlamydomonas* hemoglobin is analogous to that of rice hemoglobin, where a 5-coordinate mutant species, His(E7)→Leu, reacts more rapidly with oxygen than the native 6-coordinate ferrous species (52). Our finding bolsters the suggestion, made below, that 5-coordinate and 6-coordinate forms are in equilibrium, and only the 5-coordinate form is able to combine directly with ligands.

**Alkaline Ferrous Chlamydomonas Hemoglobin**—The clearly resolved optical spectrum of alkaline ferrous *Chlamydomonas* hemoglobin is similar to those of a number of 6-coordinate low spin ferrous derivatives of heme proteins and model compounds with oxygen, nitrogen, or sulfur atoms of the distal ligand coordinated to the heme iron. These include ferrous hemochromogens, ferrous cytochromes b and c, barley (53) and rice (52) hemoglobins, ferrous myoglobin or horseradish peroxidase cyanides (54), ferrous myoglobin or leghemoglobin nicotinates (55, 56), and aquo ferrous myoglobin (57). The resonance Raman spectra are also consistent with 6-coordinate low spin heme in the alkaline form of ferrous *Chlamydomonas* hemoglobin.

Although we cannot make a definitive determination of the nature of the sixth ligand in alkaline ferrous *Chlamydomonas* hemoglobin, we can rule out several possibilities. Occupancy of the sixth coordination position by a component of the buffer is ruled out by the insensitivity of the optical and resonance Raman spectra to the nature of the buffers. Imidazole from histidine is an unlikely candidate, as there are no histidines in the distal pocket based on the amino acid sequence alignment (see Fig. 1). Resonance Raman data make hydroxide or water unlikely potential sixth ligands, because spectra taken in H₂O (Fig. 8) or H₂¹⁸O were identical. Site-directed mutagenesis of heme pocket residues suggests that the Tyr-63 and Lys-87 groups may either be implicated in formation of the distal ligand of the alkaline ferrous form or may interact with that ligand, perhaps by formation of hydrogen bonds.

The 335 cm⁻¹ line in the resonance Raman spectrum of Chlamydomonas hemoglobin at high pH changes significantly in D₂O (Fig. 8) indicating that an exchangeable proton is close to or associated with the heme moiety. Such a change has not been reported in other hemoglobins or myoglobins. The involvement of the heme protonates is ruled out because the bending mode of the protonates at 380 cm⁻¹ (13) remains unchanged in both solvents. We propose that the protonatable group may originate from a chemical species adjacent to the heme, such as the distal ligand, but not restricted to it.

**Combination of Alkaline Ferrous Chlamydomonas Hemoglobin with Ligands**—Optical and resonance Raman spectra indicate that a 6-coordinate low spin species is strongly favored in an equilibrium between the 6- and 5-coordinate forms at alkaline pH. *A priori*, an attacking ligand would be expected to react only with a 5-coordinate form, generated by prior dissociation of the sixth ligand from the heme iron atom, as has been observed in ferrous barley and other plant hemoglobins (37, 52, 53). In general, any ligand occupying the heme-binding site must leave before it can be replaced by an incoming ligand (58).

As shown in Fig. 11, the rates of combination of oxygen, carbon monoxide, and nitric oxide with alkaline ferrous *Chlamydomonas* hemoglobin tend toward a common limiting value at high ligand concentration. A simple interpretation is that, at high ligand concentration, combination of ligand with the minority 5-coordinate species is very rapid, and conversion of the dominant 6-coordinate form to the 5-coordinate form is rate-limiting.

A kinetic model describing this sequence of reactions is presented below in Scheme I (Ref. 59 and references therein).

```
6-C Hb = k₅-C Hb

5-C Hb + L = HbL
```

**Scheme I**

where 6C and 5C are the 6-coordinate and 5-coordinate ferrous forms, L is the exogenous ligand added, and HbL is the hemoglobin-exogenous ligand complex; k₁ and k₂ are the forward and backward rate constants describing the equilibrium between the 6- and 5-coordinate forms of the protein; k₃ is the second order combination rate constant for exogenous ligand binding to the 5-coordinate ferrous form; and k₋₂ is the dissociation rate constant of that ligand from the heme. The relation shown in Equation 1 describes ligand binding according to Scheme I when k₋₂ is sufficiently small to be considered negligible (59).

\[
k_{obs} = k_{1}[L]/[L] + (k_{-2}k_{3}) (Eq. 1)
\]

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constant at low concentration of ligand. Under this condition, Equation 1 becomes \( k_{\text{obs}} = \frac{k_{2}k_{1}}{k_{-1}}[\text{L}] \). The apparent bimolecular rate constant at low ligand concentration is therefore \( k_{2}k_{1}/k_{-1} \). However, in the case of Chlamydomonas hemoglobin, when the concentration of ligand is sufficiently small to be considered negligible, smaller than that at the lowest points in Fig. 11, pseudo-first order conditions are no longer attained, and this simplified equation cannot be applied. In addition, the apparent bimolecular rate constant at low concentration of ligand cannot be obtained from the initial slope of the relation shown in Fig. 11 (see “Appendix”). Therefore, kinetics of combination with ligands for Chlamydomonas hemoglobin and other proteins, for which the overall ligand binding scheme contains a rate-limiting step, are best described by \( k_{1} \) and the ratio of \( k_{-1}/k_{2} \).

Distinct, phylogenetically related gene families encode Chlamydomonas hemoglobin (1) and the nonsymbiotic higher plant hemoglobins (37, 60). Nevertheless, the two hemoglobin groups share many common properties. These include extraordinarily slow dissociation of oxygen bound to Chlamydomonas hemoglobin and to barley (53), rice (52), and Arabidopsis (37) nonsymbiotic hemoglobins; 6-coordinate, low spin ferric forms (52, 53); 6-coordinate, low spin ferrous forms (37, 52, 53) in which the sixth ligand to the heme iron must dissociate prior to combination of the hemoglobin with oxygen or CO (53); and very rapid autoxidation at low ligand pressures where the heme iron is not fully occupied.\(^7\) These common properties are achieved very differently in the two groups. The distal ligand to the heme iron atom of 6-coordinate ferrous barley\(^5\) and rice (53) hemoglobins is the distal histidine residue, while in Chlamydomonas, it must be entirely different, since there is no distal histidine. Likewise, stabilization of the bound oxygen molecule, with consequent slow dissociation of oxygen from oxyhemoglobins, is achieved very differently in the two groups. In nonsymbiotic hemoglobins, e.g. barley (53),\(^6\) rice (52), and possibly Arabidopsis (37) hemoglobins, the distal histidine residue probably forms a hydrogen bond to the bound oxygen. In contrast, stabilization of the bound oxygen of Chlamydomonas hemoglobin requires both the distal glutamine and tyrosine B10, each contributing to a network of stabilizing hydrogen bonds. This similarity of behavior suggests that, under selection pressure, proteins with different heme pocket structures adapt to achieve similar functional properties.

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\(^7\) J. B. Wittenberg and M. Couture, unpublished observations of barley and Chlamydomonas hemoglobins.

APPENDIX

For most hemoglobins and myoglobins, combination of the ferrous form with ligands follows the simple Scheme II.

\[
\text{Hb} + \text{L} \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} \text{HbL}
\]

SCHEME II

where \( k_{\text{on}} \) is the bimolecular combination rate constant and \( k_{\text{off}} \) is the dissociation constant. When the reaction is carried out under pseudo-first order conditions (high ligand concentration), the observed combination rates can be described mathematically with Equation 2.

\[
k_{\text{obs}} = k_{\text{on}}[\text{L}] + k_{\text{off}}
\]

On the graph of the apparent rate for ligand binding (\( k_{\text{obs}} \)) as a function of ligand concentration (L), the apparent rate increases linearly with increasing ligand concentration. The slope of the relation corresponds to the bimolecular combination rate constant, while the projection of the relation to the y axis corresponds to the dissociation rate constant.

For hemoglobins displaying a rate-limiting step in combination with ligands, such as Chlamydomonas hemoglobin (Fig. 11), an approximation of the apparent bimolecular combination rate constant can be obtained from the initial slope of the relation only if the apparent rates measured at relatively low concentration of ligands, where \( k_{\text{obs}} \) is not rate-limited by the conversion of the 6C species to a 5C species, follow a linear relationship. This was the case for barley hemoglobin (53). For Chlamydomonas hemoglobin, if a line is drawn over the first four points of the CO combination data, the apparent straight line obtained crosses the y axis at a value of \(-40 \text{ s}^{-1}\), a value too high to reconcile with the CO dissociation rate of 0.0022 s\(^{-1}\) measured by replacement with oxygen or nitric oxide. To extract quantitative information from the data, we have curve fit data with Equation 1 (see “Discussion”), the results are shown in Fig. 13. Inspection of the curve fit of the carbon monoxide combination data reveals that the data points at low ligand concentration do not follow a linear relationship. Quantitative information obtained from the mathematical treatment of the data is reported in Table II.

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