Identification of the Ligands to the Ferric Heme of *Chlamydomonas* Chloroplast Hemoglobin: Evidence for Ligation of Tyrosine-63 (B10) to the Heme†

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ABSTRACT: We have studied the unusual heme ligand structure of the ferric forms of a recombinant *Chlamydomonas* chloroplast hemoglobin and its several single-amino acid mutants by EPR, optical absorbance, and resonance Raman spectroscopy. The helical positions of glutamine-84, tyrosine-63, and lysine-87 are suggested to correspond to E7, B10, and E10, respectively, in the distal heme pocket on the basis of amino acid sequence comparison of mammalian globins. The protein undergoes a transition with a p*K* of 6.3 from a six-coordinate high-spin aquomet form at acidic pH to a six-coordinate low-spin form. The EPR signal of the low-spin form for the wild-type protein is absent for the Tyr63Leu mutant, suggesting that the B10 tyrosine in the wild-type protein ligates to the heme as tyrosinate. For the Tyr63Leu mutant, a new low-spin signal resembling that of alkaline cytochrome *c* (a His−heme−Lys species) is resolved, suggesting that the E10 lysine now coordinates to the heme. In the wild-type protein, the oxygen of the tyrosine-63 side chain is likely to share a proton with the side chain of lysine-87, suggested by the observation of a H/D sensitive resonance Raman line at 502 cm^{-1} that is tentatively assigned as a vibrational mode of the Fe−O bond between the iron and the tyrosinate. We propose that the transition from the high-spin to the low-spin form of the protein occurs by deprotonation and ligation to the heme of the B10 tyrosine oxygen, facilitated by strong interaction with the E10 lysine side chain.

Chlamydomonas hemoglobin, the first discovered chloroplast hemoglobin (Hb),[1] is induced by light and requires active photosynthesis for its full expression (*1*, *2*). It occurs in the chloroplast of *Chlamydomonas eugametos* at an estimated concentration of 130 nM (*3*). The rate of dissociation of hemoglobin-bound oxygen is very low ($k_{off} = 0.014$ s^{-1}, corresponding to a half-time of ∼50 s). On the basis of biophysical studies and sequence similarity with mammalian globins, this low off-rate is attributed to hydrogen-bonding stabilization of the iron-bound oxygen molecule by a distal glutamine residue at position E7 and a tyrosine residue at B10 (*3*). At neutral pH, the heme in ferrous deoxy *Chlamydomonas* Hb is pentacoordinate high-spin, while at alkaline pH (above 9), it becomes hexacoordinate low-spin. This implies that at alkaline pH a group in the distal pocket may coordinate to the heme. In fact, O_2, CO, and NO bind to alkaline ferrous *Chlamydomonas* Hb, only following prior dissociation of a distal ligand (*3*). In comparison, the ferrous form of *Chlamydomonas* Hb at neutral pH is predominantly pentacoordinate, and consequently, the ligand combination rate becomes much faster.

Alkaline ferric *Chlamydomonas* Hb is likewise six-coordinate and low-spin, implying the presence of a ligand occupying the distal coordination position of the ferric heme iron. In this study, we address the nature of this ligand. We show that the heme undergoes a pH-dependent conversion from a six-coordinate high-spin species, similar to aquomet mammalian myoglobin (Mb) at acidic pH, to a six-coordinate low-spin species but with a pK_a of 6.3 as compared to a pK_a of 8.9 for Mb (*4*). The spectroscopic signatures of the low-spin form of *Chlamydomonas* Hb are unique and have not been encountered heretofore. We present evidence from optical, resonance Raman, and EPR spectroscopy of the wild type and the mutant forms of this protein bearing on the identification of the sixth ligand on the heme in the ferric low-spin form of this protein.

EXPERIMENTAL PROCEDURES

Purification of Chlamydomonas Hb and Its Mutants. Recombinant monomeric Hb H21 was created as described elsewhere (*5*) by removing the first 24 amino acids, and by substituting a lysine for the unique cysteine found at position 41 of the parent protein (LI637). This recombinant H21 Hb is termed in this work wild-type *Chlamydomonas* Hb. The single-amino acid substitution mutants of H21 Hb (Gln84Gly, Tyr63Leu, and Lys87Ala at putative helix positions E7, B10,

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[1] Abbreviations: EPR, electron paramagnetic resonance; Hb, hemoglobin; Mb, myoglobin; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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and E10, respectively) were prepared as described previously (*3*).

pH Dependence of Optical Spectra. The instrumentation for measuring optical spectra (at 23 °C) is described elsewhere (*3*). A separately prepared solution was used for each pH and buffer combination. The midpoints of the titration curve, pK_a, and the optical spectra of components of mixtures containing multiple species were calculated by singular-value decomposition using Specfit software (Spectrum Software Associates, Chapel Hill, NC).

Resonance Raman Spectroscopy. Details of the procedures of the resonance Raman measurements have been described elsewhere (*6*). Solutions of ferric *Chlamydomonas* Hb were prepared in water with various isotopic compositions as follows: 45 μM protein, 40 mM buffer (sodium acetate at pH 5.0 and CHES at pH 9.5), and 40 μM EDTA in 80% isotope water [using either 99.9% $D_2{}^{16}O$ from Aldrich Chemical Co. (Milwaukee, WI) or 98% $H_2{}^{18}O$ from Cambridge Isotope Laboratories Inc. (Cambridge, MA)] with 20% natural water ($H_2{}^{16}O$). The protein samples were excited with ~8 mW of laser beam power at a wavelength of 413.1 nm, and the sample cell was spun at 6000 rpm to avoid laser heating.

Electron Paramagnetic Resonance Spectroscopy. EPR spectra were collected on a Varian E112 spectrometer equipped with a Systron-Donner frequency counter and a PC-based data acquisition program. Wild-type *Chlamydomonas* Hb was examined at pH 4.8 (50 mM citrate at 77 K), 8 (20 mM bicine at 4.2 K), and 9.3 [20 mM CHES, with $H_2{}^{16}O$ (77 and 6 K) and with 35 at. % $H_2{}^{17}O$ (6 K)]. The Tyr63Leu and the Lys87Ala mutants were examined at pH 8 (20 mM bicine at 4.2 K).

RESULTS

pH Dependence of Optical Spectra. Ferric *Chlamydomonas* Hb undergoes a single pH-dependent optical spectral change as shown in Figure 1. The acid form (assessed at pH 5), with absorbance maxima at 406 and 624 nm (Figure 1A), bears the spectral signature of a six-coordinate high-spin species similar to that of aquoferric Mb. The alkaline species (assessed at pH 10.5) exhibits absorbance maxima at 410 and 535 nm with a nearly concealed shoulder near 572 nm (visualized in the second-derivative spectrum). This spectrum is slightly different from those of low-spin ferric Hbs (*7*−*9*) containing bis-histidine heme that exhibit well-defined absorption bands at ~535 and ~565 nm. It also differs from that of five-coordinate high-spin ferric Mb (*10*) and the alkaline six-coordinate low-spin Mb hydroxide (with well-defined maxima at 414, 542, and 582 nm) (*11*) and Hb hydroxide (*12*, *13*).

Analysis of the titration curves at 406, 535, and 624 nm by singular-value decomposition indicates that only two species contribute to the spectra, and that a single pK_a (6.3) is required to fit the data (Figure 1B). To demonstrate the reversibility of the reaction, two titrations, one from low to high pH and the other in the reverse direction, were carried out. The results were indistinguishable whether the initial protein solution was at pH 5.7 or 10.0. It should be noted that the pK_a of the optical transition in *Chlamydomonas* Hb (6.3) is considerably lower than in Mb (8.9) (Table 1). This suggests that the high-spin to low-spin transition in *Chlamydomonas* Hb does not form an hydroxide, as it does in Mb.

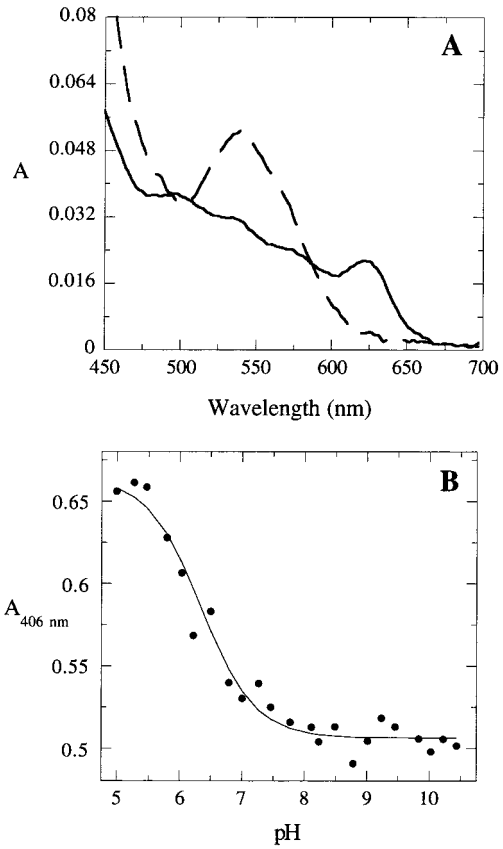

FIGURE 1: pH dependence of the optical spectrum of ferric *Chlamydomonas* hemoglobin. (A) Optical spectra of the protein (5 μM) recorded in 50 mM pH 5 (sodium acetate/EDTA) buffer (solid line) and pH 10.5 (CAPS/EDTA) buffer (dashed line). (B) Absorbance of ferric hemoglobin at 406 nm as a function of pH. The curve drawn through the data points (shown as a solid line) is calculated for a single proton equilibrium with a pK_a of 6.3.

Resonance Raman Spectra of Ferric Chlamydomonas Hb. The high-frequency region (1300−1700 cm^{-1}) of the resonance Raman spectra of heme proteins is comprised of porphyrin in-plane vibrational modes which are markers of the oxidation state, coordination state, and spin state of the central iron atom (*6*, *14*). To determine the heme coordination and spin state of the two pH-dependent forms of the ferric *Chlamydomonas* Hb, resonance Raman spectra in the high-frequency region were recorded at three different pH values (Figure 2). The data identify the high- and low-spin forms unambiguously and confirm the interconversion between these two forms as seen in the optical spectra. The oxidation state marker line (ν_4), which is sensitive to the π-electron density, appears at 1370−1374 cm^{-1}, a frequency characteristic of ferric heme proteins. Significant pH-dependent changes occur in the 1470−1505 cm^{-1} region (ν_3) as well as in the 1550−1590 cm^{-1} region (ν_2), both of which are sensitive to the spin and coordination state of the heme. In the ν_3 region, the magnitude of the major line at 1502 cm^{-1}, assigned to a six-coordinate low-spin configuration at pH 9.5 (*15*), is partially diminished at pH 6.5, and at pH 5.0, the dominant line in the ν_3 region is located at 1477 cm^{-1}. The latter line is assigned to ν_3 of the ferric six-coordinate high-spin form. The pH-dependent growth of the high-spin spectrum in the ν_3 region is accompanied by the development of a line at 1558 cm^{-1}, and a corresponding decrease in the magnitude of a line at 1572 cm^{-1} in the ν_2 region, also

Table 1: K_a of the Acid to Alkaline Transition from the High-Spin Six-Coordinate Form to the Low-Spin Six-Coordinate Form of Ferric Wild-Type *Chlamydomonas* Hb Compared to Those of Single-Amino Acid Mutants[a]

ferric *Chlamydomonas* hemoglobin	pK_a of optical change	ν_4 (cm^{-1})		ν_3 (cm^{-1})		ν_{10} (cm^{-1})
		acid	alkaline	acid	alkaline	alkaline
wild-type	6.3	1370 1374[b]	1374	1477 (6c hs) 1504[b] (6c ls)	1502 (6c ls)	1638 (6c ls)
Gln84Gly	~6.5	1372	1374	1482 (6c hs)	1504 (6c ls)	1638 (6c ls)
Tyr63Leu	~7.5	1373 1367[c]	1374	1477 (6c hs) 1500[c] (5c is)[g]	1502 (6c ls)	1635 (6c ls)
Lys87Ala	>10.0	1373	1374	1481(6c hs) 1503[c] (5c is)[g]	1503 (6c ls) 1477[b] (6c hs)	1636 (6c ls)
myoglobin	8.9[d]	1370[e]	1373[f]	1481[e] (6c hs)	1504[f] (6c ls) 1479[f] (6c hs)	1640[f] (6c ls)

[a] The resonance Raman frequencies of the oxidation state (ν_4) and spin state (ν_3) marker lines as well as of ν_{10} are given. The values of ν_4 show that the heme in all species that were examined is in its ferric state. When two values of ν_3 are observed at the same pH, the solution contains a mixture of species. The nature of the coordination and spin states of the heme inferred from Raman spectra are placed in square brackets. 6c hs and 6c ls represent six-coordinate high-spin and low-spin forms, respectively. 5c is represents a five-coordinate intermediate-spin species. Acid spectra and alkaline spectra were measured at pH 5 and 9.5, respectively, except for the Lys87Ala mutant where the corresponding pHs were 6 and 10.5, respectively. [b] Minority species. [c] Close to half of the protein population. [d] From Antonini and Brunori (*4*). [e] Sperm whale Mb, from Palaniappan and Bocian (*52*). [f] Horse heart Mb, from Feis et al. (*13*). [g] See footnote 3.

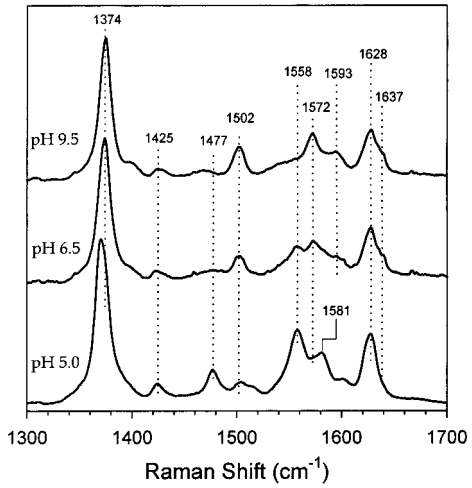

FIGURE 2: Resonance Raman spectra in the high-frequency region of ferric *Chlamydomonas* hemoglobin (30 μM) at pH 9.5 (CHES), 6.5 (bis-tris), and 5.0 (sodium acetate) (from top to bottom).

indicating a change from a low- to a high-spin six-coordinate species. At acidic pH, there is a small population from a species (ν_3 at ~1504 cm^{-1}) that has not been characterized in the work presented here. Thus, resonance Raman data in the high-frequency region demonstrate conversion of a six-coordinate high-spin species at acidic pH to a six-coordinate low-spin species at alkaline pH.

The low-frequency region of resonance Raman spectrum is useful in identifying metal−ligand vibrations (*6*). In particular, a line in the 490−575 cm^{-1} region has been assigned to the Fe−OH stretching frequency in alkaline ferric Hb, Mb, and horseradish peroxidase (*13*, *16*, *17*). Figure 3 shows the low-frequency region resonance Raman spectra of ferric *Chlamydomonas* Hb in alkaline buffered solutions of water with differing isotopic compositions. At pH 9.5, a line is detected in $H_2{}^{16}O$ at 502 cm^{-1}. Were this line to originate from an Fe−OH stretching mode, it would be expected to shift with the changing isotopic composition of the ligand, thereby indicating ligand or proton exchange with solvent. The spectrum in H_2O can be virtually superimposed with that in $H_2{}^{18}O$ (Figure 3), indicating that the oxygen atom of the ligand does not exchange with solvent and, thus, is unlikely to be a hydroxide.

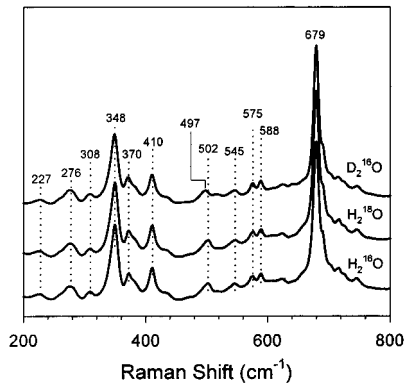

FIGURE 3: Resonance Raman spectra in the low-frequency region of alkaline ferric *Chlamydomonas* hemoglobin in water with various isotopic compositions. The protein (45 μM) was placed in buffered [40 mM CHES and 40 μM EDTA (pH 9.5)] solvent medium either in $D_2{}^{16}O$ (top spectrum), in $H_2{}^{18}O$ (middle spectrum), or in $H_2{}^{16}O$ (bottom spectrum).

However, the band at 502 cm^{-1} observed in H_2O shifts down to 497 cm^{-1} in D_2O (Figure 3). The 5 cm^{-1} downshift in the frequency of the 502 cm^{-1} band is reproducible and has been confirmed from several sets of experiments using various pH buffers (CHES, CAPS, Tris, and pyrophosphate; in the pH range of 8.5−10.5) in which optical spectra indicate that the *Chlamydomonas* Hb remains in the low-spin form. Other low-frequency modes in the 200−450 cm^{-1} region of the spectrum of the low-spin form of ferric *Chlamydomonas* Hb which are sensitive to heme propionate conformation and out-of-plane motions of the porphyrin ring do not exhibit any significant frequency shift in any of the isotopic solvents.

Resonance Raman and Optical Spectra of Single-Amino Acid Mutants. To further investigate the nature of the sixth ligand to the heme, several putative heme pocket residues were mutated, and the effects of these mutations were studied by various spectroscopic methods. Glutamine-84 (E7), the residue presumed to occupy the position distal to the heme iron, was mutated to glycine; tyrosine-63 (B10) was changed to leucine, and lysine-87 (E10), three residues removed in the backbone polypeptide chain from the presumed distal glutamine, was mutated to alanine. The pH-dependent spin transition of these mutants was followed via optical spectra

as well as via resonance Raman spectra in the high-frequency region. Table 1 lists the position of the ν_4, ν_3, and ν_{10} lines of these mutants, and also the pK_a of the high-spin to low-spin transition determined from optical spectral changes. The values of ν_4 are virtually unchanged and, as expected, are typical of ferric heme proteins. The ν_3 line of the wild type and the mutants is near 1502 cm^{-1} at the alkaline side of their respective pK_a, indicating that the alkaline species are six-coordinated low-spin forms. The alkaline species in all these proteins also exhibit the ν_{10} line near 1638 cm^{-1}, characteristic of low-spin complexes.

At acidic pH, the ν_3 line of the Gln84Gly mutant shows the presence of a six-coordinate high-spin species just as in the wild-type protein with a similar pK_a. Similar six-coordinate high-spin species are also observed in the Lys87Ala and Tyr63Leu mutants at acidic pH. However, a second species in these latter mutants (characterized by the ν_3 line at 1500 and 1503 cm^{-1} in Tyr63Leu and Lys87Ala, respectively) is increasingly populated as the pH is lowered, probably because of protein instability.[2] The pK_a of the high-spin to low-spin transition in both of these mutants is significantly changed. The Lys87Ala mutant exhibits a pK_a of >10, a value close to that of deprotonation of free tyrosine, and the Tyr63Leu mutant has a pK_a of $\sim$7.5. Interestingly, in the low-spin species of both of these mutants, no H/D frequency shift in the 502 cm^{-1} region is observed (data not shown), indicating that mutation of either of these residues results in alteration of the heme ligation structure. Thus, it implies that one of these residues may act as a heme ligand in the wild-type protein.

The Gln84Gly mutant that has high-spin to low-spin transition pK_a similar to that of the wild-type protein exhibits a similar H/D frequency shift of the 502 cm^{-1} line. The band at 502 cm^{-1} for the Gln84Gly mutant undergoes a 4 cm^{-1} downshift in frequency to 498 cm^{-1}, but does not show any frequency shift in $H_2{}^{18}O$ (data not shown). The similarity of the spectroscopic behavior of the Gln84Gly mutant to that of the wild-type protein indicates that the glutamine residue does not ligate to the ferric heme iron of the alkaline wild-type protein. However, this residue is involved in stabilization of the oxy complex of *Chlamydomonas* Hb as manifested by a significant change in the oxygen binding kinetics upon its mutation (*3*).

Electron Paramagnetic Resonance Spectra. The EPR spectrum of wild-type *Chlamydomonas* Hb contains one axial high spin ferric signal with *g* values of 5.89 and 1.99 and one rhombic low-spin ferric signal with *g* values of 2.51, 2.31, and 1.86 (Figure 4, spectra A and D). This protein has been examined at various pH values and at liquid nitrogen and helium temperatures (see Experimental Procedures). No additional high-spin or low-spin signals were resolved. High-spin signals with identical *g* values (5.89 and 1.99) were found for the Tyr63Leu (Figure 4, spectra B and E) and Lys87Ala (Figure 4, spectra C and F) mutants, although the $g = 5.89$ signal of Tyr63Leu exhibits a small increase in rhombicity (spectrum B, denoted by the asterisk) as compared to the wild-type protein. A new low-spin signal with *g* values of $\approx$3.3 and 2.2 was resolved for the Tyr63Leu mutant (the third *g* value was not resolved). The wild-type low-spin signal (*g* values of 2.51, 2.31, and 1.86) was not altered in $H_2{}^{17}O$ (35 at. %) buffer. This low-spin signal was absent in the EPR spectra of the Tyr63Leu and the Lys87Ala mutants (Figure 4, spectra E and F, respectively). It may be noted that signals from adventitious copper bound to the purified protein were found in the spectra of the two mutants.

DISCUSSION

Ferric *Chlamydomonas* Hb undergoes a high-spin to low-spin transition at an unusually acidic pH [p$K_a = 6.3$ (Figure 1)], in contrast to mammalian metMb that has a pK_a of 8.9. We shall discuss the environment of the heme in both forms, and the possible origin of the low pK_a.

The heme in *Chlamydomonas* Hb is a *b*-type heme (protoheme IX), deduced from the optical spectrum of the pyridine hemochromogen (*5*). Sequence alignment with globins suggests that the proximal residue of *Chlamydomonas* Hb is histidine (His111 at F8) and the distal residue (E7) may be glutamine (Gln84). Tyr63 is predicted to occupy the B10 position, and the phenylalanine found at position CD1 in all Hbs is also conserved in *Chlamydomonas* Hb (Phe76) (*1*). Our earlier studies, as well as those presented here, demonstrate that *Chlamydomonas* Hb contains histidine as the proximal ligand. This histidine is likely to be a neutral imidazole (*3*).

Heme Ligand Assignment in the Acid Form. At the acid extreme, pH 5, the optical spectrum of ferric *Chlamydomonas* Hb (Figure 1) is similar to that of aquoferric Mb, a high-spin six-coordinate species. Resonance Raman spectra (Figure 2) confirm this assignment and show the presence of a predominant six-coordinate high-spin species. Electron paramagnetic resonance resolves an axial high-spin ferric signal at *g* values of 5.89 and 1.99, consistent with an aquoferric heme structure. Accordingly, we suggest that water may be the distal ligand of acid ferric *Chlamydomonas* Hb. Water is also likely to be the distal ligand of high-spin forms of the Tyr63Leu and Lys87Ala mutants because high-spin ferric EPR signals identical to that of the wild-type protein were resolved for these mutants.

Heme Ligand Assignment in the Alkaline Form. In the alkaline form, a single species is observed via optical, resonance Raman, and EPR spectra. The optical spectrum (Figure 1) and the resonance Raman spectrum (Figure 2) of alkaline ferric *Chlamydomonas* Hb show that it has a six-coordinate low-spin heme structure. Methionine is ruled out as the sixth ligand because a 695 nm absorption band, characteristic of methionine ligation to ferric heme (*18*, *19*), could not be detected in the optical spectrum. Cysteine is ruled out because there is none in the primary sequence of the recombinant protein used in this study. Resonance Raman

[2] At acidic pH, the observation of a second species in the wild type (Figure 2) and the two ferric mutants is reminiscent of the acidic ferrous species in *Chlamydomonas* Hb in which the iron−proximal histidine bond breaks (*3*). In the case presented here, the second acidic species is identified as the one in which the proximal histidine bond is broken and a weak axial ligand (or no ligand) binds to the ferric heme. In such cases, the ferric ion would attain an intermediate spin state, in which the dz^2 orbital is occupied, but the dx^2-y^2 orbital remains empty. This leads to a decrease in the length of the bond between Fe and the pyrrole nitrogens, thus resulting in a shortening of the porphyrin core size, just as in the low-spin complexes. Observation of the spin state marker (ν_3) frequencies at 1500 and 1503 cm^{-1} for Tyr63Leu and Lys87Ala mutants, respectively, is consistent with the fact that the acidic species in these two mutants are five-coordinate intermediate-spin complexes in which the proximal histidine ligation to Fe is replaced by a weak ligand, presumably, water.

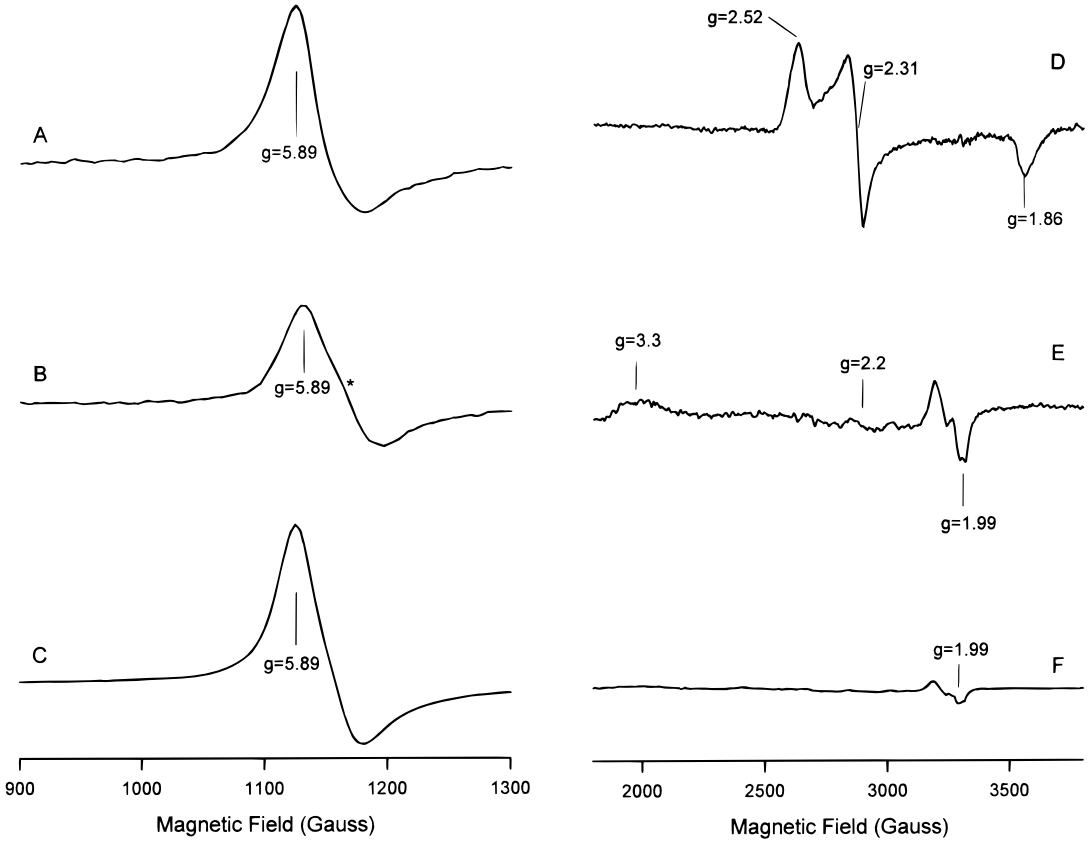

FIGURE 4: EPR spectra of wild-type (A and D), Tyr63Leu (B and E), and Lys87Ala (C and F) *Chlamydomonas* hemoglobin. Protein samples were in 20 mM HEPES at pH 8 (A−C, E, and F) and 20 mM CHES at pH 9.3 (D). Spectra A−C, E, and F were collected at a microwave frequency of 9.24 GHz, a power of 5 mW, a modulation amplitude of 10 G, and a temperature of 4.2 K. Spectrum D was collected at a microwave frequency of 9.23 GHz, a power of 10 mW, a modulation amplitude of 5 G, and a temperature of 77 K.

data show that the Lys87Ala and the Tyr63Leu mutants exhibit isotope effects different from those of the wild-type *Chlamydomonas* Hb and suggest that one of these two residues may be a ligand to the heme iron.

Assignment of the sixth ligand to the heme in *Chlamydomonas* Hb as an exchangeable water or hydroxide is highly improbable because ligand exchange with isotopic water could not be demonstrated by resonance Raman or EPR spectroscopy. Indeed, the resonance Raman spectrum of alkaline ferric *Chlamydomonas* Hb, generated[3] de novo in an $H_2{}^{18}O$ solution from either the six-coordinate high-spin aquoferric species or the ferrous CO adduct, was identical to that in $H_2{}^{16}O$. Several lines of evidence, discussed below, indicate that the sixth ligand in alkaline ferric *Chlamydomonas* Hb is the oxygen atom of the tyrosine-63 side chain. Furthermore, the data indicate that lysine-87 interacts strongly with the tyrosine.

[3] More stringent conditions for exchanging the ferric low-spin species with D_2O or $H_2{}^{18}O$ for resonance Raman experiments were as follows. Three procedures were used. (a) The ferric protein was transferred to D_2O or $H_2{}^{18}O$ at acidic pH, where a presumed aquoferric species predominates, and the pH was subsequently adjusted to regenerate the six-coordinate low-spin ferric species. (b) The ferric low-spin species was reduced with a small amount of dithionite in an anaerobic buffered D_2O or $H_2{}^{18}O$ solution at pH 7.5, where it forms a five-coordinate ligand-free ferrous deoxy species, and subsequently oxidized by exposure to air to regenerate the ferric low-spin species. (c) The low-spin ferric protein in a buffered CO-saturated D_2O or $H_2{}^{18}O$ solution was reduced with dithionite to generate the CO derivative, which was subsequently oxidized with ferricyanide to regenerate the ferric low-spin species. In all cases, no ^{16}O or ^{18}O shift in frequency was observed.

The low-spin ferric signal resolved in the EPR spectrum of *Chlamydomonas* Hb (at both 77 and 6 K) has *g* values of 2.52, 2.31, and 1.86. The **g** tensor anisotropy is characteristic of N and O ligation to heme (*20*, *21*). This rules out the possibility that a lysine amine is coordinated because a His−heme−Lys complex will give rise to a low-spin EPR signal having much greater anisotropy (*22*). The low-spin signal observed for the wild-type protein was not observed in the EPR spectrum of the Tyr63Leu mutant. Accordingly, it is reasonable to suggest that Tyr63 is the distal heme ligand. This view is supported by the lack of an ^{16}O or ^{18}O effect in the resonance Raman spectra and an ^{16}O or ^{17}O effect in the EPR spectra. These findings also support a model in which Lys87 interacts directly with the bound tyrosinate as shown in Figure 5. Although lysine-87 is not a heme ligand in the wild-type protein, the mutation of this residue to alanine increases the pK_a of the high-spin to low-spin transition from 6.5 to >10, suggesting that lysine-87 plays a significant role in the formation of the low-spin ferric complex in the wild-type protein. Indeed, in the absence of lysine-87, tyrosine-63 does not coordinate to the heme iron below pH ∼10. This is confirmed not only by the absence of a low-spin signal in the EPR of the Lys87Ala mutant but also by the presence of a high-spin signal identical to that of the wild-type protein. This suggests that the distal ligand in the Lys87Ala mutant protein is a water molecule and not a tyrosine. Were tyrosine-63 to coordinate to the heme iron in the Lys87Ala mutant and to form a high-spin complex, the EPR signal would be expected to be rhombic, not axial (*23*−*26*). The high-spin

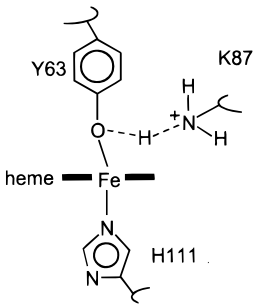

FIGURE 5: Proposed structural model of the heme ligands in ferric *Chlamydomonas* Hb. The heme, the proximal ligand (His111), the distal ligand (Tyr63), and the nearby Lys87 are shown. The amine side chain from lysine shares a proton (shown as a dashed line) with the tyrosinate side chain.

form of the Tyr63Leu mutant is also likely to be a six-coordinate species with water as its distal ligand. The rhombicity in the $g = 5.89$ signal (Figure 4B, asterisk) for this mutant is too small to arise from a five-coordinate high-spin ferric heme, such as those in distal histidine mutants of myoglobin (*27*).

The origin of the 502 cm^{-1} line in the Raman spectrum of the low-spin form of *Chlamydomonas* Hb is clearly very different from that of alkaline mammalian Mb in which the line in this region is assigned as the Fe−OH stretching mode primarily because it displays isotope sensitivity in both D_2O and $H_2{}^{18}O$ (*13*). Since in *Chlamydomonas* Hb, this mode does not show ^{16}O or ^{18}O dependence but does show H/D dependence, and since it does not respond to any of the rigorous $H_2{}^{18}O$ exchange experiments,[3] we tentatively assign the 502 cm^{-1} line to the Fe−O stretching frequency (ν_{Fe-O}) of an oxygenous ligand, namely a tyrosinate, that shares a proton with its neighboring group. The presence of a shared proton on the tyrosinate group explains the observation of a relatively large H/D shift (5 cm^{-1}) of the ν_{Fe-O} frequency. It is unlikely that the 502 cm^{-1} line corresponds to a porphyrin mode or an internal mode of Tyr63 resonance-enhanced by proximity to the heme, as in that case, a large H/D isotope shift would not be expected.

The Fe−O stretching frequency in a related but non-heme Fe(III)−cresolate model complex was assigned at 568 cm^{-1} by isotope substitution measurements (*28*). In the abnormal subunits of some naturally occurring mutant hemoglobins that form stable met forms (HbMs) (*29*, *30*) and in synthetic mutant myoglobins, containing tyrosine in either the distal or the proximal position (*31*, *32*), tyrosine binds to the ferric heme iron with either five- or six-coordinate states. In analogy to the occurrence of the $Fe-O_2$ stretching frequency at ∼570 cm^{-1} in the oxy complex of mammalian hemoglobins and myoglobins, it was argued that the appearance of a band in the 578−603 cm^{-1} region in HbMs is due to the Fe^{III}−O(tyrosinate) stretching mode (*29*). The ν_{Fe-O} frequency in non-heme iron−tyrosinate proteins has also been reported in a similar frequency region (575 cm^{-1}) (*33*, *34*). In catalases that contain tyrosinate-ligated five-coordinate heme, the Fe^{III}−O(tyrosinate) stretching mode could not be assigned because of the overlap of several porphyrin modes and a complex dependence of the lines in the low-frequency region on the excitation wavelength (*35*). The lower frequency for the Fe−O mode that we report here for *Chlamydomonas* Hb is attributed to the six-coordinate low-spin configuration of the heme group. Finally, tyrosine internal modes were observed in the tyrosine-containing proteins noted above (*29*−*36*). In *Chlamydomonas* Hb, however, no such modes were detected with the excitation frequency at 413 nm, probably because of the presence of the intense porphyrin bands in the high-frequency region of the spectrum. However, upon excitation at 441 nm, the Raman lines at 1308, 1500, and 1595 cm^{-1} were enhanced (relative to ν_4, data not shown) and may correspond to tyrosine internal modes such as those [ν_{C-O}(Tyr) at ∼1244−1310 cm^{-1} region and $\nu_{C=C}$(Tyr) at ∼1500 and ∼1600 cm^{-1}] observed in HbMs, catalases, mutant myoglobins, and non-heme iron−tyrosine proteins and model complexes (*28*−*36*).

It should be noted that unlike the tyrosine-ligated mutant myoglobins and hemoglobins which are all high-spin, tyrosine-bound ferric *Chlamydomonas* Hb is a low-spin complex and thus suggests a different nature of coordination. In accord with the conclusions drawn from the EPR spectra and the structure proposed in Figure 5, we postulate that one axial heme ligand in *Chlamydomonas* Hb is the tyrosinate form of the B10 tyrosine residue located in the distal pocket and the other ligand is the proximal histidine, resulting in a six-coordinate low-spin complex. Furthermore, we propose that the tyrosinate shares a proton with its neighboring group, Lys87. In other words, it is the interaction of Lys87 that help stabilizes the Fe−O bond by inhibiting complete protonation of the tyrosinate even at pH ∼7.

Although the assignment of Tyr63 as the distal heme−iron ligand is consistent with mutagenesis and isotope studies, it should be pointed out that the EPR properties of *Chlamydomonas* Hb are unlike those of heme proteins known to contain His−heme−Tyr centers. Some of these heme proteins undergo a high-spin to low-spin transition upon deprotonation of the tyrosine hydroxyl, whereas for others, only the high-spin form has been observed, presumably due to the high p*K* of the tyrosyl hydroxyl proton (*23*). However, in *Chlamydomonas* Hb, Tyr63 is believed to coordinate to the heme iron as a tyrosinate. The crystal field parameters of the low-spin ferric *Chlamydomonas* Hb ($V/\Delta = 0.96$; $\Delta/\lambda = 4.05$) places this protein in a structural grouping in the Blumberg−Peisach truth tables (*20*, *21*) different from those of known His−heme−Tyr centers (Table 2). This may also be the result of the strong interaction between the tyrosinate ligand and a nearby proton. It is proposed that the low pK_a of the transition between tyrosine and tyrosinate ($pK_a = 6.3$; Table 1) is a result of strong interactions with Lys87, as shown in Figure 5. Complete protonation of the tyrosine leads to its dissociation from the heme and its replacement by a water molecule. An unusually low pK_a of the tyrosine side chain has also been anticipated in other proteins where the tyrosine is either post-translationally modified (*37*−*39*) or strongly hydrogen-bonded to its surrounding groups (*40*).

The fact that mutation of Lys87 drastically changes the pK_a of the high-spin to low-spin transition (from 6.3 to >10), even though this residue is not an axial ligand to the heme in the wild-type protein, is consistent with the loss of its interaction with the tyrosine. This results in a protonated tyrosine whose pK_a closely matches that of tyrosine in solution (pK_a ∼ 10.1) and that no longer binds to the heme below pH ∼10. On the other hand, mutation of the axial tyrosine residue results in a low-spin species whose resolved

Table 2: EPR and Crystal Field Parameters of Low-Spin Ferric Heme Proteins with N−Fe^{III}−O Centers[a]

protein[b]	axial ligands[b]	g_z	g_y	g_x	V/λ[c]	Δ/λ[c]	V/Δ[c]	group[d]	ref
*Ch*Hb	His/Tyr^-	2.52	2.31	1.86	3.89	4.04	0.96	H	*e*
Lb-PFP	His/Ph^-	2.73	2.26	1.75	2.66	4.38	0.61	O	*f*
Lb-PNP	His/Ph^-	2.64	2.22	1.80	3.01	5.15	0.59	O	*f*
Lucina Hb II (maj)	His/OH^-	2.61	2.20	1.82	3.16	5.66	0.56	O	*g*
Lucina Hb II (min)	His/Tyr^-	2.76	2.20	1.75	2.53	5.22	0.48	O	*g*
P. epiclitumin Hb	His/Tyr^-	2.67	2.13	1.78	2.76	6.62	0.42	O	*h*
T. pantotropha d_1	His/Tyr^-	−1.84	2.19	−2.52	−3.98	−5.60	0.71	?	*i*
MbOH	His/OH^-	2.55	2.17	1.85	3.49	6.61	0.53	O	*j*
LbOH	His/OH^-	2.54	2.19	1.84	3.51	5.89	0.60	O	*k*

[a] V/λ and Δ/λ refer to crystal field parameters that define structural groupings in the Blumberg−Peisach truth tables. [b] *Ch*Hb, *C. eugametos* chloroplast hemoglobin; Lb-PFP, perfluorophenol complex of soybean leghemoglobin; Lb-PNP, *p*-nitrophenol complex of soybean leghemoglobin; *P. epiclitumin* Hb, *Paramphistomum epiclitum* hemoglobin; *Lucina* Hb II (maj) and (min), major and minor low-spin forms of *Lucina pectinata* hemoglobin II, respectively; *T. pantotropha* d_1, *Thiosphaera pantotropha* nitrite reductase d_1 heme; MbOH, hydroxide complex of myoglobin; LbOH, hydroxide complex of soybean leghemoglobin; His, histidine; Tyr^-, tyrosinate anion; Ph^-, phenolate. [c] Palmer (*53*). [d] The designation of O and H groups is based on the Blumberg−Peisach crystal field analysis of EPR parameters and follows their notation for low-spin ferric complexes with O and N donors, respectively, at the sixth coordination position (*20*, *21*). [e] This work. [f] H. C. Lee, J. Peisach, and J. B. Wittenberg, unpublished results. [g] Kraus et al. (*26*). [h] H. C. Lee, J. Peisach, and J. M. Friedman, unpublished results. Tyrosine ligation to the heme iron has been proposed for *P. epiclitumin* Hb, which contains tyrosine residues at positions E7 and B10. [i] Cheesman et al. (*54*). [j] Blumberg and Peisach (*20*). [k] Appleby et al. (*55*).

g values at 4 K (3.3 and 2.2) resemble those of the His−heme−Lys center of alkaline cytochrome *c* (*22*). Thus, in the absence of the native tyrosine ligand, Lys87 coordinates to the heme.

Conclusions. Chlamydomonas Hb shares the putative B10 tyrosine with legHbs and the globins of the nematodes, trematodes, clams, protists, yeasts, and some bacteria (*3*, *41*−*46*). These Hbs, however, do not contain a unique distal residue, and their oxygen affinities encompass a very wide range of values. Furthermore, the structural disposition and the function of the B10 Tyr appear to be different among these Hbs. The iron-bound oxygen in soybean legHb is stabilized by the distal histidine (*47*) as in vertebrate globins, and probably not by the B10 Tyr. It may be noted that the cyanobacterium hemoglobin from *Nostoc commune* whose sequence is reasonably similar to that of *Chlamydomonas* Hb does not contain a Tyr at the B10 position, and it has a distal pocket highly accessible to ferrous ligands (*48*). The Hb from a nematode, *Ascaris suum*, on the other hand has very high oxygen affinity which is the result of strong stabilization of the bound oxygen by both the distal Gln and the B10 Tyr (*49*, *50*). The Hb from a clam, *Lucina pectinata* (types II and III), contains the same E7 and the B10 residues, but exhibits moderate oxygen affinity which is explained by the absence of direct hydrogen bonding of the B10 Tyr to the bound oxygen (*51*). The trematode Hbs have Tyr at the E7 and B10 positions, both of which are thought to donate hydrogen bonds to stabilize bound oxygen (*42*). Moreover, at alkaline pH, the distal Tyr in the trematode, *Paramphistomum epiclitum*, is able to serve as a heme axial ligand in the ferric state, as also does the B10 Tyr in the minor population of the alkaline *Lucina* Hb II (Table 2). The *Chlamydomonas* Hb clearly is very different from all of the B10 Tyr-containing Hbs described above, in that the B10 Tyr and not the E7 Gln residue ligates to the ferric heme iron to form a single stable species at alkaline pH. Second, in the oxy form, both the B10 Tyr and the E7 Gln residues stabilize the bound oxygen (*3*). Third, the E10 Lys, a residue normally distant from the distal pocket in other hemoglobins, has a close link to the B10 Tyr and controls its reactivity in the ferric state. Thus, the function of amino acid residues in the distal pocket does not depend on their helical position but rather on their structural coordinates relative to the heme−iron or the iron-bound ligand.

In summary, the heme pocket of *Chlamydomonas* Hb has a unique structure with a distal tyrosine residue serving as an axial ligand to the heme. Also, this protein exhibits a very low rate of oxygen dissociation. These facts raise a legitimate question as to what the physiological function of this protein could be. The spectroscopic and kinetic properties of *Chlamydomonas* Hb differ from those of mammalian hemoglobins, but resemble those of some nonsymbiotic plant hemoglobins. The very low concentration of *Chlamydomonas* Hb in the chloroplast, together with very slow oxygen dissociation, implies that this hemoglobin does not transport molecular oxygen within the cell or surrender bound oxygen to a target protein. The presence of this hemoglobin in the chloroplast might suggest that it safeguards the photosynthetic machinery from small oxygen leaks in the chloroplast. A mechanism by which *Chlamydomonas* Hb could eliminate oxygen would be through the formation of hydrogen peroxide that is subsequently destroyed by a peroxidase. We note here that the oxy complex of *Chlamydomonas* Hb is readily converted to its ferric form in the presence of electron donors (*5*). Thus, a cycle can be envisioned in which limited amounts of dioxygen are removed efficiently from the system by *Chlamydomonas* Hb with the generation of hydrogen peroxide that is subsequently converted to water by peroxidases. Alternatively, the very low chloroplastic concentration makes it likely that *Chlamydomonas* Hb participates in some process involving amplification; perhaps oxygenation or deoxygenation initiates a cascade of reactions such as the ones involved in many sensory processes. Whatever the function, *Chlamydomonas* Hb appears to be involved in photosynthesis since it is located within the chloroplast and is expressed strongly only during active photosynthesis. It remains to be tested whether certain hemoglobins have multiple physiological functions and how these proteins evolved to adapt to an increasingly oxygen-rich atmosphere presumably with altered physiological functions.

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