Residues in the Distal Heme Pocket of Neuroglobin

IMPLICATIONS FOR THE MULTIPLE LIGAND BINDING STEPS*

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Amino acid residues in the ligand binding pocket of human neuroglobin have been identified by site-directed mutagenesis and their properties investigated by resonance Raman and flash photolysis methods. Wild-type neuroglobin has been shown to have six-coordinate heme in both ferric and ferrous states. Substitution of His by alanine leads to complete loss of heme, indicating that His is the proximal ligand. The resonance Raman spectra of M69L and K67T mutants were similar to those of wild-type (WT) neuroglobin in both ferric and ferrous states. By contrast, H64V was six-coordinate high-spin and five-coordinate high-spin in the ferric and ferrous states, respectively, at acidic pH. The spectra were pH-dependent and six-coordinate with the low-spin component dominating at alkaline pH. In a double mutant H64V/K67T, the high-spin component alone was detected in both the ferric and the ferrous states. This implies that His is the endogenous ligand and that Lys is situated nearby in the distal pocket. In the ferric and ferrous states, the v(Fe-His) stretching frequency appears at 221 cm\(^{-1}\), which is similar to that of deoxymyoglobin. In the ferrous CO-bound state, the v(Fe-CO) stretching frequency was detected at 251 and 494 cm\(^{-1}\) in WT, M69L, and K67T, while only the 494 cm\(^{-1}\) component was detected in the H64V and H64V/K67T mutants. Thus, the 521 cm\(^{-1}\) component is attributed to the presence of polar His. The CO binding kinetics were biphasic for WT, H64V, and K67T and monophasic for H64V/K67T. Thus, His and Lys comprise a unique distal heme pocket in neuroglobin.

Neuroglobin (Ngb) is a new member of the vertebrate globin family, which is expressed predominantly in the brain and nerve tissues (1). Although Ngb displays the structural determinants of the globin fold (2), the identity in amino acid sequence is low (20–25%) compared with vertebrate hemoglobin (Hb) and myoglobin (Mb) (3–5) (Fig. 1). Sequence analyses also show that Ngb is more primitive than Mb (1, 6). The function of Ngb is a matter of debate. Ngb is up-regulated in response to hypoxia, and this protects neurons against hypoxic damage (7). High concentrations (~100 μM) of Ngb are observed in the retina and its subcellular distribution correlates with the localization of the mitochondria (8). These observations point to a role for Ngb in intracellular O\(_2\) supply (1, 9, 10). However, the low concentration of Ngb in non-pathological brain tissue (in the micromolar range; Ref. 1) is hard to reconcile with an O\(_2\) storage or carrier function. Indeed, the Hbs of the nervous tissue of several invertebrates (11–13), which are known to be involved in O\(_2\) transport, occur in much higher concentrations (millimolar concentrations) than Ngb. Only if massive induc tion occurs can it be envisaged that the concentration of Ngb would reach levels that are sufficient to sustain an aerobic metabolism during temporary hypoxia.

Ngb may display as yet unknown enzymatic activities. Cytochrome c, another newly identified member of the globin family (14) ubiquitously expressed in human tissues (3, 15), was found to have a peroxidase activity in vitro and to be up-regulated during hepatic inflammation and fibrosis (14, 16). Ngb could be an O\(_2\) sensor protein activating other proteins with regulatory functions (17, 18). Ferric Ngb, which is generated spontaneously as a result of the rapid auto-oxidation of oxy form, binds exclusively to the GDP-bound form of the α subunit of heterotrimeric G protein (19). Finally, Ngb might be involved in NO metabolism as has been shown for Mb (20, 21), a number of flavo-Hbs (22), a truncated Hb (23), and Ascaris (Nematoda) Hb (24). Whatever its precise role, the function of Ngb is linked to the binding of gaseous ligands.

A key feature of Ngb is its six-coordinate heme structure, both in the ferric and ferrous states, with the distal histidine (His) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26). Amino acid sequence alignments with other globins indicate that the other iron-coordinating ligand is His (1) forming an endogenous ligand (3, 10, 25, 26).

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¶ The abbreviations used are: Ngb, neuroglobin; Mb, myoglobin; Hb, hemoglobin; WT, wild-type; W, watts.
Here we present an extensive resonance Raman analysis of recombinant wild-type (WT) and a series of mutant human Ngb in their ferric, ferrous, and ferrous-CO bound forms. Through the analysis of various mutants, the endogenous axial ligands have been assigned unequivocally as His64 and His96 in both ferric and ferrous states, as suggested previously. Surprisingly, ligation in the H64V mutant Ngb was found to be pH-dependent, and a further distal residue, Lys67, appears to be situated close to the heme, giving rise to a six-coordinate low-spin structure in both the ferric and ferrous states at alkaline pH. Flash photolysis studies on the CO-bound mutants revealed that both His64 and Lys67 contribute equally to CO binding in the slower phase. Thus, Ngb is revealed to have a distal environment unique among the globins. The role of these residues is discussed in the light of the possible biological functions of Ngb.

MATERIALS AND METHODS

Cloning and Purification of Ngb—To achieve high expression, a gene encoding WT Ngb was synthesized using a modification of the recursive PCR strategy (29, 30). Based on the known amino acid sequence of human Ngb (1), six forward (f1–f6) and six reverse (r1–r6) primers with lengths ranging from 55 to 60 bases were designed (Fig. 2) and custom-synthesized. Two termination codons (TAA and TGA) were placed in tandem. Each pair of forward and reverse primers overlaps by about 20 bases, enabling chain extension by KOD-Plus-DNA polymerase. The full-length PCR product was further amplified by the two flanking primers (F and R). Unique restriction sites used for subsequent manipulation of the coding sequence are underlined.

Fig. 1. Multiple amino acid sequence alignment of human globins. Residues identical to those in Ngb are shown in green. The distal and proximal histidines are shown in red. Amino acids that were mutated in this study are indicated by a plus sign.

Fig. 2. Designed gene for human Ngb. Forward (f1–f6, cyan) and reverse (r1–r6, green) primers were custom synthesized. The full-length PCR product was further amplified by the two flanking primers (F and R). Unique restriction sites used for subsequent manipulation of the coding sequence are underlined.
The high fidelity of this enzyme enabled perfect match of the synthesized and designed DNA sequences. The full-length PCR product was selectively amplified in a second PCR with a forward primer F which creates tandem Xhol and NdeI sites and a reverse primer R, which generates a BamH site (Fig. 2). The resultant product was inserted at the Xhol/BamH site of the plasmid pBluescriptII KS (+) (Stratagene), and the ligation products were used to transform Escherichia coli XL-1 Blue MRP (Stratagene). The DNA sequence of the recombinant plasmid product was confirmed with a Li-Cor model 4200S2 DNA sequencer. The QuikChange system (Stratagene) was used to introduce mutations into the Ngb coding sequence, which directed the expression of Ngbs with H64V, K67T, M69L, H96A, and H64V/K67T mutations. The Ndel/BamHI insert was excised from the pBluescript derivatives and ligated into a pET3a-based expression vector (pBEX). The ligation products were introduced into E. coli strain BL21Gold(DE3) (Stratagene). Cell growth and protein purification were performed as described previously (1). Proteins were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis (PAGE) criteria (Fig. 3). Ngb concentrations were evaluated from the heme content which was measured by a pyridine hemochrome assay.

**Absorption Spectroscopy**—The absorption spectra of the purified proteins were recorded on a Beckman DU640 spectrophotometer. pH titration of the ferric Ngb was performed in 10 mM sodium phosphate and 0.1 M NaCl. Aliquots of HCl or NaOH were added to solutions containing ~10 μM protein. The pH in the cuvette was monitored directly using a microelectrode (Hi-Tech). The ferrous proteins were also titrated with CO saturated in 10 mM sodium phosphate and 0.1 M NaCl (pH 7.0).

**Resonance Raman Spectroscopy**—Spectra were recorded using a double monochromator (Jasco R-800) with a slit width of 6 cm⁻¹, following excitation by a krypton ion laser (406.7-nm line, Coherent 1-302) or by a He-Cd laser (441.6-nm line, Kimmon IK4121R-G). A photomultiplier detector was used (Hamamatsu Photonics, R595), and the frequencies were calibrated with indene. A spinning Raman cell was used throughout the measurements. The samples contained 100 μM protein in the buffers specified in the respective figure legends. Ferrous proteins were prepared by the addition of sodium dithionite, after purging extensively with nitrogen gas. The carbonmonoxy forms were prepared by the addition of sodium dithionite under 1 atm CO. The spectra of the CO forms were obtained with a defocused laser beam.

**Flash Photolysis**—CO rebinding kinetics were monitored at 25 °C using a laser flash photolysis system (Unisoku, Osaka, Japan). After photolysis of ferrous CO-bound Ngb with a 5-ns pulse at 532 nm from a Continuum Q-switched Nd:YAG laser, ligand recombination was followed by monitoring absorbance changes at 420 nm. These experiments were performed with 10 μM Ngbs in 10 mM sodium phosphate and 0.1 M NaCl (pH 7.0) under 1 atm of CO. Selected proteins were photolyzed in 0.1 M Tris-HCl (pH 9.5) under 1 atm of CO.

**RESULTS**

**His**⁹⁶—In Fig. 1, the amino acid sequences of selected human globins are compared. Residues identical to those of Ngb are marked in green. The conserved histidines at positions 64 and 96 in Ngb (red) correspond to the distal (E7) and proximal (F8) histidines in Hb and Mb, respectively. The F8 histidine is the sole endogenous axial ligand of the heme iron in the ferric and ferrous states of Hb and Mb, and it is assumed that His⁹⁶ is the proximal ligand in Ngb. To test this hypothesis, we replaced histidine 96 with alanine (H96A) whose side chain cannot coordinate the iron. This H96A mutant was purified and its mobility in SDS-PAGE was compared with that of WT Ngb (Fig. 3). The absorption spectrum of the ferric WT Ngb is shown (Fig. 3, right) and is essentially the same as that reported previously (10). In contrast the H96A mutant is colorless, and the spectrum indicates the complete absence of heme in this mutant. It is concluded that the His⁹⁶ is essential for heme retention in Ngb, consistent with this residue forming an axial ligand.

**Lys**⁶⁷ and Met⁶⁹—It has been proposed that His⁶⁴ (E7) constitutes the second axial ligand of the heme. In support of this suggestion, the absorption spectrum of the H64L mutant reveals loss of His/His coordination (10). However, the Soret peak associated with the ferrous H64L mutant at 423 nm is blue-shifted relative to most five-coordinate Hbs and Mbs (430 nm), and the observed shoulder to this band suggests the presence of two forms, perhaps indicating the presence of an alternative distally coordinated ligand (10). To clarify the identity of the distal ligand in WT Ngb, we prepared further mutant Ngbs. Three residues, His⁶⁴, Lys⁶⁷, and Met⁶⁹, are expected to be capable of iron coordination on the distal side of heme (Fig. 1, marked with +). These three residues were mutated to Val, Thr, and Leu, respectively. Thr⁶⁷ and Leu⁶⁹ occur in Mb, and the H64V mutant of Mb has been studied extensively (31, 32).

In Fig. 4, resonance Raman spectra of ferric WT, M69L, and K67T Ngbs are shown. Resonance Raman spectroscopy is a powerful tool for revealing the oxidation states, spin states, and...
coordination numbers of heme iron (33, 34). In the higher frequency region, the $v_2$, $v_3$, $v_4$, and $v_4'$ lines are observed at 1579, 1547, 1504, and 1374 cm$^{-1}$, respectively. These frequencies are typical of six-coordinate ferric low-spin hemes and similar to those reported previously for the WT Ngb (25). In the lower frequency region, the broad line around 420 cm$^{-1}$ is again very similar to those reported previously for the WT Ngb (25). In the lower frequency region, the broad line around 420 cm$^{-1}$ appears to be slightly affected by the mutation. This line can be assigned to the in-plane bending vibration of the porphyrin vinyl groups (35), and the slight perturbations upon mutation indicate that Met$^{69}$ and Lys$^{67}$ are situated close to the heme, though these are not the Fe-coordinating ligands.

In Fig. 5, resonance Raman spectra of the ferrous mutants are shown. In the spectrum of WT Ngb, the $v_2$, $v_{38}$, $v_3$, and $v_4$ lines at 1580, 1557, 1493, and 1380 cm$^{-1}$, respectively, indicate the presence of a six-coordinate ferrous low-spin heme (25). Since these frequencies in the spectra of M69L and K67T are identical with those of WT Ngb, Met$^{69}$ and Lys$^{67}$ cannot be Fe-coordinating ligands even in the ferrous state. However, the line around 420 cm$^{-1}$ is again affected by the mutation, suggesting that Met$^{69}$ and Lys$^{67}$ are close to the heme in both ferric and ferrous states.

$His^{64}$—The last candidate for the endogenous axial ligand, His$^{64}$, was examined. In Table I, the absorption maxima of the ferric mutants are summarized. The wavelengths of these maxima are identical in WT, M69L, and K67T Ngbs, indicating unequivocally that replacement of His$^{64}$ is the origin of the alkaline transition. The spectral similarity between the H64V mutant of Ngb and WT Mb and their common pH behavior suggest strongly that each contains an iron-coordinated water in the ferric state at acidic pH.

The pH-dependent characteristics of H64V Ngb were further studied by resonance Raman spectroscopy. At acidic pH, ferric H64V Ngb has a six-coordinate high-spin heme, showing Ramans at 1561, 1518, 1484, and 1374 cm$^{-1}$ (Fig. 7). The profile is very similar to that of WT porcine Mb at pH 6.0 (1564, 1514, 1482, and 1372 cm$^{-1}$) as reported previously (38). At alkaline pH, the spectra changed drastically, showing lines at 1573, 1540, 1503, and 1374 cm$^{-1}$, which indicate that H64V contains a six-coordinate low-spin heme at alkaline pH. These frequencies are distinct from those exhibited by Mb at alkaline pH (1587, 1565, 1482, and 1375 cm$^{-1}$) (38) and rather closer to those in WT Ngb (Fig. 4). This suggests that the sixth heme ligand of ferric alkaline H64V is not a hydroxide but instead is an endogenous group that is pH-labile.

To identify the sixth ligand in H64V, we studied the alkaline transition of this mutant. This transition has been investigated in mammalian Mbs where it is established that the dissociation of one proton equivalent converts a water ligand to a hydroxide ligand at alkaline pH (37). As seen in Fig. 6 (upper panel), the absorption spectrum of the ferric H64V Ngb mutant is pH-sensitive in the range pH 6–10. Since one set of isosbestic points was observed, the titration curve was analyzed in terms of a simple equilibrium between the acidic and alkaline forms of the protein. The absorbance changes at the Soret maxima of the two forms were normalized and the molar fraction of the alkaline form was calculated and plotted against pH in Fig. 6 (lower panel). Theoretical curves drawn assuming that the alkaline transition involves one proton equivalent provide a satisfactory fit to the titration data. The apparent $pK_a$ value of the alkaline transition in H64V was calculated to be 7.57. This pH-dependent behavior of the H64V is not observed in the WT, K67T, and M69L Ngbs, indicating unequivocally that replacement of His$^{64}$ is the origin of the alkaline transition. The spectral similarity between the H64V mutant of Ngb and WT Mb and their common pH behavior suggest strongly that each contains an iron-coordinated water in the ferric state at acidic pH.

Since the structure of ferric H64V Ngb is pH-sensitive, resonance Raman spectra of the ferrous H64V were also measured at acidic and alkaline pH (Fig. 8). At acidic pH, lines were observed at 1564, 1470, and 1356 cm$^{-1}$, indicating the presence of five-coordinate high-spin heme. In the lower frequency region, the strong Raman line at 221 cm$^{-1}$ can be assigned to the $\nu$(Fe-His) stretching mode. This observation is consistent with the interpretations of the data presented in Fig. 3, where the endogenous ligand was assigned as His$^{64}$. The frequency is again very similar to that of ferrous WT porcine Mb (38). The $v_3$ and $\nu$(Fe-His) are still prominent at alkaline pH, indicating that five-coordinate high-spin heme is the dominant species. However, an additional Raman line at 1494 cm$^{-1}$ ($v_3'$) is observed, and this line signals the presence of six-coordinate low-spin heme as a minor component of ferrous H64V at alkaline pH.
His$^{64}$ and Lys$^{67}$—The electronic structure of the heme in H64V Ngb is pH-dependent, indicating that a water ligand is bound to the ferric heme at acidic pH. However, at alkaline pH, the six-coordinate low-spin component appeared in both ferric and ferrous states, suggesting that an endogenous strong-field ligand is commonly coordinated to the iron. We examined the sequence of Ngb to identify putative distal pocket residues that could gain/release a proton and account for the pH profile. An obvious candidate is Lys$^{67}$. Thus, Lys$^{67}$ was mutated to produce a double mutant H64V/K67T, and the pH dependence of the resonance Raman spectrum of the double mutant was examined. As seen in Figs. 7 and 8, the spectra of H64V/K67T are insensitive to pH and similar in form to the spectrum of H64V at low pH in both ferric and ferrous states. Thus Lys$^{67}$ must be the endogenous ligand that is responsible for the low-spin component at alkaline pH in H64V (Fig. 9).

CO Probe—The $\nu$(Fe-CO) stretching frequency is sensitive to the polarity of the residues around the bound CO. It is therefore an excellent probe of the distal environment (39–43). As seen in Fig. 10, two Raman lines are observed at 521 and 494 cm$^{-1}$ in the WT CO-Ngb, which have been assigned to the $\nu$(Fe-CO) stretching mode (25). This indicates two conformations for the Fe-CO bond (25). In the M69L and K67T mutants, two lines are observed at the same frequencies, indicating that the ligand binding pocket in these mutants is similar to that in the WT Ngb. In contrast the 521 cm$^{-1}$ line is lost in both the H64V and H64V/K67T mutants, and a single prominent Raman line is observed at 494 cm$^{-1}$. Thus, the 521 cm$^{-1}$ line is associated with the presence of His$^{64}$, which presumably interacts with the bound CO, after the former has been displaced by the exogenous ligand.

During the resonance Raman measurement, the CO-bound forms of the WT, M69L, and K67T Ngbs were found to be very photo-labile, and low laser power and a defocused beam were required for recording the spectra. In contrast, the CO-bound forms of H64V and H64V/K67T mutants were very stable, suggesting much higher CO affinities. As revealed by the titration experiments (Table II), CO clearly binds with higher affinity to the H64V and H64V/K67T mutants than to the WT and K67T Ngbs. This is reasonable since there is no requirement for the ligand to displace the coordinating His64 side chain in the H64V and H64V/K67T mutants. The equilibrium CO dissociation constant ($K_d$) for WT Ngb measured by titration (174 nM) is much larger than that obtained from the ratio of the rate constants $k_{off}/k_{on}$ (0.21 nM) (10). This could be attributable to the competitive binding of His64, suggesting that this
endogenous ligand controls the ligand binding affinity of Ngb. To examine the roles of distal residues in determining the accessibility of an exogenous ligand, rate constants of CO association to the ferrous proteins were measured (Fig. 11). At neutral and alkaline pH, CO rebinding to WT Ngb is biphasic in the time domain of flash photolysis as reported previously (9, 10, 17, 28). K67T showed similar faster ($k_f$) and slower ($k_s$) rates (Table II). The faster phase is reported to depend on the CO concentration and is assigned to bimolecular binding, while binding of the endogenous His64 ligand, which ultimately has to be replaced by the CO, accounts for the slower phase. The similar biphasic kinetics of WT and K67T Ngb is consistent with the proposed role of His64, which is present in both proteins. By contrast, the double mutant H64V/K67T showed monophasic CO association kinetics, with the apparent pseudo-order rate of 370 ms$^{-1}$ under 1 atm of CO ($k_{CO}/H_11002 370$ M$^{-1}$s$^{-1}$). This value is close to that for the faster rate constant ($k_f$) in the H64V mutant at pH 7.0 (350 ms$^{-1}$). The monophasic H64V kinetics indicate that the slower phase (5.6 ms$^{-1}$) in the H64V mutant is attributable to the presence of, but not to the coordination of, Lys$^{67}$. This is because $k_s$ is not affected by pH elevation, even though Lys$^{67}$ partly coordinates the ferrous heme at alkaline pH (Fig. 8). The observed absorbance change for photolyzed H64V is not satisfactorily accounted for by biphasic analysis ($k = 350$ and 67 ms$^{-1}$) but rather by triphasic kinetics (Table II). The second $k_f$ phase (45 ms$^{-1}$) in alkaline H64V may be attributed to the partial coordination of Lys$^{67}$ (Fig. 8), which reduces the CO binding rate in a similar way to His64 in WT Ngb.

**DISCUSSION**

**Coordination Structure**—Through measurements of absorption and resonance Raman spectra, the heme axial ligands of Ngb have been unequivocally assigned as His$^{96}$ and His$^{64}$ in both the ferric and ferrous states (Fig. 9), matching the findings from the recent x-ray structure of ferric state (27). Ferrous Ngb is expected to be the active oxygen storage/carrier form, if indeed this is the function of the protein. Lys$^{67}$ is located sufficiently close to the heme to allow its coordination by the iron in the H64V mutant at alkaline pH. Since the $\nu$(Fe-CO) stretching frequency (Fig. 10), the CO association kinetics, and the CO affinity (Table II) were changed drastically upon mu-
Absorbance changes in the visible region were recorded and analyzed with 1 atm of nitrogen) with CO-saturated buffer solutions.

The samples contained 100 μM proteins in 10 mM sodium phosphate and 0.1 mM NaCl (pH 7.0) or in 100 mM Tris-HCl (pH 9.5) under 1 atm of CO.

Extensive electron donation leads to activation of the bound exogenous ligand. The assignment of His\(^{64}\) as the proximally coordinating ligand is supported by the observation of the \(\nu(\text{Fe-CO})\) stretch at 221 cm\(^{-1}\) (Fig. 8). The extent of electron donation from the proximal histidine is believed to be one of the determinants of protein function. This \textit{trans} effect is reflected in the \(\nu(\text{Fe-His})\) stretching frequency, which is observed at about 240 cm\(^{-1}\) in peroxidases, 220 cm\(^{-1}\) in Mb and Hb, and 210 cm\(^{-1}\) in terminal oxidases (34). More extensive electron donation leads to activation of the bound oxygen molecule \textit{trans} to the proximal histidine. The \(\nu(\text{Fe-His})\) frequency for Ngb falls in the range observed for oxygen storage/carrier proteins, and this is consistent with a proposed role of Ngb, which is to supply oxygen for the brain.

OxyNgb is, however, very unstable and spontaneous auto-oxidation is fast (10), contradicting the notion that Ngb is an oxygen storage protein. In Mb, His\(^{64}\) is situated in the distal heme pocket, where it plays a key role in stabilizing the bound oxygen through the formation of a hydrogen bond with it (44, 45). His\(^{64}\) in WT Ngb appears to have two conformations when exogenous CO is bound, giving rise to two \(\nu(\text{Fe-CO})\) stretching lines (Fig. 10). The close proximity of the dissociated His\(^{64}\) to the bound exogenous ligand is revealed by the \(\nu(\text{Fe-CO})\) stretching frequency at 521 cm\(^{-1}\). This frequency is higher than that for porcine Mb-CO (511 cm\(^{-1}\)) (38), indicating that the bound CO in Ngb is in a more positively polar environment, which may be attributable to the presence of His\(^{64}\). A second \(\nu(\text{Fe-CO})\) stretching line was observed at 494 cm\(^{-1}\) in WT Ngb, and this line is prominent in both the H64V and H64V/K67T mutants, which have an apolar side chain at position 64. This frequency is similar to the \(\nu(\text{Fe-CO})\) in H64V Mb of 492 cm\(^{-1}\) (38). Thus, the His\(^{64}\) side chain may be located too close to (closed conformation for 521 cm\(^{-1}\) line) and too remote from (open conformation for 494 cm\(^{-1}\) line) the Fe for efficient stabilization of bound oxygen.

**Ligand Binding**—His\(^{64}\) in Mb stabilizes a coordinated water in the ferric state through the formation of a hydrogen bond. In the H64V mutant of Mb, this water is not stabilized in the hydrophobic pocket, resulting in a five-coordinate ferric heme (31). The H64V mutant of Ngb retains the water ligand at acidic pH (Fig. 9), suggesting, by analogy with Mb, that a group capable of stabilizing the coordinated water is present in the distal pocket of this mutant. The stabilizing group cannot be provided by Lys\(^{67}\), since the H64V/K67T mutant at both acidic and alkaline pH exhibits spectra similar to H64V Ngb at acidic pH (Fig. 7). Since ferric H64V/K67T is pH-insensitive between pH 6 and 10, we propose an acidic residue, with a \(pK_a\), lower than 5, may be stabilizing the water ligand. The identity of this residue is unknown, although Glu\(^{60}\) could be a possible candidate.

This water ligand in H64V Ngb is not a kinetic barrier to exogenous ligand entry (32) and it is possibly lost in the ferrous state, since the \(k^{\text{CO}}\) for H64V Ngb (350 μM\(^{-1}\) s\(^{-1}\)) is much larger than that for porcine H64V Mb (6.4 μM\(^{-1}\) s\(^{-1}\)) (46). This large difference in \(k^{\text{CO}}\) may be attributed to a more open entrance to the distal pocket in Ngb relative to Mb. The distal His\(^{64}\) in Mb acts as a “gate” for ligand entry, its mobility being controlled by the nearby Phe\(^{46}\) (47). The corresponding residue in Ngb is the less bulky Asn\(^{45}\) (Fig. 1), which may allow the distal His\(^{64}\) to swing out more easily in Ngb.

Lys\(^{67}\) has been shown to coordinate the heme iron at alkaline pH.
Lys67 is deprotonated and able to coordinate the ferric heme. In the ferrous state, coordination results in the formation of six-coordinate heme in which an equilibrium mixture of high-spin and low-spin components co-exist even at pH 5.9. Neutral amines normally prefer ferrous to ferric heme, since the former is neutralized by the negative porphyrin pyrrole. It would seem that the H64V Ngb mutant is flexible and able to change its conformation depending on the oxidation state.

The His64(H64) features in WT Ngb and K67T are similar, as are those in H64V and H64VK67T Ngb. Thus, Lys67 does not affect the Fe(CO) geometry (Fig. 10), although it does affect the CO association kinetics. The double mutant H64VK67T showed monophasic kinetics, while the other proteins showed biphasic behavior (Fig. 11). In the previous kinetic analyses (1, 9, 10), the biphasic kinetics of WT Ngb were analyzed in terms of an internal His coordination scheme. Now that the axial ligands are identified, we can rewrite the following equation.

$$k_H^{\text{His}_{64}} \cdot Fe^{3+} \cdot His_{64} = k_C^{\text{CO}} \cdot His_{64} \cdot CO$$

(Eq. 1)

Conflicting values of the rate constant for His64 dissociation have been reported ($k_H$), ranging from 4.5 s$^{-1}$ (1, 10) to 8200 s$^{-1}$ (9). As a result, the internal His64 affinity, $K_H = k_H/k_C$, is reported to be between 440 (1, 10) and 1.2 (9). This equilibrium exists even in the absence of CO. The dissociated product H64VFe$^{3+}$, which is expected to be five-coordinate and characterized by a $v_{5}$ line at 1470 cm$^{-1}$ and a strong Fe(CO) stretch at 221 cm$^{-1}$, as found in H64V (Fig. 8). However, in the ferrous WT Ngb, no traces of these lines are observed (Fig. 5). Thus, $K_H$ must be in the range 100–1000.

Biphasic kinetics have been reported for other six-coordinate heme proteins. However, six-coordination does not, of itself, imply biphasic kinetics. In the H64V668I double mutant of porcine Mb, monophasic kinetics were observed (48). We also reported monophasic kinetics for the H64V668H/H93A Mb triple mutant (38). These Mb mutants are six-coordinate with His/His coordination as in Ngb, which also shares the globin fold (27). Although endogenous His64 binding is not necessarily the origin of the biphasic CO association, the four combination of His plus one histidine group will slow down the association rate without changing the dissociation rate. However, the His/His coordination will slow the rate constant for His dissociation, which is in turn dependent on the rate constant for His coordination. Therefore, the His/His coordination is not the origin of the biphasic CO association.

In summary, we have been able to assign unequivocally both axial ligands of Ngb to His64 and His58. Lys67 is situated close to the heme iron, enabling ligation at alkaline pH in H64V. His64 and Lys67 comprise the distal pocket of Ngb, which is highly flexible. Since Lys67 coordination is incomplete in ferrous H64V at alkaline pH, the distal environment of Ngb is suggested to be sensitive to the iron oxidation state.

REFERENCES