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Tyrosine B10 Inhibits Stabilization of Bound Carbon Monoxide and Oxygen in Soybean Leghemoglobin†

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ABSTRACT: Detailed comparisons of the carbon monoxide FTIR spectra and ligand-binding properties of a library of E7, E11, and B10 mutants indicate significant differences in the role of electrostatic interactions in the distal pockets of wild-type sperm whale myoglobin and soybean leghemoglobin. In myoglobin, strong hydrogen bonds from several closely related conformations of the distal histidine (HisE7) side chain preferentially stabilize bound oxygen. In leghemoglobin, the imidazole side chain of HisE7 is confined to a single conformation, which only weakly hydrogen bonds to bound ligands. The phenol side chain of TyrB10 appears to “fix” the position of HisE7, probably by donating a hydrogen bond to the Nδ atom of the imidazole side chain. The proximal pocket of leghemoglobin is designed to favor strong coordination bonds between the heme iron and axial ligands. Thus, high oxygen affinity in leghemoglobin is established by a favorable staggered geometry of the proximal histidine. The interaction between HisE7 and TyrB10 prevents overstabilization of bound oxygen. If hydrogen bonding from HisE7 were as strong as it is in mammalian myoglobin, the resultant ultrahigh affinity of leghemoglobin would prevent oxygen transport in root nodules.

Myoglobin (Mb) and leghemoglobin (Lb) perform similar physiological roles in their respective environments. Both facilitate the diffusion of oxygen by increasing its effective concentration in either muscle (Mb) or root nodule (Lb) tissue. Each protein exhibits the requisite rate constants for transport, but Lb has a 20-fold higher affinity for oxygen because it must maintain lower free oxygen concentrations to prevent inhibition of the nitrogenase complex in the root nodules (1, 2). The two proteins share a globin fold that originally led to the assumption that they function similarly at the molecular level (3). However, recent detailed studies of soybean leghemoglobin (Lba) indicate that it uses a mechanism to regulate ligand binding distinct from that of Mb (4–9).

Mutagenesis studies have shown that proximal contributions to O2 affinity are quite different in Lba compared to those in sperm whale Mb (6). In Lba, the plane of the HisE8 imidazole side chain is staggered with respect to the pyrrole nitrogen atoms of the porphyrin ring. This orientation favors in-plane movement of the iron atom and strong axial ligand coordination. In all mammalian Mbs, the HisE8 side chain is in an eclipsed geometry that inhibits ligand coordination (10–12). These ideas were confirmed directly by swapping the F helices between Lba and sperm whale Mb (6). Thus, both stereochemical arguments and direct experimental measurements show that the Lba proximal heme pocket favors high oxygen affinity.

Stabilization of bound ligands by hydrogen bonding in the distal pocket of Lba is weakened to prevent an ultrahigh O2 affinity that would inhibit transport in root nodules. This idea is supported by comparative mutagenesis experiments with recombinant Lbs and Mbs containing altered distal pockets (4, 5, 13–15). HisE7 in Mb forms a strong hydrogen bond with bound oxygen, and replacement with non-hydrogen-bonding amino acids greatly decreases O2 affinity. In contrast, homologous HisE7 substitutions in Lba have very little effect on ligand affinity. Comparison of rate constants for ligand binding to wild type and distal pocket mutants of soybean Lba suggests that the unusual combination of HisE7 and TyrB10 works together to prevent HisE7 from forming a strong hydrogen bond with bound O2 (5). To test this hypothesis, we have used FTIR spectroscopy to examine electrostatic fields and hydrogen-bonding interactions in the vicinity of bound CO for a complete set of Lba and Mb distal pocket mutants.

The IR spectrum of bound CO is a sensitive measurement of hydrogen-bonding and electrostatic interactions near the ligand (16–24). In native mammalian MbCO, the C–O
arising from a sum of smaller interactions at a distance, as enhancement, weakening, or loss of interaction with this with the bound ligand due to its proximity to CO and that it was assumed that the distal His dominates the interaction 30 movement away from the bound ligand at low pH (18). They concluded that, if the proximal geometry remains proton donors adjacent to iron atom. They suggested that proton donors adjacent to the carbonyl O atom. The CO complexes of many Hbs and Mbs display multiple stretching frequencies between 1900 and 2000 cm\(^{-1}\), with the major \(v_{\text{CO}}\) peaks designated \(v_o\), \(v_1\), \(v_2\), etc., in the descending order of their frequencies (19). The structural origins of these multiple conformational substates have been studied in great detail for sperm whale MbCO. The designations \(A_0\), \(A_1\), etc. (Figure 1) represent discrete conformations of ground-state native MbCO with \(v_{\text{CO}}\) peaks at 1965, 1945, and 1932 cm\(^{-1}\), respectively, based on low-temperature time-resolved IR experiments by Frauenfelder’s group and deconvolution analyses of room temperature IR spectra by Caughey and co-workers (18, 19, 23, 25–28). Li and Spiro (29) interpreted the various \(v_{\text{CO}}\) bands in terms of different extents of back-bond donation from the iron atom. They suggested that proton donors adjacent to the O atom of the bond ligand enhance the degree of back-bonding, increasing the order of the Fe–C bond and decreasing the order of the C–O bond due to the formation of Fe\(^{3+}\)=C=O\(^{-}\) resonance structures. The loss of hydrogen bonding or the presence of a negative electrostatic potential would reverse these effects. Oldfield et al. proposed short-range electric field-induced \(v_{\text{CO}}\) frequency shifts due to 180° ring flips of the tautomers of the distal His and its movement away from the bound ligand at low pH (30, 31). It was assumed that the distal His dominates the interaction with the bound ligand due to its proximity to CO and that enhancement, weakening, or loss of interaction with this residue accounted for all four conformers of MbCO.

More recently, Phillips, Olson, Franzen, and co-workers considered longer range interactions from internal fields arising from a sum of smaller interactions at a distance, as well as specific short-range hydrogen-bonding interactions with His\(^{E7}\) and other adjacent amino acid side chains (19, 32). They concluded that, if the proximal geometry remains invariant, \(v_{\text{CO}}\) is a reflection of the electrostatic fields and hydrogen-bonding interactions exerted by amino acid side chains close to bound CO and by itself does not unambiguously define a conformational substate (18, 19, 33). A positive potential or strong hydrogen bond donation lowers \(v_{\text{CO}}\), whereas a negative potential or loss of hydrogen bonding increases \(v_{\text{CO}}\). Phillips et al. (19) also showed that there is a strong correlation between the average peak value, \(\bar{v}_{\text{CO}}\), and the logarithm of the rate constant for \(O_2\) dissociation, \(k_{O_2}\), for a series of 20 different Mbs. Thus, in Mb both \(v_{\text{CO}}\) and \(k_{O_2}\) reflect the strength of hydrogen-bonding interactions between distal pocket amino acids and bound ligands.

In sperm whale MbCO, the principal CO stretching bands are \(v_o = 1965\) cm\(^{-1}\), \(v_1 = 1949\) cm\(^{-1}\), \(v_2 = 1942\) cm\(^{-1}\), and \(v_3 = 1932\) cm\(^{-1}\), which have been assigned to the \(A_0\), \(A_1\), \(A_2\), and \(A_3\) conformational substates of the His\(^{E7}\) side chain (Figure 1; 16, 21, 22, 25, 26, 34, 35). At neutral pH, the dominant band is at 1945 cm\(^{-1}\) and represents an average peak position for the \(A_1\) and \(A_2\) conformers, often simply called \(A_1\). A minor \(A_3\) substate is observed at \(v_{\text{CO}} \approx 1932\) cm\(^{-1}\) (18). At low pH, the distal His swings out into the solvent, creating an apolar active site with \(v_{\text{CO}} \approx 1960\) cm\(^{-1}\). This Mb conformer is designated the \(A_0\) substate.

In contrast, Lba shows a narrow CO stretching frequency band at 1948 cm\(^{-1}\), indicating a single well-defined conformation at neutral pH (Figure 1) (36). This result suggests that the distal His in LbaCO is more restricted under physiological conditions than in sperm whale Mb, which exhibits significant conformational heterogeneity. In a series of high-resolution NMR studies, Mabbutt et al. (37, 38) observed that the imidazole ring of His\(^{E7}\) in LbaCO appears to be “flipped” with respect to the orientation in sperm whale MbCO by a 180° rotation about the Cβ–Cγ bond. As a result, Nδ points upward, away from the heme plane and toward the protein interior, but Nε is still close to the bound ligand. The Ne–H tautomer of His\(^{E7}\) must still be dominant because the \(v_{\text{CO}}\) peak of LbaCO (1948 cm\(^{-1}\)) is similar in position to \(\bar{v}_{\text{CO}}\) for MbCO (1941 cm\(^{-1}\)), even though the 7 cm\(^{-1}\) shift to higher frequency does indicate a weaker hydrogen-bonding interaction in the plant protein.

Regulation of the position of His\(^{E7}\) and its hydrogen-bonding potential has been suggested as a possible mechanism for attenuating ligand affinity in Lba (5). As described above, FTIR spectroscopy is an ideal tool for assessing the effects of distal pocket mutations on the electrostatic and/or hydrogen-bonding environment of bound CO and for testing structural mechanisms for regulation of ligand binding. In the present study, CO stretching frequencies for a comprehensive set of distal pocket mutants of Lba have been measured. The results have been compared to IR spectra of equivalent mutants of sperm whale Mb. In both proteins, Tyr\(^{B10}\) appears to inhibit hydrogen-bonding interactions between His\(^{E7}\) and bound CO and \(O_2\) (5, 13). The new IR and previous \(O_2\) binding and NMR results suggest that there is a direct interaction between the His\(^{E7}\) and Tyr\(^{B10}\) side chains in Lba that weakens stabilization of bound ligands.

**MATERIALS AND METHODS**

*Preparation, Expression, and Purification of Proteins.* Site-directed mutant cDNAs were constructed, and recombinant Mbs and Lbs were expressed and purified as described previously (4–6, 18). Mutations were introduced at the key helix positions E7, E11, and B10 (Figure 2). Yellow lupine leghemoglobin I (lupin Lb) cDNA in pET 3a was provided by Dr. Pawel M. Strozycki, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poland. The lupin Lb
The protein sample was rapidly added to a CaF$_2$ BioCell gas, was then used to draw LbaCO or deoxyLba from the any precipitate. An airtight syringe, equilibrated with nitrogen was vortexed and then spun in a microcentrifuge to remove any oxidized iron and to remove molecular oxygen. The tube phosphate buffer, pH 7.0, was added to the tube to reduce One microliter of a 200 mM dithionite solution in 100 mM tube was then re-equilibrated with either pure CO or N$_2$ gas.

**FTIR Spectroscopy and Kinetic Measurements.** Samples of CO-bound and deoxygenated Lba and Mb were prepared in stoppered Eppendorf tubes equilibrated with 1 atm of either CO or N$_2$. Approximately 20 $\mu$L of 2–3 mM protein was aliquoted into this tube with a syringe. The Eppendorf tube was then re-equilibrated with either pure CO or N$_2$ gas. One microliter of a 200 mM dithionite solution in 100 mM phosphate buffer, pH 7.0, was added to the tube to reduce any oxidized iron and to remove molecular oxygen. The tube was vortexed and then spun in a microcentrifuge to remove any precipitate. An airtight syringe, equilibrated with nitrogen gas, was then used to draw LbaCO or deoxyLba from the tube. The protein sample was rapidly added to a CaF$_2$ BioCell IR cuvette (5 mm thickness x 50 mm diameter, separated by a 40 $\mu$m spacer; BioTools, Inc.) to obtain a uniform, bubble-free film. Then the windows of the cuvette were quickly sealed. The cuvette was placed in the sample chamber of a Nicolet Nexus 470 FTIR spectrometer (Nicolet Instrument Corp., Middleton, WI), which was purged with nitrogen gas 1 h prior to and then during data collection. Spectra were recorded from 1800 to 2100 cm$^{-1}$ at 1 cm$^{-1}$ resolution. Up to 128 interferograms were averaged for both the HbCO and deoxyHb control samples. The final FeCO FTIR spectra were corrected for buffer and protein background by computing LbaCO minus deoxyLba difference spectra.

FTIR spectroscopy from 1800 to 2100 cm$^{-1}$ was used to quickly seal the cuvette. The cuvette was placed in the sample chamber of a Nicolet Nexus 470 FTIR spectrometer (Nicolet Instrument Corp., Middleton, WI), which was purged with nitrogen gas 1 h prior to and then during data collection. Spectra were recorded from 1800 to 2100 cm$^{-1}$ at 1 cm$^{-1}$ resolution. Up to 128 interferograms were averaged for both the HbCO and deoxyHb control samples. The final FeCO FTIR spectra were corrected for buffer and protein background by computing LbaCO minus deoxyLba difference spectra.

**RESULTS**

His$^{E7}$ Hydrogen Bonds to Bound CO Less Strongly in Lba than in Mb. CO bound to heme within a nonpolar distal pocket typically yields a single IR peak near 1965 cm$^{-1}$ and is shifted to an average value of 1941 cm$^{-1}$ in wild-type Mb (Figure 3; Table 1) (18). This blue shift is due to the positive electrostatic field created at the ligand by a hydrogen bond donated from the distal His $^{18}$–$^{20}$. The absorption at 1948 cm$^{-1}$ by wild-type LbaCO also implies a hydrogen bond to the bound ligand, although a weaker one compared to Mb. In Lba, His$^{E7}$ and Tyr$^{B10}$ could potentially serve as proton donors (Figure 2).

The Phe$^{E7}$, Leu$^{E7}$, and Val$^{E7}$ mutants of Lba show single bands at 1960, 1968, and 1969 cm$^{-1}$, respectively, indicating the loss of a hydrogen bond from the distal His (Figure 3;
Table 1: IR CO Stretching Bands for Soybean Lba, Sperm Whale Mb, and Their Mutant Proteins at pH 7 and 25 °C

<table>
<thead>
<tr>
<th>Protein</th>
<th>( \nu_0 ) (cm(^{-1}))</th>
<th>( \nu_{1,2} ) (cm(^{-1}))</th>
<th>( \nu_1 ) (cm(^{-1}))</th>
<th>( \nu_{CO} ) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Lba</td>
<td>1948 (100)</td>
<td>1948</td>
<td></td>
<td></td>
</tr>
<tr>
<td>His61 (E7) mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly( ^{E7} )</td>
<td>1961 (48)</td>
<td>1952 (52)</td>
<td>1956</td>
<td>1965</td>
</tr>
<tr>
<td>Ala( ^{E7} )</td>
<td>1969 (49)</td>
<td>1951 (51)</td>
<td>1959</td>
<td>1966</td>
</tr>
<tr>
<td>Val( ^{E7} )</td>
<td>1969 (100)</td>
<td>1968</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu( ^{E7} )</td>
<td>1968 (100)</td>
<td>1968</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe( ^{E7} )</td>
<td>1960 (100)</td>
<td>1960</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp( ^{E7} )</td>
<td>1959 (38)</td>
<td>1940 (62)</td>
<td>1947</td>
<td>1958</td>
</tr>
<tr>
<td>Tyr( ^{E7} )</td>
<td>1968 (55)</td>
<td>1953 (45)</td>
<td>1961</td>
<td>1966</td>
</tr>
<tr>
<td>Gln( ^{E7} )</td>
<td>1957 (63)</td>
<td>1943 (37)</td>
<td>1952</td>
<td>1945</td>
</tr>
<tr>
<td>Lyr( ^{E7} )</td>
<td>1968 (42)</td>
<td>1959 (58)</td>
<td>1962</td>
<td>1961</td>
</tr>
<tr>
<td>Arg( ^{E7} )</td>
<td>1957 (30)</td>
<td>1944 (34)</td>
<td>1940 (36)</td>
<td>1958</td>
</tr>
<tr>
<td>Leu65 (E11) mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val( ^{E11} )</td>
<td>1948 (100)</td>
<td>1948</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe( ^{E11} )</td>
<td>1948 (100)</td>
<td>1948</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr30 (B10) mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly( ^{B10} )</td>
<td>1966 (39)</td>
<td>1949 (26)</td>
<td>1927 (35)</td>
<td>1948</td>
</tr>
<tr>
<td>Ala( ^{B10} )</td>
<td>1963 (29)</td>
<td>1949 (34)</td>
<td>1926 (37)</td>
<td>1944</td>
</tr>
<tr>
<td>Val( ^{B10} )</td>
<td>1967 (3)</td>
<td>1950 (41)</td>
<td>1927 (56)</td>
<td>1938</td>
</tr>
<tr>
<td>Leu( ^{B10} )</td>
<td>1973 (5)</td>
<td>1951 (30)</td>
<td>1928 (65)</td>
<td>1937</td>
</tr>
<tr>
<td>Phe( ^{B10} )</td>
<td>1960 (13)</td>
<td>1951 (44)</td>
<td>1923 (43)</td>
<td>1940</td>
</tr>
<tr>
<td>Trp( ^{B10} )</td>
<td>1961 (52)</td>
<td>1948 (39)</td>
<td>1929 (53)</td>
<td>1953</td>
</tr>
<tr>
<td>Arg( ^{B10} )</td>
<td>1965 (75)</td>
<td>1948 (17)</td>
<td>1929 (8)</td>
<td>1958</td>
</tr>
<tr>
<td>Double mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu( ^{B10} )Ala( ^{E7} )</td>
<td>1965 (100)</td>
<td>1965</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Data taken from Li et al. (18) unless mentioned otherwise. *The frequency of the IR band was determined by the peak position, with estimated experimental error \( \pm 1 \text{ cm}^{-1} \); the intensity of the IR band was determined by the peak height and was normalized to the strongest band with estimated experimental error \( \pm 10\% \). *The value of \( \nu_{CO} \) is a weighted measurement of the IR CO spectral components, as computed by \( \nu_{CO} = \sum_i \nu_i \), where \( \nu_i \) is the fraction of intensity measured by peak height and \( \nu_i \) is the peak frequency of spectral component \( i \). *Four conformers were tentatively assumed in Lba for comparison with Mb. *Measured by the authors. *Data not available.

Table 1: These peaks are similar to those observed for CO–heme complexes within a completely apolar binding pocket and imply that Tyr\( ^{B10} \) makes no significant direct contribution to the electrostatic environment of the bound ligand. In the wild-type metLba structure (4), the Tyr\( ^{B10} \) side chain is too far away from the iron atom to hydrogen bond directly with bound ligands without significant movements of the B and E helices (Figure 2). This conclusion is supported by the close similarity of the \( \nu_{CO} \) bands observed for the Phe\( ^{E7} \), Leu\( ^{E7} \), and Val\( ^{E7} \) mutants, which contain Tyr\( ^{B10} \), and the \( \nu_{CO} \) band at 1965 cm\(^{-1} \) seen in the completely apolar Leu\( ^{B10} \), Ala\( ^{E7} \) double mutant of Lba (Figure 4; Table 1). Conversely, Leu\( ^{B10} \) (which still contains His\( ^{E7} \)) shows an IR band at much lower frequency, indicating that a strong hydrogen bond can be formed between bound CO and the distal His in the absence of the Tyr side chain (Figure 4).

Water molecules in the exposed distal pockets of Gly\( ^{E7} \) and Ala\( ^{E7} \) MbCO broaden the observed \( \nu_{CO} \) peaks (18, 19), and a similar explanation probably accounts for the heterogeneity observed in the IR spectra of Gly\( ^{E7} \) and Ala\( ^{E7} \) LbaCO (Figure 3). The spectrum of Gly\( ^{E7} \) LbaCO is unusually broad, with two obvious shoulders at 1952 and 1961 cm\(^{-1} \) (Table 1). The Ala\( ^{E7} \) LbaCO spectrum has two distinct peaks. The narrow band at 1969 cm\(^{-1} \) indicates a conformation with a completely apolar environment near bound CO. The broad band centered at \( \sim 1951 \text{ cm}^{-1} \) (similar to that seen in Gly\( ^{E7} \)) indicates favorable hydrogen-bonding interactions, presumably with distal pocket water molecules. This interpretation is supported by the IR spectrum of the Leu\( ^{B10} \)Ala\( ^{E7} \) LbaCO double mutant, which has no polar residues or stabilized water molecules and shows a single narrow band centered at \( \nu_{CO} = 1965 \text{ cm}^{-1} \) (Figure 4; Table 1). The lack of any hydrogen-bonding interactions in the double mutant is also apparent from its much larger rate constant for \( \text{O}_2 \) dissociation (\( k_{d2} \approx 100 \text{ s}^{-1} \)) compared to those for wild-type LbaO\( _2 \) (\( k_{d2} \approx 6 \text{ s}^{-1} \)) and the two corresponding single mutants (\( k_{d2} \approx 1–3 \text{ s}^{-1} \)) (Table 2). Thus, although His\( ^{E7} \) is the main source of polarity around bound ligands in Lba, hydrogen bonding is relatively weak, and Tyr\( ^{B10} \) can have a significant indirect influence. This results in differences in the electrostatic interactions with bound ligands in the distal heme pockets of Lba and Mb.
Role of the B10 Side Chain in Positioning HisE7 in Mb and Lba. An important difference between Lba and Mb is the Tyr found at the B10 helical position in the former instead of an apolar Leu in the latter (Figure 2). Replacement of TyrB10 in LbaCO by any other amino acid, irrespective of size or polarity, results in the appearance of multiple νCO peaks (Figure 5; Table 1). In general, the IR spectra of the B10 mutants are mixtures of high- and low-frequency bands, which are completely absent in wild-type LbaCO. The low-frequency νCO peaks for the B10 LbaCO mutants are at ~1926 cm⁻¹, which is significantly lower than those seen in the B10 mutants of sperm whale MbCO (~1932 cm⁻¹, Figure 5; Table 1). The low-frequency peaks in the B10 mutants are mixtures of high- and low-frequency bands, from all of the B10 mutants compared to wild-type LbaO₂ (Table 1).

The average νCO for the apolar B10 LbaCO mutants is lower than that of wild-type LbaCO (Table 1). However, there are also bands in the 1960–1970 cm⁻¹ range, indicating conformations with completely apolar binding sites and no interaction with the distal His. Thus, when TyrB10 in Lba is replaced by other amino acids, the side chain of HisE7 adopts multiple orientations, some of which involve formation of strong hydrogen bonds with bound ligands and some of which involve no interaction at all. In contrast, apolar B10 mutations in Mb only enhance the low-frequency band associated with the A₂ conformer (νCO ≈ 1932 cm⁻¹). Little or no intensity associated with high-frequency bands at νCO = 1960–1970 cm⁻¹ is seen (Figure 5; Table 1). The IR spectrum of PheB10 MbCO has a single band at 1932 cm⁻¹.

The crystal structures of PheB10 MbCO and MbO₂ show that HisE7 is fixed in a more A₂-like conformation and the positive edge of the benzyl side chain points toward the bound ligand (18). This conformation causes a ~10-fold decrease in the rate of O₂ dissociation from PheB10 MbO₂ compared to that from the wild-type protein (Table 2; 43).

The TrpB10 mutations in both LbaCO and MbCO cause a shift in νC=O to higher frequencies, and there is minimal appearance of strong low-frequency bands in the 1920–1935 cm⁻¹ region (Table 1). In these cases, the large size of the indole ring appears to be “pushing” the distal His away from bound ligands. The ArgB10 mutation in LbaCO causes a more significant effect, increasing νC=O from 1948 to 1958 cm⁻¹, suggesting that the HisE7 side chain has been pushed out into the solvent. Because the B10 side chain in Lba is too far from the active site, the guanidino group of ArgB10 cannot access the bound ligands either to interact favorably with them (Figure 2).

The TrpB10 mutation produces the largest effect on the IR spectrum of MbCO, causing the appearance of multiple bands from 1931 to 1980 cm⁻¹ (Figure 5). Thus, a Tyr side chain at the B10 position in Mb has the opposite effect of that observed in Lba. The phenolic side chain in Mb causes multiple conformations with positive and negative fields adjacent to the bound ligand. This interpretation is supported by the multiple phases seen for O₂ dissociation from TrpB10 MbO₂ (13; Table 2). In contrast, the TyrB10 side chain in native Lba “traps” HisE7 in a single conformational state, which can only donate a weak hydrogen bond to bound ligands. When TyrB10 is replaced by Leu in LbaCO (a distal pocket akin to Mb), HisE7 is able to adopt a conformation with a much stronger hydrogen bond to bound ligands as
The effects of the HisE7→LysE7 mutation on the IR spectra of MbCO and LbaCO are much more similar than in the case of the ArgE7 replacement (Figure 6). In both proteins, most of the absorbance occurs at higher frequencies, indicating that the dominant conformations have the primary amine group pointing out of the distal heme pocket. GlnE7 MbCO and LbaCO show peak positions similar to those of the wild-type proteins, but the bands are broader. TyrE7 in MbCO exclusively provides an apolar environment to bound CO, whereas TyrE7 in LbaCO provides some positive potential as well. TrpE7 MbCO and LbaCO show a conformer with lower νCO in addition to a higher one. The spectra for ArgE7 and LysE7 MbCO mutant proteins were measured for this work, and the others were taken from Li et al. (18).

Can either swing out into solvent, causing an apolar active site and an increase in νCO, or remain in the pocket, causing an increase in the electrostatic field near the bound ligand and a decrease in νCO. The latter “in” conformation appears to be the dominant one when ArgE7 Lba binds O2, as judged by the dramatic decrease in kO2 (Table 2). The TyrB10 hydroxyl O atom probably helps to stabilize the “in” conformation by acting as a hydrogen bond acceptor for the guanidino group, and the partial negative charge on bound O2 provides additional electrostatic stabilization. In Mb, the naturally occurring LeuB10 side chain cannot facilitate internalization of the ArgE7 side chain, and the free energy released by solvation of the guanidino group out-competes any weak, favorable interaction with bound ligands. In addition, the size of the distal pocket in Mb is smaller and probably cannot easily accommodate the large ArgE7 side chain.

The effects of the HisE7→LysE7 mutation on the IR spectra of MbCO and LbaCO are much more similar than in the case of the ArgE7 replacement (Figure 6). In both proteins, most of the absorbance occurs at higher frequencies, indicating that the dominant conformations have the primary amine pointing out of the pocket, increasing the apolar character of the ligand binding site. The GlnE7 mutations in both proteins cause little change in the average νCO value but do

**Figure 5:** IR spectra of B10 mutants of soybean LbaCO and sperm whale MbCO. All of the Lba mutants show multiple peaks, including at least one low-frequency band. Most of the B10 mutations in Mb change only in the proportion of the ν1 versus ν3 bands, with PheB10 MbCO showing only a single conformer at ν3 = 1932 cm⁻¹. In contrast, the TyrB10 mutation in MbCO causes the appearance of a major high-frequency band at 1969 cm⁻¹. The spectra for the Ala, Val, Ile, and Phe B10 Mb mutant proteins were taken from Li et al. (18); the other spectra were measured in this work.

**Polar Mutations at the E7 Position.** As shown in Figure 6, the native TyrB10 side chain in Lba also affects the position of polar amino acids when they are inserted into the E7 helical position as compared to their positions in analogous E7 mutants of Mb. The most dramatic differences between the two proteins are observed for the single ArgE7 mutants. In ArgE7 MbCO, a single, narrow high-frequency band at 1958 cm⁻¹ is observed (Figure 6) and has been interpreted in terms of the ArgE7 guanidino group pointing into the solvent, as has been observed in Hb Zurich (β HisE7→Arg) (44–47). In contrast, several bands are observed for ArgE7 LbaCO (Table 1). The majority of the conformers occur at relatively low frequencies in the 1940–1945 cm⁻¹ region (Figure 6), indicating a positive electrostatic field adjacent to bound CO. More remarkably, the HisE7→Arg mutation in Lba causes the rate constant for O2 dissociation to decrease by ~30-fold, indicating that an even stronger stabilizing interaction occurs when dioxygen is bound (Table 2). In contrast, the HisE7→Arg mutation in Mb causes kO2 to increase from 15 to ~1000 s⁻¹, indicating the loss of stabilizing electrostatic or hydrogen-bonding interactions.

Taken together, these spectroscopic and ligand-binding data suggest that the guanidino group of ArgE7 can remain in the distal pocket of Lba. In the CO complex, the side chain adopts multiple conformations. The guanidino group

**Figure 6:** IR spectra of LbaCO and MbCO mutants with charged or polar residues at position E7. ArgE7 MbCO shows a single, high-frequency peak indicating that this residue does not interact with bound CO. In contrast, ArgE7 LbaCO shows multiple bands with significant populations of conformers having lower νCO, suggesting that this side chain can form hydrogen bonds with bound CO. The LysE7 mutants for both, however, are predominantly in conformations with higher νCO and possibly have their side chains swinging out of the distal heme pocket. GlnE7 MbCO and LbaCO show peak positions similar to those of the wild-type proteins, but the bands are broader. TyrE7 in MbCO exclusively provides an apolar environment to bound CO, whereas TyrE7 in LbaCO provides some positive potential as well. TrpE7 MbCO and LbaCO show a conformer with lower νCO in addition to a higher one. The spectra for ArgE7 and LysE7 MbCO mutant proteins were measured for this work, and the others were taken from Li et al. (18).
dominant conformer in LbaCO has a low-frequency band at 1940 cm$^{-1}$, which results in the appearance of bands in the 1940 cm$^{-1}$ region of both proteins, suggesting subtle effects on protein stability and ligand-binding kinetics.

Val$^E_{11}$ in Lba Has Little Effect on Hydrogen Bonding. Val$^E_{11}$ in Mb is important for maintaining the appropriate distal pocket volume for ligand binding and prevention of autoxidation (47), and mutations at this position have been found to influence the MbCO IR spectra significantly (18, 19, 47–49). As seen in Figure 7A and Table 1, even relatively subtle mutations at the E11 position in Mb affect its IR spectrum. However, the corresponding Lba mutant proteins leave the IR spectra completely unaltered (Figure 7B). Apolar replacement of Val$^E_{11}$ in Lba also has only small, subtle effects on protein stability and ligand-binding kinetics (5). The crystal structure of Lba shows that the naturally occurring Val$^E_{11}$ side chain is behind the distal His, almost on the far interior side of the ligand-binding site compared to the position of Val$^E_{11}$ in Mb (4). Therefore, the importance of this amino acid in Mb is not shared in the distal pocket of Lba.

Role of the B10 Amino Acid in Other Leghemoglobin.

Tyr is not conserved at position B10 in all leghemoglobins. Other species, most notably lupin, contain Phe at this site (50). To examine the importance of this naturally occurring Tyr$^P_{10}$ replacement, IR spectra for cowpea LbII (Tyr$^P_{10}$) and lupin LbI (Phe$^B_{10}$) were measured (Figure 8). Cowpea LbII has an IR spectrum identical to that of soybean Lba, with a single conformer at 1948 cm$^{-1}$ (Table 3). Both of these Lbs have a distal His$^E_{7}$–Tyr$^B_{10}$ pair, nearly identical rate constants for oxygen binding (Table 3) (51), and presumably similar mechanisms for regulating ligand binding.

As shown in Figure 5, replacing Tyr$^B_{10}$ with Phe causes profound changes in the IR spectrum of soybean LbaCO, leading to the appearance of a large low-frequency band centered at 1923 cm$^{-1}$ and the retention of a peak at 1950 cm$^{-1}$. The IR spectrum of lupin LbICO is different from those of both native soybean Lba and its Phe$^B_{10}$ mutant. However, a major band (~75%) is observed at 1951 cm$^{-1}$, which is similar in position to that for cowpea and soybean LbCO. Thus, in lupin LbICO there must be constraints other than Tyr$^B_{10}$ that position His$^E_{7}$ in a conformation that allows only weak hydrogen bonding to bound CO. Harutyunyan et al. (52) have suggested that the His$^E_{7}$ side chain in the lupin LbICO crystal structure appears to be fixed in position by a lattice of hydrogen bonds linking N$\ddot{o}$ of the imidazole side chain to well-defined water molecules, Glu$^{\delta_{3}}$, and one of the heme propionates. However, these constraints do not appear to be as strong as those imposed by Tyr$^B_{10}$ in the other Lbs, because lupin LbICO also shows minor low- (7%) and high-frequency (18%) bands at 1920 and 1962 cm$^{-1}$, respectively (Table 3).

The Tyr$^B_{10}$–His$^E_{7}$ Combination Destabilizes Bound Ligands. The side chain at the B10 position in Mb is close enough to make direct contact with bound ligands. However, multiple $v_{CO}$ bands and heterogeneous ligand binding are observed for the single Tyr$^B_{10}$ Mb mutant, and there is a net increase in $v_{CO}$ and the average value of $k_{O2}$ (Table 2; Figure 9A). Draghi et al. (13) have proposed that Tyr$^B_{10}$ and His$^E_{7}$ CO FTIR Spectroscopy of Leghemoglobin

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**Table 3: IR CO Stretching Bands for Three Leghemoglobins at pH 7 and 25 °C**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$v_0^c$ (%$^c$)</th>
<th>$v_1^c$ (%)</th>
<th>$v_2^c$ (%)</th>
<th>$v_3^c$ (%)</th>
<th>$v_{CO}$ (%)</th>
<th>$k_{CO}$ ($s^{-1}$)</th>
<th>$k_{O2}$ ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean Lba</td>
<td>1948 (100)</td>
<td>0.0084</td>
<td>5.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cowpea LbII</td>
<td>1947 (100)</td>
<td>0.014</td>
<td>5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lupin LbI</td>
<td>1962 (18)</td>
<td>1951 (75)</td>
<td>1951 (7)</td>
<td>0.014</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ See footnotes b and c of Table 1. $^b$ Data taken from Gibson et al. (51).
results suggest that in native Lba the phenol side chain holds HisE7 in a fixed location farther away from bound ligands than the position of HisE7 in Mb. In almost all of the Lba B10 mutants, a low-frequency band is observed at \(~1925\) cm\(^{-1}\), indicating that a stronger hydrogen bond can form between HisE7 and bound CO in the absence of TyrB10. (2) The B10 Lba mutations also cause significant decreases in \(k_{\text{O}_2}\), indicating a strengthening of the hydrogen bond to bound O\(_2\) when HisE7 is “freed” from its interaction with the phenol side chain of TyrB10. (3) Replacement of HisE7 with large aliphatic residues (Val and Leu) causes a 20 cm\(^{-1}\) increase in the peak position of the major \(v_{\text{CO}}\) band, indicating loss of hydrogen bonding to bound ligands. However, the increases in the rate constants for O\(_2\) dissociation from these LbaO\(_2\) mutants are only 5-fold, as compared to the 200–500-fold increases observed for the ValE7 and LeuE7 replacements in sperm whale Mb. Thus, the hydrogen-bonding interaction between bound ligands and HisE7 in wild-type Lba is significantly weaker than in Mb, presumably due to interactions with TyrB10.

In the absence of crystal structures of CO and O\(_2\) complexes of soybean Lba, structural modeling was used to investigate possible interactions between TyrB10 and HisE7 that would explain the IR spectra of native and mutant LbaCOs and the rates of O\(_2\) dissociation from the corresponding oxygenated complexes. In the crystal structure of acetate-bound soybean metLba, TyrB10 is much closer to HisE7 than to the heme iron or the first two atoms of the bound ligand (4). The O atom of the TyrB10 side chain is ~3.3 Å away from the edge of the imidazole ring of HisE7 and >4.0 Å away from the second ligand atom. Even in the absence of a bound ligand, the phenol side chain cannot move closer to the iron atom unless there is substantial movement of the entire B helix toward the heme plane, and such a large-scale movement would also require significant movement of the E helix. However, the TyrB10 and HisE7 side chains both appear to be flexible enough to interact with each other while allowing only the E7 imidazole to interact directly with bound ligands.

The model shown in Figure 10A was generated from the acetate-bound metLba crystal structure (4) by using the “flipped” orientation of the E7 imidazole ring described by Mabbutt et al. (37, 38) and then rotating both the HisE7 and TyrB10 side chains about their Cα–Cβ and Cβ–Cy bonds in the program O without violating van der Waals restraints. In this conformation, the Nδ atom of HisE7 can accept a hydrogen bond from the OH group of TyrB10, and the Ne–H group of HisE7 can still donate a hydrogen bond to the bound ligand. This model predicts one HisE7 conformer for the CO complex because only one orientation could be found that allows formation of a strong hydrogen bond between HisE7 and both the TyrB10 side chain and the bound ligand. In support of the model, the IR spectrum of LbaCO shows a single, narrow band with a peak at a slightly higher \(v_{\text{CO}}\) than that for Mb. The model is also consistent with multiple \(v_{\text{CO}}\) peaks in the IR spectra of TyrB10 mutants of LbaCO because, in the absence of hydrogen bonding to the phenol side chain, HisE7 is “free” to adopt other orientations.

The model in Figure 10A requires a 180° rotation of the HisE7 side chain about the Cβ–Cy bond compared to the orientation reported for the metLba acetate (4) and nicotinate (54) crystal structures. As mentioned previously, this “flipped”...
conformation is supported by NMR investigations by Wright’s group, who showed that the imidazole ring of His E7 is oppositely oriented in LbaCO compared to that found in MbCO (37, 38). In solution, the C=H proton in Lba is closer to the heme than the C=O proton, whereas the reverse is true in MbCO (38). The C=H proton resonances for the side chains of His E7 and Tyr B10 can be calculated using a combination of the program SHIFTs (42), the PDB files for the observed crystal structure, and the model in Figure 10A. These calculated chemical shifts can then be compared to those reported by Morikis et al. (53) for LbACO.

The chemical shifts calculated for the His E7 C=H protons in our model of LbaCO are 2.72 and 2.89 ppm and nearly identical to the values observed experimentally by Morikis et al. [2.73 and 2.88 ppm (53)]. The calculated C=H shifts for the acetate—metLba structure are 3.26 and 3.27 ppm, indicating that the side chain is further from the heme plane when the larger acetate ligand is bound. More importantly, the predicted chemical shifts of the His E7 C=O and C=H protons, 6.75 and 4.82 ppm, respectively, are much closer to the observed values of 7.15 and 5.26 ppm for LbaCO in solution than those calculated for acetate—metLba crystal structure, 7.04 and 8.11 ppm. The observed values for the His E7 C=O and C=H protons require that the imidazole ring be rotated 180° with respect to the orientation found in the metLba Figure 10B) and sperm whale MbCO (Figure 10B) crystal structures (37, 38). The predicted chemical shifts of the Tyr B10 C=H, C=O, and C=H protons for the metLba crystal structure (2.54, 3.00, 6.58, and 6.38 ppm) are also closer to the observed values [53] than those calculated for the acetate—metLba crystal structure (2.79, 3.32, 7.09, and 6.69 ppm).

Thus, our structural interpretation in terms of direct hydrogen bonding between His E7 and Tyr B10 is consistent with all reported IR, ligand binding, and solution NMR data. However, more systematic modeling using molecular dynamics and energy minimization is needed. The ultimate verification of the model will come with NMR or crystal structure determinations of the wild-type LbaO2 and LbaCO complexes.

The unusual properties of Arg E7 Lba can also be rationalized by a distal pocket structure similar to that proposed for the native protein (Figure 10C). In Lba, the guanidino group can potentially swing inward and form hydrogen bonds with both the phenol side chain of Tyr B10 and bound ligands. In the case of the relatively apolar FeCO complex, the interaction with the bound ligand is weak, and 15–20% of the population of mutant conformers has an apolar pocket with νCO ≥ 1960 cm−1. However, in combination with the Tyr B10 side chain, the partial negative charge on the ligand atoms of the FeO2 complex appears to cause Arg E7 to adopt a completely “in” conformation (Figure 10C). As a result, the Arg E7 mutation in Lba O2 causes a 30-fold decrease in kO2, producing a protein with ultrahigh O2 affinity (P50 ≤ 1 nM; Table 2).

Physiological Importance of the B10 Amino Acid. The role of the B10 amino acid in regulating ligand binding in Hbs is now widely accepted (43, 55, 56). As described in this work, the naturally occurring Tyr B10 side chain in soybean and cowpea Lb serves to “trap” His E7 in a single conformation that provides weaker stabilization to bound ligands than is observed in mammalian Mbs. This helps Lb maintain an oxygen affinity appropriate for its physiological function. In almost all mammalian Mbs and Hbs, the B10 residue is
of Lb mutants listed in Table 2 (Figure 11). The cause of the variance is flexibility of the distal pocket, particularly the conformation of the E7 side chain, which probably changes between the CO and O₂ complexes of Lba.

Arg²⁷ Lba provides a good example of large apparent differences between the O₂ and CO complexes. The IR spectrum of the CO complex shows conformational heterogeneity with a broad peak at moderately low frequency and another broad peak at moderately high frequency. Because \( v_{CO} \) is 1946 cm⁻¹, only a small decrease in \( k_{O2} \) is predicted. However, the observed rate constant decreases 30-fold, suggesting strongly that the Arg²⁷ side chain has completely swung into the distal pocket, stabilizing bound O₂ due to its partial negative charge (Figure 10C). A distal Arg side chain has been shown to stabilize bound oxygen in Aplysia limacina Mb (62) and in the DOS-oxygen sensor protein from Bradyrhizobium japonicum, which has a very low rate of O₂ dissociation (63).

The nearly parallel fitted lines in the plots of \( \log(k_{O2}) \) versus \( v_{CO} \) shown in Figure 11 demonstrate that the general electrostatic/hydrogen bonding mechanism for preferentially regulating O₂ dissociation applies to both Mb and Lba. The line is displaced in Lba probably due to proximal stabilization of ligand binding, causing lower \( k_{O2} \) values. The electrostatic mechanism and the proposed structure in Figure 10A explain qualitatively all of the large changes produced by distal pocket mutations in Lba. Replacement of the distal His with large apolar residues that exclude water from the distal pocket causes increases in \( v_{CO} \) and \( k_{O2} \) (Table 2). Replacement of Tyr²¹⁰ with apolar amino acids causes the appearance of strong low-frequency \( v_{CO} \) bands and decreases in \( k_{O2} \) (Table 2) because the distal His is free to adopt multiple conforma-
tions, some of which form strong hydrogen bonds with bound ligands. When both Tyr\(^{110}\) and His\(^{17}\) are replaced with apolar amino acids, the resulting Lba double mutant shows an IR spectrum identical to that of Mb with a hydrophobic distal pocket and a large \(O_2\) dissociation rate constant.

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