Controlling Ligand Binding in Myoglobin by Mutagenesis

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A quadruple mutant of sperm whale myoglobin was constructed to mimic the structure found in Ascaris suum hemoglobin. The replacements include His(E7) → Gln, Leu(B10) → Tyr, Thr(E10) → Arg, and Ile(G8) → Phe. Single, double, and triple mutants were characterized to dissect out the effects of the individual substitutions. The crystal structures of the deoxy and oxy forms of the quadruple mutant were determined and compared with that of native Ascaris hemoglobin. Tyr(B10) myoglobin displays low O2 affinity, high dissociation rate constants, and heterogeneous kinetic behavior, suggesting unfavorable steric interactions between the B10 phenol side chain and His(E7). In contrast, all mutants containing the Tyr(B10)/Gln(E7) pair show high O2 affinity, low dissociation rate constants, and simple, monophasic kinetic behavior. Replacement of Ile107 with Phe enhances nanosecond geminate recombination singly and in combination with the Tyr(B10)/Gln(E7)/Arg(E10) mutation by limiting access to the Xe4 site. These kinetic results and comparisons with native Ascaris hemoglobin demonstrate the importance of distal pocket cavities in governing the kinetics of ligand binding. The 150-fold higher O2 affinity of Ascaris hemoglobin compared with that for Tyr(B10)/Gln(E7)-containing myoglobin mutants appears to be the result of favorable proximal effects in the Ascaris protein, due to a staggered orientation of His(F8), the lack of a hydrogen bonding lattice between the F4, F7, and F8 residues, and the presence of a large polar Trp(G5) residue in the interior portion of the proximal heme pocket.

More than 50 years ago, Davenport (1) showed that the ultrahigh affinity of Ascaris suum hemoglobin for O2 is due to a remarkably small rate constant for O2 dissociation (<0.01 s−1). In 1994, De Baere et al. (2) and Kloek et al. (3) suggested that the structural cause of the low dissociation rate was electrostatic stabilization of bound O2 by two hydrogen bonds, a very strong one with Tyr(B10) and a weaker one with Gln(E7).

These conclusions were based on the effects of Tyr(B10) → Phe and Gln(E7) → Leu mutations on the rate of O2 dissociation from recombinant Ascaris heme domain I. In an attempt to test these conclusions and to mimic the high O2 affinity, Leu(B10) → Tyr, His(E7) → Gln, and Thr(E10) → Arg mutations were introduced into sperm whale myoglobin (4–6).

The site-directed mutagenesis studies on mammalian myoglobin were also carried out to examine pathways for ligand movement inside the protein using laser photolysis techniques. At low temperatures, the large Tyr(B10) side chain prevents rebinding of photolyzed CO, which can then migrate into internal cavities within the protein and be visualized by x-ray crystallography (5).

The effects of multiple mutations at the E7 and B10 positions are also important for designing safer and more efficient hemoglobin-based blood substitutes. Replacements of Leu(B10) with large aromatic amino acids have been used to inhibit NO scavenging by recombinant myoglobins and hemoglobins (7, 8). Most workers believe that the hypertensive side effect caused by infusion of extracellular hemoglobins in animal models and in humans is due to interference with NO signaling in blood vessel walls. Doherty et al. (8) have shown that there is a strong correlation between the magnitude of blood pressure elevation in vivo and the bimolecular rate constant for NO scavenging measured in vitro.

De Baere et al. (2) and Kloek et al. (3) identified four key amino acid differences between the active site of Ascaris Hb and that of sperm whale myoglobin: Leu(B10) → Tyr (Y), 1 His(E7) → Gln (Q), Thr(E11) → Arg (R), and Ile(G8) → Phe (F). Replacements already reported in sperm whale myoglobin include single Y (9), Q (10), R, and F (11, 12) and multiple QR and YQR mutants (4, 6). This paper collates all of the previous data and describes the ligand binding properties of the double YQ and quadruple YQRF mutants. The previous Y, Q, R, F, QR, and YQR mutants were used as controls. The functional results are correlated with the deoxygenated and oxygenated crystal structures of YQRF myoglobin and those reported previously for A. suum hemoglobin (13).

The results are discussed in relation to pathways of ligand migration within globins and to general protein engineering principles. The F mutation clearly inhibits movement of ligands away from the distal pocket and into the Xe4 binding site, regardless of the nature of other distal pocket amino acids (11, 12, 14). The YQRF series myoglobin mutants exemplify the successes and problems associated with rational protein design. Unlike the individual Y and Q mutants, the YQ double...
mutant shows a marked decrease in the rate of O₂ dissociation, providing an example of positive interactions between side chains that are in close proximity to each other.

None of the YQ-containing Mb² mutants has an oxygen affinity as high as that of Ascaris hemoglobin. Detailed comparison of the structure of native Ascaris Hb and that for the YQRF Mb mutant suggests four key differences that account for the >150-fold difference in O₂ equilibrium constants. First, the side chains of Gln(E7) and Tyr(B10) in Ascaris Hb and in YQR Mb are closer to bound O₂ than in the YQRF mutant. Second, the distal cavity immediately adjacent to the bound ligand is larger in Ascaris Hb, and return to the iron is less hindered, although further migration into the protein interior is prevented. Third, in Ascaris Hb, Leu(G5) is replaced with a more polar Trp residue in the cavity underneath the C ring of the heme in Ascaris Hb. Fourth, the proximal His(F8) side chain in Ascaris Hb adopts a more favorable staggered orientation with respect to the pyrrole nitrogen atoms. All of these differences have been shown to enhance ligand affinity, increase geminate recombination, and lower dissociation rate constants in myoglobin and leghemoglobin model systems (15–20).

**Experimental Procedures**

**Mutagenesis, Expression, and Purification**—The coding sequence for YQR Mb, cloned in the vector pUC19, was used as a template to introduce the fourth mutation, Ile(G8) → Phe, by means of PCR. The quadruple mutant was checked by automated nucleotide sequencing. The site-directed YQR Mb mutant, together with the other six recombinant Mbs investigated, was expressed in *Escherichia coli* and purified as previously described (4, 21).

**Kinetic Measurements**—All of the experiments were carried out in 0.1 M phosphate buffer, pH 7.0, 2 mM EDTA, 20 °C. Reagents were of analytical grade. O₂ association time courses were measured by laser photolysis of MbO₂ samples using a 300-ns excitation pulse from a Phase-R model 2100B dye laser as described by Rohlfis et al. (10) and by mixing deoxy-Mb samples with low [O₂] in a stopped-flow, rapid mixing spectrometer. The myoglobin samples were prepared by adding a trace of dithionite to the oxidized or partially reduced protein, which was then run through a G-25 column equilibrated with various high concentrations of O₂ (1 atm of O₂ to 1 atm of N₂ for anaerobic conditions). Samples from the column were withdrawn with a gas-tight syringe and inserted into a sealed cuvette (1-mm path length) previously equilibrated with the same partial pressure of O₂. O₂ dissociation rate constants were determined by analyzing time courses for ligand recombination and overall ligand dissociation rate constants in myoglobin and leghemoglobin model systems (15–20).

**Scheme for Interpreting Geminate Recombination and Overall Ligand Binding Kinetics**—Laser spectroscopy has been used successfully to study the dynamics of ligand migration in Mb. At room temperature, O₂ and NO serve as useful probes to study the events that occur after photolysis laser on nanosecond and picosecond time scales (26, 27). CO is much less reactive, and as a result, the amount of internal recombination is <10% before ligand escape from the protein occurs. The side path scheme shown in Scheme 1 has been used successfully to interpret time courses for internal geminate rebinding in wild-type and mutant sperm whale myoglobins (11, 16).

**Photodissociation of the ligand is associated with an ultrafast "jump" from the bound position to a location parallel to the heme plane and directly below the side chain of Leu(B10) (see Ref. 28). At room temperature, ligands then either rebind from this position on picosecond time scales or migrate further into the protein. If further migration occurs, then internal rebinding becomes a slower process, since the ligands must to return to the iron from more remote positions (25).

On nanosecond time scales, the ligand may occupy any one of several rapidly interconverting positions. The more rapid and dominate nanosecond phase shows rate constants in the range of ~5–100 μs⁻¹. This phase is due to ligand rebinding from positions in the distal pocket above the porphyrin ring in the space circumscribed by Leu(B10), Phe(CD1), His(E7), Val(E11), and Ile(G8), including the initial "docking" site seen at low temperature. This collection of ligand positions is designated as the primary or "A" nanosecond state in Scheme 1.

The slower phases at room temperature show rate constants on the order of 1 to 0.1 μs⁻¹ and have been assigned to ligands rebinding from more interior positions. These locations are often designated as secondary docking sites. State C in Scheme 1 represents a collection of these more remote ligand positions, which have been identified as the Xe binding pockets based on molecular dynamics simulations (6) and analyses of laser photolysis experiments carried out under high pressures of xenon gas (16, 17). Recent low temperature crystallographic experiments with photolytic intermediates have provided direct structural confirmation of the location of dissociated CO in the distal pocket and in these secondary sites (5, 29–31).

**Crystallization and Structure Determination**—Crystals were grown at 20 °C as previously described (6). Crystals grow in ~3 weeks and belong to the hexagonal group P6. YQRF deoxy-Mb was obtained by soaking the crystals in a solution of mother liquor containing sodium dithionite. Oxy and CO derivatives were obtained by soaking fully reduced crystals in a mother liquor solution equilibrated with each gas, separately. X-ray diffraction data were collected from frozen crystals at 120 K, using 29% glycerol as cryoprotectant. Data collection was performed at the synchrotron ELETTRA in Trieste (Italy), using the MAR imaging plate system. 1° oscillation images were collected, and the data were processed with DENZO/Scalepack (32). The refinement was accomplished using the maximum likelihood program REFMAC (32) of the CCP4 suite (33). Graphical display and addition of water molecules were performed using Xtalview (33).

**Results**

Rate constants for O₂, CO, and NO binding were measured for Mb mutants containing all four Y, Q, R, and F replacements with different secondary sites (5). The slower phases at room temperature show rate constants on the order of 1 to 0.1 μs⁻¹ and have been assigned to ligands rebinding from more interior positions. These locations are often designated as secondary docking sites. State C in Scheme 1 represents a collection of these more remote ligand positions, which have been identified as the Xe binding pockets based on molecular dynamics simulations (6) and analyses of laser photolysis experiments carried out under high pressures of xenon gas (16, 17). Recent low temperature crystallographic experiments with photolytic intermediates have provided direct structural confirmation of the location of dissociated CO in the distal pocket and in these secondary sites (5, 29–31).

**Scheme 1. Side path scheme.**

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² The abbreviation used is: Mb, myoglobin.
**Table I**

O<sub>2</sub> binding to Mb mutants in 0.1 M phosphate, pH 7.0, 2 mM EDTA, 20 °C

<table>
<thead>
<tr>
<th>Protein</th>
<th>〈k&lt;sub&gt;↑&lt;/sub&gt;/&lt;K&lt;sub&gt;↑&lt;/sub&gt;〉&lt;sup&gt;μ&lt;/sup&gt; M&lt;sup&gt;−1&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>k&lt;sub&gt;↑&lt;/sub&gt; s&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>〈k&lt;sub&gt;↓&lt;/sub&gt;/&lt;K&lt;sub&gt;↓&lt;/sub&gt;〉&lt;sup&gt;μ&lt;/sup&gt; M&lt;sup&gt;−1&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>17</td>
<td>15</td>
<td>1.1</td>
</tr>
<tr>
<td>MB</td>
<td>Y 25 (~50%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~20,000 (33%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Q 24</td>
<td>130</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>R 12</td>
<td>11</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>F 11</td>
<td>7.1</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>QR 19</td>
<td>89</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>YQR 2.8</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>YQRF 1.4</td>
<td>0.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Ascaris Mb</td>
<td>1.5</td>
<td>0.004</td>
<td>370</td>
</tr>
</tbody>
</table>

<sup>a</sup> The single L29Y mutant shows multiple phases for both O<sub>2</sub> association and dissociation. Roughly 30–50% of the reaction occurs quickly, and the remaining phases are ~5–100 times slower. The equilibrium constants assume that the larger 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 value corresponds with the larger k<sub>↑</sub> value; if not, then the K<sub>↑</sub> values could range between 0.0001 and 1 μM<sup>−1</sup>.

and various combinations. Overall rate constants and went recombination parameters for O<sub>2</sub>, CO, and NO binding are reported in Tables I–III for the complete set of eight mutants and Ascaris Mb.

**O<sub>2</sub> Binding**—The single Q mutation causes a 10-fold increase in the O<sub>2</sub> dissociation rate constant, 〈k<sub>↑</sub>/<K<sub>↑</sub>〉, and a 50% increase in the association rate constant, 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 (Fig. 1, Table I). The increase in k<sub>↑</sub> is due to a decrease in the strength of hydrogen bonding to the bound ligand, and the increase in k<sub>↑</sub> is due to the enhanced flexibility of the E7 side chain and the partial loss of distal pocket water molecule (34). The single R and F mutations cause small decreases in both k<sub>↑</sub> and k<sub>↑</sub> with little change in O<sub>2</sub> affinity. As shown in Fig. 1, the single Y mutation causes complex kinetic behavior (see also Ref. 9). About 50% of Y Mb molecules show very large oxygen dissociation rate constants, and the other 50% show lower, more normal k<sub>↑</sub> values (Fig. 1A). After laser photolysis, the Y single mutant shows ~50% rapid bimolecular O<sub>2</sub> rebinding, with a rate constant similar to that observed for wild-type and Q Mb, and ~50% slow rebinding, with a rate constant that is ~10-fold smaller (Fig. 1B).

Remarkably, the double YQ mutant shows 5- and 10-fold lower association and dissociation rate constants, respectively, than those of the wild-type protein or either of the corresponding single mutants. The O<sub>2</sub> affinities of Q Mb and Y Mb are 5–100-fold lower, respectively, than for wild-type myoglobin, whereas the O<sub>2</sub> affinity of the YQ double mutant is 2-fold higher (Table I). Clearly, the effects of the single mutations are not additive for either the kinetic barriers or the equilibrium free energy changes.

The time courses for the Y mutant indicate multiple conformations for the Tyr(B10) and His(E7) side chains in both the deoxygenated and the ligand-bound states. In the case of association (Fig. 1B), one set of conformers show barriers to oxygen binding that are about the same as those seen in wild-type myoglobin, whereas the remaining conformers show much higher barriers. In the case of dissociation (Fig. 1A), an even larger range of rate constants (~20,000 to 10 s<sup>−1</sup>) is seen, suggesting no, weak, and strong hydrogen bonding to bound O<sub>2</sub>. Unfortunately, a detailed structural interpretation is impossible because crystals have not been obtained for the single Y mutant, presumably as a result of this conformational heterogeneity.

The simple kinetic behavior observed for the YQ, YQR, and YQRF multiple mutants indicates single or very rapidly interconverting conformations for the Tyr(B10) and Gln(E7) side chains. As discussed previously for the YQR mutant (4–6), the small association and dissociation rate constants imply a closely packed distal pocket and favorable hydrogen bonding to bound O<sub>2</sub>. These features are observed in both the deoxy-Mb and MbO<sub>2</sub> crystal structures of the YQR and YQRF mutants (see Ref. 6; Fig. 3).

The QR mutant also shows simple kinetic behavior. In this case, the effects of the single Q and R mutations are additive. The R mutation decreases 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 and k<sub>↑</sub> by about 20–30% with little change in net affinity when introduced into either Q, YQ, or wild-type Mb (Table I). The F mutation also has the same relative effect on the O<sub>2</sub> binding properties of the wild-type protein and the YQR triple mutant: 20–50% decreases in 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 and small increases in O<sub>2</sub> affinity.

**CO Binding**—The effects of the single and multiple Y, Q, R, and F mutations on CO binding are similar to those seen for O<sub>2</sub> binding, except that much smaller changes are observed for the CO dissociation rate constants (Table II). Because bound CO is stabilized only weakly by hydrogen bonding interactions and little or no gominate recombination is observed for any MbCO derivative at room temperature, k<sub>CO</sub> is not significantly influenced by the Y and Q mutations (35, 36).

In the case of CO association, the nonadditivity of the single Y and Q replacements is less dramatic. The 10-fold decrease in CO affinity caused by the single Y mutation dominates in the YQ, YQR, and YQRF multiple mutants. As a result, the M values (K<sub>CO</sub>/K<sub>↑</sub>) for the YQ mutants are markedly lower than those of the wild-type protein or either of the single Y and Q mutants.

**NO Binding**—The effects of the Y, Q, R, and F mutations on the association rate constant for NO binding are much smaller than those observed for 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 and 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 (Table III). Unlike O<sub>2</sub> and CO binding, where the overall association rate constant is governed in part or completely by the speed of internal iron-ligand bond formation, NO binding is limited only by the rate of bimolecular entry into the protein (25, 26). Only in the YQRF quadruple mutant does 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 decrease significantly (~2-fold) compared with wild-type myoglobin.

Again, the single Y mutant shows complex heterogeneous behavior with a large fraction of the molecules showing a ~3-fold higher rate of NO association than wild-type protein. This result seems counterintuitive because of the large volume of the Tyr(B10) side chain compared with that of the native Leu(B10). An unambiguous explanation is not possible without a crystal structure of Y Mb. However, in the structures of Phe(B10) and YQR deoxy-Mb, the large B10 side chain displaces a well defined distal pocket water molecule that is normally found hydrogen-bonded to Nε of His(E7) in wild-type deoxy-Mb (6, 37). Thus, it is very likely that in the single Y mutant, the tyrosine side chain also displaces distal pocket water. The rate of water from the distal pocket enhances the rate of ligand entry into deoxy-Mb ~10-fold (11, 38). As in the case of the single Phe(B10) mutant, this favorable effect is offset by the smaller “free space” available in the anhydrous distal cavity for ligand entry. The net result is little change in 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 for the single Phe<sup>29</sup> and Tyr<sup>29</sup> mutants, respectively (Table I (7, 11)).

The effects of the R and F mutations are additive to the multiple mutants. Both replacements cause 20–30% decreases in 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 regardless of the starting myoglobin molecule. The net result is that YQR mutant shows the lowest 〈k<sub>↑</sub>/<K<sub>↑</sub>〉 value, indicating a sterically crowded distal pocket. The functional space available for ligand entry in Ascaris Mb is
even smaller, as judged by its very low NO association rate constant (Table III).

**Nanosecond Geminate Rebinding of O₂ and NO to YQ Mutants**—Intramolecular O₂ rebinding was measured for all of the mutants using a 9-ns YAG laser photolysis pulse. Normalized time courses are shown in Fig. 2A. The total fractional amount of geminate recombination was defined by amplitude of rebinding at 1.5 μs and is listed in Table I. More detailed analyses in terms of Scheme 1 have been reported previously for all of the single mutants and wild-type MbO₂ (9, 11, 14).

The single F mutant shows the largest amount of geminate recombination (Fig. 2A, Table I). The time course for this mutant shows a predominantly rapid phase, and recently, Morishima and co-workers (12) observed the same result for the Ile<sup>107</sup> to Phe mutation in human myoglobin. This result indicates that photodissociated O₂ is kept near the iron atom in the B state and is inhibited from migrating in the Xe<sup>4</sup> pocket by the large Phe(G8) side chain (9). The R and QR mutants show behavior similar to that of wild-type MbO₂.

**TABLE III**

<table>
<thead>
<tr>
<th>Protein</th>
<th>k&lt;sub&gt;NO&lt;/sub&gt;</th>
<th>k&lt;sub&gt;O₂&lt;/sub&gt;</th>
<th>F&lt;sub&gt;geminate&lt;/sub&gt; at 1.3 μs</th>
<th>k&lt;sub&gt;entry&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td>Wild-type Mb</td>
<td>22</td>
<td>17</td>
<td>0.48</td>
<td>35</td>
</tr>
<tr>
<td>Y</td>
<td>60 (75%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 (~50%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.36</td>
<td>~69</td>
</tr>
<tr>
<td>Q</td>
<td>45</td>
<td>24</td>
<td>0.36</td>
<td>67</td>
</tr>
<tr>
<td>R</td>
<td>19</td>
<td>12</td>
<td>0.49</td>
<td>24</td>
</tr>
<tr>
<td>F</td>
<td>11</td>
<td>11</td>
<td>0.60</td>
<td>18</td>
</tr>
<tr>
<td>QR</td>
<td>27</td>
<td>19</td>
<td>0.48</td>
<td>40</td>
</tr>
<tr>
<td>YQ</td>
<td>29</td>
<td>2.8</td>
<td>≤0.05</td>
<td>≥56</td>
</tr>
<tr>
<td>YQR</td>
<td>17</td>
<td>1.8</td>
<td>≤0.10</td>
<td>≥18</td>
</tr>
<tr>
<td>YQRF</td>
<td>12</td>
<td>1.4</td>
<td>≤0.10</td>
<td>≥14</td>
</tr>
<tr>
<td>Ascaris Hb</td>
<td>5</td>
<td>1.5</td>
<td>≤0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≥7.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> The single L29Y mutant also shows multiple phases for NO and O₂ binding (Table I and Gibson et al. (9)). As result, a quantitative interpretation of the geminate time course is difficult. The k<sub>entry</sub> value was estimated using F<sub>geminate</sub> for O₂ rebinding and the larger k<sub>O₂</sub> value for the Tyr<sup>29</sup> single mutant.

<sup>b</sup> The fractional amount of nanosecond geminate recombination was taken from Gibson et al. (9). Significant picosecond O₂ recombination was also observed. Thus, the true value of F<sub>geminate</sub> for Ascaris HbO₂ is probably somewhat greater than 0.2, and the rate of ligand entry is lower than 7.5 μM⁻¹ s⁻¹.

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**Fig. 1.** Oxygen reaction kinetics for sperm whale Mb and its mutants. Left panel, oxygen dissociation time courses. The rate constants were determined by stopped-flow analyzing the time courses of replacement of bound O₂ (O₂ panel). In the oxygen combination time courses. The rate constants were determined by laser photolysis of samples in the oxygenated form ([O₂] = 1.25 mM, 270 μM, 125 μM) with 1 mM CO. Right panel, oxygen combination time courses. Protein samples were 10 μM before mixing, in 0.1 M sodium phosphate buffer, pH 7.0, T = 20°C.
for NO photodissociation to form a nanosecond intermediate are ~0.05, which are very similar to those observed for the $O_2$ complexes. Somewhat similar behavior is observed for the L29F single Mb mutant, except in this case, the quantum yield only increases 3-fold compared with wild-type MbNO. In the Phe(B10) mutant, the aromatic side chain traps some photodissociated NO adjacent to the iron, causing very rapid picosecond geminate recombination (25). However, those ligands that do escape the immediate vicinity of the iron atom rebind much more slowly due to hindrance by the large B10 side chain. As a result, the rate of internal ligand recombination becomes much smaller; there is much less geminate $O_2$ re-binding on nanosecond time scales, and the secondary NO re-binding phases occur on nanosecond, not picosecond time scales as is observed for wild-type myoglobin.

This bimodal behavior is exaggerated in the YQ mutants. Perhaps the most remarkable result is that the overall quantum yields for $O_2$ and NO photodissociation from the YQR mutant are of the same order, 0.07 and 0.04, respectively (6). This result indicates that the $O_2$ and NO ligands trapped next to the iron atom by the large Tyr(B10) side chain rebind or escape to the same extent, regardless of the reactivity of the ligand. Thus, the intrinsically higher reactivity of NO for bond formation is partially masked and not manifested as an extremely low quantum yield.

Those ligands that escape to the protein interior during the photolysis process are greatly hindered from returning to the iron atom, presumably because the Tyr(B10) side chain relaxes back to the position directly above the iron atom that is seen in the structures of YQR and YQRF deoxy-Mb (Fig. 3A). This behavior is also seen with the Trp(B10) single mutant (11), although NO re-binding to this mutant has not been as carefully analyzed. For all Mb mutants with large aromatic amino acids at the B10 position, more NO rebinds geminately than $O_2$ (Fig. 2), and $k_{NO}$ is still $> k_{O_2}$.

The rate of internal NO re-binding in Mb-YQR is increased by the presence of xenon, but the fraction of ns geminate recombination is unaffected (6). This result suggests that, like NO, NO also migrates to the secondary Xe4 docking site and that slow NO geminate re-binding represents re-binding from this or adjacent positions in the protein (Scheme 1). Crystallographic experiments with YQR Mb at ultralow temperature (5) showed that photolyzed ligands migrate into the cavity corresponding to the Xe4 site. Parallel work by others (29, 30) using both wild-type Mb and a Trp(B10) single mutant showed that the exact location of the photodissociated ligand depends on temperature. In the Trp(B10) mutant at $\sim$110 but below $\sim$220 K, the Xe1 site preferentially hosts the photodissociated CO (30). In the most recent work, Moffat and co-workers (39) used time-resolved x-ray crystallography to show that CO migrates into the Xe4 and Xe1 cavities at room temperature, confirming the physiological relevance of these pathways.

Comparison of the time courses for NO geminate recombination in YQR and YQRF MbNO demonstrates that the side chain at position G8 regulates ligand migration from the distal pocket to the Xe4 cavity (Fig. 2B and Scheme 1). The total amplitude of slow, nanosecond NO geminate re-binding in YQR MbNO drops from 43% to ~20% in the YQRF mutant. This YQRF mutant has significantly lower overall quantum yields for both $O_2$ and NO. This result indicates an increase in ultrafast, picosecond re-binding of ligands that are kept next to the iron atom by the Tyr(B10) side chain. In turn, the Tyr side chain is held in place by the large Phe(G8) side chain. The addition of Xe to YQRF Mb decreases the observable amplitude of NO recombination but at the same time increases the rate of re-binding, most of which occurs during the light pulse (Fig. 2B).

![Fig. 2. Time courses of geminate recombination with $O_2$ and NO of wild type (wt) and mutant Mbs. Top panel, time courses of geminate $O_2$ re-binding to wild type sperm whale Mb and eight mutants. Protein samples were equilibrated with either $[O_2] = 1.25$ mM or $[O_2] = 270$ m$m$M in a sealed cuvette (0.2 cm). Bottom panel, time courses for NO re-binding to YQR and YQRF, the latter in the presence and absence of xenon (12 atm). Protein samples were 50 $\mu$M; NO concentration was 110 $\mu$M. Experiments were carried out in 0.1M sodium phosphate buffer, pH 7.0; $T = 20$ $^\circ$C.](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5785285/figure/fig2/)

This bimodal behavior is exaggerated in the YQ mutants. Perhaps the most remarkable result is that the overall quantum yields for $O_2$ and NO photodissociation from the YQR mutant are of the same order, 0.07 and 0.04, respectively (6). This result indicates that the $O_2$ and NO ligands trapped next to the iron atom by the large Tyr(B10) side chain rebind or escape to the same extent, regardless of the reactivity of the ligand. Thus, the intrinsically higher reactivity of NO for bond formation is partially masked and not manifested as an extremely low quantum yield.

Those ligands that escape to the protein interior during the photolysis process are greatly hindered from returning to the iron atom, presumably because the Tyr(B10) side chain relaxes back to the position directly above the iron atom that is seen in the structures of YQR and YQRF deoxy-Mb (Fig. 3A). This behavior is also seen with the Trp(B10) single mutant (11), although NO re-binding to this mutant has not been as carefully analyzed. For all Mb mutants with large aromatic amino acids at the B10 position, more NO rebinds geminately than $O_2$ (Fig. 2), and $k_{NO}$ is still $> k_{O_2}$.

The rate of internal NO re-binding in Mb-YQR is increased by the presence of xenon, but the fraction of ns geminate recombination is unaffected (6). This result suggests that, like NO, NO also migrates to the secondary Xe4 docking site and that slow NO geminate re-binding represents re-binding from this or adjacent positions in the protein (Scheme 1). Crystallographic experiments with YQR Mb at ultralow temperature (5) showed that photolyzed ligands migrate into the cavity corresponding to the Xe4 site. Parallel work by others (29, 30) using both wild-type Mb and a Trp(B10) single mutant showed that the exact location of the photodissociated ligand depends on temperature. In the Trp(B10) mutant at $\sim$110 but below $\sim$220 K, the Xe1 site preferentially hosts the photodissociated CO (30). In the most recent work, Moffat and co-workers (39) used time-resolved x-ray crystallography to show that CO migrates into the Xe4 and Xe1 cavities at room temperature, confirming the physiological relevance of these pathways.

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Engineering $O_2$ Binding to Mb

A striking result is that the YQ mutation lowers the association rate constant for $O_2$ binding by $\sim 10$-fold without greatly affecting the rate of ligand entry estimated from $k'_{NO}$ or $k'_{O}/F_{geminates}$.

Crystallographic Structures—Data collection statistics of the structures of the deoxy- and oxy-Mb YQRF are given in Table IV. The crystallographic R factors varied from 17.3 to 22.8% for the deoxy and oxy structures, and deviations from ideal stereochemistry and cell parameters were within experimental errors. The corresponding structures of YQR Mb and YQRF Mb is shown in Fig. 3. Fig. 3A shows a close up of the distal heme pocket of the deoxy forms of the mutants. The overall root mean square between the triple and quadruple mutant is 0.18 Å and decreases to 0.08 Å for protein atoms located at a radial distance more than 8 Å away from the heme iron. There is a slight rotation of Tyr(B10) to avoid bumping with Phe(G8), and the side chain of Arg(E10) is extended into the solvent. Otherwise, the structures of the deoxy forms of YQR and YQRF are superimposable.

The oxygenated structures are also very similar (Fig. 3B). The overall root mean square deviation is $\pm 0.11$ Å for the entire structures and $\pm 0.13$ Å in the heme pocket. The Ile(G8) $\rightarrow$ Phe mutation causes a small rotation of bound oxygen and changes in the hydrogen bonding distances between bound $O_2$ and the Tyr(B10) and Gln(E7) side chains (Fig. 3B). The distance between the hydroxy group of Tyr(B10) and the bound oxygen (2) atom increases from 2.7 Å in YQR to 3.2 Å in YQRF MbO$_2$, and the distance between the NH$_2$ of Gln(E7) and the second ligand atom increases from 3.4 to 3.8 Å.

The apparently weaker hydrogen bonding interactions in YQRF MbO$_2$ explain why the addition of the F mutation did not produce a further reduction in the $O_2$ dissociation rate constant. It was expected that $k_{NO}$ would decrease significantly due to trapping of the ligand near the iron atom by the large Phe(G8) side chain (6, 40). However, this caging effect does appear in the form of decreases in the overall quantum yields for $O_2$ and NO photodissociation from YQR Mb compared with the triple mutant. The decrease in hydrogen bonding stabilization due to steric crowding in the active site, offsets the trapping effect, and only a small, $\sim 20$% decrease in the overall value of $k_{NO}$ is seen for the YQRF quadruple mutant (Table I).

Superposition of the oxy and deoxy structures of YQRF Mb is shown in Fig. 3C. In contrast to wild type but like YQR myoglobin, large movements of the mutated side chains are required to allow the $O_2$ binding to the iron atom. The Tyr(B10) side chain moves up and away from the bound ligand, and the

<table>
<thead>
<tr>
<th>Crystallographic parameters</th>
<th>Deoxy</th>
<th>Oxy</th>
</tr>
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<tbody>
<tr>
<td>Resolution (Å)</td>
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<td>19.0–1.8</td>
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<tr>
<td>Completeness</td>
<td>97.6% (95.4%)</td>
<td>98.7% (96.2%)</td>
</tr>
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<td>(last shell)</td>
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<tr>
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<tr>
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<td>Space group</td>
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<tr>
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<tr>
<td>$R_{cryst}$ ($R_{free}$) (%)</td>
<td>17.3 (22.2)</td>
<td>18.6 (22.8)</td>
</tr>
</tbody>
</table>

FIG. 3. Three-dimensional structure of Mb YQR and YQRF (superimposed). A, the deoxygtenanted derivative of both mutants, with residues B10, E7, E10, F8, G8 highlighted. Green, YQR; blue, YQRF. B, same view as in A but with the oxygenated structures and hydrogen bond distances to bound $O_2$. Green, YQR; red, YQR; YQRF. C, superposition of oxy (red) and deoxy (blue) structures of YQRF.

Xenon addition also further decreases the overall quantum yield (not shown), also indicating an increase in nonobservable, ultrafast picosecond rebinding.

Scott et al. (11) have shown that the rate of ligand entry into myoglobin ($k'_{entry}$ in Scheme 1) is defined by the ratio of the overall association rate constant and the total fractional amount of geminate recombination observed in laser photolysis experiments (i.e. $k'_{entry} = k'_{O}/F_{geminates}$; Table III). This calculated value can be verified independently by measuring the association rate constant for NO binding, $k_{NO}$, which should represent the same kinetic process. Normally, the fraction of NO rebinding is close to 1 because of the high intrinsic reactivity of the ligand, and the overall bimolecular rate is limited by the speed of ligand entry into the protein. Comparisons of $k'_{NO}$ and $k'_{entry}$ for the YQRF series of mutants are given in Table III. Unfortunately, the absence of nanosecond geminate $O_2$ recombination by the YQ, YQR, and YQRF mutants makes it impossible to calculate the bimolecular rate of ligand entry into these proteins, and only a lower limit can be estimated. However, the trends follow those seen for the more direct $k'_{NO}$ measurements, and the effects seen with the single mutants also occur when the YQ double mutation is present. The most striking result is that the YQ mutation lowers the association...
amide group of Gln(E7) moves toward the heme propionates. Direct access to the iron atom is sterically hindered in the deoxy-Mb state by the phenol oxygen atom of Tyr(B10) as was seen previously for the YQR mutant (6). The proximity of the Tyr side to the iron atom accounts for the very small overall association rate constant for O2 binding to the YQ and YQR mutants, and the further small decrease for YQRF Mb is a result of the additional reduction in free space available to incoming ligands.

Molecular dynamics simulations have shown that, after photo or thermal dissociation in YQR Mb, ligands either stay very close to their original bound position or jump quickly to the back of the pocket in a region near or in the E4(e) pocket (6). In the latter case, the Tyr(B10) side chain relaxes back to a position right above the iron, and the Gln(E7) side chain also moves inward, a conformation similar to that seen in the static deoxy structure (6). These concerted movements of the B10 and E7 residues toward the iron atom squeeze the dissociated deoxy structure (6). These concerted movements of the B10 and E7 residues toward the iron atom squeeze the dissociated ligand away from the heme, inhibit geminate recombination from internal positions, and favor escape to the solvent rather than rebinding. Those ligands that are initially trapped next to Tyr residues toward the iron atom squeeze the dissociated ligand away from the heme, inhibit geminate recombination from internal positions, and favor escape to the solvent rather than rebinding. Those ligands that are initially trapped next to the iron atom are assumed to rebind extremely rapidly on picosecond time scales or be forced out of the protein directly to rotate more freely by F46A or F46V mutations (42-44). In this context, Tyr(B10) and Tyr(E7) movements inward, a conformation similar to that seen in the static deoxy structure (6). These concerted movements of the B10 and E7 residues toward the iron atom squeeze the dissociated ligand away from the heme, inhibit geminate recombination from internal positions, and favor escape to the solvent rather than rebinding. Those ligands that are initially trapped next to the iron atom are assumed to rebind extremely rapidly on picosecond time scales or be forced out of the protein directly.

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**DISCUSSION**

**Failure of the Y Mutant to Mimic Ascaris Hb**

The single Y replacement was first prepared and characterized 8 years ago (9) in an attempt to mimic the high O2 affinity of Ascaris Hb. Surprisingly, the ligand binding properties of Y Mb show little resemblance to the behavior of Ascaris hemo-globin (Table I). Heterogeneous kinetic behavior is observed for the mutant Mb, and its O2 affinity decreases significantly with respect to that for the wild-type sperm whale myoglobin (Table I). Although Y Mb has not been crystallized, model building and molecular dynamics simulations suggest that if the distal histidine in the Y mutant were present in the same position as in wild-type myoglobin, the His(E7) and Tyr(B10) side chains would make unfavorable steric contacts, even in the deoxy structure, leading to displacement of one or both of the side chains.

Upward and outward movements of the E7 imidazole in the Y Mb would increase the association and dissociation rate constants for both CO and O2 binding by opening the gate to ligand entry and by weakening favorable hydrogen bonding interactions with the bound ligand. An outward His(E7) conformation would explain the more rapid phases seen for O2 and NO binding to the single Y Mb mutant (Fig. 1). Similar behavior is seen when the distal histidine is protonated or is allowed to rotate more freely by F46A or F46V mutations (42-44). In both cases, the imidazole side chain rotates toward the solvent in the crystal structures, and k’NO, k’O2, and kO2 all increase substantially.

The slow phases observed for ligand binding to the single Y mutant probably represent conformations where the His(E7) and Tyr(B10) side chains are tightly packed in the heme pocket, inhibiting access to the iron atom. The rate constants for these slow phases are very similar to those observed for O2, CO, and NO binding to the single Trp(B10) mutant, where steric hindrance by the larger indole side chain is clearly dominant and monophasic time courses are observed (45).

**YQ Double Mutants**

Travaglini- Allocatelli et al. (4) constructed YQR sperm whale myoglobin and observed homogeneous kinetics, marked decreases in the association and dissociation rate constants for O2 binding, and a small increase in O2 affinity (Fig. 1). The new data in Table I show that these effects can be achieved by the double YQ mutations alone and that the R replacement has only a small effect.

Gln(E7) in the YQ Mb mutants appears to have much more conformational flexibility than His(E7) in the wild-type protein. There are large conformational changes for both Tyr(B10) and Gln(E7) when O2 binds to either YQR or YQRF myoglobin (Fig. 3C). In the deoxy-Mb structures (Fig. 3A), the Tyr(B10) and Gln(E7) side chains fill the space directly above the iron atom, displacing distal pocket water. When O2 binds, the Tyr(B10) side chain rotates upward, away from the iron atom, and the Gln(E7) side chain moves toward solvent. In both cases, the Cu atoms of these amino acids also move away from the iron atom, indicating a general expansion of the distal pocket and outward movements of the B and E helices (Fig. 3C). Ligand binding is clearly hindered to a greater extent in the YQ mutants than in wild-type myoglobin, where the conformational changes required for direct access to the iron atom are much smaller (34, 46).

Evidence for steric crowding and large conformational changes is seen in the Fourier transform infrared spectra of the CO complexes of both native and recombinant Ascaris Hb. Peterson et al. (47) reported a large, symmetrical vCO band centered at 1912 cm−1 and a second set of bands of equal intensity in the 1958–1965 cm−1 region. These results imply that native Ascaris HbCO contains a roughly equal population of conformers with both Tyr(B10) and Gln(E7) hydrogen-bonding to bound CO (vCO peak at 1912 cm−1) and with the H donors of both side chains pointing away from the ligand (vCO peaks near 1960 cm−1) (36, 47). Similar evidence for steric crowding was observed in the structure of A. suum cyano- metHb (48).

In the YQ-containing MbO2 mutants, the Tyr(B10) and Gln(E7) side chains adopt conformations that result in favorable electrostatic interactions between bound O2 and both amino acids. Hindrance by the Tyr(B10) side chain is still severe. All three YQ mutants show small CO and O2 association rate constants, kO2 ~ 2 μM−1 s−1 and kCO ~ 0.05 μM−1 s−1, and very little geminate recombination on nanosecond time scales. However, in the case of O2, steric hindrance is outweighed by favorable electrostatic interactions with Tyr(B10) and Gln(E7) once the ligand is bound. The net result is a reduction in the O2 dissociation rate constant from ~15 to ~1 s−1 and an ~2-fold increase in O2 affinity. Steric hindrance by the Tyr(B10) side chain dominates in the case of the neutral FeCO complex, for which electrostatic interactions have only a small favorable effect, and CO affinity decreases 5-fold (Table II).

The Tyr(B10)/Gln(E7) combination is a common motif in many hemoglobins from lower animals and is also seen in flavohemoglobins from microorganisms (49–55). In contrast, the Tyr(B10)/His(E7) combination has only been found in plant leghemoglobins (56). In these proteins, the B helix is located farther away from the heme plane; the distal pocket is much more open; and a highly conserved Phe(B11) residue occupies an interior position that is more analogous to the Leu(B10) position in mammalian myoglobins (19, 20). In addition, the His(E7) residue in leghemoglobins is also located further away from the iron atom and is much more flexible, preventing unfavorable steric interactions with the B10 side chain (19, 20, 56).
The rarity of naturally occurring Tyr(B10)/His(E7) combinations in animal hemoglobins and myoglobins is almost certainly due to the difficulty of putting two large aromatic residues near the iron atom without generating the conformational instability and heterogeneity that is seen in the single Y Mb mutant. There is a similar lack of naturally occurring Phe(B10)/His(E7) combinations in animal myoglobins and hemoglobins, whereas Phe(B10)/Gln(E7) motifs are common, most notably in elephant myoglobin and Lucina Hb II (41, 57, 58). An exception is seen in nonsymbiotic plant hemoglobins, where the His(E7) side chain is held in the distal pocket next to a Phe(B10) side chain by direct coordination to the iron atom (59).

**Regulation of Access to the Xe4 Pocket**

Ile(G8) is in a unusual position because it circumscribes part of the primary (State B) and part of the key secondary docking site, both of which play a role in geminate recombination and overall ligand binding in myoglobin (Xe4, state C in Scheme 1). Its location suggests that the s-butyl side chain may act as a gate to the Xe4 cavity (6). This idea is consistent with the decrease in the slow O2 geminate phase following insertion of the single F mutation into wild-type Mb and the decrease in slow NO recombination caused by insertion of the F mutation into the YQR triple mutant (Fig. 2). The overall O2 and NO quantum yields for YQR Mb decrease by 30–40% compared with those for the triple mutant. The large Phe(G8) side chain appears to restrict movement of the Tyr(B10) side chain, which in turn, traps a greater fraction of the photodissociated ligands in the initial position adjacent to the iron atom. This effect has been verified directly for photodissociated CO at cryoscopic temperatures. Lamb et al. (67) used Fourier transform infrared techniques to measure the photodissociation CO into the Xe4 cavity of YQR and YQRF Mb in the temperature range 4–220 K and found that the Phe(G8) side chain markedly inhibits this movement.

In the case of the single Phe(G8) mutation, only the pocket volume is decreased. The overall association and dissociation rate constants are reduced; there is little effect on ligand affinity; and the fraction of nanosecond geminate recombination only increases from ~45 to 55% (Fig. 2A). Thus, the F mutation decreases the rate of formation and stability of ligand in the B state and inhibits access to the C state but has no effect on the inner kinetic barrier or the free energy of the final bound state. The same effects are seen when the F mutation is introduced into YQR myoglobin (Tables I and III; Figs. 1 and 2). Ishikawa et al. (12) observed the same phenomenon for the Ile(G8) to Phe mutation in human Mb and came to a similar conclusion.

Val(E11) forms the other part of the gate controlling access to the Xe4 pocket (17). When this residue is replaced with either Phe or Trp, no slow geminate phases are observed, and ~90 and 95% of photodissociated O2 molecules rebind within 10 ns after photolysis. Geminarecombination of NO is ~100% complete within 100 ps (17, 24). In this case, two effects are occurring. First, ligands are sequestered in the distal pocket and prevented from entering the Xe4 pocket, which is occupied by the large aromatic side chain of the E11 residue. Second, the Cβ atom of the Phe and Trp side chains has only one Cγ substituent. As a result, the hindrance to iron-ligand bond formation that is conferred by the Cγ methyl group of the naturally occurring Val(E11) side chain is alleviated in the Phe(E11) mutant.

**Comparison of YQRF Mb and Ascaris Hb**

Differences in O2 and CO Affinities—As described above, the presence of a large Tyr side chain at position B10 causes direct steric hindrance of the binding of all ligands. The unfavorable steric effect of Tyr(B10) is offset preferentially for O2 by formation of a strong hydrogen bond between the bound ligand and the phenolic OH group. For Ascaris Hb, there is a dramatic ~350-fold increase in O2 affinity and 2.5-fold decrease in CO affinity compared with the corresponding parameters for wild-type myoglobin (Table I). In the YQ, YQR, and YQRF Mb mutants, the increase in O2 affinity is only 2-fold, but the decrease in CO affinity is ~6-fold. The strength of hydrogen bonding with Tyr(B10) is unlikely to be the major factor contributing to the large difference between the O2 affinities of Ascaris Hb and the YQ-containing mutants (Table I). The distances between the bound O2 atoms and the Nε atom in Gln(E7) are approximately the same in Ascaris HbO2 (13), YQR MbO2 (6), and YQRF MbO2 (Fig. 3B). The distance between the OH of Tyr(B10) and second bound oxygen atom is 2.7 Å in YQR MbO2, 3.2 Å in YQRF MbO2, and 2.7 Å in Ascaris HbO2. In the case of YQRF MbO2, this weakening of hydrogen bonding interactions should have caused an increase in the O2 dissociation rate constant. However, this loss of stabilization appears to be compensated by the trapping of O2 near the iron atom due to the bulky Phe(G8) side chain, causing YQR and YQRF MbO2 to have roughly the same kO2 values. The YQR and YQRF mutants also have roughly the same O2 affinities, kO2 ~ 2 µs−1, which is 150-fold smaller than kO2 for Ascaris Hb.

Three other factors contribute to the functional differences between the YQ mutants and the Ascaris protein. First, in Ascaris Hb, Trp is present at the G5 position instead of the Leu in Mb (Fig. 4). This bulky side chain completely fills up the cavity corresponding to the Xe1 site. When the Leu(G5) → Trp single mutant is made in sperm whale myoglobin, k′O2 remains unchanged, kO2 decreases 5-fold, from 15 to 2.8 s−1; and kCO increases 5-fold (18). The structural cause of this effect is unclear, although the crystal structure of Trp(G5) metMb has been determined (1CPW (18)). The orientation of the proximal imidazole is similar to that seen in the wild-type protein structure, and only small perturbations of the heme group are seen. It is likely that the polarity of the indole ring increases the basicity of the proximal histidine, which in turn enhances the reactivity of the iron atom.

The Leu(G5) → Phe mutation in sperm whale Mb has only a small effect on O2 affinity, whereas the Leu(G5) → Asn substitution brings two water molecules into the Xe1 cavity and causes the same increase in kO2 as that
Engineering $O_2$ Binding to Mb

In lupin leghemoglobin, the proximal histidine side chain is mobile and takes up an eclipsed position in the deoxy structure and a staggered conformation in the oxy structure (19). A more reactive staggered conformation is also seen in the acetate complex of ferric soybean leghemoglobin (see Fig. 5, middle panel) (20). Harutyunyan et al. (19) argued forcefully that the flexibility of the proximal imidazole and the staggered orientation account for the high $O_2$ affinities and association rate constants of all leghemoglobins. This interpretation would also explain the much higher rates of $O_2$ geminate recombination that occur in both leghemoglobins and native $A. suum$ Mb (47, 56). Thus, it is very likely that the staggered conformation of His(F8) in Ascaris Hb contributes significantly to its abnormally high affinity for $O_2$.

Third, the unusual orientation of His(F8) in Ascaris HbO$_2$ shown in Fig. 5 appears to be due to the loss of the hydrogen bonding network that is seen in mammalian myoglobins between the backbone carbonyl of Leu(F4), the side chain of Ser(F7), His(F8), and the heme-7-propionate. The F7 residue in Ascaris HbO$_2$ is arginine, and its side chain points outward into solvent-forming salt bridges with an equally extended conformation of the heme-7-propionate (see Fig. 7 of Ref. 13). Yang et al. (13) predicted that this loss of hydrogen bonding to His(F8) and the carbonyl backbone of Leu(F4) could have significant effects on ligand binding. Smerdon et al. (60) have shown that replacing Ser(F7) with either Val or Leu does cause a ~5-fold increase in $O_2$ affinity for recombinant pig myoglobin.

Two Fe-His(F8) stretching frequencies have been reported for Ascaris deoxy-Hb that is produced 10 ns after photolysis of the CO complex (47, 61). The major $\nu_{Fe-His(F8)}$ band is at ~205 cm$^{-1}$, and a significant shoulder is observed at ~220 cm$^{-1}$, which is the value of $\nu_{Fe-His(F8)}$ seen for the mammalian deoxy-Mb. Normally, a low $\nu_{Fe-His(F8)}$ value indicates a sterically constrained proximal geometry and reduced iron reactivity, based on the values observed for R- ($\nu_{Fe-His(F8)}$ = ~233 cm$^{-1}$) and T-state ($\nu_{Fe-His(F8)}$ = ~216 cm$^{-1}$) human hemoglobin (62). However, Peterson et al. (47) pointed out that the changes in orientation of His(F8) with respect to the pyrrole nitrogen atoms and the loss of hydrogen bonding to the F7 residue may both contribute to the decrease in $\nu_{Fe-His(F8)}$ observed for Ascaris Hb. They suggested that in plane movement of the iron atom is probably still favored, as in the case of the leghemoglobins.

The three proximal effects, Trp(G5) substitution, staggered orientation of the F8 imidazole and loss of hydrogen bonding to the F7 side chain, should all enhance ligand affinity and by roughly the same amount for $O_2$, CO, and NO. These effects cannot provide an explanation for the extremely small $M_{O2}/M_{O2}$ observed for Ascaris Hb, particularly Phe(G8), decreases the flexibility of the Tyr(B10) side chain and fixes it in position 2.7 Å away from the second ligand atom. This close contact will strongly inhibit the binding of all ligands and offset the favorable proximal effects, explaining why the rate and equilibrium constants for CO binding to Ascaris Hb are very similar to those of wild-type myoglobin (Tables I and II).

In the case of $O_2$ binding, distal steric hindrance caused by the close proximity of Tyr(B10) is compensated by the formation of strong hydrogen bonds between the bound ligand and the O$_2$ atom of Tyr(B10) and Ne atom of Gln(E7). The net result is a preferential, ~800-fold increase in the equilibrium association constant for $O_2$ binding to Ascaris Hb compared with that for CO binding.

This interpretation is further supported by the large steric effects that are observed for the Trp(B10) single mutant of.

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Observed for the Trp(G5) replacement (18). Since the G5 residue is far removed physically from the distal pocket, the effects of the Trp substitution are likely to be additive with the properties of the YQRF Mb. The resultant YQRFW mutant would be expected to have an affinity for oxygen more similar to that of the Ascaris protein.

Second, the orientation of the proximal histidine in Ascaris hemoglobin is significantly different from that observed in mammalian myoglobins (Fig. 5). In YQRF and native mammalian myoglobins, the C$\delta$ atoms of the proximal imidazole are in an eclipsed conformation with respect to the nitrogen atoms of the B and D pyrrole rings, even after $O_2$, CO, and NO are bound. This orientation inhibits in-plane movement of the iron-imidazole complex that, in turn, causes a reduction in overall ligand affinity and inhibition of the rate of iron-ligand bond formation measured in geminate recombination experiments. In Ascaris HbO$_2$, the plane of the His(F8) side chain has rotated almost 65$^\circ$ about the C$\gamma$-C$\delta$ bond toward the C and A pyrrole rings (Fig. 5, bottom panel). As a result, the C$\delta$ atoms of the proximal histidine in Ascaris hemoglobin occupy a more staggered position. This orientation should facilitate in-plane movement of the iron atom, increase the rate and fraction of geminate recombination, and increase $O_2$ affinity.
sperm whale myoglobin. Close contact with the indole side chain causes the CO association rate constant to decrease ~100-fold, from 0.51 μM⁻¹ s⁻¹ for wild-type Mb to 0.0039 μM⁻¹ s⁻¹ for the Trp(B10) mutant (45). CO affinity also decreases ~40-fold, since there are no favorable proximal effects in Mb.

There are also no compensating electrostatic interactions with bound O₂ and, as a result, both k₀ and O₂ affinity also decrease ~40–50-fold as a result of the Trp(B10) mutation.

**Differences in Kinetic Behavior**—Large picosecond rebinding phases are observed for O₂ and NO geminate recombination in *Ascaris* Hb (9). Small (20–30%), but rapid (~30–40 μs⁻¹) nanosecond phases are observed for both CO and O₂ recombination (9, 47). Nanosecond NO recombination follows the decay of the YAG laser excitation pulse, and the observed absorbance excursions are very small. The bulk of NO recombination occurs on picosecond time scales, suggesting that bond formation from state B in Scheme 1 has a low kinetic barrier. These data and the lack of effect of added xenon indicate that ligands do not migrate into the protein interior of the *Ascaris* protein but are, instead, sequestered very near the iron atom for both rapid rebinding and escape.

This interpretation is supported by the structures of native *Ascaris* HbO₂ and YQRF MbO₂ shown in Figs. 4 and 6. In contrast to the YQRF Mb mutant, there is a large space adjacent to the iron atom, between the edge of the Phe(G8) residue, the bound ligand, and the oxygen atom of the Tyr(B10) side chain in *Ascaris* Hb (Fig. 4, blue side chains). This space appears to be sufficient to accommodate a dissociated ligand in a position similar to that seen in wild-type MbCO at 20 K (28). The phenol group of Tyr(B10) in *Ascaris* Hb is on the solvent side of the bound ligand and crowds the exterior portion of the distal heme pocket more than the interior cavity (Fig. 4). In native *Ascaris* Hb (13), the C₆ atom of Ile(E11) points away from the iron atom toward the protein interior and does not appear to hinder ligand binding, unlike the conformation of the s-butyl side chain of Ile(E11) in the corresponding mutant of sperm whale Mb (17). This less hindered conformation can be seen in Fig. 6, where the green spheres representing the C₆ and C₃ atoms of Ile(E11) in *Ascaris* HbO₂ are much farther away from the iron atom than the C₁₂ atom of Val(E11) in YQRF MbO₂. Consequently, photodissociated ligands can stay closer to the iron atom in *Ascaris* HbO₂ without being “pushed” further into the protein interior or out toward the solvent.

The latter interpretation is supported by molecular dynamics simulations of ligands in the YQR, YQRF Mb mutants and in *Ascaris* Hb, starting from their crystal structures. In eight simulations for *Ascaris* Hb that were run for 50 ps using MOIL (see Refs. 6, 25, 40, 63), there was only one in which the ligands moved significantly more than 4 Å away from their original bound position. In eight runs for YQR and YQRF, more than half the ligands moved farther away from the iron atom, mostly toward the protein interior. If chemical reaction were allowed, almost all of the dissociated ligands in *Ascaris* Hb would have recombined very rapidly, predicting a large fraction of geminate of recombination in laser photolysis experiments and a low overall rate of thermal dissociation. In contrast, much less internal rebinding was predicted for the YQ-containing mutants.

There are no empty spaces in *Ascaris* Hb (13) that are equivalent to the Xe4 and Xe1 binding sites seen in wild-type and YQ-containing myoglobins (6, 64). A detailed comparison of the interiors of YQRF MbO₂ and *Ascaris* HbO₂ is shown in Fig. 6, where the amino acids that comprise the Xe4 binding site in myoglobin are highlighted as green spheres. In *Ascaris* HbO₂, there appears to be no free space available to photodissociated ligands in this region of the protein. This conclusion was quantified using CastP (65) to identify internal spaces, and we found that *Ascaris* Hb is very compact and lacks the main cavities that are easily detected in the YQR and YQRF mutants. In general, the amino acids that form the Xe4 site in myoglobin (Ile(B9), Leu(B10), Val(E11), Leu(E12), Ile(G8), Ser(G9), and Ile(G12)) are replaced by larger residues in *Ascaris* Hb (Leu(B9), Tyr(B10), Ile(E11), Leu(E12), Phe(G8), Trp(G9), and Phe(G12); see Fig. 6). Single mutations mimicking these differences have been characterized in sperm whale myoglobin (11). In most cases, the larger amino acids inhibit ligand movement into the Xe4 pocket.

As shown in Fig. 6, the Ile(E11) and Phe(G8) side chains of *Ascaris* Hb block inward movement and reduce the size of the distal pocket associated with state C in Scheme 1. As a result, ligands either rebind quickly or escape after photodissociation. These and the other residues in the interior of *Ascaris* Hb also reduce significantly the volume available for retaining entering ligands. This loss of distal pocket volume accounts for the low overall association rate constant for NO binding to *Ascaris* Hb and for the low value of k’_entry calculated from geminate recombination data compared with those for wild-type and most of the YQ-containing myoglobins (Table III).

**Conclusions**

The YQRF series of mutants and *Ascaris* Hb have provided important tests of heme-protein engineering principles. First, the electrostatic theory for ligand discrimination is verified in both the YQ mutants and in naturally occurring *A. suum* Hb. The Tyr(B10) side chain sterically hinders bond formation by its close proximity to the iron atom. Donation of a strong hydrogen from the Tyr(B10) OH to bound O₂ compensates for this unfavorable effect and dramatically and preferentially increases oxygen affinity. Second, B10 mutations can be used to selectively enhance or decrease the inner barrier to iron-ligand...
bond formation. Third, successful introduction of tyrosine at the B10 position requires replacement of the distal histidine with a glutamine residue to prevent steric interference between the side chains. This observation accounts for why YQ with a glutamine residue to prevent steric interference between the side chains. This observation accounts for why YQ

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