Imidazole Is a Sensitive Probe of Steric Hindrance in the Distal Pockets of Oxygen-Binding Heme Proteins†

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ABSTRACT: The FixL heme-based sensor, despite its low affinity for oxygen, is much more reactive than myoglobin toward the large polar ligand imidazole. To determine which features of a myoglobin heme pocket favor binding of imidazole, we have measured binding of this ligand to the FixL heme domain, elephant myoglobin, wild-type sperm whale myoglobin, and sperm whale myoglobins having alanine, valine, threonine, glutamine, leucine, phenylalanine, or tryptophan substitutions of the distal (E7) histidine residue. Except for histidine, the association rate constants dropped more than 3000-fold as the volume of the E7 side chain, at position 64, was expanded from alanine (10^6 M^-1 s^-1) to phenylalanine (10^3 M^-1 s^-1). There was inhibition of imidazole binding due to displacement of coordinated water from H64 and H64Q sperm whale myoglobins, where the E7 side chain hydrogen bonds directly to the bound ligand. The imidazole dissociation rate constants varied less dramatically and less consistently with any single factor, though they were measurably decreased by hydrogen bonding to an E7 glutamine or histidine. On the whole, the results for the sperm whale myoglobin E7 substitutions show that the rate constants for imidazole binding are useful and sensitive indicators of steric hindrance and polar interactions in the distal pockets of myoglobins. The combined effects of the glutamine 64 and phenylalanine 29 in elephant myoglobin largely account for its increased imidazole association and dissociation rate constants, respectively, compared to those of sperm whale myoglobin. An unhindered distal pocket not competent to stabilize positive poles is indicated by the large imidazole association (≥10^4 M^-1 s^-1) and dissociation (≥50 s^-1) rate constants, parameters that are characteristic of FixL.

A variety of heme proteins bind oxygen reversibly as part of their physiological functions. To accomplish this, the polypeptide must at least protect the heme prosthetic group from autoxidation and cause the protein-associated ferrous heme to discriminate much more strongly against carbon monoxide. In addition, the amino acid residues surrounding the heme iron have evolved to stabilize the association and dissociation rates of oxygen so as to suit the physiological functions of the proteins. In hemoglobins and myoglobins, the two residues closest to the heme iron are the proximal (F8) residue that coordinates directly to this atom and the distal (E7) residue on the opposite side of the heme. Because of its proximity to the heme iron, the E7 side chain influences considerably the binding properties of the myoglobins, both by sterically controlling access to the iron atom and by stabilizing or destabilizing the bound ligand. For example, in most mammalian myoglobins, an E7 histidine, i.e., H64, stabilizes the highly polar iron–oxygen complex by hydrogen bonding (2).

Some myoglobins optimize their oxygen binding parameters by different mechanisms. For example, if the E7 histidine in SWMb is replaced by valine, the oxygen off rate constant increases more than 100-fold (3). Yet Aplysia limacina Mb naturally has an E7 valine and an off rate constant very similar to that of SWMb. In this protein, interactions with an E10 arginine stabilize the bound oxygen (4). In elephant Mb, an interaction of the positive edge of a B10 phenylalanine with the bound oxygen compensates for the substitution of the E7 histidine with the less effective hydrogen-bond donor glutamine (5–7). This combination of interactions results in similar oxygen affinity but 2-fold greater rate constants.

The distal pocket structure of FixL is unknown, but its visible and proton NMR spectra indicate the absence of hydrogen-bond acceptors near the ligand binding site (8, 9). Therefore other features of its heme pocket must be responsible for oxygen off rates similar to those of myoglobin.
Imidazole Binding to Myoglobins

EXPERIMENTAL PROCEDURES

Preparation of Myoglobins. The R. meliloti FixL heme domain was prepared at The Ohio State University (8). Wild-type SWMb and the myoglobins having substitutions at position 64 were purified at Rice University from overexpressing strains of Escherichia coli harboring the appropriate recombinant plasmids (3, 20, 21). Purified native elephant Mb was a gift of Dr. Jonathan Wittenberg of the Albert Einstein College of Medicine. The myoglobins were oxidized by equimolar potassium ferricyanide and then filtered through a Sephadex G25 column (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 8.0, or 100 mM sodium phosphate, pH 7.0.

Affinities. Metmyoglobins (2.5–10 μM) were titrated with increasing concentrations of imidazole. Isosbestic points were observed in the 350–700 nm region of the absorption spectra. Entire spectra were used for the $K_d$ determination. The absorption spectra were decomposed by multiple linear regression analysis into the proportions of the liganded and unliganded species. The fraction of the liganded species was taken as the saturation. Values of $n$ and of $K_d$ were obtained from Hill plots (22).

Association Rate Constants. Imidazole concentrations were chosen to give the widest measurable range of speeds. As a rule, the ligand concentrations ranged from below to above the $K_d$. The rates of imidazole binding were measured with an Applied Photophysics SX17MV reaction analyzer (Leatherhead, U.K.). The reactions were followed at the wavelength of maximal difference between the unliganded and the liganded species of Mb at pH 8.0, 25 °C. Those difference maxima were at 418 nm (absorbance increase as imidazole binds) for most of the proteins and at 405 nm for elephant Mb (absorbance decrease as imidazole binds). Variations between the difference spectra were due not to the bis-imidazole complexes but to the different proportions of the aquomet form in the different proteins (7, 17). The static absorption spectra were measured with an ATI Unicam UV4 UV–Vis spectrophotometer.

Solutions of imidazole were prepared in the same buffer as the protein (20 mM Tris-HCl, pH 8.0, or 100 mM sodium phosphate, pH 7.0). The concentration of protein in the stopped-flow syringe was 5 μM, except for H64A SWMb and elephant Mb, which were at 2.5 μM. For each imidazole concentration a time course was measured at least three times, and the $k_{obs}$ was computed from the average. All of the time courses were fit to a single exponential. The association rate constants were computed from the slopes of the linear portion of plots of $k_{obs}$ versus the imidazole concentration. These lines usually included points at 5–7 ligand concentrations and had $r^2 > 0.99$. Activation energies were determined from Arrhenius plots of rate constants measured at 6, 10, 15, 25, and 37 °C in 100 mM sodium phosphate, pH 7.0; all other rate measurements were at 25 °C.

Dissociation Rate Constants. The dissociation rate constants of imidazole were determined by ligand replacement methods (10, 23, 24). High concentrations of cyanide (the displacing ligand) were added to the ferrie heme proteins after they had been saturated with imidazole in 100 mM sodium phosphate, pH 7.0. The displacement reactions were followed at 430 nm, the wavelength of maximal difference between the imidazolylmet and cyanomet species.

RESULTS

Influence of Steric Hindrance and Water Displacement on the Association Rate Constants. Figure 1A displays the inverse exponential relationship between the size of the E7
The largest imidazole association rate constant, \(1 \times 10^6 \text{M}^{-1} \text{s}^{-1}\) was observed for H64A SWMb. Even though water is coordinated to the iron atom in H64A metSWMb, its displacement does not confer much resistance to imidazole binding. The rate constant for completely pentacoordinate H64V metSWMb is actually 10-fold smaller than that for the H64A metSWMb. The opposite effect is observed for oxygen binding. The association rate constant for oxygen binding to H64A deoxySWMb is roughly half that for the H64V deoxySWMb. Even more surprising, H64T metSWMb, which is isosteric with H64V but contains coordinated water, has a 2-fold greater association rate constant for imidazole binding and 10-fold greater rate constant for azide binding.

Factors Affecting the Dissociation Rate Constants. The relationship between the E7 residue of Mb and the imidazole dissociation rate constant is much more complex, involving a combination of polarity and steric hindrance effects. E7 side chains such as alanine, threonine, glutamine, or histidine, that permit hydrogen bonding to bound ligands, whether directly or via a molecule of water, correlate roughly with lower dissociation rate constants for both oxygen and imidazole binding (Table 1) (17). This hydrogen-bonding stabilization is most apparent in the 10-fold differences between \(k_{\text{off}}\) for imidazole dissociation from H64T and H64V metSWMb and between wild-type (H64) and H64F metSWMb. Steric factors also seem to have considerable influence on imidazole dissociation. For example, increasing the size of the distal side chain from valine to leucine reduces the \(k_{\text{off}}\) for imidazole 25-fold.

Equilibrium Constants. Figure 2 shows the titration of H64V metSWMb with imidazole. Whether the metmyoglobinins were pentacoordinate or aquomet, their imidazole complexes were identical. FixL, elephant Mb, SWMb, and all the SWMb mutants had their imidazolylmet Soret peak at 415 nm and their \(\beta\)-peak at 535 nm. As expected, the binding of imidazole was noncooperative, with a Hill constant of 1. The highest affinities at pH 8.0 were observed for H64A, H64Q, and H64T metSWMb, which showed equilibrium dissociation constants equal to 7.5, 12, and 44 \(\mu\)M, respectively (Table 1). For each of these proteins, large association rate constants were augmented by their relatively small dissociation rate constants. The large association rate
constants for these mutants must be due to significantly less steric hindrance by the smaller or more flexible E7 side chains, while the lower dissociation rate constants appear to be due to stabilization of the bound ligand by either direct polar interactions or intervening water molecules. The lowest affinities were observed for the wild-type and H64F metSWMb, which showed equilibrium dissociation constants of 28 000 and 36 000 M, respectively. Despite hydrogen-bond stabilization of the bound ligand, the affinity of wild-type metSWMb is reduced markedly by an abnormally low dissociation rate constant, showing that steric hindrance by the large histidyl side chain is the dominant factor regulating the equilibrium binding of imidazole. This interpretation is supported by the low affinity of H64F metSWMb for imidazolylmet species in a stopped-flow rapid-mixing spectrometer. The time courses of imidazole dissociation were measured by following the replacement of bound imidazole by cyanide at 430 nm (23). The direct measurements of \( K_d \) were done by titrating the met species with imidazole.

Thus the large association rate constant for imidazole binding to FixL supports the assertion that the distal cavity of this protein is relatively unhindered (26). The large dissociation rate constant indicates that there is little hydrogen-bond stabilization of the bound imidazole in FixL. Overall, the kinetic parameters for imidazole binding to metFixL most closely resemble those of elephant metMb and H64V metSWMb. For both of these proteins, relatively small or flexible E7 side chains permit the rapid entry of imidazole, and there appears to be little significant stabilization of the bound ligand. Although the distal cavity in FixL readily accommodates imidazole, the heme domain of the \( R. \) meliloti FixL still discriminates in favor of oxygen and against carbon monoxide to almost the same extent as wild-type SWMb (8). This result suggests that there may be auxiliary polar interactions on the distal side of the heme group in FixL, since the \( M \) values of Mb mutants containing completely apolar distal pockets are very large, 10 000—50 000 (14).

**DISCUSSION**

**Steric Hindrance by the E7 Side Chain.** The three-dimensional structures of imidazolylmet-SWMb and \( Aplysia \) Mb indicate that the imidazole binds in a plane approximately perpendicular to the porphyrin ring (4, 15, 27). In available structures for myoglobins with an E7 histidine, this residue is positioned above the heme iron with the ring plane also approximately perpendicular to the heme. Therefore, it seemed likely that this residue would pose considerable hindrance to the entry of imidazole. Our results show that

<table>
<thead>
<tr>
<th>protein</th>
<th>( k_{on} (M^{-1}s^{-1}) )</th>
<th>( k_{off} (s^{-1}) )</th>
<th>( K_d (\text{mM}) )</th>
<th>( E^m ) (kcal/mol)</th>
<th>( A^m (M^{-1}s^{-1}) )</th>
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<tr>
<td></td>
<td>( \text{pH 7.0} )</td>
<td>( \text{pH 8.0} )</td>
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<tr>
<td>H64A SWMb</td>
<td>( 1.0 \times 10^{6} )</td>
<td>17</td>
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<tr>
<td>H64T SWMb</td>
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<td>12</td>
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<td>19</td>
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<tr>
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<td>120</td>
<td>2.2</td>
<td>17</td>
<td>8.0 \times 10^{18}</td>
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<tr>
<td>H64Q SWMb</td>
<td>( 1.7 \times 10^{4} )</td>
<td>0.9</td>
<td>0.053</td>
<td>17</td>
<td>4.6 \times 10^{16}</td>
</tr>
<tr>
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<td>( 1.6 \times 10^{2} )</td>
<td>3.6</td>
<td>22</td>
<td>17</td>
<td>10 3 \times 10^{16}</td>
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<td>4.7</td>
<td>0.91</td>
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<tr>
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<td>43</td>
<td>39</td>
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<tr>
<td>H64W SWMb</td>
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<td>19</td>
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<td>53(^b)</td>
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<td>3.6</td>
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<td>5.1</td>
<td>0.7</td>
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<tr>
<td>H64F SWMb</td>
<td>( 1.4 \times 10^{4} )</td>
<td>50</td>
<td>36</td>
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<tr>
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<td>54</td>
<td>3.6</td>
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<td>26</td>
<td>0.28</td>
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<tr>
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<td>3.7</td>
<td>28</td>
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<td>100</td>
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*Unless otherwise noted, \( k_{on} \) and \( k_{off} \) listed for \( \text{pH 7.0} \) were measured directly, and \( K_d \) was calculated as \( k_{off}/k_{on} \); the parameters listed for \( \text{pH 8.0} \) represent directly measured \( k_{on} \) and \( K_d \) and calculations of the \( k_{off} \) as \( K_d/k_{on} \). The time courses of association were measured by mixing the met proteins with various concentrations of imidazole and following the absorption at the wavelength of maximal difference between the met and imidazolylmet species in a stopped-flow rapid-mixing spectrometer.

\(^a\) Obtained by extrapolating the apparent on rates to zero ligand. \(^b\) Winkler et al. (26).
the most influential factor in the binding of imidazole to Mb is indeed steric hindrance. This is fundamentally unlike the binding of oxygen and carbon monoxide. For these smaller ligands of ferrous heme, the displacement of water from the distal pocket poses an important kinetic barrier, whereas the size of the distal side chain is less important (25). In contrast, an approximately 3-fold reduction in the volume of the E7 side chain results in a 3000-fold increase of the association rate constant for binding of imidazole (Table 1, Figure 1). A similar large effect of the size of the E7 residue has been observed for azide binding, but this ligand is charged in addition to being larger than oxygen (24). Significant but less dramatic increases in association rate constants with decreasing size of residue E7 were observed for methyl, ethyl, n-propyl, and n-butyl isocyanide binding to deoxy-SWMb (3). Thus imidazole can also be used as a simple and sensitive probe of steric hindrance in the distal heme pockets of myoglobins.

The logarithmic relationship between the size of the distal residue and the imidazole on rate constants suggests an activation free energy barrier that is proportional to the size of the distal side chain. Interestingly, identical activation energies are observed for imidazole binding to wild-type, H64V, and H64Q metSWMbs despite their considerably different $k_\text{on}$ values (Table 1). The correlation of the rate constants with the Arrhenius amplitudes, $A$, indicate that entropic effects are more influential. Those effects likely reflect the difficulties that imidazole encounters in achieving the proper orientation for binding to the heme iron. Larger E7 side chains would impede this process considerably more than smaller ones.

Given that crowding of the heme pocket dramatically interferes with the rate of imidazole binding, how then does this ligand bind more rapidly to H64W metSWMb than to H64F metSWMb? Perutz and Mathews (28) have proposed a model for ligand entry in which the E7 histidine functions as a swinging gate that occasionally allows the ligand to enter. Indeed, binding of imidazole or ethyl isocyanide causes this residue to swing toward the protein surface, into the “open conformation” (27, 29). The swinging-gate model would predict that if the E7 residue is replaced with increasingly larger side chains, a size may be reached that will not allow the “gate” to close. The data in Figure 2 indicate that E7 phenylalanine SWMb can adopt the closed conformation, whereas E7 tryptophan allows more rapid entry of imidazole.

**Influence of Water and Polar Interactions.** Displacement of water from the ferric heme iron poses a less significant barrier to imidazole binding than steric factors. Indeed, small polar E7 side chains aid the entry of this ligand. For example, imidazole as well as azide bind more rapidly to the H64T than to the H64V metSWMb, although the former is predominantly in the aquomet form, and the latter is pentacoordinate (Table 1). Brancaccio and his colleagues (24) have suggested that a polar channel involving the E7 threonine facilitates the entry of azide into the heme pocket. Such a channel could also favor imidazole and other polar ligands. Polar E7 side chains clearly stabilize the imidazole once it is bound. The stabilizing impact of polar interactions is readily illustrated by the 10-fold slower dissociation of imidazole from H64T compared to H64V metSWMb at pH 7.0. Similarly, the more than 10-fold slower dissociation of imidazole from the wild-type compared to H64F metSWMb is due to polar interactions.

**Implications for Other Oxygen Binding Heme Proteins.** The heme pocket of elephant Mb differs from that of SWMb at several positions. Of these differences the E7 glutamine and B10 phenylalanine have the most significant effect on reactions with ligands of ferrous heme (6, 7). The smaller and more flexible E7 glutamyl side chain of elephant metMb largely accounts for its larger imidazole association rate constant compared to that of metSWMb. For H64Q SWMb, H64QL29F SWMb, and elephant Mb, the imidazole association rate constants are comparable, i.e., 104 M$^{-1}$ s$^{-1}$. This represents a 100-fold increase compared to SWMb. The increase in the dissociation rate constant for elephant Mb is due to the additional presence of a B10 (i.e., residue 29) phenylalanine instead of a leucine (Table 1). The large benzyl side chain sterically hinders the bound imidazole, increasing its rate of dissociation. This phenomenon was observed for ethyl isocyanide binding to deoxySWMb, where the L29F substitution caused an approximately 15-fold increase in the rate of dissociation of this large ligand, with little or no effect on the association rate constant (21).

For metfixL, unlike metMb, there is a strong correlation between the association rates of ligands and their pK$\alpha$ values, which reflect the tendency of the ligands to donate electrons (26). This indicates that, for FixL, formation of the coordinate bond between the heme iron and the ligand dominates the kinetics of association. The results in Table 1 and Figure 1 leave little doubt that the FixL protein has an unhindered distal pocket. Thus the very small oxygen and carbon monoxide association rates of FixL are due not to steric hindrance but rather to the intrinsic reactivity of the heme iron. This reactivity is strongly influenced by the iron—F8 histidine bond, which has a trans effect on coordination of the external ligand.

In most vertebrate myoglobins, an E7 histidine or glutamine functions as a hydrogen-bond donor during its interactions with the negative pole of oxygen bound to ferrous heme iron. For the same side chain to interact with the hydrogen atoms of H$_2$O bound to ferric heme iron, they must also be competent to function as hydrogen-bond acceptors. Comparison of the ligand dissociation rates indicate a stabilizing interaction in FixL quite different from that of vertebrate myoglobins. Absorption spectra and resonance Raman spectra show that ferric FixL is pentacoordinate and without an aquomet form (8, 19). This failure to coordinate water reflects a lack of effective stabilization by a hydrogen-bond acceptor like an E7 histidine or glutamine. The relatively large imidazole dissociation rate of FixL is also consistent with the absence of stabilization by a hydrogen-bond accepting distal side chain (Table 1). Nonetheless oxygen is undoubtedly stabilized in FixL, as revealed by oxygen dissociation rate constants that are comparable to those of myoglobins (8). Unlike the E7 histidine in SWMb that can stabilize either the positive or negative end of a dipole, the interactions in FixL seem to stabilize only negative poles, such as the one in bound oxygen. This is more reminiscent of the E10 arginine in *Aplysia* Mb or the B10 phenylalanine in elephant Mb. While a guanidinium side chain or the positive edge of phenylalanine can stabilize bound oxygen, it is not competent to stabilize bound H$_2$O or imidazole.
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