A Proximal Arginine R206 Participates in Switching of the Bradyrhizobium japonicum FixL Oxygen Sensor

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In oxygen-sensing PAS domains, a conserved polar residue on the proximal side of the heme cofactor, usually arginine or histidine, interacts alternately with the protein in the “on-state” or the heme edge in the “off-state” but does not contact the bound ligand directly. We assessed the contributions of this residue in Bradyrhizobium japonicum FixL by determining the effects of an R206A substitution on the heme-PAS structure, ligand affinity, and regulatory capacity. The crystal structures of the unliganded forms of the R206A and wild-type BjFixL heme-PAS domains were similar, except for a more ruffled porphyrin ring in R206A BjFixL and a relaxation of the H214 residue and heme propionate 7 due to their lost interactions. The oxygen affinity of R206A BjFixL (Kd ∼350 μM) was 2.5 times lower than that of BjFixL, and this was due to a higher off-rate constant for the R206A variant. The enzymatic activities of the unliganded “on-state” forms, either deoxy or met-R206A BjFixL, were comparable to each other and slightly lower (twofold less) than those of the corresponding BjFixL species. The most striking difference between the two proteins was in the enzymatic activities of the liganded “off-state” forms. In particular, saturation with a regulatory ligand (the FeIII form with cyanide) caused a >2000-fold inhibition of the BjFixL phosphorylation of BjFixJ, but a 140-fold inhibition of this catalytic activity in R206A BjFixL. Thus, in oxygen-sensing PAS domains, the interactions of polar residues with the heme edge couple the heme-binding domain to a transmitter during signal transduction.

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Keywords: FixJ; PAS domain; response regulator; sensor kinase; E. coli Dos

Introduction

In the heme-based sensor proteins, a heme-binding domain controls a neighboring transmitter based on the ligation state of the iron atom.1 The FixL protein histidine kinases are the prototypes for a large category of these signal transducers that sense O2 with a heme cofactor inside a globular sensory module called a PAS domain.2,3 Prokaryotic heme-PAS domains are typically 30% identical in amino-acid sequence and show their greatest conservation in the regions that contact the heme directly.4,5 These are: the Fα helix with the histidine residue that coordinates to the heme on the proximal side, the FG loop that extends from this helix along the heme edge containing the propionate chains, and two antiparallel β-strands (Gβ, Hβ) that continue from the FG loop and line the ligand-binding pocket on the distal side of the heme.

A basic residue occupies the Fα9 position in all the known FixLs and in the Acetobacter xylinum PDEA1 and Escherichia coli Dos cyclic diguanylic acid phosphodiesterases (Figure 1). This residue is R206 in the Bradyrhizobium japonicum FixL.3 The placement of R206 near the boundary of the Fα helix and FG loop is ideal for communicating to this loop the changes triggered in the heme by binding of ligands. When the BjFixL heme is unliganded, i.e. the “on state”, the R206 side-chain forms hydrogen bonds to the FG-loop (Figure 1).3,5–8 When the heme is bound to O2 in the ferrous form or CN− in the ferric form, i.e. the “off-state”, the R206 guanido group forms a
hydrogen bond to the heme propionate group 6 (Figure 1).3,8 An ensuing displacement of the FG loop by about 2 Å, due to the changed polar interactions on binding of regulatory ligand, is postulated to play a regulatory role.3

What aspects of the heme pockets in heme-PAS proteins contribute to O2 affinity? In *Bj*FixL proteins, the distal Gβ-2 arginine (R220) is the only polar residue sufficiently near to bound O2 to interact with this ligand directly. This residue alternately interacts with the heme propionate 7 in the unliganded on-state or bound cyanide in the off-state. The usually basic Fα-9 residue (R206 in *Bj*FixL) interacts alternately with the FG loop in the *Bj*FixL on-state or the heme propionate 6 in the off-state.

Figure 1. Structural elements implicated in *Bj*FixL regulation and their occurrence in proven heme-PAS proteins. (a) A contrast of the crystal structures of the wild-type *Bj*FixL “on-state” (left) and “off-state” (right) (PDB files 1XJ3 and 1LT0; unliganded versus liganded, respectively). Secondary structural elements are denoted A–I; the heme iron is shown as a dark-red sphere; the heme carbon atoms are in green, oxygen atoms are in red, and nitrogen atoms are in blue; the important side-chains around R206 are shown explicitly, with the hydrogen bonding interactions as dotted lines and important water molecules as red spheres.

(b) An alignment of the Fα helix and FG-loop sequences for the *Bradyrhizobium japonicum* FixL, *Sinorhizobium meliloti* FixL, *Methanobacterium thermoautotrophicum* Dos, *Acetobacter xylinum* PDEA1, and *Escherichia coli* Dos sensor proteins. The absolutely conserved Fα3 residue (H200 in *Bj*FixL), or proximal histidine, coordinates the protein to the heme iron (3,32). The conserved Gβ-2 arginine residue (R220 in *Bj*FixL) strongly influences affinity and regulation (6); it alternately interacts with the heme propionate 7 in the unliganded on-state or bound cyanide in the off-state. The usually basic Fα-9 residue (R206 in *Bj*FixL) interacts alternately with the FG loop in the *Bj*FixL on-state or the heme propionate 6 in the off-state.

The isolated heme-binding domain of *Bj*FixL (i.e. R220A *Bj*FixLH compared to *Bj*FixLH), and another FixL (i.e. the R214 substitutions in *Sinorhizobium meliloti* FixLH compared to the wild-type),9,10 another arginine residue of *Bj*FixL, the proximal Fα9 residue (R206), responds to the changed heme-iron coordination by alternately forming polar bonds to a heme propionate or the FG-loop region.3,8

The R206 guanido group does not form hydrogen bonds to bound O2 and exerts its effects, if any, via other electrostatic interactions, e.g. with the heme edge. Might the R206 residue modulate the O2 affinity by influencing the heme electronic structure and the ability of the iron atom to change its coordination?

What factors govern transduction of signal, in this case the inhibition of the kinase activity of FixL by its ligand-bound heme-PAS domain? In *vivo*, one important function of *Bj*FixL and its
response-regulator partner, the transcription factor BjFixJ, is an adaptive symbiotic induction of several high-affinity terminal oxidases before nitrogen fixation can occur.\textsuperscript{11,12} These oxidases allow B. japonicum to continue respiring aerobically, despite the nearly anoxic conditions needed for fixing nitrogen. In vitro, the unliganded form of BjFixL catalyzes the phosphorylation of BjFixL with ATP.\textsuperscript{6} Saturation of the ferrous form with O\textsubscript{2} inhibits the kinase activity.

For BjFixL substitutions that dramatically lower the O\textsubscript{2} affinity, cyanide saturation of the ferric state can nevertheless provide an accurate measure of the inhibition supplied by strongly regulatory ligands.\textsuperscript{6} The oxy-form of BjFixL and many other O\textsubscript{2}-binding heme proteins resembles a superoxide-bound ferric form (i.e. Fe\textsuperscript{II}O\textsubscript{2}→Fe\textsuperscript{III}O\textsubscript{2}), and thus the O\textsubscript{2}-bound ferrous state is isoelectronic with the cyanide-bound ferric state (Fe\textsuperscript{III}CN\textsuperscript{−}). A functional and structural correspondence of the unliganded Fe\textsuperscript{II} and Fe\textsuperscript{III} forms (active) and the liganded Fe\textsuperscript{II}O\textsubscript{2} and Fe\textsuperscript{III}CN\textsuperscript{−} forms (inactive) is well established for BjFixL.\textsuperscript{3,8} In particular, cyanide is known to induce the same conformational changes in the ferric state that O\textsubscript{2} induces in the ferrous state.

The cyanide inhibition of BjFixL is reduced about 20-fold by a R220A substitution.\textsuperscript{6} Nevertheless, the presence of this residue cannot account for all the ligand-induced inhibition seen for the wild-type (>2000-fold). Thus, these results have suggested that R220 operates as part of a regulatory ensemble.\textsuperscript{6} Here, we consider the importance of the R206 residue to heme-PAS domain structure, ligand binding, and signal transduction in BjFixL.

## Results

### Ligand binding

The R206 residue makes a small stabilizing contribution to O\textsubscript{2} binding in BjFixL, despite the inability of its guanido group to interact directly with O\textsubscript{2}. This is evident from the failure to saturate the ferrous form with 1 atm (1 atm = 101,325 Pa) of pure O\textsubscript{2} (Figure 2). From a Hill plot of the data obtained by titrating the R206A mutant directly with O\textsubscript{2}, the Hill coefficient n was 1.0 and indicated no cooperativity; the dissociation equilibrium constant (K\textsubscript{d}) was 350 \(\mu\)M and thus 2.5 times higher than the value for the wild-type (Figure 2; Table 1). This slightly lower O\textsubscript{2} affinity of the R206A variant was manifested as a faster rate of O\textsubscript{2} dissociation (\(k_{\text{off}} \sim 29\ \text{s}^{-1}\)) compared to the wild-type (\(k_{\text{off}} \sim 20\ \text{s}^{-1}\)) (Figure 3; Table 1).\textsuperscript{13}

The parameters for binding of CO did not differ significantly for the R206A mutant and wild-type proteins. The CO association rate constant was 0.016 \(\mu\)M\textsuperscript{−1}s\textsuperscript{−1} (Table 1).\textsuperscript{13} The off-rate constant, measured by displacing CO with NO, was 0.063 s\textsuperscript{−1} and in the same range as the wild-type (Table 1). From these kinetic parameters, the equilibrium dissociation constant for binding of CO to the R206A variant is estimated to be around 4 \(\mu\)M, a value similar to that of the wild-type.

Cyanide or imidazole readily saturated the met form of the R206A mutant. From the measured kinetics of cyanide binding, the dissociation equilibrium constant is estimated to be about 2 \(\mu\)M, a value similar to that of the wild-type (Table 1).\textsuperscript{6} In particular, the on-rate constant for binding of cyanide (7.5 \times 10\textsuperscript{−5} \(\mu\)M\textsuperscript{−1}s\textsuperscript{−1}) was in the same range as the value obtained for the wild-type (Table 1).\textsuperscript{6} The cyanide off-rate constant of 1.3 \times 10\textsuperscript{−4} s\textsuperscript{−1}, measured by displacing the cyanide with imidazole, was also similar to that of the wild-type (Table 1).\textsuperscript{6} For binding of imidazole to the met-forms, the R206A mutant on-rate constant (\(k_{\text{on}} = 8.5\ \text{mM} \times \text{s}^{-1}\)) was in the same range as that of the wild-type (Table 1).\textsuperscript{6}

### Crystal structure

To investigate any structural consequences of the R206A substitution, we determined the crystal structure of this mutated heme-PAS domain in
the unliganded form. Crystals were obtained for the R206A BjFixLH under conditions similar to those used for wild-type BjFixLH. A crystallographic model was constructed and refined using data to a minimum Bragg spacing (d_min) of 2.30 Å (Table 2). Overall, the R206A BjFixLH structure resembled previously determined structures of unliganded BjFixLH, with root-mean-square deviation (rmsd) for all Cα atoms of 0.28 Å compared to unliganded BjFixLH (PDB entry 1XJ3) and 0.55 Å compared to the cyanide-bound BjFixLH (PDB entry 1LT0). In both the unliganded and cyanomet forms of BjFixLH, R206 interacts directly with the H214 residue of the FG loop as well as directly and indirectly (through a water molecule) with the heme propionate groups (Figure 1). The absence of these interactions from R206A BjFixLH results in two immediate structural consequences (Figure 4). First, the H214 imidazole ring in R206A BjFixLH adopts a conformation that is rotated by about 90° compared to its position in unliganded BjFixLH. Thus, in the wild-type, the H214 side-chain conformation is presumably specifically held in its rotameric conformation through its interactions with R206. Second, the heme propionate group 7 rotates about 180° around the CAA-CBA bond (Figure 4). The porphyrin ring is also slightly more curved than in wild-type BjFixLH. Specifically, the carbon atoms of the A and C pyrrole rings move upwards and away from the proximal histidine residue (H200) that is coordinated to the heme iron (Figure 4). This results in a maximum shift of about 0.35 Å for the C2A and C3C atoms. At the same time, the nitrogen atoms in rings B

### Table 1. Ligand-binding parameters of the R206A and wild-type BjFixL proteins at pH 8.0 and 25 °C

<table>
<thead>
<tr>
<th>Ligand</th>
<th>BjFixLH</th>
<th>R206A BjFixLH</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂</td>
<td>k_{on} (μM s⁻¹)</td>
<td>k_{off} (s⁻¹)</td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>20</td>
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</tbody>
</table>

^a Calculated from kinetic constant.

Gilles-Gonzalez et al. (1994).^1^ Dunham et al. (2003).^3^

<table>
<thead>
<tr>
<th>Ligand</th>
<th>BjFixLH</th>
<th>R206A BjFixLH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>k_{on} (μM s⁻¹)</td>
<td>k_{off} (s⁻¹)</td>
</tr>
<tr>
<td></td>
<td>7.5×10⁻³</td>
<td>1.3×10⁻³</td>
</tr>
<tr>
<td></td>
<td>1.1×10⁻⁴</td>
<td>1.2×10⁻⁴</td>
</tr>
</tbody>
</table>

Except where indicated, the parameters of R206A BjFixLH were measured directly. The average relative error is ±15%.

### Figure 3. Kinetic parameters for binding of O₂ to ferrous R206A BjFixLH at pH 8 and 25 °C.

The main Figure shows a kinetic trace of the dissociation of O₂ at 25 °C, as monitored after mixing, in a stopped-flow spectrometer, equal volumes of oxy-R206A BjFixLH and 10 mM sodium dithionite in 100 mM Tris–HCl (pH 8.0). The dithionite instantaneously consumed any freed O₂. The inset shows a determination of the association rate constant k_{on}=0.16 μM⁻¹ s⁻¹ for binding of O₂ from the slope of a linear plot of the dependence of k_{obs} on ligand concentration. Binding of O₂ (100–600 μM) to deoxy-R206A BjFixLH (8 μM) was followed at 438 nm after mixing, in a stopped-flow spectrometer, equal volumes of ligand and protein solutions in 100 mM Tris–HCl (pH 8.0). Each point represents three measurements of the apparent rate of association at 25 °C.

### Table 2. Data collection and refinement statistics

<table>
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<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Space group</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Wilson B-factor (Å²)</td>
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<tr>
<td>Rwork (%)</td>
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<tr>
<td>Rfree (%)</td>
<td>21.85</td>
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<tr>
<td>Average B-factor (Å²)</td>
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<td>Bond lengths (Å)</td>
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<tr>
<td>Bond angles (deg.)</td>
<td>1.884</td>
</tr>
</tbody>
</table>

Numbers in parentheses refer to the highest-resolution shell.
and D are shifted by about 0.2 Å in the same direction.

Signal transduction

From the initial rates of the BjFixJ phosphorylation with ATP, the key difference between the reactions catalyzed by R206A BjFixL versus BjFixL was their response to regulatory ligands (Figure 5; Table 3). The unliganded ferric R206A BjFixL protein had about one-half of the enzymatic turnover activity (43 h⁻¹) of the wild-type BjFixL (81 h⁻¹), suggesting that the presence of the R206 residue normally favors the on-state. This is quite unlike the effect of the R220A substitution, which results in a protein five times more active than the wild-type in the on-state, suggesting that the R220 residue normally stabilizes the off-state.6 Saturation of the heme with cyanide caused a more than tenfold lower inhibition for R206A mutant compared to wild-type BjFixL (140-fold inhibition compared to >2000 fold, respectively). These findings implicate the R206 residue in regulation.

Discussion

Ligand binding

Although it is the heme prosthetic group that binds O₂ in heme proteins, the surrounding protein envelope utterly governs the ability of the heme to do so. Indeed, heme and most of its derivatives will not bind O₂ reversibly at all, but instead become oxidized by O₂ to the ferric state, in an essentially irreversible reaction. What are the processes by which proteins direct the reactivity of the heme? Heme proteins are well known to hinder or assist O₂ binding by controlling the formation and breakage of bonds between O₂ and the protein.14 It is much less recognized and understood that heme proteins can
achieve similar results by hindering or assisting the transfer of electron density from O₂ to the heme, a process that need not involve a direct interaction of the protein with O₂. For example, compared with other heme proteins that reversibly bind O₂, FixL proteins form a bond to O₂ quite reluctantly. An average deoxy-FixL molecule will wait 19 ms in air-saturated buffer before it finally combines with O₂: about 50 times longer than a sperm-whale myoglobin molecule would hesitate (Table 1). Once the Fe-O₂ bond is formed, however, it lasts almost as long as the analogous bond in sperm-whale myoglobin (35 versus 50 ms) (Table 1). The polar interactions of bound O₂ with these two proteins fail to explain the vastly different affinities of the very same heme embedded in sperm-whale myoglobin versus that in FixL.

Our results show that the placement of a basic residue at the F₁₉ position permits FixL to achieve a fine modulation of its affinity for O₂ without marshalling a direct interaction of the protein with this ligand. The R206 guanido group at F₁₉ permits BjFixL to achieve a fine modulation of its affinity for O₂ without marshalling a direct interaction of the protein with this ligand. The R206 guanido group at F₁₉ permits BjFixL to achieve a fine modulation of its affinity for O₂ without marshalling a direct interaction of the protein with this ligand. The R206 guanido group at F₁₉ permits BjFixL to achieve a fine modulation of its affinity for O₂ without marshalling a direct interaction of the protein with this ligand. The R206 guanido group at F₁₉ permits BjFixL to achieve a fine modulation of its affinity for O₂ without marshalling a direct interaction of the protein with this ligand.
Table 3. Influence of cyanide on the kinase reactions of R206A or wild-type BjFixL at pH 8.0 and 23 °C

<table>
<thead>
<tr>
<th>Activity (h⁻¹)</th>
<th>Inhibition factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>FixL</td>
<td>BjFixL</td>
</tr>
<tr>
<td>Met (FeIII)</td>
<td>81</td>
</tr>
<tr>
<td>Cyanomet (FeCN⁻)</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

The reaction measured was the catalytic turnover of the protein substrate BjFixL to the phospho-form in the presence of ATP. Inhibition factor is defined as the activity of the unliganded form divided by the activity of a liganded form in the same oxidation state. Quantification of ligand inhibition is most valid for the ferric forms, where the activity of the unliganded state can be compared to the activity of the same oxidation state while entirely saturated with cyanide.

with the heme propionate group 6 as one of its main polar interactions. In a complementary study, Hayashi and colleagues replaced each heme propionate group of myoglobin, in turn, with a methyl group and reported similar effects on binding of O₂ to myoglobin, indicating that non-polar interactions are increasingly implicated in the propagation of regulatory conformational changes of heme proteins. Horiiuchi and colleagues have noted a 400-fold deceleration of the reduction rate compared to the wild-type, when a conserved arginine–propionate couple in the Pseudomonas putida cytochrome P450 cam was ruptured by replacing the arginine with cysteine. The reason for the slowed reduction, a diminished affinity (20-fold) for an electron-transfer mediator protein, suggested that the arginine–propionate interactions were essential for a conformational change preceding electron transfer. Garcia and colleagues have posited, on the basis of multiple available P450 structures, that the breakage or formation of an arginine–propionate couple can open or close a channel that links the heme pocket to the protein surface. Along similar lines, Ferguson-Miller, Cukier, and their colleagues showed, for the Rhodobacter sphaeroides cytochrome c oxidase, that the interactions of the R481 and R482 residues with the heme a₃ propionate groups underpin a conserved hydrogen bond network extending to the dinuclear Cu₃ center, a magnesium ion, and several subunit II amino acid residues. The disruption of this network by an R482P substitution slowed the rate of interactions with the R206 side-chain and the heme propionate groups, and led the H214 residue and the heme propionate group 7 to relax into new conformations (Figure 4). Consequently, the porphyrin ring adopted a more “ruffled” conformation (Figure 4). Thus, the direct and indirect polar interactions of R206 in the wild-type protein appear to contribute to a flattening of the porphyrin ring.

Attempts to prepare crystals of liganded forms of the mutant by diffusing cyanide into crystals of the met form, as has been done for met-BjFixLH, caused the crystals to disintegrate. Similarly, co-crystallization of the cyanomet form with ligands under the same conditions that produced diffracting crystals of cyanomet-BjFixLH, did not lead to crystal growth. Apparently, the structural changes accompanying ligand binding in the R206A mutant are incompatible with the crystal lattice. Yet, liganded, off-state structures have been observed for wild-type BjFixLH under crystallization conditions similar to those used in this study. The structural changes associated with ligand binding are therefore likely more pronounced in the R206A mutant than in the wild-type. It seems that R206 serves as a clamp that stabilizes the porphyrin ring and damps structural changes upon ligand binding.

Overall, our results are consistent with the F₉ and G₉–2 arginine–propionate couples constituting key complementary elements of the FixL switch. The F₉ arginine–propionate 6 couple, which forms in the O₂-bound state, controls the equilibrium distribution of active and inactive enzyme for the on-state. Likewise, the G₉–2 arginine–propionate 7 couple, which exists in the unliganded state, controls the proportions of active and inactive enzyme for the off-state.

As the electrostatic charges nearest to the heme center, it is not surprising that the propionate groups are increasingly implicated in the propagation of regulatory conformational changes of heme proteins. Horiiuchi and colleagues have noted a 400-fold deceleration of the reduction rate compared to the wild-type, when a conserved arginine–propionate couple in the Pseudomonas putida cytochrome P450 cam was ruptured by replacing the arginine with cysteine. The reason for the slowed reduction, a diminished affinity (20-fold) for an electron-transfer mediator protein, suggested that the arginine–propionate interactions were essential for a conformational change preceding electron transfer. Garcia and colleagues have posited, on the basis of multiple available P450 structures, that the breakage or formation of an arginine–propionate couple can open or close a channel that links the heme pocket to the protein surface. Along similar lines, Ferguson-Miller, Cukier, and their colleagues showed, for the Rhodobacter sphaeroides cytochrome c oxidase, that the interactions of the R481 and R482 residues with the heme a₃ propionate groups underpin a conserved hydrogen bond network extending to the dinuclear Cu₃ center, a magnesium ion, and several subunit II amino acid residues. The disruption of this network by an R482P substitution slowed the rate
of electron transfer from the CuA to heme a 2000-fold, in this case because of a sub-optimal orientation for the interaction between cytochrome c and cytochrome c oxidase. The pH-dependence of the slow step in the internal reductions confirmed an involvement of the R481–D-ring propionate couple (pKd ~6.5) in electron transfer and proton pumping. An allosteric enhancement of FixL autophosphorylation by FixJ, and the inhibition of this reaction by binding of O2 to the heme-PAS in FixL, suggest that well-oriented interactions of FixL with FixJ, and of the FixL heme-PAS with the kinase, are necessary for signal transduction. It is reasonable to suppose that, as in the examples described above, the arginine–propionate couples in BjFixL trigger conformational changes that propagate to the heme-PAS surface for detection by the kinase domain and the BjFixL substrate. For the broader class of heme-based sensors, such a mechanism has the appeal of being readily transposable to alternative regulatory partners and enzymatic transmitters.

Materials and Methods

Genetic manipulations

DNAs corresponding to full-length BjfixL (codons 1–505) or its heme-PAS domain-encoding region (codons 141–270) served as the templates for preparing R206A BjfixL and R206A BjfixLH by the QuickChange site-directed mutagenesis protocol (Stratagene). The mutation was confirmed by sequencing both strands of each DNA. E. coli strain TG1 was transformed with each final construct by gel-filtration through a Sephadex-G25 column (Amersham Biosciences) with 250 mM NaCl, 5% (v/v) glycerol and equilibrated with buffer containing 10 mM sodium dithionite, so that the dithionite instantaneously consumed any free O2, and the dithionite instantaneously consumed any free O2, and the dissociation of O2 was followed. Dissociation of CO was monitored by mixing the carbonmonoxy protein (immediately after ridding large excess of NO (1.95 mM) in the stopped-flow spectrometer and following formation of the nitrosyl forms) was prepared by adding KCN (10 mM) or imidazole (200 mM) to the met forms. Measurements of ligand binding

Static absorption spectra were measured with a Cary 4000 UV-Vis spectrophotometer (Varian Analytical Instruments, Walnut Creek, CA) for the purified proteins in 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5% (v/v) glycerol at 23 °C in quartz cuvettes. Laser-flash photolysis and stopped-flow measurements were done with a LKS.60 laser kinetic spectrometer fitted with a Pi-star stopped-flow drive unit (Applied Photophysics Ltd., Leatherhead, UK). For sample excitation, the LKS.60 spectrometer was coupled to a QuanTel Brilliant B Nd:YAG laser with second-harmonic generation. Data acquisition was provided by an Agilent 54830B digital oscilloscope for fast measurements or a 12-bit ADC card within the instrument workstation for slow measurements.

Association rates

All protein or ligand solutions were in 100 mM Tris–HCl (pH 8.0), and a more than tenfold excess of ligand was mixed with protein in all the experiments. Although the protein concentrations are given as the total concentration of monomer, FixL is entirely dimeric under those conditions (monomer-dimer Kg ~0.1 μM). Ligand association was followed at 25 °C, at one or two wavelengths of maximum difference between the starting and the final species. Specifically, ligand association was monitored at 438 nm for O2, 425 nm and 441 nm for CO, 396 nm and 427 nm for CN−, and 388 nm and 419 nm for imidazole. The deoxy forms (2–10 μM) were mixed rapidly in a stopped-flow spectrometer with 20–400 μM O2 or 50–500 μM CO. Alternatively, the ferrous proteins were equilibrated with 25–200 μM CO, and the rates of rebinding were followed after flash photolysis. Ligand association to the ferric forms was followed after mixing each protein (11 μM) with 1–10 mM KCN or 2.5–20 mM imidazole in the stopped-flow spectrometer. For each chosen concentration of ligand, the apparent rate of association was determined at least three times. Rate constants were calculated by linear regression from plots of Kobs versus ligand concentration.

Dissociation rates

Rates of ligand dissociation were measured for proteins in 100 mM Tris–HCl (pH 8.0) at 25 °C, as follows. One volume of oxy protein (14 μM ferrous protein equilibrated with 500 μM O2) was mixed in a stopped-flow spectrometer with one volume of 10 mM sodium dithionite, so that the dithionite instantaneously consumed any free O2, and the dissociation of O2 was followed. Dissociation of CO was monitored by mixing the carbonmonoxy protein (8 μM ferrous protein equilibrated with 50 μM CO) with a large excess of NO (1.95 mM) in the stopped-flow spectrometer and following formation of the nitrosyl form at 428 nm. Dissociation of cyanide was monitored by mixing the cyanomet protein (immediately after ridding this protein of free CN− by gel-filtration) with 250 mM imidazole and following the formation of the imidazolomet form over 16 h with a Cary 4000 UV-Visible spectrophotometer. The cyanide dissociation rates were
calculated from the saturation changes determined from multiple linear regression analysis of whole 350–700 nm absorption spectra showing clear isosbestic points.

Equilibrium binding

The affinities of the proteins for O2 were measured directly by titrating the deoxy forms (4 μM) with 50–1000 μM O2 in buffer supplemented with 4 mM β-mercaptoethanol at 25 °C. For each protein, the dissociation equilibrium constant for binding of O2 was determined from saturation changes calculated by multiple linear regression analysis of whole spectra.

Phosphorylation assays

The catalytic phosphorylation of BjFix by BjFixL was measured. These turnover assays were done by incubating 1 μM full-length BjFixL kinase (either wild-type or R206A) with 25 μM BjFix in phosphorylation buffer (50 mM Tris–HCl (pH 8.0), 50 mM KCl, 50 μM MnCl2, 5.0% (v/v) ethylene glycol) and starting the reactions by introducing 1 mM ATP/MgCl2 (unlabeled ATP from Roche and [γ-32P]-ATP from Amersham Biosciences, specific activity 0.21 Ci/mmol) at 23 °C. After 1 min, 2.5 min, 5 min, 10 min, 20 min, and 30 min, aliquots (10 μl) of the reaction were mixed with one-third volume of stop buffer (0.5 M Tris–HCl (pH 6.8), 40 mM EDTA, 0.20 M NaCl, 50% (v/v) glycerol, 2.0% (v/v) β-mercaptoethanol). For every reaction course, the status of the heme iron was verified from the 250–700 nm absorption spectra before and after the assay. The products were electrophoresed on 15% (w/v) polyacrylamide gels. The phosphorylated protein in the dried gels was quantified with a phosphorimager (Bio-Rad Personal Molecular Imager FX).

Crystallization, data collection, structure determination and refinement

The crystals of the met-R206A BjFixLH mutant were obtained at 4 °C via the hanging-drop, vapor-diffusion method by mixing equal amounts of protein (0.6–1.2 mM in 50 mM Tris–HCl (pH 8.0) and well solution (50 mM Hepes (pH 7.5), 4.5 M NaCl, 5% (v/v) 2-methyl-2,4-pentanediol) and incubating the drop over 0.7 ml of well solution. Attempts to prepare crystals of liganded forms of the R206A mutant, for example by soaking met-R206A BjFixLH crystals with cyanide or by co-crystallization, were unsuccessful. Crystals were cryo-protected by directly titrating the deoxy forms (4 μM) and introducing 2.5 min, 5 min, 10 min, 1.2 mM ATP/MgCl2 (unlabeled ATP from Roche and [γ-32P]-ATP from Amersham Biosciences, specific activity 0.21 Ci/mmol) at 23 °C. After 1 min, 2.5 min, 5 min, 10 min, 20 min, and 30 min, aliquots (10 μl) of the reaction were mixed with one-third volume of stop buffer (0.5 M Tris–HCl (pH 6.8), 40 mM EDTA, 0.20 M NaCl, 50% (v/v) glycerol, 2.0% (v/v) β-mercaptoethanol). For every reaction course, the status of the heme iron was verified from the 250–700 nm absorption spectra before and after the assay. The products were electrophoresed on 15% (w/v) polyacrylamide gels. The phosphorylated protein in the dried gels was quantified with a phosphorimager (Bio-Rad Personal Molecular Imager FX).

Equilibrium binding

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Acknowledgements

We thank Kelly Krabill-Gerber for preparing mutant genes and Sandra Hill for providing excellent technical assistance. The project was supported by a Welch Foundation grant no. I-1575, by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2002-35318-14039, and by a Spanish DGICYT grants BFI 2001-1713 and BFU 2004-06394. Use of the Argonne National Laboratory Structural Biology Center beamlines at the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Energy Research, under contract no. W-31-109-ENG-38.

References


*Edited by R. Huber*