Oxygen Sensing in Health and Disease

Signal transduction by heme-containing PAS-domain proteins

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Gilles-Gonzalez, Marie-Alda, and Gonzalo Gonzalez. Signal transduction by heme-containing PAS-domain proteins. J Appl Physiol 96: 774–783, 2004; 10.1152/japplphysiol.00941.2003.—The most common physiological strategy for detecting the gases oxygen, carbon monoxide, and nitric oxide is signal transduction by heme-based sensors, a broad class of modular proteins in which a heme-binding domain governs the activity of a neighboring transmitter domain. Different structures are possible for the heme-binding domains in these sensors, but, so far, the Per-ARNT-Sim motif, or PAS domain, is the one most commonly encountered. Heme-binding PAS (heme-PAS) domains can accomplish ligand-dependent switching of a variety of partner domains, including histidine kinase, phosphodiesterase, and basic helix-loop-helix (bHLH) DNA-binding modules. Proteins with heme-PAS domains occur in all kingdoms of life and are quite diverse in their physiological roles. Examples include the neuronal bHLH-PAS carbon monoxide sensor Npas2 that is implicated in the mammalian circadian clock, the acetoacetate oxygen sensor Aspdea1 that directs cellulose production, and the rhizobial oxygen sensor FixL, which governs nitrogen fixation. What factors determine the range of detection of these sensors? How do they transduce their signal? This review examines the recent advances in answering these questions.

escherichia coli dos; hemoglobin; response regulator; sensor kinase

THE PAS DOMAIN: A MODULE FOR SIGNAL TRANSDUCTION

The broad category of PAS-domain-containing proteins is one of over 6,000 “protein families” that bioinformaticists have identified from multiple-sequence alignments. The amino acid sequence relationships that define these families are detected by hidden Markov models and are often quite subtle. For example, each PAS domain consists of two “conserved” regions averaging only ~12% identity with the broader class of PAS domains and divided by a sequence of variable length (63, 70). Although individual PAS-domain proteins have been intensely studied since at least 1980, the bioinformatics sophistication to recognize these proteins as a family that spans all kingdoms of life did not occur until 1997 (70). The PAS acronym itself was suggested in 1991 by a sequence motif in the bacterium Acetobacter acetosum that was also found in a diatomic gas sensor of the unicellular organism Nostoc punctiforme (44). The PAS acronym comes from the PAS-A and PAS-B domains, with the NH2-terminal-most domain designated “PAS-A” and the other one “PAS-B.” The PAS-B domain is now more rigorously viewed as a pair of consecutive PAS regions already include ion channels and protein kinases (7, 44, 55). Second, eukaryotic PAS domains do not always lack a cofactor. For example, the mammalian bHLH-PAS protein Npas2 was recently shown to couple directly to a transmitter module within the same protein.
sence CO with a heme cofactor (12). Likewise, the plant kinase NPH1 is known to detect light with a FMN cofactor (7, 26).

Interestingly, the choice of transmitter for coupling to a PAS domain mainly depends on the preferred modes of signal transduction in an organism. For example, the type of kinase most commonly coupled to PAS domains in archaea and bacteria is a histidine protein kinase domain, whereas in eukarya this is a serine-threonine kinase (7, 55, 63, 70). For the heme-binding PAS (heme-PAS) domains, at least three different types of transmitters are possible: histidine protein kinase domains in prokaryotes, cyclic dinucleotide phosphodiesterase domains in mammals (Fig. 1) (6, 11, 12, 18).

THREE ACTIVITIES SWITCHED BY HEME-PAS DOMAINS

Regulation of the Histidine Protein Kinase FixL of Rhizobia

Physiological relevance. A prototype for O\textsubscript{2} signal transduction is the rhizobial two-component regulatory system comprising the heme-based sensor kinase FixL and the transcription factor FixJ (18). In Sinorhizobium meliloti, the regulatory partners RmFixL and RmFixJ were found to restrict the expression of the bacterial nitrogen fixation genes to the hypoxic regions of symbiotic root nodules (O\textsubscript{2} \leq 50 \mu M) (61). These results agree with studies showing that RmFixL has an equilibrium dissociation constant (K\textsubscript{d}) of 50 \mu M for binding of O\textsubscript{2} (21), the deoxy form of RmFixL catalyzes the phosphorylation of a transcription factor RmFixJ (19, 66, 67), and the activated RmFixJ induces transcription of the nifA and fixK genes, triggering a cascade of nitrogen-fixation gene expression under hypoxic conditions (1, 9, 50). FixL proteins from different rhizobial species typically differ in their NH\textsubscript{2}-terminal domain sequences (~140 residues) but have a conserved heme-PAS domain (~130 residues and 50% identical) immediately preceding an equally conserved COOH-terminal histidine protein kinase domain (~230 residues) (Fig. 1) (17).

Enzymatic reaction. FixL catalyzes the transfer of an ATP \gamma-phosphoryl group to the transcription factor FixJ (18). Binding of O\textsubscript{2} to the FixL heme switches off the kinase activity, and dissociation of O\textsubscript{2} from the heme fully restores it (19, 66, 67). If FixL is provided with a large excess of ATP but no FixJ, the FixL slowly (~40 min) undergoes a regulated “autophosphorylation” at a conserved histidine residue (H) of its kinase domain (H285 in RmFixL; H291 in BjFixL) (19, 36, 43). Subsequent addition of excess FixJ causes rapid “phosphoryl transfer” (~30 s) from the phosphorylated FixL to a conserved aspartate residue (D) in FixJ (D54 in RmFixJ; D55 in BjFixJ) (19, 67). Because of these observations, it was once believed that the phosphorylation of FixJ by FixL occurred as a “ping-pong bi-bi” reaction, with ATP and FixJ binding sequentially to the enzyme. Such a mechanism would involve a rate-limiting reaction with ATP and should yield no observable phosphoryl-FixL intermediate in the simultaneous presence of ATP and FixJ. In fact, despite the rapid phosphoryl transfer from phospho-FixL to FixJ, the phospho-FixL intermediate was found to accumulate more rapidly in the presence of FixJ and ATP than with ATP alone (Fig. 2) (67). This can be explained by the presence of FixJ accelerating the first phosphoryl transfer (from ATP to FixL) so dramatically that the second transfer (from phospho-FixL to FixJ) becomes rate limiting. This implies that FixJ plays two roles: it is a powerful allosteric activator of the first and it is the substrate of the second phosphoryl transfer.

The presence of FixJ also profoundly affects the sensitivity of the RmFixL/RmFixJ system toward heme ligands (66). If phosphorylation of RmFixJ is forced to occur as two separate steps, neither the reaction of RmFixL with ATP nor the transfer of the phosphoryl group to RmFixJ is influenced by the heme-iron oxidation state (19, 66, 67). In fact, the phosphoryl transfer from preformed phospho-RmFixL to RmFixJ is entirely insensitive to the heme status (19, 20). In contrast, when turnover of RmFixJ to phospho-RmFixJ is achieved with both substrates (ATP and RmFixJ) in a single reaction mixture, the reaction is specific to the Fe\textsuperscript{II} form and most stringently inhibited by O\textsubscript{2} (66). This effect is primarily on the initial...
reaction of RmFixL with ATP, even though RmFixJ is not directly involved in this phosphoryl transfer. Further evidence of sensing by a RmFixLJ complex comes from a recent finding of three substitutions in the kinase domain of RmFixL, (R269H, E278G, and E278K) that leave the kinase activity of the deoxy form unchanged but impair the O2 switch off of that activity (57). Notably, the phosphoryl transfer from ATP to these RmFixL variants is insensitive to O2 if RmFixJ is present, but it is responsive to O2 if RmFixJ is absent. Studies such as those above make it increasingly clear that the reaction of RmFixL with only ATP, and without RmFixJ, cannot be regarded as a normal step of the catalytic mechanism. This reaction does not fully recapitulate the O2 response of the RmFixLJ complex and likely occurs only in vitro, where RmFixL is easily separable from RmFixJ.

To account for the newly discovered roles of RmFixJ, the phosphoryl transfer scheme for the S. meliloti FixL/FixJ system was recently revised as follows. 1) RmFixL forms a sensing complex with RmFixJ and ATP that accomplishes the initial detection of O2. 2) The catalysis is all done within this complex; i.e., RmFixJ greatly enhances the rate at which RmFixL reacts with ATP, and then phospho-RmFixL transfers its phosphoryl group to RmFixJ. 3) The phospho-RmFixJ and ADP dissociate from the complex. This scheme, which is considerably more efficient than the sequential mechanism, can account for essentially all of the phospho-RmFixJ produced physiologically (66, 67).

Switching of FixL activity by CO. CO inhibits the kinase activities of both BjFixL and RmFixL on their respective FixJ partners, albeit much less stringently (~3-fold) than does O2 (>100-fold) (14, 66). This inhibition by CO is reproducible and reversible, and it does not depend on the method of generation of the ferrous form. The finding of CO inhibition is significant because it implies that donation of a hydrogen bond to a bound ligand is not strictly required to inactivate FixL, although this bond appears to stabilize the off-state. A reproducible twofold inhibition by nitric oxide (NO) has also been measured (14, 66). Unlike human hemoglobin, which is exposed to endogenous CO produced from heme degradation, there is no known physiological reason why rhizobial cells should design their heme proteins to discriminate against this ligand. Thus the “discrimination” against CO, i.e., the greater effectiveness of O2 as a kinase inhibitor compared with CO, may be an incidental consequence of the inactivation mechanism. On the other hand, discrimination against NO may be a necessary feature of rhizobial O2 sensors. NO is an intermediate product of denitrification, a process carried out by many rhizobia (5).

Some lessons for signal-transducing enzymes. The FixJ protein and other response regulators of its class are sometimes mistakenly described as enzymes because their phosphorylation-site aspartate residue can rapidly react with phosphoryl transfer reagents such as acetyl phosphate (1, 8, 37, 50). Given that the nature of an enzyme is to remain unchanged while causing other molecules to react, for any true enzyme provided with a large excess of substrate, a “turnover number,” i.e., the moles of product generated per unit of time per mole of enzyme, is an intrinsic parameter that should be measurable. The phospho-FixJ from the reaction of FixJ with acetyl phosphate is inert toward additional molecules of acetyl phosphate. In contrast, the phospho-FixL from the reaction of FixL with ATP transfers its phosphoryl group to FixJ, and the subsequent dissociation of phospho-FixJ returns FixL to its original state for additional reactions. A turnover number can be measured for FixL; such a parameter cannot even be defined for FixJ.

Although some aspects of enzymes, such as turnover number, are universal, not all of the expectations traditionally based on metabolic enzymes can be transferred to signal transduction enzymes. For a metabolic enzyme, the design criterion is rapid production of large quantities of a desired product. In contrast, for a signaling enzyme, the objective is a final concentration of product that varies with the signal being transduced. Compared with the turnover numbers of metabolic enzymes, the turnover numbers of signaling enzymes are quite slow because the natural concentrations of their substrates, such as proteins to be modified (e.g., phosphorylated, hydroxylated, acetylated, and so forth), are usually comparable to the enzyme concentrations. A large excess of substrate, which is physiologically normal in the case of metabolic enzymes, is the source of the assumption that the Michaelis-Menten parameter (Km) should roughly correspond to the physiological substrate levels. For signaling enzymes, a vast excess of substrate is often unnecessary, and the effective concentrations of product additionally depend on downstream steps of signal transduction.

Oxygen Regulation of the Phosphodiesterases Escherichia coli Dos and Acetobacter xylinum PDEA1

In the closely related Escherichia coli Dos (EcDos) and Acetobacter xylinum PDEA1 (AxPDEA1) proteins, an entirely different type of enzymatic module, a phosphodiesterase found only in bacterial signal-transduction systems, is coupled to a heme-PAS module 30% identical in sequence to the FixL heme-binding domain (Fig. 1) (6, 11). This phosphodiesterase cleaves the second messenger 3’,5’-cyclic diguanylic acid (c-di-GMP) to the linear dinucleotide pGpG (Fig. 3) (54).
c-di-GMP consists of two GMP moieties linked symmetrically 5′ → 3′ by two phosphodiester bonds and structured roughly like adjacent stacked bases in a polyribonucleotide strand (15). A systematic examination of the phosphodiesterase activity of this enzyme showed it to be quite specific for this particular cyclic dinucleotide (54). Activity toward cyclic mononucleotides such as cAMP was negligible, and there was no sign of competitive inhibition by those mononucleotides.

Physiological roles. Cellulose production by cultures of *A. xylinum* has long been known to depend on O2 tensions (53). The background of Fig. 3 shows the elaborate cellulose pellicle formed at the air-water interface after cultivating, for 1–2 wk, a cocktail of microorganisms, including *A. xylinum* in a sugared brew called Kombucha tea. Work by Benziman and colleagues (53, 54) showed the cellulose synthase in *A. xylinum* to be allosterically activated by c-di-GMP and identified the AxPDEA1 protein as the phosphodiesterase specific for degrading this previously unrecognized second messenger. The biochemical basis for O2 control of the c-di-GMP level would wait until AxPDEA1 could be identified, more than 10 years later, as a new kind of heme-PAS sensor (6). In the AxPDEA1 system, O2 inhibits the linearization of c-di-GMP (6). Because the cyclic form, but not the linear form, can allosterically activate the bacterial cellulose synthase 200-fold, exposure of AxPDEA1 to O2 increases the production of cellulose. Such exposure is the reason for the enhanced cellulose production at air-liquid interfaces of *A. xylinum* cultures (Fig. 3). The equilibrium $K_d$ for binding of O2 to AxPDEA1 (~10 μM) implies that under hypoxic conditions, e.g., within the culture, the active phosphodiesterase eventually converts essentially all the c-di-GMP to the linear form, stopping cellulose production (6).

So far, little has been reported on the enzymology or physiology of EcDos (11). Shimizu and colleagues (59) have measured a slight activity of EcDos toward cAMP (~4.2 pmol·min⁻¹·mg pure EcDos protein⁻¹) and concluded from their results that cAMP was the preferred substrate. The only other nucleotide examined was GMP, which, like cAMP, is not expected to be a substrate for this type of phosphodiesterase. Given that the cAMP phosphodiesterase activity that they measured for purified EcDos was about 50-fold lower than the normal background of cAMP phosphodiesterase activity in crude *E. coli* extracts (~200 pmol·min⁻¹·mg⁻¹), it is unlikely that EcDos has any effect on cellular levels of cAMP (2, 59). It is also unlikely that EcDos functions in vivo as a redox sensor, as postulated in the same study (59). EcDos is easily reduced, with a redox potential of +67 mV (59). Absorption spectra of EcDos recorded in living, intact *E. coli* in widely varied metabolic states show no evidence of oxidized EcDos (V. Delgado-Nixon and M. A. Gilles-Gonzalez, unpublished observations). In addition, loss of activity on oxidation in no way implies physiological redox sensing, even if this inactivation can be reversed by reduction. For instance, hemoglobin is reversibly inactivated by oxidation but can hardly be considered a redox sensor. AxPDEA1 has a clearly established O2-sensing role, although, like EcDos, its enzymatic activity can respond to both the oxidation state of the heme iron and its oxygenation (6). Might EcDos also control production of a biofilm? *E. coli* does not boast the impressive cellulose pellicles of *A. xylinum* (Fig. 3). Nevertheless, *E. coli*, like many other medically and economically important bacteria, naturally thrive in biofilms of a more modest sort, and formation of those structures depends on polysaccharides. Typical laboratory strains of *E. coli*, along with the procedures for growing them, are largely inappropriate for controlled studies of biofilm production. Because of this, reports of the effects of EcDos and c-di-GMP on biofilm formation in *E. coli* have had to wait for new experimental procedures.

Implications. During hypoxia, AxPDEA1 inactivates a pre-existing cellulose synthase, whereas FixL and FixJ induce new gene expression. As such, the two systems illustrate instances
of temporary vs. chronic adaptations, two quite different responses to changing O₂ tensions that are likely to operate side-by-side in many cell types. So far, all of the known proteins with phosphodiesterase modules like the ones in AxPDEA1 and EcDos occur exclusively in bacteria. These phosphodiesterases are unusually abundant in some pathogenic species, such as Vibrio cholera. Consequently, these proteins represent excellent targets for antibiotics. Knowledge of the physiological roles of c-di-GMP, a relatively unexplored second messenger, is likely to illuminate an unpredictable variety of biological phenomena, including ones that will lend themselves to biomedical applications. For example, it is reasonable to expect that O₂ should be a major physiological signal influencing biofilm formation, not only in A. xylinum but also in many other bacterial species.

**CO Regulation of DNA Binding by the Neuronal bHLH-PAS Protein NPAS2**

NPAS2 is the first heme-PAS protein to be discovered in eukaryotes and the only heme-PAS protein so far known to control gene expression by directly regulating DNA binding (12). NPAS2 is expressed principally in the forebrain of mammals and is implicated in controlling circadian rhythm (31, 49). NPAS2 and the Clock protein are closely related, and both can engage in a similar heterodimeric partnership with the BMAL1 protein (31, 56). Mice without NPAS2 have abnormal sleep patterns and fast rather than readapt to eating in daylight (13). Although the physiological effects of heme ligands on NPAS2 are not yet known, it is already clear that the heme status couples directly to DNA binding in this transcription factor. The NPAS2 protein has two PAS domains, PAS-A and PAS-B, both of which bind heme (Figs. 1 and 4A) (12). Heterodimerization of NPAS2 with a partner such as BMAL1 is required for recognition of specific binding sites in enhancer sequences in DNA (31, 56). BMAL1 on its own forms unproductive homodimers that cannot activate transcription, but when NADPH-to-NADP ratios are high NPAS2 can displace a BMAL1 molecule from its homodimeric partner to form a productive NPAS2-BMAL1 heterodimer (56). This redox-sensing function of NPAS2 is unrelated to heme and occurs equally well in the apoprotein-lacking heme or in truncated forms consisting only of the bHLH region (12, 56). If active NPAS2, in the presence of BMAL1 and pure NADPH, is exposed to CO, the NPAS2-BMAL1 heterodimers do not form (Fig. 4C, top) (12). This inactivation by CO requires the presence of heme in the protein and is entirely reversible on removal of the CO (Fig. 4C, bottom). Inhibition by CO is maximal between 1 and 3 μM, which is in good agreement with saturation of the PAS-A domain with this ligand (Fig. 4C, top) (12).

![Fig. 4. Influence of CO on the mammalian bHLH-PAS transcription factor NPAS2. Despite the absence of an exogenous ligand, the absorption spectra of deoxy forms show a doublet in the 500- to 600-nm region that is characteristic of heme-iron hexacoordination, indicating that 2 axial ligands are provided to the heme iron by residues of the protein (black traces in A) (12). Absorption spectra of the carbon monoxo forms illustrate stable binding of CO (gray traces in A). B: limiting rates of CO association (open symbols, independently measured heme-PAS domains; closed symbols, deconvoluted rates from protein having both heme-PAS domains) (12). C: in NPAS2 without heme, CO does not affect formation of the productive NPAS2:BMAL1 heterodimers (bottom), but in NPAS2 with heme a CO concentration ([CO]) range consistent with saturation of the PAS-A domain inhibits formation of NPAS2:BMAL1 (top) (12).](image-url)

**REGULATION OF O₂ AFFINITY IN HEME-PAS PROTEINS**

One of the reasons why heme-based sensors are so excellently suited for sensing of heme ligands is the ease with which the protein matrix can “tune” the sensitivity of a heme to any ligand-concentration range of interest. Heme proteins that contain the same heme (protoporphyrin IX) are known to span a range of O₂ affinities of four orders of magnitude. However, heme in aqueous solution will not bind O₂ stably at all, because under those conditions oxidation of heme iron, to the Fe³⁺ form that can no longer bind O₂, invariably accompanies the dissociation of this ligand. The protein envelope does much more than simply shield the hydrophobic heme moiety from the aqueous solution and has at least three other ways of modulating the affinity of the heme: 1) it supplements the Fe-O₂ bond with hydrogen bonds from polar distal pocket residues to the bound O₂; 2) it supplies one or two precisely oriented axial ligands to the heme iron; and 3) it limits, by both steric and electrostatic means, the accessible conformations of porphyrin substituents such as the propionates.
Supplemental Hydrogen Bonding

This is the best understood mechanism for modulating the affinity of a heme protein (45, 46). In many hemoglobins and myoglobins, a conserved histidine at the seventh residue of the E helix, i.e., the E7 histidine or distal histidine, lines the largely hydrophobic distal heme pocket where the ligand binds (47, 68). Although O2 is apolar and diffuses readily into hydrophobic heme pockets, it acquires a partial negative charge when it forms a highly polar coordination bond with the heme iron. A hydrogen bond of ~8 kcal/mol forms between the polarized O2 and the distal histidine. Because entry of O2 displaces a water molecule from the heme pocket at a cost of ~1 kcal/mol, the distal histidine stabilization is estimated to contribute a net 7 kcal/mol to the O2 binding energy. Alternative schemes have also been reported for supplemental bonding that involve other polar distal-pocket residues. These include the E7 glutamine in elephant myoglobin, the E10 arginine in Aplysia myoglobin, and the E7 glutamine and B10 tyrosine in Ascaris hemoglobin (3, 10, 48).

It is noteworthy that the contributions of supplemental bonding vary dramatically for different heme proteins and by no means account for their vast range of affinities. In particular, the distal histidine appears more important to mammalian heme proteins. None of the known heme-PAS proteins has a distal histidine, and yet the range of observed affinities is comparable to that of hemoglobins. Soybean leghemoglobin has no special stabilizing interactions, yet O2 binds to this protein ~10 times more tightly than to sperm whale myoglobin (33). Substitutions of the sperm whale myoglobin distal histidine with alanine results in a 50-fold lower affinity, but substitutions of distal residues in leghemoglobin (including the distal histidine) have no significant effect on O2 affinity (33, 45, 46). Clearly, the protein matrix can modulate the heme by means other than supplemental bonding.

Axial Coordination

Coordination on the proximal side of the heme iron. This is the strongest single interaction of the protein with the heme. The energy of binding of any exogenous ligand to a metal center is well known to depend on the number and types of other ligands coordinated. In hemoglobins, myoglobins, FixLs, EcDos, and AxFPDEA1, at least one of the residues linking the protein to the heme iron is a proximal or coordination-site histidine. In contrast to synthetic ligands that chemists may build into heme analogs, the proximal histidine is fixed to the protein, which is analogous to a solid support, and this histidine is restricted in its rotation by constraints such as hydrogen bonding, electrostatic interactions, and steric factors. Perutz and colleagues (27) proposed that the high affinity of leghemoglobin might be related to the staggered conformation of its proximal histidine with respect to the pyrrolo nitrogens. Because the FixL proteins, which have low-O2 affinities, are even more staggered than leghemoglobin, the most significant difference between leghemoglobin and other heme proteins may be the greater freedom of rotation of the leghemoglobin proximal imidazole (14). On binding of O2 to leghemoglobin, this imidazole swings (through a ~70° angle) to optimize its energy, just as heme analogs do in solution (27). In myoglobin, the proximal histidine is so restricted that there is essentially no difference in the proximal histidine orientations of the oxy and deoxy states (69).

Control of the proximal histidine rotation represents only one way for the protein to modulate the heme iron with this residue. The reactivity of the histidine, including its pKa, also depends on its interactions with other residues of the protein. Such factors directly affect donation of electron density from this coordination-site histidine to the heme iron. For binding of O2 to heme proteins, a positive "trans" effect is expected between the Fe-histidine ligation on the proximal side and the Fe-O2 ligation on the distal side. That is, a weak Fe-histidine bond is thought to correlate with a weak Fe-O2 bond and vice versa. In the absence of high-resolution structures, Fe-histidine bond strengths are usually assessed by extended X-ray absorption fine-structure spectroscopy (EXAFS) or resonance Raman spectroscopy (RR). EXAFS can provide a direct measure of bond length, and the RR stretching frequency of a bond is believed to correlate with its strength. Although EXAFS studies have shown the Fe-histidine bond in RmFixL to have exactly the same length as the one in sperm whale myoglobin, RR studies have shown this bond to be much weaker in deoxy-RmFixL (rFe-histidine = 209 cm−1, where r is the wave number) than in deoxy-myoglobin (rFe-histidine = 220 cm−1) (42). Shiro and colleagues (42) have cautioned that the RR stretching frequency is a better indicator of bond strength because the bond length does not take into account the differences in bond angles and heme distortion. Although this argument is valid, it should be pointed out that there is no correlation between the Fe-histidine stretching frequencies and the O2 affinities of the heme-PAS proteins BjFixL, EcDos, AxFPDEA1, and the Methanobacterium thermoautotrophicum Dos (MiDos) (64). It is possible that other factors obscure the contributions of the heme coordination to O2 affinity. We expect that further study will show aspects of the proximal heme iron coordination to contribute to the affinities of heme-based sensors and indeed all hemoglobins.

Displaceable coordination on the distal side of the heme iron: EcDos and NPA2. In addition to providing the heme iron with a fixed proximal axial ligand, some heme proteins supply the iron atom with a displaceable distal axial ligand, causing even more dramatic effects of protein coordination (11, 12, 23, 65). In contrast to the heme iron in proteins, such as cytochrome c, which are permanently hexacoordinate and cannot bind ligands reversibly, the heme iron in "displaceable-residue heme proteins" is in a relatively rapid equilibrium between hexacoordinate and pentacoordinate states. The equilibrium association constant for binding of the displaceable residue, KHP, is also the ratio of pentacoordinate to hexacoordinate molecules at any given time (23, 65). Because only the pentacoordinate fraction can bind ligands, KHP can be quite small, but it cannot be zero. In the simplest case, the competition between external ligands and the displaceable residue would do the following: 1) lower the equilibrium association constant (Ka) for binding of those ligands by a factor equal to KHP, 2) lower the association rate constant (kΔa) by the same factor, and 3) set an upper limit on the rate of ligand association equal to the rate at which the displaceable residue dissociates from heme iron to generate the pentacoordinate form.

Although the three-dimensional structure of EcDos is not yet known, magnetic circular dichroism spectroscopy of the near-infrared charge-transfer band showed <2% of the heme iron to
be pentacoordinate at room temperature and revealed this atom to be axially coordinated to histidine and methionine (23). If a large population of pentacoordinate EcDos is generated by photolyzing CO from the carbonmonoxy form (Fe\(^{II}\)CO) with a brief (5-ns) and intense laser pulse and a series of transient absorption spectra are immediately recorded, then the methionine, identified as residue 95, is observed first to bind at a rate of \(\sim 12,000 \text{ s}^{-1}\) and later to be displaced by CO (23). This is because at moderate CO concentrations the rate of CO association is much slower than the rate of association of the methionine, even though CO binds more tightly due to its slower dissociation rate. It is possible to calculate a dissociation rate for the displaceable methionine of \(\sim 87 \text{ s}^{-1}\) from the CO concentration dependence of the rate of CO displacement of methionine after photolysis (35). This relatively rapid rate is consistent with the earlier observation that methionine dissociation never limits the rate of binding of gaseous ligands at any experimentally practical concentration (11). The measured microrate constants for EcDos indicate a \(K_{HP}\) of \(\sim 0.0125\), that is, about 1 of every 80 EcDos molecules at any time has its H-atom coordinating to the heme iron and can bind ligands. If the only role of methionine 95 is to compete against exogenous ligands, then its substitution should uniformly increase the association rate constant and \(K_d\) for all ligands by a factor of 80. Instead, an EcDos variant with methionine 95 replaced by isoleucine had a 10-fold higher affinity for O\(_2\) but a 200-fold higher affinity for CO compared with normal EcDos (23). This indicates a more complex role for the displaceable methionine of EcDos. In addition to being a quite refined mechanism for control of detection range, displaceable residues represent a very direct way for a ligand to trigger a regulatory conformational change.

Absorption spectra of deoxy-NPAS2 and its independent heme-binding domains are shown in Fig. 4A (12). In contrast to the spectra of pentacoordinate deoxy heme proteins, which show a single broad peak in the 500- to 600-nm region, spectra of deoxy-NPAS2 show a well-defined doublet in the 500- to 600-nm region, with the first peak less intense than the second (12). These features indicate hexacoordination of the heme iron and presence of a displaceable residue. Notice that the PAS-A domain has a little more pentacoordinate heme iron than the PAS-B domain, although both domains are overwhelmingly hexacoordinate. In contrast to the displaceable methionine in EcDos, the displaceable residues of PAS-A and PAS-B dissociate relatively slowly (12, 23). For binding of CO, the association rate increases linearly with ligand concentration only over a limited range of CO concentrations. For PAS-A, the limiting rate of CO association is \(7 \text{ s}^{-1}\), whereas for PAS-B it is \(7 \text{ s}^{-1}\) (Fig. 4B) (12). These limiting rates represent the rates of dissociation of the displaceable residue and hence the rates of generation of the pentacoordinate forms, which no ligand association can exceed. Assuming that the association rates of the displaceable residues and the reactivities of the pentacoordinate states are similar for the two PAS domains, then NPAS2 nicely illustrates how displaceable residues modulate ligand affinity. A 10-fold faster dissociation rate of the displaceable residue rate in PAS-B would result in a 10-fold higher value for \(K_{HP}\). This agrees with the observation that PAS-A has a 10-fold higher CO-association rate and 10-fold higher CO affinity than PAS-B (12).

### Protein Interactions With the Heme Substituents

This is the least understood aspect of affinity modulation. The O\(_2\) affinity of BjFixL is \(\sim 100\)-fold lower than that of sperm whale myoglobin, the latter serving a convenient point of reference because it is one of the best-studied heme proteins. In fact, the \(K_d\) for binding of O\(_2\) to BjFixL (\(\sim 140 \text{ \mu M}\)) is comparable to that of sperm whale myoglobin variants entirely without hydrogen bonding to bound O\(_2\) (21). If present, supplemental hydrogen bonds slow down the off-rate of O\(_2\) because these bonds must be disrupted for the O\(_2\) to dissociate from the heme iron, but they do not influence the on-rate of O\(_2\), because this ligand is apolar before binding to the heme iron. For example, the \(K_d\) for binding of O\(_2\) to the His\((E7)\) \(\rightarrow\) Val mutant of sperm whale myoglobin is 700 times higher than that for the wild type; the kinetics of association do not contribute to the low affinity of this variant and are actually faster than for the wild type because of the elimination of a distal pocket water molecule (46). In contrast, the \(K_d\) for binding of O\(_2\) to BjFixL is only slightly higher (42%) than that of sperm whale myoglobin, and the low affinity of BjFixL is largely due to an O\(_2\) association rate constant \(\sim 80\) times lower than that of sperm whale myoglobin (14, 21). Therefore, supplemental hydrogen bonds cannot explain the kinetics of O\(_2\) binding to BjFixL. The slower association of the apolar ligand CO to BjFixL, compared with sperm whale myoglobin, also cannot be due to differences in supplemental bonding (21). Steric factors are easily ruled out as the source of slow association for BjFixL by considering that the bulky ligand imidazole, an excellent probe of steric hindrance, binds 300 times faster to ferric BjFixL than to ferric myoglobin (38). A more reasonable explanation for the slow association rates of BjFixL is a heme iron with intrinsically low reactivity. What could be the source of this low reactivity? The X-ray crystal structure of an O\(_2\)-bound form, showing hydrogen bonding from a distal arginine, i.e., the G\(_B\)-2 arginine or R220, would appear to complicate this question (25). Because even a stabilizing distal arginine fails to raise the O\(_2\) affinity of BjFixL above that of an unsupplemented heme pocket, the Fe-O\(_2\) bond itself must be extremely weak.

Consistent with this prediction, an R220A BjFixL variant did have an extraordinarily low affinity for O\(_2\) (\(K_d\) of \(\sim 1.4\) mM) (14). On examination of the kinetics, the \(k_{off}\) for binding of O\(_2\) to R220A BjFixL was found to be lower than that of BjFixL, contrary to the acceleration that would be expected from a loss of supplemental bonding (14). Therefore, although superficially the low affinity of the R220A BjFixL appeared to be due to differences in supplemental bonding (14), the low O\(_2\) affinity of R220A BjFixL compared with BjFixL, like the low O\(_2\) affinity of BjFixL compared with sperm whale myoglobin, is due to a slowed rate of ligand association that classical distal heme-pocket effects cannot explain.

How does the G\(_B\)-2 arginine in FixL modulate its affinity for heme ligands? The structure of R220A BjFixL showed the heme and its environment, even without O\(_2\), to be quite different from that of BjFixL (14). The Fe-histidine bond was 0.31 Å longer and tilted with respect to the heme plane, the proximal histidine plane was rotated 24° about its C\(_\beta\)-C\(_\gamma\) torsion angle, and the porphyrin ring was planar despite its unquestionably high-spin heme iron. The heme propionates were also repositioned. Normally, in unliganded BjFixL, a salt
bridge between the heme propionate $7\ O_2A$ atom and the $G_{\beta}-2$ arginine directly links the porphyrin ring to the distal side of the heme (22, 25). Because, during binding of $O_2$, the supplemental bond to $O_2$ from the $G_{\beta}-2$ arginine is formed at the expense of this salt bridge, it is unclear whether the net contribution to the free energy should be positive or negative. The loss of the salt bridge between the porphyrin and the $G_{\beta}-2$ arginine, and the ensuing changes of the heme shape and electrostatics, provide the most reasonable explanation for the profound effects of removing the $G_{\beta}-2$ guanido group.

**REGULATORY CONFORMATIONAL SWITCHING:**

**LESSONS FROM FIXL**

**Modulation of Domain Interactions by Ligands**

Purified *S. meliloti* and *B. japonicum* FixL form homodimers, and heme ligands do not affect this dimerization (18, 21). Contrary to one report, RmFixL does not require RmFixJ to dimerize and does not aggregate unless denatured (40). Interestingly, depending on where the heme-binding domain of RmFixL is truncated, this isolated domain can be either monomeric or dimeric, and some heme ligands can cause dissociation of the dimeric version (41, 52). Because intact FixLs are always dimeric, there must be sufficient interdimer contacts in the kinase portion of the protein to maintain it in that state regardless of any interdimer contacts in the heme-binding domain (18, 21). The dimeric heme-binding domains of RmFixL, but not the monomeric ones, include an extra region NH$_2$-terminal to the PAS domain, suggesting that this region contains interdimer contacts that are sensitive to heme ligands despite being relatively far from the heme. Unfortunately, it is not yet possible to crystallize liganded forms of the dimeric heme-binding domains to examine the structural effects of ligands on the interdimer region.

**High-Resolution Structures**

Several high-resolution structures are available for the heme-binding domains of FixL (14, 22, 25, 41). The only heme-binding domain crystallized so far with and without ligands is the one from BjFixL (22, 25). The main change detected on binding of a regulatory ligand is a 1.6-Å push of a loop (FG loop) away from the heme center, accompanied by a switch in the hydrogen-bonding interactions of the heme propionates with protein residues. The driving force for this conformational change remains controversial, and it is not yet clear whether the movement of the heme propionates causes the conformational change or vice versa.

**Regulatory Models**

The earliest mechanistic model proposed for FixL inhibition was the “spin-state hypothesis” (20). This model proposed that in FixL, just as in hemoglobin, a transition of the heme iron from high spin to low spin, on binding of $O_2$, shifts the iron atom into the heme plane. The displaced iron atom moves the proximal histidine and its helix of attachment, the $F_{\beta}$-helix in FixL, triggering a conformational change. This model was ruled out by structures of the “on” and “off” states of the BjFixL heme-binding domain, which showed their proximal sides to be superimposable (22, 25). The original hypothesis relied largely on the observation that all high-spin forms of FixL, including deoxy (Fe$^{II}$), met (Fe$^{III}$), and fluoromet (Fe$^{III}$F$^-_2$), were fully active in the autophosphorylation reaction with ATP alone, whereas all low-spin forms, such as oxy (Fe$^{II}$O$_2$), carbonmonoxy (Fe$^{II}$CO), cyanomet (Fe$^{III}$CN$^-_2$), and imidazolomet (Fe$^{III}$Imid), appeared to be equally inhibited (20). Recent studies of the more physiologically relevant turnover reaction (see Regulation of the Histidine Protein Kinase FixL of Rhizobia above) have shown no such equivalence of the activities of like spin states (66). Even if the same heme-iron spin state results from ligation with different ligands, their electronic effects are by no means equivalent. For example, ligation with $O_2$ places a high formal positive charge on the heme iron, whereas ligation with CO results in a much more electron-rich heme because of back-bonding to the $\pi$ orbitals of the iron atom. Binding of ligands alters the porphyrin shape in ways that are specific to individual proteins and that differ substantially from the distortions of heme analogs in solution (51). Although these protein-porphyrin interactions are poorly understood, they are probably an important driving force in conformational switching. For FixL, the kinetic, structural, and enzymatic studies to date point to a role of the ligand-induced changes in the porphyrin shape, and particularly the positions of the heme propionates, in the regulatory conformational switch.

**FUTURE DIRECTIONS**

**Additional Heme-PAS Sensors in Mammals**

An important advance in understanding mammalian adaptation to hypoxia has been the discovery of the protein hydroxylases and the ubiquitin ligase directing the normoxic degradation of the transcription factor HIF-1$\alpha$ (39, 60). Do there exist additional mammalian strategies to provide for a more rapid and reversible adaptation to changing $O_2$ tensions? The possibility of heme-PAS $O_2$ sensors in mammals now seems more likely in light of the recent discovery of heme-ligand regulation of NPAS2, a transcription factor belonging to the bHLH-PAS family (12).

**A Broader View of Heme-Based Sensors**

Given the modular nature of heme-PAS sensors and their known interactions with protein partners and with nucleic acids, a broader view is essential for additional progress in understanding those systems. For most heme-PAS proteins, the heme-binding domains are relatively well understood as standalone heme proteins. By contrast, the interactions between the heme-PAS domain and its regulatory targets are largely unexplored. How does binding of a small diatomic ligand to a heme-PAS domain manage to inhibit the phosphorylation of a partner protein, the hydrolysis of a cyclic-nucleotide substrate, or the assembly of a productive transcription complex? Answers to this question will likely require an understanding of the conformations of the on- and off-states of entire sensory complexes, with all their domains intact.

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