Dos, a Heme-Binding PAS Protein from *Escherichia coli*, Is a Direct Oxygen Sensor[†]

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ABSTRACT: A direct sensor of O_2, the Dos protein, has been found in *Escherichia coli*. Previously, the only biological sensors known to respond to O_2 by direct and reversible binding were the FixL proteins of *Rhizobia*. A heme-binding region in Dos is 60% homologous to the O_2-sensing PAS domain of the FixL protein, but the remainder of Dos does not resemble FixL. Specifically, the C-terminal domain of Dos, presumed to be a regulatory partner that couples to its heme-binding domain, is not a histidine kinase but more closely resembles a phosphodiesterase. The absorption spectra of Dos indicate that both axial positions of the heme iron are coordinated to side chains of the protein. Nevertheless, O_2 and CO bind to Dos with K_d values of 13 and 10 μM, respectively, indicating a strong discrimination against CO binding. Association rate constants for binding of O_2 (3 mM^{-1} s^{-1}), CO (1 mM^{-1} s^{-1}) and even NO (2 mM^{-1} s^{-1}) are extraordinarily low and very similar. Displacement of an endogenous ligand, probably Met 95, from the heme iron in Dos triggers a conformational change that alters the activity of the enzymatic domain. This sensing mechanism differs from that of FixL but resembles that of the CO sensor CooA of *Rhodospirillum rubrum*. Overall the results provide evidence for a heme-binding subgroup of PAS-domain proteins whose working range, signaling mechanisms, and regulatory partners can vary considerably.

Heme-based sensors represent an important class of signal-transducing proteins (*1, 2*). The FixL, CooA, and guanylyl cyclase heme proteins respond to the gaseous physiological messengers O_2, CO, and NO, respectively (*3*−*5*). In every case, binding of ligand to a heme cofactor within a sensory domain regulates the enzymatic or DNA-binding activity of an adjacent C-terminal domain. Here we describe an *Escherichia coli* protein, Dos,[1] initially noted for the extensive sequence homology (∼25% identical and 60% homologous) between its N-terminal region and the O_2-sensing domain of the FixL proteins of *Rhizobia*.

The sensory domain of FixL belongs to the large family of signal-transducing PAS-domain proteins that are well represented in *Eukarya*, *Archaea*, and *Bacteria* (*6, 7*). Despite their limited sequence homology (<15% identity) and their association with dissimilar cofactors, PAS domains reflect a conserved structural fold (*8, 9*). As demonstrated by the protein crystal structures, the human voltage sensor HERG, the rhizobial O_2 sensor FixL, and the bacterial light sensor PYP all contain this fold (*8*−*10*). The signaling partners of PAS domains include histidine kinases, ion channels, methyl-carrying chemotaxis receptors, and ATP-binding domains (*3, 9*−*12*). The three structures available so far for isolated PAS domains suggest that the interactions of those domains with their partners are quite diverse. For example, hydrophobic interactions between a PAS domain and the mouth of a channel are thought to regulate the K^+ channel encoded by HERG (*9*). In PYP the light-driven changes affect polar interactions primarily in the EF loop of the PAS domain (*10*). In FixL the heme-driven changes influence polar interactions in the FG loop (*8*).

Two defining features of FixLs are that their O_2-detecting PAS domain is always associated with a C-terminal histidine kinase domain, and that their physiological role is to restrict the expression of N_2 fixation genes to hypoxic conditions (*1, 13*−*16*). Thus, the existence of Dos, an *E. coli* protein having homology to FixL's heme-binding domain but not its histidine kinase domain, suggested to us that the sensory motif found in FixL might not be limited to the control of histidine kinases nor to the regulation of N_2 fixation.

Here we show that Dos is indeed a heme-containing protein and discuss which features of its PAS sensory mechanism are altered to suit its new interactions. To our knowledge, Dos is only the second heme-containing PAS-domain protein to be described and the first heme-based sensor to be discovered in *E. coli*.

EXPERIMENTAL PROCEDURES

Genetic Manipulations. On the basis of the DNA sequence reported by Blattner and colleagues (*17*), two oligonucleotide primers were designed for amplifying the gene fragment corresponding to codons 1−147 of the *E. coli yddU* gene

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[1] Abbreviations: Dos, direct oxygen sensor; Dos_H, Dos heme-binding domain; PAS, sensory domain named after the eukaryotic proteins Period, Arnt, and Simple-minded; BjFixLH, *Bradyrhizobium japonicum* FixL heme-binding domain; PYP, photoactive yellow protein; HERG, human ether-a-go-go-related gene; PDEA, phosphodiesterase A; Hb, hemoglobin; SW Mb, sperm-whale myoglobin; IPTG, isopropyl-β-D-glucopyranoside; PMSF, phenylmethylsulfonyl fluoride; FNR, regulator of fumarate and nitrate reduction; H, hexacoordinate heme iron with endogenous axial ligands; P, pentacoordinate heme iron.

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(here renamed *dos*). The source of template for the PCR amplification was *E. coli* genomic DNA. The primers were designed to introduce an *Nde*I and an *Hin*dIII site at the 5′ and 3′ ends of the amplified fragment, respectively. This amplified product was cloned into a TA cloning vector (Invitrogen). The gene was transferred to an expression vector as an *Nde*I-*Hin*dIII fragment. The resulting plasmid, pEH31, contains dos_H under control of the *tac* promoter and confers ampicillin resistance.

Gene Expression and Protein Purification. Expression of dos_H, the gene segment encoding residues 1−147 of Dos, was induced by adding 500 μM IPTG to exponentially growing TG1(pEH31). After 5 h of induction at 37 °C, the cells were cooled to 4 °C and harvested. The cell pellets were stored at −70 °C until the protein purifications. All subsequent protein manipulations were at 4 °C, and all column matrixes were from Pharmacia. The cells were lysed by sonication. Dos_H protein was purified from the cleared lysate by ammonium sulfate precipitation followed by desalting (Sephadex G25), ion-exchange fractionation (DEAE-sephacel), and gel filtration (Superdex 75). For size determinations, the gel-filtration column was calibrated with ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa) (Pharmacia). The purified protein was stored at −70 °C. Protein concentrations were determined by the Bradford protein assay, with bovine serum albumin as the standard (*18*). The heme content was measured using a pyridine hemochromogen assay, with hemin ($\epsilon_{390} = 50$ mM^{-1} cm^{-1} in 2% sodium borate, pH 9.23) as the standard (*19*).

Absorption Spectra. Unless otherwise noted, all the absorption spectra were measured for purified protein in 0.1 M NaP_i, pH 7.0, at 23 °C in a stoppered quartz cuvette. Deoxy-Dos_H was prepared by precipitating a small aliquot of the purified protein from ammonium sulfate, resolublizing it in a small volume of anaerobic 0.1 M NaP_i, pH 7.0, inside a glovebox, and reducing it with crystalline sodium dithionite. The sodium dithionite was removed on a 5 mL Sephadex G25 desalting column pre-equilibrated with deoxygenated 0.1 M NaP_i, pH 7.0, and a deoxy-Dos_H spectrum was recorded. Oxy-Dos_H was prepared by cooling a deoxy-Dos_H sample to 4 °C and equilibrating with 1 atm of O_2. Carbonmonoxy-Dos_H was prepared by equilibrating deoxy-Dos_H with 1 atm of CO. Met-Dos_H was prepared by precipitating the purified protein from ammonium sulfate, resolublizing it in 0.1 M NaP_i, pH 7.0, and adding to it an equimolar amount of potassium ferricyanide. The potassium ferricyanide was removed on a Sephadex G25 column equilibrated with 0.1 M NaP_i, pH 7.0.

Ligand Binding. All rates were followed for 2−5 μM protein with a stopped-flow spectrometer (Applied Photophysics Ltd., Leatherhead, U.K.) at a wavelength of maximum difference between the starting and final species at 25 °C. Each apparent rate was measured at least three times. Each rate constant was calculated from a linear plot of k_{obs} vs ligand concentration including at least four ligand concentrations. All protein and gas solutions for the ligand-binding reactions were in 0.1 M NaP_i, pH 7.0, and without dithionite.

To measure the association rates, deoxy-Dos_H was mixed with 80−650 μM O_2, 60−500 μM CO, or 60−1000 μM NO. Ligand association was followed by the change in absorbance at 429 nm for O_2, 421 nm for CO, and 430 nm for NO.

To measure the O_2 equilibrium dissociation constant, deoxy-Dos_H was mixed with 10−130 μM O_2 at 25 °C in a stopped-flow spectrometer. The total absorbance change ΔA of the rate traces were monitored at a maximum, a minimum, and an isosbestic wavelength of absorption (412, 430, and 442 nm, respectively) for the conversion of deoxy- to oxy-Dos_H. For measurement of the CO equilibrium dissociation constant, deoxy-Dos_H was mixed with varying concentrations of CO (10−170 μM), and the absorption spectra (350−700 nm) were recorded. The rate amplitudes from the O_2-binding measurements and the absorption spectra from the CO-binding measurements 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 for the Hill coefficient n and for the K_d were obtained from Hill plots.

RESULTS

Domain Organization. Figure 1 illustrates the relationship between the N-terminal region of Dos and the PAS domains of various sensory proteins. For the comparison, PAS proteins were chosen that have known three-dimensional structures or extensive homology to Dos. The PAS motif, comprised of a limited number of conserved residues, such as $A_\beta 2$, $B_\beta 0$, $C_\alpha 0$, $D_\alpha 0$, $E_\alpha 0$, $F_\alpha 0$, $G_\beta 0$, $H_\beta 6$, and $I_\beta 0$, is clearly in Dos (*6*). For PAS domains having dissimilar sensory functions, this motif accounts for a sequence homology of 15% or less. In contrast, the PAS domain of Dos is about 25% identical and 60% homologous to the heme-binding domains of FixLs, with the identity extending to the heme-attachment histidine $F_\alpha 3$ (i.e., H200 in BjFixL). The evolutionary relationship between the Dos and FixL PAS domains is not as close as that between the heme-binding domains of FixLs from different *Rhizobia* (50% identical and 70% homologous). This is probably because the FixL PAS domains are always coupled to a C-terminal histidine kinase domain, whereas the Dos PAS domain is associated with a C-terminal region that resembles a phosphodiesterase (Figure 2) (*20*). We found a much more extensive homology (30% identical and 50% homologous) between Dos and the *Acetobacter xylinum* cyclic-di-GMP phosphodiesterases (PDEA), not only extending to their PAS domains but also encompassing their C-terminal domains. Like the histidine kinase domain, this type of phosphodiesterase belongs to a large family (*20*). The domain organization of Dos and PDEA1 therefore suggests that they are members of a class of heme-based sensors in which an O_2-sensing PAS domain is coupled to a phosphodiesterase.

Purity, Heme Content, and Oligomerization State. The N-terminal sequence of Dos_H was determined to be MRQDAEVI (W. M. Keck Foundation). This is identical to the sequence predicted from the expressed gene fragment. Purified met-Dos_H has a UV-to-Soret absorbance ratio (A_{280}/A_{418}) of 0.39 (Figure 3a). Comparisons of heme to protein content indicate that Dos_H contains one heme per monomer (1:1) (*18, 19*). On the basis of gel filtration, the molecular mass of native Dos_H is approximately 36 kDa. Given that the calculated molecular mass of this domain is 17 kDa, this implies that Dos_H is dimeric.

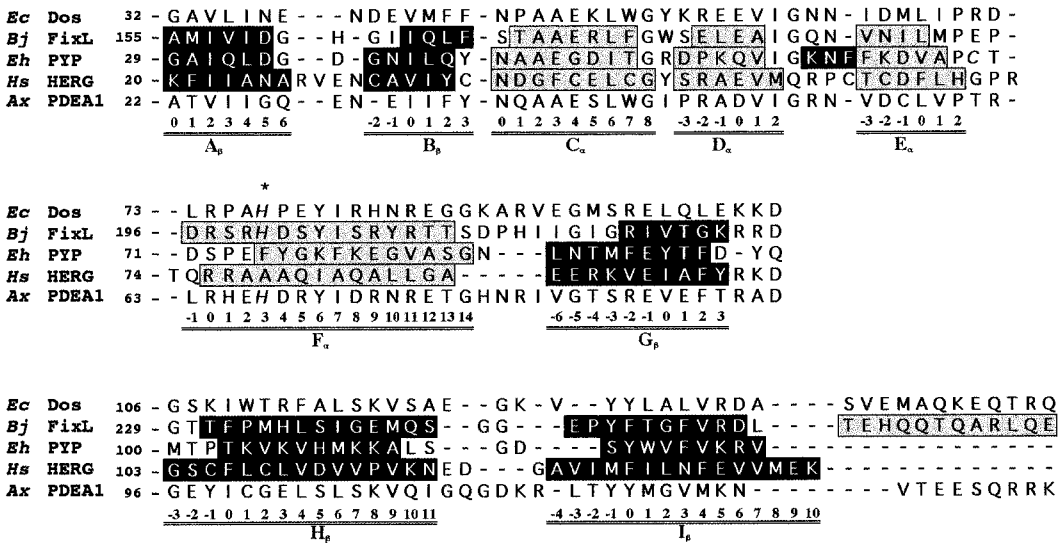

FIGURE 1: Alignment of selected PAS domains. The nomenclature is according to Gilles-Gonzalez and colleagues (*8*). The PAS-domain sequence in *E. coli* Dos (*Ec* Dos) is compared to the sequences of *A. xylinum* PDEA1 (*Ax* PDEA1) and PAS-domain proteins of known structure. The boxed regions of secondary structure are based on the crystal structures of the *B. japonicum* FixL heme-binding domain (*Bj* FixLH), the *E. halophila* PYP (*Eh* PYP), and the human ether-a-go-go-related gene product (*Hs* HERG) (*8*−*10*). Regions are labeled alphabetically with a subscript indicating the type of secondary structure. Cofactor attachment amino acids for FixL (H200) and PYP (C69) are shown in italics. The asterisk indicates the expected heme attachment site histidine in heme-binding PAS domains.

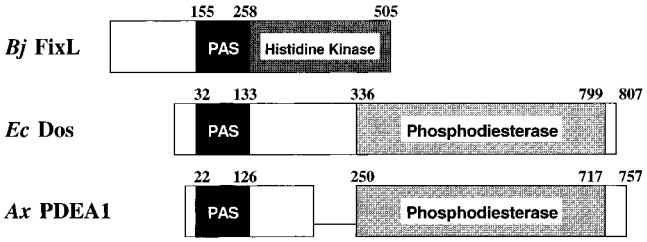

FIGURE 2: Schematic representation of the FixL, Dos, and PDEA1 proteins. The homologous PAS domains of *B. japonicum* FixL, *E. coli* Dos, and *A. xylinum* PDEA1 are indicated. The C-terminal domain of the *E. coli* Dos is aligned with the phosphodiesterase domain of the *A. xylinum* PDEA1. The numbers denote amino acid positions in the linear protein sequence.

Absorption Spectra. From the absorption spectra it is clear that despite the close sequence similarity and expected structural similarity between FixL and Dos, the environment of the heme iron in the two proteins is different (Figure 3, Table 1). Met- and deoxy-FixL have high-spin pentacoordinate heme iron. This is apparent from their absorption spectra and has been confirmed by NMR, resonance Raman spectroscopy, and X-ray crystallography (*1*, *8*, *21*−*23*). In contrast, the spectra of met- and deoxy-Dos_H are typical of heme proteins that have predominantly low-spin hexacoordinate heme iron. Instead of the broad absorption band found at ∼556 nm for deoxy-FixL, deoxy-Dos_H had well-resolved α- and β-bands in the far-visible region, with the α-band (563 nm, 26 mM^{-1} cm^{-1}) being more intense than the β-band (532 nm, 16 mM^{-1} cm^{-1}). Met-FixL has a broad band around 500 nm and the broad blue-shifted Soret band at 396 nm typical of pentacoordination, whereas met-Dos_H has distinct α- and β-bands in the 500−600 nm region and a sharp Soret band at 416 nm. Overall, the 400−700 nm absorption spectra of met- and deoxy-Dos_H are most similar to spectra reported for the CO sensor CooA, and the barley, rice, and yeast YNL234wp Hbs (*24*−*27*). The latter proteins function in reversible binding of ligands, and in every case where their coordination has been investigated in more detail, they have been confirmed to have hexacoordinate heme iron (*26*, *28*, *29*). By removing all traces of light-absorbing oxidizing or reducing agents from our preparations, we were able to reveal an additional ligand-sensitive δ-band in the 330−370 nm region of the met-, deoxy-, carbonmonoxy-, and oxy-Dos_H spectra. This region of the spectrum has not yet been explored for CooA, barley Hb, rice Hb1, or YNL234wp, whose ferrous species are generally prepared with an excess of dithionite. Even the forms of Dos_H containing exogenous ligands had absorption spectra less similar to FixL spectra than to those of proteins such as CooA and the cereal Hbs. For example, the absorption maxima of carbonmonoxy-Dos_H were within 2 nm of the corresponding bands in carbonmonoxy-CooA (*24*), whereas they differed by as much as 10 nm from the corresponding peaks in carbonmonoxy-FixL (*1*).

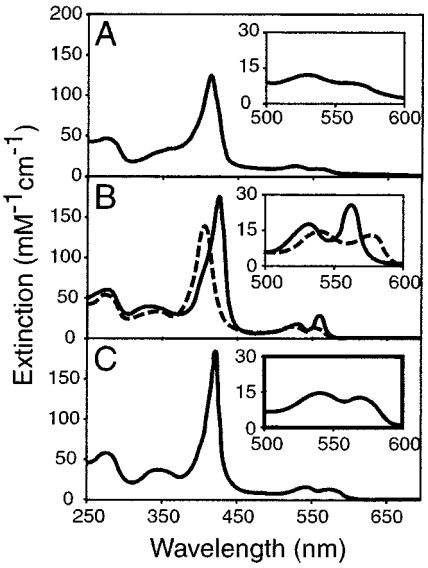

FIGURE 3: Absorption spectra of purified Dos_H. Panel A shows a spectrum of purified met-Dos_H. Panel B shows the spectra of deoxy- and oxy-Dos_H. Panel C shows the spectrum of carbonmonoxy-Dos_H.

O_2, CO, and NO binding. Data in Figures 4 and 5 and Table 1 highlight the remarkably similar binding of O_2, CO, and NO to Dos_H. Each panel of Figure 4 shows a kinetic

Table 1: Properties of Selected Heme Proteins

Absorption Maxima

protein	met (Fe^{III}) δ	γ	β	α	deoxy (Fe^{II}) δ	γ	β	α	carbonmonoxy ($Fe^{II}CO$) δ	γ	β	α	oxy ($Fe^{II}O_2$) δ	γ	β	α
Dos_H	367 (36)[a]	416 (124)	530 (12)	564 (9)	329 (37)	427 (172)	532 (16)	563 (26)	349 (37)	423 (189)	540 (15)	570 (13)	352 (32)	417 (140)	541 (14)	579 (13)
CooA[b]	361	424	541	566		426	529	559		422	540	568		ND[c]		
barley Hb[d]		411	534	565		425	539	563		417	537	567		412	540	576[c]
BjFixL[e]		395	509	645		437	556			427	548	560		419	545	562

O_2- and CO-Binding Parameters at pH 7.0 and 25 °C

protein	$k_{on}(O_2)$ (μM^{-1} s^{-1})	$k_{off}(O_2)$ (s^{-1})	$K_d(O_2)$ (μM)	$k_{on}(CO)$ (μM^{-1} s^{-1})	$k_{off}(CO)$ (s^{-1})	$K_d(CO)$ (μM)
Dos_H	0.0026	0.034[f]	13	0.0011	0.011[f]	10
BjFixL[e]	0.14	20	140	0.0050	0.045	9.0
RmFixLH[e]	0.22	6.8	31	0.017	0.083	4.9
barley Hb[d]	9.5	0.027	0.0029	0.57	0.0011	0.0019
rice Hb1[g]	68	0.038	0.000 56	7.2	0.0010	0.000 14
SW Mb[h]	14	12	0.86	0.51	0.019	0.037

[a] Values in parentheses are the extinctions (mM^{-1} cm^{-1}) for Dos_H derivatives. [b] Shelver et al. (*24*). [c] Not determined. [d] Duff et al. (*25*); the oxy-Hb spectrum is calculated. [e] Gilles-Gonzalez et al. (*1*). [f] Calculated from k_{on} and K_d. [g] Arredondo-Peter et al. (*26*). [h] Quillin et al. (*39*).

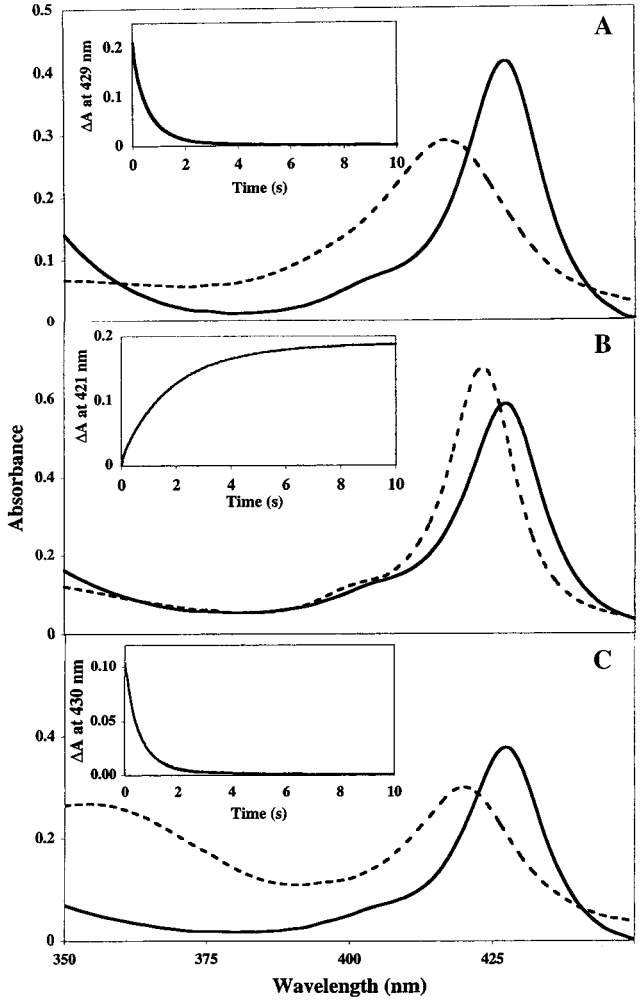

FIGURE 4: Binding of ligands to deoxy-Dos_H at 25 °C, pH 7.0. Panels A, B, and C show binding to O_2, CO, and NO, respectively. Each panel shows the absorption spectra recorded in the stopped-flow of deoxy-Dos_H prior to (solid line) and after (dashed line) mixing with ligand-saturated buffer. Each inset shows the corresponding kinetic trace and fit for the conversion of deoxy-Dos_H to the liganded species.

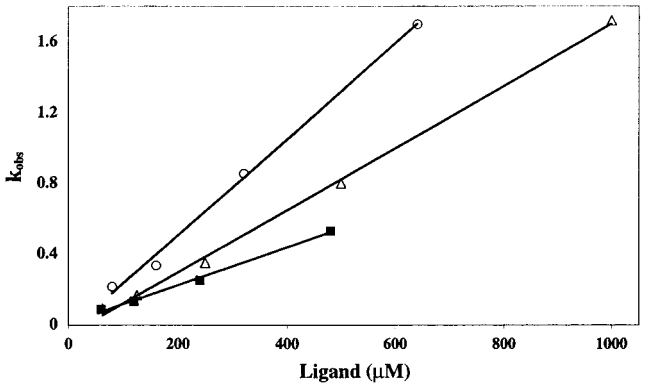

FIGURE 5: Observed rates of association of deoxy-Dos_H with ligands at 25 °C, pH 7.0. Rates of O_2 (○), CO (■), and NO (△) association are shown.

trace of the association of a ligand with deoxy-Dos_H along with absorption spectra verifying in the stopped-flow spectrometer the species present before and after the deoxy-Dos_H was mixed with a ligand. For each ligand, the observed time courses were dominated by a single-exponential process and were linearly dependent on the ligand concentration up to the highest concentrations achievable. The association rate constants were unusually small: 2.6×10^{-3} μM^{-1} s^{-1} for O_2, 1.1×10^{-3} μM^{-1} s^{-1} for CO, and 1.8×10^{-3} μM^{-1} s^{-1} for NO. To our knowledge, the latter represents the smallest association rate constant for NO binding reported for an O_2-binding heme protein. For SW Mb, NO binding is over 1000-fold faster (*30*). Direct measurements of equilibrium O_2 or CO binding revealed that for both ligands binding is noncooperative (Hill constant $n \approx 1$). The equilibrium dissociation constants were similar, $K_d(O_2) = 13$ μM and $K_d(CO) = 10$ μM, implying an unusually strong discrimination of Dos against CO [partition constant $M = K_d(O_2)/K_d(CO) = 1.3$]. Barley Hb has an equally low partition constant (*25*). The rate of O_2 dissociation from Dos_H was very slow (0.034 s^{-1}) and only 3-fold faster than the rate of CO dissociation.

DISCUSSION

Subgroups within the PAS Superfamily. Given that the heme-binding PAS domains of FixL and Dos are highly similar in sequence and function, we propose that these proteins belong to an O_2-sensing subgroup within the PAS

superfamily. An additional member of this subgroup is likely to be the PDEA1 protein that is proposed to regulate the buoyancy of *A. xylinum* on the basis of the available O_2 (*20*). Despite the limited biochemical and structural information available so far on PAS domains, it is already apparent that, within this large family, subgroups with similar sensing functions will share specific structural features. For example, there is a subgroup of blue-light sensors, exemplified by PYP from *Ectothiorhodospira halophila* and the recently discovered Ppr from *Rhodospirillum centenum* (*10, 31*). These proteins share >60% homology, including the site of chromophore attachment. The greater than 50% homology found for those subgroups is a far closer relationship than that found for randomly chosen PAS domains (<15% homology). The flavin-binding proteins nph1 of *Arabidopsis thaliana*, NifL of *Azotobacter vinelandii*, and Aer of *E. coli* indicate yet another subgroup of very homologous PAS domains (*11, 12, 32*). Comparison of the structures of PYP and FixL suggests that the core EFG region, which includes the key contacts with cofactor, is the most distinctive region of a PAS domain (Figure 1) (*8*). We propose that better than 50% homology within this EFG core will likely indicate a similar sensing function, though not necessarily a similar coupling partner or signal-transduction mechanism. For example, the redox-sensing PAS in NifL is coupled to an ATP-binding domain, whereas the related PAS in Aer is coupled to a methyl-accepting chemotaxis receptor (*11, 12*). Analogously, the O_2-sensing PAS in FixL is coupled to a histidine kinase, whereas the similar PAS in Dos or PDEA1 is coupled to a phosphodiesterase (Figure 2).

A Direct O_2 Sensor in E. coli. The related PAS domains in FixL and Dos together with the strong discrimination of Dos_H against other ligands of ferrous heme [e.g., $M = K_d(O_2)/K_d(CO) \approx 1$] suggest strongly that Dos functions in *E. coli* as a direct sensor of O_2. Thus far, studies of *E. coli* adaptation to hypoxia have focused on the FNR protein (regulator of fumarate and nitrate reduction) and the ArcA/ArcB (aerobic respiration control) two-component regulatory system (*33*−*38*). However, these two systems account neither for all the hypoxic gene expression in *E. coli* nor for a possible requirement for direct sensing of O_2 by some processes. Sawers and colleagues have noted that about half of the anaerobically induced genes in *E. coli* do not require functional FNR for their induction (i.e., occur in *fnr*$^-$ strains) (*35*). Thus, FNR does not regulate all anaerobically induced *E. coli* genes, although it does induce the expression of genes that permit anaerobically growing *E. coli* to transfer electrons to alternative terminal acceptors such as nitrate or fumarate (*36*). Mössbauer spectroscopy studies have indicated that in vivo O_2 can catalyze a reversible conversion of the FNR protein from an active species having $[4Fe-4S]^{2+}$ clusters to an inactive one having $[2Fe-2S]^{2+}$ clusters (*33*). Much remains to be learned about the mechanism of this reaction, and it is unlikely to involve direct and reversible binding of O_2. In vitro, FNR is denatured by even moderate levels of O_2; conversion of the $[4Fe-4S]^{2+}$ to a $[2Fe-2S]^{2+}$ cluster is only achieved at substoichiometric levels of O_2 and only reversed by strong reducing agents. In contrast to the positive regulatory role of FNR, the ArcA/ArcB system is thought to be primarily responsible for repressing genes involved in aerobic respiration (*37, 38*). It has not been possible to show that the ArcA/ArcB system senses O_2 directly, and it probably does not. Thus, the Dos protein, as demonstrated by its homology to PAS-domain proteins and its ligand-binding parameters, is the first *E. coli* sensor shown to bind O_2 directly and reversibly. While Dos may also be involved in respiratory control, it is more likely that it regulates a cellular process that requires direct sensing of O_2, rather than indirect sensing by way of the redox potential, the proton motive force, or ATP availability. A direct O_2 sensor would be important for processes that are specifically poisoned by O_2, such as rhizobial nitrogen fixation. Alternatively, an O_2-monitoring system may regulate the expression of genes that are important for reactions that directly consume O_2.

A Novel Variation on Heme Proteins. The most familiar heme proteins with endogenous sixth ligands, such as cytochrome *c*, do not bind ligands stably or reversibly. Several heme proteins are now known to have an endogenous sixth ligand that readily relinquishes its place on the heme iron to exogenous ligands, forming stable oxy-heme or carbonmonoxy-heme complexes (*24*−*27*). This variation is likely to be quite widespread and is manifested in heme proteins that differ greatly in their origins and structural folds. Among these are the globin fold of nonsymbiotic plant Hbs, the PAS fold of *E. coli* Dos, and possibly an additional fold for *Rhodospirullum rubrum* CooA. The ligand-binding parameters of these proteins span a considerable range. For example, the O_2 affinity of Dos is relatively low (15-fold lower than that of SW Mb), but the O_2 affinity of rice Hb1 is among the highest known for heme proteins (1500-fold higher than that of SW Mb) (Table 1) (*39*). The rates of ligand association to displaceable-residue heme proteins have a similarly impressive range. For example, O_2 association rates range from fast (68 μM^{-1} s^{-1} for rice Hb1) to extremely slow (0.0026 μM^{-1} s^{-1} for Dos_H), a range of 4 orders of magnitude. Given this variety in their other properties, it is remarkable that all the O_2 dissociation rates so far reported are near the exceptionally low value of 0.03 s^{-1}. Mutagenesis and resonance Raman data indicate that rice and barley Hb have bis-imidazole coordination (*26*, *29*). For Dos it is unlikely that a histidine could serve as the endogenous sixth ligand. Modeling of the structure of Dos_H based on that of the FixL heme-binding PAS domain leads us to predict that of all the residues on the distal side capable of coordinating to heme iron only Met 95 could approach the heme iron close enough to serve as the endogenous ligand.

Cost of Displacing an Endogenous Ligand. In the heme-proteins discussed above the displaceable residue exists in an equilibrium between the pentacoordinate (P) and hexacoordinate (H) states. Thus

$$H \underset{k_{PH}}{\overset{k_{HP}}{\rightleftharpoons}} P, \quad K_{HP} = [P]/[H]$$

where k_{HP} and k_{PH} are the rates of the H → P and P → H transitions, respectively. If K_{HP} at room temperature is 1.0, then both states are equivalent in energy, and the HP equilibrium has no effect on the affinity. If K_{HP} is reduced to 0.001, then the coordination bond to the displaceable residue is strengthened to ∼4 kcal. The affinities for all ligands become 1000 times lower because the coordination bond must be broken before an exogenous ligand can bind and constitutes an unfavorable contribution to the free energy for binding exogenous ligands. To achieve high ligand

affinities, the energy cost of dissociating the displaceable residue from the heme iron must be compensated by favorable interactions. These could be either "extra" interactions stabilizing the exogenous ligand or interactions stabilizing the displaceable residue in its displaced position, or both. Stabilization of bound ligands by distal side chains such as His (E7) in SW Mb, Gln (E7) and Phe (B10) in elephant Mb, and Gln (E7) and Tyr (B10) in *Ascaris* Hb is well characterized (*30*, *40*, *41*). This kind of stabilization is ligand specific; for example, polar interactions stabilize O_2 but not CO (*30*). An alternative to distal residues interacting favorably with the exogenous ligand is for those residues to interact favorably with the displaceable residue when it dissociates. In contrast to the first approach, an alternative "binding site" for the displaceable residue would raise the affinity equally for all exogenous ligands. For the protein to be predominantly hexacoordinate in the absence of exogenous ligands, the alternative site for the displaceable residue must be inaccessible whenever no exogenous ligand is bound; otherwise this site would compete for the displaceable residue and shift the equilibrium toward the pentacoordinate form. Therefore, there must be a protein conformational change associated with ligand binding simultaneous with the displacement of the displaceable residue.

Possible Influences of the Hexa ↔ Penta Equilibrium on the Observed Kinetics of Exogenous-Ligand Binding. For displaceable-residue heme proteins, the observed kinetics of exogenous-ligand binding really represent the competitive displacement of one ligand by another:

$$\mathrm{H} \underset{k_{\mathrm{PH}}}{\overset{k_{\mathrm{HP}}}{\rightleftharpoons}} \mathrm{P} + \mathrm{L} \underset{k}{\overset{k'}{\rightleftharpoons}} \mathrm{PL}$$

where k' is the association rate constant for binding of the exogenous ligand L to the pentacoordinate species and k is the dissociation rate of the exogenous ligand. Since only the pentacoordinate form of the heme iron can bind ligands, the observed rate of reaction with ligands is $fk'[O_2] + k$, where f is the fraction of protein in the pentacoordinate state. If the two equilibria have comparable rates, then f varies throughout the reaction and the kinetics are complex. Fortunately, there are two cases in which f remains essentially constant throughout the reaction, and one or the other case appears to apply to all displaceable-residue heme proteins studied so far.

If the rate at which the P ↔ H equilibrium recovers from a perturbation is much faster than that of the ligand-binding equilibrium, i.e., $k_{\mathrm{HP}} + k_{\mathrm{PH}} \gg k'[\mathrm{L}] + k$, then P and H are in equilibrium at all times and $f = K_{\mathrm{HP}}/(K_{\mathrm{HP}} + 1)$. The overall rate of reaction is then $k_{\mathrm{obs}} = (K_{\mathrm{HP}}/(K_{\mathrm{HP}} + 1))k'[\mathrm{L}] + k$. The observed rates are linear with respect to concentration, but slowed by a factor that increases with the fraction of protein in the H state. These are the kinetics seen for Dos and for rice Hb (Figure 5) (*26*).

Not all displaceable-residue heme proteins have such simple kinetics. If the rate of binding of ligands to the P state of the protein is not slower than the equilibration between H and P states, then the fraction of protein in the P state will be less than its equilibrium value. However, if the fraction of unsaturated protein in the P state is negligible, it is possible to derive the following equation for k_{obs}: $k_{\mathrm{obs}} = (k_{\mathrm{HP}}k'[\mathrm{L}] + kk_{\mathrm{PH}})/(k_{\mathrm{PH}} + k'[\mathrm{L}])$. This is the case for the barley Hb (*25*). At high ligand concentrations, the observed rates for all ligands will approach a limiting value of k_{HP} (the observed rate is simply the rate of generation of the P state, which reacts as rapidly as it is formed). For barley Hb, the deduced value of k_{HP} is $\sim 40\ s^{-1}$. This rate is slow compared to the observed ligand association rates for barley Hb, but much faster than any rates observable with Dos_H. So we cannot at this time say how k_{HP} of Dos compares with that of other displaceable residue heme proteins.

A Common Rate-Limiting Step for Ligand Association to Dos. CO, O_2, and NO differ greatly in their intrinsic reactivity toward heme iron, yet in Dos they have essentially the same rate of association. Since the observed rates are linearly dependent on ligand concentration, the common rate-limiting step cannot be a process that is independent of ligands, such as the H to P transition.

Sensing Mechanism. For the rhizobial O_2-sensing FixLs, a change of the heme iron from high to low spin on binding of O_2 has been proposed as the key initial step in the sensing mechanism (*42*). A spin-state change is also thought to trigger the well-known allosteric changes in the T → R transition of mammalian hemoglobins (*43*). The heme iron in Dos_H appears to be hexacoordinate and low spin whether or not an exogenous ligand is present. Therefore, another triggering mechanism must operate in this protein. We propose that a key initial step in O_2 sensing by Dos is the movement of the displaceable residue from the sixth coordination position of the heme iron to an alternative site after binding of O_2. The likely displaceable residue in Dos, Met 95 (Ile 218 in BjFixL), is expected to occur within a region corresponding to the regulatory FG loop in FixL (Figure 1) (*8*). Displacement of Met 95 by O_2 could result in a movement of the FG loop in Dos comparable to that which occurs in FixL.

Why a Displaceable Residue? Displacement of a protein residue is a very direct and straightforward way for ligand binding to induce a conformational change. The application of this phenomenon to sensing in Dos has already been discussed. Another exciting possibility is that displaceable-residue heme proteins could exploit the competition between endogenous and exogenous ligands to achieve a variable-affinity O_2 sensor or carrier. Relatively small differences in the arrangement of a heme pocket could have a large impact on the ability of the displaceable residue to compete with exogenous ligands. For example, the distal His (E7) in mammalian Hbs is never coordinated to the heme iron, even though it is close enough to form strong hydrogen bonds to bound ligands, yet the His (E7) in cereal Hbs coordinates iron readily. The value of K_{HP} should be extremely sensitive to even minute changes in the Fe−His distance and orientation, and especially to alternative interactions that stabilize the His (E7) while in the P conformation. Hill and colleagues have proposed that the cereal Hbs might function as scavengers of O_2 for delivery to terminal oxidases during periods of hypoxia (*44*). If so, the affinities of the Hbs would need to be modified at the mitochondria. A conformational change, on docking of a displaceable-residue heme protein to a target, that favors the H state could convert a protein with very slow dissociation into one with rapid dissociation. Such a heme protein could scavenge vanishingly low concentrations of O_2 and deliver the O_2 to a location within a cell as precise as a single molecule.

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