Accelerated Publications

Phosphodiesterase A1, a Regulator of Cellulose Synthesis in *Acetobacter xylinum*, Is a Heme-Based Sensor†

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ABSTRACT: The phosphodiesterase A1 protein of *Acetobacter xylinum*, *Ax*PDEA1, is a key regulator of bacterial cellulose synthesis. This phosphodiesterase linearizes cyclic bis(3′→5′)diguanylic acid, an allosteric activator of the bacterial cellulose synthase, to the ineffectual pGpG. Here we show that *Ax*PDEA1 contains heme and is regulated by reversible binding of O_2 to the heme. Apo-*Ax*PDEA1 has less than 2% of the phosphodiesterase activity of holo-*Ax*PDEA1, and reconstitution with hemin restores full activity. O_2 regulation is due to deoxyheme being a better activator than oxyheme. *Ax*PDEA1 is homologous to the *Escherichia coli* direct oxygen sensor protein, *Ec*Dos, over its entire length and is homologous to the FixL histidine kinases over only a heme-binding PAS domain. The properties of the heme-binding domain of *Ax*PDEA1 are significantly different from those of other O_2-responsive heme-based sensors. The rate of *Ax*PDEA1 autoxidation (half-life > 12 h) is the slowest observed so far for this type of heme protein fold. The O_2 affinity of *Ax*PDEA1 ($K_d \sim 10\ \mu M$) is comparable to that of *Ec*Dos, but the rate constants for O_2 association ($k_{on} = 6.6\ \mu M^{-1}\ s^{-1}$) and dissociation ($k_{off} = 77\ s^{-1}$) are 2000 times higher. Our results illustrate the versality of signal transduction mechanisms for the heme-PAS class of O_2 sensors and provide the first example of O_2 regulation of a second messenger.

A single *Acetobacter xylinum* cell can polymerize to cellulose up to 200 000 glucose molecules per second (*1*). Activation of the native cellulose synthase of this bacterium requires the dinucleotide c-di-GMP[1] as an allosteric effector (*2*, *3*). The cellular level of c-di-GMP depends on a balance between the activity of a diguanylate cyclase and the opposing action of the phosphodiesterase *Ax*PDEA1 (reviewed in ref *3*). The diguanylate cyclase synthesizes c-di-GMP from 2 molecular equiv of GTP, whereas the *Ax*PDEA1 hydrolyzes c-di-GMP to linear pGpG. The highly pure and crystalline *A. xylinum* cellulose has found numerous industrial applications, including use in speaker diaphragms, food thickeners, paper additives, clothing, wound-care products, and, more recently, artificial arteries (*3*−*6*). For the bacteria, the thick cellulosic mats they generate are thought to provide a specialized protective environment.

Although the phosphodiesterase activity of *Ax*PDEA1 has been extensively characterized, the possible heme protein nature of *Ax*PDEA1 was not appreciated until the corresponding gene was cloned and sequenced (*7*). *Ax*PDEA1 is highly homologous over its entire length to the *Ec*Dos protein of *Escherichia coli*, including an N-terminal domain of *Ec*Dos that binds heme (*7*, *8*). The O_2-sensing FixL proteins of *Rhizobia* have the homologous heme-binding domain, but FixLs have a C-terminal histidine kinase instead of a phosphodiesterase (*9*−*12*). Conversely, there exists a large family of bacterial proteins that share the phosphodiesterase domain of *Ax*PDEA1 and *Ec*Dos, though not their N-terminal domains (*7*).

The N-terminal 140 amino acids of *Ax*PDEA1 contain a PAS motif. The PAS sequence motif occurs in more than 800 proteins in *Bacteria*, *Archaea*, and *Eukarya*, where it is

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[1] Abbreviations: *Ax*PDEA1, *Acetobacter xylinum* phosphodiesterase A1; c-di-GMP, cyclic bis(3′→5′)diguanylic acid; *Ec*Dos, *Escherichia coli* direct oxygen sensor protein; PAS, sensory domain named after the eukaryotic proteins *p*eriod, *a*rnt, and *s*imple-minded.

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frequently correlated with signal transduction (*13*). PAS domains typically share less than 15% sequence identity, and they are often coupled to enzymatic or DNA-binding domains. Oxygen, light, voltage, redox potential, and aromatic hydrocarbons are among the signals that PAS domains detect. Crystal structures are known for the widely divergent PAS domains of the FixL O_2 sensor, the human ether-a-go-go-related gene-encoded (HERG) voltage sensor, and the photoactive yellow protein (PYP) light sensor (*14*−*17*). Their strikingly similar architectures imply that all PAS domains share a common $\alpha\beta$-fold. Subgroups with similar sensing functions exist that share specialized structural features and >30% sequence identity (*8*). For example, there is redox-sensing subgroup that binds flavin and a light-sensing subgroup that binds *p*-hydroxycinnamate (*17*−*21*). We have established a heme-binding subgroup of PAS domains, represented in three evolutionarily distant eubacterial genera and one *Archaeon* (*8*).

The heme-binding PAS proteins are all "heme-based sensors". This term was coined in 1994 by Gilles-Gonzalez and colleagues to describe a functional class of heme proteins in which heme is used for chemosensing rather than electron or oxygen transfer (*10*). The heme-based sensors have proved to be a very diverse group of heme proteins that bind heme in at least three different structural folds and employ a variety of enzymes for signal transduction. They respond to a variety of heme ligands and regulate diverse activities. Some previously described heme-based sensors are the soluble guanylyl cyclase (sGC), FixL, CooA, and HemAT proteins (*9*, *22*−*24*). Binding of NO to sGC activates production of cGMP; in mammals the best understood response is vasodilation (*25*). Binding of O_2 to FixL inhibits an autophosphorylation reaction that normally leads to phosphorylation of the transcription factor FixJ (*9*, *11*). Phospho-FixJ induces expression of the nitrogen fixation genes in *Rhizobia* (*26*, *27*). Association of CO to CooA enables this protein to bind DNA (*23*). The DNA-bound CooA induces transcription of the operons for oxidation of CO to CO_2 by the photosynthetic bacterium *Rhodospirullum rubrum* (*28*). Binding of O_2 to HemAT regulates chemotactic activity (*24*). HemAT is likely to be a methyl-accepting chemotaxis protein that controls the direction of *Halobacterium salinarum* and *Bacillus subtilis* movement in an O_2 gradient. The latest activity to be found coupled to heme is the c-di-GMP phosphodiesterase of *A. xylinum* PDEA1, presented here.

Along with the great functional diversity of heme-based sensors, the heme-binding domains show a much greater structural diversity than the heme proteins that function to transfer O_2, atomic oxygen, or electrons. Of the known heme-based sensors, only HemAT is likely to have a myoglobin fold (*24*). CooA binds heme in a structure similar to the cAMP-binding region of the catabolite gene activator protein (CAP) (*29*). The sequence of guanylyl cyclase suggests that its structure will be unlike other known heme proteins. So far, the most common structural motif for binding of heme by heme-based sensors is the PAS fold (*14*, *15*).

Here we show that *Ax*PDEA1 is a heme-based sensor belonging to the PAS superfamily. We report the ligand-binding parameters of *Ax*PDEA1 and present for the first time the regulation of the level of an allosteric effector by an O_2-sensing protein.

MATERIALS AND METHODS

Genetic Manipulations. Oligonucleotide primers were designed for amplifying the full-length *A. xylinum pdeA1* and the heme-binding-domain-encoding *pdeA1H* fragment (codons 1−141), based on the gene sequence (*7*). The source of template for the PCR amplifications was plasmid pUC57 (R. Mayer and M. Benziman, unpublished results). The PCR primers introduced a *Nde*I site overlapping a start codon at the 5′ end of each amplified fragment and a *Hin*dIII site overlapping a stop codon at the 3′ end. The *Nde*I−*Hin*dIII treated PCR products were ligated into an expression vector, with the *pdeA1* gene sequences optimally oriented for expression from a *tac* promoter. The resulting plasmids, pAX31 and pAX61, contain *AxpdeA1* and *AxpdeA1H*, respectively. Both plasmids confer ampicillin resistance. All clones for subsequent experiments were verified to be without polymerization error (*30*).

Gene Expression and Protein Purification. Protein manipulations were at ≤4 °C, and extended storage of cells or proteins was at −70 °C. Column matrices were from Pharmacia. *AxpdeA1H* expression was induced by adding 500 μM isopropyl β-D-thiogalactopyranoside to exponentially growing TG1(pAX61). After 3 h of induction at 37 °C, the cultures were cooled on ice, and the cells were harvested. Lysis of the cells was by sonication in 25 mM Tris-HCl, pH 8.5, 125 mM NaCl, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride. *Ax*PDEA1H was precipitated from the cleared lysate with 1.6−2.8 M ammonium sulfate, pH 7.5, and 1 mM EDTA. Further purification was by gel filtration (Superdex 75 equilibrated with 25 mM Tris-HCl, pH 8.0, and 50 mM NaCl) and stepwise elution from an anion-exchange matrix (DEAE-Sephacel and 50−125 mM NaCl in 25 mM Tris-HCl, pH 8.0). Expression of *AxpdeA1* was done similarly, except that induction of the TG1(pAX31) cells required 5 h. Following sonication of the cells, *Ax*PDEA1 was recovered from the cleared lysate as an insoluble pellet. Solubilization and purification of the *Ax*PDEA1 were as described (*7*). The purified protein, made up mostly of apo-*Ax*PDEA1, was titrated with hemin (*31*). Protein concentrations were determined by the Bradford assay, with bovine serum albumin as the standard (*32*).

Absorption Spectra. Unless otherwise noted, all the absorption spectra were measured for purified protein in 25 mM Tris-HCl, pH 8.5 at 25 °C, in a stoppered quartz cuvette. Deoxy derivatives were prepared by adding dithiothreitol (10 mM) to an anaerobic solution of protein inside of a glovebox. The dithiothreitol was removed with a 5 mL Sephadex G25 desalting column pre-equilibrated with deoxygenated 25 mM Tris-HCl, pH 8.5, and the spectra of the deoxyheme proteins were recorded. Oxy-*Ax*PDEA1 was prepared by equilibrating the deoxyheme protein with 1 atm of O_2 at 4 °C. Carbonmonoxy-*Ax*PDEA1 was prepared by equilibrating the deoxyheme protein with 1 atm of CO at 4 °C. For calculations of the extinction coefficients, the determinations of heme content were based on the amount of pyridine hemochromogen, taking hemin ($\epsilon_{390} = 50$ mM^{-1} cm^{-1} in 2% sodium borate, pH 9.23) as the standard (*33*).

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 five ligand concentrations. All the linear plots for determination of the rate constants had *r*-squared values of 0.99 or greater. Protein and gas solutions for the ligand-binding reactions were in 25 mM Tris-HCl, pH 8.5 at 25 °C. To measure the association rates, deoxy-*Ax*PDEA1 was mixed with 30−150 μM O_2 or 30−480 μM CO. Ligand association was followed by the change in absorbance at 438 nm for O_2 or 424 nm for CO. Dissociation of O_2 was followed by the change in absorbance at 578 nm after mixing oxy-*Ax*PDEA1 with 5 mM ferricyanide, which oxidizes deoxy-*Ax*PDEA1 essentially instantaneously. Dissociation of CO was followed by the change in absorbance at 424 nm after mixing carbonmoxy-*Ax*PDEA1 with a saturated solution of NO.

Phosphodiesterase Assays. These assays were adapted from methods previously described (*2*, *7*). Anaerobic reactions were in a glovebox with an N_2 atmosphere; oxic reactions were in air. Prior to the reactions, the *Ax*PDEA1 was verified to be homogeneously oxy or deoxy based on the absorption spectra. The reaction mixtures contained 0.5 μM *Ax*PDEA1 in 100 μL of 50 mM Tris-HCl, pH 8.5, 0.50 mM $MnCl_2$, 10 mM dithiothreitol, and 12 μM [^{32}P]-c-di-GMP (2.4 Ci/mmol) at 23 °C. All reactions were begun with the radiolabeled nucleotide. At 15 s time intervals, aliquots were withdrawn and mixed with one-third volume of 4 mM EDTA. The products of the reactions (~3000 cpm from each reaction) were spotted onto Baker-Flex polyethyleneimine−cellulose plates (J. T. Baker, Phillipsburg, NJ) that had been prerun in water and air-dried. For plates developed in 1.5 M KH_2PO_4, pH 3.65, c-di-GMP migrates with an R_f of 0.54 and linear pGpG with an R_f of 0.69. After development, the plates were air-dried, and the radioactivity in spots containing the relevant nucleotides was quantitated with a phosphoimager (Molecular Dynamics). Reaction mixtures lacking *Ax*PDEA1 and spots having known quantities of radiolabel provided the controls for the quantitations.

RESULTS

Domain Organization. Figure 1 highlights the modular nature of *Ax*PDEA1. Regions highly homologous (~40% identical) to the C-terminal phosphodiesterase domain occur in more than 100 different bacterial proteins. Organisms having this type of phosphodiesterase, e.g., the pathogen *Vibrio cholerae* and the nitrogen-fixing bacterium *Azorhizobium caulinodans*, differ greatly in their niches and lifestyles. The phosphodiesterase can occur independently or with other identifiable domains. The N-terminal region of *Ax*PDEA1 contains a heme-PAS domain, a more closely related subgroup (~30% identity) within the PAS superfamily (Figure 1B). So far, a heme-PAS has been found in association with three PDEA and six histidine kinase domains. In addition to the bacterial heme-PAS proteins that have been reported, there is at least one proven archaeal heme-PAS and several probable eukaryal heme-PAS proteins (A. L. Chang and M.-A. Gilles-Gonzalez, unpublished results). The best understood heme-PAS proteins are the FixL O_2 sensors of *Rhizobia*. FixL contains a C-terminal histidine kinase activity instead of a phosphodiesterase. All three types of domains, the histidine kinase, PAS, and PDEA, are

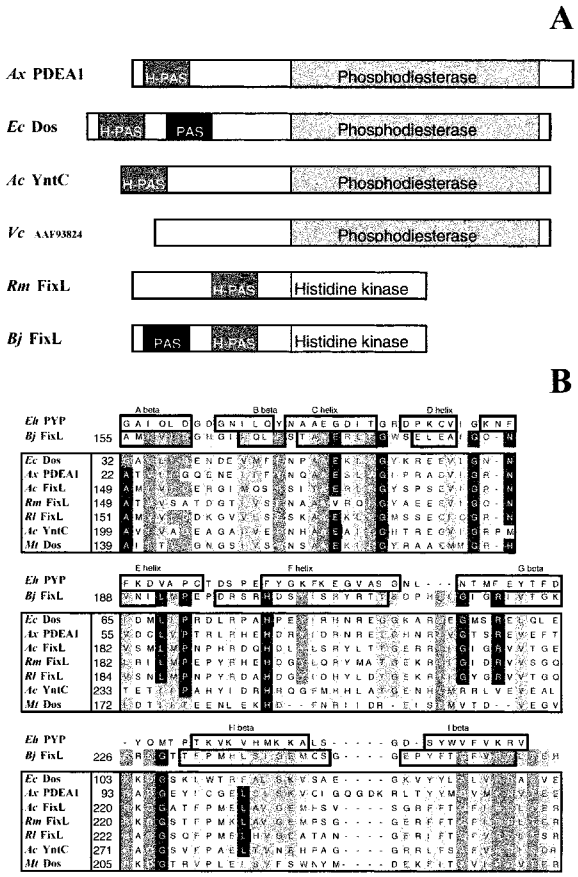

FIGURE 1: Modular organization of O_2-sensing phosphodiesterases and kinases. Part A shows a schematic organization of the enzymes. The heme-PAS domains (H-PAS) of *B. japonicum* FixL, *Ec*Dos, and *Ax*PDEA1 are ≥30% identical in sequence; unrelated PAS domains (PAS) are <15% identical when compared to each other or to heme-PAS domains; the phosphodiesterases are 40% identical over the enzymatic regions shown, as are the histidine kinases. Part B shows the heme-PAS sequence alignments for *Ec*Dos; *Ax*PDEA1; FixLs from *A. caulinodans*, *Rhizobium meliloti*, *and Rhizobium leguminosarum*; *A. caulinodan*s YntC; and *Methanobacterium thermoautotrophicum* Dos. A secondary structure alignment for the *Ectothiorhodospira halophila* photoactive yellow protein (*Eh*PYP) and the heme-PAS of *B. japonicum* FixL is provided at the top of part B.

indispensable for response to a great variety of environmental signals.

Absorption Spectra. Except for the deoxy form, the optical spectra of *Ax*PDEA1 derivatives closely match those of *Ec*Dos (Figure 2) (*8*). Deoxy-*Ax*PDEA1 has a strong Soret absorption band at 432 nm (112 mM^{-1} cm^{-1}) and a broader but less intense band between 550 and 573 nm (~13 mM^{-1} cm^{-1}). This is typical of heme proteins, such as FixL or sperm whale myoglobin, that have predominantly high-spin and pentacoordinate heme iron in their deoxy state (*9*, *10*, *34*). In contrast, ferrous *Ec*Dos without exogenous ligand has well-resolved α and β absorption bands (563 and 532 nm, respectively) that are characteristic of "deoxy" heme proteins having low-spin hexacoordinate heme iron (*8*). Nevertheless, for liganded states *Ax*PDEA1 compares best with *Ec*Dos. The oxy- and carbonmonoxy-*Ax*PDEA1 absorption bands occur within 2 nm of the corresponding peaks in *Ec*Dos and have similar extinctions. Compared to *Bradyrhizobium japonicum* FixL, the α-bands of oxy- and carbonmonoxy-*Ax*PDEA1 are red shifted by 17 and 10 nm, respectively (*10*).

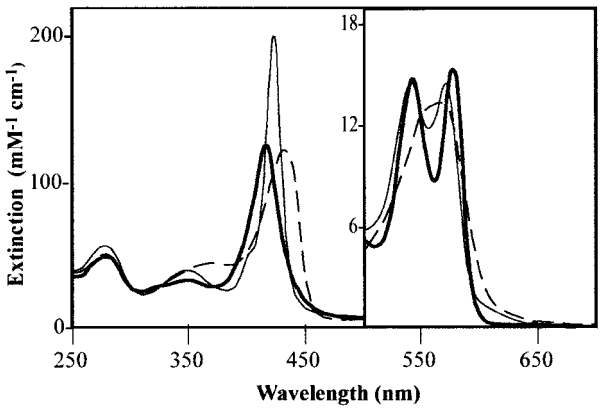

FIGURE 2: Absorption spectra of *Ax*PDEA1. Spectra are shown for the deoxy (broken line), oxy (thick line), and carbonmonoxy (thin line) derivatives of the *Ax*PDEA1 heme-binding domain.

Table 1: O_2 and CO Binding by Selected Heme Proteins[a]

	O_2			CO		
protein	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)
*Ax*PDEA1H[b]	6.6	77	12	0.21	0.058	0.28
*Ec*DosH[c]	0.0026	0.034	13	0.0011	0.011	10
*Bj*FixLH[d]	0.14	20	140	0.0050	0.045	9.0
SW Mb[e]	14	12	0.86	0.51	0.019	0.037

[a] Heme-binding domains of *A. xylinum* PDEA1, *E. coli* Dos, and *B. japonicum* FixL are compared to sperm whale myoglobin (SW Mb). [b] pH 8.5 and 25 °C; K_d values were calculated from k_{on} and k_{off}. [c] From Delgado-Nixon et al. (*8*). [d] From Gilles-Gonzalez et al. (*10*). [e] From Quillin et al. (*52*).

O_2 and CO Binding. The data in Table 1 highlight the broad range in the ligand-binding parameters observed for the heme-PAS fold. The association rate constants span at least 3 orders of magnitude for binding of O_2 and 2 orders of magnitude for binding of CO. Although *Ax*PDEA1 and *Ec*Dos both bound O_2 noncooperatively and with essentially the same affinity, for *Ax*PDEA1 the association and dissociation rate constants were 2000-fold higher. Binding of CO to *Ax*PDEA1 was also different from binding to FixL or *Ec*Dos. Though the differences were not as dramatic as for binding of O_2, the association and dissociation rate constants for binding of CO were still higher for *Ax*PDEA1 than for *Ec*Dos. In contrast to *Ec*Dos, which bound O_2, CO, and NO within a narrow range of association ($1-3$ nM^{-1} s^{-1}) and dissociation rate constants ($0.01-0.03$ s^{-1}), *Ax*PDEA1 bound O_2 30 times faster than CO. Nevertheless, because of a 1300-fold lower off-rate constant of CO, *Ax*PDEA1 had much greater affinity for CO than for O_2. This is analogous to the behavior of myglobins, for which the much slower dissociation of CO is largely responsible for tighter binding of CO compared to O_2.

Influences of Heme and O_2. *E. coli* cells overproducing the isolated heme-binding domain of *Ax*PDEA1 were noticeably red, and the purified protein contained a 1:1 ratio of iron protoporphyrin IX to protein. Preparations of the full-length *Ax*PDEA1 had less heme but could be fully reconstituted with 1 molar equiv of hemin to apoprotein. Once reconstituted, the holoprotein showed no tendency to lose heme and had an activity [~86 nmol of pGpG min^{-1} (mg of protein)$^{-1}$] more than 50-fold that of apoprotein (Figure 3A). Binding of O_2 to the ferrous heme reduced the enzymatic activity to about one-third the value measured for deoxy-*Ax*PDEA1 (Figure 3B).

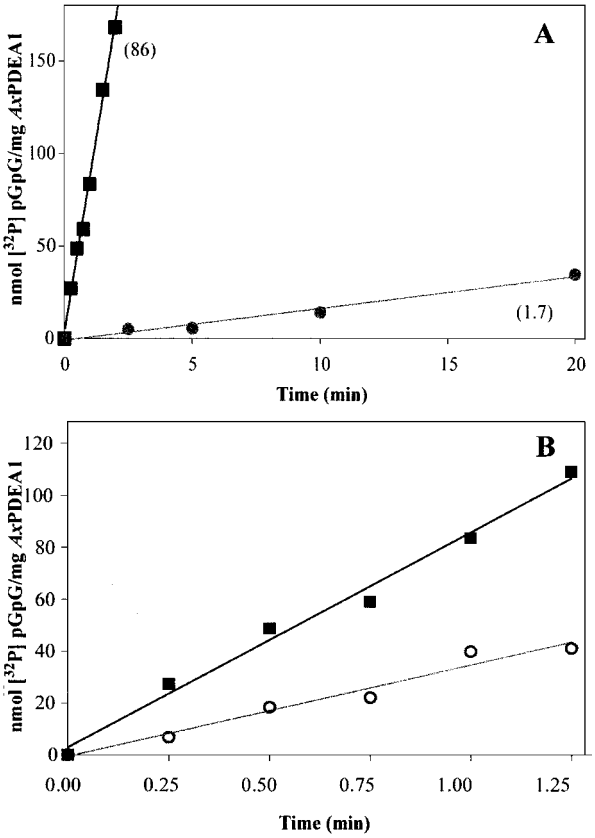

FIGURE 3: Influence of heme and O_2 on the c-di-GMP phosphodiesterase activity of *Ax*PDEA1. Part A shows the initial rates of anaerobic reactions for apo-*Ax*PDEA1 (closed circles) and deoxy-*Ax*PDEA1 (squares). Part B shows the initial rates for the reactions of oxy-*Ax*PDEA1 (open circles) or deoxy-*Ax*PDEA1 (squares). The reaction mixtures contained 0.5 μM *Ax*PDEA1 in 100 μL of 50 mM Tris-HCl, pH 8.5, 0.50 mM $MnCl_2$, 10 mM dithiothreitol, and 12 μM [^{32}P]-c-di-GMP at 23 °C. Each experiment was repeated three times. For each measurement, the deviations from the average were 15% or less.

*Ax*PDEA1 was remarkably stable against electron transfer to O_2, as measured by its autoxidation in air. Oxy-*Ax*PDEA1 was only 30% oxidized to the ferric form after 8 h in air-saturated Tris, pH 8, buffer at 23 °C. Even after 24 h, much of the protein was still ferrous. Aggregation of some of the *Ax*PDEA1 during extended incubation at 23 °C prevented an accurate measurement of the k_{ox}, but the half-life for the oxidation reaction was >12 h. This represents the slowest rate of autoxidation so far measured for a heme-PAS.

DISCUSSION

A Heme-PAS for Phosphodiesterase Control. The latest activity to be found coupled to heme is the c-di-GMP phosphodiesterase of *Ax*PDEA1 (Figures 1−3). Two other proteins with a closely related enzymatic domain (~40% identical and 60% homologous), the *Ec*Dos protein of *E. coli* and the YntC protein of *A. caulinodans*, also contain a heme-binding PAS domain (*8*; unpublished results). Surprisingly, the heme-PAS domains that control a phosphodiesterase are not significantly more homologous to each other than to the heme-PAS domains that control histidine kinases (Figure 1B) nor are they similar in their ligand-binding parameters (Table 1). Coupling of a heme-PAS to a phosphodiesterase may be

a more widespread method for O_2 sensing than coupling to a histidine kinase. The latter scheme is so far observed in targeted control of nitrogen fixation gene expression by the FixL proteins of *Rhizobia*. In contrast, the three organisms employing a phosphodiesterase for O_2 sensing have radically different habitats. *A. xylinum* is notorious as a contaminant of wine and beer, but its natural habitat is a biofilm of cellulose fibrils that it constructs on the surfaces of fruits. In this organism, *Ax*PDEA1 clearly regulates the synthesis of cellulose (*3*). *A. caulinodans* is a soil bacterium that can form nitrogen-fixing nodules on the tropical leguminous shrub *Sesbania rostrata* (*35*). *A. caulinodans* has at least one other O_2 sensor, a FixL (*36*). The role of its *Ac*YntC protein, an O_2-sensing phosphodiesterase, is not certain. As discussed below, *Ac*YntC may also control cellulose production. *E. coli* is primarily an enteric bacterium. This organism has at least two O_2 sensors (*8*, *37*). The physiological function of *Ec*Dos is not yet known, but this is certain to be a new role for heme-PAS, since *E. coli* is neither a nitrogen fixer nor a cellulose producer. Second-messenger control would provide for rapid allosteric regulation of pre-existing proteins as well as global regulation of cellular processes by O_2.

A Microaerobic Sensor with Myoglobin-like Kinetics. Although the kinetics of ligand binding have been reported for only four heme-PAS proteins, it is already apparent that they are at least as diverse in their properties as hemoglobins (Table 1). Previously studied O_2 sensors have had extremely slow association rates compared to myoglobins. In contrast, *Ax*PDEA1 is much more similar in its ligand-binding properties to sperm whale myoblogin than to its fellow O_2 sensors. For example, the association rate constant for binding of O_2 to *Ax*PDEA1 is about half that of sperm whale myoglobin but 2000-fold higher than that of *Ec*Dos and 50 times higher than that of *B. japonicum* FixL. The dissociation rate constant for binding of O_2 to *Ax*PDEA1 is 2000-fold lower than that of *Ec*Dos but only 3-fold lower than that of *B. japonicum* FixL and 6-fold lower than that of sperm whale myoglobin. There are no unique residue substitutions in the sequence of *Ax*PDEA1 to explain why its O_2-binding kinetics are so much faster than those of homologous O_2 sensors. Variations in the mechanisms coupling the hemes to the phosphodiesterases may contribute to these kinetic differences. In particular, *Ec*Dos, although coupled to an enzymatic domain very similar to the one in *Ax*PDEA1, probably achieves a heme-driven conformational change in a very different way from that of *Ax*PDEA1. In deoxy-*Ec*Dos, both axial positions of the heme iron are coordinated to protein side chains, resulting in a distinctive hemochromogen absorption spectrum for the deoxy form (*8*). O_2 displaces one of those side chains but causes no net change in coordination number of the heme iron. Consequently, protein conformational change in *Ec*Dos is directly and inextricably linked to O_2 binding but is insensitive to coordination number. In contrast, the heme iron in deoxy-*Ax*PDEA1 has a free axial position for binding of O_2. Therefore, as in FixL, the regulatory changes in *Ax*PDEA1 may be driven by subtle distortions of the heme arising from changes in coordination number (*14*, *38*).

A High-Stability Microaerobic Sensor. A major consideration when engineering O_2-binding heme proteins is to achieve the highest possible stability toward autoxidation while keeping the O_2 affinity within a desired range. For

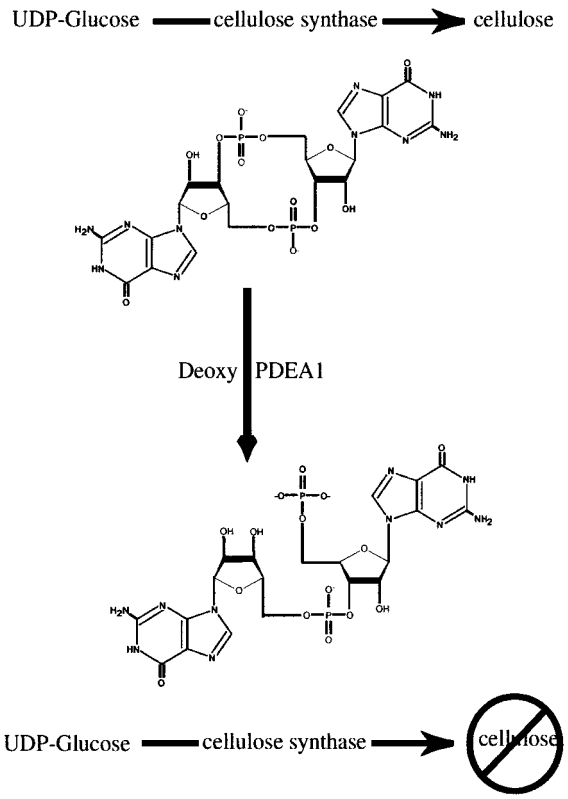

FIGURE 4: Schematic diagram of O_2 control of cellulose synthesis in *A. xylinum*. c-di-GMP is necessary for allosteric activation of the cellulose synthase. The most active form of *Ax*PDEA1 is the holoprotein with deoxyheme.

sperm whale myoglobins with amino acid substitutions, there is a rough correlation between the autoxidation rate constants (k_{ox}) and the K_d for binding of O_2 (*39*). It is tempting to presume that autoxidation is generally correlated with K_d for all O_2-binding heme proteins (*40*). Two examples show that K_d and k_{ox} are not inextricably linked. The hemoglobin from the cyanobacterium *Nostoc commune* has a high affinity for O_2 ($K_d \sim 0.2\ \mu M$), yet it autoxidizes rapidly (half-life in air ~ 3.5 h) (*41*). At the other extreme, the present work shows that ferrous *Ax*PDEA1 has a relatively low affinity for O_2 ($K_d \sim 10\ \mu M$) but is nonetheless stable to autoxidation (half-life in air > 12 h).

O_2 Signal Transduction via a Second Messenger. As in the soluble guanylyl cyclase, *Ax*PDEA1 signal transduction is by way of a second messenger. There are two significant differences: (i) the second messenger is a cyclic *di*nucleotide instead of a mononucleotide and (ii) the regulated enzyme, *Ax*PDEA1, generates the inactive linear form of the messenger rather than its cyclic form (Figure 4). The results in Figure 3 clearly show that in *Ax*PDEA1 the active site of the phosphodiesterase is tightly coupled to the heme-PAS. Apo-*Ax*PDEA1 had less than 2% of the phosphodiesterase activity of holo-*Ax*PDEA1. It is probable that even this trace activity was due to a minor contamination of the apoprotein with holoprotein. Reconstitution of apo-*Ax*PDEA1 resulted in a fully active protein with stably-bound heme. These results further imply that in *Ax*PDEA1 the heme-PAS activates the phosphodiesterase, and O_2 regulation comes about because deoxyheme is a more effective activator than oxyheme. There is some evidence to suggest that apo-FixL works in the opposite way. An apo-FixL produced by substitution of the heme-coordinating residue had some

enzymatic activity, suggesting that in FixL the heme-PAS inhibits the kinase and oxyheme is a more effective inhibitor than deoxyheme (*42*).

Physiological Importance of O_2 for A. xylinum. Our results indicate that *Ax*PDEA1 is least enzymatically active when aerobic and most active when anaerobic. Consequently, c-di-GMP and cellulose levels would be expected to rise in air and drop during O_2 depletion. This is consistent with the observation that, in static cultures of *A. xylinum*$_2$, cellulose is produced by the uppermost, better aerated cells. Cells anchored to this cellulosic pellicle would stay near the surface where they have access to nutrients as well as sufficient O_2 for respiration. Bacteria of the genus *Acetobacter* are obligate aerobes. For *A. xylinum*, production of cellulose is costly, in terms of both carbon sources and energy. Shutdown of cellulose production when O_2, and therefore energy, is low may simply be a way of conserving resources.

It should be noted that *Ax*PDEA1 is not the sole regulator of c-di-GMP levels in *A. xylinum*. Like *Ax*PDEA1, the diguanylate cyclase, DGC1, that synthesizes c-di-GMP from GTP also contains an N-terminal PAS domain. The PAS domain in DGC1 is about 25% identical to a domain in the NPH1 protein of *Arabidopsis thaliana* and 30−35% identical to domains in the NifL protein of *Klebsiella pneumoniae* and the Aer protein of *E. coli*. The NPH1, NifL, and Aer proteins have been implicated in regulation of phototropism, nitrogen fixation, and aerotaxis, respectively (*18*−*20*). All of these proteins contain flavin-binding PAS domains (*18*−*20*). NPH1 may represent a eukaryotic adaptation of the flavin-PAS to function as a blue-light detector. The more closely related prokaryotic flavin-PAS domains in NifL and Aer have been shown to respond to redox. Given that the degree of homology between DGC1, NifL, and Aer implicates them as members of the same PAS subgroup, it is likely that DGC1 binds flavin, and therefore it is possible that *A. xylinum* c-di-GMP levels are regulated by redox potential as well as O_2 concentration.

Cyclic Diguanylic Acid, a Broadly Occurring Second Messenger. c-di-GMP regulation of cellulose synthesis is not limited to *A. xylinum*. Cellulose fibrils are common in bacteria, including *Agrobacteria* and *Rhizobia* (*3*, *43*, *44*). *Agrobacterium tumefacien*s is known to produce c-di-GMP and to synthesize cellulose fibrils in response to exogenous addition of c-di-GMP (*43*). The best support for the role of those fibrils in attachment to plants during tumor formation comes from microscopy and the finding that *A. tumefaciens* mutants in cellulose production (*cel*$^-$) are avirulent (*44*). *Rhizobia* have homologous *cel* genes that may be important for the analogous attachment of these bacteria to plants when forming symbiotic nodules (*45*). The *Ac*YntC protein of *A. caulinodans*, which has an enzymatic domain similar to the one in *Ax*PDEA1, is a heme protein (Figure 1). It is therefore possible that *Ac*YntC is an *A. caulinodans* O_2 sensor similar in function to *Ax*PDEA1. Plant cellulose synthesis may also involve regulation by c-di-GMP (*46*).

Regulation by c-di-GMP is not limited to cellulose synthesis. Many bacteria that do not produce cellulose have proteins with *Ax*PDEA1-like phosphodiesterase domains. The functions of most of those proteins are unknown. So far, three are known to be global regulators of a transition to a new phase of growth. These are VirC, PleD, and YdaK. Mutations of the *virC* gene in *Vibrio anguillarum* reduce by 10 000-fold their ability to infect fish (*47*). The *pleD* gene of *Caulobacter crescentus* is necessary for the characteristic differentiation of these bacteria from "swarmer" to "stalked" cells (*48*). When expressed in *E. coli*, the *ydaK* gene of *B. subtilis* can functionally substitute for *lrp* (leucine responsive protein) in shutting down the biosynthesis of isoleucine, leucine, and valine (*49*). *E. coli* Lrp is important for the transition to the stationary phase. Although *E. coli* does not produce cellulose, the closest relative of *Ax*PDEA1 is the *Ec*Dos protein, which is also poised to detect microaerobic levels of O_2 ($K_d \sim 10\ \mu M$) (*8*). The widespread distribution of regions homologous to the *Ax*PDEA1 phosphodiesterase in bacteria argues for a much wider role for cyclic dinucleotides than is commonly believed. Amikam and colleagues have shown that c-di-GMP enters mammalian lymphoblastoid cells quite easily and binds to the growth-promoting $p21^{ras}$ protein, causing profound changes in the cell cycle (*50*, *51*). Apart from suggesting that c-di-GMP or a structurally similar molecule may play a role in mammalian cell replication, these results highlight the fact that much remains to be learned about the physiological roles of cyclic dinucleotides. The dearth of evidence for cyclic dinucleotides as second messengers, compared to cyclic mononucleotides, may simply reflect the much greater availability of cyclic mononucleotides to experimenters.

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