Novel Heme-based Oxygen Sensor with a Revealing Evolutionary History*

To monitor fluctuations in oxygen concentration, cells use sensory proteins often containing heme cofactors. Here, we identify a new class of heme-binding oxygen sensors, reveal their unusual phylogenetic origin, and propose a sensing mode of a member of this class. We show that heme is bound noncovalently to the central region of AppA, an oxygen and light sensor from *Rhodobacter sphaeroides*. The addition of oxygen to ferrous AppA discoordinated the heme, and subsequent oxygen removal fully restored the heme coordination. In *vitro*, the extent of heme discoordination increased gradually with the rise in oxygen levels over a broad concentration range. This response correlated well with the gradual decrease in transcription of photosynthesis genes regulated by AppA and its partner repressor PpsR. We conclude that the AppA-PpsR regulatory system plays a key role in this process. By repressing photosynthesis genes at high oxygen conditions as well as at high light conditions in low oxygen, this regulatory system prevents the wasteful production of a photosynthetic apparatus under inappropriate conditions and protects cells from (photo)oxidative damage (4, 5, 7).

PpsR is a transcriptional repressor, and AppA is an antirepressor capable of sensing not only oxygen but also light (8). AppA senses light with a FAD chromophore bound to its amino-terminal BLUF2 (sensor of blue light using FAD) domain (9–13) (see Fig. 1A). Light causes dissociation of the AppA-PpsR complex, releasing PpsR to repress the transcription of photosynthesis genes (11). How AppA senses oxygen has remained unclear. It was suggested that AppA senses oxygen indirectly, via its carboxyl-terminal Cys-rich domain (11). This approximately 50-amino-acid long fragment contains 6 Cys residues (4). These residues were proposed to respond to changes in the cellular redox state by reversible formation of disulfide bonds (11).

Here, we show that the central domain of AppA binds heme noncovalently and is required for oxygen sensing. We find that this heme-binding domain is distantly related to the vitamin B12-binding domain. We investigate effects of oxygen on AppA in *vitro* and *in vivo*.

**EXPERIMENTAL PROCEDURES**

**Microbiological and Molecular Genetic Methods**—The growth conditions for *R. sphaeroides*, genetic manipulations, and complementation of the anaerobic photosynthetic growth of the *appA* null mutant, APP11, were performed as described earlier (9). Plasmid p484-Nco5ΔC expressing the AppA derivative lacking the Cys-rich domain was constructed as follows. Plasmid p484Nco5 (4) containing the full-length *appA* gene

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Oxygen plays a central role in influencing lifestyles in this facultatively phototrophic proteobacterium, which is renowned for its metabolic versatility (3). In the presence of oxygen, *R. sphaeroides* derives energy by aerobic respiration. Decreased oxygen levels signal potential threats to the energy supplies and induce development of the photosynthetic apparatus: an alternative energy-generating system that operates in sunlight in the absence of oxygen. The formation of the photosynthetic apparatus requires significant up-regulation of expression of numerous photosynthesis genes. The AppA-PpsR system plays a key role in this process. By repressing photosynthesis genes at high oxygen conditions as well as at high light conditions in low oxygen, this regulatory system prevents the wasteful production of a photosynthetic apparatus under inappropriate conditions and protects cells from (photo)oxidative damage (4, 5, 7).

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2 The abbreviations used are: BLUF, sensor of blue light using FAD; MBP, maltose-binding protein; SCHIC, sensor containing heme instead of cobalamin; CO, carbon monoxide; NO, nitric oxide.
was subjected to site-directed mutagenesis (QuikChange kit, Stratagene) to construct a stop codon at the boundary of the Cys-rich domain (between amino acids 396 and 397) followed by an EcoRI restriction site. The following primers were used for mutagenesis: APP-DCYS-F, 5′-GGCCCGCCTTCGCCGCTGAAATTGCTGGGCACATGCCG-3′, and APP-DCYS-R, 5′-CGGCAATTGGCCGGGCCAGAATTCACGGGCGAGGGCCTGG-agc-3′, where the EcoRI site is shown in bold and the stop codon is underlined. The truncated appA derivative was cut out (using HindIII and EcoRI) from the mutagenized plasmid and cloned into broad host range vector pRK415 (14) to generate p484-Nco5Δ.

Plasmid pMappA for overexpression of the full-length AppA protein was constructed by cloning the appA gene (using SpeI and XbaI) from plasmid pLappA (9) into the XbaI site of vector pMAL-c2X (New England Biolabs). Plasmid pMapp-TEPY for overexpression of the central region of AppA was constructed by cloning of the PCR fragment corresponding to amino acids 197–392 of AppA into pMAL-c2X (digested with EcoRI and XbaI). The following primers were used for fragment amplification: App-TEPY-R1, 5′-CGGAAATTCCTCTCGGATCTGCTGAGCAC-3′, and App-TEPY-Spe, 5′-GGACTAGTCCAGGCGGAGCAAGCGCAGATG-3′, where restriction sites are shown in bold.

Protein Overproduction and Purification—AppA and its central region were purified as carboxyl-terminal fusions to MBP using amylose affinity chromatography according to specifications of the manufacturer (New England Biolabs). Induction of protein expression was done at 24 °C by using isopropyl-1-thio-β-D-galactopyranoside (0.25 mM, final concentration) for 4 h. Following elution from the amylose column, the proteins were collected and either used immediately or stored at −70 °C in 20% v/v glycerol (final concentration).

Protein-Cofactor Interactions—Protein-hemin reconstitutions in vitro were performed as follows. The freshly purified protein (in 50 mM Tris-HCl, pH 8.0, 10 mM maltose) was transferred into an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Residual oxygen was removed by the addition of 4 mM glucose, 0.25% glucose oxidase (Sigma, 200 units ml−1), and 0.125% catalase (Sigma, 10,000 units ml−1) followed by a 15-min incubation at room temperature. Then glycerol was added to protein solution to achieve 20% v/v (final concentration). Following elution from the amylose column, the proteins were collected and either used immediately or stored at −70 °C in 20% v/v glycerol (final concentration).

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Spectroscopy—Electronic absorption UV-visible spectra were recorded using a Cary 4000 spectrophotometer (Varian, Inc). Laser flash photolysis measurements were done with an LKS.60 laser kinetic spectrometer from Applied Photophysics Ltd. (Leatherhead, UK). The spectrometer was fitted with a Pi-star stopped-flow drive unit for stopped-flow and flow-flash measurements. For sample excitation, the LKS.60 spectrometer was coupled to a Quantel Brilliant B Nd:YAG laser with second harmonic generation. Data acquisition was provided by an Agilent 54830B digital oscilloscope for fast measurements or a 12-bit ADC card within the instrument work station for slow measurements. Kinetic traces of CO binding were fit into exponential models to calculate the observed binding rate.

DNA Microarray Experiments—These were performed using R. sphaeroides whole genome oligonucleotide microarrays, GeneChips (Affymetrix), as described earlier (3). RNA was extracted from cultures grown under continuous sparging with gas mixtures containing appropriate oxygen concentrations. The growth conditions, protocols for RNA extraction, and processing were described earlier (3, 6). The GeneChips were hybridized using a fluidics station and scanned using a GeneChip scanner (Affymetrix) at the University of Colorado Cancer Center Microarray Core Facility, according to specifications provided by the manufacturer. Expression values were generated using robust multiarray analysis with quantile normalization (15). Differential gene expression was evaluated as described earlier (3). The DNA microarray data are deposited in the Gene Expression Omnibus (GEO) data base, platform GPL162, series GSE7004.

Phylogenetic Analysis—The amino acid sequence of the central domain of AppA was submitted to psi-BLAST protein-protein search. Considering the high GC content of R. sphaeroides (67%), we applied a conditional compositional score matrix adjustment (16), which improved the search sensitivity. Sequences obtained after the second psi-BLAST iteration were aligned using ClustalW (17). The alignment was restricted to the region conserved in all the tested sequences. A draft phylogenetic tree was constructed from 196 restricted sequences using the neighbor-joining method (18). Sixty sequences representing all major branches and covering the widest range of taxa were selected. The phylogenetic tree was constructed using the minimum evolution method (19) with the close neighbor interchange algorithm implemented in the MEGA3 package (20). Robustness of the tree was evaluated using the interior branch test with 50,000 replications. The overall tree architecture matched the architecture of the draft tree and remained essentially unchanged when the maximum parsimony or maximum likelihood methods were substituted for minimum evolution. Alignments were prepared for presentation using the BioEdit software (21).

RESULTS

Localization of the Oxygen-sensing Domain in AppA—We knew from our earlier studies that the amino-terminal 190 residues of AppA (including the BLUF domain) (Fig. 1A) were dispensable for oxygen sensing (9). To investigate the
role of the Cys-rich domain, we constructed AppAΔC, an AppA derivative lacking this domain (residues 397–450) (Fig. 1A). We tested whether or not AppAΔC would still respond to changes in oxygen levels in vivo. To this end, we introduced this truncated derivative into the R. sphaeroides appA null mutant, which cannot up-regulate photosynthesis genes under anoxic conditions and, therefore, is impaired in phototrophic growth (4). We found that the expression of AppAΔC in the appA null mutant rescued photosynthetic apparatus development (not shown) and anaerobic photosynthetic growth (Fig. 1B). This indicates that the Cys-rich domain is either fully or partially dispensable for oxygen sensing. Therefore, the central region of AppA (Fig. 1A) must be involved in oxygen sensing.

**Central Region of AppA Binds Heme**—We overexpressed in *Escherichia coli* the central AppA region (residues 197–392) as a fusion to the maltose-binding protein, MBP (Fig. 1A). The absorption spectra of the purified protein showed a peak at 415 nm (Fig. 2A, black trace). This peak was small and must have been masked by the spectrum of FAD bound to the BLUF domain in the preparations of the full-length protein studied earlier. The addition of sodium dithionite resulted in a shift of the 415 nm peak to 427 nm, as well as in the appearance of new bands at 560 and 530 nm, respectively (Fig. 2A, red trace). This absorption pattern is characteristic of heme-protein complexes. However, it appeared that only ~5% of the purified protein contained heme.

When we purified the central region of AppA under highly reducing conditions from anaerobically grown *E. coli* supplemented with a common tetrapyrrole precursor, δ-amino levulinate, the relative amount of bound heme dramatically increased (not shown). We cleaved off the MBP and separated the proteins by ion exchange chromatography. As expected, all the heme was associated with AppA and not with MBP (not shown). This suggests that AppA is a heme-binding protein and that heme may serve as an oxygen-sensing cofactor. Interestingly, the central region of AppA shows no sequence similarity to previously described heme-containing oxygen sensors (1, 22–26) or any known heme-binding proteins. When we overexpressed the full-length AppA protein (as an MBP fusion), we found that it also contained heme (not shown), as expected.

**Reconstitution of the Apo-AppA with Hemin**—When we reconstituted the apo-MBP-AppA with hemin (iron protoporphyrin IX) *in vitro*, we could achieve a 1:1 molar hemin:protein ratio if the reconstitution was performed under reducing and anaerobic conditions (Fig. 2B). The hemin-protein complex reconstituted under these conditions was relatively stable after subsequent exposure to oxygen. Importantly, all hemin remained protein-bound after gel filtration, indicating a high affinity of AppA for hemin. However, reconstitutions performed on air resulted in unstable heme-AppA complexes. The nature of this instability is not yet fully understood.

Under anoxic conditions, hemin bound so rapidly to the AppA that the protein became saturated with hemin within 2 s after mixing with this cofactor in a stopped-flow spectrometer (Fig. 2E). The α (560 nm) and β (530 nm) peaks present in the absorption spectra of deoxy-AppA suggest a hexacoordinate state for the heme iron (Fig. 2C, black trace). As discussed below, the axial heme iron ligand is likely to be a His residue.

**Ligand Binding by the Holo-AppA Protein**—The addition of CO to the deoxy-AppA resulted in a characteristic carbonmonoxy heme spectrum with a sharp Soret peak at 421 nm, as well as broad α and β bands (Fig. 2C, orange trace). The association rate constant for binding of CO to deoxy-AppA, as measured by flash photolysis, was $1.03 \pm 0.02 \mu M^{-1} s^{-1}$ (Fig. 2E), similar to the on-rate constants reported for binding of CO myoglobin and hemoglobins (27–29) but about 1000-fold faster than the rate constants reported for the oxygen sensor Dos from *E. coli* that contains a heme-binding PAS domain (30).
FIGURE 2. Characterization of AppA as a heme-binding protein. A, absorption spectra of the central region of AppA. Black trace, as isolated from E. coli; red trace, after reduction with dithionite. Inset, expanded versions of the 500–600-nm region. B, in vitro reconstitution of the full-length AppA with hemin. Shown are differential spectra versus buffer containing free hemin. Inset, molar hemin:protein ratios. C, interactions of AppA with CO and NO. Black trace, reduced deoxy form of the reconstituted AppA protein; orange trace, carbonmonoxy form; blue trace, nitrosyl-heme complex. D, interaction of oxidized AppA with cyanide under anoxic conditions. Black trace, oxidized (FeIII, met) form of AppA; cyan trace, cyanomet form of AppA. E, kinetics of AppA-hemin reconstitution. AppA and hemin solutions of equimolar concentrations were rapidly mixed in a stopped-flow spectrophotometer. Shown is the change in absorbance at 417 nm. F, linear regression of the observed rate of CO binding to AppA measured using flash photolysis versus CO concentration. A representative experiment is shown. The observed rate was calculated as an exponential fit of the rebinding kinetic trace.
The addition of NO to deoxy-AppA did not result in a typical nitrosyl heme protein spectrum. Instead, the Soret peak broadened and shifted to 401 nm and decreased in amplitude (Fig. 2C, blue trace). Similar changes occur in the absorption spectra of soluble guanylate cyclase protein on the addition of NO. These changes in guanylate cyclase are due to decoordination of the heme iron from the axial His residue and the formation of a pentacoordinate nitrosyl–heme complex (31–33). The similarities in the absorption spectra suggest that AppA undergoes similar changes.

The oxidized (FeIII, met) form of holo-AppA was obtained from the deoxy form by oxidation with potassium ferricyanide under the anoxic conditions followed by ferricyanide removal by gel filtration. The met-AppA had a Soret absorption peak at 415 nm (Fig. 2D, black trace). MBP-AppA protein purified from the aerobically grown E. coli showed the same absorption band (Fig. 2A), indicating that it contained an oxidized heme. After the addition of cyanide, a known ligand of ferric heme, to met-AppA, the Soret peak shifted to 420 nm (Fig. 2D, cyan trace), consistent with production of the cyanomet form.

**Interactions of AppA with Oxygen in Vitro and in Vivo**—The addition of oxygen to deoxy-AppA resulted in a decrease in the Soret peak amplitude, a blue shift in its position, and the appearance of a shoulder at 380 nm (Fig. 3A). At the same time, α and β peaks disappeared and were replaced by a new shallow peak at 540 nm (Fig. 3A, inset), which indicated a loss of coordination of the heme iron at the axial position and the formation of a pentacoordinate oxygenated complex. Importantly, the absorption changes caused by oxygen were fully reversible; the amplitude and the position of the Soret peak as well as the amplitude and positions of α and β peaks were restored upon removal of oxygen by the glucose/glucose oxidase/catalase system (Fig. 3, A and B).

Interestingly, the response of AppA to oxygen was concentration-dependent. The extent of heme iron decoordination increased gradually as oxygen levels increased over a broad range of concentrations, from trace amounts of oxygen introduced by passing a few air bubbles through the anoxic protein solution to 600 μM, which was the highest oxygen concentration used in this experiment (Fig. 3C). Subsequent removal of oxygen by the glucose/glucose oxidase/catalase system resulted in full restoration of the spectrum of hexacoordinate deoxy form regardless of the initial oxygen concentration (not shown). Therefore, coordination of the axial heme ligand in AppA can apparently be ruptured by binding of either NO or oxygen, but not CO, and heme decoordination is proportional to oxygen concentration.

To test whether oxygen binding resulted in the oxidation of the ferrous heme iron to the ferric state, we examined the interactions of the holo-AppA protein with oxygen in a background of potassium cyanide. Since cyanide binds to the ferric iron (Fig. 2D), we expected that it would “freeze” the heme in the cyanomet form whenever oxidation occurred. We found that the absorption changes of holo-AppA in response to varying oxygen concentrations were identical with or without potassium cyanide (data not shown). Since oxygen binding to deoxy-AppA does not result in oxidation of the heme iron, the proposed mechanism of heme decoordination upon oxygen binding appears the most plausible route for oxygen sensing by AppA.

The ability of holo-AppA to respond gradually and reversibly to changing oxygen levels place this protein in a good position to function as an oxygen sensor. Heme decoordination is known to result in significant changes in protein conformation (33). We propose that these regulatory conformational changes in AppA promote dissociation of the AppA-PpsR complexes, thus allowing the released PpsR protein to repress photosynthesis genes. Since the amount of the decoordinated heme is proportionate to the oxygen concentration in vitro (Fig. 3C, inset), one may expect that in vivo, expression of the AppA-PpsR-dependent genes would also respond gradually to changing oxygen concentration.

To test this prediction, we measured, using DNA microarrays, gene expression in the R. sphaeroides cultures grown at different steady-state concentrations of oxygen. Fig. 3D shows transcript abundance of several photosynthesis genes, which are directly controlled by the AppA–PpsR system (6), as a function of oxygen concentration. It is apparent that transcript abundance of all these genes indeed changed gradually over the range of 0.5–30% oxygen concentrations. This response was independent of the absolute transcript levels, which varied by up to 2 orders of magnitude. The gene expression measurements revealed that the AppA–PpsR system functions in vivo as a fine-tuned oxygen-dependent transcriptional rheostat as opposed to an on/off switch.

We consider the strong correlation between the reversible and gradual heme decoordination in rising oxygen in vitro and gradual gene expression changes in vivo to constitute a physiological verification of the proposed oxygen sensing mechanism by AppA. However, detailed understanding of this mechanism clearly requires further investigation.

**SCHIC, a Novel Heme-binding Domain**—To define the protein module responsible for heme binding and oxygen sensing, we analyzed the sequence of the central region of AppA. We identified a similarity of the fragment encompassing residues 274–392 to the vitamin B12-binding domain (Pfam database: PF02310). The e-value of 8.90 × 10−2 indicates low similarity level.

We tested whether AppA can bind vitamin B12. Since E. coli does not synthesize cobalt-containing tetrapyroles, cobalamins, we grew the overexpressing strain in the presence of exogenously added adenosylcobalamin. We found that the purified protein contained no bound adenosylcobalamin. We also tested the ability of AppA to bind adenosylcobalamin and other cyclic (cyanocobalamin, protoporphyrin IX) and linear (biliverdin) tetrapyroles but detected no specific binding (not shown). These results suggest that AppA readily discriminates between heme and other tetrapyroles. We designated the protein domain between residues 274 and 392 (Fig. 1A) SCHIC for sensor containing heme instead of cobalamin.

An in-depth phylogenetic analysis of the SCHIC domains revealed that they form a cluster that is related to, but distinct from, the cluster of B12-binding domains (Fig. 4A). The SCHIC and B12-binding domains apparently belong to a larger domain...
superfamily that includes proteins from bacteria, Archaea, and Eukarya. The identities of cofactors for some of the branches of this superfamily remain unknown.

The multiple sequence alignment of the SCHIC and B12-binding domains revealed several conserved residues shared by the two groups, among which is the His residue that functions as an axial ligand to cobalt in the B12-binding proteins (Fig. 4B). We therefore predict that the corresponding His residue in the SCHIC domains (His284 in AppA) is a candidate for serving an axial ligand to heme iron. The alignment also revealed sequence motifs that distinguish one domain from another. It will be important to resolve the roles that...
these motifs play in determining the differential tetrapyrrole specificity of protein members of this superfamily.

**DISCUSSION**

We described a new class of heme-binding domains involved in oxygen sensing and investigated a sensory mechanism for the first representative of this class, *R. sphaeroides* AppA. The central region of AppA is required for oxygen sensing *in vivo*. It contains an ~120-amino-acid-long SCHIC domain (Fig. 4A) that binds heme and is sufficient for oxygen sensing. To ensure that the uncovered role of the SCHIC domain is applicable to other SCHIC proteins, we recently analyzed the *R. sphaeroides* PpaA protein (34) (Fig. 4B). We found that it also binds heme and responds to changes in oxygen levels *in vitro*.3

The presence of heme in AppA has been overlooked for quite some time by several research groups that study this unique sensor, probably because heme content in the protein purified from aerobically grown *E. coli* is low. Further, the absorption of the light-sensing cofactor, FAD, bound to the amino-terminal BLUF domain of AppA, confuses the spectrum of heme. In addition, the most efficient heme binding occurs under reducing and anoxic conditions, whether the protein is reconstituted

3 O. V. Moskvin, M. A. Giles-Gonzalez, and M. Gomelsky, unpublished data.
with heme in vitro or expressed in anaerobically grown E. coli. Once formed, the heme-AppA complexes remain relatively stable in the presence of oxygen.

SCHIC is the sixth kind of protein module that employs heme for oxygen sensing. These modules are structurally diverse, suggesting that heme has been recruited for this function independently many times over the course of evolution. Four out of five types of heme-based oxygen sensors described prior to SCHIC involve noncovalently bound b-type hemes, i.e. a heme-PAS domain (1, 23, 30, 35, 36), a modified globin-coupled sensor domain (24, 37), HNOX domain (25, 38), and heme-GAF domain (26). In this group, only PAS (39) and GAF (40) domains share some structural similarity. However, these domains can bind various small ligands in addition to heme. Therefore, they may have acquired heme binding properties independently of each other. The fifth member of this group, represented by Desulfovibrio vulgaris DcrA, contains a covalently bound c-type heme (22, 41). The diversity of heme-based sensory modules extends further if one considers sensors covalently bound PpsR-dependent genes at varying oxygen levels.

Mechanisms of oxygen sensing have been explored in detail for only a limited number of heme proteins. Most commonly, oxygen binding to a heme iron results in the formation of an oxi-heme species, which brings about conformational changes in the protein and initiates a signal transduction cascade (2). In the c-type heme containing DcrA, oxidation of the heme iron is important for oxygen sensing (41). AppA appears to sense oxygen by a different mechanism. Oxygen binding to the heme iron discoordinates the heme as evidenced from the loss of the spectral features that characterize protein-coordinated heme and the appearance of features that characterize disordinated heme (Fig. 3, A–C). Most likely, oxygen binding results in rupture of the bond to the protein. NO binding has the same outcome, whereas CO binding is apparently insufficient to break that axial coordination. This suggested mechanism of oxygen sensing by AppA is reminiscent of NO sensing by HNOX guanylate cyclases (31–33). The elucidation of the details of this sensing by AppA is reminiscent of NO sensing by HNOX guanylate cyclases (31–33). The elucidation of the details of this process, electron transport; hemoglobins and myoglobin to oxygen transport and storage; chlorophyll-containing proteins to photosynthesis. However, origins of these groups of proteins remain murky. Are any of them evolutionary related, or did tetrapyrrole binding arise independently in different protein lineages? The domain superfamily identified here has a potential to improve our understanding of how protein-tetrapyrrole specificity is determined, how it may have evolved, and how it can be manipulated.

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