The PpaA/AerR Regulators of Photosynthesis Gene Expression from Anoxygenic Phototrophic Proteobacteria Contain Heme-Binding SCHIC Domains[▽]

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The SCHIC domain of the B$_{12}$-binding domain family present in the *Rhodobacter sphaeroides* AppA protein binds heme and senses oxygen. Here we show that the predicted SCHIC domain PpaA/AerR regulators also bind heme and respond to oxygen *in vitro*, despite their low sequence identity with AppA.

Cells monitor fluctuations in oxygen tension by means of oxygen-sensing proteins, many of which contain heme (10, 11). We recently described a new class of heme-binding protein domains designated SCHIC (sensor containing heme instead of cobalamin) (18, 23). SCHIC comprises approximately 120 amino acids and belongs to the vitamin B$_{12}$-binding domain family (Pfam: PF02310 [9]).

The only SCHIC domain protein characterized thus far, AppA (14, 15), functions as a light- and oxygen-sensing antirepressor, which, in partnership with the repressor PpsR, controls photosynthesis gene expression in *Rhodobacter sphaeroides* (4, 12, 16, 20, 22). The SCHIC domain of AppA responds gradually to changes in oxygen concentrations, which allows the AppA-PpsR system to function as an oxygen-dependent transcriptional rheostat (23). The AppA proteins are found exclusively in the *R. sphaeroides* species. However, our sequence and phylogenetic analyses (23) predicted that aerobic regulators of photosynthesis gene expression of the PpaA/AerR family (7, 13, 21) also contained SCHIC domains. The PpaA proteins lack the light-sensing BLUF and the Cys-rich domains of AppA but often contain Arg-rich C termini (Fig. 1A). Because sequence identity among the SCHIC domains can be as low as 20% (23) (Fig. 1B), it has been unclear whether members of the PpaA/AerR family indeed bind heme instead of cobalamins.

To address this question, we characterized predicted SCHIC domain PpaA proteins from two groups of the anoxygenic photosynthetic proteobacteria—those that use photosynthesis only under anaerobic conditions (e.g., *Rhodobacter*) and those that require the presence of oxygen (e.g., *Jannaschia*). The PpaA proteins from *R. sphaeroides* 2.4.1 and *Jannaschia* sp. CCS1 were chosen because they share relatively little similarity to each other, i.e., 35% identity. Here we report that, in spite of low sequence similarity to each other and even lower simi-

larity to the SCHIC domain from AppA (23% identity), the SCHIC domains of the PpaA proteins bind heme. Further, the SCHIC domain of *R. sphaeroides* PpaA, SCHIC$_{Rs}$, responds to oxygen and CO *in vitro* in a manner that is remarkably similar to that of AppA.

The SCHIC domains of the PpaA proteins bind heme. We cloned and overexpressed fragments of the *R. sphaeroides* PpaA (residues 55 to 249) and *Jannaschia* sp. CCS1 PpaA (residues 60 to 258) proteins encompassing predicted SCHIC domains (Fig. 1A) as C-terminal fusions to the maltose-binding protein (MBP). The *R. sphaeroides ppaA* gene fragment was PCR amplified using plasmid pSmNo (13) and the primers Ppa-TEPY-RI (5′ CGGA ATTCCGCTCGATGGATCGCGCCC) and Ppa-TEPY-Spe (5′ GGACTAGTCACTGCAGCGCCGCCTCAATG) and cloned in vector pMAL-c2x (New England Biolabs). The *Jannaschia* sp. *ppaA* fragment was amplified from genomic DNA using the primers CCS1_F (5′GCCGCATGCCCTGTGAGGCGTTTCTGTC CGACAGC) and CCS1_R (5′CAGGAAGCTTTAATCCACG CCGGTCGCCTTCTTC) and cloned into a modified pMAL-c2x vector. *E. coli* DH5α cells containing the MBP-SCHIC plasmids were used for protein overexpression and purification. The fusion proteins were purified using affinity chromatography on amylose resin essentially as described previously (23).

The purified SCHIC domain from *R. sphaeroides* PpaA, MBP-SCHIC$_{Rs}$, demonstrated a Soret absorption peak at 413 nm, indicative of a bound tetrapyrrole (Fig. 2A); however, the amount of bound tetrapyrrole was relatively low (A_{413}/A_{280} ratio, 0.11). To test for the chemical nature of the tetrapyrrole, we added a strong reductant, sodium dithionite, to the protein preparation. As a result, the Soret peak was shifted to 427 nm, and two additional prominent peaks, α (560 nm) and β (530 nm), appeared. These spectral changes are typical of the conversion of a hexacoordinated heme-protein complex from the met, Fe^{3+} form to the deoxy, Fe^{2+} form. Further, the positions of the Soret, α, and β peaks of the deoxy, Fe^{2+} form of MBP-SCHIC$_{Rs}$ were identical to those of the deoxy, Fe^{2+} form of the SCHIC domain from AppA (23).

To compare the affinities of MBP-SCHIC$_{Rs}$ for heme and vitamin B$_{12}$, we expressed MBP-SCHIC$_{Rs}$ in *E. coli* grown anaerobically in media supplemented with high (50 μM) concentration of vitamin B$_{12}$ (cyanocobalamin; Sigma). Vitamin B$_{12}$ is not synthesized by *E. coli* but can be taken up from the

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A

PpaA

SCHIC | Arg
134-246 | 247-264

AppA

BLUF | SCHIC | Cys
274-392

B

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PpaA R. sphaeroides     134 PAVLVLLPPGEQH--TLGAMVAVAKLRRLGVSVCLRMSTGPAEDRELFGKRR--FDAIMI
AppA R. sphaeroides     274 GAVLVAGVPG--HKPILEAALAAEMLRAVGWSTSVVHPESVAALAARLKTSRTSTLVVAP
AerR R. capsulatus      133 ATVLLIVPPGEQH--TLGALIVAMELRRRGVSRIVFAPGLSDLSRLMATTR--FDAALI
BAG34613 Rhodospirillum 170 RRILLVTVPGEQH--SFGLLMVAEFFRRACWDVTTAMPTSRDDLSTLVRSEW--FALVGL
ZP_01041675 Erythrobacter 172 ARALFAPMPGDQH--NFGALMMDEIFARAGWNSRVLTKPLRKELLDALSREP--FDVVGL
ABD53080 Jannaschia     155 PKVLLLLVRPEHH--TLGAFVAADQFRREGYDVDIAVDQHPKQVAATLRDRR--YCMIGI
YP_680562 Roseobacter   134 LNALVVIPQNEQH--FLGASVVAAQLRRLGCEVAMSYDEDHGALTARLINDV--PDLVLV
Consensus                   L          H    G          R G
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PpaA R. sphaeroides     190 SLAHAEMLEVGRKLVKTLKDMTGGR--IPVAMGGALFLDGTEAASIPEADIVTNDIEAA
AppA R. sphaeroides     330 SLLEGTEQEADTLRFVSALRARTDLPGLSILVGGRLAQLPPSKLKDSGADAGFAHLALL
AerR R. capsulatus      189 TVGSMDRVEICAKLVKTLSSLTKGR--MRVAIGGAIVSQRAEALARTGADLVTNDLSLV
BAG34613 Rhodospirillum 226 SASCDSRLDTMAAAIRSVRRASRNR-LVGILVGGPVFDGHPEYVTAVGADIMGRNGQEA
ZP_01041675 Erythrobacter 228 TLSRDCPSAAVTNIIKAMRSVSANP-HLSILIGGRMINQNPALVAEVGADGTGADARAA
ABD53080 Jannaschia     211 SIAGRRTLASTRELVDIIRATVTR--VTPIVLGGSLLETDQDLKKATGVDYVARTVRDA
YP_680562 Roseobacter   190 TCGRLETLPSVANTIQVVRKAVGN--DPTLAVGGACQVNKQRVMEITGADIVTSVAEEA
Consensus                   S            R           GG      GAD      A
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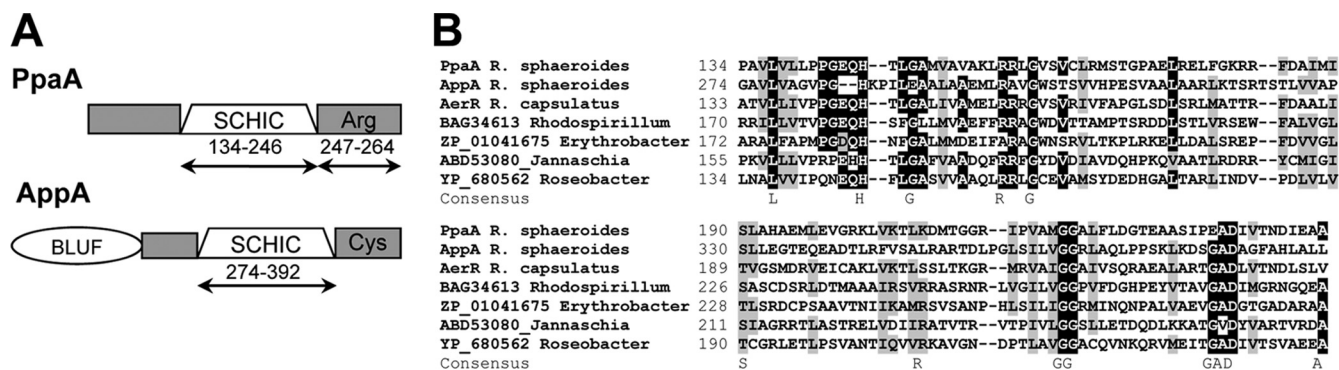
FIG. 1. (A) Domain structure of the *R. sphaeroides* AppA and PpaA proteins. SCHIC, sensor containing heme instead of cobalamin (23); BLUF, sensor of blue light using FAD (17); Cys, cysteine-rich domain; Arg, arginine-rich domain. Domains of unknown function are shown on a gray background. (B) Alignment of the SCHIC domains from anaerobic (top four) and aerobic anoxygenic phototrophic proteobacteria. Black shading indicates identities within the group; gray shading indicates similarities. The consensus is derived from a larger (58 sequences) alignment of the SCHIC domains.

medium, as evidenced by the pinkish color of the anaerobically grown *E. coli* DH5α cells and presence of cobalamin in crude cell extracts (data not shown). We found that MBP-SCHIC$_{Rs}$ purified from these cells and cells not exposed to cyanocobalamin had identical spectra indicative of bound heme, not cyanocobalamin (Fig. 2B). This observation suggests that MBP-SCHIC$_{Rs}$ readily discriminates between heme and cobalamin, even when the latter compound is present in excess.

Next, we reconstituted MBP-SCHIC$_{Rs}$ with hemin *in vitro*, under the anaerobic conditions (anaerobic chamber), using methods described by us previously (23). The met, Fe^{3+} and deoxy, Fe^{2+} forms of the holoprotein showed the same absorbance peaks as those of MBP-SCHIC$_{Rs}$ purified from *E. coli* (Fig. 2C), which suggested that reconstitution *in vitro* had reproduced the heme-protein complex present in the purified MBP-SCHIC$_{Rs}$.

The PpaA fragment from *Jannaschia* sp. CCS1, MBP-SCHIC$_{Js}$, as purified from *E. coli*, also contained relatively small amounts of bound heme, similar to MBP-SCHIC$_{Rs}$ (data not shown). We reconstituted MBP-SCHIC$_{Js}$ with hemin *in vitro* (to approximately 1:1 heme-protein molar ratio). The Soret peak of MBP-SCHIC$_{Js}$ in the met, Fe^{3+} form was 414 nm, i.e., within 1 nm of the SCHIC domains of *R. sphaeroides* AppA (415 nm) and PpaA (413 nm). Upon reduction with dithionite, the Soret peak was shifted to 420 nm, and the α and β peaks emerged at 538 and 567 nm, respectively (Fig. 2D). These peaks are 7 to 8 nm shifted compared to the SCHIC domain proteins from *R. sphaeroides*. While the observed spectral differences between the SCHIC domains suggest slightly different heme environments, it is clear that all studied SCHIC domains specifically bind heme and that heme environment is overall quite similar.

It is noteworthy that the SCHIC domain of AppA overexpressed in *E. coli* and purified on air contained only small amounts of heme (23), similar to the PpaA proteins described above. We do not fully understand the reasons for relatively low heme content in the proteins purified from aerobically grown *E. coli*. Several factors may contribute to incomplete heme saturation, e.g., insufficient heme supply in *E. coli*, instability of the heme-protein complexes under the aerobic conditions of protein expression and purification, and loss of heme

during purification. Our attempts to date to overexpress the PpaA or AppA proteins in *R. sphaeroides* have been unsuccessful because of protein toxicity.

Interactions of the SCHIC domain from R. sphaeroides PpaA with gaseous ligands. We further explored interactions of MBP-SCHIC$_{Rs}$ with gaseous ligands, carbon monoxide (CO) and molecular oxygen. The addition of CO to the deoxy, Fe^{2+} form of MBP-SCHIC$_{Rs}$ resulted in a characteristic carbonmonoxy heme spectrum with a sharp Soret peak at 421 nm, indicative of specific binding (data not shown). We determined kinetic parameters for binding of CO using laser flash photolysis measurements as described previously (23). We found that the CO on and off rate constants for MBP-SCHIC$_{Rs}$ were very similar to those of AppA (Table 1). Although many heme proteins bind CO, the kinetics of interactions with CO vary considerably among heme-binding proteins (1, 6, 8, 24). Similar kinetics suggests that the heme-binding pockets of the SCHIC domains from AppA and PpaA share key structural features and, by extension, that *R. sphaeroides* AppA and PpaA share similar functional capabilities.

Next, we tested the interactions of the deoxy, Fe^{2+} form of MBP-SCHIC$_{Rs}$ with oxygen. Upon addition of oxygen, the amplitude of the Soret peak decreased and its position shifted to the blue region, while a shoulder at 380 nm characteristic of a loosely bound heme increased. The absorbance of the α and β peaks also decreased (Fig. 3, red traces). These changes indicate a loss of coordination (i.e., discoordination) of the heme iron from the axial position following oxygen binding. Importantly, these absorption changes were reversible. We observed restoration of the deoxy, Fe^{2+} form over time in the presence of the oxygen-scavenging system (a mixture of glucose, glucose oxidase, and catalase [3]) (Fig. 3). Also noteworthy is the observation that the magnitude of the spectral changes in MBP-SCHIC$_{Rs}$ was proportional to oxygen concentration. For example, addition of 100 μM oxygen decreased the Soret peak by approximately 0.25 units (α peak by 0.03), while addition of 200 μM oxygen decreased the Soret peak by approximately 0.5 units (α peak by 0.06) (Fig. 3A and B). This graded oxygen response of MBP-SCHIC$_{Rs}$ resembles the response of the SCHIC domain of AppA (23) and suggests that PpaA
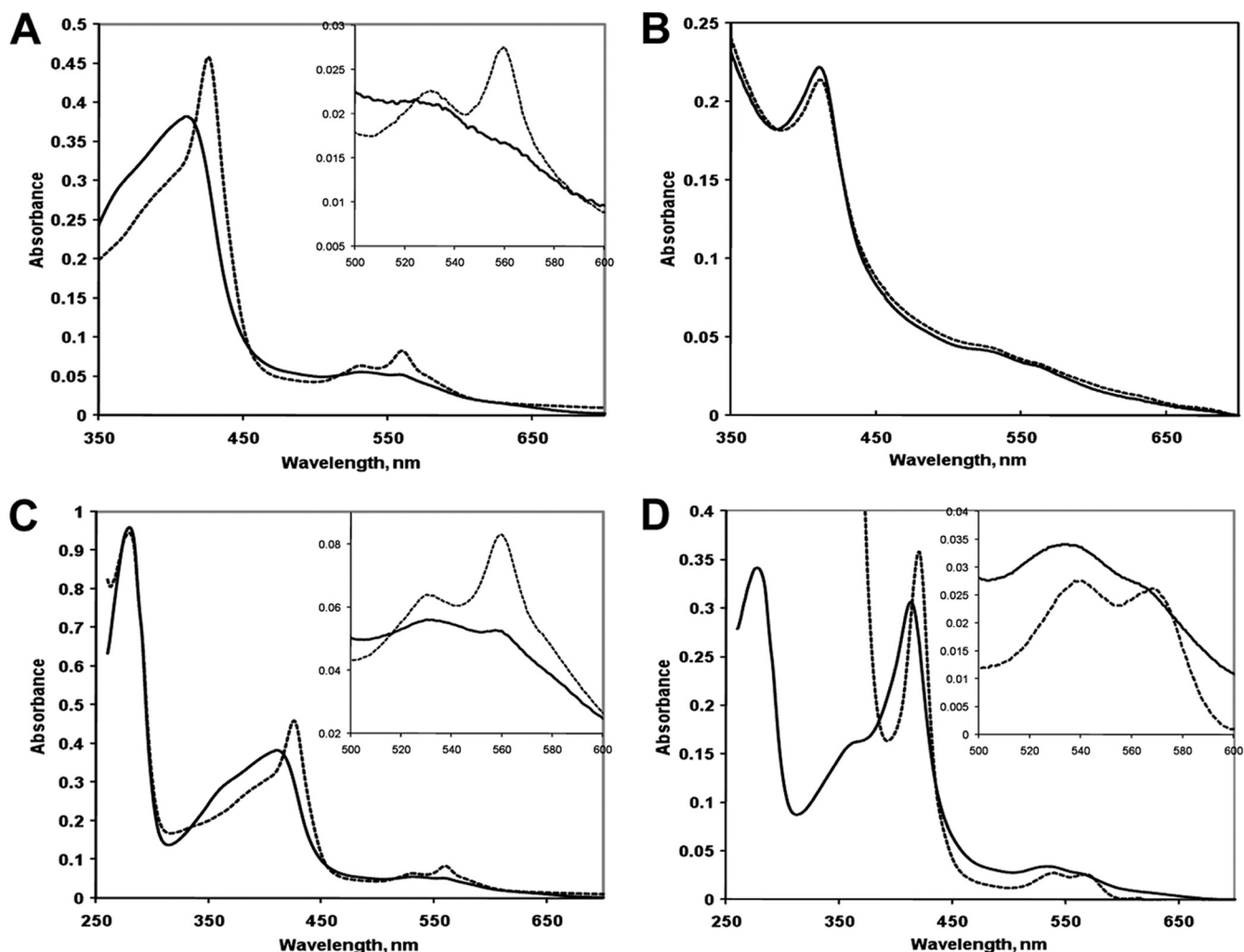
FIG. 2. (A) Electronic absorption spectra of the MBP fusion to the SCHIC domain of the *R. sphaeroides* PpaA protein, MBP-SCHIC$_{Rs}$, as purified from *E. coli*. Solid trace, original spectrum; dashed trace, spectrum obtained after addition of dithionite. (B) Spectra of MBP-SCHIC$_{Rs}$ purified from anaerobic *E. coli* cells grown in the absence (solid trace) and presence (dotted trace) of 50 μM cyanocobalamin. (C) Spectra of MBP-SCHIC$_{Rs}$ after reconstitution with hemin *in vitro*. Solid trace, original spectrum; dashed trace, spectrum obtained after reduction with dithionite (and dithionite removal by size exclusion chromatography). (D) Spectra of MBP-SCHIC$_{Js}$ after reconstitution with hemin *in vitro* (approximately 1:1 molar ratio). Solid trace, original spectrum; dashed trace, spectrum obtained after reduction with dithionite. Insets show magnified long-wavelength regions.

may function as "oxygen rheostat," similar to AppA. However, the details of the sensory mechanism of *R. sphaeroides* PpaA and functional significance of this observation remain to be investigated.

TABLE 1. Kinetics of CO binding and dissociation in heme-containing SCHIC domain AppA and PpaA proteins from *R. sphaeroides*[a]

| Protein | Constant for CO binding | | Equilibrium dissociation constant (μM) |
	On rate (μM^{-1} s^{-1})	Off rate (s^{-1})	
AppA	1.03 ± 0.02[b]	0.77 ± 0.09	0.75
PpaA	0.95 ± 0.04	0.70 ± 0.09	0.74

[a] MBP-AppA (data from reference 23) and MBP-SCHIC$_{Rs}$. Data are means ± standard deviations, determined based on kinetic traces that were recorded at least four times using two independently reconstituted protein samples.

The PpaA/AerR protein family. The PpaA/AerR proteins are present in all sequenced genomes of the anoxygenic phototrophic proteobacteria (purple nonsulfur bacteria), which is in contrast to AppA, found exclusively in the *R. sphaeroides* species. The *ppaA* genes are located in the photosynthesis gene clusters of both anaerobic (*Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, etc.) and aerobic (*Roseobacter*, *Erythrobacter*, *Jannaschia*, etc.) anoxygenic photosynthetic proteobacteria. The representatives of the latter bacterial group are particularly abundant in marine habitats from coastal waters to deep-sea sediments, from the poles to the tropics, and are found either free living or associated with phytoplankton (2, 5). *R. sphaeroides* PpaA (13) and its counterparts from *Rhodobacter capsulatus*, AerR (7), and from *Rhodocystis centenum*, AerR (21), function as oxygen-dependent regulators of photosynthesis gene expression. The PpaA protein from *R. sphaeroides* and *R. centenum* function as aerobic activators of photosynthesis gene expression (13, 21), whereas AerR functions
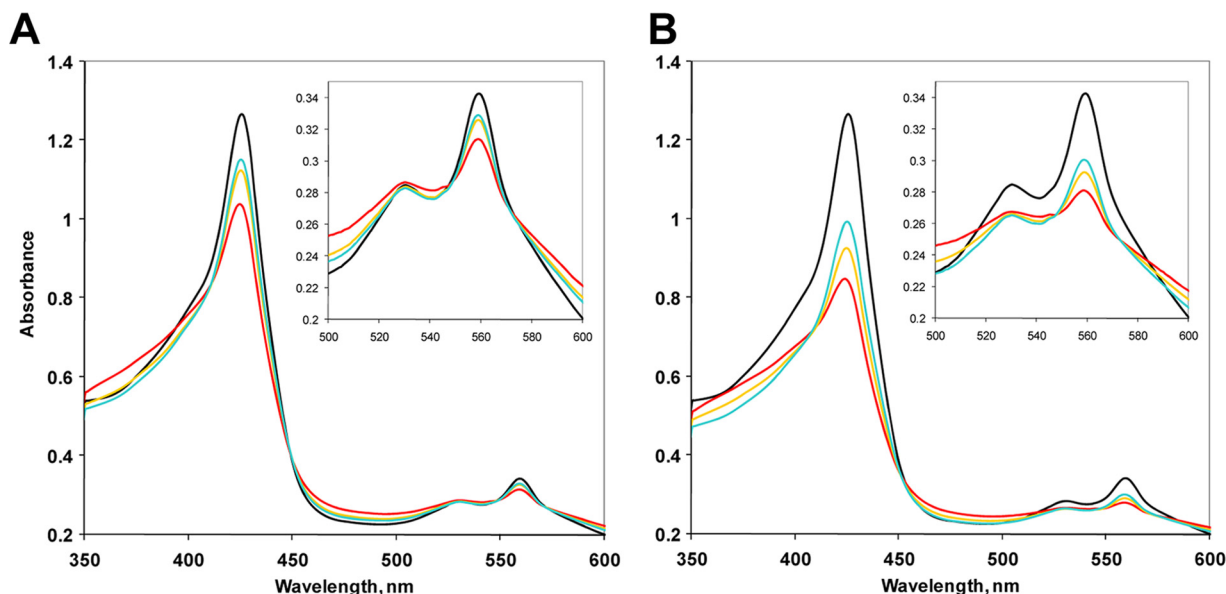
FIG. 3. Electronic absorption spectra of MBP-SCHIC$_{Rs}$ before and after exposure to 100 μM oxygen (final concentration) (A) and 200 μM oxygen (B). The medium contained 5 mM dithiothreitol and a glucose–glucose oxidase–catalase system of oxygen removal (3). Black traces, deoxy, Fe^{2+} protein; red traces, protein immediately after addition of oxygen; gold traces, protein after 3 min; cyan traces, protein after 15 min. Insets show magnified long-wavelength regions. The experimental details are given in reference 23.

as an aerobic repressor (7). Presence of the heme-containing, oxygen-responsive SCHIC domain may explain the oxygen sensitivity of the PpaA/AerR family members that appear to be important for photosynthesis regulation on the global scale.

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