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Novel FixL homologues in *Chlamydomonas reinhardtii* bind heme and O_2

U.M. Narayana Murthy [a], Matt S.A. Wecker [b], Matthew C. Posewitz [c], Marie-Alda Gilles-Gonzalez [d], Maria L. Ghirardi [a,*]

[a] Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, United States
[b] GeneBiologics LLC, Boulder, CO 80303, United States
[c] Department of Chemistry and Geochemistry, Colorado School of Mines, Golden, CO 80401, United States
[d] Department of Biochemistry, UT Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd, TX 75390-9038, United States

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ABSTRACT

Genome inspection revealed nine putative heme-binding, FixL-homologous proteins in *Chlamydomonas reinhardtii*. The heme-binding domains from two of these proteins, FXL1 and FXL5 were cloned, expressed in *Escherichia coli*, purified and characterized. The recombinant FXL1 and FXL5 domains stained positively for heme, while mutations in the putative ligand-binding histidine FXL1-H200S and FXL5-H200S resulted in loss of heme binding. The FXL1 and FXL5 [Fe(II), bound O_2] had Soret absorption maxima around 415 nm, and weaker absorptions at longer wavelengths, in concurrence with the literature. Ligand-binding measurements showed that FXL1 and FXL5 bind O_2 with moderate affinity, 135 and 222 μM, respectively. This suggests that Chlamydomonas may use the FXL proteins in O_2-sensing mechanisms analogous to that reported in nitrogen-fixing bacteria to regulate gene expression.

1. Introduction

Chlamydomonas is able to perform photosynthesis and aerobic respiration, transition into strictly anaerobic fermentations when O_2 is unavailable, and balance fermentation, photo-fermentation and respiration under conditions of sulfur deprivation in the light [1–3]. The anaerobic metabolism of phototrophic microorganisms has been of particular interest for the production of organic acids, alcohols and H_2, all of which can be used in strategies for the production of renewable fuels [4–8]. Several studies have defined aspects of these metabolic capabilities in Chlamydomonas; however, relatively little is known about the mechanisms of metabolite sensing or the signal-transduction events that occur in response to O_2 levels. Recent data indicate that significant changes occur in the abundance of several transcripts encoding fermentative enzymes as Chlamydomonas acclimates to anoxia [6,9]. Therefore, we analyzed the available Chlamydomonas genome for homologues of known O_2-sensing proteins and signal-transduction components that have been characterized in other organisms. We identified a group of Chlamydomonas genes that are predicted to encode proteins with strong amino acid similarity to the Rhizobial heme-binding, O_2-sensing PAS domains. The expected proximal histidine residue (H200 in BjFixL) is present in all of the Chlamydomonas FXL homologues, as are two highly conserved arginines (R206 and R220 in BjFixL) known to be involved in hydrogen-bonding interactions with the heme. From this set of Chlamydomonas FixL-like (FXL) homologues, we chose two members, FXL1 and FXL5 for further studies regarding their potential role as O_2 sensors and gene-expression regulators in Chlamydomonas.

The full-length versions of FXL1 and FXL5 proteins in Chlamydomonas are very large (2072 and 2299 amino acids, respectively) and each of the putative homologues has multiple transmembrane-spanning domains, which are typical of the bacterial FixL homologues. To better understand the role of heme proteins in Chlamydomonas, and to determine whether the identified PAS domains were able to bind O_2, the putative heme-binding domains from FXL1 and FXL5 proteins were cloned, heterologously expressed in *Escherichia coli*, and purified. The purified FXL1 and FXL5 proteins were then characterized for their heme and O_2-binding properties. Our results clearly indicate that FXL1 and FXL5 bind heme and, like their Rhizobial homologues, could be involved in heme-based O_2-sensing and the regulation of associated metabolic pathways in Chlamydomonas. However, since the Chlamydomonas FXL homologues lack canonical autophosphorylation and signal transmitter domains, they must utilize an unusual signal transduction mechanism involving additional residues/domains.

* Corresponding author. Address: National Renewable Energy Laboratory, 1617 Cole Blvd, Golden, CO 80401, United States. Fax: +1 303 384 7836.
E-mail addresses: maria.ghirardi@nrel.gov, maria_ghirardi@nrel.gov (M.L. Ghirardi).

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2. Materials and methods

2.1. Sequence analysis, alignments and protein modeling

Chlamydomonas FXL sequence analysis was performed using the DNASTAR expert sequence analysis software (Madison, WI). Searches for sequence similarity were performed using the BLAST network service provided by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Sequence alignment was performed using the MegAlign tool of DNASTAR. Chlamydomonas FXL proteins models were analyzed using the Chlamydomonas v4.3 genome portal at http://www.phytozome.net/chlamy. Sequence for the FXL6 homologue was obtained using the Chlamydomonas version 3.0 gene model since the current model of FXL6 excludes a stretch of nucleotide sequence at scaffold_48:736979–737039 by instead assigning these nucleotides to an intron that shows high sequence similarity to the other represented FXL proteins. Regions of identity in protein sequence were assessed by alignment using EMBL-EBI clustalW v2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and overall alignment identity percentages were assessed with Kalign (http://msa.cgb.ki.se/cgi-bin/msa.cgi) using a gap open penalty of 11, a gap extension penalty of 0.85, a terminal gap penalty of 0.45 and a bonus score of 0 [10].

2.2. Plasmid construction

The primer sequences used in this work are listed in Supplementary Table 1. RNA was extracted from Chlamydomonas strain CC425 and reverse-transcribed as described [6]. For expression in *E. coli*, the heme-binding domain of FXL1 was amplified using primers FXL1BamR and FXL1NdeF. Similarly, the heme binding domain of FXL5 was amplified using primers FXL5BamR and FXL5NdeF. The corresponding PCR products were digested with BamHI and NdeI and then ligated into pET28a (Novagen, Madison, WI) for expression as His_6-tagged proteins. FXL1 and FXL5 mutant constructs were also generated in which the putative heme-binding histidine was changed to serine, creating FXL1-H200S and FXL5-H200S. To change the single histidine to serine in the FXL domain of FXL1 and FXL5 recombinant PCR was performed using the appropriate primer sets shown in Supplementary Table 1 in two consecutive reactions that generated the mutated domains and then annealed and further amplified them with the appropriate T7 primers. The resulting PCR products were digested with BamI and NdeI and then ligated into pET28*a* (Novagen, Madison, WI) for expression as His_6-tagged proteins.

2.3. Protein purification

For purification of the FXL1 and FXL5 heme-binding domains, the plasmids containing the FXL1 or FXL5 sequences were transformed into *E. coli* BL21 (DE3) codon$^+$ (Stratagene, La Jolla, CA) and expression was induced by IPTG. The FXL1 and FXL5 domains were purified by the procedure developed by Murthy et al. [11]. The purified proteins were dialyzed against 25 mM Tris–HCl, pH 7.4. Homogeneity and purity assessments of all proteins employed SDS–PAGE with Coomassie blue staining. In addition, LC–MS/MS (done by the Colorado State University at Fort Collins, CO according to the method described in http://www.pmf.colostate.edu/) and UV–visible spectroscopy were carried out after the final elution.

2.4. Gel heme assays

Proteins were analyzed using 15% Tris–Tricine mini gels with a 6% stacking gel in Tris–Tricine buffer containing 0.05% SDS. Protein bands were visualized after staining for heme with *o*-dianisidine (DMB) according to the procedure developed by Francis and Becker [12].

2.5. Spectrophotometric studies and estimation of O_2 dissociation constants

Absorption spectra (350–700 nm) of samples at 25 °C in 50 mM phosphate buffer (pH 7.4) were recorded with a Varian 4000 spectrophotometer. The deoxygenated [Fe(II), no bound O_2] spectra were recorded after reduction with a twofold molar excess of sodium dithionite followed by removal of the reductant through a 3 mL G-25 column equilibrated with degassed buffer at 4 °C. To obtain the oxy-FXL [Fe(II), bound O_2] absorption spectra while avoiding auto-oxidation, one atmosphere of O_2 was layered over the deoxygenated samples and the solutions were equilibrated by shaking immediately before the spectra were recorded. Oxidized spectra [Fe(III)] for FXL1 and FXL5 were obtained by exposing the samples to air for 30 min or treating them with potassium ferricyanide at 25 °C. Apoproteins were prepared by extracting heme from FXL1 and FXL5 by cold acid-acetone treatment as described previously [13]. The determination of the stoichiometric amount of heme bound to each protein was done according to the method of Atassi and Childress [14].

In order to estimate O_2 dissociation constants, a stock solution of 1.3 mM O_2 in 50 mM phosphate buffer (pH 7.4) was prepared by bubbling with O_2 at room temperature for 1 h in a septum-sealed glass vial. The stock solution was then transferred to an anaerobic chamber, and various aliquots were transferred to deoxygenated buffer samples in individual sealed glass vials using a gas-tight Hamilton syringe to prepare an O_2 dilution series. Purified FXL1 and FXL5 protein samples in the deoxy state were added to respective vials of the dilution series and their absorption spectra recorded. Spectra for deoxy and oxy states of the protein using titrations from 0 to 1280 μM O_2 were used to determine O_2 saturation [15], and O_2 equilibrium dissociation constants (K_d values).

2.6. Quantitative real-time PCR

RNA was extracted from *Chlamydomonas reinhardtii* strain CC425 as described [6] and DNaseI was used to remove DNA before reverse transcription. Real-time PCR (RT-PCR) was performed using a LightCycler 480 in combination with the SYBRGreen I Master kit (Roche, Germany) according to the supplier's protocol.

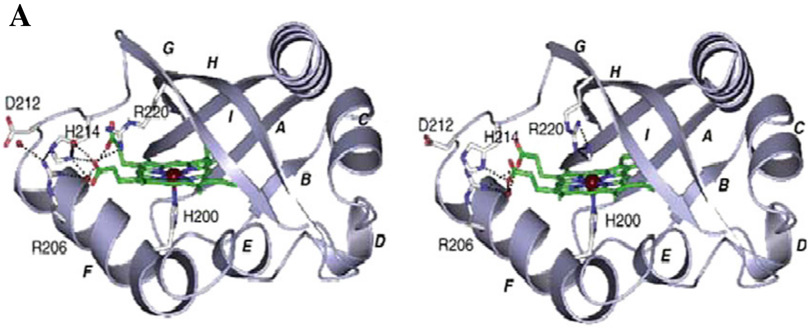
3. Results

3.1. Identification of FXL homologues in Chlamydomonas and sequence characteristics

Nine FXL-like sequences were identified by BLAST searches of the Chlamydomonas genome data base JGI v4.3 using *Rhizobium* FixL as a query. Interestingly, these genes aligned closely with at least five hypothetical genes (VOLCADRAFT-104960, -104036, -93382, -91579 and -93917) from *Volvox carteri*, a colonial chlorophyte alga, closely related to the single-celled Chlamydomonas. These potential *Volvox* FXL genes retained the canonical heme-binding histidine of the Chlamydomonas FXL proteins (not shown). As with the Chlamydomonas FXL genes, these potential *Volvox* genes did not contain the FixL histidine phosphorylation site.

Each Chlamydomonas protein contained a FixL-like heme-binding PAS consensus site (Figs. 1B and 2). The nine FXL protein PAS core regions, comprising about 100 residues between the two ends of the predicted β-sheet structures of the PAS domain shared 76% overall amino acid identity. These same regions showed a 59%

A



B

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                          Aβ      Bβ    Cα        Dα      Eα       Fα
FXL1      PEFLTSMSMEDDAVIVINAEGTIMMVSQAVQKVFGYPKTELEGANVALLMPQPSSQRHPS 600
FXL2      PEFLTSMSMEDDAVIVINAEGTIMMVSQAVQKVFGYPKAELEGANVSLLMPQPFSQRHPS 662
FXL3      PEFLTSMSMEDDAVIVINAEGTIMMVSQAVQKVFGYPKTELEGANVSLLMPQPFSQRHPS 571
FXL4      PDFLTSMSMEDDAVIVINAEGTIMMCSQAVSRVFGYGKGELEGSNVSVLMPPPFSQRHAS 557
FXL7      PDFLTSMSMEDDAVIVINAEGTIMMCSQAVNSVFGYAKNELEGMNVSLLMPQPFSQRHPS 647
FXL6      PEFLTSMSIQDDAVVVINAEGTIMMTSQAVQTVFGYTKNELEGVNVALLMPQPFSQRHAS 630
FXL5      PDFLTSMSMEDDAVIVVDAEGTIMMVSQAVQKTFGYAKIELEGTNVSALMPQPFSQRHPG 474
FXL8      PDFLTSMDLHEDACMVINHEGNIMMVNSCVTPLLGYLRSELEGASVSLIMPQPFAGRHAG 491
FXL9      PDFLTSMDLHEDACMVINHEGNIMMVNSCITNLLGYAKTELEGNNVSMIMPQPFSGRHAG 765
BjFixL    ----SILHTIPDAMIVIDGHGIIQLFSTAAERLFGWSELEAIGQNVNILMPEPDRSRHDS 202
SmFixL    ----SILDTVPDATVVSATDGTIVSFNAAAVRQFGYAEEEVIGQNLRILMPEPYRHEHDG 196
RlFixL    ----SILDTVPDAMVVIDDKGVVLSFSKAAEKLFGMSSEQICGRNVSNLMPNPYRDAHDG 198
              : :    ** 1*2  .* :   . *.   1*  . :  * .3  23* *    4 .

             Fα    FG-loop    Gβ            Hβ            Iβ
FXL1      YLTRYVNGGEPHILDTVREVVGLHKERYVFPMQLCVTRMSGTGSDSVFLGVARPMVANSL 660
FXL2      YMQRYVAGGEPHILDTVREVLALHKERYVFPMQLCVTKMSGTGTDSIFLGVARPMAPNSM 722
FXL3      YLTRYVNGGEPHILDTVREVVALHKERYVFPMQLCVTKMSGTGSDSVFLGVVRPLGSNLL 631
FXL4      YLQRYAGGGEPRILDSVREVIALHKERYVMPMSLCVTKMSGTGTDSVFLGVVRPMASSTR 617
FXL7      YLSRYVNGGEPHILDTVREVVGLHKDRFVFPLSLCVTKMSGTGTDSVFLGVARPMASNSL 707
FXL6      YLTRYVATAEPRILDSVREVVALHKERYVFPLSLCVTKMSGTGSDSVFLGVMRPLPPSSL 690
FXL5      YMQRYVGGGEPHMLDSVREVVALHKERYVFPVQLCVTKLSGSGSDSIFLGVIRPLPPNPH 534
FXL8      YMQRYVQGGEPHILDSVRDVVALHRDRVVFPLQLCVTKLSGIGTDSIFLGVMRPAPLDPH 551
FXL9      YMQRYIQGGEPHILDSVRDVVALHRDRVVFPLQLCVTKLSGIGTDSIFLGVMRPVPLDAH 825
BjFixL    YISRYRTTSDPHIIGIGRIVTGKRRDGTTFPMHLSIGEMQ-SGGEPYFTGFVRDLTEH-- 259
SmFixL    YLQRYMATGEKRIIGIDRVVSGQRKDGSTFPMKLAVGEMR-SGGERFFTGFIRDLTER-- 253
RlFixL    YIDHYLDTGEKRIIGYGRVVTGQRADGSQFPMELHVGEATANGER-IFTGFVRDLTSR-- 255
          33 51   .: 666.  7 8 1   :   :*8 8 8.   . *    1 *. * :

FXL1      NVRAWVSPNGTFLCGDQMFASMCGIAESDLVGRSLASLSAGPEIDIETLLDRCRDATAAD 720
FXL2      NKRAWIAPNGIFMCGDQQFASMCGITEGELVGRTLASLMEATDVEVELLLERCHAASASD 782
FXL3      NVRAWVAPNGVFLCGDNNFASMCGATENELVGRSLSTLLDNDQMEVEQLLERCRDAPAAE 691
FXL4      NVRCWVAPSGVILCGDQQFASMIGVTEGEVVGRTLLSLAASPPSDIELLLERCRDATASE 677
FXL7      NVRAWIAPNGVFLCGDTQFASMCGIAESELVGCTLEGISLTAAVEVQQLLEQCRAASEVE 767
FXL6      RVRAWVAPNGAFLCGDQQFASMCGLAEKDLVGCTLSSLVANPEEEVNDLLERFRSATAAE 750
FXL5      VMRAWLSPNGTFLCADQQFASAVGRSEGELVGHALLALAVS-AEAVEALLERCKAATAAE 593
FXL8      NVRAWVAPNGLVLCTDPQFASLTGLTSEEMLAS--------------------------D 610
FXL9      NVRAWVAPNGLVLCTDPQFASLTGLTSEEMVGLTVQSMCVD-GTAIEDLLDYCKNAPYEE 884
BjFixL    ----------------QQTQARLQELQSELVHVSRLSAMGEMASALAHELNQPLAAISNY 303
SmFixL    ----------------EESAARLEQIQAELARLARLNEMGEMASTLAHELNQPLSAIANY 297
RlFixL    -----------------------YKIEEDLRQSQKMEAVGQLTGGIAHDFNNLLTVISGN 292
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Fig. 1. Structural elements implicated in *Bj*FixL regulation and their occurrence in other heme-PAS proteins. (A) Contrast of the crystal structure of the wild-type *Bj*FixLH "on-state" (left) and "off-state" (right). This figure is adopted with permission from Gilles-Gonzalez et al. [29]. (B) Amino acid sequence similarity of the nine FXL Chlamydomonas proteins to the PAS domains of selected Rhizobia FixL. The position numbering at the end of each line refers to the amino acid position number for the modeled protein. Arrows above the notations refer to the α-helix turns and β-sheet structures. Number notations below the sequences refer to the following conserved heme-PAS residues (1) residues lying in the plane of the porphyrin ring. (2) Residues that are peripheral to heme coordination. (3) Residues that orient *Bj*H200 to the proximal side of the heme group. (4) Strictly conserved histidine that is required for coordination to heme. (5) Arginine residue that binds the edge of the heme and coordinates ligand detection with the FG loop transmitter. (6) Residues that bind propionate group on heme. (7) Arginine that binds the heme propionate in the "on-state" or O_2 in "off-state" and transmits its status to the FG loop in *Bj*. (8) Residues oriented on the distal side of the heme. (9) Phosphorylation site. The sequences for the FXL1PAS and FXL5PAS truncates begin at the 1st amino acid shown and extend to the end of the I β-sheet structure.

overall identity when three Rhizobial FixL heme-binding PAS domains were included in the alignment. Nearly all of the main features of the FixL heme-binding sites are present (see Fig. 1 legend). Importantly, the *Bradyrhizobium japonicum Bj*FixL H200 PAS residue is conserved, as is a putative axial binding Histidine ligand to the Fe atom. As mentioned above, all nine homologues lacked canonical phosphorylation and DNA-binding domains, although it is worth noting that the FXL6 C-terminal domain has high sequence similarity to ankyrin protein–protein interaction domain (Supplementary Fig. 1), and a potential Ser phosphoryla-

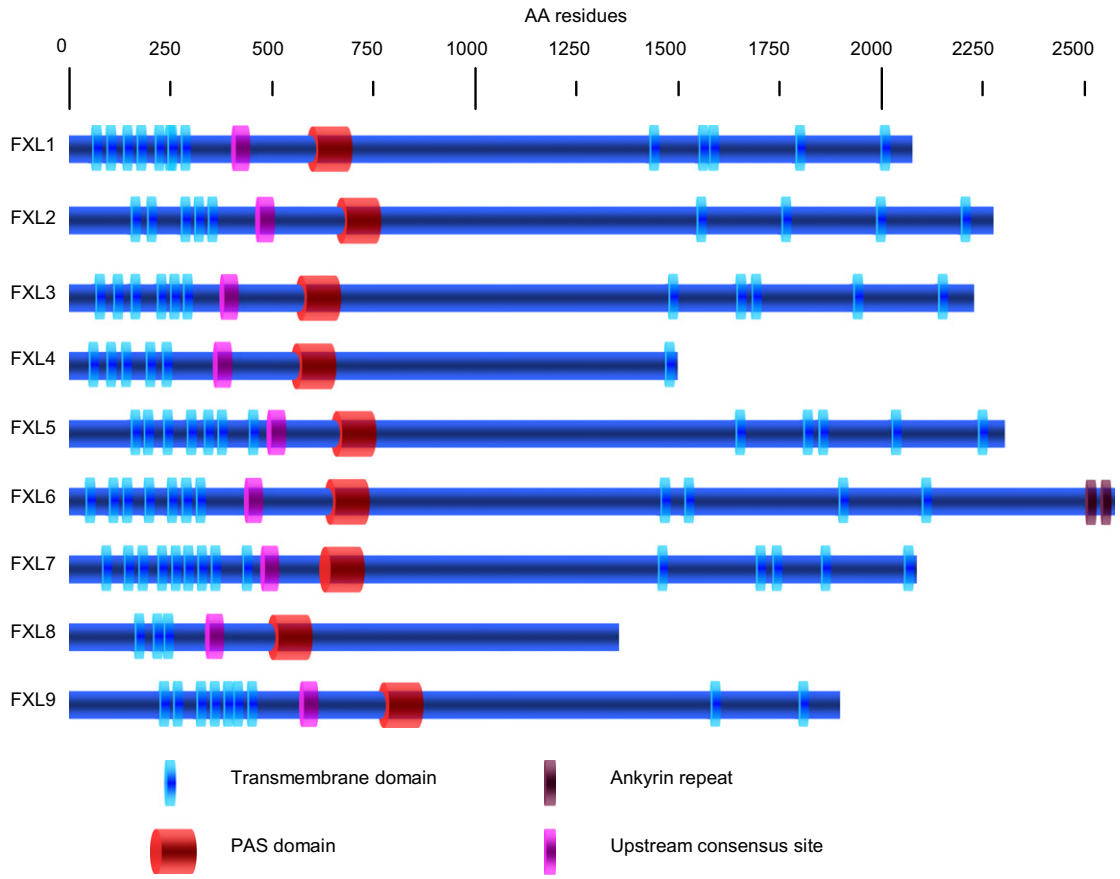

Fig. 2. Schematic representation of the nine *C. reinhardtii* FXL homologues, as predicted by Chlamydomonas V4.3 website. Only FXL1 and FXL5 have been accurately sequenced from cDNA. Approximate locations of the functional elements described in Fig. 1 are indicated.

tion residue is observed downstream from the PAS consensus domain in FXL3. Schematic representation of the deduced sequence of the nine *C. reinhardtii* FIXL homologues and the locations of the predicted functional elements are indicated in Fig. 2.

3.2. Characteristics of heterologously-expressed FXL heme-binding domains

The purified FXL1 and FXL5 were orange–red proteins with masses of about 12.5 kDa as judged by SDS–PAGE (Supplementary Fig. 2A). The purified FXL1-H200S and FXL5-H200S proteins, with substitutions of the proximal histidine to serine were colorless, indicating the absence of heme. LC–MS/MS analysis of FXL1 and FXL5 indicated that the respective proteins were correctly purified (Supplementary Fig. 3A and B). His_6 tag-cleaved FXL1 and FXL5 were used for all biochemical characterizations. Purified FXL1 and FXL5 carried heme throughout the purification step. Purified FXL1 and FXL5, as well as FXL1H-200S and FXL5-H200S were assayed for heme-binding by using a non-denaturing gel heme assay (Supplementary Fig. 2B). As expected, FXL1 and FXL5 both stained positively for heme, whereas FXL1-H200S and FXL5-H200S showed no heme reaction.

Hemin titrations show that FXL1 and FXL5 interact with hemin in a 1:1 M ratio of hemin to protein (Supplementary Fig. 2C and D). The concentration of the FXL1 and FXL5 protein calculated from the absorbance at 415 nm agree with the protein concentration determined from the protein assay, suggesting that FXL1 and FXL5 bind one heme per monomer.

3.3. Spectral properties and ligand binding properties

Absorption spectra were collected in the range of 350–700 nm before and after reduction of FXL1 and FXL5 proteins from the ferric form [Fe(III)] to the ferrous form [Fe(II)]. The optical absorption of met [Fe(III)], deoxy [Fe(II), no bound O_2] and oxy [Fe(II), bound O_2], spectra complexes of FXL1 and FXL5 are shown in Fig. 3A and B. The Soret and visible absorption peaks of oxy (415 nm) and deoxy (430 nm) Fe(II) complexes of FXL1 and FXL5 are characteristic of protein-bound heme and are similar to those of deoxy and oxy *Rm*FixL [15]. Upon reduction with 10 mM sodium dithionite the primary 415 nm Soret bands of both FXL1 and FXL5 shift about 15 nm downfield, and the ratio of the absorbance of the 560 nm band relative to the 530 nm band increases.

Linear combinations of spectra for deoxy and oxy states of the protein were used to determine the saturations at varying O_2 concentrations (Fig. 3C and D). FXL1 and FXL5 showed hyperbolic O_2-saturation curves (Fig. 3E and F). From this study, we determined the equilibrium K_d for O_2 binding to FXL1 to be ~135 μM. For FXL5, the K_d values were determined to be ~222 μM.

4. Discussion

The presence of nine FXL homologues in Chlamydomonas suggests that this alga may use heme-based O_2 sensing to regulate aspects of metabolism in response to O_2. Particularly intriguing is a possible role in sensing and responding to a transition from an aerobic to an anaerobic metabolism.

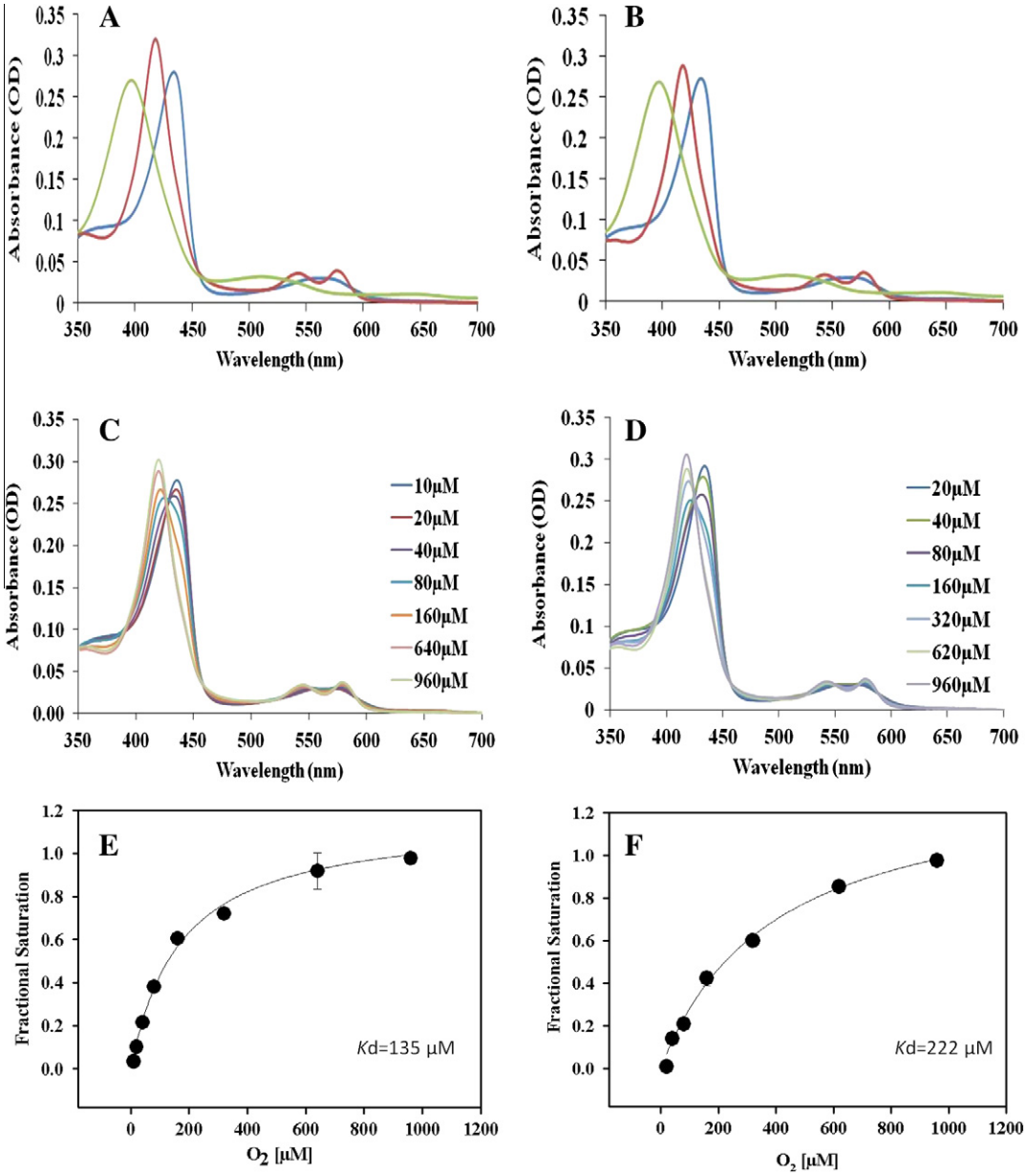

Fig. 3. Spectral properties and ligand binding properties of the FXL homologues. Characteristic UV–visible absorption spectra of FXL1 (A) and FXL5 (B) proteins. Blue line, deoxy [(Fe(II), no bound O_2]; red line, oxy [Fe(II), bound O_2]; green line, met [Fe(III)]. Changes in FXL1 (C) and FXL5 (D) absorption spectra upon titration with O_2. Absorption spectra of FXL1 and FXL5 are shown at various O_2-saturation levels (in μM). For FXL1, the spectrum under 320 μm O_2 is not included. However, the absorption values from the second replicate were used to calculate the K_d values. For FXL5, only one replicate was used at the 20 μM O_2 concentration. All other spectra contain at least two replicates for FXL1 and FXL5. Details are in Section 2. Determination of the FXL1 (E) and FXL5 (F) O_2 affinities. K_d values of 135 and 222 μM (average of two replicates) for FXL1 and FXL5, respectively were determined by directly titrating both proteins with 0–1280 μM O_2, deconvoluting the deoxy and oxy fractions in the absorption spectra, and fitting the data to a hyperbolic equation for single binding, using least-square analysis.

4.1. Heme-binding proteins in Chlamydomonas: sequence analysis

Despite retaining many features central to the function of the bacterial FixL proteins, several unique features are observed in the Chlamydomonas homologues that may be a consequence of expression and function in an eukaryotic host. The histidine required for autophosphorylation and signal transduction in bacterial FixL is absent from the Chlamydomonas FXL homologues. Therefore it is not clear how the putative Chlamydomonas FXL PAS domains might relay the state of the heme ligand to a regulatory element. Chlamydomonas FXL proteins are the only known members of the heme family lacking histidine kinase domains for autophosphorylation (although the five hypothetic genes from Volvox that show homology to FXL sequences also lack the canonical His phosphorylation residue). We predict that other functional domains exist in these FXL proteins, which might relay the state of the heme ligand. For example, bacterial PAS elements lacking histidine kinase domains are often associated with phosphodiesterase or DNA-binding domains [16]. We speculate on the possibility that signal transduction functions are performed by other residues/domains present in the Chlamydomonas FXL homologues, such as the ankyrin domain present in the model of FXL6 (Supplementary Fig. 1A). Ankyrin domains have been shown to bind to a number of plasma membrane-associated proteins [17], including the Na/K-ATPase [18], the amiloride-sensitive sodium channel [19] and the voltage-dependent sodium channel [20]. Recently, it has been

shown that ankyrin also interacts with intracellular calcium channels such as the IP_3 receptor [21]. Indeed, ankyrin domains are found in the Chlamydomonas copper responsive regulator 1 (CRR1) that is associated with anoxic response [22]. Finally, the ~100 amino acid consensus sequence closely upstream of the PAS domain in each of the FXL proteins (see Supplementary Fig. 1B), does not share strong identity to any known protein. Further work is needed to identify the elements of the full signal transduction pathway involving FXL proteins in Chlamydomonas.

4.2. FXL1 and FXL5 are heme-binding proteins

Recent microarray data indicated that only slight increases occur in the abundance of two of the nine FXL proteins, namely FXL1 and FXL5, as Chlamydomonas acclimates to anoxia [6]. In this study, using highly purified proteins, we showed that their expression level remains unchanged upon anaerobiosis (not shown) and that they are heme-binding domains (Supplementary Fig. 2B). A change of histidine to serine eliminates heme-binding for FXL1-H200S and FXL5-H200S mutants. This position is thus likely to be the site of heme–iron coordination and provides further evidence for the importance of this histidine residue in heme coordination. Finally, we show that FXL1 and FXL5 each bind one mole of hemin per mole of protein to form a stable hemin-protein complex (Supplementary Fig. 2C and D).

4.3. Spectral properties of the FXL1 and FXL5 heme-binding domains are typical of FixL proteins

Spectral measurements of the recombinant FXL1 and FXL5 heme-binding domains show intense absorption at around 415 nm (the "Soret" band), followed by weaker absorptions at longer wavelengths. These are characteristic bands of protein-bound heme in the oxy Fe[II] form and are very similar to absorption spectra of *Rhizobium* FixL protein [15] both in the presence and absence of O_2. This was confirmed by the 15 nm downfield shift seen upon reduction (deoxy [FeII] form), which is also typical of heme-binding proteins. Heme proteins with cysteines, methionine or tyrosine as proximal ligands, on the other hand, have very different absorption spectra [23]. Our absorption data and the sequence homology with *Rhizobium* FixL suggest that the heme moieties in FXL1 and FXL5 are present in a binding environment similar to that of the FixL heme.

4.4. Possible physiological roles of FXL proteins in Chlamydomonas

The K_d values for O_2 binding to FXL1 and FXL5 were 135 and 222 μM, respectively. The estimated K_d value for FXL1 is close to that found in the Bradyrhizobium FixL (140 μm) but much higher than in Rhizobium FixL (0.003 μm) [15,24]. The K_d value for FXL5 is much higher than the values calculated for Bradyrhizobium and Rhizobium but much lower than *E. coli DOSH* (340 μm) [15,25,26] (see Supplementary Table 2). It is also important to note that the K_d values, while serving well to indicate the saturation state of the hemes, do not necessarily relate linearly to the activities of the proteins. In the case of RmFixL, pronounced hysteretic behavior was reported, such that relatively low saturation of the heme could completely shut down the kinase activity. Based on sequence similarity to the Rhizobium FixL protein, and from the results presented above, it is reasonable to assume that the two proteins described here function as heme-binding proteins in vivo. Given the metabolic flexibility of Chlamydomonas and its ability to transition quickly from an aerobic to an anaerobic environment (and vice versa), it will be critical to understand the mechanisms by which the organism senses O_2 levels and initiates the appropriate transcriptional, translational and posttranslational responses. The K_d values for both FXL domains are near the concentration of O_2 dissolved in water in equilibrium with the atmosphere. The relatively high K_d values for O_2 suggest that the FXL proteins are used to respond to changing levels of O_2 at the soil surface or to O_2 produced during photosynthesis. Since Chlamydomonas can generate significant quantities of photosynthetic O_2, the expression of O_2-sensitive proteins must be tightly controlled to ensure that cellular energy is not wasted on the synthesis of O_2-intolerant proteins during aerobic growth. Similarly, it is essential for aerobically growing Chlamydomonas to down-regulate the expression of proteins that are required to be functional only during anaerobiosis. For example, the [FeFe]-hydrogenases are irreversibly inhibited by O_2 and their transcription is down-regulated by O_2 [27,28]. Similar challenges are faced by N_2-fixing Rhizobia, some of which use the heme-based, O_2-sensing FixL proteins to detect O_2 levels and initiate signal transduction events that ensure the synthesis of N_2 fixation proteins only when O_2 levels are sufficiently low to prevent enzyme inactivation. It is thus tempting to propose that the Chlamydomonas homologues are involved in regulating transcription of genes in response to changes in intracellular O_2 levels.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.06.052.

References

[1] Antal, T.K., Krendeleva, T.E., Laurinavichene, T.V., Makarova, V.V., Ghirardi, M.L., Rubin, A.B., Tsygankov, A.A. and Seibert, M. (2003) The dependence of algal H2 production on Photosystem II and O2 consumption activities in sulfur-deprived *Chlamydomonas reinhardtii* cells. Biochim. Biophys. Acta 1607, 153–160.
[2] Fouchard, S., Hemschemeier, A., Caruana, A., Pruvost, J., Legrand, J., Happe, T., Peltier, G. and Cournac, L. (2005) Autotrophic and mixotrophic hydrogen photoproduction in sulfur-deprived chlamydomonas cells. Appl. Environ. Microbiol. 71, 6199–6205.
[3] Melis, A., Zhang, L., Forestier, M., Ghirardi, M.L. and Seibert, M. (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol. 122, 127–136.
[4] Gfeller, R.P. and Gibbs, M. (1984) Fermentative metabolism of *Chlamydomonas reinhardtii*: I. Analysis of fermentative products from starch in dark and light. Plant Physiol. 75, 212–218.
[5] Kruse, O., Rupprecht, J., Mussgnug, J.H., Dismukes, G.C. and Hankamer, B. (2005) Photosynthesis: a blueprint for solar energy capture and biohydrogen production technologies. Photochem. Photobiol. Sci. 4, 957–970.
[6] Mus, F., Dubini, A., Seibert, M., Posewitz, M.C. and Grossman, A.R. (2007) Anaerobic acclimation in *Chlamydomonas reinhardtii*: anoxic gene expression, hydrogenase induction and metabolic pathways. J. Biol. Chem. 282, 25475–25486.
[7] Posewitz, M.C., King, P.W., Smolinski, S.L., Zhang, L., Seibert, M. and Ghirardi, M.L. (2004) Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. J. Biol. Chem. 279, 25711–25720.
[8] Posewitz, M.C., Smolinski, S.L., Kanakagiri, S., Melis, A., Seibert, M. and Ghirardi, M.L. (2004) Hydrogen photoproduction is attenuated by disruption of an isoamylase gene in *Chlamydomonas reinhardtii*. Plant Cell 16, 2151–2163.
[9] Dubini, A., Mus, F., Seibert, M., Grossman, A.R. and Posewitz, M.C. (2009) Flexibility in anaerobic metabolism as revealed in a mutant of *Chlamydomonas reinhardtii* lacking hydrogenase activity. J. Biol. Chem. 284, 7201–7213.
[10] Lassmann, T. and Sonnhammer, E. (2005) Kalign – an accurate and fast multiple sequence alignment algorithm. BMC Bioinformatics 6, 298.
[11] Murthy, U.M.N. et al. (2009) Characterization of *Arabidopsis thaliana* SufE2 and SufE3. Functions in chloroplast iron-sulfur cluster assembly and NAD synthesis. J. Biol. Chem. 284, 27020.

[12] Francis, R.T. and Becker, R.R. (1984) Specific indication of hemoproteins in polyacrylamide gels using a double-staining process. Anal. Biochem. 136, 509–514.
[13] Suquet, C., Savenkova, M. and Satterlee, J.D. (2005) Recombinant PAS-heme domains of oxygen sensing proteins: high level production and physical characterization. Protein Expr. Purif. 42, 182–193.
[14] Atassi, M.Z. and Childress, C. (2005) Oxygen-binding heme complexes of peptides designed to mimic the heme environment of myoglobin and hemoglobin. Protein J. 24, 37–49.
[15] Gilles-Gonzalez, M.A., Gonzalez, G., Perutz, M.F., Kiger, L., Marden, M.C. and Poyart, C. (1994) Heme-based sensors, exemplified by the kinase FixL, are a new class of heme protein with distinctive ligand binding and autoxidation. Biochemistry 33, 8067–8073.
[16] Taylor, B.L. and Zhulin, I.B. (1999) PAS domains: internal sensors of oxygen, redox potential, and light. Microbiol. Mol. Biol. Rev. 63, 479–506.
[17] Davis, L.H., Otto, E. and Bennett, V. (1991) Specific 33-residue repeat(S) of erythrocyte ankyrin associate with the anion-exchanger. J. Biol. Chem. 266, 11163–11169.
[18] Shull, M.M., Pugh, D.G. and Lingrel, J.B. (1989) Characterization of the human Na, K-Atpase alpha-2 gene and identification of intragenic restriction fragment length polymorphisms. J. Biol. Chem. 264, 17532–17543.
[19] Smith, P.R., Saccomani, G., Joe, E.H., Angelides, K.J. and Benos, D.J. (1991) Amiloride-sensitive sodium-channel is linked to the cytoskeleton in renal epithelial-cells. Proc. Natl. Acad. Sci. U.S.A. 88, 6971–6975.
[20] Kordeli, E. and Bennett, V. (1991) Distinct ankyrin isoforms at neuron cell-bodies and nodes of Ranvier resolved using erythrocyte ankyrin deficient mice. J. Cell Biol. 114, 1243–1259.
[21] Bourguignon, L.Y.W. and Jin, H.T. (1995) Identification of the ankyrin-binding domain of the mouse T-lymphoma cell inositol 1,4,5-trisphosphate (Ip3) receptor and its role in the regulation of Ip3-mediated internal Ca2+ release. J. Biol. Chem. 270, 7257–7260.
[22] Kropat, J., Tottey, S., Birkenbihl, R.P., Depege, N., Huijser, P. and Merchant, S. (2005) A regulator of nutritional copper signaling in Chlamydomonas is an SBP domain protein that recognizes the GTAC core of copper response element. Proc. Natl. Acad. Sci. U.S.A. 102, 18730–18735.
[23] Quillin, M.L., Arduini, R.M., Olson, J.S. and Phillips, G.N. (1993) High-resolution crystal structures of distal histidine mutants of sperm whale myoglobin. J. Mol. Biol. 234, 140–155.
[24] Monson, E.K., Ditta, G.S. and Helinski, D.R. (1995) The oxygen sensor protein, FixL, of Rhizobium meliloti. Role of histidine residues in heme binding, phosphorylation, and signal transduction. J. Biol. Chem. 270, 5243–5250.
[25] Balland, V., Bouzhir-Sima, L., Kiger, L., Marden, M.C., Vos, M.H., Liebl, U. and Mattioli, T.A. (2005) Role of arginine 220 in the oxygen sensor FixL from *Bradyrhizobium japonicum*. J. Biol. Chem. 280, 15279–15288.
[26] Taguchi, S. et al. (2004) Binding of oxygen and carbon monoxide to a heme-regulated phosphodiesterase from *Escherichia coli*. Kinetics and infrared spectra of the full-length wild-type enzyme, isolated PAS domain, and Met-95 mutants. J. Biol. Chem. 279, 3340–3347.
[27] Adams, M.W. (1990) The structure and mechanism of iron-hydrogenases. Biochim. Biophys. Acta 1020, 115–145.
[28] Ghirardi, M.L., Togasaki, R.K. and Seibert, M. (1997) Oxygen sensitivity of algal H-2-production. Appl. Biochem. Biotechnol. 63–65, 141–151.
[29] Gilles-Gonzalez, M.A., Caceres, A.I., Sousa, E.H., Tomchick, D.R., Brautigam, C., Gonzalez, C. and Machius, M. (2006) A proximal arginine R206 participates in switching of the *Bradyrhizobium japonicum* FixL oxygen sensor. J. Mol. Biol. 360, 80–89.