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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%