Control of cAMP in Human Cells with a Photoactivated Adenylyl Cyclase

Cyclic-AMP is one of the most important second messengers, regulating many crucial cellular events in both prokaryotes and eukaryotes. The photoactivated adenylate cyclase (PAC) from the photosynthetic cyanobacterium *Oscillatoria acuminata* is a small homodimer eminently suitable for this task, requiring only a simple flavin as chromophore. Here we describe its structure using X-ray crystallography and solution microspectrophotometry. Site-directed mutants show signal transduction over 30 Å across the protein with minimal conformational rearrangement. The use of the protein in living human cells is demonstrated with cAMP-dependent luciferase, showing stability of response to light over many hours and activation cycles.

Naturally occurring light sensor domains are able to control many biological processes such as plant development and the behavior of microbes by utilizing the photochemical response of prosthetic flavins, and in recent years there has been growing interest in understanding and exploiting these proteins for synthetic biology [1]. One of the most studied photoreceptor families contains the BLUF domain [2], which responds to blue light and either regulates the activity of an attached enzyme domain or changes its affinity for a repressor protein. OaPAC, a previously undescribed photoactivated adenylate cyclase from *Oscillatoria acuminata*, is a homodimer of a 366 amino acid residue protein carrying an N-terminal BLUF domain and a C-terminal class III adenylate cyclase (AC) domain. Its small size and substantial activation by light (up to 20-fold over basal levels in the dark) make it of particular interest for biotechnology. OaPAC shows 57% sequence identity with a PAC from the soil bacterium *Beggiatoa*, bPAC, but neither structure has so far been solved by crystallography. The OaPAC structures determined in this study will assist future efforts to create artificial light-regulated control modules as part of a general optogenetic toolkit.

We describe its structure using X-ray crystallography (**Fig. 1a**) and solution microspectrophotometry (**Fig. 1b**, **c**) in showing that the dimer structure is completely different from BlrP1, with the monomers arranged in a parallel fashion, the two BLUF domains sandwiching a pair of helices (α3 and its symmetry mate, **Fig. 1d**) that hold the AC domains distant from the flavins [3]. The two flavin mononucleotide (FMN) binding sites are on opposite sides of the dimer, over 30 Å apart, yet they apparently act cooperatively on the active sites, which are themselves a similar distance from the light-sensing prosthetic groups (**Fig. 1a**). BLUF domains consist of a five-stranded β-sheet flanked by helices [4, 5], and use conserved tyrosine and glutamine residues adjacent to the bound FMN to sense light. The simplest proposed sensing mechanism is a rotamer shift of the glutamine, so that after stimulation this side-chain donates a hydrogen bond to the C4=O carbonyl and accepts one from the tyrosine, whereas in the dark state the glutamine donates to the tyrosine [6].

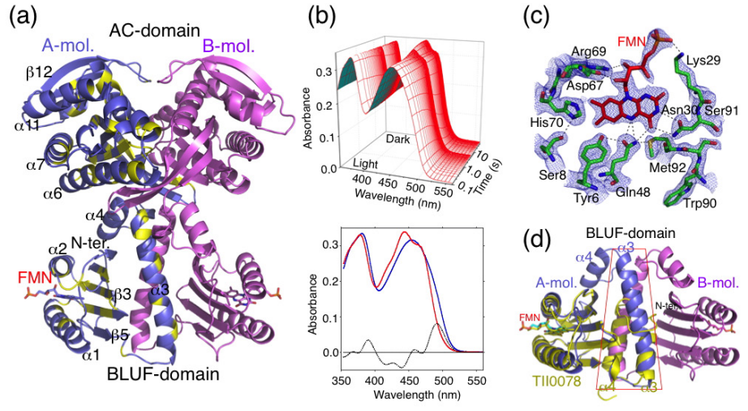

Figure 1: Overall structural properties and solution absorption spectra of OaPAC. **(a)** The chains are shown as ribbons, with the dyad axis vertical and the BLUF domains at the bottom. One monomer is shown in pink, and the other in purple and yellow, yellow indicating conserved residues between OaPAC and bPAC. **(b)** Absorption spectra of OaPAC in solution. (upper panel) Rapid scan spectrophotometry of the switch from light-adapted to dark-adapted conditions, with time shown on a logarithmic scale. (lower panel) The difference is indicated as a dotted black line (red: dark-state, blue: light-state). **(c)** The 1.8 Å 2mFo-DFc electron density map, contoured in purple at 1 σ level in the FMN binding site. **(d)** Superposition of the BLUF domain of OaPAC (monomers shown in purple and pink) with the monomer BLUF domain of TII0078 (yellow). Reprinted with permission from PNAS.

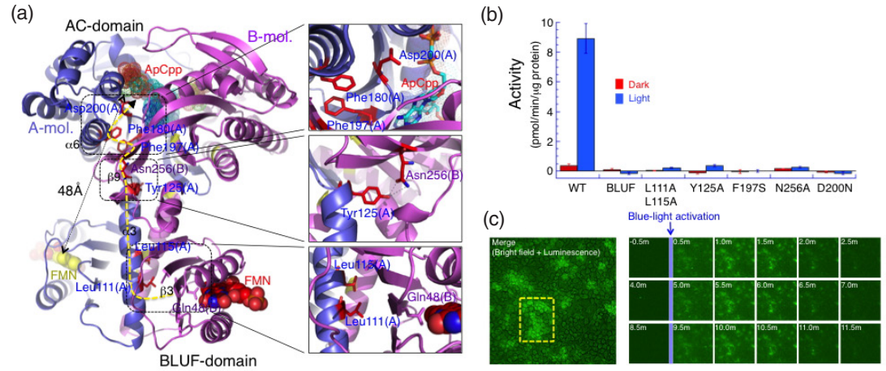

Figure 2: Enzyme assays and proposed photoactivated regulation pathway of OaPAC. **(a)** The positions of mutated residues in the structure. **(b)** Adenylate cyclase activity assay. Activity of wild-type (WT) and mutant OaPAC was determined as described in the Methods section. Red bars indicate cyclase activity under dark conditions, blue bars indicate activity with light exposure. The BLUF domain alone and every mutant indicated showed no significant activity under any conditions. **(c)** OaPAC expressed in HEK 293 (human embryonic kidney) cells was activated by blue light, and the rise in intracellular cAMP was monitored by luminescence imaging of cAMP-dependent luciferase. A merged micrograph of cAMP-dependent luminescence and bright-field image of the cells. Cellular luminescence is shown in green. The dotted square indicates the area selected for quantification of luminescence. Reprinted with permission from PNAS.

To clarify the path by which information travels from the FMN to the active site, a number of mutants were constructed and tested for light-stimulated cyclase activity (**Fig. 2a**, **2b**). The wild-type protein shows low activity in the dark state, and the BLUF domain alone none at all. Cyclase activity requires a metal ion coordinated by Asp 200, and replacing this residue with asparagine abolishes all activity under any conditions. Other mutations between the FMN and active sites are also found to block activation, and are difficult to reconcile with a simple, mechanical conformational control. The α3 helices are tightly associated by hydrophobic faces, with Leu 111 and Leu 115 facing their symmetry partners across the dimer two-fold symmetry axis. To be of use as an optogenetic tool, a light-stimulated cyclase must show steady responses over time. This requires stability of the protein itself as well as constant activation responses to a given stimulus. HEK 293 cells, a cultured cell line derived from human embryonic kidney cells, were used to demonstrate OaPAC function in living tissue. OaPAC was expressed with glosensor-22F, a luciferase-based cAMP reporter, allowing OaPAC stimulation to be measured directly by luminescence (**Fig. 2c**). In each experiment, emitted light was constantly monitored while stimulating blue light was applied in 30-second pulses every 4 minutes. Intracellular cAMP concentration showed a highly reproducible response, rising immediately on blue-light exposure and continuing to rise for a further minute afterwards, before peaking and returning to basal level over the course of several minutes. An identical pattern was observed over a period of hours. The structures determined in this study will assist future efforts to create artificial light-regulated control modules as part of a general optogenetic toolkit.

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BEAMLINES

BL-1A and BL-17A

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