A Noncanonical Chromophore Reveals Structural Rearrangements of the Light-Oxygen-Voltage Domain upon Photoactivation


†School of Chemistry, Cardiff University, Park Place, Cardiff CF10 3AT, United Kingdom
‡School of Medicine, University Hospital Wales, Main Building, Heath Park, Cardiff CF14 4XN, United Kingdom

Supporting Information

ABSTRACT: Light-oxygen-voltage (LOV) domains are increasingly used to engineer photoresponsive biological systems. While the photochemical cycle is well documented, the allosteric mechanism by which formation of a cysteinyl-flavin adduct leads to activation is unclear. Via replacement of flavin mononucleotide (FMN) with 5-deazaflavin mononucleotide (5dFMN) in the Aureochromel1 (Au1a) transcription factor from Ochromonas danica, a thermally stable cysteinyl-5dFMN adduct was generated. High-resolution crystal structures (<2 Å) under different illumination conditions with either FMN or 5dFMN chromophores reveal three conformations of the highly conserved glutamine 293. An allosteric hydrogen bond network linking the chromophore via Gln293 to the auxiliary A′α helix is observed. With FMN, a “flip” of the Gln293 side chain occurs between dark and lit states. 5dFMN cannot hydrogen bond through the CS position and proved to be unable to support Au1a domain dimerization. Under blue light, the Gln293 side chain instead “swings” away in a conformation distal to the chromophore and not previously observed in existing LOV domain structures. Together, the multiple side chain conformations of Gln293 and functional analysis of 5dFMN provide new insight into the structural requirements for LOV domain activation.
Aureochromes comprise a family of LOV domain-containing transcription factors found in photosynthetic stramenophiles that regulate the cell cycle and photomorphogenesis. Au1a consists of an N-terminal unstructured region, followed by a basic leucine zipper (bZIP) domain and a C-terminal LOV domain. This domain topology is inverted compared to those of most other photoreceptors and means that the A′α helix, instead of the C-terminal Jα helix, connects effector and LOV domains. Spectroscopic and biochemical measurements of the isolated LOV domain from Phaeodactylum tricornutum and Vaucheria frigida Au1a suggest that stepwise unfolding of A′α and Jα helices upon illumination results in LOV domain dimerization. Single-crystal X-ray structures of light-grown LOV domain crystals at 2.7 Å suggested the availability of the core β-sheet for use as a dimerization interface. In dark-state structures, this dimerization site is obscured by the A′α helix. Full-length Au1a has resisted crystallization, but small-angle X-ray scattering (SAXS) of constructs whose unstructured region has been truncated shows significant volume changes that suggest intramolecular bZIP–LOV interactions. Steric caging of the bZIP domain may therefore complement LOV domain-driven dimerization, which is proposed to be the driving force behind Aureochrome DNA binding. Here, we present functional analysis and the first high-resolution crystal structures of a LOV domain with 5dFMN, identifying three conformations for Gln293 of Au1a and the allosteric network linking the chromophore to the A′α helix. This glutamine is widely conserved among LOV domains, and as there are several examples in which truncations of the A′α helix directly influence effectors connected through the Jα helix, these results may have wider implications beyond the Au1a family.

## MATERIALS AND METHODS

### Protein Expression and Purification

Standard molecular biology techniques were employed to generate OdAu1αLOV and OdAu1αbZIPLOV constructs from the wild-type O. danica Au1α gene (UniProt, CSNSW6_OCHDN) using oligonucleotides detailed in Table S1. OdAu1α-derived proteins were obtained by heterologous expression in BL21 (DE3) Escherichia coli in either minimal and autoinduction medium supplemented with glucose [1% (w/v)] and kanamycin (50–100 µg/mL). Cultures were grown at 37 °C until an OD₆₀₀ of 0.8 was reached, induced with isopentenyl thiogalactose (0.5 mM, IPTG), Melford), and grown at 25 °C for a further 16 h. OdAu1α proteins were purified by Ni²⁺-NTA (5 mL, GE Healthcare) affinity chromatography followed by Resource Q anion exchange (GE Healthcare) chromatography using purification buffer: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 20 mM, pH 7.8), sodium chloride [20 mM (OdAu1αLOV) or 150 mM (OdAu1αbZIPLOV)], tris(carboxymethyl) phosphine (TCEP, 0.3 mM), and gradients of imidazole (from 20 to 500 mM) and sodium chloride (from 0 to 1 M). Chromophore exchange was performed by applying the clarified cell lysate to Ni²⁺-NTA resin (5 mL, GE Healthcare) and washing with 5 column volumes of purification buffer. Proteins were partially unfolded by passing this buffer supplemented with guanidine hydrochloride (6 M) over the resin. To complete FMN elution, a guanidinium thiocyanate switching have been proposed. MD simulations of phototropin LOV domains generated a different conformation for the conserved glutamine side chain, altering the hydrogen bonding network to flanking helices. Other recent reports propose that further glutamine side chain orientations are involved in LOV domain activation through hydrogen bonds with O4 of the flavin ring. Given the importance of the potential hydrogen bonding associated with N5 of the flavin and the challenges associated with studying the lit state of thermally reverting LOV domains, we used S-deazaflavin mononucleotide [5dFMN (Figure 1B)], an analogue that had previously been suggested to form a stable photochemical cysteinyl-flavin adduct in BsYtvA and successfully employed to alter the redox potentials of other flavoproteins. At present, there are no experimental data to indicate whether the lit states of 5dFMN-containing LOV photoreceptors function like FMN-containing examples. We therefore decided to examine the effect of 5dFMN incorporation on the photochemistry and function of Aureochromela1 (Au1a) of Ochromonas danica.

Figure 1. (A) Formation of a cysteinyl-FMN covalent adduct occurs upon absorption of blue light by flavin mononucleotide (FMN). Spontaneous thermal reversion re-forms the dark-adapted state. (B) Structure of S-deazaflavin mononucleotide (5dFMN) with a carbon atom (blue) at position S. (C) Domain topology of O. danica Aureochromela1. Au1αbZIPLOV comprises bZIP and LOV domains, and Au1αLOV comprises only the LOV domain. UV–vis spectra of thermal reversion from the lit to dark state of (D) FMN-containing (red–green) and (E) 5dFMN-containing (orange–blue) OdAu1αLOV. Spectra were recorded every hour for the first 3 h and then every 2 h. Reversion kinetics were monitored at 448 nm for FMN-containing OdAu1αLOV and 406 nm for 5dFMN-containing OdAu1αLOV. Lit-state FMN OdAu1αLOV reverts to its dark state with a half-life of 112 min. No reversion to the dark state is observed for lit-state 5dFMN-Au1αLOV.

Biochemistry 2019, 58, 2608–2616

DOI: 10.1021/acs.biochem.9b00255
solution (3 M) was applied until no flavin was observed in the eluent by ultraviolet–visible (UV–vis) spectroscopy. Proteins were refolded by sequentially applying lower concentrations of guanidine hydrochloride (one column volumes of concentrations of 6, 5, 4, 3, 2, and 0 M). The resin was then washed with 5 column volumes of purification buffer, followed by incubation with 1 column volume of purification buffer containing 5dFMN (0.1–0.5 mM) for 30 min. Protein samples were eluted and then further purified as previously described.

Solution Characterization. For circular dichroism (CD) experiments, purified protein samples were dialyzed overnight at 4 °C against potassium phosphate buffer (10 mM, pH 7.0). Spectra were collected with an Applied Photophysics Chirascan spectrophotometer. For analytical gel filtration experiments, protein samples were exchanged into gel filtration buffer [HEPES (20 mM, pH 7.4), sodium chloride (100 mM), magnesium chloride (10 mM), and TCEP (0.3 mM)] in centrifugal filter columns. All protein samples were handled in dim red light. For photoactivation, protein samples were illuminated with 450 nm light-emitting diodes (LEDs) until a steady state was reached as determined by UV–vis spectroscopy. For gel filtration experiments, analytical gel filtration columns were either wrapped in aluminum foil for dark experiments or illuminated with 450 nm LEDs for lit-state experiments. For nuclear magnetic resonance (NMR) studies, protein samples were transferred into clear NMR tubes and into amber-colored NMR tubes. For lit-state experiments, protein samples were transferred into 450 nm LEDs for lit-state experiments. For dark-state experiments, protein samples were transferred into clear NMR tubes and illuminated with 450 nm LEDs. NMR spectra were recorded on a DPX-600 MHz Bruker NMR spectrometer equipped with a cryo probe and preamplifiers.

DNA Binding. Light-dependent DNA binding was characterized by electrophoretic mobility gel shift assays (EMSA) using TAMRA-labeled double-stranded DNA containing an OdAu1a recognition site (5′-TGAGGGTC-GACGTGGTTCCCAC-3′). EMSA experiments were performed at 4 °C. Dark-state experiments were performed in a room illuminated by dim red light, while for lit-state experiments, protein samples were illuminated for 5 min prior to commencing the experiment and throughout the electrophoresis experiment with 450 nm LEDs. Gels were imaged using a Bio-Rad ChemiDoc MP system (Bio-Rad Laboratories) and software provided by the manufacturer.

Crystallography. Purified FMN- or 5dFMN-containing OdAu1aLOV was exchanged into crystallization buffer [2-(N-morpholino)-ethanesulfonic acid (MES, 20 mM, pH 6.0), sodium chloride (100 mM), magnesium chloride (20 mM), sodium acetate (20 mM), dithiothreitol (DTT, 5 mM), and EDTA (5 mM)] and concentrated to 10–15 mg/mL. Dark-state crystals were grown in plates wrapped with aluminum foil by the hanging drop method. Drops consisted of protein (2 μL, 10 mg/mL) mixed with a reservoir solution [2 μL, 10–20% (w/v) polyethylene glycol (PEG) with an average molecular weight of 2000 or 3000, ammonium chloride (0.1 M), and sodium acetate (0.1 M, pH 4.5–4.9) or disodium citrate, (0.1 M, pH 4.5–4.9)] suspended over further reservoir buffer (100 μL) in 96-well plates (Screw Top Hanging Drop Plate, Molecular Dimensions). Crystal growth was usually evident after 16 h with maximum growth observed after 7 days. For dark-state structures, crystals were cryoprotected with ethylene glycol, harvested, and flash-frozen in liquid nitrogen under dim red light. For illumination experiments, crystals were illuminated with 450 nm LEDs for 30 min, cryoprotected with ethylene glycol, harvested, and flash-frozen in liquid nitrogen. Light-grown crystals were obtained by mixing light-state FMN- or 5dFMN-containing OdAu1aLOV (1.7 μL of a 15 mg/mL solution) with a reservoir solution [2 μL, disodium malonate (1.5–3 M, pH 7.0) and TRIS acetate (0.1 M, pH 7.5–8.0)]. Drops were supplemented with hexaamine cobalt(III) (0.3 μL, 0.1 M). Crystals were grown under blue light and appeared after 1–7 days. Crystals were harvested without cryoprotection and flash-frozen in liquid nitrogen. Data sets were collected from a single crystal each at the Diamond Light Source synchrotron at beamlines I02, 103, and I24. Initial structures were obtained from PhaserSS or MolReps55,56 using the dark-state Phacocystis tricornum Au1abZIP domain (Protein Data Bank entry SAB8) as a search model. Structures were determined by subjecting initial models to cycles of model building with COOT57 and refinement using REFMACS.55 For final Rwork and Rfree values, see Tables S4 and S5.

RESULTS

OdAu1a with 5dFMN Incorporated Forms a Thermally Stable Cysteinyl-Flavin Adduct. To investigate the mechanism of LOV domain activation and the effects of introducing 5dFMN (Figure 1B), two truncated versions of O. danica Au1a containing the isolated LOV domain (OdAu1aLOV) and the LOV domain with the DNA binding bZIP domain (OdAu1abZIPLOV) were constructed. The FMN cofactor of the expressed proteins was replaced with 5dFMN by binding the protein to Ni-NTA resin and washing with guanidine thiocyanate followed by removal of the denaturant and incubation with 5dFMN. Refolding of OdAu1aLOV and OdAu1abZIPLOV in the presence of 5dFMN produced the characteristic blue-shifted vibrational triplet of oxidized 5dFMN with absorbance maxima at 385, 406, and 423 nm (Figure 1E and Figure S1C–F).41 Comparing the absorbance at 406 and 475 nm indicated that >99% of the cofactor had been exchanged. OdAu1aLOV containing FMN reverted from its lit state to its dark state, with a half-life of 112 min, but no reversion was observed for OdAu1aLOV containing 5dFMN even after 7 days (Figure S1C,D). Cycling between lit and dark states using 450 and 330 nm light was possible with no significant photobleaching for at least five cycles (Figure S1E,F). The stability of the 5dFMN adduct was further demonstrated in liquid chromatography–mass spectrometry experiments in which species corresponding to the stable covalent cysteinyl-5dFMN conjugate for OdAu1aLOV were observed but no cysteinyl-FMN conjugates were evident (Figures S2 and S3).

FMN to 5dFMN Exchange Prevents Light-Induced Dimerization of OdAu1aLOV. 1H–15N heteronuclear single-quantum coherence NMR spectra of 15N-labeled proteins confirmed that refolding with 5dFMN did not lead to any large-scale structural perturbation. Illumination of OdAu1aLOV bound to 5dFMN resulted in chemical shift perturbations similar to those observed for FMN (Figure S4). CD spectra also indicated that the secondary structure after refolding of 5dFMN-containing OdAu1aLOV was the same as that of native OdAu1aLOV (Figure S5). Both FMN- and 5dFMN-containing

DOI: 10.1021/acs.biochem.9b00255
Biochemistry 2019, 58, 2608–2616
OdAu1αLOV samples exhibited changes in their CD spectra when photoactivated. FMN-containing OdAu1αLOV displayed a 14.2 ± 0.8% decrease in mean residue ellipticity at its 220 nm minimum (Figure 2A and Figure S5C), whereas 5dFMN-containing OdAu1αLOV showed a 5.1 ± 0.6% decrease at 280 nm (Figure 2B and Figure S5D). Size-exclusion chromatography of OdAu1αLOV unique samples showed a peak at a lower elution volume (12.8 mL) for the dark-state condition, whereas the illuminated state eluted at a higher volume (13.5 mL) (Figure 2C). FMN-containing OdAu1αLOV samples appeared to elute uniformly at volumes consistent with a dimer [200 to 10 μM (Figure 2D)]. Although 5dFMN incorporation did not affect the protein’s overall dimerization properties, the size-exclusion chromatography showed a shift to a higher elution volume for the 5dFMN-containing OdAu1αLOV construct, indicating a potential change in protein-protein interactions.

Electrophoretic mobility shift assays were used to examine DNA binding by OdAu1αabZIPLOV containing FMN or 5dFMN in the dark and under illuminated conditions (Figure 2E–H). Lit-state FMN-containing OdAu1αabZIPLOV uniquely showed a slowly migrating “supershifted” band (Figure 2E), while experiments with dark-state FMN (Figure 2F) and both dark and illuminated 5dFMN (Figure 2G,H) showed evidence of only a single slower-migrating shifted band. Having demonstrated that 5dFMN incorporation creates a protein that can form a stable cysteinyl-5dFMN adduct that shows some structural features of a lit-state FMN-containing protein but with incomplete control over the longer-range interactions that direct DNA binding and dimer stability. 5dFMN retained the capacity to induce OdAu1αLOV dimerization in response to light that has been shown for other isolated Aureochrome1α LOV domains.21,22,49,50 FMN-containing OdAu1αLOV (100 μM) showed clear light-dependent dimerization as determined by size-exclusion chromatography. Dark-state FMN-containing OdAu1αLOV eluted at 13.7 mL with an estimated mass of 20.4 kDa with a slight shoulder toward a larger volume, whereas the lit-state equivalent elution maxima shifted to 12.8 mL in agreement with dimerization (Figure 2C). However, 100 μM 5dFMN-containing OdAu1αLOV showed very little shift of the elution volume with peaks at 13.5 and 13.3 mL for the dark and lit states, respectively (Figure 2D). Such an intermediate elution volume most likely represented a monomer–dimer exchange on the time scale of the size-exclusion experiments.

To probe this observation further, we conducted concentration-dependent experiments. The position of the lit-state 5dFMN-containing OdAu1αLOV peak was strongly concentration-dependent (Figure S6), showing earlier elution at higher concentrations. In an identical concentration range, equivalent FMN-containing samples appeared to elute uniformly at volumes consistent with a dimer [200 to 10 μM (Figure S6)]. Although 5dFMN was unable to effect efficient dimerization in OdAu1αLOV, the longer OdAu1αabZIPLOV construct consistently eluted from the size-exclusion column at volumes corresponding to a dimer with both cofactors in the dark and lit states (Figures S7–S9). Strong DNA binding was observed in both states regardless of the cofactor used for 50 μM protein samples. To further probe the light responsiveness of 5dFMN, lower concentrations were utilized in DNA binding assays.

Electrophoretic mobility shift assays were used to examine DNA binding by OdAu1αabZIPLOV containing FMN or 5dFMN in the dark and under illuminated conditions (Figure 2E–H). Lit-state FMN-containing OdAu1αabZIPLOV uniquely showed a slowly migrating “supershifted” band (Figure 2E), while experiments with dark-state FMN (Figure 2F) and both dark and illuminated 5dFMN (Figure 2G,H) showed evidence of only a single slower-migrating shifted band. Having demonstrated that 5dFMN incorporation creates a protein that can form a stable cysteinyl-5dFMN adduct that shows some structural features of a lit-state FMN-containing protein but with incomplete control over the longer-range interactions that direct DNA binding and dimer stability.

5dFMN Forms Cysteinyl-Flavin Adducts at the C4a Position but Induces No Rearrangement of the A’α Helix. To understand how 5dFMN can mimic FMN photochemistry but is incapable of complete OdAu1α activation, high-resolution single-crystal X-ray structures for FMN- and 5dFMN-containing OdAu1αLOV, were determined for crystals grown in the dark, in the dark and then illuminated with blue light (“illuminated”), and under steady strong blue-light exposure (“light-grown”). The highest-resolution structure of dark-state FMN-containing OdAu1αLOV was obtained at 1.37 Å from a single crystal in space group P2₁,2,2, with four monomers per asymmetric unit as parallel dimers (Figure 3A).
Crystals in space group $P3_121$ were also observed, but these diffracted poorly. A 1.97 Å structure of dark-state 5dFMN-containing OdAu1aLOV was obtained from crystals grown under identical conditions in space group $P3_121$ indicating a parallel dimer per asymmetric unit (Figure 3D). The identity of the cofactor had little effect on the overall LOV domain structure or the chromophore binding pocket (Figure 4A,D), confirming an identical mode of chromophore binding and no rearrangement of the surrounding environment. When dark-grown crystals were illuminated, the space group changed to $P6_22$ with a single monomer per asymmetric unit (Figure 3B,E). However, once symmetry partners were considered, symmetrical dimers almost identical to the dark-adapted state could be identified with symmetry equivalents. Inspection of the cofactor binding site of the illuminated crystals showed electron density for approximately 30% occupancy of a covalent bond between Cys230 and the cofactor for both FMN and 5dFMN structures (Figure 4B,E). This occupancy that is significantly lower than indicated by UV−vis spectroscopy and MS (Figure 1D,E, and S10) is likely to be the result of a photochemical scission of the covalent adduct during data collection as reported for other LOV domain proteins.29 Although the usual approach under such circumstances is to record multiple data sets from a single crystal, this usually yields much poorer resolution and was therefore not attempted. We hypothesized that higher-resolution data sets could provide unique insights into structural change. To ensure minimal bias in the cycles of structural refinement, we modeled covalent adduct structure at 30% occupancy (cysteinyl-flavin photoadduct) and the dark state at 70% occupancy, yielding two flavin and cysteine orientations. Electron density for a partial occupancy of a cysteinyl-flavin adduct at the C4a position of the isoalloxazine ring for 5dFMN-containing OdAu1aLOV was clearly observed, confirming that 5dFMN forms a photochemical adduct structurally equivalent to the native chromophore.

Figure 3. Dimer arrangements for X-ray crystal structures of FMN- and 5dFMN-containing OdAu1aLOV under dark (left), illuminated (middle), and light-grown (right) conditions. (A) The 1.37 Å structure of dark-state FMN-containing OdAu1a. The asymmetric unit contained four monomers as parallel dimers (green) with $\alpha\alpha$ positioned across the $\beta$-sheet surface (half black box). Loops of each monomer lie close to each other (black curved line). (B) The 1.50 Å structure of illuminated crystals of dark-grown FMN-containing OdAu1a. The asymmetric unit contained a single monomer (pink), forming a parallel dimer similar to that in the dark state when considering a symmetry equivalent (gray). (C) The 1.66 Å structure of light-grown FMN-containing OdAu1a featuring a unique dimer arrangement with $\alpha\alpha$ being repositioned across a $\beta$-sheet surface (black box) and loop region rearrangement (dashed arrow). (D) The 1.97 Å structure of dark-state 5dFMN-containing OdAu1a with a dimer similar to dark-state FMN. (E) The 1.43 Å structure of illuminated 5dFMN-containing OdAu1a with a symmetry partner equivalent to a dimer colored gray. (F) The 2.00 Å structure of light-grown 5dFMN-containing OdAu1A showing a similar loop (black curved line) and $\alpha\alpha$ helix arrangement (black box) as for dark-state and illuminated proteins with a symmetry equivalent colored gray.

Figure 4. Electron density maps (gray mesh) for FMN or 5dFMN, Cys230, and residues forming hydrogen bonding networks among O4 of FMN, Asn272, Gln293, and Asn194 are displayed at the $\sigma = 1$ level. Partial occupancies are colored by characteristic conformations observed for dark-state (green), illuminated (orange), or light-grown (purple) structures. Yellow dashed lines indicate predicted hydrogen bonding. (A) Dark-state FMN (1.36 Å). (B) Illuminated FMN (1.50 Å). (C) Light-grown FMN (1.67 Å). (D) Dark-state 5dFMN (1.97 Å). (E) Illuminated 5dFMN (1.43 Å). (F) Light-grown 5dFMN (2.00 Å).
Light-grown crystals could not be obtained under the conditions used for the dark state, but alternative conditions produced monoclinic crystals in space group C121 for the FMN sample and hexagonal crystals in space group P6₁22 for 5dFMN. For light-grown FMN-containing OdAu1a₅LOV, no cysteinyl-FMN adduct was evident in the electron density map. Electron density corresponding to a cysteinyl-5dFMN adduct was observed but was less prominent than in maps from illuminated crystals. Notably, light-grown FMN-containing OdAu1a₅LOV contained four monomers per asymmetric unit as parallel dimers. Two of the monomers appeared to be identical to the dark-state structure, while the second pair showed a different Aα arrangement across the β-sheet surface. Compared with the dark-state structures, a change in the relative positions of strand βf (287–293) of the β-sheet and Aα (183–189) of 12° is observed. This rearrangement does not occur in illuminated structures, probably due to crystal lattice constraints. Light-grown 5dFMN-containing OdAu1a₅LOV maintained a single monomer per asymmetric unit, resembling the arrangement of illuminated structures. This supported solution data that although 5dFMN undergoes photochemistry similar to that of FMN, it is unable to fully activate OdAu1a₅LOV. Taken together, this suggests that the Aα rearrangement, observed for only light-grown FMN-containing OdAu1a₅LOV, could correlate with dimerization in solution (Figure 3A,C).

**Adduct Formation Populates Different Conformations of Gln293.** Dark-state structures gave single well-defined populations of Gln293, Asn272, and Asn194 for both FMN and 5dFMN, but close examination of electron density maps from illuminated and light-grown conditions yielded multiple conformations for these residues (Figure 4). In dark-state structures, Gln293 lies close to the chromophore and forms a probable hydrogen bond to the O4 position (Figure 4A,D). For illuminated structures, a 20% occupancy of a new conformation of Gln293, with its side chain away from the FMN binding pocket, was evident (Figure S22). Additional conformations of Asn194 and Asn272 are also observed. Formation of a new hydrogen bond network among these three residues creates a route for the conformation of Gln293 to be communicated to the Aα helix through Asn194 (Figure 4B,E), which is located in the loop connecting Aα with the LOV domain core. Examination of the FMN binding pocket of the light-grown crystal structure revealed a third arrangement for Gln293 and Asn194. In the parallel dimer with a unique Aα arrangement, Asn194 moves in toward Gln293. This coincides with a probable change in the orientation of the Gln293 side chain and the polarity of the hydrogen bond network due to flavin protonation (Figure 4C). In contrast, 5dFMN-containing OdAu1a₅LOV did not form this “flip” conformation but closely resembled the structure of illuminated 5dFMN-containing OdAu1a₅LOV with identical Asn194 and Gln293 conformations (Figure 4F). This suggests that the structural changes that we observed were not an artifact of different crystallization conditions, as identical space groups were achieved for 5dFMN under both conditions. It appears that only growing crystals of FMN-containing protein under constant illumination allow the structural reorientation of the allosteric Aα helix to support dimer rearrangement (Figure 3).

**DISCUSSION**

Reconstitution of truncated versions of the light-dependent transcription factor OdAu1a₅LOV with 5dFMN led to proteins that undergo photoadduct formation to produce a thermally stable cysteinyl-5dFMN adduct. Despite clear evidence of adduct formation captured by UV spectroscopy and mass spectrometry and subsequent light-induced changes determined by CD and NMR spectroscopy, 5dFMN-containing OdAu1a₅LOV does not dimerize under the conditions where dimerization of FMN OdAu1a₅LOV occurs. High-resolution crystal structures show identical FMN and 5dFMN binding modes in dark-grown crystals, suggestive of identical chromophore binding characteristics. Crystal structures of illuminated and light-grown OdAu1a₅LOV containing 5dFMN provide conclusive evidence of cysteinyl photoadduct formation at the C4a position in apparent support of the radical-based mechanism proposed for the native chromophore. However, in our and other published light-grown structures, the distance between Gln293 and N5 of FMN is longer than might be expected for a hydrogen bond (~3 Å). 5dFMN forms the equivalent covalent adduct, but in contrast to FMN, Gln293 does not appear to “flip” in...
light-grown crystals (Figure 4C,F). Taken with 5dFMN’s inability to induce light-dependent dimerization, this furthermore suggests that N5 protonation is a prerequisite for locking the lit-state conformation of OdAu1αLOV. Comparison of illuminated and light-grown FMN-containing OdAu1αLOV structures (Figure 4B,C) suggests that a key aspect of the Gln293 “flip” is to engage an alternative conformation of the side chain of Asn194, located between the Aβ strand and Aα helix, in hydrogen bonding. This change in the Asn194 conformation may be the key to propagating the effects of adduct formation beyond the LOV domain, by rearranging the domain to favor dimerization and/or by releasing the Aα helix. Notably, this key Asn residue is also found in other Au1a homologues34,49,50 and in AsLOV2.52

To the best of our knowledge, our structures of illuminated crystals of 5dFMN-containing OdAu1αLOV provide the first experimental evidence for a further arrangement of Gln293 and Asn194. For both FMN and 5dFMN, Gln293 “swings” away from the chromophore when illuminated (Figure 5). The persistence of a “swing” conformation in 5dFMN light-grown crystals suggests that it is not a crystallographic artifact generated by illuminating LOV domains trapped in the crystal lattice or a product of a different space group due to changes in crystallographic conditions but that 5dFMN is unable to support progress to the “flip” state. CD measurements indicate that illumination of 5dFMN-containing OdAu1αLOV results in some secondary structural changes, although not to the extent seen with FMN. Likewise, size-exclusion chromatography revealed intermediate changes upon illumination for 5dFMN. These results infer the “swing” conformation of Gln293, formed in the absence of larger-scale secondary structural changes, provides a degree of activation. It therefore seems probable that the “swing” state represents an intermediate stage in activation rather than an unproductive conformation (Figure 5).

The key determinants of success for LOV domain-based optogenetic systems are the dynamic ranges of affinities and activities in the dark and lit states. Most previous work has relied on using molecular modeling to guide alterations to helix docking propensities to improve the dynamic range of optogenetic tools. Here we provide experimental insight into the molecular basis of LOV domain photoactivation. The nature of the “swing” state may be crucial for improving LOV domains by rational design; if the “swing” state is an on-path intermediate, then encouraging its formation is of key importance, placing a greater emphasis on the O4=Gln293−Asn194 axis for initial activation. This is in agreement with MD simulations and Fourier transform infrared spectroscopy experiments that identified hydrogen bond changes to O4 of the FMN ring being important for the regulation of LOV domain activation especially in the early stages after photoduction formation.33−37,39,40 Indeed, results that inferred a role for N5 protonation by generation of a neutral flavin semiquinone radical33,51 and N5-protonated reduced flavins52 also predict significant polarity changes at O4.44 Alternatively, if the “swing” state is an off-path intermediate, its destabilization may lead to improved switches with higher dynamic ranges. Understanding these fundamental aspects of domain activation has a potentially enormous impact for the design of new tools based on LOV domains and may allow researchers to improve the performance of designed LOV domains with multiple optogenetic applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.9b00255.

Experimental procedures, including purification, characterization, and crystallization methods; UV−vis spectroscopy, mass spectrometry, NMR, CD, and SEC data of OdAu1a (Figures S1−S10, S22) and 5-deazaflavin mononucleotide chemoenzymatic synthesis (Figures S11−S21); and tables of DNA oligonucleotides, protein sequences, and structural refinement statistics (Tables S4 and S5) (PDF)

Accession Codes

Protein Data Bank entries 6l20, 6l21, 6l22, 6l23, 6l24, and 6l25.

AUTHOR INFORMATION

Corresponding Author

*E-mail: allemannrk@cardiff.ac.uk.

ACKNOWLEDGMENTS

The authors thank Dr. Harald Janovjak (IST Austria) for providing the DNA encoding full-length O. danica Aureochrome1a, Dr. Yi Jin for help with structure determination and helpful discussions, and Tom Williams for assistance with mass spectrometry. The authors thank the Diamond Light Source for beamtime (Proposal mx14843) and the staff of beamlines I02, I03, and I24 for assistance with data collection.

ABBREVIATIONS

OdAu1a, O. danica Aureochrome1a; LOV, light-oxygen-voltage; bZIP, basic leucine zipper; FMN, flavin mononucleotide; PAS, Per-ARNT-Sim; 5dFMN, 5-deazaflavin mononucleotide; CD, circular dichroism; SAXS, small-angle X-ray scattering; MD, molecular dynamics; NMR, nuclear magnetic resonance.

REFERENCES


