Photocontrolled Exposure of Pro-apoptotic Peptide Sequences in LOV Proteins Modulates Bcl-2 Family Interactions

Robert J. Mart, Dilruba Meah, and Rudolf. K. Allemann*[a]

LOV domains act as biomolecular sensors for light, oxygen, or the environment’s redox potential. Conformational changes upon the formation of a covalent cysteinyl flavin adduct are propagated through hydrogen-bonding networks in the core of designed hybrid phototropin LOV2 domains that incorporate the Bcl homology region 3 (BH3) of the key pro-apoptotic protein BH3-interacting-domain death agonist (BID). The resulting change in conformation of a flanking amphiphilic α-helix creates a light-dependent optogenetic tool for the modulation of interactions with the anti-apoptotic B-cell leukemia-2 (Bcl-2) family member Bcl-xL.

Light-oxygen-voltage (LOV) domains are molecular switches that act as internal sensors of oxygen, redox potential and light in cells.[1–3] Some LOV domains function as reversible photoswitches[4] and underpin a range of blue-light responses in plants, fungi and bacteria including phototropism[5, 6] and regulation of the circadian rhythm.[7] LOV photosensors share a common mechanism by which a noncovalently bound flavin cofactor absorbs blue light (450–475 nm) to enter an excited electronic state; this leads to the formation of a covalent adduct between flavin mononucleotide (FMN) and the sulfur atom of a cysteine residue,[8] and significant conformational changes occur.[9] The FMN adduct spontaneously reverts to its noncovalently bonded dark state with rates that reflect the function of the individual LOV domain. Phototropin was one of the first blue-light receptors discovered in plants, and LOV2 from Avena sativa (AsLOV2) has previously been used for phototropin engineering. In its dark form, the C-terminal Jα helix of AsLOV2 is tightly bound to the β-sheet (Figure 1), but upon light-activated adduct formation, the 20 residues of the C-terminal Jα helix are displaced from the β-sheet, thereby exposing the amphiphilic helix (Figure 2).[10–12]

LOV domains have been used to create light-responsive DNA-binding motifs[13–15] and transcriptional activators,[16, 17] as well as to control the activity of enzymes[18, 19] and the subcellular location[20] and degradation rates[21] of proteins by domain fusion or insertion. We have previously modified peptide sequences from proapoptotic proteins with azobenzene crosslinkers to create biomolecular nanoswitches (BNs), whose conformations and binding properties change in response to light.[22–24] A LOV-derived protein of equivalent functionality could be genetically encoded and photoactivated in vivo through transient expression or gene integration. A LOV/caspase 7 hybrid has previously been shown to cause cell death;

Figure 1. Overall structure of Avena sativa Phototropin 1 LOV2 domain (PDB ID: 2V1A)[12] with the Jα helix in green.

Figure 2. Cartoon illustrating the dark and irradiated forms of AsLOV2 and the four hybrids between AsLOV2 and BID, LOV-BID1 to LOV-BID4, with different locations of the BH3 recognition element of BID (red) within the Jα helix of AsLOV2 (green).

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however, overexpression of Bcl-2, which is common in many
types of cancer cells, diminished its proapoptotic effect.\(^{[25]}\)

Previous work has sought to maximise the dynamic range
between the light- and dark-state affinities of LOV–peptide fu-
sions,\(^{[21,26]}\) but controlling potentially irreversible apoptotic pro-
cesses with an expressed protein requires stringent “caging” of
the active epitope. Caging efficiency is affected by the position
of the binding epitope in the Jx helix; residues incorporated
closer to the body of the protein are better caged in the dark
state but pay a steric penalty in the light state. Well-
characterised protein–protein interaction motifs have
been introduced into LOV domains to generate ge-
eric photoassociation tools. Incorporating an amino
acid sequence that is strongly bound by PSD-95/discs
large/zona occludens 1 (PDZ) domains into sites be-

tween residues 540 and 545 of the Jx helix of
AsLOV2 (Table S1) led to proteins with increased af-
finities for PDZ in the light-activated state.\(^{[27]}\) Introdu-
cing the recognition sequence at residue 542 led to the
widest dynamic range between dark-
and light-state affinities. A protein database search re-
vealed that peptide sequences similar to AsLOV2 Jx
have been crystallised bound to interacting part-
ners.\(^{[28]}\) Elements of one such sequence, the SsrA
peptide, were incorporated at residues 523, 535, 538
and 542 of the Jx helix of AsLOV2. The abilities of
these proteins to bind SspB, the cognate partner of
SsrA, were compared by fluorescence polarisation; whilst
the sequence inserted at 538 showed the tightest binding affinity,
insertion at 542 led to the greatest difference between light
and dark states. Proteins could be marked for light-dependent
proteasomal degradation by inserting a four-amino acid de-
grons, RRRG, at residue 543 of
AsLOV2 (Table S1) to generate LOV–peptide fu-
dictate specificity for anti-apoptotic protein subfamilies.\(^{[31]}\) We

<table>
<thead>
<tr>
<th>Protein (^a)</th>
<th>Partial sequence (Jx region)</th>
<th>(T_{1/2}) [°C]</th>
<th>(t_{1/2}^{UV}) [min]</th>
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</thead>
<tbody>
<tr>
<td>His(_{6})-AsLOV2</td>
<td>DAAERGVLKKA/NIARHLAQVGDSIDRSI</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td>BID BH3</td>
<td>DAAERGVLKKA/NIARHLAQVGDSIDRSI</td>
<td>105</td>
<td>101</td>
</tr>
<tr>
<td>LOV-BID1</td>
<td>DAAERGVLKKA/NIARHLAQVGDSIDRSI</td>
<td>104</td>
<td>100</td>
</tr>
<tr>
<td>LOV-BID2</td>
<td>DAAERGVLKKA/NIARHLAQVGDSIDRSI</td>
<td>103</td>
<td>80</td>
</tr>
<tr>
<td>LOV-BID3</td>
<td>DAAERGVLKKA/NIARHLAQVGDSIDRSI</td>
<td>102</td>
<td>75</td>
</tr>
<tr>
<td>LOV-BID4</td>
<td>DAAERGVLKKA/NIARHLAQVGDSIDRSI</td>
<td>101</td>
<td>70</td>
</tr>
</tbody>
</table>

\(^{[a]}\) All LOV-BID proteins described include the V416I mutation to stabilise the cystein-
yl-FMN adduct. |

Despite these compromises, a fluorescently labelled peptide
corresponding to the proposed Jx region of the hybrid LOV-
BID1 (Table 1) showed only a twofold reduction in affinity for
Bcl-x\(_L\) (\(K_D = 46 \pm 2.6\) nM) relative to the parent sequence
(Table S4). A plasmid containing DNA encoding AsLOV2 fused
to the C terminus of a domain used for affinity purification,
hisactophilin-C495,\(^{[32]}\) was used to construct hybrids between
AsLOV2 and BID BH3. Although wild-type Hisact-AsLOV2 re-

tered with a half-life of approximately 1 min after photoactiva-
tion, a mutant with a different hydrophobic side chain near the
active site, Hisact-AsLOV2-V416L, had a significantly longer-
lived (\(t_{1/2} = 7.7\) min) photoactivated state (Table S5).\(^{[33\text{--}34]}\) The hi-
sactophilin prosthetic domain was replaced by a His-tag, and
BH3-like sequences were added at different positions of the Jx
helix (Table 1) to generate LOV-BID1–4. Solutions of these hy-

\(10.4 (LOV-BID1) \text{ and } 7.5 \text{ min (LOV-BID3; Table 1 and S5). }\)

The thermal stabilities of the hybrids, as measured by CD
spectroscopy, decreased from LOV-BID1 to LOV-BID4 (Table 1).

The degree of structural change shown by CD spectroscopy
upon photoactivation is strongly reduced in LOV-BID1 com-
pared to AsLOV2-V416L (Figure 3). Comparison of the mean resi-

\(10.4 (LOV-BID1) \text{ and } 7.5 \text{ min (LOV-BID3; Table 1 and S5). }\)

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pared to AsLOV2-V416L (Figure 3). Comparison of the mean resi-

\(10.4 (LOV-BID1) \text{ and } 7.5 \text{ min (LOV-BID3; Table 1 and S5). }\)
dark state, as both the Jα helix in the light state of wild-type AsLOV2\textsuperscript{[34]} and BID BH3 peptides\textsuperscript{[22]} are unstructured. LOV-BID2 shows little difference in structure between the dark and photoactivated states, but single-wavelength monitoring could be used to fit a decay curve to a change at 222 nm. As the value obtained matches the half-life of the other hybrids, it appears the Jα helix is either disordered in the dark state or maintains helicity in the photoactivated state rather than cysteinyl-FMN adduct formation becoming decoupled from structural changes. Much larger changes were observed in the CD spectra for light-activated and dark-state LOV-BID1, LOV-BID3 and LOV-BID4 samples.

A fluorescence anisotropy assay was used to measure the ability of the hybrids LOV-BID1–4 in their light-activated and dark states to target fluorescently labelled loop-truncated Bcl-x\textsubscript{L}.\textsuperscript{[22–23]} As expected, no binding was observed for AsLOV2 (V416I) either before or after photoactivation (Figure 4). LOV-BID hybrids on the other hand, showed high-nanomolar affinities for Bcl-x\textsubscript{L} in their photoactivated states. Light-state dissociation constants decreased as the length of hybrid Jα helix increased (Table 2); this possibly reflects the increased accessibility of the BH3 motif. No binding to Bcl-x\textsubscript{L} could be measured in the dark-adapted states of LOV-BID1, LOV-BID3 and LOV-BID4, but LOV-BID2 bound to Bcl-x\textsubscript{L} in the dark with threefold re-

Figure 3. Circular dichroism spectra of proteins in the dark-adapted (blue) and lit states (red) after 30 s of irradiation with a 1 W 455 nm LED in sodium phosphate (50 mm, pH 7.5) buffer containing sodium chloride (10 mm). A) AsLOV2 (V416I) B) LOV-BID1 C) LOV-BID2 D) LOV-BID3 E) LOV-BID4.

Figure 4. Normalised fluorescent anisotropy binding curves of proteins to TAMRA-labelled Bcl-x\textsubscript{L} (S2C) (10 nm) in sodium phosphate buffer (50 mm, pH 7.5) containing NaCl (10 mm) at 15 °C to minimise relaxation during the experiment in the dark-adapted (○) and lit states (●) after 30 s irradiation with a 1 W, 455 nm LED A) AsLOV2 (V416I); B) LOV-BID1; C) LOV-BID2; D) LOV-BID3 and E) LOV-BID4.
duced affinity compared to that in its photoactivated state. This suggests that the Jc region of LOV-BID2 is poorly caged in the dark state rather than remaining structured in the light-activated state.

Without calculating the dark-state affinities, it is impossible to calculate the dynamic range of LOV-BID1, LOV-BID3 and LOV-BID4. However, the best dynamic range obtained in previous peptide experiments was a 23-fold difference in affinities for an i/i+4 azobenzene-conjugated BID peptide. An equivalent dynamic range would equate to a dark state affinity of approximately 2 μM for LOV-BID4, which it greatly exceeds. Even without further mutations, such as those used elsewhere to modify the strength of Jc-helix docking, the switching magnitude of the LOV-BID proteins reported here is better than in many previous reports and similar to the best reported values for LOV-SsrA variants optimized by phage display (36- and 58-fold). The penalty for embedding the BH3 sequence closer to the core of the LOV domain is relatively low (~2.5-fold) compared to LOV-SsrA proteins (~16-fold). This might reflect the structure of the binding site of the target protein; Bcl-x, presents a shallow groove across one face with space at either end for overhanging protein.

Incorporating Bid BH3-derived sequences into the Jc helix of AsLOV2 did not alter the photochemistry of the LOV domain; it was generally well tolerated, resulting in proteins that underwent conformational changes in response to irradiation with blue light. The affinity of the embedded BH3 sequences for Bcl-xL was dependent on the conformational state of the LOV-BID fusions, which offer significant potential as optically controlled intracellular modulators of protein–protein interactions. The relative ease of integration of peptide sequences based on amphiphilic helices (in contrast with previous work incorporating more polar sequences) suggests wider applications of LOV photoswitches to rapidly and reversibly control protein levels and activities with light at the post-translational level. Photo-exposure of peptide epitopes in LOV domain hybrids introduced into transiently or stably transfected cells will generate potent optogenetic tools that avoid the difficulties of trafficking peptides across the cell membrane and offer a complementary approach to the use of azobenzene photoswitches.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dark-adapted $K_d$ [nM]</th>
<th>Light-activated state $K_d$ [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsLOV2 (V416I)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>LOV-BID1</td>
<td>n.d.</td>
<td>216 ± 16</td>
</tr>
<tr>
<td>LOV-BID2</td>
<td>998 ± 111</td>
<td>271 ± 14</td>
</tr>
<tr>
<td>LOV-BID3</td>
<td>n.d.</td>
<td>167 ± 3</td>
</tr>
<tr>
<td>LOV-BID4</td>
<td>n.d.</td>
<td>89 ± 5</td>
</tr>
</tbody>
</table>

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Photocontrolled Exposure of Pro-apoptotic Peptide Sequences in LOV Proteins Modulates Bcl-2 Family Interactions

LOV2 die for: Genetically encoded photoswitches display pro-apoptotic BH3 sequences in response to blue light. The design, synthesis and structure of a series of hybrids are examined to find the optimal position within the $\alpha$ helix of *Avena sativa* Phot1 LOV2 for incorporation of photo-uncaged peptides.