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High-resolution Crystal Structures of Transient Intermediates in the Phytochrome Photocycle

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29 **Abstract**

30 Phytochromes are red/far-red light photoreceptors in bacteria to plants, which elicit a variety of

31 important physiological responses. They display a reversible photocycle between the resting (dark)

32 Pr state and the light activated Pfr state, in which light signals are received and transduced as

33 structural change through the entire protein to modulate the activity of the protein. It is unknown

34 how the Pr-to-Pfr interconversion occurs as the structure of intermediates remain notoriously

35 elusive. Here, we present short-lived crystal structures of the classical phytochrome from

36 myxobacterium *Stigmatella aurantiaca* captured by an X-ray Free Electron Laser 5 ns and 33ms

37 after light illumination of the Pr state. We observe large structural displacements of the covalently

38 bound bilin chromophore, which trigger a bifurcated signaling pathway. The snapshots show with

39 atomic precision how the signal progresses from the chromophore towards the output domains,

40 explaining how plants, bacteria and fungi sense red light.

41

42 Keywords

43 Bacteriophytochrome, Photocycle, Pr, Pfr, Lumi-R, X-ray Free Electron Lasers, Photoconversion,

44 Infrared Fluorescent Protein (IFP) Tissue Markers, PCM-Photosensory Core Module, Time-

45 resolved serial femtosecond crystallography

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Introduction

Phytochromes are red-light protein photoreceptors, initially discovered in plants[1] where they regulate essential physiological responses such as shade avoidance and etiolation[2]. With that they are critical to the thriving of all vegetation on earth. Homologous proteins exist in bacteria[3,4], cyanobacteria[5,6] and fungi[7]. In photosynthetic bacteria, they regulate the synthesis of light-harvesting complexes[8-12]. In non-photosynthetic bacteria their role is less understood, but they are involved in various processes such as the regulation of carotenoid pigments, which protect from harmful light exposure[4], in conjugation[13], plant colonization[14], quorum sensing and multicellular fruiting body formation[15,16]. Bacteriophytochromes (BphP) have also been successfully used as infrared fluorescent tissue markers in mammals[17].

Phytochromes consist of 2 modules, where the N-terminal photosensory core module (PCM) is attached to a C-terminal effector module[18]. The latter module provides enzymatic activity together with a so-called N-terminal extension[19]. In plant and class I BphPs the PCM consists of three domains called PAS (Per-ARNT-Sim), GAF (cGMP phosphodiesterase/ adenylate cyclase/FhlA), and PHY (phytochrome-specific) (Fig. 1 a, b). The module is conserved from bacteria to plants and holds a covalently bound bilin chromophore, an open chain tetrapyrrole, which is biliverdin IXα (BV) in bacteria (Fig. 2 b). Hallmark features are a conserved water molecule in the center of the biliverdin, called the pyrrole water (PW)[20], the so-called PHY (sensory) tongue, which changes fold in the Pr-to-Pfr transition[21], and the long helix along the dimer interface, which spans the entire PAS/GAF and PHY domains[22,23]. The C-terminal effector domain is divergent between species and is often a histidine kinase in bacteriophytochromes[18,24,25] Full length phytochromes are difficult to crystallize, but the PCM forms crystals that diffract to 2 Å resolution and beyond[26]. They are particularly suited for time-resolved crystallographic investigations.

Phytochromes display a photocycle (Fig. 2 a) with two half-cycles that are driven by two different wavelengths of light. In classical phytochromes, the dark-adapted state, denoted as Pr, absorbs red light (λ ~700 nm), which causes a Z to E isomerization of the C15=C16 double bond within its bilin chromophore. Subsequent conformational changes of the entire protein end in a far-red light absorbing state, denoted as Pfr. The Pfr state either relaxes thermally back to Pr, or

82   can be driven back to Pr by far-red light (λ ~750 nm). The structural changes associated with the

83   Pr to Pfr transition modulate the enzymatic activity of the phytochrome[27,28]. Although the Pr and

84   Pfr states have been structurally characterized in detail using the *Deinococcus radiodurans*

85   *(Dr)*BphP PCM[21-23,29], structures of the nanosecond intermediates Lumi-R and Lumi-F as well as

86   those of the longer-lived intermediates in each photo-halfcycle (Fig. 2 a) are missing. In the Lumi-

87   R intermediate, the BV chromophore is in the electronic ground state. The 15*Z* anti (Fig. 2 b) to

88   15*E* anti isomerization of the C15=C16 double bond between rings C and D of the BV

89   chromophore should have taken place, resulting in a nearly 180° rotation of the D-ring[30-32]. (Fig.

90   2 a).

91   Through the latest developments in time-resolved serial x-ray crystallography (TR-SFX),

92   the 1 ps structure of the truncated *Dr*BphP chromophore binding domain (CBD) that consists only

93   of the PAS and GAF domains was determined[33]. 1ps after photoexcitation the BV D-ring in the

94   *Dr*BphP CBD rotates counter-clockwise, while the PW becomes photodissociated from the

95   chromophore binding pocket. Displacements of important, conserved amino acid residues are

96   observed already at 1 ps. For example, the conserved Asp-207 in the GAF domain moves

97   significantly, which could imply signaling directed towards the PHY-sensory tongue. However,

98   the PHY domain is not present in the CBD construct. Experiments on the entire PCM including

99   the critical PHY domain and sensory tongue are necessary to understand how the light signal is

100   transduced to the C-terminal enzymatic domain.

101   Previous attempts to initiate the photocycle in PCM crystals of various BphPs at room

102   temperature were unsuccessful presumably because the PCM constructs were not photoactive in

103   the crystal form, the illumination protocol was sub-optimal and/or the spatial resolution reached at

104   room temperature was not sufficient[16,34]. Recently, we published the structure of a classical

105   phytochrome from non-photosynthetic myxobacterium *S. aurantiaca*, denoted *Sa*BphP2 PCM

106   solved to a resolution of 1.65 Å at cryogenic temperatures (100 K) in the Pr form. *Sa*BphP2 PCM

107   microcrystals are photoactive (Fig. 2 c) and diffract to 2.1 Å resolution at room temperature[26]

108   which provides an opportunity to describe the Pr to Pfr transition by TR-SFX experiments.

109   The TR-SFX experiments on the *Sa*BphP2 PCM reported here were conducted at the

110   Japanese XFEL, the Spring-8 Angstrom Compact X-ray Laser (SACLA). They resulted in room

111   temperature structures 5 ns and 33 ms after light illumination of the Pr (dark) state with 640 nm

112   laser pulses (Methods and Extended Data Tab. 1). Results are discussed in terms of extensive

113   rearrangements of BV, specifically the D-ring, the PW and neighboring water network and

114   conserved amino acids in the GAF and PHY domains.

115   **Results**

116   **Difference Electron Density at 5ns and 33 ms.**

117   The difference electron density (DED) maps calculated at the 5 ns and 33 ms time delays

118   shows a large number of correlated positive and negative DED features in the PAS-GAF as well

119   as in the PHY domains (Fig. 3, Extended Data Tab. 2, see Methods and Extended Data Table 3 for

120   a statistical assessment). These features indicate structural changes through the entire SaBphP2

121   PCM dimer. The control map at 66 ms only contains spurious features, supporting this assignment

122   (Fig. 3). In all previous ns time-resolved crystallographic experiments on photoactive yellow

123   protein[35,36], myoglobin[37,38], and others [39,40]DED features are mostly localized to the chromophore

124   and a few residues. As a consequence, the DED map sigma level is determined by the noise in the

125   DED map as has been shown previously[41]. Here, this is different. The map sigma level is

126   determined by both the noise and the signal. The large number of difference features poses a

127   formidable challenge for the interpretation of the DED maps, as well as for structure determination.

128   The features must be interpreted locally near the chromophore and the chromophore pocket, and

129   more globally for the entire SaBphP2 PCM dimer (see Methods). Standard deviations (σ) of the

130   DED maps are determined by both the noise and the signal. We therefore use the σ values of the

131   66 ms control DED map to contour the 5ns and 33ms maps and to identify chemically meaningful

132   signals.

133   **Ring-D Orientations at 5ns and 33ms**

134   Substantial DED features are observed on and near the BV chromophore (Fig. 3, 5 ns insert

135   and Fig. 4). Strong negative DED features on the D-ring carbonyl, methyl and vinyl mark

136   substantial structural rearrangements in both subunits. Interestingly, positive features that identify

137   D-ring orientations upon light illumination differ in subunit A and in B. In subunit A, there are

138   strong lateral features (β_1 to β_4) that support a clockwise ~90º twist of the D-ring (Extended Data

139   Fig. 1 b-e) when viewed along the chromophore axis from the D to the A-ring. These features can

140   be reproduced by calculated difference maps (compare Extended Data Fig. 1 b,c and d,e). In

141 addition, positive features ξ (Fig. 4 a) are oriented in a way that support a 180° rotation. For the

142 interpretation of features ξ, extrapolated electron density (EED) maps were necessary since the

143 strong negative density on the D-ring carbonyl tends to eliminate close-by positive features. Ring-

144 like electron density appears in the EED maps (Fig. 4 b) indicating a fully isomerized D-ring.

145 Accordingly, two conformations of the chromophore are need to interpret the positive DED to

146 completion, a ~90° clockwise D-ring twist and a fully isomerized ~180° clockwise D-ring rotation

147 (Fig. 4 b, Extended Data Tab. 4).

148   In subunit B, features ξ are absent (Fig. 4 c). In accordance, EED maps (Fig. 4 d) do not

149 support a fully isomerized configuration (as in subunit A) for both the 5 ns and the 33 ms time

150 delays. Instead, strong positive features determine the geometry of the BV A to C-rings; see Fig.

151 4 a and c for a comparison of the DED in both subunits. In subunit B the entire BV pivots about

152 the B-ring (Fig. 4 c) which leads to strong C-ring and D-ring displacements. To interpret positive

153 features β_{c1} and β_{c2} ('c' for clockwise) C-ring must be tilted backwards (blue arrow in Fig. 4 c)

154 and the ring-D can only be oriented clockwise (light blue BV structure in Fig. 4 c,d) to fit the DED.

155 This leaves a strong feature β_{a1} which is located behind the C-ring plane. To reproduce this feature,

156 the ring C propionyl must tilt in the opposite direction (purple arrow in Fig. 4 c) which leads to a

157 displacement of carbon atom C_{15} forward. Then, the counter-clockwise D-ring orientation (in pink)

158 fits the DED.

Amino acid and water network rearrangement in the chromophore binding pocket and the

sensory tongue

161   Strong negative DED features indicate that the PW photo-dissociated from BV in both

162 subunits at 5ns and 33ms (Fig. 4, Extended Data Tab. 2). Moreover, significant displacements of

163 the conserved Asp-192 of the PASDIP consensus sequence in the GAF domain and the Arg-457

164 of the PRXSF motif[22,42] in the PHY domain are observed in subunit A (and at 33 ms also in subunit

165 B). Asp-192 and Arg-457 form a salt bridge, anchoring the PHY tongue to the chromophore region

166 in the Pr state. This connection is broken at 5ns. A strong positive DED feature between these two

167 amino acids is observed, indicating a water molecule (Fig. 5 a). Furthermore, the conserved Tyr-

168 248 in proximity to Asp-192 adopts a dual conformation at 5 ns and 33 ms (Extended Data Fig.

169 2). Similarly, a dual conformation is observed for the conserved His-275 (at 33 ms) that forms a

170 hydrogen bond to the D-ring carbonyl in the Pr state of the *Sa*BphP2 and other classical BphPs. In

171 contrast to these amino acid rearrangements, the structure of the sensory tongue is only locally

172 affected (Fig. 5 c). A β-sheet to α-helix transition is not observed which coincides with only minor

173 changes of the PHY domain position (Fig. 5 d,e).

174 On the other side of the chromophore, opposite to the tongue region, strong features in the

175 DED maps at 5ns and 33 ms indicate that the BV C-ring propionyl drags the conserved Ser-257

176 and Ser-259 along at 5ns and 33 ms. (Fig. 5 b, see also Extended Data Tabs. 2 and 4). A positive

177 DED feature in the B subunit indicates the appearance of a water molecule that may form hydrogen

178 bonds with the C-ring propionyl as well as with Ser-257 and Ser-259. Moreover, the DED maps

179 show correlated negative and positive features within the PAS-GAF domains and along the long

180 helices that form the dimer interface (Fig 3), pushing outwards the C-terminal helix that connects

181 to the output module (Fig 5 d and e). We propose that the structural changes in the chromophore

182 pocket initiate the signal that is transduced along the long helices 'wiring' together the BV

183 chromophore and effector domains.

Discussion

Global and local structural relaxations at 5 ns and 33 ms post illumination

186 In the *Dr*BphP CBD protein relaxations can already observed at 1 ps and 10 ps[33]. Although

187 individual BV ring displacements are observed, the chromophore essentially stays at the position

188 that it also occupies in the dark structure (Extended Data Tab. 5). Despite this, the signal has

189 penetrated deep into the BV-pocket of the GAF domain[33]. On the ns time-scale the signal is

190 expanding further through the entire PCM of the classical *Sa*BphP2. In contrast to smaller proteins,

191 such as PYP and myoglobin, the *Sa*BphP2 is large and flexible and can react readily and fast to

192 local chromophore perturbations. In the *Sa*BphP2 PCM structures shown here, protein relaxations

193 are advanced enough that large chromophore displacements are observed in both subunits.

194 Especially in subunit B, large chromophore geometry distortions are present (Extended Data Tab.

195 4) as the C-ring tilts out in both directions. It appears as if the energy of the absorbed photon is

196 stored in a distorted BV geometry that drives protein relaxations. Chromophore geometry

197 distortions are also found in early intermediates of unrelated proteins such as the PYP[35].

198 Distortions of the BV chromophore of the phytochrome photocycle have been also predicted by

199 time-resolved spectroscopy[31]. They were directly observed in temperature scan cryo-

200 crystallography experiments performed on the bathy phytochrome *Pa*BphP from *Pseudomonas*

201   *aeruginosa*[43] (Extended Data Tab. 5) further suggesting that distorted chromophore conformations

202   are part of an important mechanism to advance photochemical reactions. As the BV chromophore

203   position changes, it strongly affects amino acid residues near the BV propionyl moieties that shift

204   in unison with the chromophore. Examples are listed in Extended Data Tab. 2 and 4, and are

205   discussed further down. The species with the full 180º rotation of the D-ring might be associated

206   with a key Lumi-R like intermediate in the phytochrome photocycle (Fig. 2 a). To determine the

207   specific time point where the 180º rotation begins, additional data collected at different time delays

208   are required.

209   The absence of the fully isomerized D-ring isoform in subunit B can likely be explained

210   by differences in the subunits related by non-crystallographic symmetry. As the structure of the

211   sensory tongue is essentially identical in both subunits, it is unlikely the reason for this behavior.

212   By inspecting the region near Cys-13 to which the BV chromophore is bound, differences are

213   found. Distances to symmetry related molecules are different for subunit A and B. In subunit A

214   the distances from Arg-15 to Gln-139 and Cys-13 (S) to Lys-136 (N_z) (Gln-139 and Lys-136

215   belong to the molecule related by crystallographic symmetry) are 6.0 Å and 7.2 Å, respectively.

216   These distances are smaller in subunit B (5.2 Å, and 4.0 Å, respectively). These differences likely

217   have an impact on chromophore relaxations, as in subunit B BV structure appears more distorted

218   than in subunit A (see twisting angles for subunits A and B in Extended Data Tab. 4).

Rearrangement of water network and neighboring amino acids

220   The PW forms a stable hydrogen bond network with BV rings A-C in both the Pr and Pfr

221   states, but it is absent at 5 ns and 33 ms. It photo-dissociates already within 1 ps in the *Dr*BphP

222   CBD fragement[33]. While the twisting motion of D-ring has been the working model for

223   phytochrome activation and now has been confirmed, the disappearance of the PW is surprising.

224   Given the large sliding motions of the chromophore (Fig. 4 a and c), this now makes sense as the

225   absence of the PW most likely enables these displacements. It is interesting to note that the PW

226   dissociates very early[33] and rebinds back to BV in Pfr[21,29]. The PW may have a dual role in

227   facilitating the structural transition and stabilizing the reaction product (Pr as well as Pfr) in both

228   halves of the reaction cycle. Both, the rotation of the D-ring together with photodissociation of the

229   PW likely are the main triggers for subsequent protein structural changes. Together, they transduce

230   the light signal to the sensory tongue of the PHY domain and cause relaxations of the GAF domain

231   that propagate further up the long helices along the dimer interface.

Sensory tongue and the PHY domain

233   The sensory tongue connects the PHY domain directly with the chromophore region (Fig.

234   5). During the full Pr the Pfr transition the sensory tongue undergoes extensive structural

235   transitions from a beta sheet to an alpha helix[21,29]. In the Pfr state, Pro-456 becomes adjacent to

236   the D-ring and forms a hydrogen bond with Tyr-248. This is stabilizing the D-ring in E

237   configuration. It is interesting to ask how this shift is initiated. In the present microcrystals, the

238   transition is not observed (Fig. 5 c). We ascribe this to the crowded environment of the crystals.

239   However, the tight Asp-192 to Arg-457 salt bridge is already broken at 5 ns and a water is inserted

240   in between the residues. This is an important first step to enable the sensory tongue to rearrange.

241   We therefore conclude that the signal is transduced to the PHY tongue via the displacement of the

242   chromophore that enforces the movement of Asp-129 and the photo-dissociation of the PW.

Propagation of the light signal

244   Caused by D-ring rotation, the conserved Tyr-248 moves (Extended Data Fig. 2). This

245   destabilizes interactions with the neighboring amino acids and the water network. Arg-457 and

246   Aps-192 form new hydrogen bonds with a water molecule (Fig. 5 a). As the chromophore slides

247   substantially (Fig. 4), it induces structural changes in the GAF domain sensed by the conserved

248   serines 257, 259 and 261 and multiple other amino acids near the chromophore. His-275 loses

249   contact with the ring-D carbonyl (distance: > 4 Å) and with the more distant Arg-157 (now ~4.0

250   Å) that lead to substantial GAF domain relaxations which are ultimately relayed to the PHY

251   domain through the long dimer-interface helices. As the speed of sound in protein crystals is about

252   2000 m/s[44], heat expansion through 100 Å of protein (roughly the length of the *Sa*BphP2 PCM) in

253   5 ns cannot be excluded. However, relaxations at 33 ms are very similar to those at 5 ns. Heat

254   produced locally after chromophore light absorption should have dissipated by then, and the DED

255   features at 5 ns rather represent genuine protein relaxations.

256   The changes on the long dimer helix and the C-terminal helix (Fig. 3) suggest a mechanism

257   of signal transduction that does not rely exclusively on the opening of the PHY domains (Fig. 1

258   b). It seems as the long helices, and not so much the sensory tongues, translate the signal towards

259 the small C-terminal helices that are connected to the coiled-coil linker region of the effector

260 domain (Fig. 5 d,e, red arrows). This confirms a suggestion that was made based on the static

261 crystal structures of the bathy *Pa*BphP PCM[23] and compares favorably to signal transduction in

262 transmembrane sensory proteins[45]. Only small PHY domain displacements (Fig. 5 d,e) are

263 necessary for signal transduction. Since the linker helices of the sister monomers are at an angle,

264 translations along their axes will slightly change the relative orientation of the effector domains,

265 and hence their activity, possibly modulated by a shift in register of the coiled coil linker [27].

Summary and outlook

267 The short-lived structural intermediates presented here establish that it is indeed the

268 isomerization of the D-ring, which drives the photoconversion in phytochromes. The remaining

269 rings of the chromophore move notable distances and the movements are heterogeneous between

270 the different subunits. The presence of unproductive BV conformations, may explain the relatively

271 low quantum yield for the Pr to Pfr transition (approximately 10-15%). Nevertheless, we observe

272 a fully isomerized BV configuration and establish that photodissociation of the PW and the

273 displacements of the strictly conserved Asp-195 and Tyr 248 lead to a disconnection of the PHY

274 sensory tongue from the chromophore region. Finally, the data show strong evidence for a

275 structural change along the long helices at the dimer interfaces that transduce the signal further

276 towards the PHY domains.

277 Earlier time points within the *Sa*BphP2-WT PCM photocycle should be collected to assess

278 when the large BV chromophore displacements begin. Large scale structural changes are limited

279 by the crystal packing. Therefore, methods, which act on proteins in solution should be explored

280 to make further progress. Recently, solution NMR spectra of a full PCM was assigned for a

281 phytochrome[46] and new developments in cryoEM[47] bring atomic resolution of macromolecular

282 structures within reach without the need for crystals. Calculations are underway[48] explaining how

283 to obtain structures from single biological macromolecules, such as the full-length, intact BphPs

284 at XFELs.

Methods

Protein purification and crystallization

287   Microcrystals of the SaBphP2-PCM were grown as described[49] by mixing a mother liquor

288   consisting of 0.17 M Ammonium acetate, 0.085 M Sodium citrate tribasic dihydrate pH 5.6, 25.5%

289   w/v Polyethylene glycol 4000, 15% v/v Glycerol (cryo-screen solution) and 3 % w/v Benzamidine

290   Hydrochloride, with 60 mg/mL protein (3:2 protein to mother liquor ratio). The mixture was

291   seeded with finely crushed macrocrystals. After 4 days, the microcrystals were collected and

292   concentrated to about 10^{11} crystals /ml and subsequently folded into a tenfold amount of nuclear

293   grade grease[50,51]. All steps in crystallization and tray observations were performed under green

294   safety light.

Experimental Setup.

296   Pump-probe experiments were conducted at beamline BL2 at SACLA using a nanosecond

297   laser[52,53]. For our nanosecond TR-SFX experiments a two-sided laser illumination geometry was

298   used where a split laser beam intercepts the X-rays and the viscous jet at a 90° angle from opposite

299   sides. A relatively large laser fluence of 3.5 mJ/mm^2 was chosen for each side, respectively. The

300   laser fluence was chosen based on absorption measurements on grease crystal mixtures (Extended

301   Data Fig. 3, see also below). For femtosecond TR-SFX experiments X-rays and laser illumination

302   are parallel [33,39,54-57]. Then, the effective 'hit-rate' of the laser illumination is equivalent to the X-

303   ray hit-rate [54,55] and shading by other crystals in the viscous jet does not play a role. This has

304   consequences for the selection of the laser fluence, especially for fs laser illumination, which are

305   discussed [54,55]. In contrast, for a perpendicular geometry as employed here, the entire path of the

306   X-ray beam through the crystal must be illuminated by the laser. This leads to an effective laser

307   beam size that is much larger than the X-ray beam (Extended Data Fig. 3 b). The large effective

308   laser beam size is likely intercepted by other crystals in the relatively thick (100 μm) viscous jet.

309   This results in substantial shading by crystals not exposed to the X-rays. To roughly estimate this

310   shading, the crystal-grease mixtures were sandwiched between cover slides kept apart by 50 μm

311   washers to match the optical path through half of the 100 μm thick viscous jet. Absorption was

312   measured with a microscopectrophotometer located at BioCARS (APS, Argonne National

313   Laboratory). Grease mixed with SaBphP2 PCM microcrystals shows an absorption of 0.75 at 640

314   nm (Extended Data Fig. 3 a), which corresponds to a 5 fold reduction of the incident fluence. In

315   addition, at 640 nm the absorption is only 40% of that at the maximum at 700 nm. Accordingly, a

316   3.5 mJ/mm^2 fluence at 640 nm is equivalent to only about 0.28 mJ/mm^2 at the absorption

317   maximum. Because of the 2-sided illumination the total fluence at a crystal probed in the middle

318   of the jet is 0.56 mJ/mm^2 (with reference to the absorption maximum). Given the previous

319   experiences with photoactive yellow protein[36,56], 0.56 mJ/mm^2 is well below the threshold to

320   generate damage even with femtosecond laser pulses [58], and does not play any adverse role with

321   nanosecond laser pulses. We believe that for our experimental geometry strong laser excitation has

322   been essential to boost excitation levels to the extent that analyzable signal is obtained.

TR-SFX data acquisition and processing

324   200 µl of the crystal-grease mixture were transferred into an injector reservoir[59] and

325   extruded into air at ambient temperatures (293 K) through a 100 µm wide nozzle with a flow rate

326   of about 4 µl/min. The photoreaction was started with 5 ns lasers pulses of 640 nm wavelength

327   with a full width half maximum (FWHM) of 52 µm. The laser repetition rate was varied between

328   15 Hz and 10 Hz (Extended Data Fig. 4). A flow rate of 4 µl/min displaces at least a 300 µm

329   column of grease between the high frequency (15 Hz) laser pulses. 5 ns after the laser pulse the

330   stream of microcrystals was exposed in air to intense X-ray pulses ($\lambda = 1.38$ Å) of < 10 fs duration

331   with either 10 Hz or 15 Hz repetition rates. The scattering background was minimized by using a

332   helium-purged collimator. We used a pump-probe, 33 ms, 66 ms (Extended Data Fig. 4 b) data

333   collection strategy to assess whether a once laser illuminated/excited viscous jet volume has left

334   the X-ray interaction region and moved sufficiently that multiple laser excitations of the X-ray

335   probed volume are avoided.

336   Reference data which are free of laser excitation have been collected previously without

337   the laser[26]. For all experiments, diffraction patterns were collected on a CCD detector with eight

338   modules[60] and analyzed with a user-friendly data-processing pipeline[61] consisting of hit-finding

339   with Cheetah[62], and indexing and Monte Carlo integration by CrystFEL[63]. The hit rate was about

340   30%. About 50 % of diffraction patterns were successfully indexed. Mosflm and DirAx both were

341   used for indexing. The extracted partial intensities were merged to full reflection intensities using

342   the 'partialator' program in CrystFEL. For data statistics, see Extended Data Tab. 1. The full

343   intensities were converted to structure-factor amplitudes by software based on the CCP4 suite of

344   programs [64].

Computation of Difference Electron Density Maps

346   Weighted difference electron density (DED) maps were calculated as described [36,65]. The

347   DED maps at 33 ms and 66 ms were inspected for strong DED features near the chromophore. At

348　33 ms clear signal is present (Extended Data Fig. 5 b). At 66 ms, only spurious and randomly

349　distributed features could be detected (Extended Data Fig. 5 c). This demonstrates that the viscous

350　jet is extruded fast enough to cope with a data collection strategy shown in Extended Data Fig. 4

351　a (pump-probe, dark) as with this strategy the next laser pulses arrives 66 ms after the previous

352　one. This way, the laser excites a pristine jet volume that is free from contaminations from earlier

353　laser pulses. Data for two time-delays are collected from the same experimental setup, as the first

354　X-ray pulse after laser excitation contributes diffraction patterns for a 5 ns dataset, the second X-

355　ray pulse contributes to a 33 ms dataset. Needless to say, when only one intermittent X-ray pulse

356　is used (Extended Data Fig. 4 a, the reference data must be collected separately with the laser

357　switched off (see above).

Statistical Analysis of DED Features

359　　　The signal content and signal variance (sigma values) in the DED maps were analyzed by

360　histograms. Gaussian fits to the histogram should reproduce the root mean square deviation

361　(RMSD) values of the fft program that calculates the DED when the signal is purely random. When

362　the signal is weak and localized, it only changes the distribution in the flanks of the Gaussian[66],

363　but not the sigma value. If the signal is strong and everywhere, the fitted Gaussian becomes broader

364　and does not fit the flanks. The noise originates from the experimental error in the difference

365　amplitudes and errors introduced by the Fourier approximation[67]. In the presence of localized

366　signal, the noise and not the signal determines the sigma value of a DED map[66]. Here, this is not

367　the case, and occurs for the first time in time-resolved crystallography. In Extended Data Fig. 6 b,

368　a histogram of DED values derived from difference amplitudes $\Delta F = F_{66ms}-F_{Dark}$ is shown. The

369　histogram is fit by a Gaussian with a sigma of 0.0125 $e^-/Å^3$. The same value also reported by the

370　fast Fourier program ('fft') from the ccp4 suite of programs. The Gaussian fits the histogram

371　perfectly which outlines the random nature of the DED features. A histogram prepared from the

372　5ns-F_{dark} DED_{5ns} map is shown in Extended Data Fig. 6 a. The Gaussian is broader as in Extended

373　Data Fig. 6 b, and the flanks of the histogram are not fit properly by the Gaussian. If the DED

374　features containing signal would be sparse, the sigma from the fit would be essentially identical to

375　the value obtained from the 66 ms control data only. However, it is larger, 0.0144 $e^-/Å^3$. The form

376　of the histogram is an indication of strong signal throughout the map. For the DED_{5ns} map the

377　sigma value is determined by all of the noise sources described above plus the signal.

378　Consequently, for meaningful comparisons, the DED_{5ns} map must be contoured as a multiple of

379   the sigma value found in the DED_{66ms} control map, as this reflects the error level in a DED map
380   without signal.

381   As large numbers of DED features were found, it is useful to estimate how many of these
382   features might be generated by the noise sources mentioned above. For this, DED maps were
383   sampled on a 3D grid no larger than $2(h_{max}+1)$ with h_{max} the maximum h,k and l values within the
384   resolution limit. As an example: at a resolution of 2 Å and a unit cell axis a = 80 Å, h_{max} is 40.
385   Assuming similar values for the other cell axes, the unit cell and its DED content is sampled on a
386   82 x 82 x 82 grid. This way the DED peaks found in such a map correspond to independent
387   features. Extended Data Tab. 3 contrasts the number of DED features observed at 33 ms to the
388   ones expected to occur randomly in the DED map. On the 3 sigma level, for example, 1839 features
389   are expected to be purely random, and 3167 features are observed. On the 5 sigma level, the
390   probability of a feature to be random is so low that not even one feature is expected in the entire
391   DED map, yet 264 features (132 for a *Sa*BphP2 PCM dimer located in the asymmetric unit) are
392   observed. To bring this into perspective, in the strong difference map determined for the
393   photoactive yellow protein at a 3 ps pump-probe time delay[56], only 12 features, two per symmetry
394   related PYP molecule, are observed on the 5 sigma level (at 2.1 Å resolution to be compatible with
395   the resolution achieved here, and h_{max} determined as described).

Structure determination

397   Structural models were derived from extrapolated maps calculated by adding N*DF to the
398   structure factors calculated from an accurately refined dark state *Sa*BphP2 PCM model. The factor
399   N_C required to extrapolate the fraction of excited molecules to 100 % was determined by
400   integrating negative density in the extrapolated maps until the values diverge (Extended Data Fig.
401   7). For the 5 ns time delay N_C is 19 which corresponds to a population of 10.5 % activated
402   molecules in the crystal [65]. For the 33 ms time delay N_C is 22 (about 9% of molecules are activated).
403   The chromophore was moved by hand into the extrapolated maps calculated at 5 ns and 33 ms
404   time delays. The D-ring was rotated about the double bond Δ15,16 to achieve maximum agreement
405   with the DED maps as well as with the extrapolated density. Multiple D-ring orientations were
406   accommodated by generating chromophore double conformations. The apo-SaBphP2 PCM
407   structures were determined by using the real space (stepped) refinement option in 'coot'. This was
408   followed by a scripted 'zoned' refinement, also performed in real space in 'coot'. For this, the

409   script activates α-helical and β-strain restraints when needed. After the real space refinement the

410   agreement with the difference map was inspected, and if necessary corrected further by hand.

411   Double conformations for certain residues (His275, Tyr 248) were introduced to accommodate

412   DED features that result from the various D-ring orientations. If in doubt, extrapolated maps with

413   higher N (N ~ 40) were computed to verify the presence or to clarify the absence of individual

414   structural moieties at specific locations in space. A final reciprocal space refinement was

415   conducted using phased extrapolated structure factors (PESF) calculated as described

416   previously[33,65,68]. As calculated structure factors of the reference structure are used to determine

417   the PESFs, the final refinement is biased towards the reference (dark) structure. Therefore, refined

418   differences are (i) real, and (ii) sometimes tend to be underdetermined by a fraction of an Å.

419   Extrapolated amplitudes also amplify errors in the difference amplitudes N_c times. Due to this,

420   structures were refined to 2.4 Å which is lower than the resolution limit of the data. Due to the

421   same reasons, occupancies for the chromophore and other amino acid residues double

422   conformations were not refined. Occupancies were rather distributed on equal par among the

423   double conformations.

424   **Pearson Correlation Coefficient to estimate ring-D orientation.**

425   Once a model that interprets the DED is determined, calculated DED maps can be

426   computed from this model and the reference model [33,68] by subtracting structure factors calculated

427   from both models. In order to corroborate the assessment of clockwise and counter-clockwise D-

428   ring rotation, the Pearson Correlation Coefficient was used. For the correlation coefficient the

429   observed and calculated DED maps are compared. Since negative DED is always on top of the

430   reference model, the negative DED does not add information to distinguish competing models.

431   Accordingly, only positive DED features near the ring-D region are compared. A Fortran program

432   was developed that reads difference maps in ccp4 format, masks out a specific volume around a

433   pdb-file provided to the program, and calculates the Pearson correlation coefficient (PCC) within

434   this masked volume. Here, the mask volume is determined by the coordinates of the D-ring in both

435   clockwise and counter-clockwise orientations. Within this volume, the PCC was determined by a

436   grid-wise comparison of positive observed and corresponding calculated DED features as:

437

438
$$PCC = \frac{\sum_i \left(\Delta\rho^{obs} - \left\langle\Delta\rho^{obs}\right\rangle\right)\left(\Delta\rho^{calc} - \left\langle\Delta\rho^{calc}\right\rangle\right)}{\sqrt{\sum_i \left(\Delta\rho^{obs} - \left\langle\Delta\rho^{obs}\right\rangle\right)^2}\sqrt{\sum_i \left(\Delta\rho^{calc} - \left\langle\Delta\rho^{calc}\right\rangle\right)^2}} \; .$$

439

440   The terms in the bracket are the averages computed from all grid points i in the mask. Although
441   the average DED in an entire difference map is zero, the averages computed here are not zero,
442   since only positive features are evaluated. In addition, map grid points i were selected only when
443   the observed DED values are larger than a certain sigma value. With this, the PCC can be plotted
444   as a function of increasing sigma values (see Extended Data Fig. 8). In subunit A, at 5 ns, only the
445   clockwise rotation is supported in particular by high DED features. In subunit B, the PCC is similar
446   regardless whether a model with a clockwise, a counter-clockwise or a double conformation is
447   examined. This demonstrates that both clockwise and counter-clockwise ring-D rotations produce
448   calculated DED features that explain the observed density equally well. The PCC cannot
449   distinguish between the two ring-D orientations.

450   **Detailed Views of Structural Moieties.**

451   Structural views were generated by UCSF Chimera[69].

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References

454   **References**

455   1   Butler, W. L., Norris, K. H., Siegelman, H. W. & Hendricks, S. B. Detection, assay, and
456   preliminary purification of the pigment controlling photoresponsive development of plants.
457   *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6 (1959).

458   2   Batschauer, A. Photoreceptors of higher plants. *Planta* **206**, 479-492, doi:DOI
459   10.1007/s004250050425 (1998).

460   3   Jiang, Z. *et al.* Bacterial photoreceptor with similarity to photoactive yellow protein and plant
461   phytochromes. *Science* **285**, 406-409 (1999).

462   4   Davis, S. J., Vener, A. V. & Vierstra, R. D. Bacteriophytochromes: Phytochrome-like
463   photoreceptors from nonphotosynthetic eubacteria. *Science* **286**, 2517-2520, doi:DOI
464   10.1126/science.286.5449.2517 (1999).

465   5   Hughes, J. *et al.* A prokaryotic phytochrome. *Nature* **386**, 663, doi:10.1038/386663a0 (1997).

466   6   Yeh, K. C., Wu, S. H., Murphy, J. T. & Lagarias, J. C. A cyanobacterial phytochrome two-
467   component light sensory system. *Science* **277**, 1505-1508, doi:10.1126/science.277.5331.1505
468   (1997).

469   7   Blumenstein, A. *et al.* The Aspergillus nidulans phytochrome FphA represses sexual development
470   in red light. *Curr Biol* **15**, 1833-1838, doi:10.1016/j.cub.2005.08.061 (2005).

471   8   Yang, X. *et al.* Light Signaling Mechanism of Two Tandem Bacteriophytochromes. *Structure* **23**,
472   1179-1189, doi:10.1016/j.str.2015.04.022 (2015).

473   9   Toh, K. C., Stojkovic, E. A., van Stokkum, I. H. M., Moffat, K. & Kennis, J. T. The
474   Photochemistry of Bacteriophytochrome: Key to its Use as a Deep-Tissue Fluorescence Probe.
475   *Biophysical journal* **100**, 174-174 (2011).

476   10   Giraud, E. *et al.* Bacteriophytochrome controls photosystem synthesis in anoxygenic bacteria.
477   *Nature* **417**, 202-205, doi:10.1038/417202a (2002).

478   11   Fixen, K. R., Baker, A. W., Stojkovic, E. A., Beatty, J. T. & Harwood, C. S. Apo-
479   bacteriophytochromes modulate bacterial photosynthesis in response to low light. *Proceedings of*
480   *the National Academy of Sciences of the United States of America* **111**, E237-244,
481   doi:10.1073/pnas.1322410111 (2014).

482   12   Yang, X., Stojkovic, E. A., Kuk, J. & Moffat, K. Crystal structure of the chromophore binding
483   domain of an unusual bacteriophytochrome, RpBphP3, reveals residues that modulate
484   photoconversion. *Proceedings of the National Academy of Sciences of the United States of*
485   *America* **104**, 12571-12576, doi:10.1073/pnas.0701737104 (2007).

486   13   Bai, Y., Rottwinkel, G., Feng, J., Liu, Y. & Lamparter, T. Bacteriophytochromes control
487   conjugation in Agrobacterium fabrum. *Journal of photochemistry and photobiology. B, Biology*
488   **161**, 192-199, doi:10.1016/j.jphotobiol.2016.05.014 (2016).

489   14   McGrane, R. & Beattie, G. A. Pseudomonas syringae pv. syringae B728a Regulates Multiple
490   Stages of Plant Colonization via the Bacteriophytochrome BphP1. *MBio* **8**,
491   doi:10.1128/mBio.01178-17 (2017).

15 Barkovits, K., Schubert, B., Heine, S., Scheer, M. & Frankenberg-Dinkel, N. Function of the bacteriophytochrome BphP in the RpoS/Las quorum-sensing network of Pseudomonas aeruginosa. *Microbiology* **157**, 1651-1664, doi:10.1099/mic.0.049007-0 (2011).

16 Woitowich, N. C. *et al.* Structural basis for light control of cell development revealed by crystal structures of a myxobacterial phytochrome. *IUCrJ* **5**, 619-634, doi:10.1107/S2052252518010631 (2018).

17 Shu, X. *et al.* Mammalian expression of infrared fluorescent proteins engineered from a bacterial phytochrome. *Science* **324**, 804-807, doi:10.1126/science.1168683 (2009).

18 Auldridge, M. E. & Forest, K. T. Bacterial phytochromes: More than meets the light. *Crit Rev Biochem Mol* **46**, 67-88, doi:Doi 10.3109/10409238.2010.546389 (2011).

19 Legris, M., Ince, Y. C. & Fankhauser, C. Molecular mechanisms underlying phytochrome-controlled morphogenesis in plants. *Nat Commun* **10**, 5219, doi:10.1038/s41467-019-13045-0 (2019).

20 Wagner, J. R., Brunzelle, J. S., Forest, K. T. & Vierstra, R. D. A light-sensing knot revealed by the structure of the chromophore-binding domain of phytochrome. *Nature* **438**, 325-331, doi:Doi 10.1038/Nature04118 (2005).

21 Takala, H. *et al.* Signal amplification and transduction in phytochrome photosensors. *Nature* **509**, 245-248, doi:10.1038/nature13310 (2014).

22 Essen, L. O., Mailliet, J. & Hughes, J. The structure of a complete phytochrome sensory module in the Pr ground state. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 14709-14714, doi:10.1073/pnas.0806477105 (2008).

23 Yang, X., Kuk, J. & Moffat, K. Crystal structure of Pseudomonas aeruginosa bacteriophytochrome: photoconversion and signal transduction. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 14715-14720, doi:10.1073/pnas.0806718105 (2008).

24 Gourinchas, G. *et al.* Long-range allosteric signaling in red light-regulated diguanylyl cyclases. *Sci Adv* **3**, e1602498, doi:10.1126/sciadv.1602498 (2017).

25 Otero, L. H. *et al.* Structure of the Full-Length Bacteriophytochrome from the Plant Pathogen Xanthomonas campestris Provides Clues to its Long-Range Signaling Mechanism. *Journal of molecular biology* **428**, 3702-3720, doi:10.1016/j.jmb.2016.04.012 (2016).

26 Sanchez, J. C. *et al.* High-resolution crystal structures of amyxobacterial phytochrome at cryo and roomtemperatures. *Struct Dyn-Us* **6**, doi:10.1063/1.5120527 (2019).

27 Gourinchas, G., Heintz, U. & Winkler, A. Asymmetric activation mechanism of a homodimeric red light-regulated photoreceptor. *eLife* **7**, doi:10.7554/eLife.34815 (2018).

28 Giraud, E. *et al.* A new type of bacteriophytochrome acts in tandem with a classical bacteriophytochrome to control the antennae synthesis in Rhodopseudomonas palustris. *Journal of Biological Chemistry* **280**, 32389-32397 (2005).

29   Burgie, E. S., Zhang, J. & Vierstra, R. D. Crystal Structure of Deinococcus Phytochrome in the Photoactivated State Reveals a Cascade of Structural Rearrangements during Photoconversion. *Structure* **24**, 448-457, doi:10.1016/j.str.2016.01.001 (2016).

30   Mathes, T. *et al.* Femto- to Microsecond Photodynamics of an Unusual Bacteriophytochrome. *J. Phys. Chem. Lett.* **6**, 5, doi:10.1021/jz502408n (2014).

31   Toh, K. C., Stojkovic, E. A., van Stokkum, I. H., Moffat, K. & Kennis, J. T. Proton-transfer and hydrogen-bond interactions determine fluorescence quantum yield and photochemical efficiency of bacteriophytochrome. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 9170-9175, doi:10.1073/pnas.0911535107 (2010).

32   Schmidt, M., Patel, A., Zhao, Y. & Reuter, W. Structural basis for the photochemistry of alpha-phycoerythrocyanin. *Biochemistry* **46**, 416-423, doi:10.1021/bi061844j (2007).

33   Claesson, E. *et al.* The primary structural photoresponse of phytochrome proteins captured by a femtosecond X-ray laser. *eLife* **9**, doi:10.7554/eLife.53514 (2020).

34   Roessler, C. G. *et al.* Acoustic Injectors for Drop-On-Demand Serial Femtosecond Crystallography. *Structure* **24**, 631-640, doi:10.1016/j.str.2016.02.007 (2016).

35   Jung, Y. O. *et al.* Volume-conserving trans-cis isomerization pathways in photoactive yellow protein visualized by picosecond X-ray crystallography. *Nature chemistry* **5**, 212-220, doi:10.1038/nchem.1565 (2013).

36   Tenboer, J. *et al.* Time-resolved serial crystallography captures high-resolution intermediates of photoactive yellow protein. *Science* **346**, 1242-1246, doi:10.1126/science.1259357 (2014).

37   Schmidt, M. *et al.* Ligand migration pathway and protein dynamics in myoglobin: a time-resolved crystallographic study on L29W MbCO. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 11704-11709, doi:10.1073/pnas.0504932102 (2005).

38   Srajer, V. *et al.* Photolysis of the carbon monoxide complex of myoglobin: Nanosecond time-resolved crystallography. *Science* **274**, 1726-1729, doi:10.1126/science.274.5293.1726 (1996).

39   Nogly, P. *et al.* Retinal isomerization in bacteriorhodopsin captured by a femtosecond x-ray laser. *Science* **361**, 145-+, doi:10.1126/science.aat0094 (2018).

40   Skopintsev, P. *et al.* Femtosecond-to-millisecond structural changes in a light-driven sodium pump. *Nature* **583**, 314-+ (2020).

41   Srajer, V. *et al.* Protein conformational relaxation and ligand migration in myoglobin: a nanosecond to millisecond molecular movie from time-resolved Laue X-ray diffraction. *Biochemistry* **40**, 13802-13815 (2001).

42   Toh, K. C., Stojkovic, E. A., van Stokkum, I. H. M., Moffat, K. & Kennis, J. T. M. Fluorescence quantum yield and photochemistry of bacteriophytochrome constructs. *Physical Chemistry Chemical Physics* **13**, 11985-11997, doi:10.1039/C1cp00050k (2011).

43   Yang, X., Ren, Z., Kuk, J. & Moffat, K. Temperature-scan cryocrystallography reveals reaction intermediates in bacteriophytochrome. *Nature* **479**, 428-432, doi:10.1038/nature10506 (2011).

20

566 44   Speziale, S. *et al.* Sound velocity and elasticity of tetragonal lysozyme crystals by Brillouin
567   spectroscopy. *Biophysical journal* **85**, 3202-3213 (2003).

568 45   Gushchin, I. *et al.* Mechanism of transmembrane signaling by sensor histidine kinases. *Science*
569   **356**, doi:10.1126/science.aah6345 (2017).

570 46   Gustavsson, E. *et al.* Modulation of Structural Heterogeneity Controls Phytochrome
571   Photoswitching. *Biophysical journal* **118**, 415-421, doi:10.1016/j.bpj.2019.11.025 (2020).

572 47   Nogales, E. The development of cryo-EM into a mainstream structural biology technique. *Nature*
573   *methods* **13**, 24-27, doi:10.1038/nmeth.3694 (2016).

574 48   Poudyal, I., Schmidt, M. & Schwander, P. Single-particle imaging by x-ray free-electron lasers-
575   How many snapshots are needed? *Struct Dyn* **7**, 024102, doi:10.1063/1.5144516 (2020).

576 49   Sanchez, J. *et al.* High-resolution crystal structures of a myxobacterial phytochrome at cryo and
577   room temperatures. *Structural Dynamics* **(accepted)** (2019).

578 50   Sugahara, M. *et al.* Grease matrix as a versatile carrier of proteins for serial crystallography.
579   *Nature methods* **12**, 61-63 (2015).

580 51   Sugahara, M. *et al.* Hydroxyethyl cellulose matrix applied to serial crystallography. *Scientific*
581   *reports* **7**, 703, doi:10.1038/s41598-017-00761-0 (2017).

582 52   Kubo, M. *et al.* Nanosecond pump-probe device for time-resolved serial femtosecond
583   crystallography developed at SACLA. *J Synchrotron Radiat* **24**, 1086-1091,
584   doi:10.1107/S160057751701030x (2017).

585 53   Nango, E. *et al.* A three-dimensional movie of structural changes in bacteriorhodopsin. *Science*
586   **354**, 1552-1557 (2016).

587 54   Grunbein, M. L. & Nass Kovacs, G. Sample delivery for serial crystallography at free-electron
588   lasers and synchrotrons. *Acta Crystallogr D Struct Biol* **75**, 178-191,
589   doi:10.1107/S205979831801567X (2019).

590 55   Grunbein, M. L. *et al.* Illumination guidelines for ultrafast pump-probe experiments by serial
591   femtosecond crystallography. *Nature methods*, doi:10.1038/s41592-020-0847-3 (2020).

592 56   Pande, K. *et al.* Femtosecond Structural Dynamics Drives the Trans/Cis Isomerization in
593   Photoactive Yellow Protein. *Science* **352**, 725-729 (2016).

594 57   Barends, T. R. *et al.* Direct observation of ultrafast collective motions in CO myoglobin upon
595   ligand dissociation. *Science*, doi:10.1126/science.aac5492 (2015).

596 58   Hutchison, C. D. M. *et al.* Photocycle populations with femtosecond excitation of crystalline
597   photoactive yellow protein. *Chem Phys Lett* **654**, 63-71, doi:10.1016/j.cplett.2016.04.087 (2016).

598 59   Shimazu, Y. *et al.* High-viscosity sample-injection device for serial femtosecond crystallography
599   at atmospheric pressure. *J Appl Crystallogr* **52**, 1280-1288 (2019).

600 60   Kameshima, T. *et al.* Development of an X-ray pixel detector with multi-port charge-coupled
601   device for X-ray free-electron laser experiments. *Review of Scientific Instruments* **85**,
602   doi:10.1063/1.4867668 (2014).

603 61   Nakane, T. *et al.* Data processing pipeline for serial femtosecond crystallography at SACLA. *J
604   Appl Crystallogr* **49**, 1035-1041, doi:10.1107/S1600576716005720 (2016).

605 62   Barty, A. *et al.* Cheetah: software for high-throughput reduction and analysis of serial
606   femtosecond X-ray diffraction data. *J Appl Crystallogr* **47**, 1118-1131, doi:Doi
607   10.1107/S1600576714007626 (2014).

608 63   White, T. A. *et al.* Recent developments in CrystFEL. *J Appl Crystallogr* **49**, 680-689,
609   doi:10.1107/S1600576716004751 (2016).

610 64   Winn, M. D. *et al.* Overview of the CCP4 suite and current developments. *Acta Crystallogr D* **67**,
611   235-242, doi:10.1107/S0907444910045749 (2011).

612 65   Schmidt, M. Time-Resolved Macromolecular Crystallography at Pulsed X-ray Sources.
613   *International Journal of Molecular Sciences* **20**, doi:https://doi.org/10.3390/ijms20061401
614   (2019).

615 66   Srajer, V. *et al.* Extraction of accurate structure-factor amplitudes from Laue data: wavelength
616   normalization with wiggler and undulator X-ray sources. *J Synchrotron Radiat* **7**, 236-244,
617   doi:10.1107/S0909049500004672 (2000).

618 67   Henderson, R. & Moffat, K. The difference Fourier technique in protein crystallography: errors
619   and their treatment. *Acta Crystallogr B* **27**, 1414-1420 (1971).

620 68   Pandey, S. *et al.* Time-resolved serial femtosecond crystallography at the European XFEL.
621   *Nature methods* **17**, 73-78, doi:10.1038/s41592-019-0628-z (2020).

622 69   Pettersen, E. F. *et al.* UCSF chimera - A visualization system for exploratory research and
623   analysis. *Journal of computational chemistry* **25**, 1605-1612, doi:10.1002/Jcc.20084 (2004).

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Author Contributions

Conceptualization, E.A.S. and M.S.; Methodology, S.W., E.A.S. and M.S.; Samples, M.C., J.S., M.N., L.A., D.F. and E.A.S.; Data Collection, M.C., S.P., J.S., M.N., I.P., L.A., T.N.M., E. C., W. Y. W., D. F., V. Š., M.M., L.C., S.I., E.N., R.T., T.T., L.F., K.T., S.O., S.W., E.A.S. and M.S.; Data Processing: I.P., L.C. and S.P.; Data Analysis, S.P., E.A.S. and M.S.; Writing – Original Draft, E.A.S and M.S.; Writing – Review & Editing, E.A.S. and M.S. with input from all authors.; Funding Acquisition, S.I., S.W., E.A.S. and M.S.; Resources and Supervision, S.I., S.W., E.A.S. and M.S..

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Declaration of Interests

The authors declare no competing interests.

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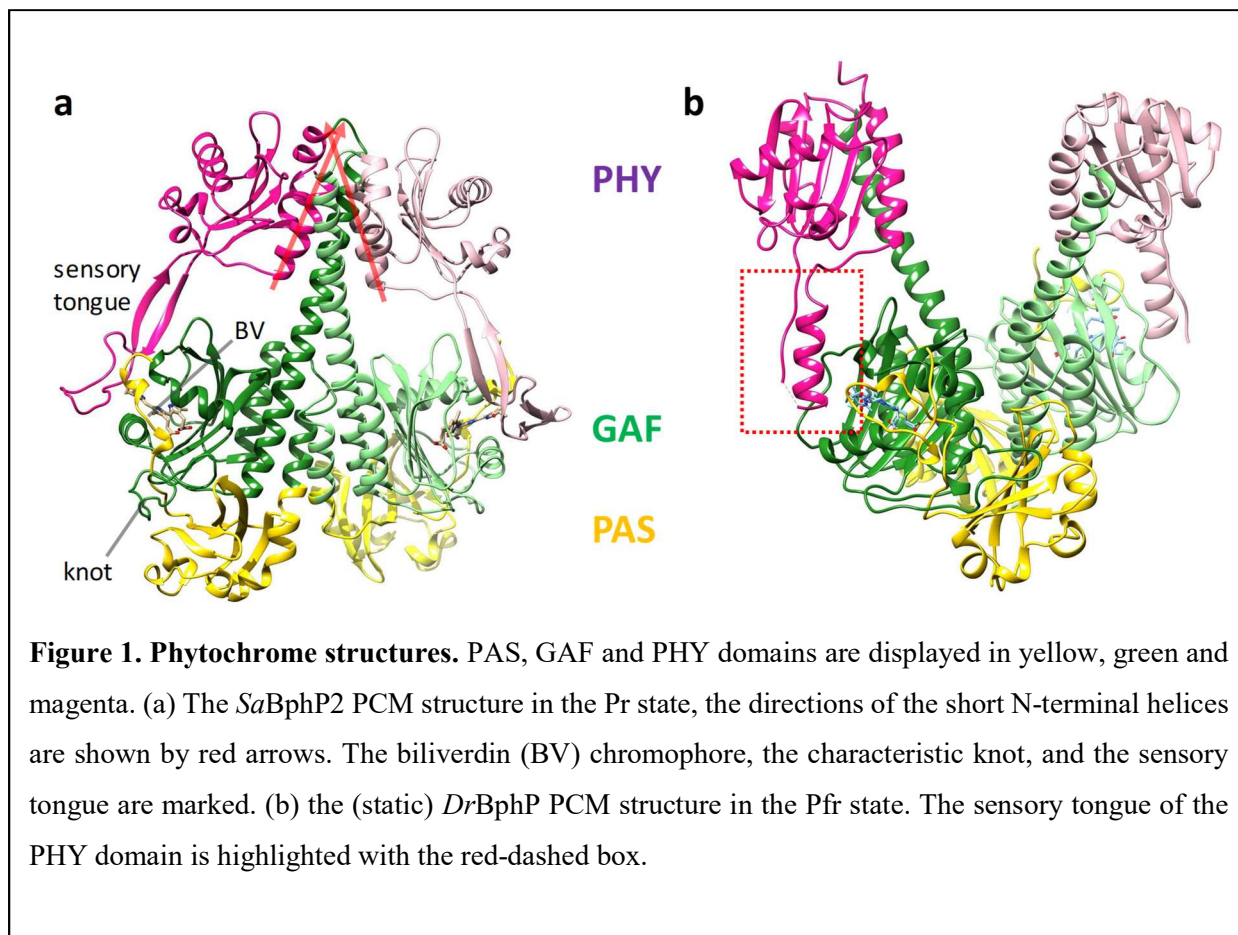
Figure 1. Phytochrome structures. PAS, GAF and PHY domains are displayed in yellow, green and magenta. (a) The *Sa*BphP2 PCM structure in the Pr state, the directions of the short N-terminal helices are shown by red arrows. The biliverdin (BV) chromophore, the characteristic knot, and the sensory tongue are marked. (b) the (static) *Dr*BphP PCM structure in the Pfr state. The sensory tongue of the PHY domain is highlighted with the red-dashed box.

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Figure 2. Phytochrome Photocycle, and UV-vis absorption spectra of *Sa*BphP2. (a) Phytochrome photocycle, approximate time scales for intermediate (Lumi-R, Meta-Ra and Meta-Rc) formation are shown. Pfr is formed after about 50 ms. The two half cycles can be driven by illuminating the stable Pr and Pfr states (displayed for the *Sa*BphP2 and *Dr*BphP PCMs, respectively) by red and far red light. (b) The chemical structure of BV bound to Cys-13 in the phytochrome. The torsional angle φ_T that defines isomerization/rotation about the double bond $\Delta 15,16$ is marked. In Pr the structure is all-*Z* syn-syn-anti. (b) *Static* absorption spectra of the *Sa*BphP2 Pr (solid line) to Pfr (dashed line) transition in solution. The difference is shown in red. (c) As (b) but in the crystal. The transition is initiated by 640 nm LED light (arrow). The difference (red line) is enhanced 3-fold.

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Figure 3. Difference maps for 5 ns and 66 ms time delays overlaid on the *Sa*BphP2 PCM dimer. The 5 ns DED map is displayed with subunit A, the 66 ms map on subunit B (gray). Contour levels: red -3σ, green 3σ. PAS, GAF and PHY domains of subunit A in yellow, green, magenta. Red arrow: direction of the C-terminal helix that connect to the coiled-coil linker in the full length BphP. Note the numerous DED features along the dimer interface helix into the PHY domain and the C-terminal helices at 5 ns. Inserts show the corresponding difference maps in the biliverdin (BV) binding pocket. At 66 ms, only spurious, random DED features are present. The chromophore is essentially free of signal.

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Figure 4. Chromophore displacements and D-ring rotations. DED is displayed in red and green, contour levels: red, -2.7 σ (- 3 σ of DED_{66ms}), green, 2.7 σ (3 σ of DED_{66ms}), EED is shown in blue with $N_c = 22$ (b) and $N_c = 19$ (d), contour level 1.5 σ. (a) overall chromophore configuration in subunit A at the 33 ms time delay. Gray: reference (dark), orange: intermediate at 33 ms. subunit A, reference structure in gray, 90° clockwise D-ring rotation (light blue), full isomerization (orange). Chromophore slides in the direction of the red arrows. (b) EED (blue), $N_c = 22$, on the D-ring in subunit A, colors as in (a). (c) Chromophore configuration in subunit B at 5 ns. Positive features β determine the ring positions. Feature $β_a$ (behind the chromophore plane) enforce a C-ring tilt resulting in a counter-clockwise rotation (purple and purple arrow). Clockwise rotation (blue arrow) shown in light blue. (d) EED (blue), $N_c = 19$, on the D-ring in subunit B, colors as in (c).

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Figure 5. Local and global structural changes. DED in red and green (-/+ 3σ contour), EED in blue (1.2 σ contour). (a) Separation of the sensory tongue from the BV binding region (dotted arrow). Asp-192 and Arg-457 are marked. The BV chromophore with the 90° twisted and fully isomerized D-ring is shown in yellow and green, respectively. β_1, positive DED feature interpreted by a water molecule. (b) The BV (green) C-ring propionyl detaches from Ser257 and Ser259 which coordinate a water (feature β_2, green positive DED) instead. The structure is displayed for subunits B (33 ms) where the D-ring twists ~90° both clockwise and counter-clockwise. (c) The sensory tongue region. Gray: structure of the reference state, orange: structure at 33 ms. Residues at the beginning and the end of the region are marked. The chromophore is shown with the twisted D-ring (green) and the fully isomerized form (orange). (d) The PHY domain region. Comparison of the 5ns structure (green) to the reference structure (gray). Sequence numbers are marked. The PHY domain centroid (black dot) moves by 1.3 Å (black arrow), and rotates (red curved arrow) by 1.3°. The connection to and from the sensory tongue is marked. (e) PHY domain at 33 ms versus reference. Similar displacements as in (a) are observed. Displacement of the c-terminal helix is marked by the red dashed arrow in (d) and (e).

669   **Extended Data Figures and Tables**

Extended Data Figure 1. Chromophore isomerization 5 ns after excitation in subunit A. DED is displayed on contour levels: red, $-2.7 \, \sigma$ ($- 3 \, \sigma$ of DED_{66ms}), green, $2.7 \, \sigma$ ($3 \, \sigma$ of DED_{66ms}). (a) overall chromophore configuration. Gray: reference, dark, yellow: early intermediate at 5 ns. Red arrows show the direction of the chromophore sliding, and the direction of the rotation. (b) BV D-ring enlarged, side view as in (a) to emphasize the negative DED features, (c) corresponding calculated DED on the $4 \, \sigma$ contour level. (d) top view to emphasize positive DED features, and (e) corresponding calculated DED. The observed negative features $\alpha_1 - \alpha_3$ and the positive features $\beta_1 - \beta_4$ are reproduced, features ξ not shown in b $-$ e for clarity.

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Extended Data Figure 2. Close views of the BV chromophore and Tyr-248. Red and green maps: DED map (-/+ 2.7 σ contour level). Faint blue maps: extrapolated maps (N=19 and 22, for 5ns and 33 ms, respectively, contour level 1.5 σ). Only the negative feature α_1 is displayed to avoid overcrowding as the reference structure is not shown. α_1 is occupied by the Tyr248 hydroxyl of the reference. BV and Tyr248 are marked. (a) 5ns, subunit A. Chromophore in green: 90° ring-D rotation, clockwise; yellow: *E*-configuration. (b) 5ns, subunit B: double conformation of ring-D, clockwise and anticlockwise rotation. (c) 33 ms, subunit A. Green and gold chromophore conformations as in (a), respectively. Multiple positive features β indicate a double conformation of Tyr248 (β_1 and β_2, respectively) which is corroborated by the extrapolated maps.

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Extended Data Figure 3. Absorption measurement on crystal-grease mixtures and perpendicular beam geometry. (a) At 640 nm (the excitation wavelength, marked) the grease is almost free of absorption (red line). With an optical path of 50 μm, coverslides, grease and crystals result in about an absorption of 1 at 640 nm (green line). (b) The effective (probed) illuminated crystal volume is given by the X-ray beam cross-section and the crystal size. The effective laser cross section is given by the crystal size and the X-ray beam diameter. The situation is depicted here with a 10 μm crystal and a 1 μm^2 X-ray beam cross-section.

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Extended Data Figure 4. Data collection schemes. (a) Pump-probe, dark, pump-probe dark sequence. 30 Hz X-ray pulse repetition. The pump laser repetition rate is 15 Hz with 66 ms between laser excitations (b) Pump-probe, dark, dark, data collection sequence with a 10 Hz laser repetition rate with 100 ms between excitations.

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Extended Data Figure 5. Difference maps near the chromophore. 5 ns (a), 33 ms (b) and 66 ms (c) after laser excitation (the reference structure is shown in gray). Green and red DED features are contoured at 2.7/-2.7 sigma for (a) and (b) and at 2.5/-2.5 sigma for (c), respectively. As there is signal in (a) and (b), at 66 ms the signal has vanished, and only spurious, random features persist.

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Extended Data Figure 6. Histograms from DED maps. (a) and (b) Histograms derived from DED maps at 5ns. The Gaussian fit is shown in red. (b) enlargement of the fit of the Gaussian at the flanks of the histogram. (c) and (d) Histogram derived from the 66 ms – dark DED map. The fit of a Gaussian is perfect also in the flanks (d).

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Extended Data Figure 7. Factor N determination for extrapolated maps. The negative density $\sum |\Delta\rho|$ found in extrapolated maps in a sphere of 6 Å about the ring D double bond is plotted against N. (a) The characteristic N (N_C) for 5 ns is 19, (b) N_C is 22 for the 33 ms time delay.

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Extended Data Figure 8. Pearson correlation coefficients as obtained by various models. (a) Subunit A, black line: ring-D clockwise rotation, red line: ring-D anticlockwise rotation. (b) Subunit B: black and red as in (a), green: double conformation.

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Extended Data Table 1. Data collection statistics.

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	Dark	5 ns	33 ms	66 ms
Beamline	SACLA BL2			
Resolution	2.1 Å			2.3 Å
Temperature	285 K			
Space group	$P2_1$			
Unit-cell parameters (,°)	a = 83.7 Å b = 83.4 Å c=86.9 Å α=90° β=107.6° γ=90°			
Observations	35,170,843	30,128,819	30,917,561	10,193,417
No of unique reflections	68,911	68,919	68,919	53,717
Redundancy	510 (111)	437 (135)	449 (154)	190 (124)
Completeness (%)	100.0	100.0	100	100
CC1/2 at d_{min}	0.11	0.16	0.21	0.31
R_{split} (%)	11.1 (221.9)	9.4 (109.4)	9.4 (101.6)	18.1 (157.2)
Max/min Δρ	reference	7.0/-8.6	6.7/-8.6	5.2/-4.9
R_{cryst}/R_{free} (%)	19.5/23.7	31.4/34.1	29.3/33.3	--
D-ring torsion	32°/26°	A: -60°/-151° B: 134°/-88°	A: -52°/-160° B: 132°/-100°	--
RMSD to reference [Å]	n/a	1.4	1.5	--

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Extended Data Table 2. Difference electron density features and displacements. Values are listed for selected functional groups and atoms at the 5 ns time-delay. I applicable, displacements are averaged over double conformations.

| | Prominent DED Features, 5 ns | | Displacements [Å] | |
| | Subunit A Negative/positive [sigma] | Subunit B Negative/positive [sigma] | 5ns | |
			Subunit A	Subunit B
A-ring carbonyl	-5.5 / 3.5	-7.3 / n.o.	1.3	1.6
B-ring methyl	-3.4/2.8	-3.3/4.3	1.4	1.5
B-ring nitrogen	-4.5/3.7	-4.0/4.0	0.7	1.2
B-ring propionate carboxyl	-5.4/4.6	-5.4/5.0	1.5	1.5
C-ring propionate carboxyl	-6.1/3.9	-7.0/7.0	1.3	1.3
D-ring carbonyl	-8.5/3.8	-6.6/3.9	2.0	1.8
D-ring methyl	-4.0/3.4	-5.7/4.3	3.5	2.4
D-ring vinyl (double bond)	-3.3/3.2	-3.7/2.5	3.1	2.2
D-ring nitrogen	-5.3/3.2	-3.1/3.5	3.4	2.3
Tyr-201-OH	-6.0/4.2	-5.8/4.8	1.4	1.3
Tyr-248-OH	-5.5/4.1	-5.5/3.0	1.3	2.0
Ser-257-OG	-5.9/4.2	-6.1/3.7	1.6	1.4
Ser-259-OG	-4.6/4.0	-5.0/3.5	1.2	2.5
Ser-261-OG	-5.4/3.3	-6.3/4.0	1.8	1.0
Pyrrole water*	-6.1	-5.4	ex	ex

* photo-dissociated from binding pocket

n/o: not observed (positive and negative DED cancel)

710 **Extended Data Table 3. Observed DED features and expected random DED features.** Random features

711 were estimated as a function of a multiple (x) of sigma (sigma level) in the unit cell of the *Sa*BphP2

712 difference map calculated on a 88 x 88 x 88 grid (N = 681472). The number of observed peaks was

713 determined by the ccp4 program *peakmax* as a function of the DED sigma value. PM is the probability to

714 observe at least one random feature equal or more than a given sigma level in the *entire* map, as opposed

715 to the probability PI to observe this random feature in an individual voxel. Note, we write (1 – erf) rather

716 than using the equivalent erfc, where erf is the error-function.

Feature [sigma]	Observed	$\mathrm{erf}(x/\sqrt{2})$	PI $1-\mathrm{erf}(x/\sqrt{2})$	$\mathrm{erf}(x/\sqrt{2})^N$	PM $1-\mathrm{erf}(x/\sqrt{2})^N$	expected random features in the DED map $N * \left(1.0 - \mathrm{erf}(x/\sqrt{2})\right)$
1	> 8000	0.68	0.31	0.0	1.0	216238
2	> 8000	0.95	0.05	0.0	1.0	31007
3	3167	0.997	0.002	0.0	1.0	1839
4	1018	1.0	0.0	0.0	1.0	43
5	264	1.0	0.0	0.67	0.33	0.4
6	44	1.0	0.0	1.0	0.0	0.0

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719 **Extended Data Table 4. Comparison of the reference (dark) and the 5 ns and the 33 ms *Sa*BphP2**

720 **structures.** Bold entries: important interactions. Bold numbers: large changes relative to the reference

721 structure.

	reference (Pr)		5 ns		33ms	
	A	B	A	B	A	B
Asp192-O/A-ring N	3.0	3.3	3.1/3.1	4.4/4.0	**3.8/4.0**	**4.1/4.6**
Asp192-O/A-ring carbonyl	4.5	4.4	4.1/4.1	5.5/5.8	4.5/5.1	5.4/5.6
Asp192-OD2/A-ring carbonyl	3.2	3.2	**4.1/4.1**	**4.1/4.1**	3.4/**4.6**	**3.9/4.1**
Asp192-O/B-ring N	3.0	3.0	2.9/3.0	3.1/3.3	**4.2/4.3**	**3.3/3.3**
Asp192-O/C-ring N	2.9	3.0	**3.8/4.1**	**5.1/5.2**	**5.1/5.1**	**4.7/5.0**
Asp192-OD2/Arg457-NH	2.6	2.9	**5.4**	3.0	**4.4**	**4.8**
Asp192-OD2/Tyr161-OH	5.6	5.7	5.5	4.9	5.6	4.2
Asp192-OD2/Tyr248-OH	2.7	2.7	**3.5/3.5**	**5.8/5.3**	**5.3/5.5**	**3.9/4.4**
Tyr248-OH/D-ring methyl	3.5	3.5	2.7/4.2	3.4/>5.0	2.6/3.9	2.9/5.3
Tyr248-OH/Arg457-NH1	4.2	4.4	3.5/>**5.0**	3.5/4.3	5.3/5.5	4.8/5.5
Ser242-N/B-ring propionyl O2	2.9	2.8	**5.1/5.1**	**4.3/>5.0**	**3.3/3.3**	**5.2/5.7**
Ser257-OG/C-ring propionyl O2	3.2	2.5	2.3/2.6	2.6/**3.9**	2.6/**3.3**	**3.5/5.0**
Ser259-OG/C-ring propionyl O2	2.7	2.7	3.1/3.3	2.6/2.5	2.1/**3.5**	2.3/2.4
Ser259-OG/Arg207-NH2	2.9	2.8	**5.1**	**4.7**	**4.4**	**5.4**
His275-NE2/C-ring propionyl O2	4.7	6.2	6.0	7.1/7.2	6.7/6.9	6.2/7.5
His275-NE2/D-ring carbonyl	3.0	2.8	**6.0**	2.8/**4.3**	**4.3/4.8**	3.3/**4.4**
His275-ND1/Arg157-NH2	2.9	2.9	**3.6**	**3.8**	2.7/**4.8**	**4.5**
His245-ND1/B-ring N	3.5	3.6	3.6/3.8	3.7/3.8	3.7/3.7	5.1/5.3
His245-ND1/C-ring N	3.4	3.6	3.7/3.8	4.2/4.7	3.7/3.8	4.6/5.4
His245-NE2/C-ring propionyl O1	2.6	2.8	2.7	2.9/**3.8**	3.0/**3.5**	**3.4/5.4**
Arg457-NH1/A-ring carbonyl	4.5	4.5	6.0	5.4/5.5	7.4/7.4	7.0/7.2
Tyr201-OH/Arg207-NH1	4.0	3.9	3.7	4.0	4.9	**3.0**
Tyr201-OH/B-ring propionyl O1	2.7	2.8	2.9	2.7/3.1	2.8/2.9	2.3/3.1
Tyr201-OH/C-ring propionyl O1	5.9	5.9	4.4/5.9	5.0/5.0	5.9/6.1	5.7/5.7
Ser261-OG/ Arg207-NE	3.1	2.7	**3.6**	**4.0**	**3.6**	**4.0**
Arg207-NH2/C-ring propionyl O1	5.4	5.3	4.9/6.1	4.1/5.7	4.1/5.1	3.9/4.2
Arg239-NH2/B-ring propionyl O1	2.7	2.9	2.4	2.5/2.6	**3.8/3.9**	2.8/2.9
A-Ring torsion D-Ring torsion	7 32	9 27	12/20 -61/-151	40/40 134/-88	7/-1 -62/-160	15/39 132/-100
B/C-Ring relative tilt*	11	14	25/25	43/37	32/46	43/46
Δ D-ring rotation**	-	-	-93/ **-183**	102/ -120	-94/ **-192**	105/ -127

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723 * separately for the two alternate conformations

724 **difference between D-ring torsion in the dark and the D-ring torsion at measured time delays. Negative
725 values: clockwise rotation relative to dark, positive values: counterclockwise rotation relative to dark,
726 bold red: *E*-configuration.

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728 **Extended Data Table 5. Important distances in other BphP structures** (identified by pdb entry).

729 Sequence numbers are in the *Sa*BphP2 PCM convention (directly comparable with Extended Data Tab.

730 4), they denote equivalent amino acid residues found in the various structures. L3 is an intermediate

731 determined by temperature scan crystallography.

	1ps 6TSU		*Dr*BphP (Pfr) 5C5K		Bathy *Pa*BphP (Pfr) 3C2W		L3 (bathy) 3NOU
	A	B	A	B	A	B	A
Asp192-O/A-ring N	2.7	3.2	2.8	2.8	3.2	3.1	3.0
Asp192-OD2/A-ring carbonyl	4.3	4.8	4.1	4.2	3.5	2.8	3.3
Asp 192-O/A-ring carbonyl	3.8	3.8	3.9	3.6	4.1	3.4	3.9
Asp192-O/B-ring N	3.0	3.4	3.1	3.3	3.1	3.6	2.7
Asp192-O/C-ring N	3.4	3.5	3.5	3.4	3.0	3.3	2.7
Asp192-OD2/Arg457-NH	n/a	n/a	16.1	15.8	nd	nd	nd
Asp192-OD2/Tyr248-OH	5.9	5.2	3.8	3.8	4.0	4.5	3.9
Tyr248-OH/D-ring carbonyl	4.1	4.0	3.5	3.1	3.2	3.9	N/A
Ser242-N/B-ring propionyl O2	3.2	3.3	4.9	4.7	5.1	4.8	5.5
Ser257-OG/C-ring propionyl O2	3.7	4.0	3.1	2.2	2.5	3.5	4.7
Ser259-OG/C-ring propionyl O2	4.5	3.6	6.8	3.9	6.7	6.2	2.5
Ser259-OG/Arg207-NH2	3.2	3.7	7.5	7.7	nd	nd	nd
His275-NE2/C-ring propionyl O2	5.4	5.7	3.8	5.0	2.8	3.4	4.1
His275-NE2/D-ring carbonyl	3.9	4.3	7.7	8.6	nd	nd	6.6
His275-ND1/Arg157-NH2	4.9	4.3	6.9	6.5	4.7	4.2	4.8
His245-ND1/B-ring N	3.6	3.5	3.6	4.5	3.7	3.7	4.2
His245-ND1/C-ring N	4.0	4.2	3.6	3.7	3.8	3.9	4.3
His245-NE2/C-ring propionyl O1	2.8	2.7	2.8	3.7	4.2	4.2	5.6
Tyr201-OH/Arg207-NH	4.7	4.4	7.5	7.2	nd	nd	nd
Tyr201-OH/B-ring propionyl O1	2.7	3.8	3.8	3.1	3.8	2.8	2.8
Tyr201-OH/C-ring propionyl O1	7.3	6.0	5.7	3.3	5.4	5.1	2.9
Ser261-OG/ Arg207-NH1	2.7	2.8	nd	nd	nd	nd	nd
Arg239-NH2/B-ring propionyl O1	3.4	3.0	3.5	2.7	4.0	4.1	4.6
Ring-A torsion	19	40	-7	10	11	30	24
Ring-D torsion	58	89	-142	-161	-150	-143	-25***
B/C-Ring relative tilt	8	9	0	0	5	6	35
Δ ring D rotation	33*	69*	-179**	-198**	na	na	125

732 *difference from comparing pdb entries 6TSU/6T31, counterclockwise rotation (positive)

733 ** difference from comparing pdb entries 5C5K/4Q0J, clockwise rotation (negative)

734 *** bathy phytochrome: -150º in the dark, rotation relative to dark is counterclockwise (positive)

735 nd: not done, n/a not applicable because the residue is missing.