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Ultrafast structural changes within a photosynthetic reaction centre

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41

42 **Photosynthetic reaction centres harvest the energy content of sunlight by transporting**
43 **electrons across an energy transducing biological membrane. We use time-resolved**
44 **serial femtosecond crystallography¹ at an X-ray free electron laser² to observe light-**
45 **induced structural changes in the photosynthetic reaction centre of *Blastochloris viridis***
46 **on a time-scale of picoseconds. Structural perturbations are first centred upon the**
47 **protein's special pair of chlorophyll molecules that are photo-oxidized by light. Electron**
48 **transfer to the menaquinone acceptor on the opposite side of the membrane induces a**
49 **movement of this cofactor in concert with lower amplitude protein rearrangements.**
50 **These observations reveal how proteins utilize conformational dynamics to stabilize the**
51 **charge separation steps of electron transfer reactions.**

52

53 Our biosphere depends upon the electron transfer reactions of photosynthesis as a primary
54 source of energy. Photosystems and photosynthetic reaction centres form a family of integral
55 membrane protein complexes found in plants, algae, cyanobacteria and photosynthetic
56 bacteria that convert the energy of a captured photon into a charge separated state. The
57 photosynthetic reaction centre of the purple non-sulphur bacterium *Blastochloris viridis*
58 (RC_{vir}) contains three transmembrane subunits called H, L and M and a periplasmic subunit C.
59 These subunits support four bacteriochlorophyll molecules (BCh), two bacteriopheophytin
60 molecules (BPh), a tightly bound menaquinone (Q_A), a mobile ubiquinone (Q_B), a single non-
61 haem iron and four haem co-factors (**Fig. 1**). Electron transfer reactions originate at a special
62 pair (SP) of strongly interacting bacteriochlorophylls which in *Bl. viridis* have an absorption
63 maximum at 960 nm. Photo-oxidation of the SP liberates an electron which is transferred to
64 the active branch BPh_L within a few picoseconds, is transferred to the tightly bound
65 menaquinone (Q_A) in less than a nanosecond, and is transferred to the mobile ubiquinone (Q_B)
66 in microseconds. SP^+ is reduced by subunit C and a second photo-oxidation event transfers a

67 second electron to Q_B^- , which is protonated from the cytoplasm and released into the
68 membrane as ubiquinol (H_2Q). Other proteins participate in a cyclic flow that returns
69 electrons to subunit C and the net effect is that two protons are transported across an energy-
70 transducing membrane for every photon absorbed.

71

72 Electrons may tunnel between cofactors when they are separated by approximately 10 Å or
73 less.³ The primary electron transfer step from SP to BPh_L occurs in 2.8 ± 0.2 ps⁴ over a
74 distance of 10 Å by means of a two-step hopping mechanism *via* the monomeric BCh_L ⁵ and is
75 more rapid than conventional Marcus theory. By contrast, the 9 Å electron transfer step from
76 BPh_L to Q_A has a single exponential decay time of 230 ± 30 ps⁶ which is consistent with
77 conventional Marcus theory. Coherent nuclear motions⁷ and protein structural changes⁸ have
78 been suggested to influence the initial charge-transfer reactions of photosynthesis yet the
79 specific nature of these putative protein motions is unknown. Flash-freeze crystallographic
80 trapping studies,⁹ time-resolved Laue diffraction¹⁰ and time-resolved serial femtosecond
81 crystallography¹¹⁻¹⁴ (TR-SFX) have revealed structural changes in bacterial photosynthetic
82 reaction centres^{9,10} and cyanobacterial photosystem II¹¹⁻¹⁴ that occur on the late microsecond
83 to millisecond time-scale, yet no time-resolved crystallographic studies on the time-scale of
84 the primary charge separation reactions of photosynthesis have been reported.

85

86 Here we apply time-resolved serial femtosecond crystallography¹ at an X-ray free electron
87 laser (XFEL) to probe the ultrafast structural response of RC_{vir} to light. We photo-excited the
88 special pair with 150 fs pulses centred at 960 nm (Extended Data Fig. 1). X-ray pulses 40 fs in
89 duration were generated at the LCLS² and were used to record diffraction patterns from tens
90 of thousands of microcrystals for the time points $\Delta t = 1$ ps, 5 ps (two repeats), 20 ps, 300 ps
91 (two repeats) and 8 μ s after photoexcitation (Extended Data Table 1). The time point $\Delta t = 1$ ps

92 populates the photo-excited charge transfer state of the SP in which charge rearrangements
93 have occurred within the bacteriochlorophyll dimer but are prior to the primary electron
94 transfer step; $\Delta t = 5$ and 20 ps are after the initial charge-transfer step and SP is oxidized and
95 BPh_L is reduced; $\Delta t = 300$ ps is longer than the time constant for electron transfer to Q_A and
96 menaquinone is reduced; and $\Delta t = 8 \mu\text{s}$ corresponds to a meta-stable charge separated state.

97

98 Extended Data Fig. 2 presents overviews of the $|F_{\text{obs}}|^{\text{light}} - |F_{\text{obs}}|^{\text{dark}}$ isomorphous difference
99 Fourier electron density maps (“light” corresponds to data collected from photo-activated
100 microcrystals whereas “dark” corresponds to data collected from microcrystals that were not
101 photo-activated) for all time points. Difference electron density features are visible above 4.0
102 σ (σ is the root mean square electron density of the map) near SP for all time points and
103 strong features associated with Q_A are visible for $\Delta t \geq 300$ ps (Extended Data Table 2). In
104 contradistinction with ultrafast TR-SFX studies of bacteriorhodopsin,¹⁵ photoactive yellow
105 protein,¹⁶ rsEGFP¹⁷ and bacterial phytochromes¹⁸ in which ultrafast structural changes are
106 driven by the movements of atoms due to a photo-isomerization event, TR-SFX
107 measurements of RC_{vir} reveal a knock-on effect on protein structure due to the light-induced
108 redistribution of charge. Electric-field induced conformational changes have been observed
109 when fields of the order 10^8 V/m are applied across a protein-crystal¹⁹ and this is the same
110 order of magnitude as electric field perturbations due to the movement of an electron within
111 RC_{vir}.

112

113 Recurring changes of electron density are visible as positive difference electron density in the
114 region of overlap between the two bacteriochlorophylls SP_L and SP_M of the special pair, and
115 complementary negative difference electron density features are visible primarily associated
116 with SP_M (Fig. 2, Extended Data Fig. 3, Extended Data Table 2, Supplementary Video 1).

117 Singular value decomposition (SVD) of all seven difference Fourier electron density maps
118 (Fig. 2e) reveals that the strongest positive and several of the strongest negative difference
119 electron density features of the principal SVD component are associated with the SP
120 (Extended Data Table 2). Quantification of electron density changes²⁰ within the RC_{vir} co-
121 factors (Fig. 2f) and statistical tests against control difference Fourier electron density maps
122 (Methods) provides a very high-level of confidence (p -value ≤ 0.001 , Extended data Table 3)
123 that these recurring difference electron density features do not arise by chance. Thus
124 photoexcitation causes the bacteriochlorophylls of SP to move closer together and the bending
125 (an out of plane distortion) of SP_M could explain these observations. An out of plane
126 distortion was used to model difference electron density features observed as carbon
127 monoxide was photo-dissociated from the haem of myoglobin²¹ (Extended Data Fig. 4).
128 Nonplanar distortions of chlorin and bacteriochlorin rings are observed in photosystem II and
129 RC_{sph} due to interactions with the surrounding protein²² and nonplanar porphyrins are also
130 more easily oxidized than planar porphyrins.^{23,24} This suggests that the distortion of SP in
131 advance of the primary charge separation event (Fig. 2a) could enhance the yield of the
132 primary charge-transfer reaction, which has been optimized by evolution to achieve almost
133 perfect quantum efficiency.²⁵

134

135 When the C-subunit is fully reduced, an electron is transferred from haem₃ to SP⁺ in less than
136 a microsecond.²⁶ The above reasoning implies that SP⁺ may be more easily reduced should
137 SP_M return to a planar geometry before this electron transfer occurs. This hypothesis is
138 consistent with our experimental observations since the amplitude of the positive difference
139 electron density feature between the SP bacteriochlorophylls increases from $\Delta t = 1$ ps to 20
140 ps, decreases for $\Delta t = 300$ ps and is insignificant for $\Delta t = 8$ μ s (Fig. 2, Extended Data Fig. 3,
141 Extended Data Table 2). Moreover, neither TR-SFX studies of the S2→S3 transition of

142 cyanobacteria PSII¹³ ($\Delta t = 150 \mu\text{s}$ and $400 \mu\text{s}$) nor TR-Laue diffraction studies of RC_{vir}¹⁰ ($\Delta t =$
143 3 ms) report a positive difference electron density feature in the region of overlap between the
144 special pair of (bacterio)chlorophylls, implying that this feature has decayed.

145

146 Charge rearrangements cause SP⁺ to move up to 0.3 \AA towards the M-subunit by $\Delta t = 300 \text{ ps}$
147 and the side-chains of both His173_L and His200_M adjust to preserve their ligating interactions
148 with the magnesium ions of SP⁺, as do His168_L and Tyr195_M adjust conformation in order to
149 maintain their H-bond interactions to SP_L and SP_M respectively. These structural perturbations
150 are revealed by paired negative and positive difference electron density features on the side-
151 chain of His173_L in the principal SVD components calculated from both the early (1ps, $2 \times$
152 5 ps , 20 ps) and late ($2 \times 300 \text{ ps}$, $8 \mu\text{s}$) sub-sets of TR-SFX data, whereas positive difference
153 electron density features associated with the side-chains of His200_M and Tyr195_M become
154 noticeably stronger for the later sub-set of data (Extended Data Fig. 3i,j; Extended Data Table
155 2; Supplementary Video 1). These observations suggest that SP_L moves towards subunit M
156 slightly in advance of SP_M, which may be due to dielectric asymmetry within photosynthetic
157 reaction centres.^{27,28} Dielectric asymmetry is believed to underpin the phenomenon that
158 electron transfer occurs only along the A-branch²⁷ in purple bacteria RCs and PSII (Fig. 1).

159

160 An electron moves from SP to BPh_L in $2.8 \pm 0.2 \text{ ps}^4$ and from BPh_L to Q_A in $230 \pm 30 \text{ ps}^6$
161 The tightly bound menaquinone is therefore neutral for $\Delta t = 1 \text{ ps}$, 5 ps and 20 ps ; three-
162 quarters of the photo-activated population are reduced to semiquinone by $\Delta t = 300 \text{ ps}$; and
163 essentially all photo-activated molecules have Q_A reduced at $\Delta t = 8 \mu\text{s}$. Our difference
164 Fourier electron density maps confirm these expectations since the few difference electron
165 density features visible within the Q_A binding pocket for $\Delta t \leq 20 \text{ ps}$ are isolated whereas more
166 continuous paired positive and negative difference electron density features are visible for Δt

167 ≥ 300 ps (Fig. 3, Extended Data Fig. 5). These recurring features of the later sub-set of TR-
168 SFX data (2×300 ps, $8 \mu\text{s}$) produce strong difference electron density features in the
169 principal SVD component that are associated with Q_A and its H-bond interaction with
170 His217_M (Extended Data Table 2, Fig. 3d, Supplementary Video 2) and statistical tests
171 establish that these recurring changes cannot be ascribed to noise ($p\text{-value} \leq 0.0125$, Extended
172 Data Table 3). Structural refinement models these observations as due to a twist and
173 translation of the semiquinone that brings the negatively charged head-group approximately
174 0.2 \AA closer to the positive charge of the non-haem Fe^{2+} (Fig. 3f) and thereby stabilizes the
175 reduced form of this cofactor. This interpretation receives support from QM/MM calculations
176 that predict that the Q_A to His217_M H-bond is shortened by 0.17 \AA when Q_A is reduced
177 (Extended Data Fig. 6f) and suggest that semiquinone binding is stabilized by approximately
178 36 kJ.mol^{-1} due to structural changes (Extended Data Fig. 6g,h, Methods), which is a sizeable
179 fraction of the energy (125 kJ.mol^{-1}) of a 960 nm photon. Similar conclusions were drawn
180 from an earlier analysis using a Density Functional Theory (DFT) formalism.²⁹ Light-induced
181 electron density changes were visible for Q_A in TR-SFX studies of the S2→S3 transition of
182 cyanobacteria PSII¹³ for the time points $150 \mu\text{s}$ and $400 \mu\text{s}$, light-induced movements of the
183 mobile quinone Q_B were also observed in PSII¹¹⁻¹⁴ for delays of hundreds of milliseconds, and
184 larger light-induced motions of Q_B were reported in freeze-trapping studies of the
185 *Rhodobacter sphaeroides* photosynthetic reaction centre.⁹

186

187 For $\Delta t = 300$ ps, paired negative and positive difference electron density features are
188 associated with the cytoplasmic portions of TM helices D_M and E_M (Fig. 3e) and indicate that
189 RC_{vir} adjusts its structure in response to the movement of the semiquinone within the Q_A
190 binding pocket (Fig. 3f). A more quantitative analysis (Methods, Extended Data Fig. 7,
191 Supplementary Video 3) suggests that low-amplitude protein motions begin to arise already

192 by $\Delta t = 1$ ps (Fig. 4a) as observed in TR-SFX studies of bR^{15,20} and Mb:CO²¹; the amplitude
193 of these motions increase with time and by $\Delta t = 5$ ps larger displacements are observed near
194 the SP⁺ and BPh_L⁻ cofactors (Fig. 4b); and for $\Delta t = 300$ ps protein conformational changes
195 extend throughout the A-branch of the electron transfer pathway from SP⁺ to Q_A⁻ (Fig. 4c).
196 When the same representation is used to depict protein conformational changes predicted
197 from QM/MM calculations (Supplementary Video 4) almost no structural changes are
198 expected for the photo-excited charge transfer state (Fig. 4d); protein movements arise near
199 the charged cofactors in the SP⁺:BPh_L⁻ charge-separated state (Fig. 4e); and structural changes
200 extend throughout the A-branch in the SP⁺:Q_A⁻ charge-separated state (Fig. 4f). These
201 findings demonstrate that RC_{vir} is not a passive scaffold but rather low-amplitude protein
202 motions engage in a choreographed dance with electron movements taking the lead and
203 protein conformational changes following. Conversely, as the protein's structure adjusts to
204 stabilize these charge rearrangements the energetic barriers hindering the reverse electron
205 transfer reaction increase, thereby extending the lifetime of the charge separated species and
206 enhancing the overall efficiency of photosynthesis.

207

208 In Marcus theory the total potential energy of an electron donor and its surroundings must be
209 equal to that of the electron acceptor and its surroundings if an electron is to tunnel from
210 donor to acceptor.³ Fluctuations in the organizational energy around protein cofactors are
211 therefore essential to facilitate electron transfer reactions. Efforts aimed at understanding how
212 protein conformational dynamics control the rates of electron transfer between cofactors^{8,30}
213 have been hampered by a lack of experimental tools that characterize protein structural
214 changes on the relevant time-scales. Our observations provide an experimental framework for
215 extending the standard description of electron transfer reactions in photosynthesis³ to
216 explicitly incorporate protein structural changes. Electron transfer reactions are ubiquitous in

217 nature and therefore a more nuanced understanding of the interplay between protein structural
218 dynamics and the movement of electrons has far-reaching biochemical significance.

219

220

221 **Extended Data** is available for this manuscript.

222

223 **Supplementary Information** is available for this manuscript.

224

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243

244 **Author Information**

245 These authors contributed equally: Robert Dods, Petra Båth.

246

247 **Contributions**

248 R.N. conceived the experiment, which was designed with input from G.B., R.D., J.D., S.B.,
249 M.L., S.C., D.M., M.S.H., G.J.W., J.D., D.P.D. and A.B. Samples were prepared by R.D.,
250 P.Bå., D.A. and R.B. Crystallization was supervised by G.B.. Time-resolved serial
251 femtosecond crystallography experiments were performed at the LCLS by R.D., P.Bå., D.A.,
252 R.B. K.R.B., G.N., M.L., D.M., J.R., R.H., P.Be., E.M., L.J., R.A., S.C., E.C., C.E.C., P.D.,
253 G.H., M.S.H., C.L., S.L. C.S., A.S., G.J.W., C.W., J.D., D.P.D., A.B., G.B. and R.N. The CXI
254 instrument was set up and run by M.L., M.S.H., G.J.W. and S.B.. The photoexcitation laser of
255 960 nm was operated and aligned by D.M., J.R., S.C. and J.D.. Sample delivery was
256 performed by K.R.B., G.N., R.H., P.Be., P.D. and D.P.D. Data were analysed by R.D., P.Bå.,
257 A.R., O.Y., A.B., G.B. and R.N. Structural refinement was performed by R.D. and P.Bå.
258 Resampling, full occupancy structural refinement and analysis were performed by V.A.G,
259 G.K. and A.V. Integration within a sphere and statistical tests were performed by C.W., R.N.
260 and P.Bå.. SVD analysis was performed by A.V. Quantum Mechanics/Molecular Mechanics
261 analysis were performed by D.M., H.L.L and G.G.. Time-resolved IR spectroscopy
262 measurements were performed by J.K, M.M and S.W. The manuscript was prepared by R.N.,
263 P.Bå., R.D. and G.B. with additional input from all authors.

264

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267

268 **Code Availability**

269 Software used for SVD analysis is available at <https://github.com/Neutze-lab/SVD>. Code
270 written in MATLAB to analyse difference electron density amplitudes is available at

271 <https://github.com/Neutze-lab/maptool>. Software associated with the resampling of X-ray
272 diffraction data is available at https://github.com/Katona-lab/CFEL_tools.

273

274 **Data Availability**

275 Atomic coordinates and structure factors have been deposited in the Protein Data Bank,
276 www.pdb.org. PDB ID codes are the following: 5O4C the dark-conformation (a); 5NJ4 the
277 dark conformation (b); 6ZHW the time-point $\Delta t = 1$ ps; 6ZI4 the time-point $\Delta t = 5$ ps (a);
278 6ZID the time-point $\Delta t = 5$ ps (b); 6ZI6 the time-point $\Delta t = 20$ ps; 6ZI5 the time-point $\Delta t =$
279 300 ps (a); 6ZI9 the time-point $\Delta t = 300$ ps (b); 6ZIA the time-point $\Delta t = 8$ μ s. Difference
280 Fourier electron density maps and stream files containing X-ray diffraction intensities are
281 deposited at the CXI database (<http://www.cxidb.org/>) with identification number ID 161.

282

283 **Competing Interests**

284 The authors declare no competing interests.

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367 **FIGURE LEGENDS**

368

369 **Figure 1: Electron transfer steps of the photosynthetic reaction centre of *Bl. viridis*.**

370 Cartoon representation of the H, L, M and C subunits. Cofactors are shown in black including
371 the special pair of bacteriochlorophylls (SP), two monomeric bacteriochlorophylls (BCh), two
372 bacteriopheophytins (BPh), a tightly bound menaquinone (Q_A), a mobile ubiquinone (Q_B), a
373 non-haem iron (Fe²⁺) and four haems. The approximate boundaries of the membrane are
374 suggested in blue. The electron transfer pathway: SP → BPh_L → Q_A is referred to as the A-
375 branch. Approximate timescales for the first two electron transfer events, from SP to BPh_L
376 and from BPh_L to Q_A, are depicted.

377

378 **Figure 2: Light-induced electron density changes in RC_{vir} at the site of photo-oxidation.**

379 **a**, Experimental $F_{\text{obs}}^{\text{light}} - F_{\text{obs}}^{\text{dark}}$ difference Fourier electron density map for $\Delta t = 1$ ps. **b**,
380 Difference Fourier electron density map for $\Delta t = 5$ ps (data set a). **c**, Difference Fourier
381 electron density map for $\Delta t = 20$ ps. **d**, Difference Fourier electron density map for $\Delta t = 300$
382 ps (data set a). **e**, Principal component from SVD analysis of all seven experimental
383 difference Fourier electron density maps. All maps are contoured at $\pm 3.2 \sigma$ (blue, positive
384 difference electron density; gold, negative difference electron density; σ is the root mean
385 square electron density of the map). **f**, Relative amplitudes of difference electron density
386 features integrated within a 4.5 \AA sphere²⁰ centred upon the RC_{vir} co-factors (Extended Data
387 Fig. 3j). The colour bars represent: cyan, $\Delta t = 1$ ps; blue, $\Delta t = 5$ ps a and b (in that order);
388 purple, $\Delta t = 20$ ps; red, $\Delta t = 300$ ps b and a (in that order); mustard, $\Delta t = 8 \mu\text{s}$.

389

390 **Figure 3: Light-induced electron density changes in RC_{vir} within the menaquinone**

391 **binding pocket. a**, Experimental $F_{\text{obs}}^{\text{light}} - F_{\text{obs}}^{\text{dark}}$ difference Fourier electron density map for

392 $\Delta t = 5$ ps (data set a). **b**, Difference Fourier electron density map for $\Delta t = 300$ ps (data set a).

393 **c**, Principal component from SVD analysis of the first four experimental difference Fourier

394 electron density maps ($\Delta t = 1$ ps, 5 ps (a), 5 ps (b), 20 ps). **d**, Principal component from SVD

395 analysis of the final three experimental difference Fourier electron density maps ($\Delta t = 300$ ps

396 (a), 300 ps (b), 8 μ s). All maps are contoured at $\pm 3.0 \sigma$ (blue, positive difference electron

397 density; gold, negative difference electron density). **e**, Difference Fourier electron density

398 map for $\Delta t = 300$ ps (data set a) showing the protein immediately surrounding Q_A and

399 contoured at $\pm 3.5 \sigma$. **f**, Superposition of the refined structures for the dark structure (yellow,

400 Q_A in black) and $\Delta t = 300$ ps (purple structure).

401

402 **Figure 4: Structural response of RC_{vir} to electron transfer events. a**, Recurring

403 movements of C α atoms for $\Delta t = 1$ ps quantified by full occupancy structural refinement

404 against 100 randomly resampled TR-SFX data sets. **b**, Recurring movements of C α atoms for

405 $\Delta t = 5$ ps (a) using the same representation. **c**, Recurring movements of C α atoms for $\Delta t = 300$

406 ps (a) using the same representation. Recurring movements are represented as error weighted

407 mean ratios relative to 100 control structural refinements (Methods) coloured from grey (< 80

408 % of the maximum error weighted mean ratio) to red (≥ 95 % of the maximum error weighted

409 mean ratio). An identical representation is given for all time points in Extended Data Fig. 7. **d**,

410 Movements of C α atoms estimated from QM/MM energy minimization calculations

411 associated with the SP photo-excited and all other cofactors in resting state: SP*:BPh_L⁰:Q_A⁰

412 (Methods). **e**, Movements of C α atoms estimated from QM/MM energy minimization

413 calculations associated with the SP photo-oxidized and BPh_L reduced: SP⁺¹:BPh_L⁻¹:Q_A⁰. **f**,

414 Movements of C α atoms estimated from QM/MM energy minimization calculations
415 associated with SP photo-oxidized and Q_A reduced: SP⁺¹:BPh_L⁰:Q_A⁻¹. Movements are
416 coloured from grey (no movements) to red (maximum C α motions). Transmembrane helices
417 are drawn as rods.

418 **METHODS**

419 *Protein production and purification*

420 The expression and purification of photosynthetic reaction centre from *Bl. viridis* cells was
421 adapted from Wöhri et al.³¹. Cells were disrupted by three rounds of sonication followed by
422 centrifugation in a JA20 rotor at 15000 rpm for 20 minutes to recover the membrane
423 suspensions. Membranes were then purified by ultracentrifugation at 45000 rpm for 45 min in
424 a Ti45 rotor. Membranes were homogenized in 20 mM Tris-HCl, pH 8.5 and diluted to
425 OD₁₀₁₂ = 10. Membranes were then solubilized in 4 % lauryldimehtylamine-N-oxide (LDAO)
426 for 3 hours at room temperature. Unsolubilized membranes were removed by
427 ultracentrifugation at 45000 rpm for 75 min in a Ti70 rotor. RC_{vir} protein was purified by
428 loading the supernatant onto a 250 ml POROS 50- μ m HQ ion-exchange medium equilibrated
429 with wash buffer (20 mM Tris-HCl, pH 8.5, 1% LDAO). The column was washed with 2 l of
430 wash buffer with 5 % elution buffer (20 mM Tris-HCL, pH 8.5, 1 M NaCl, 1 % LDAO) and
431 eluted with an increasing concentration of elution buffer over 20 column volumes. Fractions
432 with an A₂₈₀/A₈₃₀ < 3.5 were pooled and concentrated in 100 kDa MW cut off concentration
433 tubes (Vivaspin) to a volume of 10 ml. This was loaded in 5ml batches onto a HiPrep 26/60
434 Sephacryl S-300 column (GE) equilibrated with SE buffer (20 mM Tris-HCl, pH 8.5, 100 mM
435 NaCl, 0.1 % LDAO) and eluted into 1.8 ml fractions. Fractions with an A₂₈₀/A₈₃₀ < 2.6 were
436 pooled and concentrated, followed by a 20-fold dilution in final protein buffer (20mM
437 NaH₂PO₄/Na₂HPO₄, pH 6.8, 0.1 % LDAO, 10 μ M EDTA) and then concentrated again to 20
438 mg.ml⁻¹. Samples were flash-frozen in liquid nitrogen and stored at -80 °C.

439

440 *Protein crystallization*

441 20 μ l sitting drops were set up with a 1:1 ratio of protein solution (10 mg.ml⁻¹) and precipitant
442 solution (3.6 M ammonium sulphate, 6 % heptane-1,2,3-triol, 20 mM NaH₂PO₄/Na₂HPO₄, pH

443 6.8) set up against a 1 ml reservoir of 2 M ammonium sulphate. Large crystals grew at 4 °C
444 in 3 days. Crystals were harvested by pipette and crushed mechanically to create a seed stock
445 by vortexing with seed beads for approximately 20 min with occasional cooling on ice³². For
446 the XFEL experiment in April 2015 (run a) new 18.5 µl sitting drop vapour diffusion
447 crystallization drops were set up in order to yield large numbers of micro-crystals. In these
448 experiments the protein concentration was 8.5 mg.ml⁻¹ and a protein:precipitant concentration
449 of 10:7.5 was used in the drops. 1 µl of undiluted crystal seed stock was spiked into the drops
450 for a final v/v concentration of 5.4 %. Crystallization drops were then mixed by pipette and
451 covered with a glass cover slide. Rod-like crystals grew over 5 days at 4 °C and were 10 – 20
452 µm in the longest dimension. Microcrystals for the experiment in June 2016 (run b) were
453 prepared as above, but with an additional round of microseeding using crushed microcrystals
454 to seed an additional round of microcrystal growth³². Micro-crystals were harvested by pipette
455 and concentrated up to three-fold by centrifugation at 1000 g for 1 min followed by removal
456 of supernatant. These crystals were somewhat thicker and, while diffracting to higher-
457 resolution, they highlighted the compromise inherent in TR-SFX since a lower excited-state
458 occupancy was usually observed when working with crystals of higher optical density.

459

460 *Sample injection and data collection*

461 Microcrystals were transferred from Eppendorf tubes to a sample reservoir using a syringe
462 and passing the microcrystal slurries through a stainless steel 20 µm filter (VICI AG
463 International) or a 20µm nylon filter (Sysmex). The reservoir was loaded into a temperature
464 controlled rocking chamber and injected into the XFEL through a GDVN³³ using an internal
465 diameter of 75 µm. The microjet used a microcrystal suspension flow rate of 20 µl.min⁻¹ and
466 was focused to a 10 µm diameter using helium gas. The X-ray beam was aligned to interact

467 with the liquid jet as close to the tip of the GDVN as practical and before Rayleigh breakup of
468 the microjet.

469

470 Diffraction data were collected at 293 K at the CXI beam line³⁴ of the LCLS XFEL during
471 beamtime awarded in April 2015 (run a) and June 2016 (run b). Diffraction data were
472 recorded on a Cornell-SLAC Pixel Array detector³⁵. The X-ray wavelengths and equivalent
473 pulse energies were 1.89 Å (6.56 keV) in 2015 and 1.31 Å (9.49 keV) in 2016. An X-ray
474 pulse duration of 36 fs was used in 2015 and 45 fs in 2016. The XFEL beam was focused to a
475 $3 \mu\text{m}^2$ spot for both experiments. The detector was located 89 mm from the microjet in 2015
476 and 145 mm from the microjet in 2016. Diffraction data were collected at a repetition rate of
477 120 Hz from microcrystals that were not exposed to any optical laser pump (dark-state) and
478 for five time points corresponding to $\Delta t = 1 \text{ ps}$, 5 ps, 20 ps, 300 ps and 8 μs after photo-
479 excitation. The time points $\Delta t = 5 \text{ ps}$ and 300 ps were repeated in both 2015 and 2016 and are
480 referred to as data sets a and b respectively.

481

482 *Laser photoexcitation*

483 An optical Ti:Sa pump laser 150 fs in duration was focused into a spot size of 190 μm FWHM
484 ($323 \mu\text{m}^2$) and aligned to overlap with the LCLS X-ray pulse. The LCLS timing-tool³⁶
485 provided a timing accuracy of $\pm 200 \text{ fs}$ for the time point, Δt , between the arrival of the
486 optical pump laser and the X-ray probe. A pump-laser wavelength of 960 nm was used to
487 photo-excite RC_{vir} microcrystals, and this wavelength is at the absorption maximum of the
488 special pair ($\epsilon_{960} \approx 100\,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The pump laser energy per pulse was 11.8 μJ in April
489 2015 and 11.0 μJ in June 2016. For an idealized Gaussian beam, 86.5 % of this light will pass
490 through a spot with diameter $1/e^2$ and 50 % of this light will pass through a spot with diameter
491 FWHM. Thus the average fluence within the FWHM spot can be estimated as 25 mJ/cm^2 and

492 23 mJ/cm² which equates to a pump-laser power-density of 138 GW/cm² for the 2015
493 experiment and 129 GW/cm² for the 2016 experiment. This calculation defines the units used
494 throughout to specify the laser power-density. Both values are above 30 GW/cm² to 100
495 GW/cm² that has been recommended as an upper threshold to avoid nonlinear effects in
496 bacteriorhodopsin.^{37,38}

497

498 Extreme non-linear absorption was observed as ultrafast sample heating in time-resolved X-
499 ray scattering studies of RC_{vir} when pumped with 800 nm light.³⁹ When using 800 nm to
500 photo-excite RC_{vir} it is the BCh cofactors (rather than the SP) which absorb light ($\epsilon_{800} \approx$
501 180 000 M⁻¹.cm⁻¹). The pump-laser fluence used in that study³⁹ was 1560 GW/cm². Ultrafast
502 sample heating within a GDVN liquid microjet has also been measured as a function of the
503 800 nm pump-laser fluence using time-resolved X-ray scattering (Fig. 28 of reference⁴⁰).
504 These measurements show that the energy deposited into RC_{vir} samples is proportional to the
505 pump-laser fluence (a linear response) up to 270 GW/cm² and that the measured heating then
506 varies quadratically (a non-linear response) above a pump-laser fluence of 355 GW/cm². Thus
507 either an idealized assumptions of a perfectly aligned Gaussian beam may not be realistic,
508 and/or large losses occur as the incoming laser pulse is reflected from the surface of a GDVN
509 liquid microjet, and/or a thresholds^{37,38} of 30 GW/cm² to 100 GW/cm² do not apply RC_{vir}
510 when photo-excited at 800 nm. When 960 nm light is used to photo-excite the SP of RC_{vir} it is
511 likely more difficult to induce non-linear effects because the photo-excited state SP* has an
512 absorption maximum red-shifted 70 nm relative to the ground state⁴¹ and hole-burning⁴² has
513 been observed in RC_{vir} such that SP* is effectively transparent to the incoming light.
514 Moreover, the absorbance of RC_{vir} at 960 nm is only 56 % of its absorbance at 800 nm and
515 therefore non-linear effects are likely to arise at higher power densities when using 960 nm
516 rather than 800 nm to photo-excite RC_{vir}. Non-linear ultrafast heating⁴⁰ is observed in RC_{vir}

517 delivered using a GDVN liquid microjet and photo-excited at 800 nm only above a power-
518 density of 355 GW/cm². Therefore the 960 nm pump-laser power-densities of 138 GW/cm²
519 and 129 GW/cm² used in this work are below where non-linear effects may reasonably be
520 anticipated. These conclusions are supported by time-resolved IR spectroscopy measurements
521 (Extended Data Fig. 1).

522

523 *Time-resolved infrared spectroscopy*

524 Time-resolved vibrational spectroscopy measurements were performed with a near infrared
525 (NIR) pump and mid-infrared (IR) probe setup using a regenerative amplifier (Spitfire Ace,
526 Spectra Physics) to deliver pulses centred at 800 nm (1.2 mJ, 5 kHz). The amplifier output is
527 used to pump a TOPAS-TWINS (Light Conversion) capable of generating tuneable
528 femtosecond pulses at two different wavelengths. One path was used to generate mid-infrared
529 probe light centred at 6000 nm via difference frequency generation whereas the other path
530 generated 960 nm pump pulses via second harmonic generation of the idler beam. The 960
531 nm beam was chopped to 2.5 kHz and delayed in time relative to the probe pulses using an
532 optical delay line. Two weak replicas derived from the midIR beam were used as probe and
533 corresponding reference. Both probe and reference were dispersed in a Horiba spectrograph
534 (grating with 75 gr/mm) and detected and integrated on a double-row MCT array with 64
535 pixels each on a shot-to-shot basis using a commercial detection system (Infrared Systems).
536 Samples of RC_{vir} were prepared in a customized cell by enclosing ca. 15 uL of solution (RC_{vir}
537 at ca. 0.4 mM in D₂O buffer) between two 2 mm thick CaF₂ windows separated by a 25 μm
538 spacer. Probe and reference beams were focused at the sample position and collimated using
539 90° off-axis parabolic mirrors. The pump beam was focused using a 30-cm lens and
540 overlapped with the probe beam at its focus. The sample cell was placed where pump and
541 probe beams meet and translated continuously perpendicular to the beam direction during data

542 acquisition. The focal spot size of the pump beam was determined using knife-edge scans and
543 yielded perpendicular $1/e^2$ radii of 57 μm and 56 μm . Different pump fluences were adjusted
544 using reflective neutral density filters (Edmund Optics). For each fluence, twelve repeats over
545 five time points (1000 pump shots per time point and repeat, at delays of -50, 1, 2, 5 and 300
546 ps) were recorded and less than 5% of shots were rejected during data treatment. Signals were
547 calculated by subtracting consecutive pump-on from pump-off shots followed by application
548 of the noise reduction algorithm.^{43,44} The spectral resolution is $< 5 \text{ cm}^{-1}$. The results of these
549 measurements are presented in Extended Data Fig. 1.

550

551 *Data processing*

552 Images containing more than 20 diffraction spots were identified as diffraction hits by
553 Cheetah.⁴⁵ Cheetah converted the raw detector data into the HDF5 format and data were then
554 processed using the software suite CrystFEL version 0.6.2.^{46,47} Crystals were indexed using a
555 tetragonal unit cell ($a = b = 226.4 \text{ \AA}$, $c = 113.7 \text{ \AA}$, $\alpha = \beta = \gamma = 90^\circ$). Scaling and merging were
556 performed using Monte Carlo methods using the same software. Data from the dark state and
557 photo-excited states were scaled together using the custom dataset splitting option in the
558 CrystFEL partialator module. Structure factors were calculated from merged intensities by the
559 CCP4 module TRUNCATE⁴⁸ and molecular replacement was performed using the CCP4
560 module Phaser⁴⁹ using the ground-state RC_{vir} structures solved with XFEL radiation (PDB
561 codes 5O4C and 5NJ4) as a search models. Statistics for data collection and refinement are
562 detailed in Extended Data Table 1.

563

564 *Electron density difference maps*

565 Isomorphous $|F_{\text{obs}}|^{\text{light}} - |F_{\text{obs}}|^{\text{dark}}$ difference Fourier electron density maps were calculated using
566 the refined dark state structures for phases with the time-points $\Delta t = 5 \text{ ps}$ (data set a) and 300

567 ps (data set a) calculated against data and coordinates using the pdb entry 5O4C whereas the
568 time point with the time-points $\Delta t = 1$ ps, 5 ps (data set b), 20 ps, 300 ps (data set b) and 8 μ s
569 were calculated against data and coordinates using the pdb entry 5NJ4. Thus all difference
570 electron density map calculations used only data collected during the same experiment.
571 Difference Fourier electron density maps represent measured changes in X-ray diffraction
572 intensities as changes in electron density without bias towards the photo-activated state's
573 structural model. The technique is extremely sensitive to small changes in electron density⁵⁰
574 and reveals more subtle features than are apparent from $2mF_{\text{obs}}-DF_{\text{calc}}$ electron density maps
575 alone (m is the figure of merit and D is estimated from coordinate errors). A Bayesian
576 weighting calculation script⁵¹ using CNS software⁵² was also used to analyse the difference
577 Fourier electron density maps. In this procedure structure factor amplitude differences were
578 weighted by the product of the figure of merit of the ground state structure reflections and of a
579 weighting term, w (Equation 14 of reference⁵³), which was calculated
580 using Bayesian statistics developed to improve signal to noise.⁵³ For six of seven data-sets the
581 recurring difference electron density features were slightly strengthened by this step. The
582 exception was the time-point $\Delta t = 8 \mu$ s which has difference electron density features that are
583 weaker than for the other maps (Fig. 2f) and appears due to a lower occupancy of the charge
584 separated state in these microcrystals. It is possible that a fraction of the photo-oxidized SP⁻
585 population is reduced from the C-subunit by $\Delta t = 8 \mu$ s, which is longer than the time-scale of
586 this electron transfer step.²⁶ However, no efforts were made to reduce the C-subunit when
587 preparing microcrystals and a similar occupancy ($30\% \pm 5\%$) is observed to persist in time-
588 resolved spectroscopy measurements on crystals for up to millisecond delays.¹⁰

589

590 *Singular value decomposition*

591 SVD analysis of difference Fourier electron density maps was performed using an in-house
592 code written in python that is based upon an approach previously described.⁵⁴ As has been
593 discussed,⁵⁵ SVD may serve as a noise-filter to enhance the signal across a sequence of
594 difference Fourier electron density maps. This step contains the assumption that the overall
595 mechanism is linear and that changes in electron density are similar over the selected time-
596 windows. When applying SVD we evaluate the expression $[U, \Sigma, V] = \text{SVD}(A)$, where A is a
597 matrix of n difference Fourier electron density maps containing m elements; U is an $n \times n$
598 unitary matrix; Σ is an $n \times m$ rectangular matrix containing n diagonal elements (the singular
599 values) arranged in decreasing order and all other matrix elements are zero; and the first right
600 singular vector (the first row of the matrix V) is referred to as the principal component.
601 Results from SVD analysis of all seven electron density maps are presented in Fig. 2e and
602 Extended Data Fig. 3l,m. Results from SVD analysis deriving from the first four time-points
603 ($\Delta t = 1$ ps, 5 ps a and b, 20 ps) and the last three time-points ($\Delta t = 300$ ps a and b, 8 μ s) are
604 shown in Fig. 3c,d; Extended Data Fig. 3h,i; Extended Data Fig. 5h,i; Supplementary Videos
605 1 and 2. This separation of the maps is motivated by the fact that photo-activated RC_{vir}
606 molecules have menaquinone oxidized for the first sub-set of time-points yet most
607 menaquinone molecules of photo-activated RC_{vir} are reduced for the second sub-set of time-
608 points.

609

610 *Structural refinement of photo-excited states*

611 Isomorphous $|F_{\text{obs}}|^{\text{light}} - |F_{\text{obs}}|^{\text{dark}}$ difference Fourier electron density maps were inspected in
612 COOT. Structural refinement was performed using Phenix.⁵⁶ A model was first placed within
613 the unit cell using rigid body refinement followed by multiple rounds of partial-occupancy
614 refinement where the SP, BCh_L, BPh_L, Q_A, portions of TM helices E_L, D_L, E_M and D_M, as well
615 as connecting loops, and additional residues near cofactors (L153-178, L190, L230, L236-

616 248, M193-221, M232, M243-253, M257-266) were allowed to adopt a second conformation
617 with 30 % occupancy and the dark-state structure (pdb entry 5O4C) was held fixed. The
618 occupancy of 30 % was chosen by assessing the results from partial occupancy refinement
619 when the occupancy was allowed to vary and was imposed for all structural refinements for
620 consistency. Results from structural refinement were compared against the difference electron
621 densities and some manual adjustments were made using COOT.⁵⁷ Refinement statistics are
622 displayed in Extended Data Table 1. Validation of structure geometry was performed using
623 MOLPROBITY⁵⁸ and PROCHECK.⁵⁹ Structural changes were also validated by calculating
624 simulated difference Fourier electron density maps from the refined structures^{10,20} (Extended
625 Data Figs. 2i and 4j).

626

627 *Structural analysis of large-scale protein motions*

628 The high multiplicity of SFX data was exploited for structural analysis by randomly selecting
629 a sub-set of experimental observations from within each SFX data-set to create 100 separate
630 (but not independent) serial crystallography data-sets for the two resting state data-sets and
631 the seven photo-activated data-sets, amounting to 900 resampled data-sets in total. For each of
632 these resampled data-sets the mean and uncertainty estimates (σ) for every unique Bragg
633 reflection were determined. Structural refinement over a cycle of 100 rigid body and 100
634 isotropic restrained refinements with all atoms allowed to move and with every atom having
635 100 % occupancy were then performed against each of these 900 resampled data-sets using
636 pdb entry 5NJ4 as a starting model. R_{free} values ranging from 22.1 % to 23.1% were
637 recovered. Coordinate errors associated with each individual structural refinement are
638 estimated⁶⁰ to be ≤ 0.2 Å.

639

640 The distances between the Ca atoms of the photo-activated and resting RC_{vir} structures were
 641 compared pairwise using the miller package of CCTBX.⁶¹ A 100×100 Euclidian distance
 642 matrix was then calculated for every Ca atom and every time point according to: $\Delta r_{ij}^{\Delta t, \text{dark}} =$
 643 $|\mathbf{r}_i^{\Delta t} - \mathbf{r}_j^{\text{dark}}|$, where i and j vary from 1 to 100 and denote resampled dataset numbers, $\Delta r_{i,j}$
 644 depicts the distance separating the Ca coordinates of datasets i and j , and $\mathbf{r}_i^{\Delta t}$ and $\mathbf{r}_j^{\text{dark}}$ are the
 645 refined coordinates obtained from the photo-activated or dark structures, respectively. A
 646 second order Taylor series expansion was then used to estimate the mean and error associated
 647 with the ratio $\Delta r_{ij}^{\Delta t, \text{dark}} / \Delta r_{ij}^{\text{dark, dark}}$ arising from coordinate variations within each set of 100
 648 structural refinements. This expansion leads to the expression:

$$649 \quad \text{Error weighted mean ratio} = \langle \Delta r_{ij}^{\text{state, dark}} \rangle / \langle \Delta r_{ij}^{\text{dark, dark}} \rangle$$

$$650 \quad - \text{var}(\Delta r_{ij}^{\text{dark, dark}}) \times \langle \Delta r_{ij}^{\text{state, dark}} \rangle / \langle \Delta r_{ij}^{\text{dark, dark}} \rangle^3 + \text{cov}(\Delta r_{ij}^{\text{state, dark}}, \Delta r_{ij}^{\text{dark, dark}}) / \langle \Delta r_{ij}^{\text{dark, dark}} \rangle^2$$

651 where $\langle X \rangle$ is the mean of the set X, $\text{var}(X)$ is the variance of the set X, and $\text{cov}(X, Y)$ is the
 652 covariance of two sets X and Y. The resulting error weighted mean ratios are represented in
 653 Fig. 4A to 4C and Extended Data Fig. 7 in which movements are coloured from grey
 654 (movements $\leq 80\%$ of the maximum ratio) to red (movements $\geq 95\%$ of the maximum ratio).
 655 Full occupancy structural refinement avoided systematic bias in this analysis arising from
 656 partial occupancy structural refinement with a single dark-conformation held fixed, but at the
 657 cost of underestimating the magnitude of light-induced conformational changes. Despite this
 658 limitation, this analysis extracted recurring structural motions that evolve with time (Fig. 4a-c
 659 and Extended Data Fig. 7, Supplementary Video 3) and in a manner that is both consistent
 660 with the known time-scales of the electron transfer reactions (Fig. 1) and theoretical
 661 predictions (Fig. 4d-f, Supplementary Video 4).

662

663 *Tests of the statistical significance of recurring difference electron density features*

664 For each of the seven experimental difference Fourier electron density maps ($\Delta t = 1$ ps, 5 ps
665 (data set a), 5 ps (data set b), 20 ps, 300 ps (data set b), 300 ps (data set a), 8 μ s) a lower-
666 pedestal of 3.0σ was applied such that all electron density with an amplitude $< 3.0 \sigma$ was set
667 to zero. Both positive and negative difference electron densities were then integrated within a
668 4.5 \AA radius sphere about a chosen coordinate (Extended Data Fig. 3j) as described for the
669 analysis of TR-SFX data recorded from bacteriorhodopsin.²⁰ These positive (A+) and
670 negative (A-) integrated difference electron density amplitudes were merged to yield a single
671 amplitude according to: $A(\mathbf{r}) = ((A^+)^2 + (A^-)^2)^{1/2}$ about the centre of integration \mathbf{r} . The results of
672 this analysis are presented in Fig. 2f where six centres of integration, \mathbf{r} , are chosen as: the
673 centre of the BPh_M ring; the magnesium atom of BCh_M; the mid-point between the two
674 magnesium atoms of the two SP bacteriochlorophylls; the magnesium atom of BCh_L; the
675 centre of the BPh_L ring; and the centre of the ketone containing six-carbon ring of
676 menaquinone Q_A.

677

678 For tests of statistical significance (Extended Data Table 3), this set was complemented by the
679 addition of amplitudes extracted by integration about the iron atoms of haem₁, haem₂, haem₃
680 and haem₄ to create a set of ten amplitudes for each of the seven time points:

681 $[A(\text{BPh}_M), A(\text{BCh}_M), A(\text{SP}), A(\text{BCh}_L), A(\text{BPh}_L), A(\text{Q}_A), A(\text{H}_1), A(\text{H}_2), A(\text{H}_3), A(\text{H}_4)]_{\Delta t}$

682 arranged as a 10×7 element matrix. Control “noise only” $|F_{\text{obs}}|^{\text{dark}} - |F_{\text{obs}}|^{\text{dark}}$ isomorphous
683 difference Fourier electron density maps were calculated by first selecting sixteen resampled
684 data-sets from the set of 100 generated from the 2015 RC_{vir} dark data, and sixteen resampled
685 data-sets from the set of 100 generated from the 2016 RC_{vir} dark data. Eight $|F_{\text{obs}}|^{\text{dark}} - |F_{\text{obs}}|^{\text{dark}}$
686 isomorphous difference Fourier electron density maps were then calculated by pair-wise
687 comparisons between the sixteen resampled data-sets of the 2015 data, and another eight
688 difference Fourier electron density maps were calculated by pairwise-comparisons of the

689 sixteen resampled data-sets of the 2016 data. Seven control difference Fourier electron density
690 maps were then randomly selected from the set of sixteen “noise-only” maps, difference
691 electron density values with an amplitude lower than 3σ were set to zero, and a set of
692 $[A(\mathbf{r}, \text{dark-dark})]$ were created by integrating the remaining difference electron density within
693 a 4.5 Å radius sphere centred upon the RC_{vir} cofactors as described above. A two-sample t -
694 test was then performed in MATLAB to determine if the set of seven time-dependent
695 amplitudes $[A(\mathbf{r}, \Delta t)]$ and the set of seven “noise-only” amplitudes $[A(\mathbf{r}, \text{dark-dark})]$ were
696 indistinguishable from one another (the null hypothesis). The t -tests were then repeated 1000
697 times by randomly selecting a different combination of seven control amplitudes $[A(\mathbf{r}, \text{dark-}$
698 $\text{dark})]$ from the sixteen “noise-only” difference Fourier electron density maps calculated
699 above (of $16!/(9! \times 7!) = 11440$ possible different combinations of the 16 control maps). The
700 results of this analysis are summarized in Extended Data Table 3 and show that, when a
701 threshold of $p \leq 0.001$ is applied, the difference electron density amplitudes associated with
702 the SP cannot be ascribed to noise. When a threshold of $p \leq 0.0125$ is applied and the last
703 three time-points ($\Delta t = 300$ ps (a), 300 ps (b), 8 μs) are examined as a set, the difference
704 electron density amplitudes associated with the SP, BCh_L and Q_A cannot be ascribed to noise.
705 Conversely, the set of difference electron density amplitudes associated with most other co-
706 factors, as well as all sets of difference electron density amplitudes generated from noise-only
707 maps, are indistinguishable from noise according to the results of this two sample t -test
708 (Extended Data Table 3).

709

710 *QM/MM Geometry optimizations*

711 Initial coordinates were taken from PDB databank entry 5O4C and missing residues and co-
712 factor segments were retrieved from PDB entry 1PRC.⁶² Protonation states of residues were
713 chosen based on their reference pK_a values and structural criteria such as hydrogen bond

714 interactions. After the addition of protons to the structure, a 200 step steepest descent
715 geometry optimization was performed with Gromacs 4.5⁶³ to relax these coordinates. During
716 this optimization the positions of the heavy atoms were constrained to their positions in the x-
717 ray structure. As in previous work, the interactions were modelled with the Amber03 force-
718 field.^{64,65} Non-bonded Coulomb and Lennard-Jones interactions were evaluated without
719 periodic boundary conditions (PBC) and using infinite cut-offs.

720

721 After relaxing hydrogens with molecular mechanics (MM) optimization, we performed
722 several Quantum Mechanics (QM)/MM geometry optimizations of all atoms in the reaction
723 centre, using the interface between the TeraChem quantum chemistry package^{66,67} and
724 Gromacs 4.5.⁶³ These optimizations were also performed without PBC and with infinite cut-
725 offs for the Coulomb and Lennard-Jones interactions. The QM subsystems (Extended Data
726 Fig. 6a-c) were modelled with unrestricted DFT. In these DFT calculation we used the PBE0
727 functional⁶⁸ in combination with the LANL2DZ basis set.⁶⁹ Empirical corrections to
728 dispersion energies and interactions were introduced with Grimme's DFT-D3 model.⁷⁰ The
729 remainder of the protein, including crystal water molecules, was modelled with the Amber03
730 force-field,^{64,65} in combination with the TIP3P water model.⁷¹ We searched for minimum-
731 energy geometries in all relevant oxidation states of the system using the limited-memory
732 Broyden-Fletcher-Goldfarb-Shannon quasi-Newton optimization algorithm.

733

734 The goal of these optimization steps was to characterize the structural relaxation of the protein
735 in response to changes in the electronic states of the cofactors along the A-branch of the
736 photo-induced electron transfer process. We therefore examined the following electronic
737 configurations:

738 i. All cofactors in their resting states: SP^0 , BPh_L^0 , Q_A^0

739 ii. Special pair photo-excited, other cofactors in resting state: SP^* , BPh_L^0 , Q_A^0

740 iii. Special pair photo-oxidized, BPh_L reduced: SP^{+1} , BPh_L^{-1} , Q_A^0

741 iv. Special pair photo-oxidized, Q_A reduced: SP^{+1} , BPh_L^0 , Q_A^{-1}

742 Since including all co-factors into one large QM-region is computationally too demanding, we
743 performed the optimizations with a different QM subsystem for each co-factor, including
744 nearest residues, in all relevant electronic states. The structural response to the change in
745 electronic state (Fig. 4d-f) was obtained by comparing the optimized geometries and potential
746 energies in the various oxidation states.

747

748 To quantify the effect of photo-absorption by the special pair (SP, i to ii) we first optimized
749 the resting state with the SP plus nearby residues in the QM region (Extended Data Fig. 6a),
750 described at the PBE0/LANL2DZ level of theory plus D3 dispersion corrections. This
751 structure was used as a reference for the optimized structures in the excited state (SP^* , ii) and
752 after photo-oxidation (SP^{+1} , iii). Using the same QM/MM subdivision, we optimized the
753 system in the first singlet excited state (S_1) by switching the QM description to the Time-
754 dependent DFT within the Tamm-Dancoff approximation,⁷² and in the photo-oxidized state
755 by switching the spin state of the electronic wave function to the lowest energy doublet state
756 (D_0). In the QM/MM optimization of the D_0 state of the SP, we modelled the BPh_L with point
757 charges representing the reduced state of that co-factor. Only very modest protein structural
758 changes were associated with the optimized geometries with the SP in the S_1 and D_0 relative
759 to the reference structure in the resting state (S_0). Likewise, we also optimized the geometry
760 of the protein with the BPh_L and nearby residues in the QM region (Extended Data Fig. 6d) in
761 both the lowest energy singlet (S_0 , i) and doublet (D_0 , reduced, iii) states. In the optimization
762 of the D_0 state of BPh_L , the partial charges on the SP were changed to reflect its photo-

763 oxidized (D_0 , SP^{+1}) state. Again the structural response is rather minor, as the geometries are
764 very similar (Extended Data Fig. 6e).

765

766 In the next step of the electron transfer process, the electron transfers from BPh_L to Q_A (iv).
767 We optimized the protein with Q_A plus its immediate environment, including the non-heme
768 Fe^{2+} site, in the QM region. The optimized structures in the resting and reduced states are
769 compared in Extended Data Fig. 6f. Reduction of Q_A from menaquinone to (deprotonated)
770 semiquinone induces significant structural changes in the Q_A binding pocket. In line with the
771 difference densities observed at 300 ps after photo-excitation, the hydrogen bond between the
772 Q_A carbonyl and His217_M reduces by 0.17 Å. We suggest that the reduction of this hydrogen
773 bond helps stabilizing the negative charge on the Q_A .

774

775 To quantify the overall structural response to the electron transfers, we computed the
776 displacements of the atoms in the various states (ii-iv) with respect to the structure of resting
777 state (i) and recorded these displacements as B-factors to the pdb coordinate file of the resting
778 state. Because only one co-factor was included in the QM region of our QM/MM
779 optimizations, we summed up the displacements of both QM/MM optimizations of each redox
780 state. Fig. 4d-f represents these displacements as colours.

781

782 *Stabilization energies*

783 To estimate the energetic effects of the protein structural changes on the electron transfer
784 process, we computed the adiabatic and vertical electron affinities for Q_A in isolation and in
785 the optimized QM/MM protein models. These energies are shown schematically in Extended
786 Data Fig. 6g,h. For the neutral Q_A in vacuum, the electron affinity *without* structural
787 relaxation is 164.5 kJ.mol⁻¹ (vertical electron affinity, VEA). Structural relaxation in response

788 to adding the electron increases the affinity further by 24 kJ.mol^{-1} , so that the energy
789 difference between the neutral reactant minimum on the one hand and the reduced product
790 minimum is $188.5 \text{ kJ.mol}^{-1}$ (adiabatic electron affinity, AEA) on the other hand. The
791 calculated AEA is in good agreement with results from previous computations,²⁹ but is an
792 overestimation with respect to the experimental value for the related 1,4-naphthoquinone (175
793 kJ.mol^{-1}).⁷³ Inside the protein environment, the VEA is much higher (258 kJ.mol^{-1}), part of
794 which we attribute to the electrostatic interaction between the reduced Q_A co-factor with the
795 positively charged Fe^{2+} ligand site. Structural relaxation of both the Q_A cofactor and the
796 protein environment increases the electron affinity by 60 kJ.mol^{-1} to yield an AEA of 318
797 kJ.mol^{-1} . Thus, the results of the computations suggest that the structural response of the
798 protein adds another 36 kJ.mol^{-1} to the intrinsic relaxation energy of Q_A (24 kJ.mol^{-1} in
799 vacuum) as concluded in earlier computations.²⁹ We note that in this analysis we focussed
800 only on the effect of the structural response on the affinity of Q_A . To estimate the *total*
801 reaction energy associated with the photo-induced electron transfer process from the SP to
802 Q_A , we also need the absolute energies of the neutral, photo-excited and oxidized states of the
803 SP as well as the neutral and reduced states of BPh_L . However, since these energies were not
804 computed with identical QM/MM setups, we do not provide an accurate estimate here.

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809 **METHODS REFERENCES**

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927 **EXTENDED DATA FIGURE LEGENDS**

928

929 **Extended Data Figure 1: Dependence of transient infrared (IR) spectra on the pump**

930 **laser fluence. a,** Time-resolved IR difference spectra recorded from RC_{vir} in D₂O buffer for

931 four time points and variable pump-laser fluence following 960 nm excitation. Spectral

932 changes are consistent with earlier reports.^{74,75} Dominant time-dependent features are

933 highlighted and include: (1) a negative band at 1687 cm⁻¹ (9-carbonyl stretch in SP_L and SP_M)

934 and (2) a broad negative band centred at 1680 cm⁻¹ (9-carbonyl stretch in BCh_L). Transient

935 changes from $\Delta t = 2$ ps to 5 ps correlate with the time-dependent photo-oxidation of SP. Grey

936 columns indicate decreasing bands whereas cyan columns indicate increasing bands. **b,** Time

937 resolve IR difference spectra normalized and superimposed. These spectra remain

938 superimposable throughout the pump-laser fluence domain probed. **c,** Dependence of the

939 magnitude of the difference IR signal (calculated as the sum of the absolute value over all

940 pixels) on the pump laser fluence. Below 20 GW/cm² the absolute signal increases

941 approximately linearly whereas above 60 GW/cm² the signal flattens out as a plateau is

942 reached. This plateau is consistent with the complete bleaching of the special pair absorption

943 at 960 nm in the photoexcited state and correlates with the phenomenon of hole-burning in

944 RC_{vir}.⁴²

945

946 **Extended Data Figure 2: Overview of the experimental $F_{\text{obs}}^{\text{light}} - F_{\text{obs}}^{\text{dark}}$ difference**

947 **Fourier electron density maps calculated between the photo-excited and resting state**

948 **data. a,** Overview of the structure of RC_{vir} when viewed from the plane of the membrane. TM

949 helices E_L and D_M are highlighted in red and gold respectively. **b-h,** Difference Fourier

950 electron density maps for the time-points: **b,** $\Delta t = 1$ ps; **c,** $\Delta t = 5$ ps (a); **d,** $\Delta t = 5$ ps (b); **e,** Δt

951 $= 20$ ps; **f,** $\Delta t = 300$ ps (b); **g,** $\Delta t = 300$ ps (a); **h,** $\Delta t = 8$ μ s. All maps are contoured at $\pm 4.0 \sigma$

952 (σ is the root mean square electron density of the map). Positive difference electron density is
953 shown in blue and negative difference electron density is shown in gold.

954

955 **Extended Data Figure 3: Light-induced electron density changes in RC_{vir} at the site of**
956 **photo-oxidation.** Experimental $F_{\text{obs}}^{\text{light}} - F_{\text{obs}}^{\text{dark}}$ isomorphous difference Fourier electron
957 density maps for the time-points: **a**, $\Delta t = 1$ ps. **b**, $\Delta t = 5$ ps (data set a); **c**, $\Delta t = 5$ ps (data set
958 b). **d**, $\Delta t = 20$ ps; **e**, $\Delta t = 300$ ps (data set b); **f**, $\Delta t = 300$ ps (data set a); **g**, $\Delta t = 8$ μs . **h-i**,
959 Principal component from SVD analysis of difference Fourier electron density maps for: **h**,
960 the first four time-points, $\Delta t = 1$ ps, 5 ps (data set a), 5 ps (data set b) and 20 ps; **i**, the final
961 three time-points, $\Delta t = 300$ ps (data set a), 300 ps (data set b) and 8 μs . All maps are
962 contoured at $\pm 3.2 \sigma$ (blue, positive difference electron density; gold, negative difference
963 electron density). Peak heights listed in Table 2 are marked with coloured circles in panels h
964 and i. **j**, Representation of a sphere of radius 4.5 \AA used to integrate difference electron
965 density above a pedestal of 3.0 σ as described in reference²⁰. Positive difference density
966 amplitudes (A+) and negative difference density amplitudes (A-) were merged according to
967 $((A+)^2 + (A-)^2)^{1/2}$ and are displayed in Fig. 2f. These integrated difference electron density
968 values are also used in the statistical analyses presented in Extended Data Table 2. **k**,
969 Simulated difference Fourier electron density maps near the SP calculated from the refined
970 structure for $\Delta t = 20$ ps versus the refined resting state structure. Data are cut at 2.8 \AA
971 resolution for comparison with experimental data. The simulated map is contoured at ± 12.0
972 σ . **l**, Relative magnitude of the singular values resulting from SVD analysis of the difference
973 Fourier electron density maps for all seven time-points. **m**, First (blue) and second (red)
974 columns of the unitary matrix U resulting from SVD analysis of all seven time-points
975 weighted according to their corresponding singular values. The first right singular vector (the
976 principal SVD component) makes a significant and positive contribution to all time-points. In

977 contrast the difference electron density maps for $\Delta t = 5$ ps (a) and 300 ps (a) contain strong
978 negative contributions from the second right singular vector, which suggests that differences
979 between the two experimental runs cause systematic differences in the difference Fourier
980 electron density maps that are separated by SVD analysis.

981

982 **Extended Data Figure 4: Electron density changes near the active site of myoglobin.** The

983 $F_{\text{obs}}^{\text{light}} - F_{\text{obs}}^{\text{dark}}$ difference Fourier electron density map for $\Delta t = 10$ ps was calculated from
984 data (pdb entries 5CNE minus 5CMV) recorded during TR-SFX studies of the photo-
985 dissociation of carbon monoxide from the active site of myoglobin.²¹ **a**, Data from 15 Å to 1.8
986 Å were used to calculate the difference Fourier electron density map. **b**, Data from 15 Å to 3.0
987 Å were used to calculate the difference Fourier electron density map. Positive and negative
988 difference electron density features associated with the heme group indicate slight motions of
989 the protoporphyrin-IX. Blue represents positive difference density (contoured at 3.0 σ) and
990 gold represents negative density (contoured at -3.0 σ). At 1.8 Å the maximum amplitude of
991 the highlighted difference density features are: a+, 14.5 σ ; a-, 14.0 σ ; b+, 4.6 σ ; b- 4.0 σ ; c+,
992 4.1 σ , c- 4.0 σ ; d+, 3.8 σ . When the map is recalculated after data is cut to 3.0 Å resolution the
993 corresponding values are: a+, 11.9 σ ; a-, 12.4 σ ; b+, 4.2 σ ; b- 4.1 σ ; c+, 3.7 σ , c- 2.9 σ ; d+, 3.2
994 σ .

995

996 **Extended Data Figure 5: Light-induced electron density changes in RC_{vir} within the**

997 **menaquinone binding pocket.** Experimental $F_{\text{obs}}^{\text{light}} - F_{\text{obs}}^{\text{dark}}$ difference Fourier electron
998 density maps for the time-points: **a**, $\Delta t = 1$ ps. **b**, $\Delta t = 5$ ps (data set a); **c**, $\Delta t = 5$ ps (data set
999 b). **d**, $\Delta t = 20$ ps; **e**, $\Delta t = 300$ ps (data set b); **f**, $\Delta t = 300$ ps (data set a); **g**, $\Delta t = 8$ μ s. All seven
1000 maps are contoured at ± 3.0 σ (blue, positive difference electron density; gold, negative
1001 difference electron density). **h-i**, Principal component from SVD analysis of difference

1002 Fourier electron density maps for: **h**, the first four time-points, $\Delta t = 1$ ps, 5 ps (data set a), 5 ps
1003 (data set b) and 20 ps; **i**, the final three time-points, $\Delta t = 300$ ps (data set a), 300 ps (data set b)
1004 and 8 μ s. SVD difference Fourier maps are contoured at $\pm 3.2 \sigma$. Peak heights listed in Table
1005 2 are indicated in panel **i**. **j**, Simulated difference Fourier electron density maps near Q_A
1006 calculated from the refined structure for $\Delta t = 300$ ps (data set a) versus the refined resting
1007 state structure. Data are cut at 2.8 Å resolution for comparison with experimental data. The
1008 simulated map is contoured at $\pm 12.0 \sigma$.

1009

1010 **Extended Data Figure 6: Results of QM/MM energy minimization calculations. a-c**, QM
1011 subsystems used in the QM/MM optimizations of the co-factors. Atoms included in the QM
1012 region are shown in ball-and-stick representation, while the other atoms of the protein are
1013 shown as cartoons. Atoms belonging to the co-factor that are not included in the QM region
1014 are shown as sticks. Water molecules that are not part of the QM region are not shown. **a**,
1015 Atoms included in the QM region associated with the SP. **b**, Atoms included in the QM
1016 region associated with the BPh_L. **c**, Atoms included in the QM region associated with the Q_A.
1017 **d**, Structures of the energy minimized resting conformation (black: SP and BPh_L neutral) and
1018 that after the first electron transfer step (blue: SP⁺¹ and BPh_L⁻¹) shown near the special pair. **e**,
1019 Structures of the energy minimized resting conformation (black: SP and BPh_L neutral) and
1020 that after the first electron transfer step (blue: SP⁺¹ and BPh_L⁻¹) shown near BPh_L. **f**, Structures
1021 of the energy minimized resting conformation (black: SP and Q_A neutral) and that after the
1022 second electron transfer step (blue: SP⁺ and Q_A⁻) shown near Q_A. The H-bond between O₁ of
1023 the reduced semiquinone and N_{δ1} of His217_M is predicted to be shortened by 0.17 Å when
1024 menaquinone is reduced. **g-h**, Schematic depiction of the potential energy surfaces for
1025 menaquinone in vacuum (**g**) and within the protein (**h**) in the resting (neutral) electronic state
1026 (black) and the reduced electronic state (red). Vertical electron affinities (VEA), adiabatic

1027 electron affinities (AEA) and relaxation energies E_r were computed at the PBE0-
1028 D3/LANL2DZ level of DFT for isolated Q_A and at the PBE0-D3/LANL2DZ/Amber03
1029 QM/MM level for Q_A within the protein.

1030

1031 **Extended Data Figure 7: Recurring movements of $C\alpha$ atoms quantified by structural**
1032 **refinement.** Recurring movements of $C\alpha$ atoms quantified by full occupancy structural
1033 refinement against 100 randomly resampled TR-SFX data sets. Recurring movements are
1034 represented as error weighted mean ratios relative to 100 control structural refinements
1035 (Methods). Error weighted means ratios are coloured from grey (< 80 % of the maximum
1036 ratio) to red (≥ 95 % of the maximum ratio). **a**, Recurring movements of $C\alpha$ atoms associated
1037 with refinements against data for $\Delta t = 1$ ps. **b**, Recurring movements of $C\alpha$ atoms for $\Delta t = 5$
1038 ps (data set a). **c**, Recurring movements of $C\alpha$ atoms for $\Delta t = 5$ ps (data set b). **d**, Recurring
1039 movements of $C\alpha$ atoms for $\Delta t = 20$ ps. **e**, Recurring movements of $C\alpha$ atoms for $\Delta t = 300$ ps
1040 (data set b). **f**, Recurring movements of $C\alpha$ atoms for $\Delta t = 300$ ps (data set a). **g**, Recurring
1041 movements of $C\alpha$ atoms for $\Delta t = 8 \mu\text{s}$. Transmembrane helices are drawn as rods.

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1044 **FOOTNOTES TO EXTENDED DATA TABLES**

1045

1046 **Extended Data Table 1: Crystallographic data and refinement statistics**

1047 Footnotes to Extended Data Table 1

1048
$$^{\dagger}R_{\text{split}} = 1 / \sqrt{2 \frac{\sum hkl |I_{\text{even}} - I_{\text{odd}}|}{1/2 \sum hkl |I_{\text{even}} + I_{\text{odd}}|}}$$

1049 ‡ Values in parenthesis is those of the highest resolution shell.

1050 $^{\text{£}}$ Ratio of the number of indexed images to the total number of images.

1051

1052

1053 **Extended Data Table 2: Difference Fourier electron density peak amplitudes**

1054 Footnotes to Extended Data Table 2

1055 *Positive and negative difference electron density peaks are marked on Extended data Figs.

1056 3h, 3i and 5i. Difference electron density values were read out manually in COOT.

1057 Amplitudes < 2.4 σ are not shown.

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1060 **Extended Data Table 3: Two sample t-tests of integrated electron density amplitudes**

1061 Footnotes to Extended Data Table 3

1062 *Mean σ /sphere represents the root-mean-square of the difference electron density above and

1063 below a threshold of $\pm 3.0 \sigma$ within a sphere of radius 4.5 Å centred on the Mg²⁺ atoms of the

1064 BChs; the Fe atoms of the haems; and ring centres of the menaquinone and BPhe cofactors.

1065 All values are scaled relative to a mean value for SP = 1.

1066 † The number of elements in each sampled set of the two-sample t-test.

1067 ‡ Difference electron density associated with haem₃ gave the lowest mean σ /sphere (0.14)

1068 and an exceptionally low standard deviation (± 0.06). Randomly generated control data

1069 gave the corresponding values of 0.09 ± 0.22 . The coincidence of a set with a low standard

1070 deviation being compared against a control set with an exceptionally high standard

1071 deviation frequently yielded low *p*-values. Nevertheless, the experimental difference

1072 features associated with haem₃ are weak and are therefore not physically meaningful.

1073 $^{\text{£}}$ Thirty two control data sets were generated by randomly selecting a sub-set of the dark

1074 observations to generate a new data set. Sixteen control difference Fourier electron density

1075 maps were then calculated between two of the control data-sets to generate maps

1076 representing the noise inherent within the SFX experiment. Mean σ /sphere calculations and

1077 all other steps proceeded as with the light versus dark experimental difference Fourier

1078 electron density maps.

1079 $^{\text{£}}$ The reference set consisted of a set of seven maps ($N_B = 7$) randomly selected from

1080 sixteen control maps. Random selection of the control set and the t-test was repeated 1000

1081 times, from a possible set of $16!/(9! \times 7!) = 11440$ different combinations of the 16 control
1082 maps. Percentage values give the fraction of occasions when $p \leq$ threshold for the resulting
1083 two sample t-test.

1084 [§]The hypothesis that the two sets of the t-test are indistinguishable is either true (0) or false
1085 (1) at the specified confidence level.

1086 [§] Features identified as distinguishable from noise with a degree of confidence defined by
1087 the given p -value.

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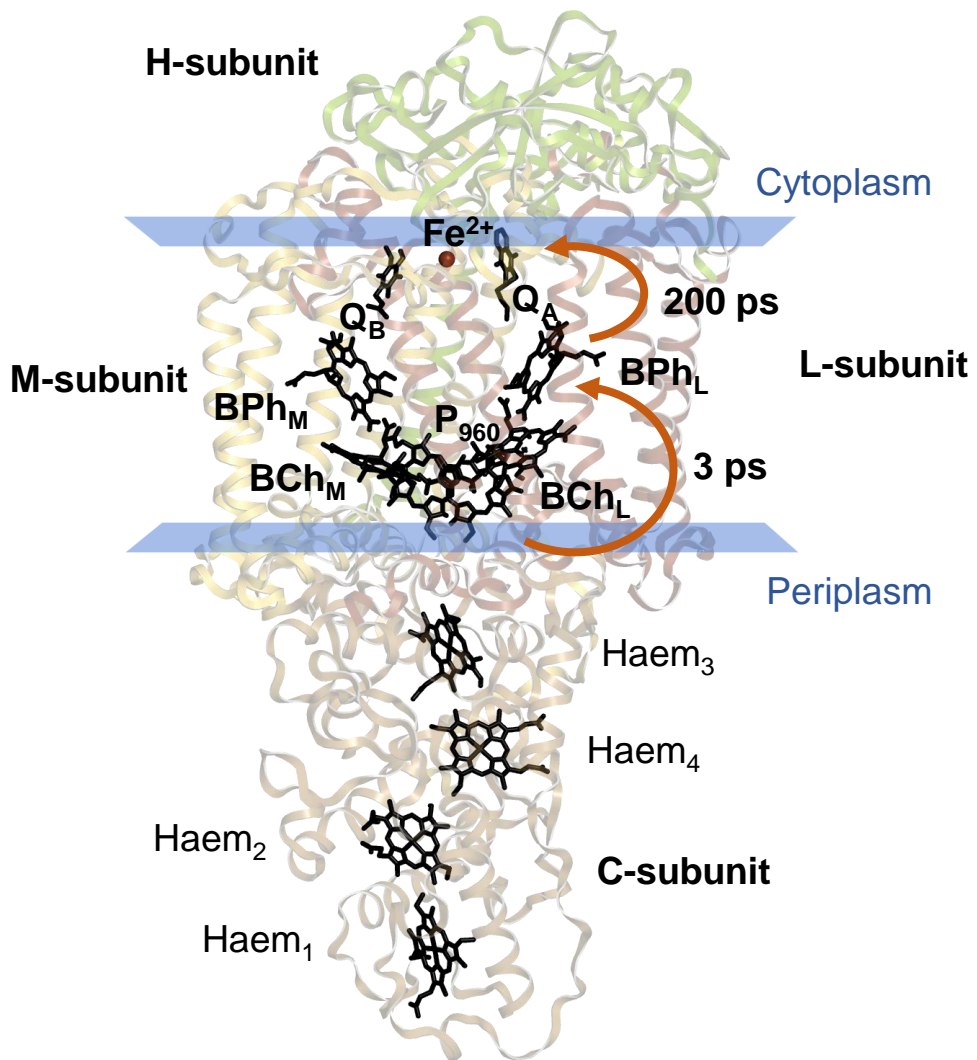


Figure 1: Dods et al.

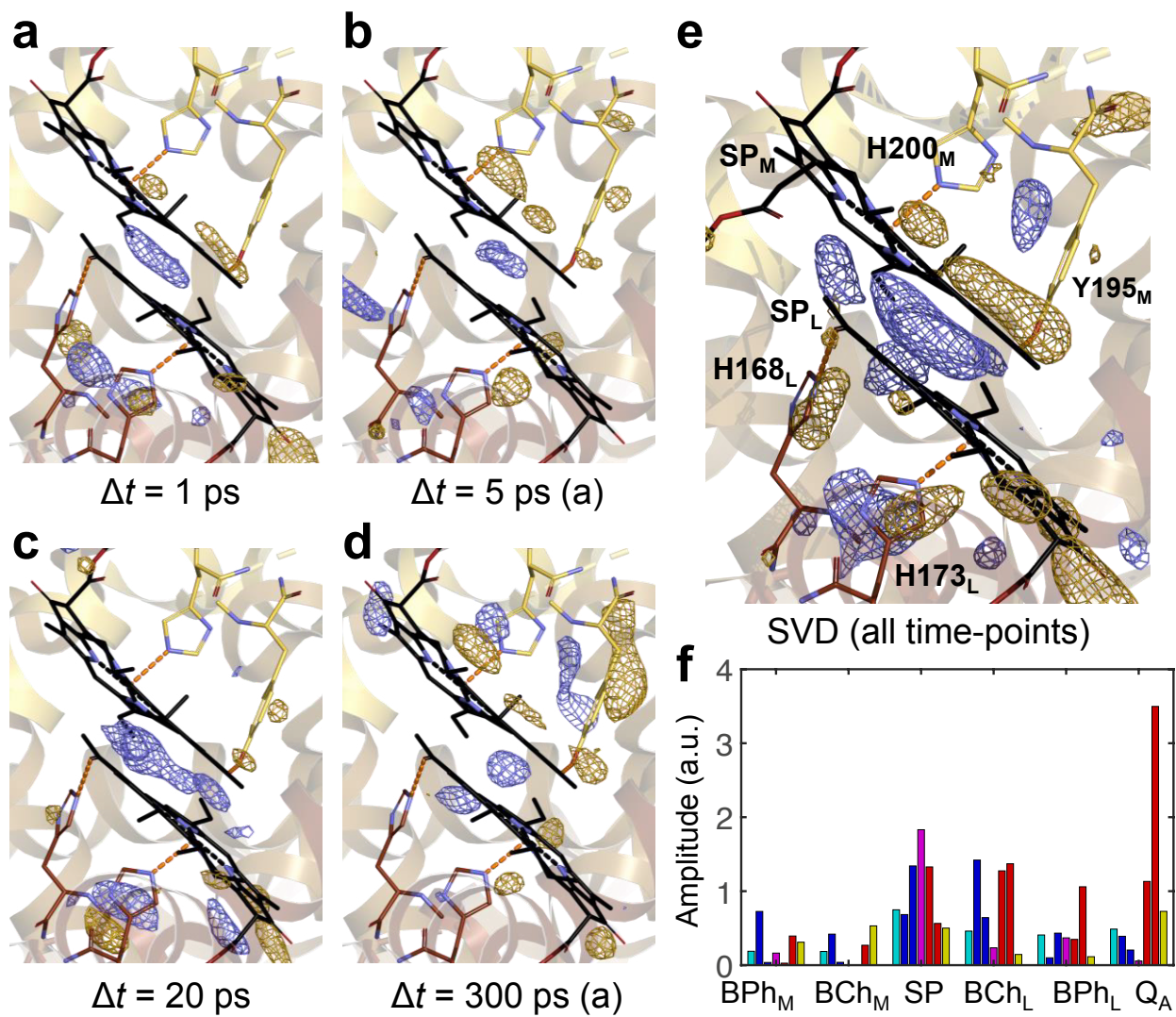


Figure 2: Dods et al.

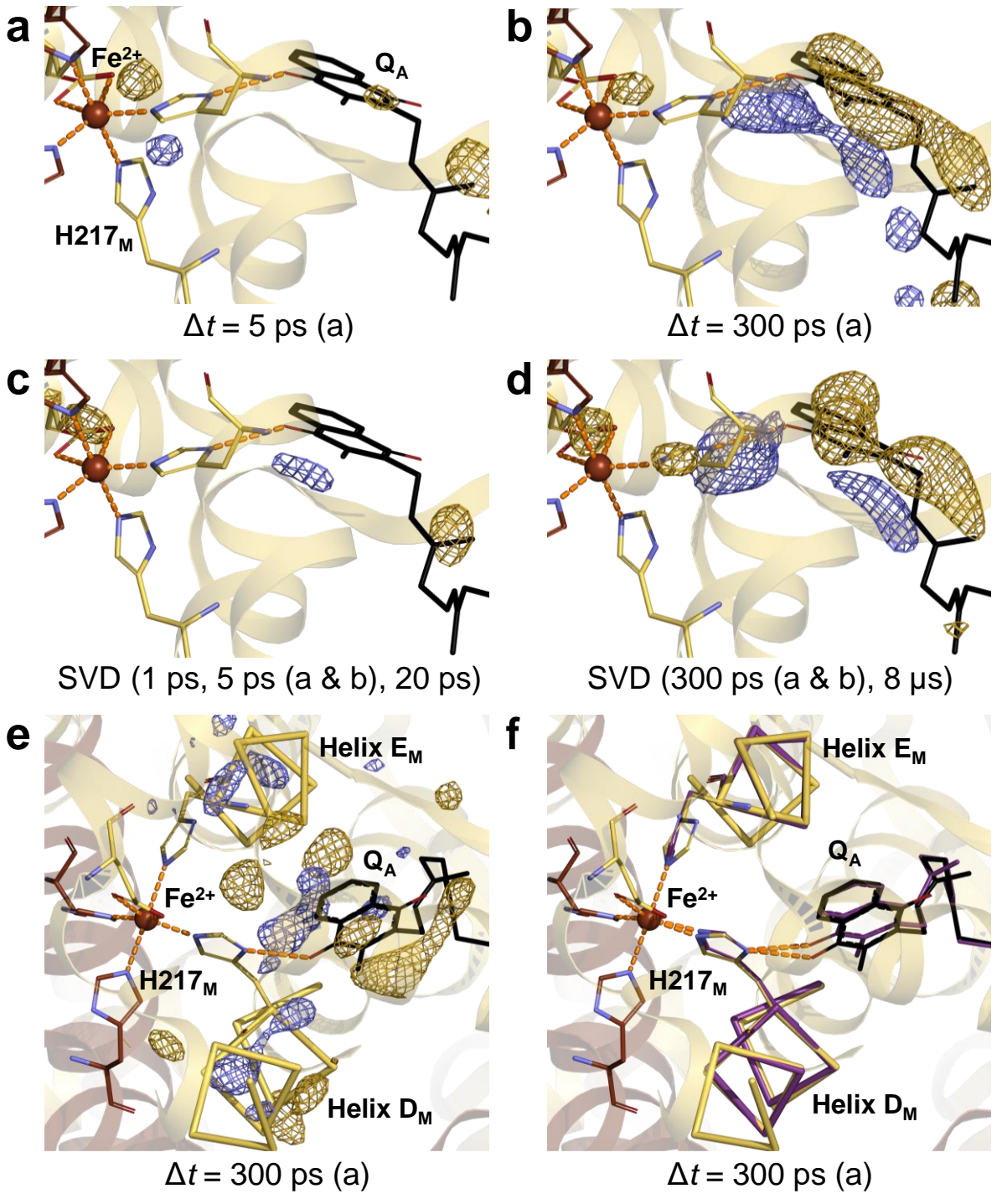


Figure 3: Dods et al.

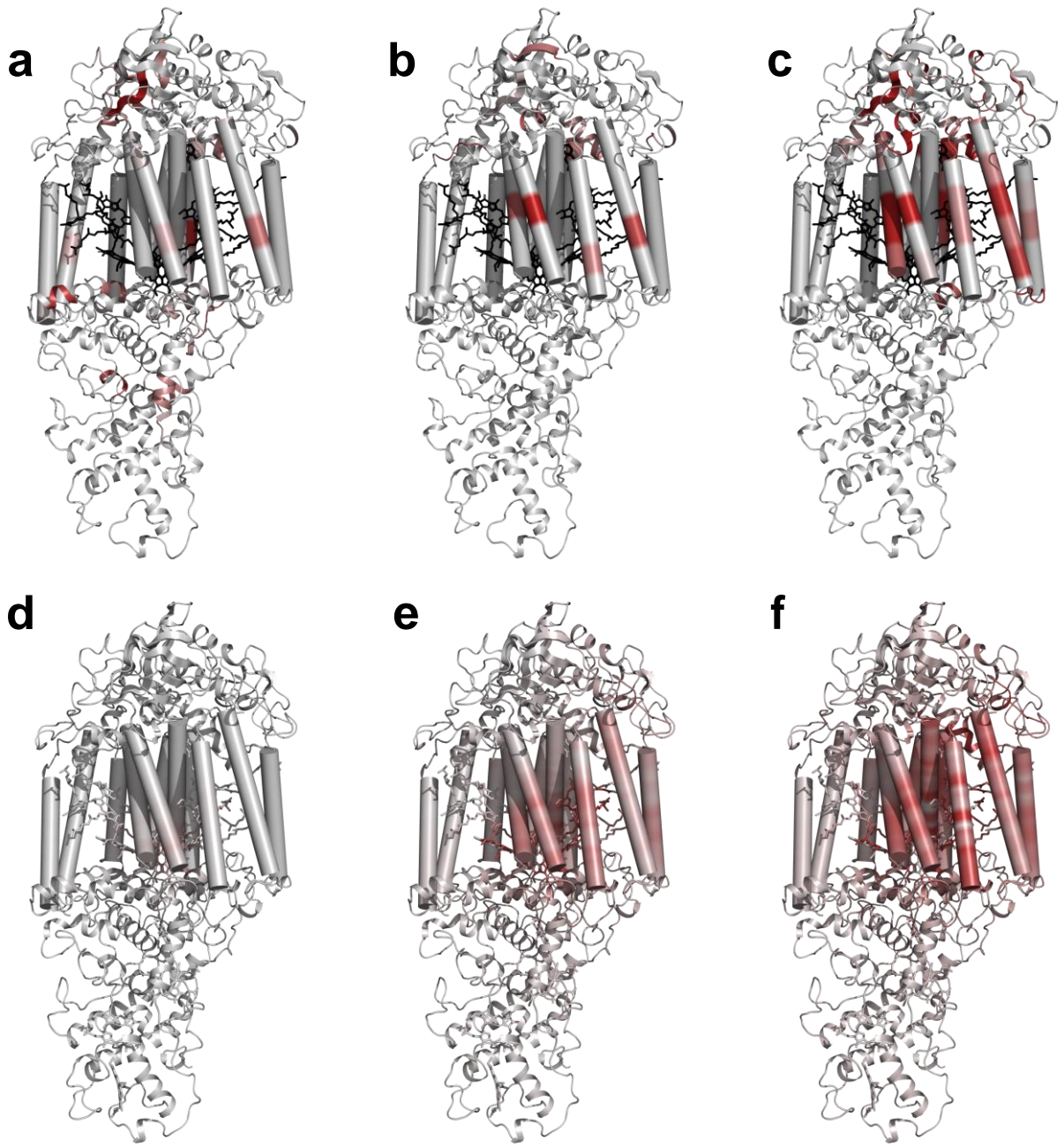


Figure 4: Dods et al.