

1 **High-resolution cryo-EM structure of photosystem II: Effects of electron beam**  
2 **damage**

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24 **Abstract**

25 Photosystem II (PSII) plays a key role in water-splitting and oxygen evolution. X-ray  
26 crystallography has revealed its atomic structure and some intermediate structures.  
27 However, these structures are in the crystalline state, and its final state structure has not  
28 been solved because of the low efficiencies of the S-state transitions in the crystals. Here  
29 we analyzed the structure of PSII in solution at 1.95 Å resolution by single-particle  
30 cryo-electron microscopy (cryo-EM). The structure obtained is similar to the crystal  
31 structure, but a PsbY subunit was visible in the cryo-EM structure, indicating that it  
32 represents its physiological state more closely. Electron beam damage was observed at a  
33 high-dose in the regions that were easily affected by redox states, which was reduced by  
34 reducing the electron dose. This study will serve as a good indicator for determining  
35 damage-free cryo-EM structures of not only PSII but also all biological samples,  
36 especially redox-active metalloproteins.

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## 39 **Introduction**

40 PSII is a multi-subunit pigment-protein complex embedded in the thylakoid  
41 membranes of higher plants, green algae and cyanobacteria, and is the only molecular  
42 machine capable of oxidizing water by use of visible light in the nature. Water molecules  
43 are split into electrons, hydrogen atoms and oxygen molecules at the catalytic center of  
44 PSII, namely, the oxygen-evolving complex (OEC), through four electron and/or proton  
45 removing steps as described in the Si-state cycle (with  $i = 0-4$ , where  $i$  indicates the  
46 number of oxidative equivalents accumulated)<sup>1</sup>.

47 In order to elucidate the mechanism of the water-splitting reaction, the structure of  
48 PSII has been studied extensively by X-ray diffraction (XRD) with synchrotron radiation  
49 (SR)<sup>2-6</sup>. The SR structure of PSII at an atomic resolution revealed that OEC is a  $Mn_4CaO_5$   
50 cluster organized into a distorted-chair form, in which the cuboidal part is composed of  
51  $Mn_3CaO_4$  and the outer manganese is attached to the cuboid via two  $\mu$ -oxo-bridges<sup>6</sup>.  
52 However, based on the extended X-ray absorption fluorescence spectra (EXAFS)  
53 analysis, the dose used for collecting the SR structure at 1.9 Å resolution may cause 25%  
54 of the Mn ions in OEC to be reduced to  $2^+$  ions, causing some elongations in the Mn-Mn  
55 distances in the structure<sup>7</sup>. This issue is overcome by the use of X-ray free electron lasers  
56 (XFEL), which provide X-ray pulses with ultra-short durations that enable collection of  
57 the diffraction data before onset of the radiation damage (diffraction before destruction)<sup>8</sup>.  
58 Using XFELs, radiation damage free structure of PSII was solved at a high resolution by  
59 an approach called fixed-target serial rotational crystallography, which uses multiple  
60 large PSII crystals by a shot-and-move/rotation method<sup>9,10</sup>. The result showed a

61 shortening of 0.1-0.2 Å in some of the Mn-Mn distances, indicating that the structure  
62 represents a damage free one<sup>10</sup>. By a combination of serial femtosecond X-ray  
63 crystallography (SFX) with XFELs and small crystals, structures of S-state intermediates  
64 up to S<sub>3</sub>-state were analyzed by pump-probe experiments where snapshot diffraction  
65 images were collected from flash-illuminated PSII crystals<sup>11-14</sup>. These results  
66 demonstrated the appearance of a new oxygen atom O6 (O<sub>x</sub>) close to O5 between Mn1  
67 and Mn4 upon two flashes, suggesting insertion of a water molecule in the S<sub>2</sub> → S<sub>3</sub>  
68 transition for O=O bond formation. However, all these studies were conducted with PSII  
69 crystals, and the efficiencies of the S-state transitions in the microcrystals were reported  
70 to be slightly lower compared with those in solution using light-induced Fourier  
71 transform infrared difference spectroscopy<sup>15</sup>. Moreover, it is unknown if the structure of  
72 PSII in the crystalline state is the same as those in the solution.

73 Cryo-electron microscopy (cryo-EM) can solve the structures of proteins in solution  
74 without crystallization, which may represent the physiological states of proteins more  
75 closely. It can also analyze the dynamic changes of proteins in solutions in the time range  
76 of ms, provided that cooling of the samples is rapid enough. In recent years, the technique  
77 of cryo-EM has been developed rapidly, and the resolutions of structures that can be  
78 solved by cryo-EM are increased dramatically<sup>16-18</sup>. However, there is also the issue of  
79 damage caused by the electron beam during cryo-EM data collection, even though the  
80 cryo-EM is usually conducted at a low temperature. Radiation damage has been  
81 extensively studied with X-rays, and it has been shown that the damage mainly manifests  
82 as breakage of disulfide bonds, decarboxylation of acidic amino and photoreduction of

83 metal centers<sup>7,19-21</sup>. The damage caused by electron beams have also been shown in  
84 cryo-EM analysis<sup>17</sup>. In order to obtain a high resolution, however, cryo-EM studies are  
85 usually conducted at a high-dose of electron beams without paying much attention to the  
86 electron beam damage. In this paper, we analyzed the structure of PSII in ice by cryo-EM  
87 at a resolution of 1.95 Å, and investigated the electron beam damage to PSII, especially  
88 its OEC, upon dose accumulation. We show that the structure of PSII analyzed by cryo-EM  
89 may represent the physiological state more closely, as it retains the PsbY subunit.  
90 However, it suffers from a severe electron beam damage at a high-dose, and this damage  
91 was reduced at a much decreased dose without a significant loss of resolution. These  
92 results are not only important for the analysis of the PSII structure in solution, but also  
93 provide important implications for all cryo-EM studies that use considerably high-doses  
94 for imaging.

95

## 96 **Result**

### 97 **High resolution single particle analysis of the PSII**

98 To obtain the high resolution structure of PSII, three data sets of single-particle  
99 images of the PSII dimer from *T. vulcanus* were collected using Thermo Fisher Scientific  
100 Titan Krios and JEOL CRYO ARM 300 at different conditions as summarized in Table 1.  
101 Because the sample for the 75 x k magnification using Titan contained 5% glycerol in the  
102 buffer, the sample was diluted ten times with the buffer without glycerol. The other  
103 samples did not contain the glycerol and were concentrated by PEG 1450 precipitation in  
104 the final step. Image processing yielded final resolutions of 2.22 Å for the data set

105 collected at 75 x k magnification using Titan Krios (Titan-75k), 2.20 Å for the data set  
106 collected at 96 x k magnification using Titan Krios (Titan-96k), and 1.95 Å for the data  
107 set collected at 60 x k magnification using CRYO ARM 300 (ARM-60k) (Table 1, Table  
108 2 and Supplementary Fig. 1-4). These results indicate that the quality of the cryo-EM  
109 density maps achieved were at the level comparable to those obtained with SR and XFEL  
110 previously<sup>6,10</sup>. The resolutions of the Titan-75k and Titan-96k data were almost the same,  
111 in spite of the different magnifications and buffers used. The resolution of the ARM-60k  
112 data was significantly better than that of the Titan-96k data, despite that the same buffer  
113 condition was used for the two data sets. The resolution achieved by cryo-EM depends on  
114 a number of factors, including sample quality, the type and preparations of cryo-grids  
115 used, the thickness of ice in the samples, microscope alignment and imaging conditions,  
116 etc. However, the major one could be the electron beam source. The CRYO ARM 300  
117 microscope has a cold field emission gun (CFEG) that produces an electron beam with a  
118 high temporal-coherence and superior high-resolution signals<sup>22</sup> over that from the  
119 Schottky emission gun equipped in the Krios microscope.

120 In Fig. 1, the squared inverse resolution of reconstructions achieved from random  
121 subsets of particles is plotted against the subset size on a logarithmic scale. This is known  
122 as Rosenthal-Henderson plot<sup>23</sup>. These plots indicated that the resolution is proportional to  
123 the log of particle size. The B-factors estimated from these plots are 60.8 for the  
124 Titan-75k data set, 74.9 for the Titan-96k data set and 43.3 Å<sup>2</sup> for the ARM-60k data set.  
125 The ARM-60k data set again shows the lowest value, in agreement with its highest  
126 resolution.

127

## 128 **Overall structure of PSII**

129       The overall atomic model of PSII was built based on the highest 1.95-Å resolution  
130 density map reconstructed from the ARM-60k data set. At this resolution, the features of  
131 cofactors and water molecules can be easily identified in the map (Fig. 2). The overall  
132 architecture of the PSII dimer from *T. vulcanus* is very similar to that of SR (PDB:  
133 3WU2)<sup>6</sup> and XFEL structures (PDB: 4UB6 and 4UB8)<sup>10</sup>, except for PsbY. The density of  
134 PsbY is present in one of the two monomers in the native (PDB: 4UB6)<sup>10</sup> and the  
135 Sr<sup>2+</sup>-substituted PSII dimer structures (PDB: 4IL6)<sup>24</sup> but absent in the SR structure (PDB:  
136 3WU2)<sup>6</sup>. However, this density was seen in both sides of the PSII dimer in the cryo-EM  
137 structure, although the density is somewhat poorer compared with that of the other  
138 assigned subunits (Fig. 2a, f). This suggests that the cryo-EM structure more closely  
139 represents the native state of PSII.

140       The root mean square deviation (RMSD) is 0.40 Å for 5227 C<sub>α</sub> atoms between the  
141 structures of cryo-EM and SR, and 0.46 Å for 5267 C<sub>α</sub> atoms between the structures of  
142 cryo-EM and XFEL. Because the RMSD was 0.32 Å for 5241 C<sub>α</sub> atoms between the SR  
143 and XFEL structures of PSII dimers, the cryo-EM structure is almost identical to the SR  
144 and XFEL structures at the backbone level. In the cryo-EM density map, we assigned  
145 2432 water molecules at a contour level of 5 σ, which are slightly less than the number of  
146 waters assigned in the SR and XFEL structures<sup>6,10</sup>. The atomic displacement parameter  
147 (ADP) of the cryo-EM structure refined with Refmac5 in reciprocal space correlated well  
148 with that of the SR structure (Supplementary Fig. 5), although it may be somewhat

149 overestimated in the cryo-EM structure. Since the cryo-EM map was subjected to  
150 B-factor sharpening with Postprocessing, it is not suitable to compare ADP values  
151 directly between cryo-EM and X-ray structures. Nevertheless, the relative ADP of the  
152 atoms in the molecule appears to reflect the characteristics of the map. The average ADP  
153 for the OEC atoms ( $13.8 \text{ \AA}^2$ ) were found to be lower than that observed in the overall  
154 protein atoms of the cryo-EM structure ( $20.6 \text{ \AA}^2$ ), suggesting that the structure of OEC  
155 was determined more reliably than that of the overall structure. This may be due to the  
156 presence of metal ions in the  $\text{Mn}_4\text{CaO}_5$  cluster, which gives rise to higher cryo-EM  
157 density than that of lighter protein atoms.

158

### 159 **Electron beam damage to the PSII structure**

160 Several regions of PSII were found to have different structures between cryo-EM  
161 and XFEL, which are considered to arise from electron beam induced damage. In the  
162 PsbO subunit, a disulfide bond between Cys19 and Cys41 was observed in the XFEL  
163 structure<sup>10</sup>, but it was completely cleaved in the cryo-EM structure (Fig. 3a). In the C  
164 terminus of D1 subunit, a part of Ala344, the C-terminal residue that coordinated to the  
165  $\text{Mn}_4\text{CaO}_5$  cluster, flipped out from the OEC and adopted an alternative conformation in  
166 the cryo-EM structure (Fig. 3b). These are the typical sign of damage caused by the  
167 electron beam irradiation during the acquirement of cryo-EM images.

168 In the OEC, the positions of heavy metals were confirmed clearly and were assigned  
169 based on their highest peaks in the cryo-EM map achieved at the high-dose (Fig. 4a). In  
170 addition, five oxo-oxygen atoms and four water molecules ligated to the OEC were



171 assigned in the difference map, which were obtained by subtracting the metal densities in  
172 a diameter of 1.5 Å of that metal from the whole cryo-EM map. The overall architecture  
173 of the OEC in the cryo-EM structure is very similar to that of the SR and XFEL structures,  
174 however, distinct differences were observed in Mn–Mn and Mn–O distances (Table 3).  
175 The Mn–Mn distances calculated from the initially assigned positions based on the  
176 cryo-EM density were 2.8 Å for Mn1–Mn2, 3.5 Å for Mn1–Mn3, 5.0 Å for Mn1–Mn4,  
177 3.1 Å for Mn2–Mn3, 5.4 Å for Mn2–Mn4, and 2.7 Å for Mn3–Mn4 (Table 3). Except the  
178 Mn1–Mn4 and Mn3–Mn4 distances, all of the Mn–Mn distances are 0.1–0.2 Å and 0.1–0.4  
179 Å longer than those of the SR and XFEL structures, respectively<sup>6,10</sup>. Most of the Mn–O  
180 distances in the cryo-EM structure were also 0.1–0.5 Å and 0.1–0.7 Å longer than those in  
181 the SR and XFEL structures, respectively (Table 3)<sup>6,10</sup>. These differences may be caused  
182 by two factors. One is the electron beam damage, and the other one is the experimental  
183 errors in determining the positions of the individual atoms based on the cryo-EM map  
184 only. Especially, the position of oxygen atoms may not be determined precisely because  
185 the map of the oxygen atoms cannot be separated from the map of metal atoms. Thus, the  
186 OEC structure was refined with the restraints for bond distances (Mn–O and Ca–O) that  
187 were taken from the initial positions. The Mn–Mn distances refined with restraints were  
188 2.8 Å for Mn1–Mn2, 3.4 Å for Mn1–Mn3, 5.0 Å for Mn1–Mn4, 3.1 Å for Mn2–Mn3, 5.6  
189 Å for Mn2–Mn4, and 3.0 Å for Mn3–Mn4 (Fig. 4b and Table 3). Except the Mn1–Mn4  
190 distance, all of the Mn–Mn distances are still 0.1–0.2 Å and 0.1–0.4 Å longer than those of  
191 the SR and XFEL structures, respectively<sup>6,10</sup>. Most of the Mn–O distances in the cryo-EM  
192 structure refined with the restraints were also 0.1–0.4 Å longer than those in the SR and

193 XFEL structures (Table 3). In addition, the occupancy of the OEC atoms refined with  
194 Refmac5 were found to be lower than 1.0 (0.87). These results indicate that the OEC is  
195 reduced by electron dose exposed, leading to the elongation of the Mn-Mn and Mn-O  
196 distances and some disorder or displacement of the metal centers during the cryo-EM  
197 data acquisition. This reduced occupancy is in agreement with the previous theoretical  
198 calculation of the cryo-EM structure of higher plant PSII-LHCII supercomplex<sup>25</sup>, which  
199 may be the reason why a part of Ala344 flipped out and does not ligate to the OEC. The  
200 reduction of metal ions with electron doses was already observed in electron  
201 crystallography previously<sup>26</sup>.

202 Further structural changes in the redox-active sites, including reaction center  
203 chlorophylls, electron transfer chain, proton channels and water channels, were not found  
204 except the water molecule near D2-Tyr160 ( $Y_D$ ) (Supplementary Fig. 6c). This water  
205 molecule was disordered at two positions with one being able to hydrogen bond to  $Y_D$  and  
206 the other one being able to hydrogen bond to D2-Arg180 in the SR structure and XFEL  
207 structures<sup>6,10</sup>. In the cryo-EM structure, this water molecule was ordered and connected to  
208 D2-Arg180. This may reflect the electron beam induced damage, which causes reduction  
209 of  $Y_D$  and broken the hydrogen-bond to  $Y_D^+$ .

210

### 211 **Reducing electron beam dosage in determining the PSII structure**

212 In order to reduce the electron beam damage, the final cryo-EM maps were  
213 calculated from only initial several frames of each movie stack. In Supplementary Fig. 7,  
214 the inverse resolutions of reconstructions achieved from decreased electron doses for the

215 ARM-60k data set, associated with the frame numbers, are plotted against the dose values  
216 on a logarithmic scale. Surprisingly, the electron doses from  $83 \text{ e}^- \text{ \AA}^{-2}$  down to  $10 \text{ e}^- \text{ \AA}^{-2}$   
217 gave rise to almost the same resolution, indicating that increase in the electron beam  
218 dosage during this range does not contribute to increase in the resolution significantly.  
219 Near atomic resolution is retained at the total dose of  $3.3 \text{ e}^- \text{ \AA}^{-2}$  for the ARM-60k data set  
220 ( $2.08 \text{ \AA}$ ), which was achieved by using the initial 2 frames of each micrograph.

221 An overall atomic model of the low-dose PSII was built based on the highest  $2.08 \text{ \AA}$   
222 resolution density reconstructed from the ARM-60k data set at the dose of  $3.3 \text{ e}^- \text{ \AA}^{-2}$   
223 (Table 2). The overall architecture of the low-dose PSII is very similar to that of the  
224 high-dose PSII, with a RMSD of  $0.21 \text{ \AA}$  for 5310  $C_\alpha$  atoms between the structures of  
225 high-dose and low-dose. However, in the regions where structural changes were observed  
226 due to electron beam damage, the disulfide bond between Cys19 and Cys41 of the PsbO  
227 was restored at a dose of  $5 \text{ e}^- \text{ \AA}^{-2}$ , and Ala344 of the D1 subunit was returned to the single  
228 conformation to ligate to the OEC similar to those seen in the crystal structures (Fig. 3, 4  
229 and 5, Supplementary Fig. 6 and 8). The ADP for the OEC atoms ( $12.8 \text{ \AA}^2$ ) were lower  
230 than that observed in the overall protein atoms of the cryo-EM structure ( $22.4 \text{ \AA}^2$ ), and the  
231 occupancy value of the OEC atoms refined with Refmac5 was returned to 1.0. These  
232 results indicate the reduction of the electron beam damage in the structure. However,  
233 similar to the high-dose structure, the water molecule near  $Y_D$  are connected to  
234 D2-Arg180 in an ordered manner and not hydrogen-bonded to  $Y_D$  (Supplementary Fig. 6),  
235 indicating that some electron beam damage remained.

236 The Mn–Mn distances calculated from the initially assigned positions based on the

237 cryo-EM density were 3.0 Å for Mn1–Mn2, 3.4 Å for Mn1–Mn3, 5.3 Å for Mn1–Mn4,  
238 2.8 Å for Mn2–Mn3, 5.4 Å for Mn2–Mn4, and 3.1 Å for Mn3–Mn4 (Table 3). All of these  
239 Mn–Mn distances are 0.1–0.3 Å longer than those of the SR and XFEL structures<sup>6,10</sup>.  
240 Most of the Mn–O distances in the cryo-EM structure were also 0.1–0.6 Å and 0.1–0.8 Å  
241 longer than those in the SR and XFEL structures, respectively (Table 3). As is done with  
242 the high-dose structure, we refined the OEC structure with the restraints for bond  
243 distances of Mn–O and Ca–O that were taken from the initial positions. The Mn–Mn  
244 distances in the OEC refined with the restraints were 2.9 Å for Mn1–Mn2, 3.2 Å for  
245 Mn1–Mn3, 5.1 Å for Mn1–Mn4, 2.9 Å for Mn2–Mn3, 5.4 Å for Mn2–Mn4, and 3.0 Å for  
246 Mn3–Mn4 (Fig. 4f, Table 3). Most of these distances are shorter than those of the  
247 high-dose structure and close to those of the XFEL structure, although most of them are  
248 still longer than the SR and XFEL structures by 0.1 Å and 0.1–0.2 Å, respectively (Table  
249 3). The Mn–O distances after refinement with the restraints also became close to the  
250 XFEL structure, indicating the necessity of refinement with restraints. However, some of  
251 the Mn–O distances were still longer or deviated from those found in the XFEL structure,  
252 which may be caused by the electron beam damage remained and/or coordinate errors in  
253 the cryo-EM analysis at the current resolution.

254

## 255 **Discussion**

256 In recent years, the resolution of single-particle cryo-EM has improved to atomic  
257 resolutions without crystallization, and it has been reported that biological samples are  
258 damaged by electron beams. In this study we elucidated the structure of the PSII at 1.95 Å

259 resolution in solution by cryo-EM, which is similar to the SR or XFEL structure in the  
260 crystalline state except the PsbY subunit, which was visible in the cryo-EM structure but  
261 absent or partially visible in the SR and XFEL structures<sup>6,10</sup>. This indicates that the  
262 structure solved by cryo-EM more closely represents the physiological state.

263 Despite the total electron dose of  $83 \text{ e}^- \text{ \AA}^{-2}$  which is commonly used in the acquisition  
264 of cryo-EM images, radiation damages are found in regions susceptible to redox changes,  
265 i.e. the disulfide bond and the redox-active metals, whereas the overall structure is very  
266 similar to those of the SR and XFEL structures<sup>6,10</sup>. The exposure of the sample to a flux of  
267 electrons is conveniently expressed in terms of electrons per  $\text{\AA}^2$  of specimen surface area  
268 ( $\text{e}^- \text{ \AA}^{-2}$ ), which is converted to the SI unit for the absorbed ionizing radiation, the Gray (Gy,  
269 with  $1 \text{ Gy} = 1 \text{ J kg}^{-1}$ ), by a factor of  $\square 3.7^{27}$ . Thus, the total electron dose of  $83 \text{ e}^- \text{ \AA}^{-2}$  is  
270 equal to the absorption of 307 MGy, which greatly exceeds the Henderson limit (20  
271 MGy) that is the X-ray dose that a cryo-cooled crystal can absorb before the diffraction  
272 pattern decays to half of its original intensity<sup>28</sup>. Nevertheless, our cryo-EM structure is  
273 almost the same to the SR and XFEL structures in the redox-active sites, including  
274 reaction center chlorophylls, electron transfer chain, proton channels and water channels,  
275 indicating that the radiation damage does not affect the structure significantly. This is  
276 considered to be the result of successful dose-weighted correction in the Bayesian  
277 polishing step. However, in the PsbO subunit, disulfide bond between Cys19 and Cys41  
278 were completely breaking (Fig. 3) in the cryo-EM structure. In the OEC structure, the  
279 Mn-Mn were 0.1–0.4  $\text{\AA}$  longer than those in the XFEL structure, and most of the Mn-O  
280 distances were also significantly longer. In addition, the occupancy of the OEC atoms

281 were lower than 1.0 (0.87), resulting in a multiple conformation of the C-terminal residue  
282 of D1, where a part of Ala344 flipped to a direction that does not ligate to the OEC.

283 We examined whether radiation damage could be reduced by reducing the total  
284 number of stacked movie frames used in the structural analysis. In the electron doses  
285 analyzed, the reconstructed map from summing the initial two frames of each micrograph  
286 ( $3 \text{ e}^- \text{ \AA}^{-2}$ , 11.1 MGy) gave rise to almost similar resolutions to that of high-dose data set  
287 ( $83 \text{ e}^- \text{ \AA}^{-2}$ , 307 MGy) (Supplementary Fig.7). The reconstructed map from the ARM-60k  
288 data set at the dose of  $3.3 \text{ e}^- \text{ \AA}^{-2}$  (11.1 MGy) gave a resolution of 2.08 Å. This structure  
289 was compared with that obtained with the high-dose, and it was found that the disulfide  
290 bond in the PsbO was recovered, and Ala344 of the D1 subunit was returned to the single  
291 conformation similar to the SR and XFEL structures (Fig. 3 and 5). This indicates a  
292 significant reduction of the electron beam damage to the structure. In the structure of  
293 OEC, most of the Mn-Mn and Mn-O distances became shorter than those observed in the  
294 high-dose structure before refinement. However, some of them are still significantly  
295 deviated from those of the XFEL structure (Table 3), which become closer after the  
296 refinement with restraints starting from the initial structure. Thus, it is advisable to refine  
297 the cryo-EM structure with restraints imposed for compounds such as the  $\text{Mn}_4\text{CaO}_5$   
298 cluster.

299 After refinement, most of the Mn-Mn and Mn-O distances of the low-dose structure  
300 are similar to those observed in the XFEL structure. However, some of the distances are  
301 still longer than or deviated from those found in the damage free XFEL structure<sup>10</sup>. This  
302 may be caused by two reasons; one is some electron beam damage remained, and the

303 other one is coordinate errors existed in the cryo-EM structure. It has been reported that  
304 about 80% of Mn of OEC in solution is reduced to divalent cations by an X-ray dose of 5  
305 MGy<sup>7</sup>. Even though it is estimated that about 90% of Mn of OEC is Mn(II) at an electron  
306 beam dose of 11.1 MGy used for the low-dose structure, the structure of the OEC retained  
307 an occupancy of 1.0. This may be contributed by the stability of the structure of OEC, as  
308 the metal ions of OEC are liganded by seven amino acid residues (D1-D170, D1-E189,  
309 D1-H332, D1-E333, D1-D342, D1-A344, and CP43-E354). However, the longer  
310 distances observed in some of the metal-oxygen distances of OEC even in the low-dose  
311 structure indicated the existence of electron beam damage. Tanaka et al. has reported that,  
312 using SR, a dose of 0.1 MGy is necessary to achieve a structure similar to that of the  
313 XFEL structure<sup>29</sup>. Thus, in order to achieve a damage free structure, the electron beam  
314 dose needs to be further reduced. Fortunately, our data indicated that the resolution  
315 depends on the particles used, and by using more images and particles, it will be possible  
316 to lower the electron beam dose and achieve the structure at a higher resolution.

317       Coordinate errors in the cryo-EM structure may be caused by the ambiguities in the  
318 orientations of particles and their averaging, as well as the subsequent structural analysis  
319 procedures. Structural analysis by cryo-EM at a higher resolution should eliminate such  
320 errors, and gives rise to a more accurate structure. It is also expected that improvements in  
321 the averaging and structural analysis algorithms of the cryo-EM data may improve the  
322 accuracy of the structures at the same resolutions.

323       In summary, we show that the electron dose commonly used in cryo-EM is damaging  
324 to protein samples. However, the damaged area was limited to redox-sensitive part. Our

325 results suggest that it is possible to obtain a structure with less damage and high  
326 resolution by reducing the total dose and increasing the number of particles. This study  
327 will serve as a good indicator for determining damage-less cryo-EM structures of PSII  
328 and all biological samples, especially redox-active metalloproteins.

329

330

### 331 **Methods**

332 **Growth of cells and purification of PSII.** Cells of *Thermosynechococcus vulcanus* (*T.*  
333 *vulcanus*) were grown in four 10 L bottles at 50°C. PSII with a high oxygen evolving  
334 activity was purified from *T. vulcanus* as described previously<sup>30-32</sup> and suspended with a  
335 buffer containing 20 mM MES-NaOH (pH 6.0), 0.04%  $\beta$ -dodecyl-D-maltopyranoside  
336 and 5% glycerol. For the Titan-96k and ARM-60k data collection, glycerol in the buffer  
337 was removed by polyethylene glycol (PEG) precipitation and the resultant PSII was  
338 re-suspended in a buffer containing 20 mM MES-NaOH (pH 6.0), 20 mM NaCl, 3 mM  
339 CaCl<sub>2</sub>, 0.04%  $\beta$ -dodecyl-D-maltopyranoside.

340

341 **Cryo-EM data collection.** For cryo-EM experiments, 3- $\mu$ L aliquots of the PSII sample  
342 at each condition (shown in Table 1) were applied to Quantifoil R1.2/1.3, Mo 300 mesh  
343 or Cu 200 mesh grids. The grids were incubated for 10 s in an FEI Vitrobot Mark IV at  
344 4°C and 100% humidity. The grids were immediately plunged into liquid ethane cooled  
345 by liquid nitrogen and then transferred into the Titan Krios (Thermo Fischer Scientific)  
346 equipped with a field emission gun, a Cs corrector (CEOS GmbH), and a direct electron



347 detection camera (Falcon 3EC, Thermo Fischer Scientific), or CRYO ARM 300 (JEOL)  
348 equipped with a cold-field emission gun and a direct electron detection camera (Gatan K2  
349 summit, Gatan Inc). These microscopes were operated at 300 kV and a nominal  
350 magnification of  $\times 75,000$  (Titan-75k),  $\times 96,000$  (Titan-96k) for Titan Krios and  $\times 60,000$   
351 (ARM-60k) for CRYO ARM 300. Images were recorded using the Falcon 3EC in linear  
352 mode or Gatan K2 summit in counting mode. Micrographs were recorded with a pixel  
353 size of  $0.870 \text{ \AA}$ ,  $0.678 \text{ \AA}$  and  $0.822 \text{ \AA}$  at a dose rate of  $40 \text{ electrons \AA}^{-2} \text{ sec}^{-1}$ ,  $40 \text{ electrons}$   
354  $\text{ \AA}^{-2} \text{ sec}^{-1}$  and  $83 \text{ electrons \AA}^{-2} \text{ sec}^{-1}$  for Titan-75k, Titan-96k and ARM-60k, respectively.  
355 The nominal defocus range were  $-1.0$  to  $-2.0 \text{ \mu m}$ ,  $-1.0$  to  $-2.5 \text{ \mu m}$ , and  $-0.8$  to  $-1.6 \text{ \mu m}$   
356 for Titan-75k, Titan-96k and ARM-60k, respectively. Each exposure was conducted for  
357  $45.11 \text{ s}$ ,  $26.64 \text{ s}$  and  $10.00 \text{ s}$ , and were dose-fractionated into 78, 39 and 50 movie frames  
358 for Titan-75k, Titan-96k and ARM-60k, respectively. We acquired 2,084, 4,237 and  
359 2,160 images for the data sets of Titan-75k, Titan-96k and ARM-60k, respectively.

360

361 **Cryo-EM image processing.** Movie frames were aligned and summed using the  
362 MotionCor2 software<sup>33</sup> to obtain a final dose weighted image. Estimation of the contrast  
363 transfer function (CTF) was performed using the CTFFIND4 program<sup>34</sup>. All of the  
364 following processes were performed using RELION3.0<sup>35</sup>. For structural analyses of the  
365 Titan-75k data set, 354,233 particles were automatically picked from 2,084 micrographs  
366 and then were used for reference-free 2D classification. Then, 309,028 particles were  
367 selected from the good 2D classes and subjected to 3D classification with a C2 symmetry.  
368 The  $1.9 \text{ \AA}$  PSII structure from *T. vulcanus* (PDB: 3WU2)<sup>6</sup> was employed for the initial

369 model for the first 3D classification with 60-Å low-pass filter. As shown in the  
370 Supplementary Fig. 1 and 2, the PSII structure was reconstructed from 90,897 particles at  
371 an overall resolution of 2.22 Å. For structural analyses of the Titan-96k data set, 612,287  
372 particles were automatically picked from 4,237 micrographs and then used for  
373 reference-free 2D classification. Then, 566,145 particles were selected from the good 2D  
374 classes and subjected to 3D classification with a C2 symmetry. The 2.22-Å map from  
375 Titan-75k data was employed for the initial model for the first 3D classification with a  
376 60-Å low-pass filter. As shown in the Supplementary Fig. 1 and 2, the PSII structure was  
377 reconstructed from 203,912 particles at an overall resolution of 2.20 Å. For structural  
378 analyses of the ARM-60k data set, 481,946 particles were automatically picked from  
379 2,160 micrographs and used for reference-free 2D classification. Then, 481,927 particles  
380 were selected from the good 2D classes and subjected to 3D classification with a C2  
381 symmetry. The 2.22-Å map from Titan-75k data was employed for the initial model for  
382 the first 3D classification with a 60-Å low-pass filter. The PSII structure was  
383 reconstructed from 174,099 particles at an overall resolution of 1.95 Å (Supplementary  
384 Fig. 3 and 4). For the low-dose maps, the summing number of movie frames were  
385 decreased in the final step of Bayesian polishing and used for reconstruction without  
386 refinement of particle positions and orientations, using RELION<sup>35</sup> with the command line  
387 option “relion\_reconstruct” and then post-processed in RELION<sup>35</sup>. All of the resolution  
388 was estimated by the gold-standard Fourier shell correlation (FSC) curve with a cut-off  
389 value of 0.143 (Supplementary Fig. 2 and 4)<sup>36</sup>. The local resolution was estimated using  
390 RELION<sup>35</sup>.

391

392 **B-factor estimation.** For the B-factor plot, the total set of all particles from the final  
393 refinement was randomly resampled into smaller subsets. These subsets were subjected  
394 to 3D auto-refinement and the resulting orientations were used to calculate  
395 reconstructions for each of the two random halves used in the auto-refinement. The  
396 squared values of the resulted, estimated resolutions were then plotted against the natural  
397 logarithm of the number of particles in the subset, and B-factors were calculated from the  
398 slope of the straight line best fitted with the points in the plot (Fig.1).

399

400 **Model building and refinement.** The 1.95-Å and 2.08-Å cryo-EM maps were used for  
401 model building of the high-dose and low-dose PSII structures, respectively. First, the  
402 crystal structure of *T. vulcanus* PSII (PDB: 3WU2) was manually fitted into each  
403 cryo-EM map using UCSF Chimera<sup>37</sup>, and then the structures were inspected and  
404 adjusted individually with COOT<sup>38</sup>. The structures of high-dose PSII and low-dose PSII  
405 were then refined with phenix.real\_space\_refine<sup>39</sup> and Refmac5<sup>40</sup> with geometric  
406 restraints for the protein-cofactor coordination. The positions of four manganese atoms  
407 and one calcium atom were clearly visible in the cryo-EM map (Fig. 2 and 4). The  
408 positions of the five oxo-oxygen atoms and four water molecules ligated to the OEC were  
409 less clear, and they were identified by the difference map in which, the maps of metal ions  
410 with a diameter of 1.5 Å from that metal ion were subtracted from the whole cryo-EM  
411 map, after placement of the manganese and calcium atoms (Fig. 2 and 4). The initial  
412 positions of metal and oxygen atoms were assigned based on the highest peaks in the

413 cryo-EM maps. This was taken as the initial structure. Subsequently, we performed the  
414 structural refinement with loose restraints ( $0.1 \sigma$ ) for bond distances (Mn–O and Ca–O)  
415 that were taken from the initial position. Then the refinement was performed with tighter  
416 restraints ( $0.05 \sigma$ ) for bond distances successively using the modified ‘new’ library for the  
417 bond distances. This geometry optimization procedure was repeated several times until  
418 the bond distances converged. However, the distance of Mn4-O4 in the high-dose  
419 structure, and the distances of Mn1-O1 and Mn3-O2 in the low-dose structure, were fixed  
420 to  $1.8 \text{ \AA}$ , because these distances were too close and could not be refined. The averages of  
421 the distances of Mn–Mn, Mn–Ca, Mn–O and Mn–ligand were calculated from each PSII  
422 in the final four refinement steps and are listed in Table 3. The final models were further  
423 validated with Q-score<sup>41</sup>, MolProbity<sup>42</sup> and EMringer<sup>43</sup>. The statistics for all data  
424 collection and structure refinement are summarized in Table 1 and 2. All structural  
425 figures are made by Pymol<sup>44</sup> or UCSF ChimeraX<sup>45</sup>.

426

427 **Difference map analysis between low-dose PSII and high-dose PSII.** A difference  
428 map was calculated by subtracting high-dose map from the low-dose map, i.e. (low-dose  
429 PSII) minus (high-dose PSII). The rotational and translational matrix was calculated  
430 based on the refined atomic coordinates using lsqkab in CCP4<sup>46</sup>. A map of low-dose PSII  
431 was superposed with a map of high-dose PSII which were applied by a low-pass filter and  
432 adjusted to  $2.08 \text{ \AA}$  resolution, with calculated rotational and translational matrix using  
433 maprot in CCP4<sup>47</sup>. The high-dose PSII map and the low-dose PSII map was normalized  
434 based on the ratio of the root mean square map density value and then the difference maps

435 were calculated using UCSF Chimera<sup>37</sup> with the command line option “vop subtract”  
436 (Supplementary Fig. 8).

437

438 **Data availability.** Atomic coordinates and cryo-EM maps for the reported structure of  
439 PSII determined from the high-dose data set of ARM-60K and low-dose data set of  
440 ARM-60k were deposited in the Protein Data Bank under an accession codes 7D1T and  
441 7D1U, respectively, and in the Electron Microscopy Data Bank under the accession codes  
442 EMD-30547, EMD-30548, respectively. The cryo-EM maps of the Titan-75k data set and  
443 Titan-96k data set were deposited in the Electron Microscopy Data Bank under the  
444 accession codes EMD-30549 and EMD-30550, respectively.

445

446

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454

#### 455 **Author Contributions**

456 J.-R.S. and K. Y. conceived the project; Y.N. and F.A. purified the PSII; N.M. and T.H.

457 collected cryo-EM images; N. M., K.K. and T.H. processed the EM data. K.K. built the  
458 structure model and refined the final models; K.K. analyzed the structure; and K.K., T.H.,  
459 N.M. and J.-R.S. wrote the paper, and all of the authors joined the discussion of the  
460 results.

461

462

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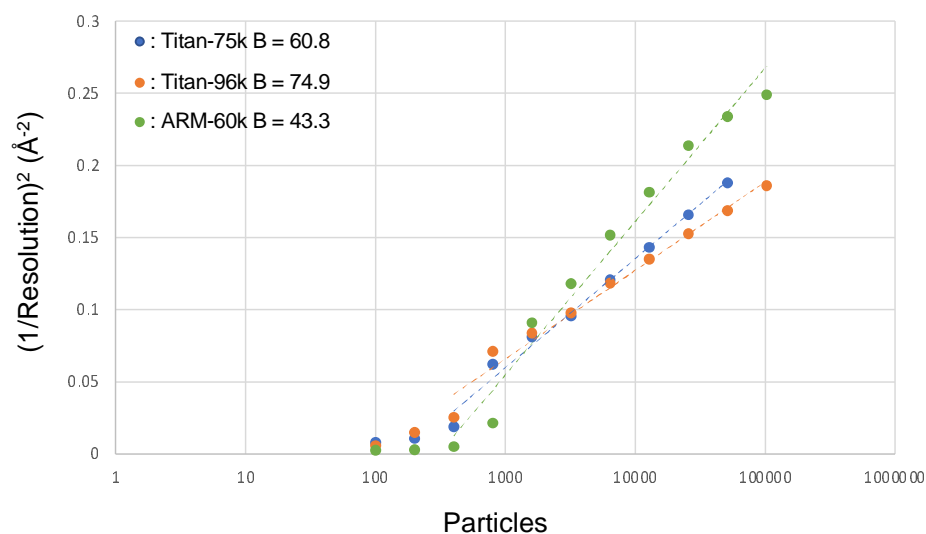
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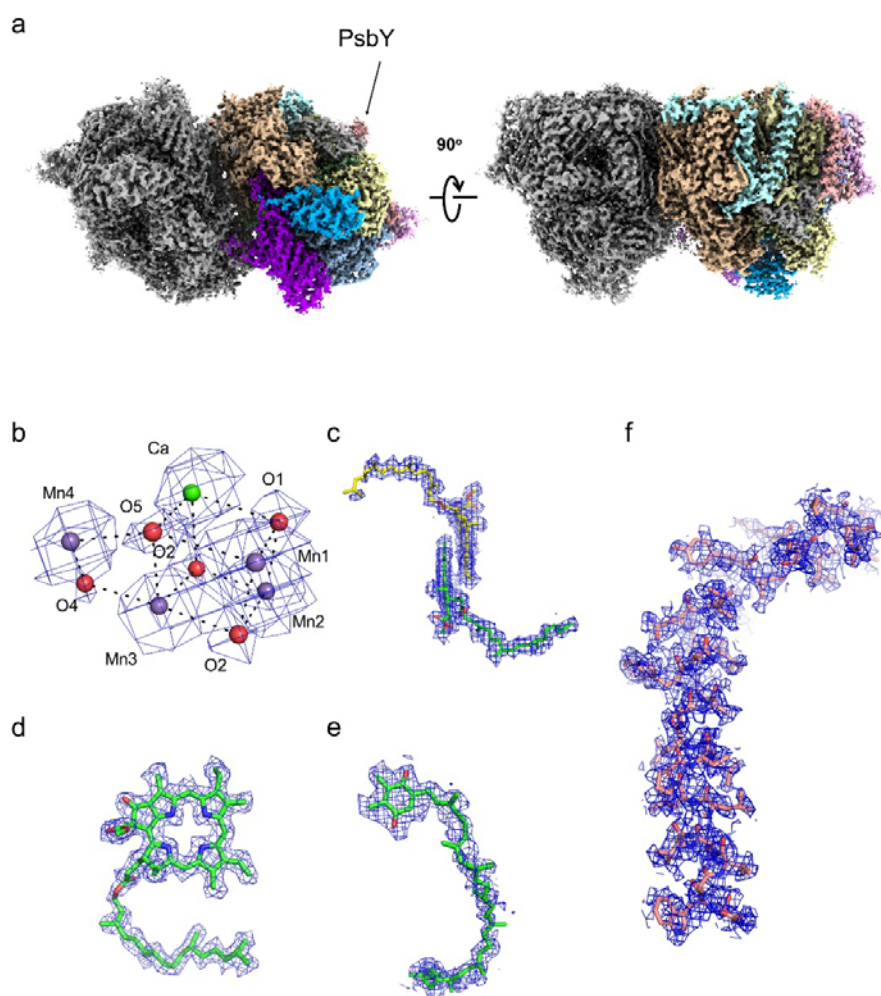
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572 48

573 49 **Fig. 1 B-factor plot for the data sets of Titan-75k, Titan-96k and ARM-60k.** B  
574 factor plot for the Titan-75k data set at a dose of  $40 \text{ e}^- \text{ \AA}^{-2}$  (blue), the Titan-96k data  
575 set at a dose of  $40 \text{ e}^- \text{ \AA}^{-2}$  (orange), and the ARM-60k data set at a dose of  $83 \text{ e}^- \text{ \AA}^{-2}$   
576 (green).

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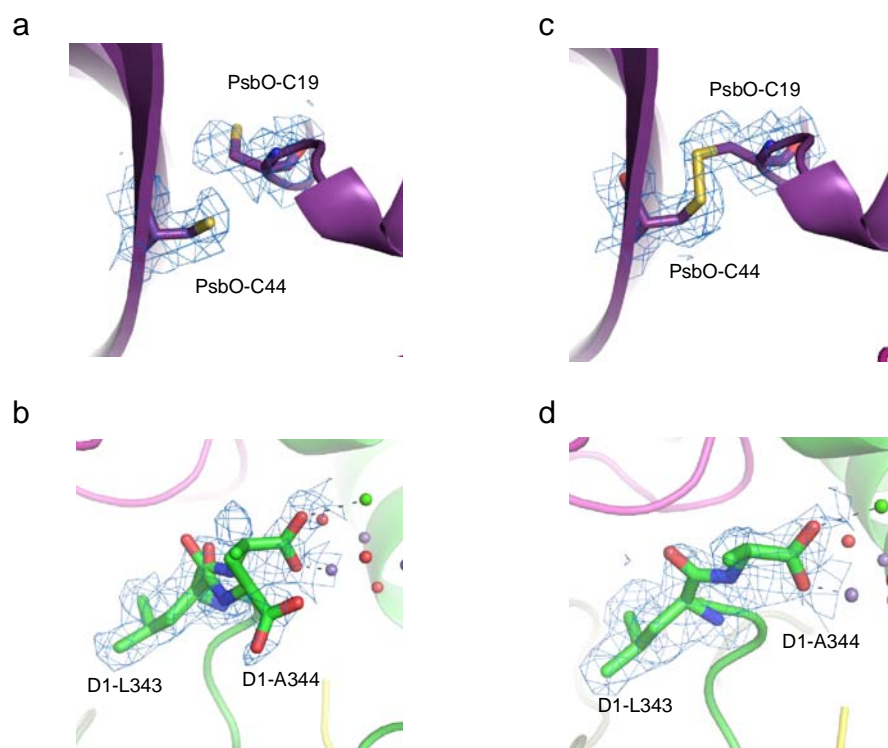


578

579 **Fig. 2 Overall structure of PSII at a high-dose.** **a** The cryo-EM density of the PSII at  
580 1.95 Å resolution from the ARM-60k data set. **b-e** The cryo-EM density of cofactors,  
581 OEC (**b**), P680 (**c**), pheophytin (**d**) and plastoquinone (Q<sub>B</sub>) (**e**), superposed with the  
582 refined model. **f** The density of PsbY superposed with the refined model. The densities  
583 were depicted at 5  $\sigma$ .

584

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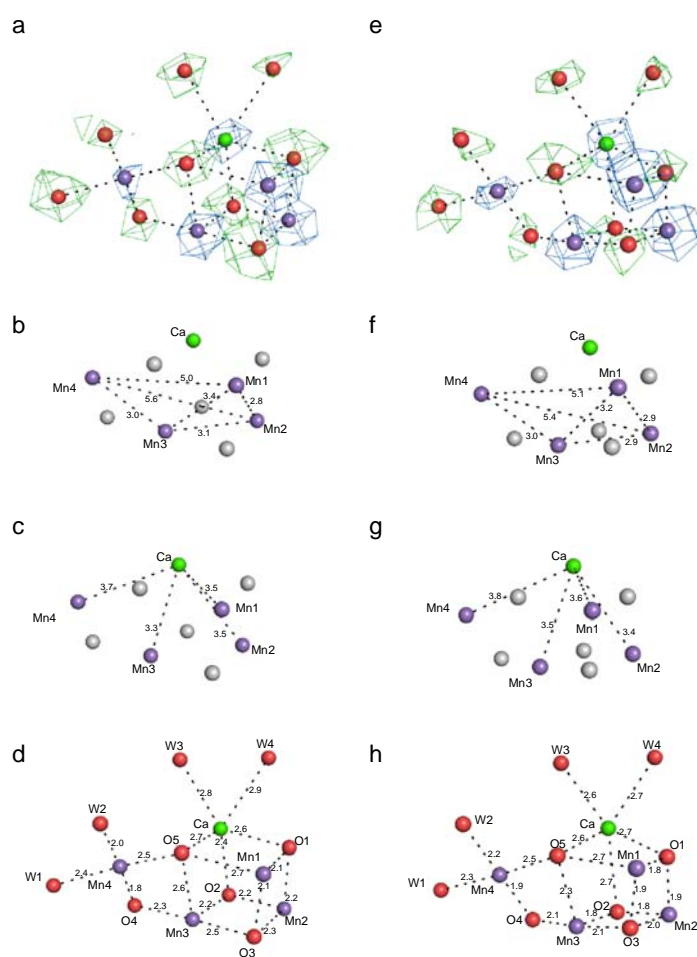


586

587 **Fig. 3 Electron beam damages in the PSII structure of the ARM-60k data set at the**  
588 **high-dose ( $83 \text{ e}^- \text{ \AA}^{-2}$ ) and low-dose ( $3.3 \text{ e}^- \text{ \AA}^{-2}$ ).** **a** The broken disulfide bond in PsbO at  
589 the high-dose. **b** The alternative conformation at D1-A344 at the high dose. **c** The  
590 disulfide bond recovered in PsbO at the low-dose. **d** The single conformation of  
591 D1-A344 at the low dose. The densities were depicted at  $5 \sigma$ .

592

593



594

595 **Fig. 4 Electron beam damages in the OEC structure solved at the high-dose (83 e<sup>-</sup>**

596 **Å<sup>-2</sup>) and low-dose (3.3 e<sup>-</sup> Å<sup>-2</sup>).** **a-d:** High dose structure. **a** The cryo-EM density (blue)

597 for manganese and calcium atoms at 17 σ and the subtracted map (green) for oxygen

598 atoms and water molecules at 7 σ. **b** Mn-Mn distances in the OEC (in Å). **c** Mn-Ca

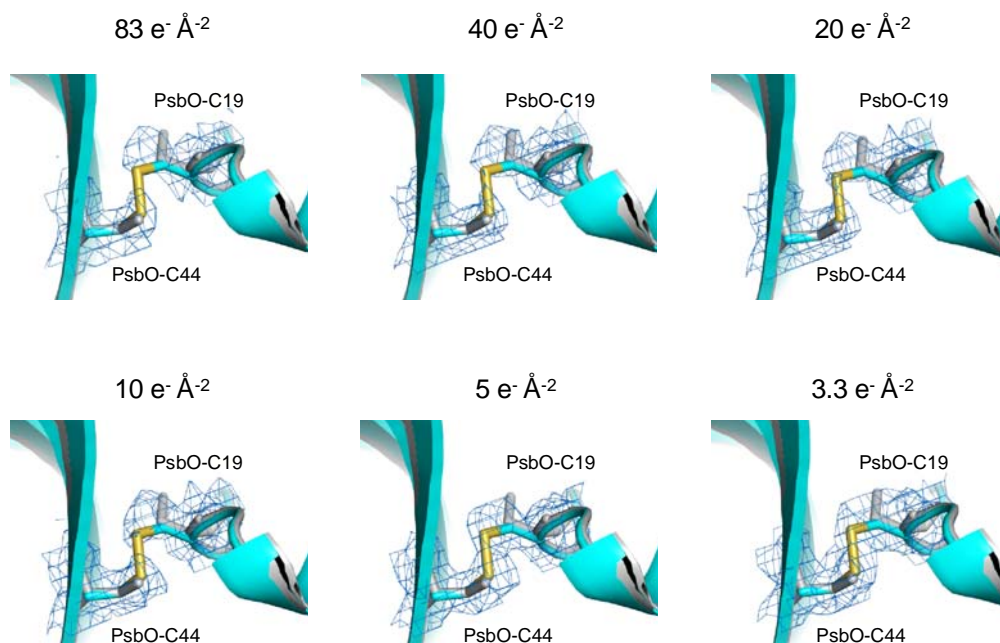
599 distances in the OEC (in Å) **d** Mn-O, Ca-O, Mn-water and Ca-water distances in the

600 OEC (in Å). **e-h:** Low dose structure. **e** The cryo-EM density (blue) for manganese and

601 calcium atoms at 17 σ and the subtracted map (green) for oxygen atoms and water

602 molecules at  $7 \sigma$ . **f** Mn–Mn distances in the OEC (in Å). **g** Mn–Ca distances in the OEC

603 (in Å). **h** Mn–O, Ca–O, Mn–water and Ca–water distances in the OEC (in Å).



604

605 **Fig. 5 Changes of the cryo-EM map in the region of the disulfide bond in PsaO**

606 **with changes of the electron beam dose.** The cryo-EM maps for each electron dose are

607 displayed as a blue mesh at  $4 \sigma$  and the corresponding models for low-dose (colored)

608 and high-dose (gray) are shown as sticks.

609



610 **Table 1 Sample preparation and Cryo-EM data collection parameters.**

Data set	Titan-75K	Titan-96K	ARM-60K
Original sample conc.	3.8 Chl mg/ml	9.7 Chl mg/ml	9.7 Chl mg/ml
Original sample buffer	20 mM MES (pH6.0), 5% glycerol, 0.04% $\beta$ -DDM	20 mM MES (pH6.0)	20 mM MES (pH6.0)
Dilution buffer	20 mM MES (pH6.0), 0.04% $\beta$ -DDM	20 mM MES (pH6.0)	20 mM MES (pH6.0)
Dilution	10-fold	5-fold	5-fold
Final sample conc.	0.38 Chl mg/ml	1.94 Chl mg/ml	1.94 Chl mg/ml
Data collection and processing			
Microscope	Titan Krios	Titan Krios	CRYO ARM 300
Detector	Falcon3EC in EC mode	Falcon3EC in EC mode	Gatan K2 summit in Counting mode
Magnification	75K	96K	60K
Voltage (kV)	300	300	300
Defocus range ( $\mu$ m)	-1.00 to -2.00	-1.00 to -2.50	-0.8 to -1.6
Pixel size ( $\text{\AA}$ )	0.870	0.678	0.822
Total electron dose ( $\text{\AA}^2$ )	40	40	83
Exposure time (s)	45.11	24.64	10
Number of frames per image	78	39	50
Number of micrographs	2,084	4,237	2,160
Initial particle images	354,233	612,287	444,729
Final particle images	90,897	203,912	174,099
Map resolution ( $\text{\AA}$ )	2.23 (2.22)*	2.26 (2.20)*	1.98 (1.95)*
Map sharpening B-factor ( $\text{\AA}^2$ )	-54 (-53)*	-60 (-56)*	-34 (-32)*
Rosenthal-Henders on B factor ( $\text{\AA}^2$ )	60.8	74.9	43.3
Applied symmetry	C2	C2	C2

611 \* After micelle-density subtraction

612

613 **Table 2 Statistics of data collection, processing and refinement.**

614

<b>Refinement</b>	<b>High-dose</b>	<b>Low-dose</b>
PDB ID	7D1T	7D1U
EMDB ID	EMD-30547	EMD-30548
Initial Model used (PDB code)	3WU2	3WU2
Model resolution (Å)	1.94	2.03
FSC threshold	0.5	0.5
No. of atoms		
Protein	41708	41680
Ligand	8854	8854
Water	2432	2121
B factors (Å <sup>2</sup> )		
Protein	20.6	21.2
Ligand	23.5	24.1
Water	26.4	23.7
R.m.s deviations		
Bond lengths (Å)	0.012	0.010
Bond angles (°)	1.46	1.31
Validation		
MolProbity score	1.3	1.5
Clashscore	1.8	2.2
Poor rotamers (%)	2.4	3.1
EMRinger score	7.0	6.8
Q-Score	0.87	0.86
Ramachandran plot		
Favored (%)	97.93	97.55
Allowd (%)	1.88	2.26
Disallowed (%)	0.19	0.19

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619

620 **Table 3 Summarization of the distances of atoms of the Mn<sub>4</sub>CaO<sub>5</sub> cluster.**

	High-dose	Low-dose	SR (3WU2)	XFEL (4UB6)	High-dose					Low-dose				
	(Average)	(Average)	(Average)	(Average)	4th	3rd	2nd	1st	Initial	4th	3rd	2nd	1st	Initial
<b>Mn1-Mn2</b>	2.8	2.9	2.8	2.7	2.81	2.83	2.82	2.82	2.81	2.88	2.87	2.89	2.8	2.95
<b>Mn1-Mn3</b>	3.4	3.2	3.3	3.2	3.35	3.36	3.35	3.36	3.46	3.20	3.20	3.24	3.18	3.37
<b>Mn1-Mn4</b>	5.0	5.1	5	5	5.04	5.02	5.03	5.03	4.99	5.10	5.09	5.08	5.04	5.32
<b>Mn2-Mn3</b>	3.1	2.9	2.9	2.7	3.08	3.10	3.09	3.09	3.06	2.88	2.88	2.88	2.85	2.75
<b>Mn2-Mn4</b>	5.6	5.4	5.4	5.2	5.65	5.65	5.65	5.63	5.42	5.47	5.46	5.42	5.43	5.40
<b>Mn3-Mn4</b>	3.0	3.0	3.0	2.9	2.97	2.97	2.98	2.94	2.67	3.04	3.03	3.01	3.00	3.05
<b>Mn1-Ca</b>	3.5	3.6	3.5	3.5	3.55	3.52	3.52	3.52	3.51	3.59	3.57	3.55	3.56	3.83
<b>Mn2-Ca</b>	3.5	3.4	3.4	3.3	3.49	3.45	3.46	3.46	3.29	3.44	3.43	3.41	3.44	3.35
<b>Mn3-Ca</b>	3.3	3.5	3.4	3.4	3.32	3.29	3.28	3.27	3.04	3.45	3.45	3.44	3.46	3.43
<b>Mn4-Ca</b>	3.7	3.8	3.8	3.8	3.73	3.69	3.70	3.68	3.57	3.76	3.76	3.70	3.80	3.86
<b>Mn1-O1</b>	2.1	1.8	1.9	1.8	2.11	2.10	2.08	2.06	2.34	1.80	1.76	1.77	1.78	2.09
<b>Mn1-O3</b>	2.1	1.9	1.8	1.9	2.09	2.10	2.10	2.09	2.29	1.89	1.90	1.91	1.92	1.90
<b>Mn1-O5</b>	2.7	2.7	2.6	2.7	2.72	2.73	2.74	2.75	2.64	2.71	2.72	2.72	2.72	2.89
<b>Mn2-O1</b>	2.2	1.9	2.1	1.8	2.18	2.17	2.17	2.16	2.19	1.98	1.95	1.92	1.86	2.12
<b>Mn2-O2</b>	2.2	1.8	2.1	1.8	2.24	2.23	2.2	2.17	2.45	1.84	1.83	1.82	1.80	2.45
<b>Mn2-O3</b>	2.3	2.0	2.1	2.0	2.28	2.28	2.27	2.26	2.36	2.01	2.01	2.01	2.00	1.95
<b>Mn3-O2</b>	2.2	1.8	1.9	1.9	2.18	2.18	2.16	2.14	2.04	1.80	1.78	1.81	1.86	2.08
<b>Mn3-O3</b>	2.5	2.1	2.1	2.1	2.48	2.47	2.44	2.42	2.48	2.05	2.06	2.06	2.07	2.02
<b>Mn3-O4</b>	2.3	2.1	2.1	1.9	2.30	2.30	2.28	2.26	2.38	2.17	2.11	2.05	1.96	2.71
<b>Mn3-O5</b>	2.6	2.3	2.4	2.2	2.58	2.58	2.56	2.54	2.58	2.35	2.32	2.29	2.23	2.57
<b>Mn4-O4</b>	1.8	1.9	2.1	2.0	1.80	1.80	1.79	1.82	1.96	1.86	1.89	1.92	1.97	2.05
<b>Mn4-O5</b>	2.5	2.5	2.5	2.3	2.49	2.46	2.45	2.44	2.59	2.5	2.48	2.45	2.40	2.67
<b>Mn4-W1</b>	2.4	2.3	2.2	2.3	2.38	2.40	2.39	2.40	2.54	2.31	2.32	2.36	2.32	2.33
<b>Mn4-W2</b>	2.0	2.2	2.2	2.1	2.05	2.03	2.03	2.07	2.11	2.18	2.18	2.18	2.15	1.84
<b>Ca-O1</b>	2.6	2.7	2.4	2.6	2.63	2.63	2.62	2.62	2.60	2.72	2.70	2.67	2.63	2.47
<b>Ca-O2</b>	2.4	2.7	2.5	2.7	2.39	2.39	2.41	2.43	2.41	2.67	2.68	2.69	2.71	2.73
<b>Ca-O5</b>	2.7	2.6	2.7	2.5	2.71	2.70	2.68	2.66	2.70	2.62	2.60	2.58	2.55	2.60
<b>Ca-W3</b>	2.8	2.6	2.4	2.6	2.76	2.79	2.78	2.78	2.80	2.56	2.56	2.57	2.53	2.64
<b>Ca-W4</b>	2.9	2.7	2.5	2.5	2.89	2.93	2.92	2.94	2.92	2.71	2.71	2.73	2.65	2.71

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