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Structural insights into photosystem II assembly

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Abstract

Biogenesis of photosystem II (PSII), nature's water splitting catalyst, is assisted by auxiliary proteins that form transient complexes with PSII components to facilitate stepwise assembly events. Using cryo-electron microscopy, we solved the structure of such a PSII assembly intermediate from *Thermosynechococcus elongatus* at 2.94 Å resolution. It contains three assembly factors (Psb27, Psb28, Psb34) and provides detailed insights into their molecular function. Binding of Psb28 induces large conformational changes at the PSII acceptor side, which distort the binding pocket of the mobile quinone (Q_B) and replace the bicarbonate ligand of non-heme iron with glutamate, a structural motif found in reaction centers of non-oxygenic photosynthetic bacteria. These results reveal novel mechanisms that protect PSII from damage during biogenesis until water splitting is activated. Our structure further demonstrates how the PSII active site is prepared for the incorporation of the Mn₄CaO₅ cluster, which performs the unique water splitting reaction.

1 Introduction

2 Photosystem II (PSII) is the only enzyme that catalyzes the light-driven oxidation of water, a
3 thermodynamically demanding reaction that drives photosynthesis, sustaining life on our
4 planet¹⁻³. This multi-subunit membrane protein complex is located in the thylakoid membranes
5 of cyanobacteria, algae and plants. PSII strips electrons from water and injects them into the
6 photosynthetic electron transport chain (PET). It forms a homodimer with a molecular mass of
7 ~500 kDa⁴, with each monomer composed of at least 20 protein subunits and numerous
8 cofactors, including chlorophylls, quinones, carotenoids, lipids, bicarbonate and the unique
9 Mn₄CaO₅ cluster⁵⁻⁷. The two core proteins D1 and D2 form a central, membrane-intrinsic
10 heterodimer, which binds all important redox cofactors involved in internal electron transfer⁸.
11 Light-excitation leads to a charge-separated state in which an electron is transferred from the
12 reaction center chlorophylls P₆₈₀⁹ to the nearby pheophytin¹⁰. Subsequently, the electron is
13 passed to the bound plastoquinone (Q_A) and then to the mobile plastoquinone molecule (Q_B),
14 which leaves the complex after accepting two electrons and two protons¹¹. The electron hole at
15 P₆₈₀ is filled by oxidation of an adjacent tyrosine residue (Tyr_Z)¹² and finally by the oxygen

16 evolving complex (OEC), which contains the Mn_4CaO_5 cluster. In cyanobacteria, the cluster is
17 shielded on the luminal side by the three extrinsic proteins, PsbO, PsbU and PsbV, which
18 regulate access to the OEC by forming an intricate network of channels for different substrates
19 and products¹³. Light energy is collected and funneled towards P_{680} by the two membrane-
20 intrinsic antenna proteins CP43 and CP47. These proteins bind most of the chlorophyll
21 molecules and are located on opposite sides of the D1/D2 heterodimer¹⁴. Moreover, at least
22 twelve small transmembrane subunits with one or two transmembrane helices have been
23 identified in PSII¹⁵, including cytochrome- b_{559} ¹⁶.

24 Structural and spectroscopic investigations have revealed these aforementioned comprehensive
25 insights into PSII function¹⁷⁻²¹, but we are far from understanding PSII biogenesis with
26 molecular detail. How nature facilitates the assembly of a multi-subunit, multi-cofactor
27 membrane protein complex is a fundamental unsolved question. The biogenesis of PSII is even
28 more challenging, as the mature complex performs sophisticated and extreme redox chemistry
29 to catalyze the light-driven oxidation of water. This can easily lead to the formation of reactive
30 oxygen species (e.g., singlet oxygen is produced by triplet chlorophyll in the PSII reaction
31 center) and subsequent loss of function due to damaged proteins and cofactors^{22,23}. Biogenesis
32 intermediates with only partially functional fragments of the redox chain are particularly prone
33 to damage, thus demanding specialized protection mechanisms for the assembly process²⁴.
34 Therefore, PSII biogenesis is not a spontaneous process but rather must be tightly regulated by
35 the action of assembly factors. Thus far, more than 20 auxiliary proteins have been identified
36 that guide the stepwise assembly of PSII subunits and cofactors via intermediate modules,
37 which are assembled independently and then joined together to produce mature PSII²⁵⁻²⁷. In
38 cyanobacteria, PSII biogenesis begins with the formation of the D1/D2 heterodimer reaction
39 center (RC) complex from the D1 precursor protein (pD1) and the D2 protein. This is assisted
40 by the PSII assembly factor Ycf48 after partial processing of the D1 C-terminal extension by
41 the D1 specific peptidase CtpA^{28,29}. In the next step, the assembly factor Psb28 helps CP47 join
42 the RC complex to form the RC47 complex, in which iD1 is further processed to its mature
43 form by CtpA^{30,31}. Almost all ligands of the Mn_4CaO_5 cluster are already present at this stage,
44 except for those provided by CP43, which comes pre-constructed with assembly factor Psb27
45 and several small subunits (together called the CP43 module)³². Psb28 is released as CP43
46 binds, and the resulting Psb27-PSII monomer is activated by maturation of the OEC and the
47 binding of the extrinsic proteins PsbO, PsbU and PsbV³³⁻³⁵. OEC assembly is a multistep
48 process that requires a functional upstream redox chain for the oxidation of Mn^{2+} to build up
49 the cluster's μ -oxo bridges between the manganese atoms^{36,37}. The mechanistic and structural

50 details of this photoactivation process are not yet understood. In the consensus ‘two quantum
51 model’³⁸⁻⁴⁰, a single Mn²⁺ ion bound to the high-affinity site (HAS)⁴¹ is oxidized to Mn³⁺. This
52 initiating light-dependent step is followed by a slow light-independent phase and further fast
53 light-dependent steps in which the remaining Mn²⁺ ions are oxidized and incorporated.
54 Understanding the light-independent slow phase is key to unraveling the mechanism of
55 photoactivation. Finally, PSII biogenesis completes with dimerization of two fully assembled
56 monomers and attachment of the soluble phycobilisome antenna complexes. Interestingly,
57 deletion of *psbJ*, which encodes a small single transmembrane helix protein at the entrance of
58 the PSII plastoquinone channel, decreases PSII-mediated oxygen evolution and increases the
59 lifetime of the reduced primary acceptor (Q_A⁻) in cyanobacterial and tobacco chloroplasts⁴².
60 These effects are caused by a massive accumulation of an intermediate monomeric PSII
61 complex, which contains both assembly factors Psb27 and Psb28⁴³. Physiological studies of
62 Psb27 and Psb28 deletion strains point towards multifaceted functions. Cyanobacterial mutants
63 lacking Psb28 exhibited slower autotrophic growth, particularly under stress conditions^{31,44}, and
64 limited synthesis of Chl-binding proteins but without decreased PSII functionality³¹. The Psb28
65 mutant also exhibited an overall increase in PSII repair and faster recovery from
66 photodamage³¹. Chemical cross-linking combined with mass spectrometry revealed that Psb28
67 binds to the cytosolic side of CP47 close to cytochrome-b₅₅₉ and the Q_B binding site. Based on
68 this, researchers postulated a protective role for Psb28, where it blocks electron transport to the
69 acceptor side of PSII to shield the RC47 complex from excess photodamage during the
70 assembly process⁴⁵. This hypothesis is strengthened by the observation that Psb28 is also found
71 in PSII repair complexes⁴⁶. The luminal PSII assembly factor Psb27 has been similarly well
72 investigated. This lipoprotein is predominantly associated with inactive PSII fractions involved
73 in assembly or repair^{32,34,46-50}, stabilizing the CP43 luminal domain and presumably facilitating
74 the assembly of the OEC.

75 Our current knowledge of PSII biogenesis mainly describes the order of events and protein
76 composition of each intermediate, as well as the general roles of PSII assembly factors.
77 However, the precise molecular functions of these intermediate complexes and the involved
78 assembly factors are still elusive due to their low abundance and intrinsic instability. High-
79 resolution structural information is of vital importance to gain a deeper understanding into the
80 molecular action of PSII assembly factors, as they are proposed to alter the structures of their
81 associated PSII proteins to provide protection or facilitate specific biogenesis transitions.

82 Here, we use cryo-EM single particle analysis to describe the first molecular structure of a PSII
83 assembly intermediate. This structure represents one of the key transitions in PSII biogenesis:

84 the attachment of the CP43 module to the pre-assembled RC47 reaction center complex, which
85 precedes incorporation and activation of the Mn_4CaO_5 cluster. We complement this structural
86 data with spectroscopic analysis, revealing the first detailed insights into the molecular
87 mechanisms of PSII assembly. Our study provides mechanistic answers to three long-standing
88 questions: i) How do assembly factors modulate the structures of PSII subunits to assist
89 biogenesis? ii) How is PSII protected from photodamage during assembly? iii) How is the PSII
90 active site prepared for incorporation of the Mn_4CaO_5 cluster?

91 **Results**

92 Stable PSII intermediates were purified from the *T. elongatus* $\Delta psbJ$ mutant⁴³ by affinity
93 chromatography using a twin-strep-tag fused to the C-terminus of the CP43 subunit and
94 subsequent ion exchange chromatography (Fig. S1a). The main peak of the IEC profile
95 corresponds primarily to monomeric PSII, which lacks the extrinsic subunits PsbO, PsbU and
96 PsbV that are indicative for water splitting activity (Fig. S1b and c). Single particle cryo-EM
97 analysis of this PSII fraction resulted in three different high-resolution maps that allowed model
98 building with high confidence and excellent statistics (Fig. S2, Table S1). In addition to the
99 protein subunits, we also faithfully assigned all essential non-protein cofactors, including
100 chlorophylls, quinones, carotenoids and lipids, which are also present in the mature PSII
101 complex (Fig. S3). Consistent with previous biochemical studies^{34,43,48}, the EM density
102 corresponding to the fully assembled, active Mn_4CaO_5 cluster is missing in the purified
103 biogenesis intermediates. The first cryo-EM map (2.94 Å), which we call PSII-I (for PSII-
104 Intermediate), provides a snapshot of the attachment of the CP43 module to the pre-assembled
105 RC47 reaction center complex (Fig. 1). This PSII intermediate contains three assembly factors
106 (Psb27, Psb28 and Psb34), as well as almost all the membrane-intrinsic subunits and cofactors
107 found in mature PSII, although CP43 is bound in an immature conformation. Psb27 and Psb28
108 are well-known assembly factors^{31,32,35,43,51}, whereas the additional single transmembrane helix
109 protein (tsl0063), which we named Psb34, has not been described before. The small subunit
110 PsbY, which is known to be loosely bound⁵², is not resolved in our structure. In addition, PsbJ
111 is not present, as the corresponding gene was inactivated to stall PSII assembly at this specific
112 transition⁴³.

113 The two additional maps serve as internal controls. PSII-I' (2.76 Å) lacks Psb27 but is otherwise
114 comparable to PSII-I; the root mean square deviation (RMSD) of the C_α atomic positions
115 between similar subunits of the two complexes is 0.4 Å. Most likely, Psb27 was partly lost

116 during sample preparation. The third cryo-EM map (2.82 Å), which we call PSII-M (for PSII-
117 Monomer), represents a monomeric PSII complex without bound assembly factors.
118 Comparison of our PSII-M structure with a crystal structure of monomeric PSII⁵² (PDB-ID
119 3KZI, 3.6 Å) reveals only minimal differences between both structures, with a C α RMSD of
120 1.3 Å, which verifies that the structural changes observed in PSII-I are not caused by the
121 deletion of PsbJ.

122 **Psb34 binds to CP47 and is involved in the RC47 transition**

123 Our PSII-I structure provides the first identification of the single transmembrane helix protein
124 Psb34 bound to a PSII assembly intermediate (Fig. 2a), which we also confirmed by mass
125 spectrometry (Fig. 2b). Psb34 was probably overlooked previously due to its hydrophobicity
126 and small size. It has a single transmembrane helix that binds to the CP47 antenna protein in
127 close proximity to PsbH (Fig. 2a). Its conserved long N-terminal arm is located at the side and
128 top of the D2 subunit (Fig. 2a). In addition, we independently confirmed the interaction of
129 Psb34 with PSII assembly intermediates by isolation of strep-tagged Psb34 complexes from
130 otherwise unmodified cells, indicating a specific function of Psb34 in the attachment of CP43
131 to RC47 (Fig. 2c). In this case, two distinct PSII intermediates were isolated via pulldown of
132 strep-tagged Psb34: the RC47 complex with bound Psb28 and the subsequent PSII intermediate
133 after attachment of CP43 and Psb27 (Fig. 2c). This observation implies that Psb28 is usually
134 released from the PSII intermediate after attachment of CP43, probably after incorporation of
135 PsbJ, as this trigger is missing in the analyzed $\Delta psbJ$ mutant. PSII-I, which only accumulates
136 in the $\Delta psbJ$ mutant, seems to represent a hybrid state of both PSII intermediates that might be
137 stabilized by the incomplete binding of CP43. Psb34 shows sequence similarity to high-light
138 inducible proteins (HLIPs), which play a role in transient chlorophyll storage and chlorophyll
139 biosynthesis⁵³. However, the chlorophyll binding motive is missing in Psb34 (Table S2),
140 suggesting a distinct function for this protein in PSII biogenesis.

141 **Psb28 induces conformational changes to the D1 D-E loop**

142 Psb28 binds on the cytosolic faces of the D1 and D2 subunits, directly above the Q_B binding
143 site (Fig. 3a), which differs from the position that was previously predicted by mass
144 spectrometry⁴⁵. Its binding induces the formation of an extended beta-hairpin structure that
145 incorporates the central anti-parallel beta-sheet of Psb28, the C-terminus of CP47 and the D1
146 D-E loop⁵⁴ (Fig. 3a). Binding of Psb28 to the C-terminus of CP47 also imparts a directionality

147 to the assembly process. In the Psb28-free complex (PSII-M), the CP47 C-terminus blocks the
148 Psb28 binding site by interacting with the D1 D-E loop, thus preventing the reverse process and
149 perturbation of active PSII by Psb28. Using nuclear magnetic resonance (NMR) spectroscopy,
150 we performed chemical shift perturbation (CSP) experiments with recombinant Psb28 and a
151 synthetic peptide of the conserved CP47 C-terminus to characterize this interaction in detail
152 and determine the dissociation constant (K_D) (Fig. 3 and Fig. S4). The CSP measurements
153 indicated significant shifts with a chemical shift difference ($\Delta\delta$) of more than one standard
154 deviation located at strands β_3 and β_4 as well at the C-terminal region of Psb28 (Fig. 3c and
155 d). Upon peptide binding, resonances for several residues gradually appeared with increasing
156 peptide concentration, which were line-broadened beyond detection for the free form of Psb28.
157 This observation indicates a less dynamic and more rigid complex structure. This is further
158 supported by the heteronuclear Overhauser effect (NOE) data, which show that the C-terminus
159 of Psb28 becomes rigid from L108 to K112 upon CP47 peptide binding due to creation of an
160 intermolecular β -sheet (Fig. 3e). 2D-lineshape analysis was performed, yielding a K_D of
161 $53.92 \pm 0.41 \mu\text{M}$ and a dissociation rate k_{off} of $10.14 \pm 0.16 \text{ s}^{-1}$, which is consistent with the
162 observed slow-exchange in the NMR spectra (Fig. 3b). The affinity of Psb28 for full-length
163 CP47 and PSII might indeed be even higher due to additional contacts between Psb28 and the
164 D-E loop of D1 (Fig 3a).

165 **Psb28 prevents full association of CP43 and distorts the Q_B -site**

166 Binding of Psb28—with support of Psb34—causes major structural perturbations at the PSII
167 acceptor side (Supplementary Movies 1 and 2), which mainly involve the D-E loops of the
168 central D1 and D2 subunits. Comparison of the CP43 structure in PSII-I with that in our
169 PsbJ-free control PSII-M (Fig. 4a-d) or with that in mature monomeric PSII (PDB-ID 3KZI)
170 (Fig. 4c and d) reveals several differences. The CP43 C-terminus is not resolved in PSII-I,
171 probably due to an immature position of the last transmembrane helix of CP43 and an altered
172 conformation of the D1 D-E loop, which may prevent binding of the CP43 C-terminus to the
173 cytoplasmic PSII surface (Fig. 4b). This region is close to the loop between helices D and E of
174 the D2 subunit, which is also altered by binding of Psb28, as clearly shown by movement of
175 D2 Arg233 (Fig. 4b, Fig. S5a and b). After dissociation of Psb28, the CP43 module undergoes
176 a rigid body rotation where it clicks into place (Fig. 4b-d, Supplementary Movie 1), whereas
177 binding of PsbJ and the extrinsic proteins PsbO, PsbV and PsbU during further maturation has
178 very little influence on the CP43 binding position (Fig. 4c and d). The part of PSII that

179 originates from RC47 shows almost no difference between PSII-I and mature PSII (Fig. 4d),
180 except for PsbE, which binds adjacent to PsbJ (Fig. 4c).

181 Most importantly, the structural changes in the D1 D-E loop may have a direct functional impact
182 on PSII electron transfer (Fig. 4e and f), as this region coordinates several important PSII
183 cofactors. In functional PSII, after charge separation at the reaction center P₆₈₀, electrons are
184 transferred via pheophytin to the bound plastoquinone (Q_A) and further to mobile plastoquinone
185 (Q_B). In our PSII-I structure, the Q_A site is fully assembled, and a well-resolved Q_A molecule is
186 bound (Fig. 4e and f, Fig. S5c and d). The nearby non-heme iron is also already in place in
187 PSII-I (Fig. 4e and f, Fig. S5e and f). The Q_B binding site of the PSII-M control is comparable
188 to mature PSII, although it is not occupied by Q_B in our preparation (Fig. S5g). In contrast, the
189 Q_B binding site of PSII-I is immature due to the Psb28- and Psb34-induced structural changes
190 in the D1 D-E loop (Fig. 4e and f, Fig. S5h). Notably, D1 Phe265, which coordinates the head
191 group of Q_B in mature PSII, is clearly at a different position²¹ (Supplementary Movie 2).

192 **Binding of Psb28 protects PSII during biogenesis**

193 A more detailed analysis of the structural environment close to the Q_A/Q_B binding sites revealed
194 differences in the coordination and the hydrogen-bond network of the adjacent non-heme iron,
195 which also indicate functional consequences for PSII electron transfer and charge
196 recombination processes. In mature PSII, the non-heme iron is coordinated by four histidine
197 residues and bicarbonate as the fifth ligand (Fig. 5a and c), whereas in PSII-I, the bicarbonate
198 molecule is replaced by the E241 side-chain of D2 (Fig. 5b and d, Fig. S5e and f, Supplementary
199 Movie 3). Other residues, including D1 E244 and Y246, which bind to the bicarbonate molecule
200 in mature PSII (Fig. 5a), are also displaced in PSII-I due to the conformational change of the
201 D1 D-E loop (Fig. 5b, Fig. S5e and f, Supplementary Movie 3). Binding of bicarbonate is
202 important for PSII efficiency⁵⁵, as it lowers the redox potential of (Q_A/Q_A⁻) to favor forward
203 electron transport^{56,57}. If charge recombination occurs, the lower redox potential favors indirect
204 charge recombination via P₆₈₀⁺/Pheo⁻. This back reaction yields triplet chlorophyll and
205 subsequently singlet oxygen⁵⁷, a highly reactive oxidizing species. Changes in the redox
206 potential of (Q_A/Q_A⁻) have been proposed to tune the efficiency of PSII depending on the
207 availability of CO₂ as the final electron acceptor and thereby protect PSII under low CO₂
208 conditions⁵⁷. Therefore, we used flash-induced variable fluorescence to monitor the stability
209 of Q_A⁻ in active PSII, in the PSII-I assembly intermediate and in inactivated, Mn-depleted PSII,
210 both of which lack a functional OEC (Fig. 5e, Fig. S6a and b). The results indicate that the

211 unique fluorescence traces observed with PSII-I are a consequence of Q_A/Q_A^- redox tuning by
212 an absent OEC, an immature Q_B -binding pocket and probably the replacement of bicarbonate
213 by glutamate as a ligand of the non-heme iron (see supplementary discussion for details). To
214 determine the functional consequences of this redox tuning in PSII-I, we measured the
215 formation of 1O_2 by EPR spectroscopy using the spin probe TEMPD. The data clearly show
216 that 1O_2 formation is reduced by ~30% in PSII-I compared to inactivated PSII (Fig. 5f).

217

218 **Psb27 binds to loop E of CP43 at the luminal PSII surface**

219 Psb27 binds to the luminal side of the PSII complex, adjacent to loop E of the CP43 subunit
220 (Fig. 6a and b). We could successfully model the Psb27 N-terminus until Cys22, which is the
221 first residue after N-terminal processing of Psb27³⁴. Psb27-Cys22 is located close to the
222 membrane plane, but the lipid moiety is not well enough resolved to build a model. In contrast
223 to previously proposed models^{58,59}, the binding site of Psb27 has little overlap with the binding
224 sites of the extrinsic subunits (PsbO, PsbV and PsbU) and has at least no direct contact to the
225 Mn_4CaO_5 cluster binding site, particularly not to the D1 C-terminus (Fig. 6a and b), which has
226 been suggested previously^{58,60}. Instead, Psb27 is bound at a remote position that might be
227 occupied by CyanoQ in the mature complex⁶¹. This position of Psb27 may still prevent proper
228 binding of PsbO³⁴ by lowering its binding affinity due to the partial overlap of the binding sites,
229 although both proteins can bind together, as PsbO-containing Psb27-PSII complexes have been
230 isolated via his-tagged Psb27⁴⁹. Additionally, Psb27 might stabilize loop E of CP43 in the
231 unassembled state and facilitate its binding to the D1 subunit. This is of particular importance,
232 as loop E of CP43 provides Arg345 and Glu342 (Arg357 and Glu354 in previous publications,
233 see figure caption), to position the Mn_4CaO_5 cluster in mature PSII (Fig. 6b, dashed box).
234 Glu342 serves a ligand, whereas Arg345 stabilizes the Mn_4CaO_5 cluster by coordinating
235 Asp170 of D1 through a hydrogen bond. Moreover, in the Psb27-bound state (PSII-I), the D1
236 C-terminus, which is directly involved in coordination of the Mn_4CaO_5 cluster²¹, is bound away
237 from the cluster (Fig. 6c, Fig. S7), which might be an additional consequence of Psb27-induced
238 stabilization of the CP43 E-loop. Thus, our PSII-I structure reveals not only how the Psb27
239 protein binds to CP43 and thus stabilizes it, but also indicates a role for the maturation of the
240 oxygen evolving cluster that is consistent with functional data from previous studies^{32,35,48}.

241 **PSII-I contains a single cation at the immature OEC site**

242 The unique Mn_4CaO_5 cluster is a key feature of PSII that splits water into oxygen and protons.
243 However, our PSII-I complex does not show any oxygen-evolving activity, suggesting that the
244 oxygen evolving complex (OEC) is not fully assembled. In mature PSII, the Mn_4CaO_5 cluster
245 is submerged in the complex and additionally capped by the extrinsic subunits PsbO, PsbU and
246 PsbV (Fig. 6a and b). In our PSII-I structure, these subunits are absent, which leaves two parts
247 of the CP43 E-loop (residues 320-327 and 397-404) in a flexible conformation, exposing the
248 binding site of Mn_4CaO_5 cluster to the lumen. There is no strong density feature at this position
249 that would correspond to the fully assembled metal-redox cofactor. Thus, our PSII-I structure
250 provides a model for an immature OEC. Comparing our structure with the high-resolution
251 crystal structure of mature PSII²¹ (PDB-ID 3WU2) provides insights into the first-steps of OEC
252 biogenesis (Fig. 7).

253 The D1 C-terminus is an essential component for formation of the of OEC, as it provides several
254 charged residues that are responsible for coordination of the chloride ion and the Mn_4CaO_5
255 cluster (Fig. 7a, b and d). The density for these C-terminal residues is weak in our PSII-I map,
256 but traceable (Fig. S7a), indicating a flexibility that confirms the absence of the OEC.
257 Compared to the mature complex, the last 12 residues of the C-terminal tail of D1 would need
258 to undergo significant conformational changes to bring the side chains of Glu333, His337,
259 Asp342, and the Ala344 C-terminus into the correct position to coordinate the Mn_4CaO_5 cluster
260 (Supplementary Movie 4).

261 Moreover, we identify a clearly visible density at the position of the chloride ion, which is
262 coordinated by Lys317 (D2) and the hydrogen atom of the backbone nitrogen of Glu333 (D1)
263 in mature PSII (Fig. 7b and e). Despite the similar position, the Cl^- is coordinated by the nitrogen
264 atom of the ring of adjacent His332 (D1) in PSII-I (Fig. 7a and e, Fig. S7d). Surprisingly, we
265 identified another density in the area where the Mn_4CaO_5 cluster is located in mature PSII
266 (Fig. 7a-c and f, Fig. S7c). However, this density is not large enough to reflect the whole cluster.
267 Based on its size and interaction partners (Fig. 7f), it corresponds to one positively charged ion.
268 In the structural context, this ion is most likely Mn^{2+} , but it could also be Ca^{2+} or any other
269 positively charged ion.

270 This ion is coordinated by the side chains of D1 Asp170, Glu189, and His332, which are already
271 in similar positions compared to mature PSII. Glu342 and Arg345 (Glu354 and Arg357 in
272 previous publications) of CP43, which are both involved in the second coordination sphere of
273 the Mn_4CaO_5 cluster, are also already pre-positioned through the interaction between Arg345

274 with D1 Asp170 (Fig. 7g). However, there are still significant conformational changes
275 necessary for the transition from PSII-I to mature PSII, as highlighted in Figure 7d and g, as
276 well as in Supplementary Movie 4. The D1 C-terminal tail must bring the side chains of Glu333,
277 His337 and Asp342, as well as the C-terminus of Ala344, into correct alignment to coordinate
278 the Mn_4CaO_5 cluster. In addition, the C-terminal tail of D2 needs to flip towards the D1 C-
279 terminus (Fig. 7c, Fig. S7b, Supplementary Movie 4). In summary, PSII-I is characterized by
280 only one positive charged ion bound instead of the complete Mn_4CaO_5 cluster, resulting in
281 significantly different conformations of the D1 and D2 C-termini compared to the structural
282 model containing a mature Mn_4CaO_5 cluster. However, the PSII-I structure seems to be
283 prepared to accept the Mn_4CaO_5 cluster, as indicated by the above described similarities in side
284 chain positioning.

285 **Discussion**

286 PSII biogenesis is a complex process that requires the action of specific assembly factors. These
287 auxiliary proteins are not present in the mature complex and interact only transiently with
288 specific subunits or preassembled PSII intermediates. Although more than 20 factors have been
289 identified and assigned to specific transitions, their precise molecular functions in PSII
290 assembly remain elusive in almost all cases. Our study provides the first detailed molecular
291 insights into the function of PSII assembly factors Psb27, Psb28 and Psb34, which are involved
292 in an important transition prior to activation of the OEC. The determined binding positions of
293 Psb27 and Psb28, which are two of the most studied PSII assembly factors, define the binding
294 with atomic resolution, in contrast to previous models^{58,59,62-66}. This provides precise
295 information about the molecular function of Psb27 and Psb28 in the assembly process.

296 Binding of Psb28 and Psb34 to the cytoplasmic side of PSII induces large conformational
297 changes in the D1 D-E loop (Fig. 4), which has been identified previously as an important
298 location for PSII photoinhibition and D1 degradation^{67,68}. Structural changes observed in the
299 PSII-I Q_B binding pocket and coordination of the non-heme iron suggest a functional impact on
300 PSII electron transfer to protect the immature complex until water splitting is activated. In
301 particular, D2 Glu241 replacing bicarbonate as a ligand of non-heme iron suggests a regulatory
302 role, as binding of bicarbonate was proposed to tune PSII efficiency by changing the redox
303 potential of (Q_A/Q_A^-)^{56,57}. As a functional consequence, PSII-I generates less singlet oxygen
304 compared to inactivated PSII (Fig. 5f).

305 Interestingly, the coordination of the non-heme iron in PSII-I resembles that in non-oxygenic
306 bacterial reaction centers (BRCs)⁶⁹ (Fig. S6c). In BRCs, the fifth ligand of the non-heme iron
307 is provided by E234 of the M subunit⁷⁰, and mutagenesis of this residue induces changes in the
308 free energy gap between the P^{•+}/Q_A^{•-} radical pair⁷¹. These findings indicate that the environment
309 of the non-heme iron is important for the Q_A redox potential, which influences charge
310 recombination and the formation of reactive oxygen species⁵⁶.

311 The Psb27-bound and -unbound structures do not differ substantially (Fig. 6b), suggesting a
312 rather subtle action in PSII biogenesis. Previous work demonstrated that Psb27 is already bound
313 to free CP43³², where it might protect free CP43 from degradation or stabilize the E-loop in a
314 specific conformation to chaperone the subsequent association with the RC47 complex. This
315 step is crucial for preparing the binding site of the Mn₄CaO₅ cluster, as the CP43 E-loop is
316 involved in its second coordination sphere⁷². Moreover, a recent high-speed AFM study with
317 spinach PSII has shown fluctuations of the C43 E-loop at room temperature after removal of
318 the extrinsic subunits and destruction of the Mn₄CaO₅ cluster⁷³, which might suggest a role of
319 Psb27 in restraining the scope of conformational fluctuations for further assembly of the OEC.
320 Consistent with this model, recent spectroscopic analysis of PSII from *Synechocystis* sp. PCC
321 6803 revealed that Psb27 facilitates photoactivation, probably by stabilization of intermediates
322 with increased accessibility for ions, especially Ca²⁺, to the site of cluster assembly⁷⁴. However,
323 our data indicate a self-organization of the CP43 E-loop, at least in *T. elongatus*. The
324 corresponding structural models for this region are almost identical, independent of binding of
325 Psb27 or the extrinsic proteins, whereas the positions of the D1 and D2 C-termini are clearly
326 different between intermediate and mature PSII. Binding of PsbO seems to be the main driver
327 for these structural differences, and its presence interferes with efficient photoactivation,
328 probably by closing the cluster binding site and thus preventing the exchange of ions⁷⁴. The
329 overlap of the Psb27 and PsbO binding sites, although small, might decrease the PsbO affinity
330 and prevent its premature binding, as initially suggested^{34,37}, or Psb27 may promote binding of
331 PsbO in a different conformation (both proteins can bind simultaneously in a stable complex⁴⁹)
332 that might support photoactivation.

333 Previous structural studies aimed to obtain mechanistic insights into the dark-rearrangement
334 during photoactivation by removing the Mn₄CaO₅ cluster from fully assembled PSII, either by
335 depleting it directly from PSII crystals by chemical treatment⁷⁵ or by cryo-EM single particle
336 analysis in manganese- and calcium-free buffer⁷⁶. The X-ray structure was indeed missing the
337 Mn₄CaO₅ cluster, but the D1 C-terminus followed mostly the same trajectory as found in the
338 mature PSII-dimer structure. The authors suggested that the D1 C-terminus might not rearrange

339 during Mn_4CaO_5 cluster assembly. However, the crystal structure was dimeric and still had the
340 extrinsic subunits PsbO, PsbU, and PsbV bound. It is known that these subunits are typically
341 not associated with Mn_4CaO_5 cluster-depleted PSII. Thus, the structure might be artificially
342 stabilized by crystal packing forces. The cryo-EM structure, on the other hand, revealed a
343 monomeric PSII that lacks extrinsic subunits and the Mn_4CaO_5 cluster⁷⁶. This structure is more
344 similar to our PSII biogenesis intermediate PSII-I, as PsbY, PsbZ and PsbJ are also missing.
345 The PsbJ subunit is surprising; it is an integral subunit of PSII and should not be easily detached,
346 yet it is missing from this structure, as we deleted it to stabilize our PSII-I complex. These
347 observations might indicate a more specific and regulatory role of PsbJ in PSII biogenesis.
348 Additionally, the D1 C-terminus is disordered in this previous cryo-EM structure, and the
349 authors suggest that the dark-rearrangement involves a transition from a disordered to an
350 ordered state.

351 Our structure now reveals the fate of the D1 C-terminus with the assembly factor Psb27 bound.
352 The D1 C-terminus follows a different trajectory compared to the mature PSII. Thus, we
353 provide structural evidence that the slow dark-rearrangement involves a conformational change
354 of the D1 C-terminus rather than the previously proposed disorder-to-order transition after
355 initial photoactivation⁷⁶. Compared to mature PSII, twelve residues of the D1 C-terminal tail
356 must undergo significant conformational changes to bridge the side chains of Glu333, His337
357 and Asp342, as well as to bring the C-terminus of Ala344 to the correct position to coordinate
358 the Mn_4CaO_5 cluster (Fig. 6c and 7d, Supplementary Movie 4), which is consistent with
359 previous models^{39,77,78}. We also identified a single positively charged ion in our PSII-I
360 structure, coordinated by Asp170, Glu189 and His332 of D1 (Fig. 7f), at the position of the
361 Mn_4CaO_5 cluster of mature PSII. This binding site most likely corresponds to the long-sought
362 single high-affinity site (HAS), where the first Mn^{2+} binds prior to the first photoactivation step
363 in OEC biogenesis⁴¹. The presence of manganese in immature PSII, due to a PrtA-facilitated
364 pre-loading mechanism, has been previously suggested⁷⁹. However, we cannot exclude binding
365 of Ca^{2+} , which was shown to bind with a much lower affinity^{77,80}, or any other positively
366 charged ion at this position. Nevertheless, Asp170 has been identified as the most critical
367 residue for the HAS^{81,82}, which supports our hypothesis. Further photoactivation steps occur
368 presumably after cooperative binding of calcium and manganese. The binding of the extrinsic
369 subunit PsbO, potentially after release of Psb27 and maturation of the water oxidizing complex,
370 is the next step of the PSII assembly line *in vivo*, which leads to the next unsolved question in
371 PSII biogenesis: what triggers the release of an assembly factor? For Psb27, its detachment
372 might be promoted by the binding of PsbO, as their binding sites partially overlap.

373 Membrane protein complexes play a fundamental role in bioenergetics to sustain and proliferate
374 life on Earth. They drive the light-to-chemical energy conversion in photosynthetic organisms
375 and are essential for energy supply in heterotrophs. These intricate molecular machines are
376 assembled from numerous single proteins in a spatiotemporally synchronized process that is
377 facilitated by a network of assembly factors. These auxiliary proteins are the key players of
378 Nature's assembly lines. Our PSII-I cryo-EM structure reveals the first molecular snapshot of
379 PSII biogenesis and, accompanied by our spectroscopic and biochemical analyses, provides
380 clear mechanistic insights into how three assembly factors (Psb27, Psb28 and Psb34) coordinate
381 the stepwise construction of this powerful catalyst of life.

382 **Methods**

383 **Cultivation of *Thermosynechococcus elongatus* BP-1**

384 Cell growth and thylakoid membrane preparation were performed as described previously⁸³. In
385 brief, *T. elongatus* mutant strains (Δ psbJ psbC-TS and psb34-TS) were grown in BG-11 liquid
386 medium inside a 25-litre foil fermenter (Bioengineering) at 45°C, 5% (v/v) CO₂-enriched air
387 bubbling and 50-200 μ mol photons m⁻² s⁻¹ white light illumination (depending on the cell
388 density). Cells were harvested at an OD₆₈₀ of ~ 2 after 5-6 days of cultivation and concentrated
389 to ~ 0.5 l, using an Amicon DC10 LA hollow fibre system, pelleted (3500 rcf, 45 min and 25
390 °C) and resuspended in 150 ml of Buffer D (100 mM Tris-HCL, pH 7.5, 10 mM MgCl₂, 10 mM
391 CaCl₂, 500 mM mannitol and 20% (w/v) glycerol). The harvested cells were flash-frozen in
392 liquid nitrogen and stored at -80 °C until further use.

393 **Preparation of *T. elongatus* mutant strains**

394 *Thermosynechococcus elongatus* Δ psbJ psbC-TS was generated based on the previously
395 described strain *T. elongatus* Δ psbJ⁴³ that was transformed with the plasmid pCP43-TS. The
396 plasmid is based on pCP34-10His³⁴. The His-tag sequence was exchanged with TwinStrep-tag
397 by PCR using the primers CP43TS_rev (5'CCCGATATCTTACTTCTCAAATTGCGGAT
398 GAGACCACGCAGAACCACGAGAACCACCGCCGCTGCCGCCGCCTTTTTTCGAACTG
399 CGGGTGGCTCC 3') and NTCP43 (5' TGCTCTAGAATGAAAACCTTTGTCTTCCCAGA
400 3'). The resulting PCR product was ligated back into an empty pCP34-10His backbone using
401 XbaI and EcoRV restriction endonucleases. *T. elongatus* BP-1 cells were transformed as
402 described previously⁸⁴. Mutant colonies were selected by frequent re-plating onto agar plates
403 with increasing antibiotic concentrations, stopping at 8 μ g/ml of chloramphenicol and 80 μ g/ml

404 of kanamycin. Complete segregation of the mutant was confirmed by PCR with the primers
405 CTCP43DS (5' CCGCTCGAGACCATCCAAGCTTGGCAGCA 3') and NTCP43 (5'
406 TGCTCTAGAATGAAAACCTTTGTCTTCCCAGA 3').

407 *T. elongatus* psb34-TS was generated by transformation with the plasmid pPsb34-TS. The
408 plasmid DNA was obtained from TwistBioscience. It consisted of psb34 (tsl0063) with a C-
409 terminal TwinStrep-tag and a kanamycin resistance cassette, flanked by tsl0063-upstream and
410 downstream regions (900 bp each). *T. elongatus* BP-1 cells were transformed⁸⁴ and mutant
411 selection took place³⁴. Complete segregation of the mutant was verified by PCR. The primers
412 used were tsl0063-up-for (5' CATATGGTCTCGCAATTATTTGCCCATGC 3') and tsl0063-
413 down-rev (5' GGTACCCCG ACACAGTTGATCACCGC 3').

414 **Purification of photosystem II assembly intermediates**

415 Thawed cells were diluted in 100 ml of Buffer A (100 mM Tris-HCL, pH 7.5, 10 mM MgCl₂
416 and 10 mM CaCl₂) and pelleted again (21 000 rcf, 20 min and 4°C). The pellet was resuspended
417 in 100 ml of Buffer A with 0.2% (w/v) lysozyme and dark incubated for 75-90 min at 37 °C.
418 This was followed by cell disruption by Parr bomb (Parr Instruments Company) and pelleting
419 (21 000 rcf, 20 min and 4°C). All following steps were performed under green illumination.
420 The pellet was resuspended in 150 ml of Buffer A and pelleted again (21 000 rcf, 20 min and
421 4°C). This step was repeated three times, with the last resuspension in 80 ml of Buffer B (100
422 mM Tris-HCL, pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂ and 500 mM mannitol). The isolated
423 thylakoid membranes were flash frozen in liquid nitrogen and stored at -80 °C.

424 Strep-Tactin-affinity purification of PsbC-TS and Psb34-TS assembly intermediates were
425 performed under green illumination. Membrane protein extraction was performed as described
426 previously⁸³, with certain adaptations. Isolated thylakoid membranes were supplemented with
427 0.05% (w/v) n-Dodecyl β-maltoside (DDM) (Glycon) and pelleted (21000 rcf, 20 min and 4°C).
428 The sample was resuspended in extraction buffer (100 mM Tris-HCL, pH 7.5, 10 mM MgCl₂,
429 10 mM CaCl₂, 1.2% (w/v) DDM, 0.5% (w/v) sodium-cholate and 0.01% (w/v) DNase) to a
430 final chlorophyll concentration of 1 mg/ml and incubated for 30 min at 20 °C. The solubilized
431 membrane proteins were ultra-centrifugated (140000 rcf, 60 min and 4 °C) and NaCl was added
432 to the supernatant to a final concentration of 300 mM.

433 The supernatant was filtered through a 0.45 μm syringe filter (Sarsted AG, Germany) and
434 applied to a 5 ml Strep-Tactin Superflow HC column (IBA Lifesciences), equilibrated in Buffer
435 W (100 mM Tris-HCL, pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂, 500 mM mannitol, 300 mM

436 NaCl and 0.03% (w/v) DDM) at a flowrate of 3 ml/min. The column was washed with Buffer
437 W until a stable baseline (A_{280}) was reached. Strep-tagged protein complexes were eluted by an
438 isocratic elution with Buffer E (100 mM Tris-HCL, pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂, 500
439 mM mannitol, 300 mM NaCl 2.5 mM desthiobiotin and 0.03% (w/v) DDM). The captured
440 fractions were equilibrated in Buffer F (20 mM MES, pH 6.5, 10 mM MgCl₂, 10 mM CaCl₂,
441 500 mM mannitol and 0.03% (w/v) DDM) with a spin concentrator (Amicon, Ultra – 15,
442 100000 NMWL), flash-frozen in liquid nitrogen and stored at -80 °C until analysis.

443 PsbC-TS containing assembly intermediates were further separated by ion exchange
444 chromatography (IEC). Captured elution fraction from the Strep-Tactin-affinity purification
445 were loaded onto a anion exchange column (UNO Q-6, Biorad) with a flowrate of 4 ml/min,
446 pre-equilibrated in Buffer F. Protein complexes were eluted by a liner gradient of MgSO₄ (0-
447 150 mM) using Buffer G (20 mM MES, pH 6.5, 10 mM MgCl₂, 10 mM CaCl₂, 500 mM
448 mannitol, 150 mM MgSO₄ and 0.03% (w/v) DDM). Fractions containing PSII assembly
449 intermediates were collected, concentrated to 100 – 10 μM reaction centers, using a spin
450 concentrator (Amicon, Ultra – 15, 100 000 NMWL), aliquoted, flash frozen in liquid nitrogen
451 and stored at -80 °C until further analysis.

452 **Cryo-electron microscopy**

453 For cryo-EM sample preparation, 4.5 μl of purified protein complexes were applied to glow
454 discharged Quantifoil 2/1 grids, blotted for 3.5 s with force 4 in a Vitrobot Mark III (Thermo
455 Fisher) at 100% humidity and 4°C, then plunge frozen in liquid ethane, cooled by liquid
456 nitrogen. Cryo-EM data was acquired with a FEI Titan Krios transmission electron microscope
457 using the SerialEM software⁸⁵. Movie frames were recorded at a nominal magnification of
458 22,500x using a K3 direct electron detector (Gatan), The total electron dose of ~55 electrons
459 per Å² was distributed over 30 frames at a calibrated physical pixel size of 1.09 Å. Micrographs
460 were recorded in a defocus range of -0.5 to -3.0 μm.

461 **Image processing, classification and refinement**

462 Cryo-EM micrographs were processed on the fly using the Focus software package⁸⁶ if they
463 passed the selection criteria (iciness < 1.05, drift $0.4 \text{ \AA} < x < 70 \text{ \AA}$, defocus $0.5 \text{ \mu m} < x < 5.5$
464 um , estimated CTF resolution < 6 Å). Micrograph frames were aligned using MotionCor2⁸⁷ and
465 the contrast transfer function (CTF) for aligned frames was determined using Gctf⁸⁸. Using
466 Gautomatch (<http://www.mrc-lmb.cam.ac.uk/kzhang/>) 693,297 particles were picked template-
467 free on 824 acquired micrographs. Particles were extracted with a pixel box size of 260 using
468 RELION 3.1⁸⁹ and imported into Cryosparc 2.3⁹⁰. After reference-free 2D classification,

469 675,123 particles were used for ab initio construction of initial models and subjected to multiple
470 rounds of 3D classification to obtain models with and without Psb28 density. Non-uniform
471 refinement in Cryosparc resulted in models with an estimated resolution of ~ 3.2 Å. Particles
472 belonging to 3D classes with and without Psb28 (150,090 and 166,411 particles, respectively)
473 were reextracted in RELION with a pixel box size of 256 and subjected to several rounds of
474 CTF-refinement (estimation of anisotropic magnification, fit of per-micrograph defocus and
475 astigmatism and beam tilt estimation) and Bayesian polishing⁹¹. Both classes were refined using
476 the previously generated starting models. 3D classification without further alignment using a
477 mask around the Psb27 region separated particles in the Psb28-containing class into distinct
478 classes with and without Psb27 (57,862 and 91,473 particles, respectively). Final refinement of
479 each of the three classes (with Psb27 and Psb28 (PSII-I), with Psb28 but without Psb27 (PSII-
480 I'), and without Psb27 and Psb28 (PSII-M)) resulted in models with global resolutions of 2.94
481 Å, 2.76 Å and 2.82 Å, respectively (Gold standard FSC analysis of two independent half-sets
482 at the 0.143 cutoff). Local-resolution and 3D-FSC plots (Extended Data Fig. 2) were calculated
483 using RELION and the “Remote 3DFSC Processing Server” web interface⁹², respectively.

484 **Atomic model construction**

485 The 3.6 Å resolution X-ray structure of monomeric PSII from *T. elongatus* with PDB-ID 3KZI⁵²
486 was used as initial structural model that was docked as rigid body using Chimera⁹³ into the
487 obtained cryo EM densities for PSII-M and PSII-I. The cofactors that had no corresponding
488 density were removed. The subunit PsbJ was also removed, as it was deleted in the experimental
489 design. By highlighting the still unoccupied parts of the PSII-I density map, we identified
490 densities that lead to the structures of Psb27, Psb28, and Psb34.

491 The 2.4 Å resolution X-ray structures of isolated Psb28 from *T. elongatus* with PDB-ID 3ZPN⁹⁴
492 and the 1.6 Å resolution X-ray structure of isolated Psb27 from *T. elongatus* with PDB-ID
493 2Y6X⁶⁵ were docked as rigid bodies into the unoccupied densities. The 1.6 Å resolution X-ray
494 structure of CyanoQ from *T. elongatus* with PDB-ID 3ZSU⁹⁵ does not fit into the density and
495 was therefore not modeled.

496 As there was no experimentally resolved structural model of Psb34 available, we first used the
497 sequence with UniProt-ID Q8DMP8 to predict structures using the webserver SWISS Model⁹⁶
498 and LOMETS⁹⁷. We also predicted the secondary structure through the meta server
499 Bioinformatics Toolkit⁹⁸ and CCTOP⁹⁹. The results of the secondary structure prediction are
500 summarized in Table S4. Combining these predictions together with the unassigned cryo-EM

501 density, we used COOT¹⁰⁰ to build an initial model of Psb34 that has one α -helix from amino
502 acid number 28 to 55.

503 **Model Refinement**

504 The initial model of the complex described above was refined in real space against the cryo-
505 EM density of PSII-I, and structural clashes were removed using molecular dynamics flexible
506 fitting (MDFF)¹⁰¹. MDFF simulations were prepared in VMD 1.9.4a35¹⁰² using QwikMD¹⁰³
507 and the MDFF plugin. The simulations were carried out with NAMD 2.13¹⁰⁴ employing the
508 CHARMM36 force field. Secondary structure, cis peptide and chirality restraints were
509 employed during 800 steps of minimization followed by a 40 ps MDFF simulation at 300K.
510 Due to the employed restraints, only conformational changes of side chains and subunit
511 movements compared to the initial structure are identified during the initial MDFF run. We
512 checked the fit to density of the structure by calculating cross-correlation values of the backbone
513 atoms. For PSII-I, we identified residues 217 to 269 from PsbA and residues 467 to 499 from
514 PsbB and PsbZ as main regions where the structural model was not yet in accordance with the
515 density after the initial MDFF run. For these three regions, we employed an iterative
516 combination of MDFF with Rosetta^{105,106}. Here, we used the optimized strategy as described
517 for model construction of the 26S proteasome^{107,108}.

518 To obtain an atomic model that fit the PSII-M density, we used the initial model based on 3KZI
519 described above, but without PsbJ, Psb27, Psb28, and Psb34. After the initial MDFF run, the
520 cross-correlation check did not reveal any regions with significant deviation between model
521 and density. Therefore, no further refinement was necessary. This fast convergence reflects that
522 there are no crucial differences between the PSII-M model and the X-ray structure 3KZI.

523 To obtain the atomic model that fit the PSII-I' density, we used the final PSII-I model without
524 Psb27 for MDFF. After the initial MDFF run, the cross-correlation check did not reveal any
525 regions with significant deviation between model and density. This fast convergence reflects
526 that there are no crucial differences between the PSII-I and PSII-I' models, except for the
527 presence of the Psb27 subunit.

528 Last, the PSII-M, PSII-I, and PSII-I' models were used to initiate one final round of real-space
529 refinement in Phenix¹⁰⁹.

530

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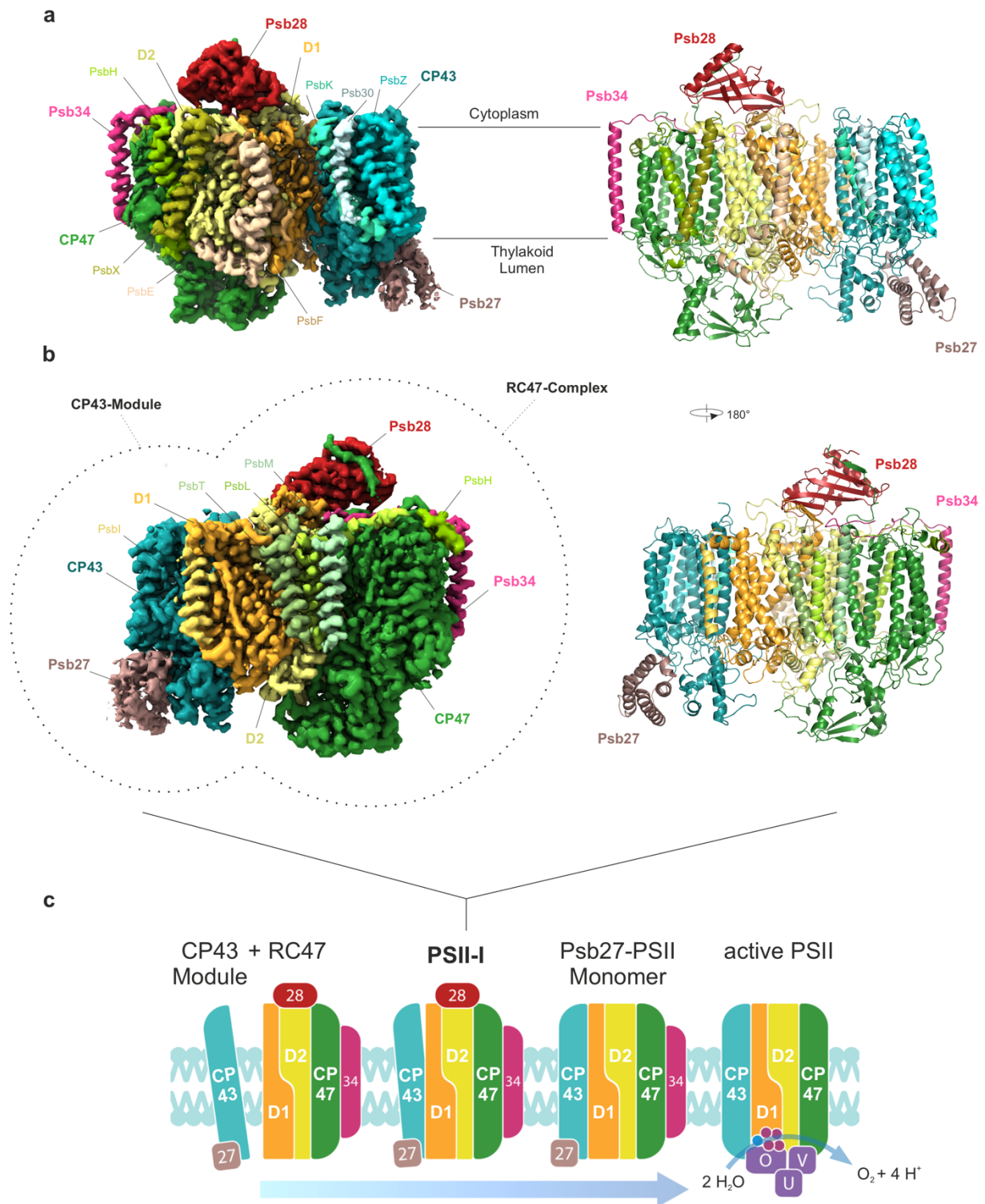


Fig. 1: Cryo-EM map of a PSII assembly intermediate (PSII-I) from *T. elongatus*, segmented by subunit. a, 15 PSII subunits and 3 assembly factors are colored and named (PSII subunits: D1, D2, CP43, CP47, PsbE, PsbF, PsbH, PsbI, PsbK, PsbL, PsbM, PsbT, PsbX, PsbZ and Psb30; assembly factors: Psb27, Psb28 and *tsl0063*, which we named Psb34) (front view). **b**, Parts of PSII that originate from the CP43 module (comprised of CP43, Psb27, PsbZ, Psb30 and PsbK) and the RC47 complex are indicated by dashed lines (back view). **c**, Schematic model of the PSII assembly process starting with the formation of PSII-I from the CP43 module and RC47. Small PSII subunits were omitted for simplicity.

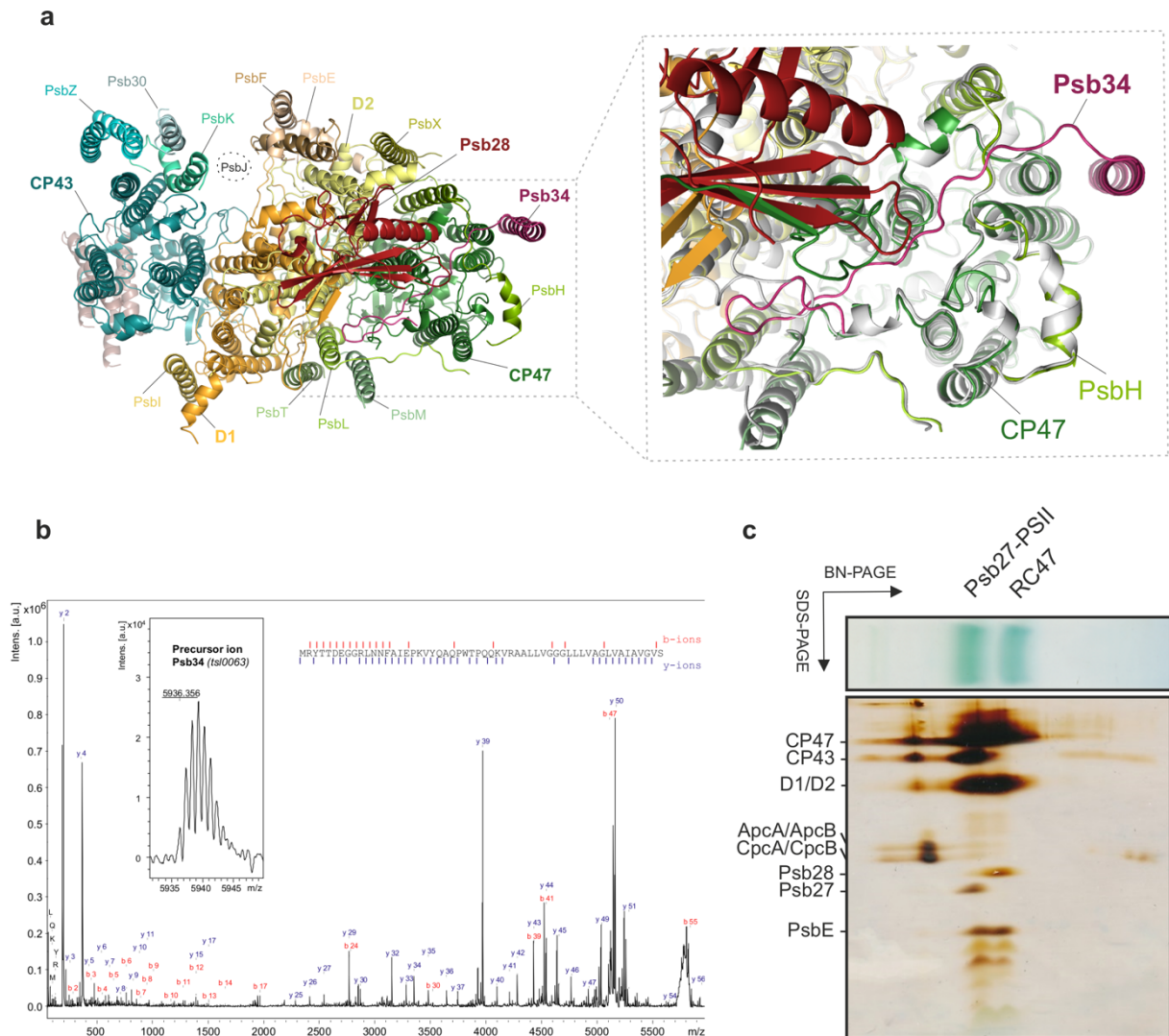


Fig. 2: Psb34 binds to RC47 during attachment of the CP43 module. a, Binding site of Psb34 at CP47, close to PsbH (top view), with extended binding of the Psb34 N-terminus along the cytoplasmic PSII surface (dashed box). **b**, MALDI-ToF analysis of PSII assembly intermediates. Mass spectrum of Psb34 (tsl0063) from the PSII complex (inset) and the fragment spectrum obtained for m/z 5936.356 with annotated b- and y-ion series matching the Psb34 sequence. Observed fragmentation sites are indicated by dashes in the sequence. Mascot score: 171. **c**, Subunit composition of Psb34-PSII assembly intermediates analyzed by 2D-PAGE.

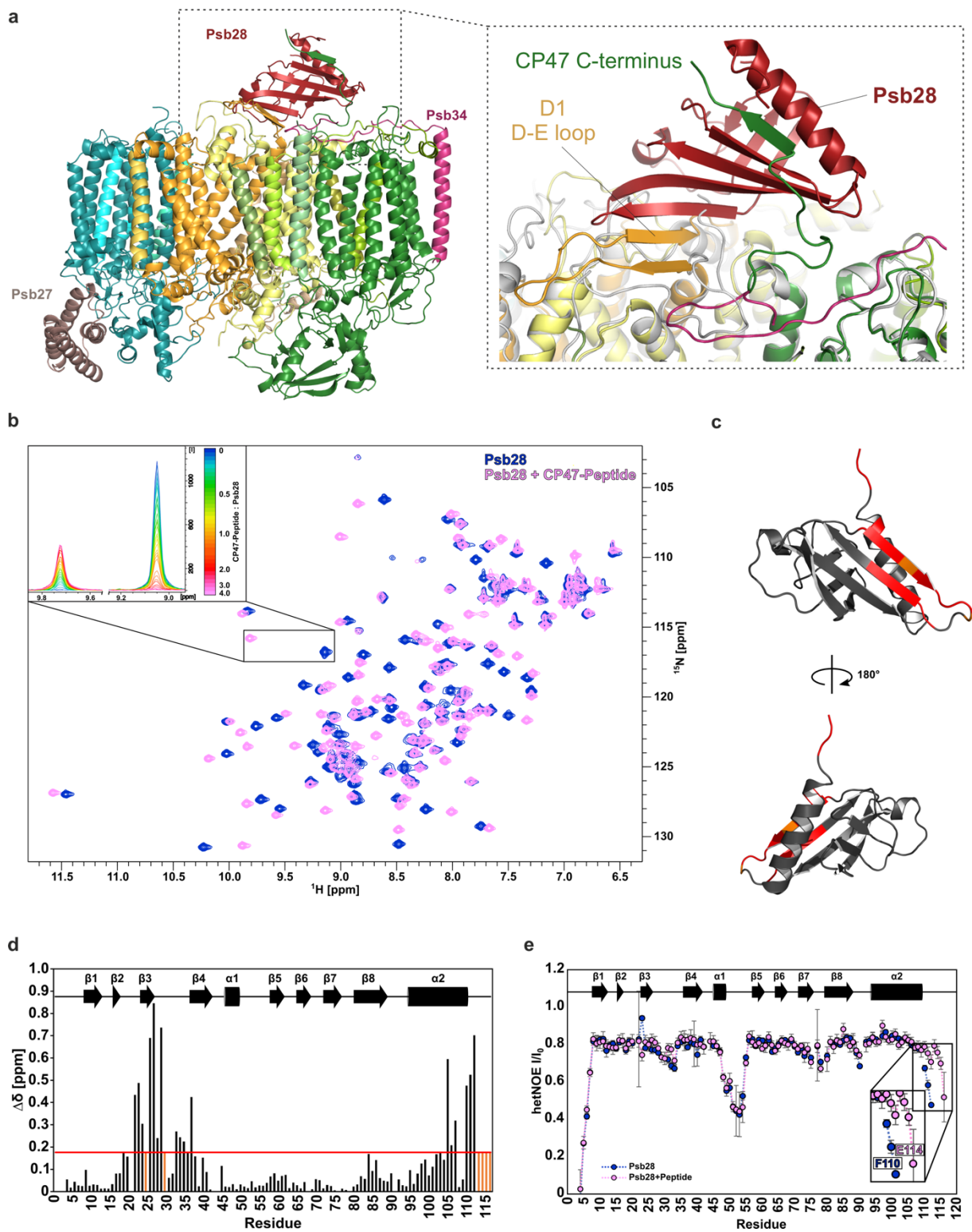


Fig. 3: The role of the CP47 C-terminus in binding of Psb28. **a**, Binding of Psb28 at the cytoplasmic/stromal PSII surface (side view, colors correspond to Fig. 1) and continuation of the central Psb28 beta-sheet by the CP47 C-terminus and the D-E loop of D1 (dashed box). For comparison, mature monomeric PSII (PDB-ID 3KZI) is shown in gray. **b**, Superimposed 2D ^1H - ^{15}N -HSQC spectra of free Psb28 (blue) and Psb28 bound to the C-terminal peptide of CP47 (magenta). Upper left inset: representation of slow exchange behavior for the proton amide resonance of T24, ranging from 126.9 ppm to 128.6 ppm in the ^{15}N dimension. **c**, CSPs of more than one SD projected onto the model representation of Psb28. **d**, Weighted $^1\text{H}/^{15}\text{N}$ chemical shift perturbations observed for

Psb28 upon binding to the CP47 peptide. Red line indicates one standard deviation (SD), residues that yield resonances only in the complex form are indicated in orange. **e**, Backbone ^{15}N $\{^1\text{H}\}$ -heteronuclear NOE of free Psb28 (*blue*) and Psb28 bound to the C-terminal region of the CP47 peptide (*magenta*). Smaller I/I_0 ratios correspond to regions that exhibit dynamics on the pico- to nanosecond timescale.

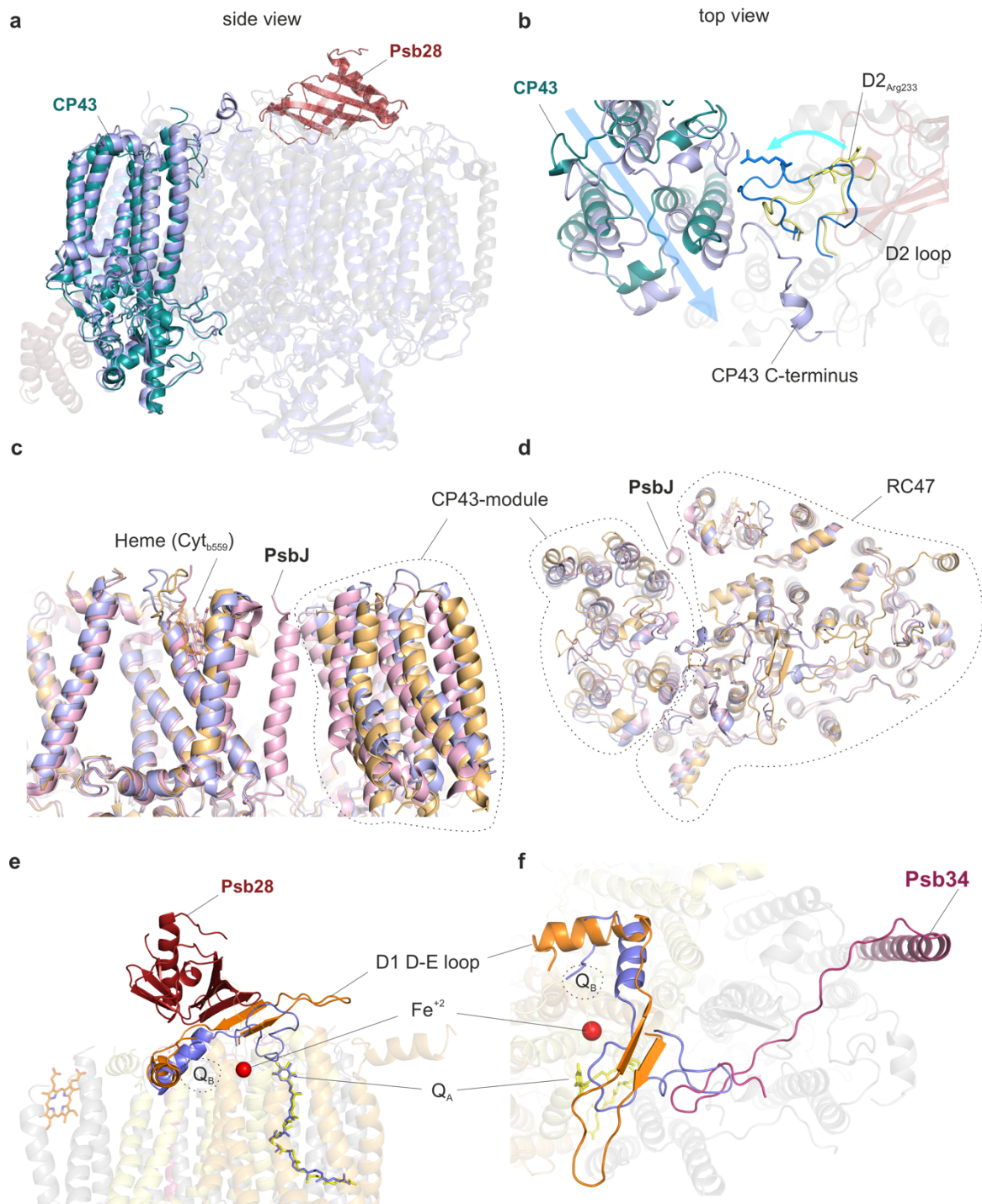


Fig. 4. Structural changes of the D1 and D2 D-E loops induced by binding of Psb28 and Psb34. **a**, Side view of the CP43 antenna protein in PSII-I (*teal*) and the PSII-M control (*light blue*). **b**, Structural changes between PSII-I and the PSII-M control in the cytoplasmic D2 D-E loop (*yellow*: PSII-I, *blue*: PSII-M) and attachment of CP43 (*teal*: PSII-I, *light blue*: PSII-M control) (top view). Details of the structural changes in the D2 loop are shown in Fig. S5A and B. **c**, Side view and **d**, top view of the PSII-I structure (*orange*) compared to the PSII-M control (*light blue*) and mature monomeric PSII (*light red*, PDB-ID 3KZI). **e**, Side view and **f**, top view of the Psb28-induced structural changes in the D1 D-E loop (*orange*) and perturbation of the Q_B binding site compared to PSII-M (*light blue*), which lacks the assembly factors. Q_A is shown in *yellow* (PSII-I) or *light blue* (PSII-M), respectively. See Fig. S5c-h for enlarged views of the Q_A and Q_B binding site and the adjacent non-heme iron.

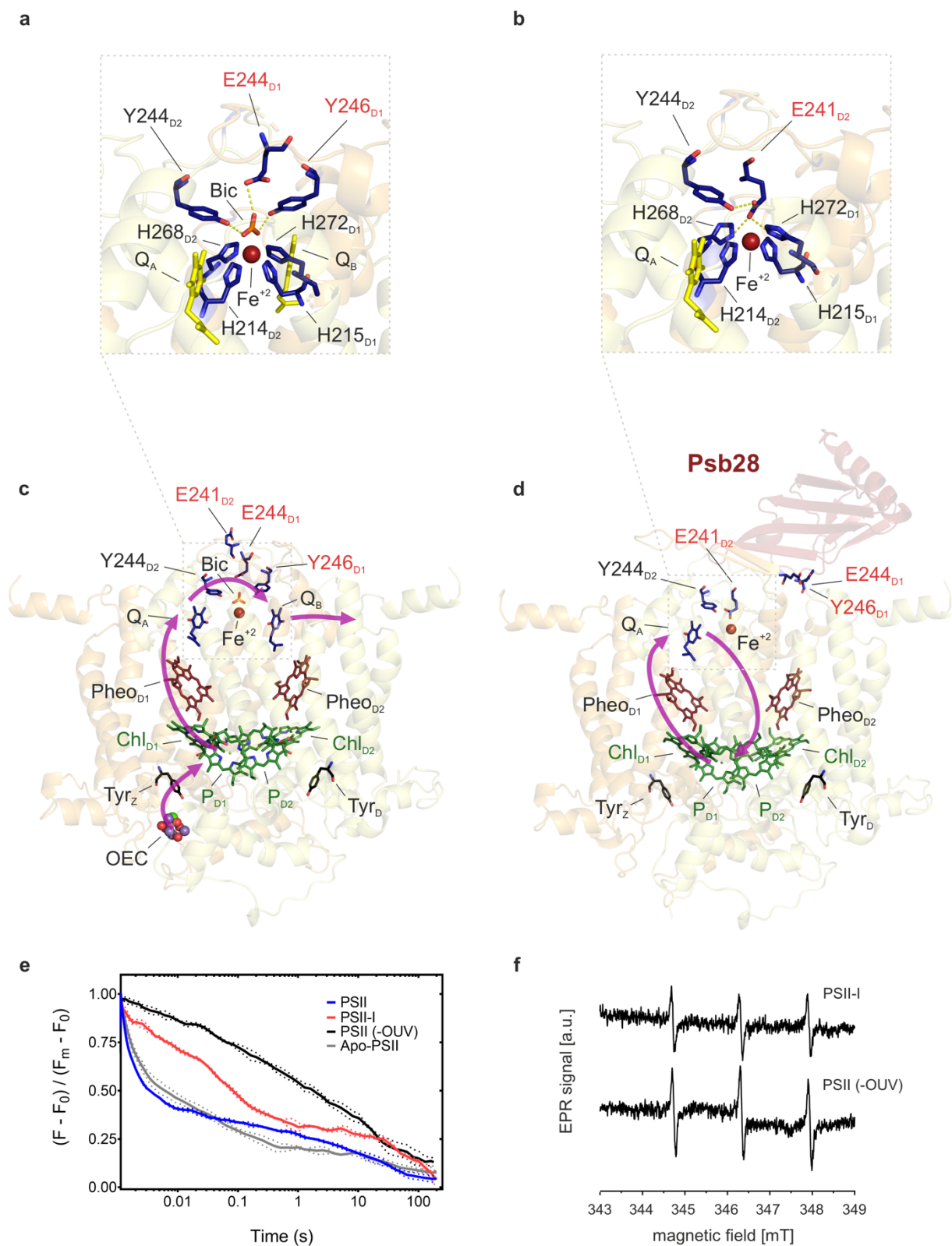


Fig. 5: Binding of Psb28 displaces bicarbonate as a ligand of the non-heme iron and protects PSII from damage. **a**, The electron transfer from PQ_A to PQ_B is coordinated by the non-heme iron (Fe^{2+}), with the binding of bicarbonate (Bic) serving as a regulatory mechanism⁵⁷ in mature PSII (PDB-ID 3WU2). **b**, Binding of Psb28 to the PSII-I assembly intermediate induces a conformational change in the cytoplasmic D2 D-E loop, where the side

chain of Glu241 replaces bicarbonate as a ligand of the non-heme iron. The respective fits of the non-heme iron binding sites are shown in Fig. S5E and F. A similar coordination is also found in non-oxygenic bacterial reaction centers⁶⁹ (Fig. S6C). **c**, Electron transfer (purple arrows) in mature PSII. Light-induced charge separation at the reaction center chlorophylls (P_{D1} , P_{D2} , Chl_{D1} , Chl_{D2}) leads to electron transfer via pheophytin ($Pheo_{D1}$) and plastoquinone A (Q_A) towards Q_B . The electron gap at the reaction center is filled by the oxygen evolving complex (OEC). **d**, Reoxidation of Q_A^- by direct and safe charge recombination is favored in the PSII assembly intermediate, as indicated by the purple arrows. **e**, Flash-induced fluorescence decay of PSII. Blue lines represent active PSII and red lines correspond to PSII-I. Black and grey lines represent PSII control samples without a functional OEC (Apo-PSII: hydroxylamine treated PSII; PSII (-OUV): extrinsic proteins removed). Dotted corridors depict SD ($n = 3$). **f**, The protective role of Psb28 binding was further confirmed by EPR spectroscopy using the spin probe TEMPd, which is specific for 1O_2 , the major reactive oxygen species in PSII generated by triplet chlorophyll (3P).

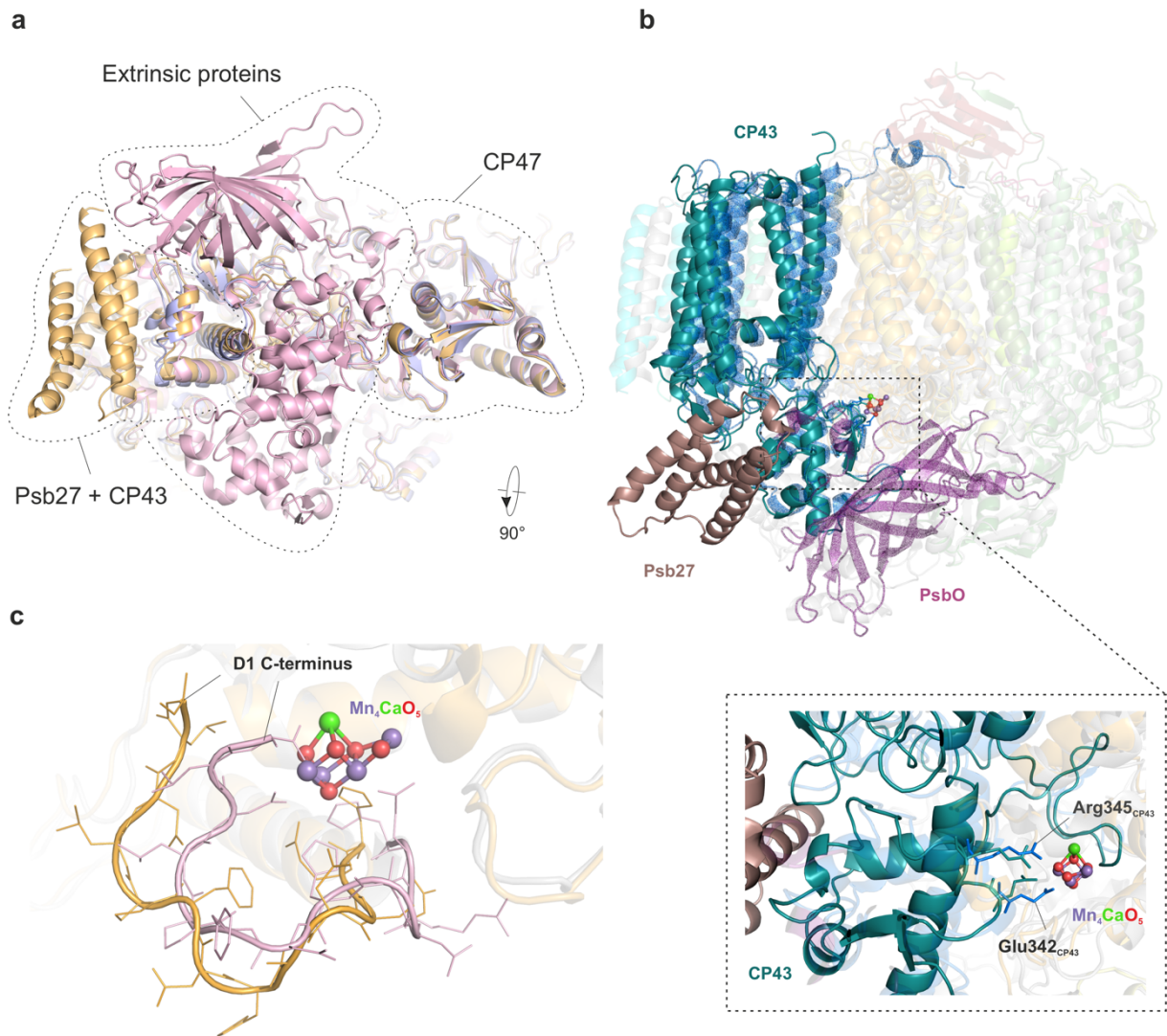


Fig. 6: The role of Psb27 in Mn_4CaO_5 cluster assembly. **a**, Bottom view of the luminal PSII surface for PSII-I (orange), the PSII-M control (light blue) and mature monomeric PSII (PDB-ID 3KZI) (light red). **b**, Side view of CP43 (teal) and Psb27 (brown) in PSII-I, as well as of CP43 (blue) and PsbO (purple) in mature monomeric PSII (PDB-ID 3KZI). Dashed box: CP43 E loop with residues Arg345 and Glu342 (shown as sticks), which form the second coordination sphere of the Mn_4CaO_5 cluster. We changed the numbering of CP43 residues due to a corrected N-terminal sequence (www.UniProt.org). The residues correspond to Arg357 and Glu354 in previous publications. The high-resolution structure of the Mn_4CaO_5 cluster is taken from Umena et al. 2011 (PDB-ID 3WU2). **c**, Position of the D1 C-terminus in PSII-I (orange) and mature monomeric PSII (PDB-ID 3KZI) (light red).

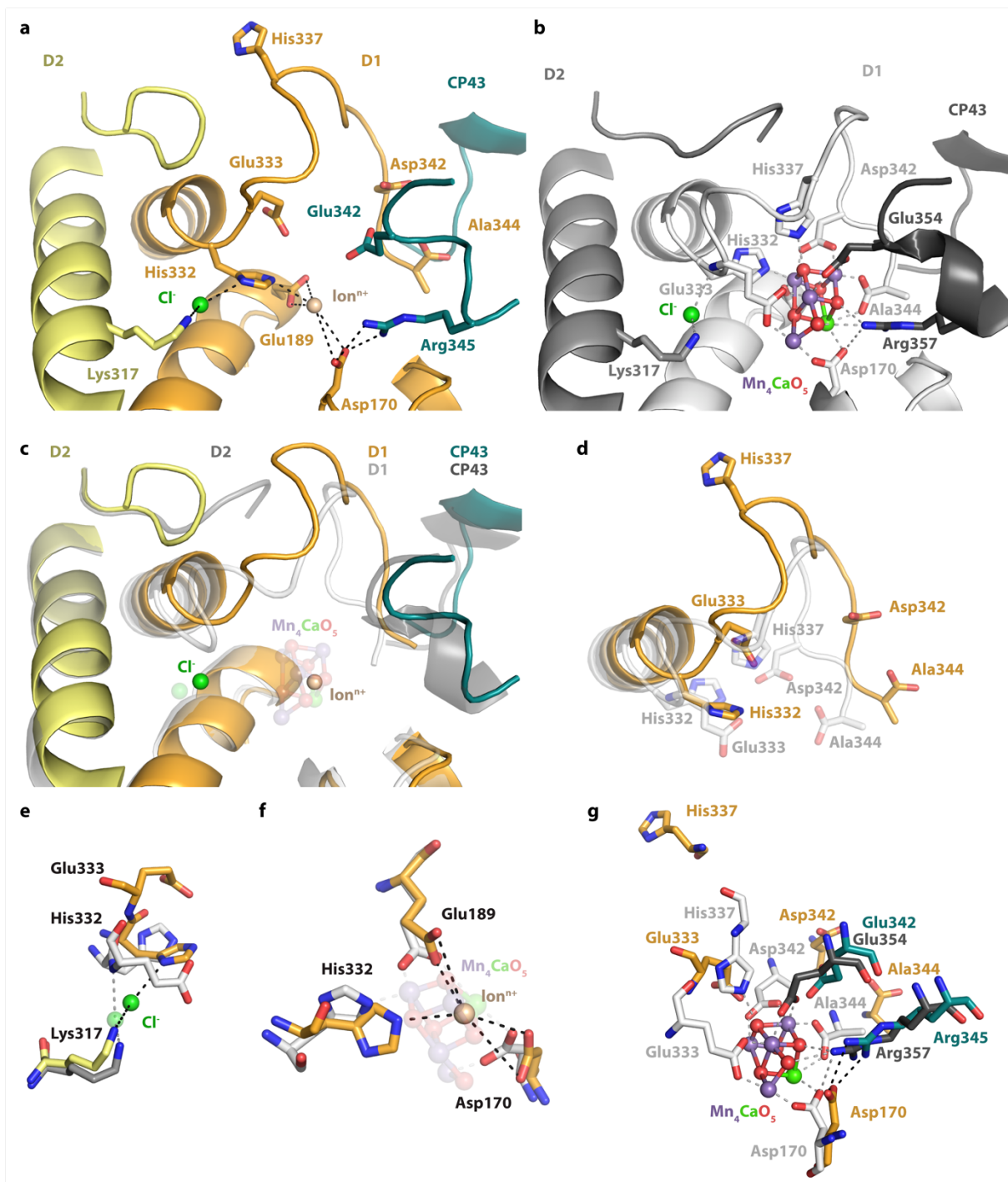


Figure 7: Conformational changes within the active site of the Mn_4CaO_5 cluster. The Mn_4CaO_5 cluster performs PSII's unique water-splitting reaction. **a**, The active site of the Mn_4CaO_5 cluster is resolved within our PSII-I structural model but is not yet oxygen-evolving. **b**, Crystal structure of the oxygen-evolving, mature PSII (PDB-ID 3WU2, resolution 1.9 Å). **c**, Overlay of both structures, illustrating significant differences in the backbone conformation of the D1 and D2 C-terminal tails. **d**, Accompanying side chain rearrangements of the D1 C-terminus. The Cl^- (**e**), Ion^{n+} (**f**) and Mn_4CaO_5 (**g**) cluster coordination partners are compared in detailed. The validation of the fit to density for the structural details shown here is provided in Figure S7.