

Hypothesis

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Hypothesis

Hypothesis: Oxidative Modification of the D1 Residue ³³²H During Photoinhibition Drives Photosystem II Turnover

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Abstract

Photosystem II (PS II) is a water-plastoquinone oxidoreductase. This photosystem has a short ½ life (<1hr) which is driven by repair of oxidative damage to the D1 protein. Reactive oxygen species (ROS) produced by the photosystem under stress conditions oxidatively modify key residues which trigger the sequential disassembly of the photosystem. We hypothesize that the principal target for ROS damage of D1 is ³³²His. This residue is a ligand to Mn1 of the Mn₄CaO₅ cluster and is the first residue exhibiting oxidative modification during photoinhibition. Recently, cryo-EM studies indicate that PS II monomers containing oxidatively modified ³³²His lose their manganese clusters and the extrinsic proteins associated with the photosystem, both hallmarks of the early stages of PS II turnover and repair.

Keywords: Photosystem II; Photoinhibition; ROS; D1:³³²His

Introduction

PS II is a water-plastoquinone oxidoreductase and is the source of virtually all O₂ in the biosphere. Functionally, excitation energy from the light-harvesting apparatus (phycobilisomes in cyanobacteria and light-harvesting chlorophyll proteins in green plants and algae) is transferred to the reaction center of the photosystem. Initial charge separation occurs between Chl_{D1} and Pheo_{D1} which yields Chl_{D1}⁺Pheo_{D1}⁻ [1]. This charge separation is stabilized by electron transfer first to Q_A and then to Q_B. The accumulation of two electrons on Q_B leads to protonation and its subsequent release as plastoquinol. Chl_{D1}⁺ is reduced by Y_Z, the residue D1:¹⁶¹Y, yielding Y_Z^{*} with the release of a proton. The subsequent reduction of Y_Z^{*} by the Mn₄CaO₅ cluster occurs via proton-coupled electron transfer and leads to the accumulation of an oxidizing equivalent at the metal cluster. The accumulation of four oxidizing equivalents, in a manner consistent with the Kok Cycle [2], ultimately leads to the oxidation of two water molecules and the release of dioxygen [3,4]. The photosystem is highly susceptible to photoinhibition and is continuously being repaired. It has been estimated that photoinhibition can reduce biomass yield by as much as 30% in some plant species [5]. In this communication we propose that oxidative modification of D1:³³²His is the principal residue damaged during photoinhibition and that this triggers the PS II repair cascade (Figure 1).

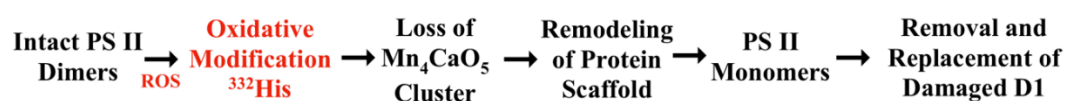


Figure 1. The Hypothesis. Intact PS II dimers are subject to photodamage by ROS which oxidatively modify D1:³³²His (shown in red), leading to the loss of the Mn₄CaO₅ cluster. This leads to remodeling of the protein scaffold which includes loss of the interaction of ³⁴⁴Ala with the metal cluster, increased structural flexibility of

the C-terminus of D1, structural reorientation of D2, CP43 and CP47 and loss of the extrinsic proteins (PsbO, PsbU and PsbV). Subsequently, the PS II dimers monomerize and the ROS-damaged monomers dissociate, yielding a CP43 repair module and a damaged CP43-less reaction center module along with their suites of accessory proteins. Subsequently, the oxidatively modified D1 protein is removed from the damaged CP43-less reaction center module and replaced by a nascent D1 subunit.

Reactive Oxygen Species (ROS) and Photoinhibition

Excitation energy transfer and electron transport within PS II are unavoidably associated with production of ROS when the absorption of light by the chlorophyll antenna exceeds the capacity for energy utilization or when the Mn_4CaO_5 cluster is damaged. PS II appears particularly susceptible to damage by ROS. A variety of different ROS species have been proposed to oxidatively damage PS II components, including $^1\text{O}_2$, $\text{O}_2^{\cdot-}$, H_2O_2 (and cofactor-bound peroxides) and HO^{\cdot} ; with a variety of mechanisms being proposed for their possible production both on the oxidizing- and reducing-side of the photosystem (22,23). ROS can oxidatively damage proteins, lipids and DNA [6]. PS II photoinhibition occurs when ROS oxidatively modify PS II component proteins, including D1, D2, CP47, and CP43. Many studies support a two-step model of PSII photoinhibition in which the earliest detectable photodamage occurs at the oxygen-evolving Mn_4CaO_5 cluster, followed by secondary reaction center damage occurring when the impaired oxygen-evolving complex can no longer efficiently reduce P_{680}^+ [7–9]. This model is supported by the observation that Mn is released during the early stages of photoinhibition, by experiments showing that visible light can directly damage the oxygen-evolving complex prior to detectable reaction-center damage [10], and the observation that the earliest detectable oxidative modification events occur in the vicinity of the Mn_4CaO_5 cluster of the D1 protein. Subsequent oxidative damage occurs in the vicinity of Pheo_{D1}, Q_A, non-heme iron, and Q_B [11,12].

PS II Repair

Photoinhibitory damage to PS II by ROS occurs at all light levels [13,14], necessitating that the photosystem be continuously repaired [15,16]. Briefly, during the repair process in cyanobacteria, functional dimeric PS II becomes oxidatively damaged during photoinhibition, leading to loss of the extrinsic proteins and subsequent monomerization of the complex. Subsequently, the damaged PS II core at least partially disassembles, yielding a CP43 repair module and a damaged CP43-less reaction center module, both of which are likely associated with suites of repair-specific proteins. These would include proteins that stabilize these dissociated sub-complexes and facilitate their interaction with other repair components. The damaged D1 protein is degraded by the FtsH and Deg proteases and is subsequently replaced by co-translational insertion of a nascent D1 chain. In *Synechocystis*, membrane integration of D1 depends on the Oxa1/Alb3/YidC homolog Slr1471 [17], while YCF48/HCF136 stabilizes newly synthesized pD1/iD1 [18] in supporting efficient PSII assembly and repair. Concomitantly, D1 chls are probably inserted as the nascent D1 elongates and associates with D2 within a repaired CP43-less reaction center module. C-terminal processing of D1 (and assembly of the $\text{Mn}_4\text{O}_5\text{Ca}$ cluster) may occur prior to re-association of the undamaged CP43-less reaction center complex with CP43, yielding PS II monomers. Subsequently, dimerization of the PS II monomers and reassociation of the extrinsic proteins with the dimeric complex and re-association with the PS II antennae completes the repair cycle. In higher plants and cyanobacteria, these repair processes are thought to be quite similar, although there are differences with respect to the specific accessory proteins functioning in both systems. So the question is: how are these events triggered? What subset of oxidative modifications within the photosystem are necessary and sufficient to induce this repair cascade?

Earlier, we have hypothesized that amino acid residues in the vicinity of the sites of ROS production would be particularly susceptible to ROS modification [19,20]. Consequently, the identification of such oxidatively modified residues should serve to identify the site(s) of ROS generation within PS II and, perhaps, assist in the identification of residues whose modification elicit PS II turnover. The PS II cofactors required for water splitting are primarily associated with the D1

protein and this component is the primary site for photodamage in PS II [21]. Due to this damage, D1 is replaced rapidly (a $t_{1/2}$ of <1 hr in *Synechocystis* [22]) during repair, which is much faster than observed for other PS II core components (D2, 3.3 hrs., CP43, 6.5 hrs, CP47, 11.5 hrs [22]). Modules containing these other PS II components have been demonstrated to be reused during the repair process [13,23]. **Consequently, this necessitates that ROS targeted residues in D1 are the primary trigger for initiating the repair cascade, while the other, more stable PS II core components serve as a scaffold for reassembly of the repaired photosystem.** If the initial triggering oxidative modification(s) resided on other PS II core proteins, removal and replacement of damaged D1 would lead to a newly repaired PS II complex already containing the triggering oxidative modification (due to reuse of the modules). This would result in a futile cycle of D1 degradation and replacement.

Modification of D1 by ROS

So, which residues of D1 are oxidatively modified? In early work, Sharma et al. [24] identified a number of D1 peptides which exhibited oxidative modifications in a higher plant PS II core preparation isolated from pea. Because of instrumental limitations in 1997 the individual residues modified were not identified. These oxidized peptides included ^{130}E - ^{136}R (+16 amu), which lies in the vicinity of Phe_{OD1} (7-16 Å), and oxidized peptides in the vicinity of the Mn₄CaO₅ cluster (4-24 Å); ^{184}Ile - ^{199}Hse (+48 amu), ^{313}Val - ^{323}Arg (+32 amu), and ^{332}His - ^{344}Ala (+32 amu). We confirmed a number of these observations, identifying several ¹natively oxidized amino acid residues (D1: ^{130}E , ^{133}L and ^{135}F) present in the D1: ^{130}E - ^{136}R peptide [20] and in the domain ^{313}Val - ^{344}Ala (^{316}T , ^{317}W , ^{319}D , ^{328}M , ^{331}M and ^{333}E , [12]). Additionally, other natively oxidized residues (D1: ^{239}F , ^{241}Q , ^{242}E) were in close proximity to the non-heme iron [20]. Barry and coworkers identified an oxidatively modified tryptophan residue, D1: ^{317}W , which appears to be a target for oxidizing-side ROS modification [25]. Kato et al. [26] identified D1: ^{317}W and D1: ^{14}W as accumulating in *Chlamydomonas* mutants lacking the FtsH protease which degrades damaged D1, speculating that D1: ^{14}W may be involved in triggering D1 degradation. This latter residue has not been identified in experiments mapping oxidative modifications in response to light treatments in either cyanobacteria or higher plants [11,12]. These studies indicate that residues in the vicinity of the Mn₄CaO₅ cluster, Phe_{OD1}, and the non-heme iron, are natively oxidized; however, the relative importance of these sites during the photoinhibition time-course was not determined. It should be noted that several studies [20,25] were performed on a standard PS II preparation (BBYs, [27]) isolated from field grown spinach which could arguably be challenged by a variety of environmental insults prior to harvesting (high light intensity, high temperatures, drought, wound damage during harvesting, etc.), all of which could elicit ROS production and protein damage. Consequently, while the accumulation of oxidative modifications at specific residues which are adjacent to various PS II cofactors suggest possible sites of ROS production, these studies do not demonstrate any direct correlation to serious photodamage which would elicit the repair cascade, nor do they necessarily address the temporal order that these oxidative modifications were produced.

More recently two studies addressed these limitations [11,12]. In *Synechocystis* 6803, Weisz et al. [11] examined the differential accumulation of oxidized residues occurring either in the absence or presence of illumination. They identified numerous oxidative modifications in the D1, D2 and CP43 proteins, some of which were present only in illuminated samples ([11], Supplementary Information, Table S2). The D1 residues which were modified only during illumination were ^{235}Y (near Q_A), ^{249}V , and ^{257}R (near Q_B). Additionally, two residues, ^{332}H and ^{333}E , which are in close proximity to the Mn₄O₅Ca cluster were oxidatively modified. These results indicated that a relatively long-term exposure (6 hr) to modest light intensities (50 $\mu\text{moles photons/m}^2/\text{s}^{-1}$) elicited significant oxidative modification of the proteins examined.

In our laboratory we examined the time-course of the appearance of oxidatively modified residues during photoinhibition [12]. In these studies, prior to the isolation of PS II membranes, the

¹Oxidized residues which are present in PS II even in the absence of experimental photoinhibitory irradiation. These accumulate due to constitutive photodamage occurring under growth light conditions.

spinach leaves were incubated overnight under dim light conditions ($<5 \mu\text{moles photons/m}^2/\text{s}^{-1}$) to facilitate repair of the photosystem. This pre-treatment markedly decreased the abundance of the natively oxidized residues that we had observed in earlier studies [19,20]. Illumination at $1,400 \mu\text{moles photons/m}^2/\text{s}^{-1}$ was carried out for 30 min, with mass spectrometry analysis performed on samples collected after 0, 15 and 30 min of illumination. Photoinhibition, as monitored by oxygen evolution, exhibited a $t_{1/2}$ of ~ 20 min. Only oxidized residues in the vicinity of the manganese cluster were observed at 0 and 15 min, with extensive modification of residues in the vicinity of Phe_{OD1}, Q_A, the non-heme iron and Q_B appearing only after 30 min of illumination ([12], Supplementary Information, Table S1). At 0 min the native oxidative modification of the D1 residues ³¹⁶T, ³¹⁷W, ³¹⁹D, ³²⁸M, ³³¹M and ³³³E were observed. After 15 min of illumination only a single additional residue was observed to be oxidatively modified, D1:³³²His.

Recently, a critically important cryo-EM study by Zhao et al. [28] has provided strong evidence that D1:³³²His is directly involved in PS II remodeling in response to photoinhibition. In the cyanobacterium *Thermosynechococcus vestitus* grown under moderate light conditions ($60 \mu\text{moles photons/m}^2/\text{s}^{-1}$), three types of PS II dimers were isolated: fully active (PDB: 9G6H), containing two active monomers; semi-active (PDB: 9G6G), containing one active and one inactive monomer; and fully inactive (PDB: 9G6F), containing two inactive monomers. These inactive monomers were likely the result of constitutive photodamage occurring under standard growth light conditions [14,29]. The inactive monomers exhibited loss of the Mn₄CaO₅ cluster, increased flexibility of the C-terminus of D1 (such that it is no longer resolved in cryo-EM maps), structural alterations in CP43 and CP47, and loss of PsbO, PsbU and PsbV. Monomerization of the inactive dimers was not observed. Importantly, all inactive monomers also exhibited oxidative modification of D1:³³²His (+16 amu, Figure 1). The authors speculated that oxidative damage to D1:³³²His was an early event in photoinactivation of the photosystem, echoing proposals in earlier studies [11,12]. Importantly, this was the only oxidized residue identifiable in their cryo-EM maps. Mass spectrometry analysis indicated that D1:³²⁸M and ³³¹M are also oxidized (+16 amu), however the cryo-EM maps associated with these residues do not show any oxidative modification in the inactive monomers, indicating low occupancy of any of these oxidized species in their cryo-EM structures. Earlier, both D1:³²⁸M and D1:³³⁸M had been identified as being natively oxidized [11,12]. The authors hypothesized that the oxidative modification to D1:³³²His is the addition of a hydroxyl group at the ring N_{δ1} position, forming a ²N_{δ1}-hydroxy-L-histidine residue, a highly unusual, and if correct, novel oxidative protein modification. Better documented oxidized histidine ring modifications include 2-oxo-histidine [30] and possibly 2-hydroxy-histidine [31]. The latter has also been proposed as a possible short-lived intermediate or transient product in histidine oxidation pathways, but is not well established as a stable protein modification. It should be pointed out that the authors did not report examining rotomers of 2-oxo-histidine (or 2-hydroxy histidine) for possible fit to their observed cryo-EM maps.

While these and earlier results are highly suggestive, an important question persists: why would oxidative modification of ³³²His lead to disassembly of PS II? The core observation in this regard is that ³³²His is an inner shell ligand to the Mn₄CaO₅ cluster [32], with the histidyl N_{ε2} nitrogen coordinating Mn1 of the cluster. Oxidative modification of this residue would, with high probability, modify this interaction. For instance, if the modification is indeed N_{δ1}-hydroxy-L-histidine, it is estimated that the approximate pK of the N_{ε2} nitrogen would shift from ~ 6.9 to ~ 4.9 (pK estimates using the MolGpK online resource [33]). Earlier, mutagenesis studies replacing ³³²His with the acidic residues Glu (pK ~ 4.5) and Asp (pK ~ 4.1) indicated that these substitutions had a dramatic effect on the structure/function of the active site [34]. For instance, replacement of ³³²His with ³³²Glu led to profoundly altered electronic and geometric structure of the manganese cluster, with the authors stating, "The substantial structural changes provide an explanation not only for the altered properties of the D1-H332E mutant but also the importance of the histidine ligand for proper assembly of the Mn₄Ca cluster

²In the SI for this paper, the authors identify the modified residue as 3-hydroxy-L-histidine, in this communication we prefer the designation N_{δ1}-hydroxy-L-histidine.

[35].” It should be noted that if the oxidative modification were to 2-oxo-histidine, similar defects might be expected. The pKs of the N_{δ1} and N_{ε2} nitrogens are estimated to be 12.3 and 12.8, respectively [15]. The protonation of these nitrogens at physiological pH would effectively preclude their coordination to Mn1 [34]. Although the 2-keto oxygen of 2-oxo-histidine could, in principle, interact with Mn1, it would not be expected to substitute effectively for the native histidine imidazole nitrogen ligand. Mutants which substitute ³³²His with ³³²Gln or ³³²Asn, both of which contain amide carbonyl substituents, exhibit very low oxygen evolution rates, assemble fewer PS II reaction centers and exhibit very rapid fluorescence relaxation kinetics, all of which are consistent with a defective Mn₄CaO₅ cluster [34]. Importantly, in active PS II monomers, the ³³²His N_{δ1} atom lies within van der Waals contact with the backbone carbonyl oxygen of D1-³²⁹Glu (~2.7 Å). This close native contact leaves little space for addition of an N_{δ1}-hydroxy substituent. Formation of N_{δ1}-hydroxy-L-histidine would therefore be expected to generate a severe steric conflict with the D1-³²⁹Glu backbone carbonyl unless relieved by local rearrangement. Comparison of the active and inactive monomers [28] suggests that such relief could involve an approximately 180° rotation of the ³³²His imidazole ring about the C_β-C_γ bond, which corresponds to a χ₂ rotamer change. This rotation would increase the N_{ε2}-Mn1 distance from ~2.2 Å to ~2.8 Å and introduce an angular distortion of >45°, changes that are consistent with severe weakening or functional loss of the normal Mn-imidazole coordination interaction. A qualitative estimate based on bond-valence distance dependence and simple angular-overlap considerations suggests that the effective coordination interaction could be reduced to roughly ~10% of that in the active monomer [36,37].

The inactive monomers observed by Zhao et al. [28] were isolated from *T. vestitis* cells, grown under standard light conditions which had apparently lost their Mn₄CaO₅ clusters due to constitutive photodamage. Earlier cryo-EM studies in *Synechocystis* indicated that removal of the Mn₄CaO₅ cluster by chemical treatment [38] also led to increased flexibility of the C-terminus of D1 (such that it was no longer resolved in cryo-EM maps), structural alterations of CP43 and CP47, and loss of PsbO, PsbU and PsbV. In this case, dimers were not observed, only inactive monomers. We speculate that in both systems the Mn2 ligand ³⁴⁴Ala (via its C-terminal carboxylate) normally locks the D1 C-terminus in position in active monomers. Removal of the Mn₄O₅Ca either during photoinactivation as a result of ³³²His oxidation or after chemical treatment disrupts this association and allows for much greater mobility of the D1 C-terminus, precluding its identification in these cryo-EM maps [28,38]. This enhanced mobility may predispose the photosystem for the structural modifications of CP43 and CP47 and loss of the extrinsic subunits and, ultimately, monomerization of the PS II dimers.

Testing the Hypothesis

The studies outlined above provide a mechanistic framework which supports the hypothesis that oxidative modification of D1:³³²His triggers the disassembly of photodamaged PS II (Figure 3). Hypotheses, however, must necessarily be tested. Additional cryo-EM and mass spectrometry studies performed on cyanobacterial and higher plant samples which initially are allowed to repair in the dark and then subsequently collected during a photoinhibitory time-course would be most welcomed. These studies could potentially confirm or reject the hypothesis. While cryo-EM studies capture protein structures which, by definition, are frozen in time, the damage repair process is highly dynamic and can potentially be studied using molecular dynamic simulation. Atomistic molecular dynamic simulations examining the stability of the Mn₄CaO₅ cluster in the presence of D1:³³²N_{δ1}-hydroxy-L-histidine or D1:³³²2-oxo-histidine could provide direct evidence that oxidation of this residue results in lower Mn₄CaO₅ cluster stability (simulated on ns-μs timescales). Additionally, course-grained molecular dynamic simulations would allow the examination of the flexibility of the D1 C-terminus, possible CP43/CP47 remodeling, and potentially, the release of the extrinsic proteins (simulated on 1-10 μs timescales), in addition to possible dimer monomerization. These molecular dynamic studies are ongoing.

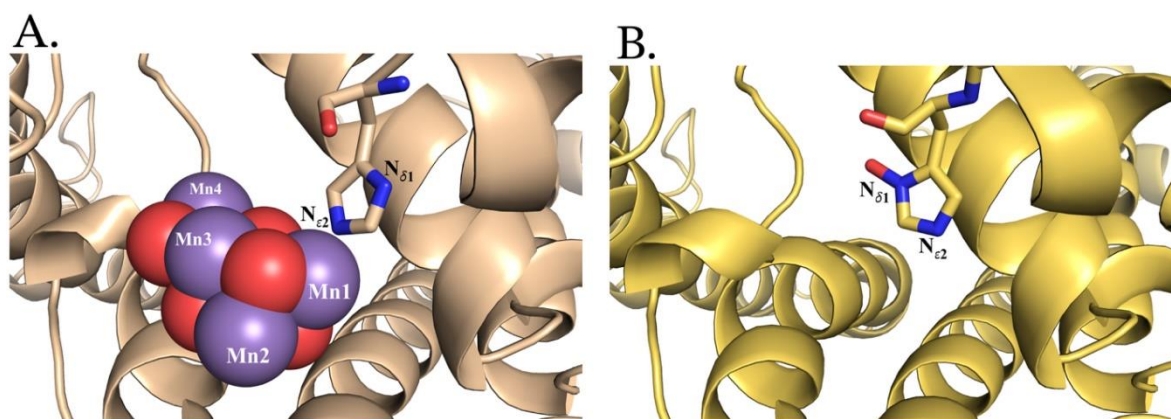


Figure 2. Orientation of ^{332}His in the presence and absence of the Mn_4CaO_5 cluster (PDB:9G6G, [28]) A. Active monomer, D1 is shown in light brown, the Mn_4CaO_5 cluster is shown as spheres and color coded by atom type with the manganese atoms (Mn1-Mn4) labeled. ^{332}His is shown as sticks and color-coded by atom type with the imidazole nitrogens shown in blue. $\text{N}_{\delta 1}$ and $\text{N}_{\epsilon 2}$ are labeled. B. Inactive monomer, D1 is shown in yellow, $^{332}\text{N}_{\delta 1}$ -hydroxy histidine is shown as sticks and color-coded by atom type, with the imidazole nitrogens shown in blue, $\text{N}_{\delta 1}$ and $\text{N}_{\epsilon 2}$ are labeled. Some regions of D1 have been omitted for clarity.

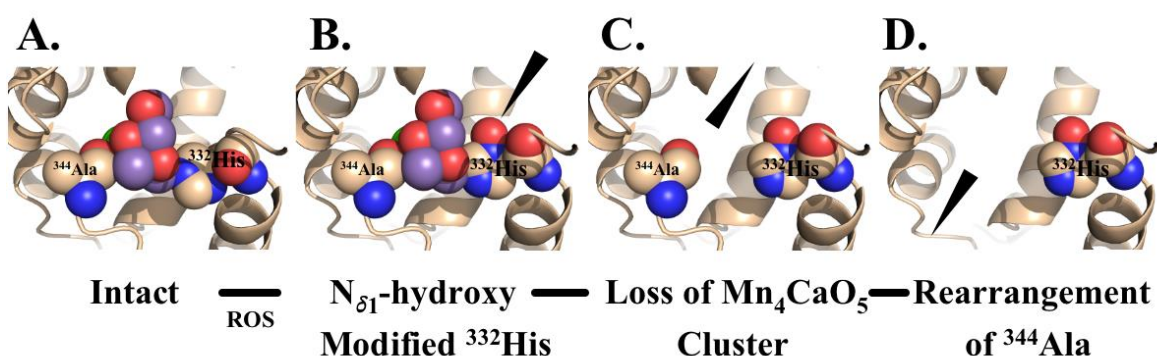


Figure 3. Proposed Sequence at the Mn_4CaO_5 Cluster Binding Site. A. Active monomer, D1 is shown in light brown, ^{332}His and ^{344}Ala and the Mn_4CaO_5 cluster are shown as spheres and color-coded by atom type. B. After exposure to ROS, $\text{N}_{\delta 1}$ -hydroxy ^{332}His is formed. The $\text{N}_{\delta 1}$ -hydroxy modification is highlighted by the arrow. C. Loss of effective ligation of Mn1 by the $\text{N}_{\delta 1}$ imidazole nitrogen leads to destabilization and loss of the Mn_4CaO_5 cluster, which is highlighted by the arrow. D. Loss of the Mn_4CaO_5 cluster allows increased flexibility of the D1 C-terminus, including ^{344}Ala , which precludes structure determination in cryo-EM. Sphere color code: carbon, light brown; nitrogen, blue; oxygen, red and manganese, purple.

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