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Radiolytic Mapping of Solvent-Contact Surfaces in Photosystem II of Higher Plants

EXPERIMENTAL IDENTIFICATION OF PUTATIVE WATER CHANNELS WITHIN THE PHOTOSYSTEM

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Background: Substrate water must reach the buried Mn$_4$O$_5$Ca cluster in Photosystem II.

Results: OH$^-$ produced by radiolysis modified buried amino acid residues. These were mapped onto the PS II crystal structure.

Conclusion: Two groups of oxidized residues were identified which form putative pathways to the Mn$_4$O$_5$Ca cluster.

Significance: Identification of water and oxygen channels is crucial for our understanding of Photosystem II function.

Photosystem II uses water as an enzymatic substrate. It has been hypothesized that this water is vectored to the active site for water oxidation via water channels that lead from the surface of the protein complex to the Mn$_4$O$_5$Ca metal cluster. The radiolysis of water by synchrotron radiation produces amino acid residue-modifying OH$^-$ and is a powerful technique to identify regions of proteins that are in contact with water. In this study, we have used this technique to oxidatively modify buried amino acid residues in higher plant Photosystem II membranes. Fourier transform ion cyclotron resonance mass spectrometry was used to identify these oxidized amino acid residues that were located in several core Photosystem II subunits (D1, D2, CP43, and CP47). While, as expected, the majority of the identified oxidized residues (≈75%) are located on the solvent-exposed surface of the complex, a number of buried residues on these proteins were also modified. These residues form groups which appear to lead from the surface of the complex to the Mn$_4$O$_5$Ca cluster. These residues may be in contact with putative water channels in the photosystem. These results are discussed within the context of a number of largely computational studies that have identified putative water channels in Photosystem II.

These are CP47, CP43, D1, D2, and the α and β subunits of cytochrome b$_{559}$. Deletion of any one of these subunits uniformly results in the loss of PS II function and assembly (1, 2). In higher plants, three extrinsic proteins, PsbO, PsbP, and PsbQ also are required for maximal rates of O$_2$ evolution under physiological inorganic cofactor concentrations (3). The PsbO protein appears to play a central role in the stabilization of the manganese cluster (4), is essential for efficient and stable O$_2$ evolution and is required, along with PsbP, for photoautotrophic growth and PS II assembly in higher plants propagated under normal growth conditions (5–8). Under low light growth conditions the PsbQ component also is required for photoautotrophy (8, 9). Over the past decade, increasingly higher resolution crystal structures of cyanobacterial PS II have significantly enhanced our understanding of the molecular organization of the constituent polypeptides of the photosystem and the active site for oxygen evolution, the Mn$_4$O$_5$Ca cluster (10–14). A high resolution, 1.9 Å crystal structure of cyanobacterial PS II has recently become available (15). Unfortunately, no crystal structures for higher plant PS II have been presented. While there are differences between the higher plant and the cyanobacterial photosystems, particularly with respect to the identity and organization of the extrinsic proteins (3), the amino acid sequences of the intrinsic components (D1, D2, CP43, and CP47) are very similar (>85% similarity (16)). Consequently, one would expect that the structural and functional organization of these proteins within PS II would be highly homologous between higher plants and cyanobacteria.

The use of the synchrotron radiolysis of water to produce OH$^-$ capable of oxidatively modifying amino acid residues in contact with water is an emerging and useful technique in structural biology (17, 18). Recently, this method has been used to identify buried water molecules which function in a channel mediating the activation of a membrane protein, the G-protein-coupled receptor, rhodopsin (19). It has also been used to identify the surface residues of proteins that are exposed to the bulk aqueous solvent (20, 21).

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Because the active site for water oxidation, the Mn₄CaO₅ cluster, is buried within the structure of the photosystem, substrate water must transit from the bulk solvent to the active site through water channels. Several largely computational studies have sought to identify these pathways. Murray and Barber (22) used the CAVER Program (23) to examine the 3.5 Å crystal structure of Thermosynechococcus elongatus (12). Gabdulkhayev et al. (24) used noble gas and dimethyl sulfoxide co-crystallization studies in combination with CAVER to examine the 2.9 Å structure of T. elongatus (14), and Ho and Stoying calculated solvent-accessible surfaces for the 3.0 Å T. elongatus structure of Loll et al. (13). Molecular dynamic simulations have also been used to probe for water channels within the photosystem (25, 26), the latter study being performed on the recent high resolution PS II structure (15).

We hypothesized that buried amino acid residues in contact with such putative water channels in the interior of PS II would be significantly more susceptible to oxidative amino acid modification during the synchrotron radiolysis of water than buried residues not exposed to water. Consequently, the identification of such oxidatively modified residues in the interior of PS II should serve to complement and extend the largely computational studies mentioned above.

EXPERIMENTAL PROCEDURES

PS II membranes were isolated from market spinach by the method of Berthold et al. as modified by Ghanotakis and Babcock (27, 28). After isolation, the PS II membranes were suspended at 2 mg chlorophyll/ml in 50 mM Mes-NaOH, pH 6.0, 300 mM sucrose, 15 mM NaCl buffer, and frozen at −80 °C until use. In these experiments, we have used the XLRM2 beamline at The J. Bennett Johnston, Sr. Center for Advanced Microstructures & Devices (CAMD) synchrotron. This beamline provides attenuated unfocused radiation which has been passed through a 100 μm beryllium and 25 μm aluminum filters to remove the low energy components. The maximum flux is near 4 keV. The photon flux absorbed by the sample was 1.58 × 10⁶ photons/sec/μm². Consequently, at our longest exposure time of 16 s, the sample absorbed 2.5 × 10⁷ photons/μm², which was equivalent to an absorbed dose of about 2.43 × 10⁴ Gray. The end-station was modified to accommodate a Plexiglas chamber with machined channels (3 mm × 60 mm × 1 mm) to contain the PS II membrane samples. The samples were covered by 75 μm Kapton foil to retain the sample (~180 μl). The individual channels were positioned in the beam by remote control and exposed for various lengths of time (0, 4, 8, 16 s) at room temperature. After exposure, the samples were immediately removed from the chamber and held on ice until being stored at −80 °C until further analysis.

The proteins in the samples were resolved on a 12.5–20% acrylamide gradient by LiDS-PAGE using a non-oxidizing gel system (29, 30). This was required, as standard PAGE is known to introduce numerous protein oxidation artifacts (29, 31). In the non-oxidizing system the gels are polymerized with riboflavin (in the presence of diphenylidonium chloride + toluene sulfinate) followed by exposure to UV light. The upper reservoir contained thioglycolate. Preliminary experiments indicated that proteins resolved in this system exhibited much lower levels of artifactual protein oxidation than proteins resolved under standard LiDS-PAGE conditions (see Ref. 30, supplemental Fig. S1). After electrophoresis, the gels were stained with Coomasie Blue, destained, and protein bands containing CP47, CP43, D1, and D2 were excised. These were then processed for trypsin digestion using standard protocols. In some cases, the tryptic peptides were processed using a C18 ZipTip® prior to mass analysis.

Reversed-phase chromatography was performed as described previously (30) using a Finnigan Surveyor MS pump and a Finnigan Micro AS autosampler. A Waters X-Bridge C18 3.5 μm 2.1 × 100 mm column was used for the reversed phase separation of the tryptic peptides. It was operated at a flow rate of 200 μl/min. The mobile phases consisted of a 95:5 water:acetonitrile with 0.1% formic acid aqueous phase and a 95:5 acetonitrile:water with 0.1% formic acid organic phase. The gradient was as follows: The organic phase composition was 10% for the first 5 min, ramped to 20% for the next 10 min, ramped to 50% for the next 25 min, ramped to 80% in the next 35 min, held at 80% for 10 min followed by a quick ramp to 10% in 5 min, and a 10 min hold to equilibrate the column.

Mass spectrometry was performed on a Thermo Scientific LTQ-FT™, a hybrid instrument consisting of a linear ion trap and an FT-ICR mass spectrometer. The experiments used the standard electrospray source operating with a source voltage of 5 keV and a capillary temperature of 275 °C. Sheath and auxiliary gas flows were 18 and 5 respectively (both Thermo Scientific instrument settings). A typical scan sequence involved a positive ion FT-ICR scan at 100 K resolution (100 K at m/z 400). During the FT-ICR acquisition, six MS/MS scans were acquired by the linear ion trap determining the parent ions from the six most abundant ions observed from a preview of the FT-ICR scan. The CID scans were acquired with an isolation width of 2 and a normalized collision energy of 35 (both Thermo Scientific instrument settings). After acquiring the tandem mass spectra twice, the ion was placed into an exclusion list for 30 s. Charge state screening was enabled with monoisotopic precursor selection.

In this study, two biological replicate experiments were performed. Identification and analysis of peptides containing oxidative modifications were performed using the MassMatrix Program ver. 1.3.1 (32, 33). The modification files were adjusted to include all of the possible oxidative modifications described in references (17, 34). A FASTA library containing all of the spinach PS II subunit protein sequences was searched. Additionally, a decoy library that contained these same proteins but with reversed amino acid sequences was examined. No hits to the decoy library were observed. For the determination of the quality of the peptide calls within MassMatrix, max(pp1, pp2) was ≥ 8.5 and pp_Tag ≥ 5.0 (32, 33). These parameters yield a p value of ≤ 0.00001; only oxidized peptides which exhibited this extremely low p value were considered for the identification of peptides containing oxidized residues. The quality of the mass spectra observed at this high stringency is illustrated in Fig. 1 of references (30) and (35). In this context it should be noted that in MASCOT (software from Matrix Sciences) searches (36), peptide identifications are typically performed with p values ≤ 0.05. The direct consequence of our using such low p values is
that in all cases high quality mass spectra were observed with nearly complete γ- and b-ion series for the oxidatively modified peptides. Given the high quality of the data used in this study, the union of the replicate data sets was examined. The identified oxidatively modified residues were mapped onto the Thermosynechococcus vulcanus PS II structure (15) of the D1, D2, CP43, and CP47 proteins using PYMOL (37).

RESULTS

As we have reported previously, even in the absence of synchrotron radiation a number of amino acid residues are found to be oxidatively modified in PS II (30, 35). In these earlier studies, seventy-three oxidatively modified amino acid residues were identified in the D1, D2, CP43, and CP47 proteins of spinach PS II at time 0, representing approximately 4% of the residues found in these four subunits. That residues are observed to be natively oxidatively modified is not surprising, since the presence of reactive oxygen species (ROS) in the cellular environment and the production of ROS by PS II itself can lead to the oxidative modification of amino acid residues within the photosystem (38–40). Several of these residues are associated with CP43 (354E, 355T, 356M, and 357R) and are buried and in close vicinity to the Mn$_4$CaO$_5$ cluster. We hypothesized that these residues are associated with an oxygen/ROS exit pathway from the photosystem (30). Additionally, we have reported that subsets of these residues that are associated with the stromal domains of the D1 and D2 proteins are in close proximity to Q$_A$ (D1 residues 237P, 239F, 241Q, 242E, and the D2 residues 237P, 239F, 241N, and 244M) and PheoD$_{D1}$ (D1 residues 130Q, 133L, and 135Y). The oxidative modification of these residues appears to indicate that both Q$_A$ and Pheo$_{D1}$ may generate ROS on the reducing side of the photosystem (35).

The oxidative modifications which were observed at the various time points are summarized in supplemental Tables S1–S4, along with the type of oxidative modifications observed and the residue location (surface, buried but in contact with cavity/channel, or buried and not in contact with an apparent cavity/channel) when mapped onto the T. vulcanus crystal structure. As noted previously (30), at time 0, the overall sequence coverage observed in this study for the examined proteins was: D1, 24%, D2, 27%, CP47, 41%, and CP43, 26%; the coverage of the residues located in the luminaly exposed extrinsic loops of these proteins, the domains of principal interest in this study, was significantly higher: D1, 35%, D2, 43%, CP47, 55% and CP43, 43%. With increasing irradiation, the sequence coverage improved, such that at 16 s irradiation the overall sequence coverage was: D1, 30%, D2, 33%, CP47, 44%, and CP43, 40% while the coverage of the residues located in the extrinsic loops of these proteins was uniformly higher: D1, 45%, D2, 51%, CP47, 60%, and CP43, 52%. This was expected since oxidative modifications yield peptides which are more hydrophilic and, consequently, more easily resolved by reversed phase chromatography. It should also be noted that not all residues observed to be modified at a particular time point are necessarily observed at all subsequent time points. For instance, 16% of the residues observed to be oxidatively modified in the proteins at 0 s irradiation are not observed at one or more subsequent time points using our stringent peptide selection criteria (i.e. $p \leq 10^{-3}$). This is due primarily to the different populations of peptides being present in the irradiated samples (see above) and that only the most abundant peptides, at any particular elution time in the reversed-phase chromatogram, are selected for fragmentation during mass spectrometry. If one lowers the selection criteria to levels typically used in MASCOT searches (i.e. $p \leq 0.05$), significantly higher proportions of the peptides are observed at other time points. Using this relaxed criteria, only 9% of the peptides initially identified at high stringency were not observed using the lower stringency criteria. It should also be noted that in no instances were hits to the decoy library observed for any of the examined proteins. In this communication, if a residue was observed to be oxidatively modified at a particular time point, it is assumed to also be modified at subsequent time points with respect to mapping onto the T. vulcanus crystal structure.

In Figs. 1–4, the accumulation of oxidized residues is presented at the various times shown. Since the D1, D2, CP47, and CP43 proteins are highly homologous between higher plants and cyanobacteria (>85% similarity), it is possible to positionally map the modified residues present on these intrinsic proteins from spinach directly to the corresponding residues in the T. vulcanus crystal structure. Of the residues which we observed to be oxidatively modified in spinach, 88% were conserved or conservatively replaced in T. vulcanus.

Fig. 1 presents an overview of the time course for the oxidative modification of proteins in the core of PS II. As expected, at all time points the vast majority ($\approx 75\%$) of the observed modifications were located on the surface of the PS II complex which is exposed to the bulk aqueous solvent. It should be noted that oxidized residues identified at the N terminus of the D1 (27T, 3A 4I, 5L, 6E, 7R, 8R, 9E, and 10S), D2 (4I, 7K, and 10T) and CP43 (4T,}
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4L, 7L, and 8R), which were observed at various time points are also probably surface-exposed; however, this could not be verified by direct comparison to the T. vulcanus structure, as these residues are not resolved. Consequently, these residues are not included in the calculation above and are not illustrated in Fig. 1. Increasing times of irradiation clearly leads to increased numbers of oxidatively modified residues, most of which are located on the surface of the complex. However, a number of buried residues are also modified. Since these are not in contact with bulk solvent water, the modifying OH− must be produced from buried water molecules located on the interior of the PS II complex. The modified residues appear to form two large groups. The first consists of D1, D2, and CP47 residues, while the second consists of CP43 residues only. Both of these groups of modified residues appear to lead from the Mn4O5Ca cluster to modified residues on the surface of the PS II complex.

Fig. 2 illustrates the time course for radiolytic modification of the modified residues which are located within a 15 Å sphere of the Mn4O5Ca metal cluster (please note that T. vulcanus numbering is used in Figs. 2–5). We had previously reported that at time 0 the residues 354E, 357T, 356M, and 357R of CP43 and residues 346R of the D2 protein are oxidatively modified. This latter residue is exposed on the surface of the complex (30). With increasing irradiation times, additional residues within this sphere become modified. After 4 s irradiation, the D2 residues 320L and 321L as well as CP43,356M of are modified. All of these residues are near the 15 Å boundary and are distant from the M4O5Ca cluster. After 8 s irradiation a number of additional residues are observed to be modified; on D1 332H, 333E, 329E, 330V, 334R, and 338M, D2:329W, CP47:384R, and CP43:395Y and 403S. After 16 s irradiation no modification of additional D2 residues was observed, however, D1:331M and the CP43 residues 349P, 320W, and 406P are oxidatively modified. In Figs. 2–4 some of the residues are not labeled because they are eclipsed by modified foreground residues. The Mn4O5Ca cluster is shown with the manganese, oxygen, and calcium illustrated as purple, red, and bright green spheres, respectively. To orient the reader, the Ca of the Mn4O5Ca cluster is labeled at the 0 s time point.
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DISCUSSION

In this communication we have used the synchrotron radiolysis of water in an attempt to experimentally identify putative water pathways within PS II. Several points must be kept in mind. First, a limitation of our study is that we do not have full mass coverage of all of the domains of the examined proteins exposed to the lumenal side of the membrane. This is, in large measure, a consequence of poor chromatographic resolution of highly hydrophobic peptides. Consequently, our catalogue of oxidatively modified residues is almost undoubtedly incomplete. Additionally, we have examined only the core subunits of PS II - D1, D2, CP43, and CP47. It is probable that residues of other proteins within the complex may participate in the formation of water channels (22, 24, 26, 41). It should also be cautioned that the lack of observed modification of an amino acid residue in no way suggests that the residue is not in contact with water. Finally, photoreduction of the manganese cluster by X-rays could lead to conformational rearrangements within the metal cluster and/or ligating amino acid environment, leading to greater exposure of residues to water and possibly their oxidative modification.

With these caveats in mind, we observe two major groups of oxidized amino acid residues. The first group contains modified amino acid residues identified on D1, D2, and CP47 (Fig. 3). This group extends from D1 residues directly associated as first sphere ligands of the Mn₄O₅Ca cluster (329H and 333E) and other nearby residues on D1 (329E, 334R, 328M, 331M, and 330V) and D2 (320L and 348R). At this point the path bifurcates with one path leading from D2:320L (D2:323E, 326R, and CP47:364S, 365G, 366F, 359M 425I, and 426F) to CP47:423K, which is exposed at the surface of the complex. Many of the residues in this path are buried and in contact with cavities and/or channels evident in the T. vulcanus crystal structure (see below). A second apparent path leads from D2:348R to the surface residues D2: 344E and 346L and CP47:383R.

The second group of oxidized residues consists only of CP43 residues (Fig. 4) and leads from the inner sphere ligand 354E and second sphere ligand 355F to the buried residues 355F and 356M and the surface-exposed 359W. At this point the group bifurcates and one branch of residues (354P, 364E, 341R, and 344E) is in direct contact with the PsbO subunit. While this branch does not appear to reach the luminal surface, it is possible that PsbO residues complete a putative pathway to the lumen. The second branch of this CP43 pathway includes the buried residues 361E, 366L, and 363G. These residues are in contact with numerous surface-exposed CP43 residues including 362R, 367E, 370R, 369L, 375L, 368P, 365W, etc. Interestingly, no residues in these regions of CP43 have been implicated in any of the proposed models for channels within the photosystem. Many of these residues, however, are in contact with non-contiguous cavities which are present in CP43 and at the CP43:PsO interface. It is possible that these residues constitute an additional novel water pathway within the PS II complex. Another possibility exists, however. As noted previously (30), the CP43 residues 354E, 355F, 355R, 356M and 356P may be associated with a putative dioxygen/ROS exit pathway. Intriguingly, many of the oxidatively modified CP43 residues identified in this communication extend directly from these four residues, which are in close proximity to the Mn₄O₅Ca cluster, to the luminal surface of PS II. It is possible that these modified residues form distal portions of a putative dioxygen/ROS egress pathway that is occupied by transient water molecules which could undergo radiolysis. In a variety of other systems the occupation of oxygen channels with water molecules has been documented (42–44). Additionally, any dioxygen remaining in a putative oxygen egress pathway would also be subject to modification during radiolysis, yielding reactive oxygen species (principally HO₂⁻ and O²⁻) capable of oxidatively modifying amino acid residues (45). These species can produce oxidative modification of amino acid residues which are indistinguishable from those produced by OH⁻. We hypothesize that the observed CP43 pathway may constitute an oxygen egress pathway leading from the Mn₄O₅Ca cluster to the luminal surface of the PS II complex. As noted above, no continuous channel connects these CP43 residues forming such a putative dioxygen exit pathway. It is possible that conformational changes occurring during S-state cycling could lead to structural alterations completing a continuous pathway from the Mn₄O₅Ca cluster to the surface of the complex (see below).

Our findings are schematically summarized in Fig. 5. Fig. 5A illustrates the oxidized residues found in the D1, D2, and CP47...
proteins while Fig. 5B shows the modified residues found in CP43. In this figure, the buried residues are highlighted in red while the surface-exposed residues are shown in blue.

Earlier computational studies have identified several putative water/oxygen/proton channels in PS II (22, 24, 41). These studies have been recently reviewed (46). Briefly, 3–5 channels were identified computationally as leading from the luminal surface water/oxygen/proton channels in PS II (22, 24, 41). These studies have provided computational methodologies which could substantially alter the overall shape and dimensions of possible water entrance pathways. A number of studies have indicated that largely undefined conformational changes do occur during S-state cycling (47–50) although the specific residues involved and the effects on putative water channels within the photosystem remain undetermined. It has been noted that even a modest conformational change involving one or a few amino acid residues could hypothetically either open or close a putative water transport (or oxygen transport) channel during S-state cycling (26).

With these caveats in mind, comparison of our results with these computational studies yields some interesting insights. First, as expected, there is no direct one-to-one correspondence between the oxidized residues which we observe and those hypothesized to be in contact with computationally identified putative channels. This is due principally to the lack of complete mass coverage of the examined proteins. Additionally, since we have collected data only on the D1, D2, CP43, and CP47 core subunits of the photosystem, residues on other PS II subunits which contribute to channel formation remain unidentified (22, 24–26, 41). Second, in the protein domains for which we do have mass coverage, comparison to the channels summarized by Ho (Table 1, Ref. 46) yields the following results: in the Back Channel, 22% of the residues were observed to be oxidized, in the Narrow Channel, 33% were modified, and in the Large Channel 35% contained oxidative modifications. Larger numbers of oxidized residues were observed in the Broad Channel, with 55% containing oxidative modifications. Similar results were obtained comparing our data to the channels identified by Vassiliev et al. (26). These investigators identified four channels possibly involved in water transit to the Mn₄O₅Ca cluster. A fifth channel, corresponding to the Back Channel of Ho and Styring (41) exhibited a very high energy barrier (22 kcal/mol) for water transport. Of the four putative water channels (designated Channels 1–4 in Ref. 26) which had lower energy barriers (10–15 kcal/mol), residues in contact with Channels 2 and 4 exhibited low amounts of oxidative modification (0 and 22%, respectively). The residues in Channels 1 and 3 which were explicitly identified by the authors exhibited a high degree (67%) of oxidative modification. Consequently, residues located in Channels 1/3, which in large measure corresponds to the Broad Channel of Ho and Styring (41), exhibit a high propensity for oxidative modification in our experiments.

CONCLUSIONS

In this study, we have presented experimental evidence which has identified residues within PS II which are susceptible to modification by OH⁻ produced by synchrotron radiation. We believe that our findings implicate the Broad Channel of Ho and Styring (41) (Channels 1/3 of Vassiliev et al. (26)) as a water
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channel functioning to deliver substrate water to the Mn$_4$O$_5$Ca cluster. A second group consisting entirely of modified CP43 residues was also identified. These residues may be associated with a hypothetical channel which has not been previously been identified. While this putative channel may be a second water channel, we hypothesize that it may constitute a dioxygen/ROS exit pathway leading from the Mn$_4$O$_5$Ca cluster to the thylakoid lumen. Experiments are currently ongoing to differentiate between these and other possibilities.

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