Amino acid residues that modulate the properties of tyrosine YZ and the manganese cluster in the water oxidizing complex of photosystem II

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Abstract

The catalytic site for photosynthetic water oxidation is embedded in a protein matrix consisting of nearly 30 different polypeptides. Residues from several of these polypeptides modulate the properties of the tetrameric Mn cluster and the redox-active tyrosine residue, YZ, that are located at the catalytic site. However, most or all of the residues that interact directly with YZ and the Mn cluster appear to be contributed by the D1 polypeptide. This review summarizes our knowledge of the environments of YZ and the Mn cluster as obtained from the introduction of site-directed, deletion, and other mutations into the photosystem II polypeptides of the cyanobacterium Synechocystis sp. PCC 6803 and the green alga Chlamydomonas reinhardtii. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The catalytic site for photosynthetic water oxidation contains four Mn ions and the redox active tyrosine, YZ (for review see [1–8]). One Ca2+ ion and one Cl− ion are required for catalytic activity and appear to be located in the vicinity of the Mn cluster. The four Mn ions are arranged as a magnetically coupled tetramer [9–11] (reviewed in [12] and see review by Peloquin and Britt in this issue). The point-dipole distance between YZ and the Mn cluster is 7–9 Å, as shown by simulations of EPR [10,13–18] and ENDOR [10] data obtained with samples trapped in the S2YZ state. Because the structure of the Mn cluster is unknown, this point-dipole distance is compatible with structural models showing either direct hydrogen bonding between YZ and a water-derived Mn ligand [7,19,20] or indirect hydrogen bonding, such as via an intervening water molecule [11,12]. Hydrogen bonding between YZ and the Mn cluster, whether direct or indirect, would be consistent with recent proposals for the mechanism of water oxidation that invoke proton-coupled electron transfer from Mn-bound water molecules or water-derived Mn ligands to YZ. In these proposed mechanisms, YZ abstracts both an electron and a proton from the Mn cluster during some [2,12,20–23] or all [19,24–30] of the S state transitions. This coupling of proton and electron transfer events has been postulated to provide the necessary driving force for oxidizing the Mn cluster in its higher S states.

The photosystem II (PS II) complex in vivo contains nearly 30 different polypeptides, including those involved in light-harvesting (for review, see [8,31,32]). The light-harvesting polypeptides of higher plants...
and green algae consist of LHC II and the minor chlorophyll $a/b$ proteins, CP29, CP26, and CP24 (for review, see [33–37]). The light-harvesting proteins of cyanobacteria and eukaryotic red algae consist of the phycobiliproteins [38,39]. About 20 polypeptides make up PS II proper and are encoded by the $psb$ genes. These are the major membrane spanning subunits known as CP47, CP43, D2, and D1, the smaller membrane spanning subunits including cytochrome $b-559$, and the extrinsic subunits including the ‘33 kDa polypeptide’ encoded by the $psbO$ gene. Residues from many polypeptides modulate the properties of $Y_Z$ and the Mn cluster. However, most or all of the residues that ligate Mn or Ca$^{2+}$ or that interact directly with $Y_Z$ appear to be contributed by the D1 polypeptide. Insight into the protein environments of $Y_Z$ and the Mn cluster has been provided in the last decade by spectroscopic analysis of cells and PS II preparations from site-directed mutants of the cyanobacterium Synechocystis sp. PCC 6803 and the green alga Chlamydomonas reinhardtii. These studies are the subject of this review.

2. Polypeptides of PS II

Single-particle image averaging and electron crystallographic studies show that PS II is dimeric in vivo and has twofold rotational symmetry (for reviews, see [32,40–42]). The three-dimensional structure of a PS II preparation containing CP47, D2, D1, cytochrome $b-559$, and several small subunits has been determined to an in-plane resolution of 8 Å [41,43]. Such PS II preparations retain the ability to oxidize $Y_Z$ (or $Y_D$) [44–46] and to reduce $Q_A$ [45,46], but do not oxidize water. A two-dimensional map of a PS II preparation that retains the ability to evolve $O_2$ has been determined to a resolution of 9 Å [47]. This PS II preparation contains, in addition to the above subunits, CP43 plus additional small subunits. The 8 Å and 9 Å structures have been modeled into 24–30 Å projection maps obtained from single particle analysis of more intact $O_2$-evolving PS II preparations containing the extrinsic subunits and some light-harvesting polypeptides to obtain three-dimensional structures. These structures show the positions of the extrinsic subunits relative to those of CP47, CP43, D2, and D1 in spinach [48], in C. reinhardtii [49] and in the cyanobacterium Synechococcus elongatus [49]. In all of these structural models, the D2 protein is placed adjacent to CP47 on the basis of cross-linking studies [50,51].

2.1. The D1 and D2 proteins

At the core of each PS II monomer is the D1–D2 heterodimer. The D1 and D2 proteins are homologous 38–39 kDa polypeptides that are encoded by the $psbA$ and $psbD$ genes, respectively. Both proteins contain five membrane-spanning helical domains. The D1 protein is post-translationally cleaved after Ala-344 [52–55]. The D1–D2 heterodimer contains the cofactors involved in light-induced electron transfer. It contains six chlorophyll $a$, two pheophytin $a$, and two $\beta$-carotene molecules [56–58]. It also contains the plastoquinones $Q_A$ and $Q_B$, the redox-active tyrosines $Y_Z$ and $Y_D$, and one atom of non-heme iron. Because of the sequence and functional similarities between the two photosystems, the bacterial reaction center has long served as a structural model for PS II [59,60]. The electron transfer cofactors are believed to be arranged in two pseudo-symmetric branches with $P_{\text{680}}$ coordinated by D1-His-198 and D2-His-198 (spinach numbering throughout this review), the non-heme iron atom coordinated by D1-His-215, D1-His-272, D2-His-215, and D2-His-269 and the pheophytin electron acceptor interacting with D1-Glu-130. The fifth and sixth coordination positions of the iron atom in PS II are occupied by bicarbonate [61]. The 8 Å structure of PS II shows that the distance between the central pair of chlorophyll molecules in PS II is greater than the distance between the special pair of bacteriochlorophyll molecules in bacterial reaction centers [43]. The greater distance is consistent with the weaker exciton coupling of the pigments comprising $P_{\text{680}}$ compared to that in bacterial reaction centers [3,62,63].

The original bacterial model for the D1–D2 heterodimer [59,60] inspired the site-directed mutagenesis studies that identified $Y_D$ as Tyr-161 of D2 [64,65] and $Y_Z$ as Tyr-161 of D1 [66,67]. Refined versions of this model [68–71] have been used as predictive tools for mutagenesis studies. Recent studies have shown that the $P_{\text{680}}$ cation is localized primarily on the chlorophyll molecule ligated by D1-His-198 [72], that D1-His-198 [73], D1-Glu-130 [73,74] and D2-Leu-206
[75] influence the quantum yield and free energy of the \( P_{680}^* \)-Pheo\(^+-\) radical pair state, that the peptide nitrogen of D2-Ala-260 forms a weak hydrogen bond to \( Q_A^* \) [76], that D1-His-118 provides an axial ligand to Chl\(_Z\) [77] (a monomeric chlorophyll that is oxidized by \( P_{680}^* \) via a molecule of \( \beta \)-carotene [78,79]), and that D2-His-118 provides an axial ligand to another monomeric chlorophyll [77,80,81] known as Chl\(_D\) [77] (for reviews of earlier mutagenesis studies, see [70,82,83]). Studies of the environments of \( Y_Z \), \( Y_D \), and the Mn cluster are described below. Unfortunately, the refined structural models [68–71] provide limited insight into the environment of the Mn cluster because bacterial reaction centers do not oxidize water and because the luminal domains of D1 and D2 are much larger than the corresponding domains of bacterial reaction centers.

2.2. The CP47 and CP43 proteins

The CP47 and CP43 proteins have molecular masses of approximately 56 and 50 kDa and are encoded by the \( psbB \) and \( psbC \) genes, respectively [1,84,85]. Both proteins contain approximately 15 molecules of chlorophyll \( a \) and two or three molecules of \( \beta \)-carotene [86]. Both proteins contain six membrane-spanning helical domains. In CP47, the chlorophyll molecules are arranged in two layers near the luminal and stromal ends of the helical domains [41,43,87], with many probably ligated to conserved histidine residues [87–90]. The 8 Å structure of PS II shows that the transmembrane helices of CP47 are arranged as a trimer of dimers [41,43,87]. The CP43 protein is believed to have a similar structure [41,47], with its chlorophyll molecules arranged similarly to those in CP47 and also ligated to conserved histidine residues [91]. The 9 Å projection map shows that CP47 and CP43 are arranged on either side of the D1–D2 heterodimer and are related by the same pseudo twofold symmetry axis that relates D1 and D2 [47]. In this regard, the structure of the CP47/CP43/D2/D1 complex resembles that of the core of Photosystem I [92–95]. In both CP47 and CP43, a large hydrophilic loop connects transmembrane helices V and VI. This loop is located on the luminal side of the thylakoid membrane and consists of \( \sim 190 \) residues in CP47 and \( \sim 130 \) residues in CP43. These loops are believed to fold over part of the luminal domains of the D1–D2 heterodimer because residues in these loops strongly influence the properties of the Mn cluster (see Section 3.3) and, because, in the absence of the extrinsic proteins, \( Y_D \) is located \( \sim 27 \) Å [96] or \( \sim 20 \) Å [97] from the lumen despite its location near the luminal end of helix C of D2. The large hydrophilic loops of CP47 and CP43 interact with the extrinsic 33 kDa protein (see Sections 2.4.1, 3.3, and 3.4).

2.3. Other membrane spanning subunits

With the exception of the \( \sim 22 \) kDa product of the \( psbS \) gene, all other intrinsic subunits of PS II have molecular masses \( \leq 10 \) kDa, and contain a single transmembrane helix. These are cytochrome \( b \)-559 and the products of the \( psbH \), \( psbI \), \( psbJ \), \( psbK \), \( psbL \), \( psbM \), \( psbN \), \( psbR \), \( psbT_V \), \( psbT_C \), \( psbW \), and \( psbX \) genes. The functions of most of these subunits are unknown (for reviews, see [8,31,32]). The PS II preparation whose structure was determined to an in-plane resolution of 8 Å [41,43] contained cytochrome \( b \)-559 and the products of the \( psbI \), \( psbK \), \( psbL \), \( psbT_C \), and \( psbW \) genes in addition to CP47, D2, and D1.

The \( psbS \) protein is evolutionarily related to the LHC II, CP29, CP26, and CP24 antenna proteins. However, it contains four helical domains rather than the three helical domains present in the other chlorophyll \( \alpha \beta \) proteins [33–37] and appears to be involved in the thermal dissipation of excess excitation energy rather than in light harvesting [98].

Cytochrome \( b \)-559 consists of two polypeptides, known as \( \alpha \) and \( \beta \), that are present in 1:1 stoichiometry. The \( \alpha \) and \( \beta \) polypeptides are encoded by the \( psbE \) and \( psbF \) genes and have molecular masses of 9.3 and 4.4 kDa, respectively [99]. The heme of cytochrome \( b \)-559 is coordinated by two histidine residues [100]. The \( \alpha \) and \( \beta \) polypeptides each contain a single histidine residue. Consequently, cytochrome \( b \)-559 is generally considered to be arranged as an \( \alpha \beta \) dimer with its heme group located near the stromal surface of the membrane (for review, see [101,102]). Cytochrome \( b \)-559 is oxidized by \( P_{680}^* \) via Chl\(_Z\) (or Chl\(_D\)) and/or \( \beta \)-carotene [78,79] and reduced by \( Q_A^* \) [103], \( Q_B^* \) [104], or Pheo\(^+-\) [105], depending on the conditions. These reactions are too slow to be part of the normal electron transfer reactions of water ox-
dation but are believed to help protect PS II from photo-oxidative damage (for review, see [101,102]).

The psbH protein has a molecular mass of ~8 kDa. The deletion of the psbH gene from Synechocystis sp. PCC 6803 slows electron transfer from Q$_A^*$ to Q$_B$ and renders PS II more sensitive to photoinactivation [106]. The psbI and psbW proteins have molecular masses of 4.2 kDa [99] and 5.9 kDa [86], respectively, and are intimately associated with the D1–D2 heterodimer. Their functions are unknown. The deletion of the psbH gene from Synechocystis sp. PCC 6803 has little effect on O$_2$ evolution but renders PS II more sensitive to photoinactivation because mutants of Synechocystis sp. PCC 6803 abolishes photoautotrophic growth [112]. The psbJ and psbK proteins have molecular masses of ~4.2 kDa [86,108]. Their functions are unknown. Neither protein is essential for O$_2$ evolution because mutants of Synechocystis sp. PCC 6803 that lack psbJ [108] or psbK [109,110] grow photoautotrophically, although at somewhat diminished rates. The psbL protein has a mass of 4.4 kDa [86]. It is required for the oxidation of Y$_Z$ by P$^*_S$ [111]. Consequently, the deletion of the psbL gene from Synechocystis sp. PCC 6803 abolishes photoautotrophic growth [112]. The psbM and psbN proteins each have molecular masses of ~4.7 kDa [113]. Their functions are unknown. Deletion of both the psbN and psbH genes from Synechocystis sp. PCC 6803 produced no effects other than those observed in the absence of psbH alone [106]. The psbR protein has a molecular mass of ~10 kDa [114]. Its function is unknown and it is absent from O$_2$-evolving PS II preparations that lack the LHC II, CP29, CP26, and CP24 antenna proteins (e.g., [115,116]). The nuclear encoded psbTo protein has a molecular mass of ~3 kDa and is believed to be an extrinsic protein [117]. Its function is unknown. The chloroplast-encoded psbTC protein has a molecular mass of 3.9 kDa [86]. Its function is also unknown, but the deletion of the psbTC gene from C. reinhardtii renders PS II more sensitive to photoinactivation [118].

2.4. The extrinsic subunits

2.4.1. The 33 kDa polypeptide

The extrinsic ‘33 kDa Polypeptide’ has a molecular mass of ~26.5 kDa [119] and is encoded by the psbO gene. It is conformationally flexible in solution [120–122] and has been referred to as a ‘natively unfolded’ protein [121] or as a molten globule [122]. Its conformation changes when it binds to the intrinsic subunits of PS II [120,123] and it binds more tightly in the presence of the Mn cluster than in its absence [124–126]. Whether each monomeric unit of PS II contains one [41,48,49,127,128] or two [126,129,130] copies of the extrinsic 33 kDa polypeptide (with one copy binding to a structural site and the other to a regulatory site [130]) remains controversial. Single particle image averaging studies incorporating the 8 A and 9 A structures of the PS II core show that the extrinsic 33 kDa polypeptide covers parts of the D1–D2 heterodimer and CP47, including the luminal ends of helices C, D, and E of D2, the luminal ends of helices V and VI of CP47, and the putative location of the loop connecting helices C and D of D1 [48,49]. Because the large hydrophilic loop of CP47 connects helices V and VI, this location is consistent with cross-linking, accessibility, deletion mutagenesis, and site-directed mutagenesis studies showing that the extrinsic 33 kDa polypeptide interacts with this loop (for reviews of these studies, see [131,132]). The extrinsic 33 kDa polypeptide is not required for O$_2$ evolution [133–136]. However, it protects the Mn cluster from endogenous reductants and optimizes its catalytic efficiency for water oxidation (for reviews, see [131,132]). In its absence, the rates of O$_2$ release [137,138] and Y$_S^*$ reduction during the S$_3$→(S$_0$)→S$_0$ transition [139] are slowed ~five-fold, the S$_2$-state is abnormally stable [137,138,140], and PS II is more susceptible to photoinactivation [134,135]. Also, unless the polypeptide-depleted preparations are maintained in the presence of high concentrations of Cl$^-$ ions [141] two Mn ions are gradually lost from the Mn cluster as Mn$^{2+}$ ions [141,142]. In protecting the Mn cluster from exogenous reductants, the extrinsic 33 kDa polypeptide serves as a diffusion barrier that impedes the access of Mn$^{2+}$ ions to sites on the apoprotein. In the absence of the Mn cluster, the efficiency of Mn$^{2+}$ photo-oxidation [143] and the quantum yield of photoreduction of the Mn cluster [144] increase substantially in the absence of the 33 kDa polypeptide.

2.4.2. The 24 kDa and 17 kDa polypeptides

In higher plants and green algae, two additional extrinsic subunits are associated with PS II. These
are the 24 and 17 kDa polypeptides, the products of the \textit{psbP} and \textit{psbQ} genes, respectively [85,131]. They are present in a 1:1:1 stoichiometry with the extrinsic 33 kDa polypeptide [145,146]. Single particle image averaging studies incorporating the 8 A\textdegree and 9 A\textdegree structures of the PS II core show that these polypeptides bind next to the extrinsic 33 kDa polypeptide near CP43, covering the luminal ends of helices A and B of the D1 protein and two unidentified helices nearby [48,49]. The 24 kDa polypeptide maintains an optimal concentration of Ca\textsuperscript{2+} ions near the Mn cluster [147], whereas the 17 kDa polypeptide (alone or in combination with 24 kDa polypeptide) maintains an optimal concentration of Cl\textsuperscript{−} ions near the Mn cluster [148] (for review, see [131]). The 24 and 17 kDa polypeptides influence the magnetic properties of the Mn cluster: the parallel polarization multilane EPR signal of the S\textsubscript{1} state only appears in the absence of these polypeptides [149] and the EPR signal of the S\textsubscript{1}Y\textsubscript{Z} state in Ca\textsuperscript{2+}-depleted samples is narrowed in their absence [150].

2.4.3. The cytochrome c-550 and 12 kDa polypeptides

Cyanobacteria and eukaryotic red algae do not contain the extrinsic 24 kDa and 17 kDa polypeptides. Instead, these organisms contain two other extrinsic polypeptides, the products of the \textit{psbU} and \textit{psbV} genes [131]. The \textit{psbU} protein has a molecular mass of \(\sim 10.5\) kDa [151], but is generally referred to as the ‘12 kDa polypeptide’. The \textit{psbV} protein is known as cytochrome c-550 and has a molecular mass of \(\sim 17\) kDa [152]. The heme group of this cytochrome has a very low potential (\(-260\) mV), but its function is unknown. Single particle image averaging studies show that the \textit{psbU} and \textit{psbV} polypeptides bind next to the extrinsic 33 kDa polypeptide [49,153], although whether they bind in the same position as the 24 and 17 kDa polypeptides in higher plants and green algae [49], or in a different position [153], remains unclear. Both the \textit{psbU} [151] and \textit{psbV} [152,154] proteins help to maintain optimal concentrations of Ca\textsuperscript{2+} and Cl\textsuperscript{−} ions near the Mn cluster, with \textit{psbV} being more essential than \textit{psbU} [154].

3. The environment of the Mn cluster

Several lines of evidence suggest that the Mn and Ca\textsuperscript{2+} ions in PS II are coordinated primarily or exclusively by residues of the D1 protein (for reviews, see [1,2,8] and see review by Diner in this issue). The single Ca\textsuperscript{2+} ion that is required for water oxidation [147,155] has been proposed to be located \(\sim 3.5\) Å from the Mn cluster on the basis of Mn- and Sr-EXAFS studies [156,157], although other workers have presented conflicting Mn-EXAFS data [158]. A recent solid-state \(^{111}\)Cd NMR study concluded that PS II contains a single Ca\textsuperscript{2+} ion that is six-coordinate, is located near the Mn cluster, and has a symmetrical distribution of oxygen and nitrogen and/or chlorine ligands [159].

The Mn ions are believed to be coordinated primarily by carboxylate residues. Coordination by both carboxylate and histidine residues has been proposed on the basis of chemical modification studies [160–168]. Coordination by at least one histidine residue has been demonstrated by ESEEM studies conducted with PS II preparations labeled with \(^{15}\)N-histidine [169]. This work extended earlier ESEEM [170,171] and ENDOR [172] studies that provided evidence of Mn ligation by at least one nitrogen atom. Subsequent ESEEM studies showed that the ligating histidine residue(s) coordinate Mn with their \(\varepsilon2\) (t) nitrogen(s) [173]. Additional evidence for coordination by histidine was provided by a recent FTIR study conducted with \(^{15}\)N-histidine [174]. Coordination by a carboxylate residue that forms a bridge to a Ca\textsuperscript{2+} ion has been proposed on the basis of an FTIR study of intact and Ca\textsuperscript{2+}-depleted PS II preparations [175], although the bridging aspect of this assignment has been questioned [176,177].

Most attempts to identify individual ligands of the Mn cluster have focused on the D1, D2, CP47, and CP43 polypeptides. It is unlikely that the extrinsic 33 kDa protein or any of the characterized smaller intrinsic subunits provide ligands to the Mn cluster because, except for the \textit{psbL} protein, photoautotrophic growth is retained when the genes encoding these proteins are deleted from \textit{Synechocystis} sp. PCC 6803. Also, the unidentified helices in the 8 Å structure of the PS II core (which must correspond to those of the cytochrome b-559, \textit{psbI, psbK, psbL, psbTc}, and \textit{psbW} proteins [43]) are located far from the expected location of Y\textsubscript{Z} [41,43]. Additional reasons that cytochrome b-559 is unlikely to contribute
ligands are that photoautotrophic growth is retained when the luminal domain of the $\alpha$ polypeptide is truncated by 31 residues [178] and that the $\beta$ polypeptide lacks a luminal domain [101,102]. The increased sensitivity to photoinactivation characteristic of mutants lacking the extrinsic 33 kDa polypeptide, psbH, psbI, or psbTc, or bearing mutations in the C-terminal domain of the $\alpha$ polypeptide of cytochrome b-559 [101], may be caused by the release of toxic, activated oxygen species from Mn clusters that have been perturbed by long-range changes in protein backbone conformation. Mutants containing perturbed Mn clusters are often much more sensitive to photoinactivation than mutants lacking Mn clusters [179,180]. Nevertheless, a terminal ligand of the Mn cluster might be functionally replaced by a water molecule, a Cl$^-\$ ion, or a residue or peptide carbonyl group recruited from another protein. Therefore, it is not possible to rigorously exclude the 33 kDa protein as potentially contributing ligands to the Mn cluster.

Similarly, until the additional helices in the 8 Å structure of the PS II core are identified, it will not be possible to rigorously exclude cytochrome b-559 or any of the other small intrinsic subunits as potentially contributing ligands.

3.1. The D1 protein

Because of the proximity of Y Z to the Mn cluster [10,15–18], most efforts to identify Mn ligands have targeted the D1 protein. Site-directed mutagenesis studies have targeted all conserved carboxylate, histidine, and tyrosine residues in the luminal domains of the D1 protein in Synechocystis sp. PCC 6803 [179–182]. The mutants Tyr73Phe, Tyr94Phe, Glu98Asn, Glu98Asp, Asp103Asn, Asp103Glu, Glu104Gln, Glu104Asp, Tyr107Phe, Asp308Asn, Asp319Asn, Asp319Glu, Glu329Gln, and Glu329Asp caused no discernible effects on photoautotrophic growth or O$_2$ evolution [179,180]. Therefore, it was concluded that neither Tyr-73, Tyr-94, Glu-98, Asp-103, Glu-104, Tyr-107, Asp-308, Asp-319, nor Glu-329 is crucial to PS II function under the conditions of the experiments [179,180]. However, the Mn clusters in all mutants are perturbed to some degree, particularly those in the Asp61Asn, Asp61Ala, Glu65Gln, and Glu65Ala mutants. These evolve O$_2$ at only $\sim 20\%$ the rate of wild-type cells [179]. In the Asp61Asn and Asp61Ala mutants, O$_2$ release is slowed $\sim 10$-fold [183]. Oxygen release is also slowed in the Asp59Asn and Asp61-Glu mutants, but to lesser extents [184]. The PS II reaction centers in the Asp59Asn and Asp61Ala mutants appear to have substantially diminished affinities for Ca$^{2+}$: in the presence of only trace quantities of Ca$^{2+}$ (i.e., if Ca$^{2+}$ is omitted from the growth medium), photoautotrophic growth of these mutants is abolished, electron transfer from Y Z to P$_{680}^+$ is slowed dramatically, and PS II becomes very sensitive to photoinactivation [179]. On the basis of these investigations, Asp-59 and Asp-61 have been identified as possible ligands of Ca$^{2+}$, and Asp-170, His-190, His-332, Glu-333, His-337, Asp-342, and the C-terminus of Ala-344 have been identified as possible ligands of Mn (Fig. 1 and see below). Histidine-337 has also been identified as a possible ligand of Mn on the basis of chemical modification and protease digestion studies [163,168].

3.1.1. Carboxylate residues

3.1.1.1. Asp-59, Asp-61, and Glu-65. All mutants constructed at Asp-59, Asp-61, and Glu-65 are photoautotrophic [179,181,182]. None of these residues appears likely to ligate Mn because none is essential for the assembly or stability of the Mn cluster in vivo [179]. However, the Mn clusters in all mutants are perturbed to some degree, particularly those in the Asp61Asn, Asp61Ala, Glu65Gln, and Glu65Ala mutants. These evolve O$_2$ at only $\sim 20\%$ the rate of wild-type cells [179]. In the Asp61Asn and Asp61Ala mutants, O$_2$ release is slowed $\sim 10$-fold [183]. Oxygen release is also slowed in the Asp59Asn and Asp61-Glu mutants, but to lesser extents [184]. The PS II reaction centers in the Asp59Asn and Asp61Ala mutants appear to have substantially diminished affinities for Ca$^{2+}$: in the presence of only trace quantities of Ca$^{2+}$ (i.e., if Ca$^{2+}$ is omitted from the growth medium), photoautotrophic growth of these mutants is abolished, electron transfer from Y Z to P$_{680}^+$ is slowed dramatically, and PS II becomes very sensitive to photoinactivation [179]. On the basis of these results, it was proposed that Asp-59 and Asp-61 may directly coordinate a Ca$^{2+}$ ion [179].
Photoactivation intermediates are greatly destabilized in the Asp59Asn and Asp61Glu mutants [184]. It has been proposed that binding Ca$^{2+}$ to a site formed by Asp-59 and Asp-61 promotes repositioning the C-terminal domain of D1 during photoactivation of the Mn cluster [185].

Both Asp-59 and Asp-61 may comprise part of an Asx turn, a motif found in Ca$^{2+}$-binding loops in most Ca$^{2+}$-binding proteins [186]. An Asx turn involves a hydrogen bond between a side-chain oxygen of an Asp or Asn residue at position ‘n’ and the peptide amide nitrogen at position ‘n+2’ [187]. In most Ca$^{2+}$-binding proteins, the Asx residue is Asp [186]. A Pro or Gly residue is located adjacent to the turn and within three residues of the Asx residue [186]. This residue acts in conjunction with the Asx turn to fold the Ca$^{2+}$-binding loop around the Ca$^{2+}$ ion and to correctly position the oxygen ligands. Both Asp-59 and Asp-61 are located near the center of a 14-residue domain that is conserved in all known D1 sequences [188] and includes Pro-56, Pro-57, and Gly-62. Therefore, Asp-59 may serve as Asx residue in a Ca$^{2+}$-binding loop. If this residue normally coordinates a Ca$^{2+}$ ion with both side-chain oxygens, then replacing Asp with Asn would likely decrease the affinity of the site for Ca$^{2+}$ ions (e.g., [189,190]). Similarly, if Asp-61 coordinates a Ca$^{2+}$ ion via a single oxygen, replacing this residue with Asn may not seriously diminish binding (e.g., [190]), but replacing it with Ala would (e.g., [190–193]).

On the other hand, both Asp-59 and Asp-61 are located near the base of the A helix, probably at least 15 Å from Asp-170 [68–71], a likely ligand of the Mn cluster (see Section 3.1.1.2). If Asp-170 ligates the Mn cluster and the PS II core contains only one cluster (see Section 3.1.1.2). If Asp-170 ligates the first Mn$^{2+}$ ion photo-oxidized during photoactivation of the Mn cluster, in contradiction to the widely held notion that the Ca$^{2+}$ ion is located near the Mn cluster [1–8] and in contradiction to the Mn- and Sr-EXAFS data showing that Mn and Ca$^{2+}$ are in close proximity [156,157]. Therefore, an alternate possibility is that mutations at Asp-59 and Asp-61 cause structural changes in PS II that weaken the affinity of Ca$^{2+}$ for a distant site. As an example of such a long-range structural change, the Asp199Asn mutation in chloramphenicol acetyltransferase disrupts a buried salt bridge, resulting in the reorientation of two loops, including residues located 20 Å from the mutation site [194].

### 3.1.1.2. Asp-170

At least 15 mutations have been constructed at this position: Glu, His, Cys, Met, Asn, Gln, Ser, Thr, Ala, Gly, Arg, Tyr, Phe, Trp, and Pro [179,195–198]. The Glu and His mutants are photautotrophic, whereas O$_2$ evolution is abolished in the Ala, Ser, Thr, Phe, and Pro mutants, nearly abolished in the Asn mutant, and severely diminished in most others. The Mn content of PS II particles isolated from the Asn mutant is also diminished substantially [196] and a high-affinity Mn binding site on the apoprotein is abolished in at least the Asn, Ala, and Ser mutants [195,199]. When Mn-depleted wild-type PS II particles are illuminated in the presence of Mn$^{2+}$ ions, Y$_{Z}$ oxidizes Mn$^{2+}$ to Mn$^{3+}$ and a parallel polarization EPR signal of the Mn$^{3+}$ ion is observed [200]. This signal shows a high-field structure consistent with either an elongated six-coordinate octahedral Mn$^{3+}$ geometry or a five-coordinate square-pyramidal Mn$^{3+}$ geometry [200]. In Mn-depleted Asp170Glu and Asp170His PS II particles, Y$_{Z}$ is also capable of oxidizing Mn$^{2+}$ ions [195,199,200]. However, a different Mn$^{3+}$ EPR signal, without a resolved hyperfine structure, is observed in Asp170His PS II particles, and no Mn$^{3+}$ EPR signal is observed in Asp170Glu PS II particles. Instead, the illuminated Asp170Glu PS II particles exhibit a perpendicular polarization signal suggestive of Mn$^{3+}$ [200]. These results show that Asp-170 forms part of the binding site for the first Mn$^{2+}$ ion photo-oxidized during photoactivation of the Mn cluster and that the residue present at this position influences the coordination environment and redox properties of the photo-oxidized Mn ion.

If Asp-170 ligates the first Mn$^{2+}$ ion photo-oxidized during photoactivation of the Mn cluster, it would seem likely that this residue would ligate the assembled Mn cluster. Preliminary ESEEM and FTIR data support this impression. Intact Asp170His PS II particles exhibit normal multiline EPR spectra in both the S$_1$ state (R.J. Debus, K.A. Campbell, and R.D. Britt, unpublished observations) and the S$_2$ state (X.-S. Tang and B.A. Diner, personal communication; R.J Debus, K.A. Campbell, and R.D. Britt, unpublished observations). Preliminary
ESEEM spectra of the $S_2$ state multiline EPR signal of Asp170His PS II particles show that the amplitude of the 5 MHz nitrogen modulation increases relative to that of wild-type when normalized to the amplitude of the modulations of nearby protons at 14–15 MHz (R.J. Debus, K.A. Campbell, W. Gregor, and R.D. Britt, unpublished observations). Because this modulation arises from magnetic coupling between the Mn cluster and 1-2 histidyl ligands [169,173], its increased amplitude relative to that of the proton modulations in Asp170His PS II particles is consistent with ligation of the assembled Mn cluster by Asp-170. However, an alternate possibility is that Asp-170 does not ligate the Mn cluster but that the Asp170His mutation alters proton modulations sufficiently to decrease the amplitude of the 14–15 MHz feature relative to that of the 5 MHz feature. Additional experiments are planned to distinguish between these possibilities.

The $S_2$–$S_1$ FTIR difference spectrum of wild-type PS II particles from *Synechocystis* sp. PCC 6803 closely resembles that of spinach PS II particles in both the mid-frequency (2000–1000 cm$^{-1}$) [201,319] and low-frequency (1000–350 cm$^{-1}$) [319] regions of the IR spectrum except for slight differences in the amide region. The spectrum of Asp170His PS II particles shows no appreciable changes in the mid-frequency region [319], implying that the carboxylate vibrational modes of Asp-170 are unchanged during the $S_1 \rightarrow S_2$ transition. However, in the low-frequency region, a band at 606 cm$^{-1}$ shifts to $\sim$612 cm$^{-1}$ [319]. Because the 606 cm$^{-1}$ band has been assigned to a Mn$_2$O$_2$ diamond mode of a bis-$\mu$-oxo-bridged [Mn($\mu$-O)$_2$] structure [177,208], Asp-170 must be coupled to the [Mn($\mu$-O)$_2$] structure that gives rise to this band. Because this band also shifts when Ca$^{2+}$ is replaced by Sr$^{2+}$ (from 606 cm$^{-1}$ to $\sim$618 cm$^{-1}$) [177,208], Asp-170 must either directly ligate Mn or Ca$^{2+}$ or participate in a hydrogen bond to the (Mn)$_4$–Ca cluster. However, if Asp-170 ligates Mn, the absence of changes in the mid-frequency region of the $S_2$–$S_1$ FTIR difference spectrum of Asp170His PS II particles shows that Asp-170 does not ligate the Mn ion that is oxidized during the $S_1 \rightarrow S_2$ transition.

In apparent contradiction to data supporting ligation of the assembled Mn cluster by Asp-170, the Asp170Val mutant is photoautotrophic and the Leu, and Ile mutants evolve O$_2$ at $\sim$20% the rate of wild-type cells [179]. One explanation is that Asp-170, although required for efficient photo-oxidation of the first Mn ion ligated during assembly of the Mn cluster, does not remain as a ligand to the assembled Mn cluster. However, if Asp-170 does not ligate the assembled Mn cluster, it is difficult to explain why replacement of this residue with Asn is more deleterious to O$_2$ evolution than replacement with Val, Leu, or Ile. To resolve this paradox, it was proposed that Val, Leu, and Ile, being bulky and hydrophobic, cause structural perturbations that permit the missing carboxylate moiety to be replaced by another residue, a peptide carbonyl group, or a water molecule [179]. Compensatory, mutation-induced structural rearrangements have been observed in other systems. In ferredoxin I of *Azotobacter vinelandii*, replacing Cys-20 with Ala caused a structural rearrangement that permitted Cys-24 to replace Cys-20 as a ligand to the [4Fe–4S] cluster [209]. In ricin A, replacing the active-site residue Glu-177 with Ala caused Glu-208 to rotate into the active site with partial restoration of catalytic activity [210]. In human alcohol dehydrogenase, replacing the pyrophosphate-binding residue Arg-47 with Gly caused structural rearrangements that moved Lys-228 into the pyrophosphate site with partial restoration of activity [211].

### 3.1.1.3. Glu-189

At least 17 mutations have been constructed at D1-Glu-189: Gln, Asp, Asn, His, Ser,
Thy, Ala, Gly, Cys, Lys, Arg, Leu, Ile, Val, Met, Phe, and Tyr [179,212,213]. Only the Gln, Lys, Arg, Leu, and Ile mutants are photoautotrophic and none of the others evolve O₂ to any significant degree. The influence of the Glu-189 mutants on the assembly or stability of the complex is minor, so this residue is not believed to ligate Mn [179,213]. The EPR and electron transfer properties of PS II preparations isolated from the Gln, Leu, Asp, Asn, His, Gly, and Ser mutants have been examined recently. Intact PS II preparations isolated from Glu-189 mutants that evolve no O₂ also exhibit no S₁ or S₂ state multiline EPR signals and are unable to advance beyond an altered S₂ YZ state, as shown by the accumulation of narrow S₂ YZ EPR signals under multiple turnover conditions [213]. In the Gly and Ser mutants, the quantum yield for oxidizing the S₁ state Mn cluster is very low, corresponding to a ≥1400-fold slowing of the rate of Mn oxidation by YZ. Charge recombination between Q₅⁻ and YZ is also accelerated, showing that the mutations alter the redox properties of YZ in addition to those of the Mn cluster [213]. These results are consistent with Glu-189 participating in a network of hydrogen bonds that modulates the properties of both YZ and the Mn cluster [213] and are consistent with proposals [25,27,29] that Glu-189 positions a group that accepts a proton from His-190 (see Section 4.2).

As noted above, the Gln, Lys, Arg, Leu, and Ile mutants are photoautotrophic. A hydrogen-bonded network involving Glu-189 could be maintained by Gln: in dihydrofolate reductase, a hydrogen bond network involving Asp-27 is retained in the Asp-27Asn mutant because an O–HN hydrogen bond [214]. The flexibility and hydrogen bonding properties of Lys and Arg may similarly permit these residues to maintain a network of hydrogen bonds in place of Glu-189 [215]. None of the other residues substituted for Glu-189 would be likely candidates for maintaining such a network of hydrogen bonds. To rationalize the photoautotrophic growth of the Leu and Ile mutants, it was proposed that Leu and Ile cause structural perturbations that permit the missing carboxylate moiety to be replaced by another residue or by a water molecule [213]. Such an explanation was proposed previously to rationalize the significant O₂-evolving activities of the Val, Leu, and Ile mutations of Asp-170 (see Section 3.1.1.2).

Intact Glu189Gln PS II particles exhibit normal rates of electron transfer from Mn to YZ and from YZ to P₆₈₀ [216]. The benign nature of this mutation implies that (1) Glu-189 does not accept or donate protons during proton transfer reactions involving YZ or His-190, and (2) Glu-189 is protonated at neutral pH values. The sizeable apparent shift in the pKₐ value of Glu-189 probably arises from an interaction with nearby charged groups.

3.1.1.4. Glu-333. At least six mutations have been constructed at Glu-333: Gln, Asp, Asn, His, Ala, and Tyr [180,182]. Only the Gln mutant is photoautotrophic. This mutant evolves O₂ at ∼36% the rate of wild-type cells. In all mutants, substantial fractions of PS II complexes lack photo-oxidizable Mn ions in vivo [180], showing that Glu-333 influences the assembly or stability of the Mn cluster. However, the high-affinity Mn-binding site identified in the Asp-170 mutants [195,199,200] remains intact [182]. All of the Glu-333 mutants are extremely sensitive to photoinactivation, possibly because toxic, activated oxygen species are released from perturbed Mn clusters [180]. Because the Gln mutant evolves O₂ and because Gln can functionally replace His as a ligand to Fe in cytochrome c peroxidase [217], Glu-333 has been proposed to be a possible ligand of the Mn cluster [180]. This residue may also ligate a Ca²⁺ ion because electron transfer from YZ to P₆₈₀ is slowed dramatically when Glu-333 mutants are propagated in the presence of only trace quantities of Ca²⁺ (i.e., when Ca²⁺ is omitted from the growth medium) [180]. However, this apparently diminished affinity for Ca²⁺ could be caused by the perturbations to the Mn cluster [180]. Another possibility is that Glu-333 participates in a crucial network of hydrogen bonds that can be maintained by Gln, but not by the shorter and less flexible Asp and Asn residues. An additional possibility is that Glu-333 helps orient a His residue that ligates the Mn cluster. In proteins, histidine typically ligates metal ions with its ε2 (τ) nitrogen [218]. The δ1 (τ) nitrogen generally forms a hydrogen bond to a peptide carbonyl group or a carboxylate residue [218]. This hydrogen bond helps orient the His residue and its strength modulates the strength of the His-metal interaction [218–
220]. The improper positioning of this residue in the Glu-333 mutants could decrease the stability of the Mn cluster or the probability of its correct assembly.

3.1.1.5. Asp-342. At least five mutations have been constructed at Asp-342: Glu, Asn, His, Ala, and Val [180,182]. Only the Glu mutant is photoautotrophic, although the Asn mutant evolves O$_2$ at 33% the rate of wild-type cells when propagated in dim light. In all mutants, some fraction of PS II complexes lack photo-oxidizable Mn ions in vivo [180], showing that Asp-342 influences the assembly or stability of the Mn cluster. However, the high-affinity Mn-binding site identified in the Asp-170 mutants [195,199,200] remains intact [182]. Most of the Asp-342 mutants are extremely sensitive to photo-inactivation, possibly because toxic, activated oxygen species are released from perturbed Mn clusters [180]. Because Asn can replace Asp as a ligand to Fe in cytochrome $b_6$ ubiquinol oxidase [221], Asp-342 has been proposed to be a possible ligand of the Mn cluster. This residue has also been proposed as a possible Ca$^{2+}$ ligand because, when Asp342Glu and Asp342Asn cells are propagated in the presence of only trace quantities of Ca$^{2+}$ ions, electron transfer from $Y_Z$ to $P_{680}$ is slowed dramatically [180]. A third possibility is that Asp-342 performs an important structural role in PS II that is partly satisfied by the carboxylate moiety of Glu, but much less effectively by Asn and His, and not at all by Ala and Val. Perhaps Asp-342 helps orient a His residue that ligates the Mn cluster. As noted earlier, the $\delta 1$ ($\tau$) nitrogen of a metal-ligating histidine residue typically forms a hydrogen bond with a peptide carbonyl group or a carboxylate residue [218]. This hydrogen bond helps orient the His residue and its strength modulates the strength of the His-metal interaction [218-220]. In cytochrome $c$ peroxidase, Asp-235 and His-175 form such an interaction, with His-175 ligating the heme Fe atom with its e2 ($\tau$) nitrogen. The Asp235Glu mutation introduces only slight structural perturbations into the enzyme active site, but significantly weakens the strength of the carboxylate-histidine hydrogen bond. This weakened hydrogen bond, in turn, weakens the His–Fe interaction, increasing the heme midpoint potential by approximately 70 mV [220].

3.1.2. Histidine residues

3.1.2.1. His-92. Only the His92Leu mutant has been reported [179,181]. It is photoautotrophic and contains Mn clusters that are perturbed only minimally. Consequently His-92 has been considered unlikely to ligate Mn or to have a major role in water oxidation. Nevertheless, in view of the relatively benign nature of the Asp170Leu and Glu189Leu mutations discussed previously (Sections 3.1.1.2 and 3.1.1.3, respectively), the examination of additional His-92 mutants would seem worthwhile. The His-92Leu mutation does appear to diminish the affinity of PS II for Ca$^{2+}$ ions [179], but to a lesser extent than mutations at Asp59, Asp61, or Asp342.

3.1.2.2. His-190. At least 15 mutations have been constructed at His-190: Lys, Arg, Asp, Glu, Asn, Gln, Ser, Thr, Cys, Tyr, Phe, Leu, Val, Ala, and Gly [179,222-225]. All abolish photoautotrophic growth. Because of this, several authors have suggested that His-190 may ligate the Mn cluster [2,169,179,182,222]. Both the Arg and Lys mutants evolve O$_2$, but at only 13% the rate of wild-type cells [179,224]. Because Arg can replace His [226] and Lys can replace Met [227,228] as ligands to Fe in cytochrome $c$, it was proposed that His-190 may indeed ligate the Mn cluster [179]. A dual role for His-190, interacting with both $Y_Z$ (see Section 4.2) and the Mn cluster, has been proposed [2]. However, it will be difficult to test whether His-190 ligates the Mn cluster because only the Arg and Lys mutants contain photo-oxidizable Mn ions in vivo and these contain photo-oxidizable Mn ions in only a minority of reaction centers [179].

3.1.2.3. His-332. At least 10 mutations have been constructed at His-332: Gln, Ser, Asn, Asp, Glu, Lys, Arg, Leu, Tyr, and Gly [180,182]. None are photoautotrophic. Only the Gln and Ser mutants evolve O$_2$, and at only 10–15% the rate of wild-type cells. In all mutants except Asp and Glu, substantial fractions of PS II complexes lack photo-oxidizable Mn ions in vivo [180], showing that His-332 influences the assembly or stability of the Mn cluster. However, the high-affinity Mn-binding site identified in the Asp-170 mutants [195,199,200] remains intact [182]. Several His-332 mutants are extremely sensitive
to photoinactivation, possibly because toxic, activated oxygen species are released from perturbed Mn clusters [180]. Because Gln and Glu functionally replace His as a ligand to Fe in cytochrome c peroxidase [217,229,230], and because Asp and Ser are potential ligands to Mn, it was proposed that His-332 may ligate the Mn cluster and that its redox properties are substantially altered in the Asp and Glu mutants [180]. Intact His332Glu PS II preparations exhibit an altered S2 state multiline EPR signal that has more hyperfine lines and narrower splittings than the S2 state multiline EPR signal observed in wild-type PS II preparations [231]. However, the quantum yield for oxidizing the S1 state Mn cluster is very low, corresponding to an 8000-fold slowing of the rate of Mn oxidation by Y* [231]. In addition, the temperature threshold for forming the S2 state is approximately 100 K higher than in wild-type PS II preparations. Furthermore, His332Glu PS II preparations are unable to advance beyond an altered S2Y* state, as shown by the accumulation of a narrow S2Y* EPR signal under multiple turnover conditions [231]. Charge recombination between QA* and Y* is also accelerated, showing that the Glu mutation alters the redox properties of YZ in addition to those of the Mn cluster [231]. These results are consistent with His-332 being close to the Mn cluster and participating in a network of hydrogen bonds that modulates the properties of both YZ and the Mn cluster [231].

Recent experiments show that the nitrogen modulation observed near 5 MHz in ESEEM spectra of the S2 state multiline EPR signal in wild-type PS II particles [169,173] is diminished substantially in His332Glu PS II particles [320]. Because this modulation arises from magnetic coupling between the Mn cluster and 1-2 histidyl ligands [169,173], its diminished amplitude in His332Glu PS II particles is consistent with ligation of the Mn cluster by His-332. The diminished amplitude could represent either loss of the ligating D1-His332 nitrogen, decreasing the coordination number of one Mn ion from six to five, or replacement of the ligating nitrogen with an oxygen from glutamate, or from another residue, a peptide group, or a water molecule. However, another possibility is that His-332 does not ligate the Mn cluster, but that minor structural perturbations associated with the His332Glu mutation alter or abolish the magnetic couplings between the Mn cluster and the actual histidine ligand(s). An example with some relevance is provided by the α-His195Asn mutation in nitrogenase from A. vinelandii. The ESEEM spectrum of nitrogenase shows deep nitrogen modulation that arises from magnetic coupling between α-Arg359 and the Fe7Mo cofactor [232]. This residue forms a hydrogen bond to a sulfide that bridges between Fe and Mo. Several other residues, including α-His-195, form single hydrogen bonds to sulfides that bridge between pairs of Fe ions. The α-His195Asn mutation eliminates the hydrogen bond between α-His-195 and one of these sulfide bridges [232,233]. The loss of this hydrogen bond causes the Fe7Mo cofactor to reorient sufficiently to alter the hydrogen bond between α-Arg-359 and its sulfide, thereby abolishing the magnetic coupling between α-Arg-359 and the Fe7Mo cofactor [232,233]. Therefore, it is possible that His-332 serves in a network of hydrogen bonds connecting YZ and the Mn cluster without ligating the Mn cluster. The altered properties of the Glu mutant might only reflect perturbations associated with the His332Glu mutation. In this scenario, His-332 would serve as a crucial hydrogen bond donor that can be partly replaced by Gln (and by Ser via an immobilized water molecule). In several systems, Gln functionally replaces His as a hydrogen bond donor. In nitrogenase, the nitrogen modulation that is abolished by the α-His195Asn mutation is not abolished by the α-His195Gln mutation [233]. In sperm whale myoglobin, His-64 provides a crucial hydrogen bond to O2 that is retained in the His64Gln and His64Gly mutants (the latter via an immobilized water molecule), but not in the His64Leu mutant [234]. In ribulose-1,5-bisphosphate carboxylase/oxygenase from Ana
cystis nidulans, His-324 provides a hydrogen bond to substrate that is retained in the His324Gln mutant with retention of some catalytic activity [235]. To determine whether or not His-332 ligates the Mn cluster, ESEEM analysis of additional His-332 mutants will be necessary. One candidate is the Gln mutant. Because His332Gln cells evolve O2, perturbations associated with the His332Gln mutation may be fewer than those possibly associated with the His332Glu mutation. In cytochrome c peroxidase, Gln replaces His-175 as a ligand to heme and
is believed to ligate the Fe with its oxygen atom [229].

3.1.2.4. His-337. At least 10 mutations have been constructed at His-337: Arg, Gln, Asn, Glu, Asp, Tyr, Val, Leu, Phe, and Gly [180,182]. The Arg, Gln, and Phe mutants are photoautotrophic and the Glu, Asp, Asn, and Leu mutants evolve O₂ [180]. In many mutants, substantial fractions of PS II complexes lack photo-oxidizable Mn ions in vivo [180], showing that His-337 influences the assembly or stability of the Mn cluster. However, the high-affinity Mn-binding site identified in the Asp-170 mutants [195,199,200] remains intact [182]. Several His-337 mutants are extremely sensitive to photoinactivation, possibly because toxic, activated oxygen species are released from perturbed Mn clusters [180]. Because Arg can replace His as a ligand to Fe in cytochrome c [226] and Gln can replace His as a ligand to Fe in cytochrome c peroxidase [217,229,230], it was proposed that His-337 is a possible ligand of the Mn cluster [180]. Ligation of Mn by His-337 has also been proposed on the basis of chemical modification and proteolysis studies [163,168]. The Mn clusters in the Val mutant are severely perturbed, whereas those in the Leu mutant evolve O₂ and those in the Phe mutant support photautotrophic growth [180]. To explain why progressively larger hydrophobic residues cause progressively fewer perturbations, the bulky Leu and Phe residues were proposed to cause structural perturbations that permit the missing imidazole moiety to be replaced by another residue, a peptide carbonyl group, or a water molecule [180]. Such an explanation was proposed previously to rationalize the significant O₂-evolving activities of the Val, Leu, and Ile mutations of Asp-170 (Section 3.1.1.2) and the photoautotrophic growth of the Leu and Ile mutations of Glu-189 (Section 3.1.1.3).

An alternate possibility is that His-337 does not ligate the Mn cluster but rather serves as a crucial hydrogen bond donor whose influence on the Mn cluster is indirect. Both Arg and Gln are good hydrogen-bond donors that can potentially substitute for His. As noted earlier, Gln can replace His as a hydrogen bond donor in nitrogenase from A. vinelandii [232,233], in sperm whale myoglobin [234], and in ribulose-1,5-bisphosphate carboxylase/oxygenase from A. nidulans [235].

3.1.3. The C-terminus of Ala-344

The mutations Ala344stop and Ser345Pro abolish photoautotrophic growth and O₂ evolution [54,143]. However, the high-affinity Mn-binding site identified in the Asp-170 mutants [195,199,200] remains intact [54]. Because the D1 polypeptide in the Ala344stop mutant is shortened by one residue and the C-terminal domain is not post-translationally processed in the Ser345Pro mutant, it has been proposed that the C-terminus of the D1 polypeptide at Ala-344 may be a ligand to the assembled Mn cluster [54].

3.2. The D2 protein

The mutations Glu70Gln and Glu70Val abolish photoautotrophic growth [236]. The Glu70Gln mutant evolves O₂, but the rate declines rapidly during illumination, presumably because of photo-oxidative damage. Because the addition of Mn²⁺ ions stabilized the rate of O₂ evolution in this mutant, D2-Glu70 was proposed to be a ligand of the Mn cluster [236]. This residue is located in the loop connecting helices A and B of the D2 protein. However, in the recent 8 Å structure of the PS II core [41,43], this loop seems far from the expected location of the Mn cluster near YZ. Furthermore, exogenous Mn²⁺ ions may protect against light-induced damage by donating electrons to Y₂, in the same manner that some other electron donors offer protection (see discussion in [1]). Therefore, it seems unlikely that D2-Glu-70 is a ligand to the Mn cluster.

On the basis of chemical modification and proteolytic digestion studies, D2-His-337 has been proposed to ligate the Mn cluster [168]. However, neither the His337Tyr mutation [82] nor any of the other mutations constructed in the lumenal domains of the D2 protein by site-directed [82] or random chemical [237] mutagenesis, including mutations constructed at all conserved carboxylate and histidine residues, abolish photoautotrophic growth, with the exception of the mutation Ser80Phe [237]. Like the Glu70Gln mutant, the Ser80Phe mutant evolves O₂ but is sensitive to photoinactivation [237]. Several deletion mutants have been constructed in the D2 protein. Photoauto-
trophic growth is retained in mutants lacking the C-terminal 11 residues or containing seven or eight amino acid deletions within the C-terminal domain [238], but is lost in mutants containing seven or eight residue deletions in the loop connecting the C and D helices [239]. The photoautotrophic deletion mutants are also sensitive to photoinactivation [238].

On the basis of the available data and the 8 Å and 9 Å structures of the PS II core, it seems unlikely that the D2 protein provides any ligands to the Mn cluster. Instead, mutations or deletions in the luminal domains of the D2 protein probably perturb the Mn cluster by perturbing the backbone conformations of this and other polypeptides. Nevertheless, because a terminal ligand of the Mn cluster might be functionally replaced by a water molecule, a Cl⁻ ion, or a repositioned residue or peptide carbonyl group from another protein, it is not possible to rigorously exclude the D2 protein as a potential source of one or more terminal ligands of the Mn cluster.

3.3. The CP47 protein

The large hydrophilic loop of CP47 interacts with the extrinsic 33 kDa protein and is believed to cover part of the luminal domains of the D1–D2 heterodimer. The importance of specific domains in this loop has been probed by segment deletion [240–244], site-directed [241,244–251], combinatorial [252], and random [90] mutagenesis. Segment deletion mutagenesis identified the regions extending from Ala-373 to Asp-380 and from Arg-384 to Val-392 as crucial for tight association of the extrinsic 33 kDa protein and for the stability of a functional Mn cluster [242,243]. Site-directed mutagenesis studies show that Arg-384 and Arg-385 are required for the proper binding of the extrinsic 33 kDa protein [250]. Mutation of these residues slow O₂ release [247], stabilize the S₂ state [247], increase sensitivity to photoinactivation [245,247], and increase the quantum yield of photoactivation of the Mn cluster [245,247], and increase the Cl⁻ requirement for O₂ evolution. Mutations at these residues increase the sensitivity of PS II to photoinactivation and abolish O₂ evolution at low Cl⁻ concentrations. Both Gly-342 [90] and Glu-364 [244] are also critical: the Gly342Asp mutation impairs photoautotrophic growth [90], while the Glu364Gln mutation abolishes photoautotrophic growth in the absence of the psbV protein [244]. Also, mutation of Trp-167, a residue in the smaller loop connecting helices III and IV of CP47, severely retards photoautotrophic growth, decreases the amount of PS II stability incorporated in the thylakoid membrane, and increases the sensitivity of PS II to photoinactivation [248].

In contrast to the D1 protein, no individual residues on CP47 have been identified that abolish O₂ evolution under normal growth conditions without concurrently destabilizing the structure of PS II. Therefore, no specific residues on CP47 have been proposed as potential ligands for Mn. Nevertheless, because a terminal ligand of the Mn cluster might be functionally replaced by a water molecule, a Cl⁻ ion, or a repositioned residue or carbonyl group from another protein, it is not possible to rigorously exclude CP47 as potentially contributing one or more terminal ligands to the Mn cluster.

3.4. The CP43 protein

The large hydrophilic loop of CP43 also interacts with the extrinsic 33 kDa polypeptide. The importance of specific domains in this loop has been probed by segment deletion [253] and site-directed [254,255] mutagenesis. Photoautotrophic growth and O₂ evolution are abolished in all of the deletion mutants [253]. The mutations Arg305Ser and Glu293Gln slightly impair O₂ evolution, the mutations Glu339Gln and Arg342Ser severely impair O₂ evolution and eliminate photoautotrophic growth, and the mutation Glu352Gln prevents stable assembly of PS II reaction centers [254,255]. The Glu293Gln, Glu339Gln, and Arg342Ser mutants are sensitive to photoinactivation [254,255]. The Arg342Ser mutant is so sensitive to photoinactivation that O₂ evolution can only be detected when cells are propagated photoheterotrophically in media containing DCMU [254].

The CP43 protein is present in every O₂-evolving
PS II complex yet isolated. However, photoactivation of the Mn cluster has been reported to occur in the absence of CP43 in vitro [256]. Therefore, it seems unlikely that CP43 provides any ligands to the Mn cluster. Nevertheless, as with other subunits, because a terminal ligand of the Mn cluster might be functionally replaced by a water molecule, a Cl\(_3\) ion, or a repositioned residue or carbonyl group from another protein, it is not possible to rigorously exclude CP43 as potentially contributing one or more terminal ligands to the Mn cluster, particularly in view of the proximity of CP43 to the C and E helices of the D1 protein in the 8 Å and 9 Å structures of the PS II core [41,43,47].

4. Environments of Y\(_D\) and Y\(_Z\)

4.1. Tyrosine Y\(_D\)

Tyrosines Y\(_D\) and Y\(_Z\) correspond to D2-Tyr-161 [64,65] and D1-Tyr-161 [66,67], respectively, and are located symmetrically in the D1–D2 heterodimer [257] near the luminal ends of the C helices. The Y\(_D\) radical is extremely stable under physiological conditions. It is well shielded from solvent, as shown by \(_2\)H\(_2\)O/\(_1\)H\(_2\)O exchange [258,259] and reductant accessibility [260,261] studies. In the absence of the extrinsic subunits, Y\(_D\) is located \(~27\) Å [96] or \(~20\) Å [97] from the lumen, probably because the luminal end of the C helix of D2 is covered by the luminal domains of D1 and D2 and by the large hydrophilic loops of CP47 and CP43. Modeling studies predict that Y\(_D\) is located in a hydrophobic environment, surrounded by D2-Phe-170, D2-Phe-182, D2-Phe-189, D2-Leu-290, D2-Ala-291, and D2-Phe-186 (the latter is a Leu residue in cyanobacteria) [68–70]. A recent combinatorial mutagenesis study concluded that D2-Phe-170 is not permissive to substitution [239]. Tyrosine Y\(_D\) is predicted to form hydrogen bonds to D2-His-190 and D2-Gln-165, to be spatially positioned by D2-Pro-162, and to be in van der Waals contact with D2-Phe-186 and D2-Ala-291 [70]. The presence of a hydrogen bond between Y\(_D\) and D2-His-190 has been confirmed by CW-ENDOR [258] and high-field EPR [262] studies and by a pulsed ENDOR study conducted with PS II preparations of Synechocystis sp. PCC 6803 labeled with \(^{15}\)N-histidine [263]. The latter study demonstrated that Y\(_D\) accepts a hydrogen bond from the \(\epsilon2\) (\(\tau\)) nitrogen of D2-His-190. A subsequent FTIR study has shown that Y\(_D\) donates a hydrogen bond to D2-His-190 [264]. The environment of Y\(_D\) differs in Synechocystis and spinach in that the \(^{15}\)N couplings to Y\(_D\) are substantially different [265] and that Y\(_D\) accepts two hydrogen bonds in Synechocystis but only one in spinach [259].

The EPR line-shape of Y\(_D\) is altered in all D2-His-190 mutants [258,262,266]. The spin density distributions of tyrosine radicals in proteins are largely independent of the radical’s hydrogen bonding status [267–269]. Therefore, the altered Y\(_D\) EPR line-shapes must be caused by perturbations in the environment or orientation of Y\(_D\) in the absence of the hydrogen bond from D2-His-190. These perturbations may explain why both hydrogen bonds to Y\(_D\) in Synechocystis [259] are lost in the D2-His190Gln mutant [258,262–264], even though only one is provided directly by D2-His-190. Perturbations in protein backbone conformation or side-chain interactions were invoked previously to explain the altered Y\(_D\) EPR line-shapes observed in mutants of D2-Pro-162 and D2-Gln-165 [270]. Similar perturbations probably also explain why the Y\(_D\) EPR line-shapes observed in mutants of D2-Arg-181 are altered [271]. Mutations of this residue presumably perturb the structure of the loop connecting helices C and D of the D2 polypeptide. These mutations also appear to influence the redox potential of P\(_{680}\) [271] (also see [272]).

4.2. Tyrosine Y\(_Z\)

In contrast to Y\(_D\), tyrosine Y\(_Z\) is readily accessible to solvent, as shown by \(^2\)H\(_2\)O/\(^1\)H\(_2\)O exchange [259,273] and reductant accessibility [260,261] studies. Modeling studies predict that Y\(_Z\) is located in a hydrophilic environment, with D1-Gln-165, D1-Ser-169, D1-Asp-170, D1-Glu-189, and D1-His-190 located nearby and D1-Phe-186 and D1-Ala-294 making van der Waals contact [68–70]. A combinatorial mutagenesis study has shown that residues 160, 162, and 163 of the D1 polypeptide are permissive to substitution [274].

The oxidation of Y\(_Z\) requires its deprotonation by D1-His-190. In all D1-His-190 mutants examined, the oxidation of Y\(_Z\) by P\(_{680}^+\) is slowed more than
1000-fold at neutral pH values [179,222–225,275,276], but can be accelerated substantially by the addition of small organic bases [224,276] or by raising the pH [275,276]. In D1-His190Ala PS II preparations from *Synechocystis* sp. PCC 6803, the efficiency of YZ oxidation increased significantly above pH 9, exhibiting an apparent pK_A of ~10.3. This apparent pK_A value decreased in the presence of imidazole and ethanolamine, becoming the same as that of the added base. These observations were taken as further evidence that the oxidation of YZ requires its deprotonation. The value of ~10.3 was assigned to the pK_A of YZ in the absence of the D1-His-190 imidazole moiety [276].

In the absence of the Mn cluster, the reduction of Y_Z requires its reprotonation by D1-His-190. In all D1-His-190 mutants examined, the reduction of Y_Z was slowed dramatically [224,275] but was accelerated substantially by the addition of small organic bases [224]. It was concluded that the hydroxyl proton of YZ remains bound to D1-His-190 during the lifetime of Y*_Z* and that reprotonation of Y*_Z* is required for its reduction [224]. However, deprotonation of the distal nitrogen of D1-His-190 in concert with YZ oxidation would likely prevent the energetically unfavorable [277] formation of the imidazolium cation [224]. These data were taken as additional evidence that Y_Z and D1-His-190 are functionally coupled [224,275].

In intact PS II preparations, the kinetics of Y_Z oxidation are multiphasic, with the dominant phases having half-times of 20–40 ns and 100–300 ns and the remainder having half-times of 2–5 and 20–40 μs [273,278–287]. The ns phases are electron-transfer-limited, exhibiting no deuterium isotope effect [273,283,285,286]. They have been described in terms of non-adiabatic electron transfer theory [29,288,289] and have been proposed to occur in reaction centers where the hydroxyl proton of YZ is either rapidly delocalized after transfer to D1-His-190 [290] or rapidly transferred from D1-His-190 to the luminal surface via one or more proton transfer pathways [29]. In these reaction centers, D1-His-190 is postulated to accept a hydrogen bond from YZ [29,283,289–291], and the amino acid residues within the putative proton transfer pathways are postulated to be in protonation states that are optimal for rapid proton transfer [29]. The μs phases of Y_Z oxidation are proton-transfer-limited, exhibiting significant deuterium isotope effects [286,292]. These phases have been postulated to represent either (i) relaxation processes involving proton movements that shift the equilibrium, P≠_680Y_Z ↔ P≠_680Y*_Z, to the right [286,290,292] or (ii) rate-limiting deprotonation events in proton pathways branching from D1-His-190 to the luminal surface [29]. In the former model, the relaxation processes take place in all reaction centers and correspond to rearrangements of a hydrogen bond network that includes YZ and D1-His-190 [290]. In the latter model, the deprotonation events occur in a fraction of reaction centers: those with proton pathways whose protonation states at the time of the actinic flash are sub-optimal for rapid proton transfer from D1-His-190 to the luminal surface [29]. Proton transfer from D1-His-190 to the luminal surface is envisioned to take place by a concerted ‘bucket brigade’ or ‘domino’ (Grotthuss) mechanism [29].

In Mn-depleted PS II preparations, the oxidation of YZ by P≠_680* is slowed 100–1000-fold [224,259,273,276,285,293–298], shows a deuterium isotope effect, k_H/k_D, of 2–3 [259,273,276,285,298], and is accelerated substantially by the addition of imidazole and other exogenous bases [224,276]. These observations show that, in the absence of the Mn cluster, the interaction between YZ and D1-His-190 is altered so that the oxidation of YZ becomes rate-limited by its deprotonation. The alteration could correspond to an elongation or angular distortion of the hydrogen bond between YZ and His-190. An elongation of a hydrogen bond between an OH group and a nitrogen atom can increase the activation energy (ΔG°) for proton transfer by as much as 10 kcal/mol [299]. If the hydrogen bond is also distorted from its optimal geometry, ΔG° can be even higher [300]. Even when proton tunneling dominates, a 5 kcal/mol increase in ΔG° could slow the rate of proton transfer by several orders of magnitude [301]. Therefore, an elongated or angularly distorted hydrogen bond between YZ and D1-His-190 could explain the slowed, proton-transfer-limited, oxidation of YZ that is observed in the absence of the Mn cluster. However, pulsed EN-DORe measurements conducted with [15N]histidine-labeled, Mn-depleted, PS II preparations from *Synechocystis* sp. PCC 6803 detect no strong, isotropic couplings between [15N]histidine and Y*_Z* analogous to those previously detected [263] between [15N]-
histidine and \(Y^*_D\) [265]. These data imply that no direct hydrogen bond exists between \(Y^*_Z\) and D1-His-190 in the absence of the Mn cluster.

To account for the ENDOR data, three models have been proposed. In one model [259], \(Y_Z\) and D1-His-190 interact intermittently in the absence of the Mn cluster, so that each forms hydrogen bonds with other partners in a significant percentage of reaction centers. In this model, the oxidation of \(Y_Z\) is rate-limited by the transient formation of a hydrogen bond between \(Y_Z\) and D1-His-190. In the absence of the Mn cluster, the hydrogen bonds formed by \(Y_Z\) [302] and \(Y^*_Z\) [259,262,303–305] are known to be heterogeneous compared to those formed by \(Y^*_P\). In a second model, the distance between \(Y_Z\) and D1-His-190 increases substantially (to \(\approx 5\) Å) in the absence of the Mn cluster so that the hydrogen bond between these two residues is lost [276]. In this model, proton transfer from \(Y_Z\) to D1-His-190 takes place via a hydrogen-bonded chain of at least two water molecules that transiently connects \(Y_Z\) with D1-His-190. An analogous situation is found in carbonic anhydrase, where the distance between the proton donor (His-64) and the proton acceptor (a Zn-bound hydroxyl group) in the active site is \(\approx 8\) Å [306–308]. In this model, the activation energy for proton transfer is dominated by the energy required to properly orient \(Y_Z\), D1-His-190, and the hydrogen-bonded water chain that transiently connects them. In a third model, \(Y_Z\) forms a hydrogen bond to D1-His-190, but \(Y^*_Z\) and D1-His-190 form hydrogen bonds with other partners after \(Y_Z\) has been oxidized [265]. This is an example of a dissociative proton-coupled electron-transfer reaction [309]. This model is of particular interest in regard to proposed mechanisms for water oxidation in 'intact' PS II preparations. These mechanisms postulate that, during some [2,12,20–23] or all [19,24–30] of the S state transitions, \(Y^*_Z\) and \(Y_Z\) interact primarily with different species, with \(Y^*_Z\) abstracting a proton from a Mn-bound water molecule or a water-derived Mn ligand and \(Y_Z\) donating this proton to D1-His-190. A dissociative proton-coupled oxidation of \(Y_Z\) in 'intact' PS II preparations could readily be accommodated in the proposed mechanisms.

The data discussed in the last paragraph were acquired with Mn-depleted PS II preparations. In intact PS II preparations, \(Y_Z\) is believed to form a hydrogen bond to D1-His-190 [29,283,289–291]. Some authors have proposed that this bond is so strong that \(Y_Z\) is deprotonated at neutral pH values [289,291]. However, this proposal conflicts with FTIR data showing that the \(\delta C-O-H\) bending mode of \(Y_Z\) is present at pH 6.0 in PS II preparations that retain the Mn cluster [201,302]. Acetate-treated PS II preparations are certainly more intact than Mn-depleted preparations. However, no strong, isotropic couplings between \(^{15}\text{N}\) and \(Y^*_Z\) were detected in acetate-treated, globally \(^{15}\text{N}\)-labeled, PS II preparations from spinach that had been poised in the \(S_2Y^*_Z\) state [265]. This observation implies that no direct hydrogen bond exists between \(Y^*_Z\) and D1-His-190 in acetate-treated PS II preparations, despite the presence of the Mn cluster. However, \(Y_Z\) oxidation is slowed even more dramatically in the presence of high concentrations of acetate [310,311] than in the absence of the Mn cluster. Consequently, the interaction between \(Y_Z\) and D1-His-190 may be significantly distorted or disrupted in acetate-treated PS II preparations.

In Mn-depleted PS II preparations, the rate of \(Y_Z\) oxidation is critically dependent on pH [259,273, 276,294,297]. Depending on buffer conditions, time resolution, and deconvolution procedures, pK\(_A\) values of 7.0–8.3 have been extracted from measurements of the rate of \(Y_Z\) oxidation (\(P^*_\text{\(680\)}\text{\(\rightarrow\)}\)) as a function of pH. Debus and coworkers extracted a pK\(_A\) value of \(\approx 7.5\) from their kinetic data [276] and found a pH-independent deuterium isotope effect, \(k_H/k_D \approx 1.7\), that persisted up to the highest pH value examined (pH = 10). If \(Y_Z\) was deprotonated, one would expect that \(k_H/k_D = 1.0\). Accordingly, \(Y_Z\) was assigned a pK\(_A\) of \(\approx 10.3\), the same value as in the D1-His190Ala mutant (see above) and that of tyrosine in solution. The pK\(_A\) value of 7.5 was then assigned to D1-His-190 [276]. A similar pK\(_A\) value of \(\approx 7.6\) was assigned to D1-His-190 by Styring and coworkers from analyses of the flash-induced yield and subsequent decay of chlorophyll \(a\) fluorescence in \(C.\text{\reinhardtii}\) thylakoid membranes [275]. Junge and coworkers obtained data similar to that of Debus and coworkers but found that \(k_H/k_D < 1.1\) [273]. Accordingly, these workers assigned \(Y_Z\) a pK\(_A\) value of \(\approx 7.0\). Diner and coworkers, working at a lower time resolution and deconvoluting their data differently, extracted a pK\(_A\) value of \(\approx 8.3\) and found that
$k_{H}/k_{D}$ was sharply pH dependent, being maximal at pH 7 ($k_{H}/k_{D} = 3.6$) and decreasing at both lower and higher pH values, with the decrease at higher pH values having an apparent $pK_A$ value of 8.0 [259]. Accordingly, these workers assigned $Y_Z$ a $pK_A$ value of 8.0–8.3. Additional data will be required to determine the actual $pK_A$ value for $Y_Z$ in Mn-depleted PS II preparations and the degree to which it is altered by D1-His-190.

5. Conclusions

Our understanding of the protein environment of the water oxidizing complex has increased steadily over the last decade. The overall structure of PS II is becoming clearer, amino acid residues that modulate the properties of $Y_Z$ and the Mn cluster have been identified, and new models for water oxidation have been proposed. Although no amino acid ligand of the Mn cluster has been identified unambiguously, many candidates have been identified and spectroscopic characterization of site-directed mutants is being pursued vigorously. The next 5 years should see continued progress as advanced spectroscopic methods such as pulsed and parallel polarization EPR [12] and FTIR difference spectroscopy [177] continue to be applied to mutant and isotopically labeled PS II preparations and as new methods (e.g., low frequency FTIR [177,205,208,312,313], low frequency resonance Raman [314], and solid-state NMR [159]) are developed and applied. The recent application of His-tag methodology to the isolation of intact PS II particles [315–318] should provide further impetus to these studies.

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References
