An Activated Glutamate Residue Identified in Photosystem II at the Interface between the Manganese-stabilizing Subunit and the D2 Polypeptide

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Photosystem II (PSII) catalyzes the oxidation of water during oxygenic photosynthesis. PSII is composed both of intrinsic subunits, such as D1, D2, and CP47, and extrinsic subunits, such as the manganese-stabilizing subunit (MSP). Previous work has shown that amines covalently bind to amino acid residues in the CP47, D1, and D2 subunits of plant and cyanobacterial PSII, and that these covalent reactions are prevented by the addition of chloride in plant preparations depleted of the 18- and 24-kDa extrinsic subunits. It has been proposed that these reactive groups are carbonyl-containing, post-translationally modified amino acid side chains (Ouellette, A. J. A., Anderson, L. B., and Barry, B. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2204–2209 and Anderson, L. B., Ouellette, A. J. A., and Barry, B. A. (2000) J. Biol. Chem. 275, 4920–4927). To identify the amino acid binding site in the spinach D2 subunit, we have employed a biotin-amine labeling reagent, which can be used in conjunction with avidin affinity chromatography to purify biotinylated peptides from the PSII complex. Multidimensional chromatographic separation and multistage mass spectrometry localizes a novel post-translational modification in the D2 subunit to glutamate 303. We propose that this glutamate is activated for amine reaction by post-translational modification. Because the modified glutamate is located at a contact site between the D2 and manganese-stabilizing subunits, we suggest that the modification is important in vivo in stabilizing the interaction between these two PSII subunits. Consistent with this conclusion, mutations at the modified glutamate alter the steady-state rate of photosynthetic oxygen evolution.

In plants, algae, and cyanobacteria, oxygenic photosynthesis converts solar energy into chemical energy. Photosystem II (PSII) is one of two photosynthetic reaction centers in the thylakoid membrane of photosynthetic organisms. PSII catalyzes the light-driven oxidation of water and reduction of plastoquinone. X-ray structures of PSII are available from the cyanobacterium, Thermosynechococcus, at resolutions of 3.8–3.0 Å (1–4). The structures reveal a dimeric organization. Each monomer is formed by more than 15 intrinsic subunits and three extrinsic subunits (5, 6). For higher plants, PSII structures are still limited to ~8 Å (7), but intrinsic plant PSII subunits display a high homology to their cyanobacterial homologs. Together with the small polypeptides cyt b559 α, cyt b559 β and PsbI (8), D1 and D2 form the PSII reaction center complex, which contains the redox-active prosthetic groups involved in photo-induced electron transfer. The oxidation of water to molecular oxygen occurs within the PSII oxygen-evolving complex, a tetranuclear manganese-calcium metallocluster (1–4, 9). Chloride also plays an important, but poorly understood role, in oxygen production (10).

Modifications of amino acid side chains play essential roles in enzymatic catalysis (11), regulation (12), and protein turnover (13). For the intrinsic PSII subunits, proteolytic processing of the N (14) and C terminus (15, 16), N-acetylation (14), palmitylation (17), and several phosphorylation sites (18) have been identified. A generalized susceptibility of the reaction center proteins D1 and D2 to oxidation, after treatment with high concentrations of detergents and chaotropic agents, has been reported (19). However, the locations of the modified amino acids in the protein sequence were not determined, and it is not known if those putative oxidative modifications occur under more physiological conditions. Other amino acid modifications, which have been identified within the PSII subunits, CP43 and CP47, (20, 21) are also a consequence of redox reactions. Because experimental evidence suggests that oxidative damage may trigger degradation of subunit D1 and D2 subunits (22), identification of oxidized amino acids residues in D1 and D2 is an important goal. These oxidized sites may constitute a specific site, which triggers turnover under native conditions.

Biochemical evidence has shown that amines covalently label the D1 and D2 subunits in cyanobacterial and plant PSII (23, 24). The reactive groups were attributed to a modified, carbon

cytochrome; MS, mass spectrometry; MudPIT, Multidimensional Protein Identification Technology; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WT, wild type; MSP, manganese-stabilizing subunit; MALDI, matrix-assisted laser desorption/ionization.
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 activated amino acid side chain in each subunit. These covalent reactions were observed in plant PSII only in the absence of extrinsic PSII subunits; this result was attributed to a change in access to the labeling sites after extrinsic subunit removal. Labeling was observed both in subunit-depleted oxygen-evolving preparations and in manganese-depleted PSII preparations. In the oxygen-evolving preparations, addition of chloride inhibited binding; the functional dependence suggested that the binding reactions occurred at or near the water splitting site. Because the oxidative deamination of primary amines was also observed in PSII (23), at least one of the D1- and D2-modified amino acids must be redox-active.

Modified amino acids, which contain carbonyl groups and react with amines, have been identified previously. Examples of such carbonyl-containing amino acids in other enzymes include trihydroxphenylalaninequinone (11), lysine tyrosylquinone (25), tryptophan tryptophylquinone (26), and cysteine tryptophylquinone (27). The barley lipid transfer protein has a lipid molecule attached through an ester linkage to a modified aspartate, which can be displaced by hydroxylamine (28). In PSII, previous work has identified an aspartyl aldehyde in the PSII CP47 subunit as an amine-binding site (21).

For the elucidation of side chain modifications, the resolutions of the PSII structures (1–4) are still insufficient. Therefore, mass spectrometric methods play a valuable role. Based on the MS/MS spectra, programs using algorithms based on partial de novo sequencing (29, 30) or spectral alignment (31, 32) enable the identification of novel post-translational modifications. In this work, we use a biotin-amine reagent, avidin affinity purification, and multistage mass spectrometry to identify the site of covalent amine modification in the D2 subunit.

EXPERIMENTAL PROCEDURES

Isolation of PSII Membranes—PSII membranes were isolated according to Berthold et al. (33) with modifications (24). The preparation was carried out under green safelight illumination and at 4 °C. PSII membranes with steady-state rates of oxygen evolution in the range of 800–1000 μmol O₂/(mg chl·h) were obtained (34). The isolated PSII membranes were directly treated with 2 M NaCl to remove the extrinsic 18- and 24-kDa subunits (35). Salt-washed PSII membranes were further depleted of MSP and manganese by incubation in 800 mM Tris, pH 8.0 at room temperature in the light for 45 min (36). The PSII membranes were washed three times in the 25-fold volume of 400 mM sucrose, 50 mM HEPES, pH 7.5. After the final wash step PSII membranes were resuspended in the same buffer to yield a chlorophyll concentration of 2 mg/ml, resulting in a Tris concentration below 50 μM and a chloride concentration below 10 μM. The Tris-washed PSII membranes were frozen in N₂(g) and stored at −70 °C.

Sample Preparation and Electrophoretic Separation—Binding of 4 mM 5-(biotinamido)-pentylamine (Invitrogen, Carlsbad, CA) or 4 mM [14C]methylamine to Tris-treated PSII membranes was performed under room light and at room temperature as described previously (23, 24). Solubilization of PSII membranes was performed at 4 °C for 30 min at a chlorophyll concentration of 0.55 mg/ml in a medium containing 25% (w/v) glycerol, 50 mM Bis-Tris·HCl, pH 6.0, and 2% (w/v) n-dodecyl-β-D-maltoside (Anatrace, Maumee, OH) in the dark under continuous agitation. After insoluble material was removed by centrifugation (30,000 × g, 30 min), the sample was applied to a gradient gel with a total polyacrylamide concentration of 3.5–16% and separated in the first dimension by blue-native gel electrophoresis (BN-PAGE) as described in Ref. 37. 3 mg of chlorophyll was loaded per gel (16 × 16 × 0.3 cm). Relevant parts of the BN-gel were excised and analyzed by Tricine-SDS-PAGE in the second dimension as described in Ref. 38. Gels were either stained with Coomassie R250 or were blotted onto polyvinylidene difluoride membranes (39). An avidin-alkaline phosphatase conjugate (Invitrogen, Carlsbad, CA) was used for selective detection of biotinylated proteins.

In-gel Digestion and Peptide Isolation—The D1/D2 spot was cut out of the two-dimensional gel and digested with 2.5 μg/ml porcine trypsin (modified by reductive methylation and TPCK treatment (Promega, Madison, WI) overnight at 37 °C (40). The peptides, extracted from the gel, were dried completely by lyophilization. The peptides were reconstituted in phosphate buffer, loaded onto a monomeric avidin resin, and subjected to affinity purification in spin columns with a bed volume of 100 μl as described in Ref. 41, 42. For detection and quantification of the biotinylated peptides, an ELISA-like affinity reaction between biotin and a strepavidin-horseradish peroxidase-conjugate (Pierce) (43) was employed to monitor elution of the affinity column (data not shown). Affinity-purified samples were dried completely by lyophilization and stored at −20 °C.

LC-MS Analysis—Lyophilized peptide samples were reconstituted in 50 μl of buffer A (95% water, 5% acetonitrile, 0.1% acetic acid) and analyzed on a triphasic MudPIT (44, 45) column with 4 cm of a C18 resin and 4 cm of SCX resin, coupled to a 10 cm analytical C18 column, which had been pulled to a 5-μm emitter tip. For MudPIT analysis, six ammonium acetate salt steps from 0 mM to 500 mM in buffer A were employed. For reverse phase chromatography, a gradient of buffer A (95% H₂O, 5% acetonitrile, 0.1% acetic acid) and buffer B (20% water, 80% acetonitrile, 0.1% acetic acid) was used. For MS analysis, a Thermo LTQ Orbitrap mass spectrometer was operated in 8-s cycles, consisting of one 400–2000 m/z FTMS and eight MS-MS LTQ scans.

Data Analysis—For analysis of the LC-MS/MS data, Sequest (46) was applied for peptide identification versus a data base, consisting of all spinach protein sequences in the NCBI data base and typical contaminants. The detection of unknown post-translational modifications was performed employing Inspect (32). An upper mass limit of 800 Da was set for the post-translational modification, because of performance constraints from the software. This takes into account post-translational modifications with masses of up to 476 Da prior to derivatization with the biotin-amine compound. The output from Inspect was then validated by submission to Sequest, with the assigned mass shift from Inspect as the mass of the putative modification.

Mutants—The Synechocystis sp. PCC 6803 mutants, E302V (D2) and E307V (D2), were a generous gift from Prof. W. Vermaas (47). These mutants and a wild-type strain of Synechocystis were grown under photoautotrophic conditions in BG11 medium, supplemented with 5 mM TES-NaOH, pH 8.0 (48), under constant illumination for 7 days. After the cell harvest,
steady-state rates of oxygen evolution were measured in BG-11 containing 0.8 mM recrystallized 2,6-dichlorobenzoquinone and 2 mM potassium ferricyanide, as exogenous electron acceptors (34).

RESULTS

For our investigation, PSII membranes were isolated from market spinach (33) and were incubated with the primary amine 5-(biotinamido)-pentylamine (B5A, Fig. 1), after the removal of extrinsic subunits and manganese (35, 36, 49). The reactivity of the biotin compound was estimated in a competition experiment with [14C]methylamine, which had been employed in earlier investigations (20, 21). The autoradiogram (supplemental Fig. S1) showed a 40% decrease in [14C]methylamine labeling, when the B5A concentration was raised to 20 mM (at 4 mM methylamine). The reduction in labeling efficiency, when B5A and methylamine are compared, is attributed to the larger size of the biotin reagent. Notice that all three amine labeling sites, in the CP47, D1, and D2 subunits, react with B5A (supplemental Fig. S1).

After labeling with the biotin-amine reagent, the PSII membranes were solubilized in 2% dodecylmaltoside and subjected to BN-PAGE (37). BN-PAGE is a native electrophoresis technique, which separates membrane-integral protein complexes by their size and their tendency to bind the anionic dye, Coomassie G250 (50). After separation in the first dimension by BN-PAGE, the PSII dimer lanes were subjected to SDS-PAGE as a second dimension of analysis. In the first dimension, the solubilized PSII membranes were separated into different PSII complexes (Fig. 2A, vertical lanes A–C and E) and the highly abundant LHCII complex (Fig. 2A, lane G). By comparison to results in the literature (51), lanes A–C were assigned to dimeric PSII and lane E was assigned to monomeric PSII. Lanes C lacks the minor light-harvesting proteins CP29, CP26, and CP24, which are present, to some extent, in lanes A and B (see discussion of two-dimensional gel below). In addition, minor amounts of cytochrome b_{6f} complex (Fig. 2A, lane F) and trace amounts of PSI (Fig. 2A, lane D) were observed in the gel.

In the second dimension, the protein subunits of each individual complex species were separated under denaturing conditions, and the subunits of each type of protein complex appear as spots in vertical lines in the two-dimensional gel (Fig. 2A). To unambiguously identify the protein complexes present in the first dimension, the spots on the two-dimensional gel were subjected to in-gel digestion and identification by MALDI-MS/MS. For each PSII complex species (Fig. 2A, vertical lanes A–C and E), the identity of all significantly stained spots was determined. For the other complexes, only one protein spot was analyzed. The identified proteins are described in the Fig. 2 legend.

Fig. 2B shows a Western blot, which was used to display the degree of biotinylation. While the degree of biotinylation relative to the amount of protein appears to be higher for the monomeric PSII species (Fig. 2B, lane E), the absolute amount is highest in lane C. Therefore, subsequent steps of our investigation, focused on the double spot 3, 4 in lane C. These spots were identified as the D1 and D2 subunits by MS, as described below. These subunits were subjected to in-gel tryptic digestion, and
avidin affinity chromatography was employed for the enrichment of biotinylated peptides. The resulting sample was analyzed by two-dimensional LC-MS/MS, using MudPIT (44, 45), Sequest (46), and DTASelect (52). This approach led to unambiguous identification of the D1 and D2 proteins, with a sequence coverage of 39 and 30%, respectively, when accepting matches with cross-correlation-coefficients of 2.5 and above for doubly charged peptides. This analysis showed that the D1 and D2 subunits in spots 3,4 were free of common contaminating proteins, such as keratin, proteins used during the biochemical procedures, such as BSA, trypsin, and avidin, as well as other, non-PSII-derived thylakoid proteins. A few peptides from other PSII subunits, such as CP47, CP43, and MSP, were occasionally found in the D1/D2 samples.

Because programs frequently used for analysis of MS and MS/MS data, including Sequest or Mascot (54), are unable to detect unknown post-translational modifications, Inspect (31, 55), was employed. Inspect is specialized for the detection of unknown post-translational modifications using a spectral alignment algorithm (31, 55). Because the site of interest is biotinylated, candidate modifications were constrained to be mass shifts of greater than or equal to 227 Da. This mass shift corresponds to the mass of biotin label without the 1,5-pentandiamine-linker (C10H15N2O2S).

The modified peptide, AYDVSQE*IR, was transferred from the SCX part of the MudPIT column at the 500 mM ammonium acetate step onto the analytical C18 column, and eluted from the C18 column after 59.6 min in the reversed phase gradient, when an acetonitrile concentration of 19% was reached (Fig. 3, inset, red chromatogram). The unmodified peptide, AYDV-SQEIR, was also detected by both programs with a cross-correlation coefficient of 3.3. This mass shift is expected from the binding of the biotin label, when a loss of one water molecule is assumed during the binding reaction. This identification was subsequently validated, performing a Sequest search (Xcorr: 3.3) with a predefined differential mass shift of 310.2 for glutamate. A complete set of b- and y-ions (b2–b9, y2–y9) was observed in the MS/MS spectrum. Additional signals in the mass spectrum can be assigned to singly charged a-ions, as well as double charged b- and y-ions.

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**DISCUSSION**

To identify amine binding sites in the heterodimeric D1/D2 core of PSII, we have conducted experiments with a biotinylated amino compound. None of the twenty proteinogenic amino acids side chains forms stable covalent products with primary amines without a preceding post-translational modification. In this and prior investigations (23, 24), the PSII amine adducts have been shown to be stable to biochemical manipulations, withstanding pH values from 3 to 9 during isolation and detection. Substantial (60–80%) labeling stoichiometries have been reported for methylamine and phenylhydrazine (24).

By employing two-dimensional electrophoresis prior to digestion and affinity purification of biotinylated PSII peptides, the complexity of the peptide analysis was decreased. This was demonstrated by the subsequent MudPIT analysis, which showed that the extracted mixture contained primarily tryptic peptides of D1 and D2. This purification enabled an efficient use of Inspect software, which profits significantly from the use of the NCBi data base (supplemental materials, Fig. S2), showed that the D2 residue Glu303, identified by our affinity-labeling approach, was located within the PSII subunits. Glu302 is located at the interface between the D2 and MSP subunits, with Lys186 in the MSP subunit and Tyr315 in the D2 subunit as the two closest neighbors.

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**FIGURE 4.** A, activated glutamate within the cyanobacterial structure of PSII (PDB ID:2axt (4); D1: blue; D2: purple; MSP: yellow; CP43, CP47: black). The residues Glu302, Tyr315 in D2, and Lys186 in MSP from *T. elongatus* are the homologs to residues Glu303, Tyr316, and Lys843, respectively, found in the *Spinacia oleracea* PSII subunits. Glu302 is located at the interface of the MSP and D2 at a distance of 23 Å and 20 Å from manganese cluster and tyrosine D, respectively. B, local environment surrounding glutamate 302 (Glu302–Lys186: 2.7 Å, Glu302–Tyr315: 2.8 Å).
303 from the D2 subunit with a mass shift of 310.2 Da in the MS/MS data (Fig. 3). This mass shift was confirmed by the mass difference of 310.1830 Da deduced from the Orbitrap-MS spectra, which was obtained immediately prior to the MS/MS spectra. No combination of amino acids in the protein sequence adjacent to the peptide on the N- or C-terminal site can be employed to explain this mass shift. Because of potential drifts in the mass analyzer caused by electric field and temperature changes, the LTQ-Orbitrap MS instrument was externally calibrated prior to each experiment. A mass accuracy within 5 ppm is expected with nominal mass resolving power of 60,000. If, as an upper limit, a mass accuracy of 10 ppm is assumed, and the composition of the covalent modification is limited to the elements C, H, N, O, S, and P, the number of potential modifications can be limited to just four different sum formulas: C_{15}H_{26}N_{4}O_{1}S_{1} (310.1825 Da, relative mass deviation 1.60 ppm), C_{18}H_{32}N_{10}O_{4} (310.1825 Da, relative mass deviation 1.60 ppm), C_{15}H_{26}N_{4}O_{1}S_{1} (310.1838 Da, 2.58 ppm), C_{11}H_{26}N_{4}O_{6} (310.1882 Da, 7.09 ppm), and C_{15}H_{26}N_{4}O_{1}S_{1} (310.1827 Da, 0.97 ppm). Of these sum formulas, only C_{15}H_{26}N_{4}O_{1}S_{1} provides the potential for stable chemical structures under the conditions employed here and is also the most probable modification based on the measured mass shift. The sum formula of C_{15}H_{26}N_{4}O_{1}S_{1} and mass shift of 310.1827 Da correspond to the labeling of the 5-(biotinamido)-pentamine tag at the side chain of glutamate 303, with the loss of one water molecule (Fig. 1). Based on the identification of the peptide and modification site with two independent algorithms, the high accuracy of the mass obtained by Orbitrap-MS, and the correspondence with the chemical formula of the biotin-labeling reagent, both the location and the identity of the labeled glutamate can be deduced with high confidence.

A multiple alignment study shows that glutamate 303 is conserved in all known, complete D2 sequences. In Synechocystis sp. PCC 6803, a mutant, E302V, of the homologous glutamate 302 was isolated during a screen for photoheterotrophic mutants in the C-terminal region of the D2 subunit (47). Because E302V grows photautotrophically, the mutant was not further analyzed in the previous study. In the present investigation, however, we were able to show that this mutant is significantly impaired in oxygen evolution, when compared either to wild type or to a E307V D2 mutant (47).

In the structure of T. elongatus PSII (4), glutamate 302 (D2) is found at the interface between the D2 and MSP subunits (Fig. 4A). Glu^{302} (D2) is 23 Å from the manganese cluster and 20 Å from tyrosine D (Tyr^{160}, D2). At a distance of 2.7 Å from MSP residue Lys^{186}, Glu^{302} establishes the closest contact site between D2 and MSP. Like Glu^{302}, Lys^{186} (MSP) is strongly conserved in the vast majority of published MSP sequences and is homologous to Lys^{243} in the spinach sequence. The proximity of Lys^{186} (MSP) to Glu^{302} (D2) suggests that the environment of Glu^{302} (D2), and its second nearest neighbor Tyr^{315} (D2), will be significantly altered by depletion of the MSP subunit (Fig. 4B). Because high resolution structures for PSII are still limited to a resolution of 3.0 Å and are only available for cyanobacterial systems, information on the orientation of amino acid side chains is limited. This is especially true in the proximity of the manganese cluster, which has been structurally compromised by radiation damage (56, 57). Distances between individual side chains might be subject to change in a future improved structure of PSII. However, it should be noted that E302 is consistently placed at the MSP-D2 interface in existing structural models (1–4).

In previous work, it was shown that removal of MSP increases amine labeling of PSII subunits (23, 24). This result is consistent with the identification of Glu^{302} (D2) as an amine labeling site, because MSP provides close contacts with this amino acid side chain (Fig. 4B). The yield of amine labeling was found to be higher at low chloride concentrations (in the absence of the 18- and 24-kDa subunits) and under illumination (23, 24). These changes are attributable to conformational changes in the reaction center, which increase amine access to the D2 subunit. Note that reaction-induced FT-IR spectroscopy has shown that chloride depletion results in structural changes in the oxygen-evolving center (58).

Because carboxylic acids and their anions, as glutamate or aspartate, do not form adducts with amines in aqueous environment, a direct reaction between the glutamate side chain with the biotin-amine compound is highly unlikely. For example, in a control experiment with the unmodified AYDVSQEIR peptide, obtained by solid-phase synthesis, no reactivity of the biotin label toward the glutamate residue could be observed by ESI-MS or by MALDI-MS (data not shown). In order for amine labeling to occur, the electrophilicity of the carboxylate group must be enhanced by converting the negatively charged oxygen into a better leaving group. For example, in solid phase peptide synthesis, reactants like dicyclohexylcarbodiimide are used to activate carboxylic acids for amide bond formation.

The identity of the group that activates the glutamate is of interest but cannot be addressed definitively with our approach, because amine binding displaces this functional group. However, the PSII structure suggests one possible activation mechanism, involving the aromatic side chain of Tyr^{315} (D2), which is 2.8 Å from the labeled glutamate (Fig. 4B). In the activating mechanism shown in Fig. 5, tyrosine Tyr^{315} and glutamate Glu^{302} are covalently linked originally to form an aromatic ester. One electron oxidation of the linked aromatic ester generates a radical cation, which in turn may dissociate to give a neutral tyrosyl radical and carboxonium ion at the Glu^{302} side chain (Fig. 5). The carboxonium ion reacts with 5-(biotinamido)-pentamine to form the biotinylated glutamate. Reaction with water will compete with amide formation to give a product indistinguishable from the unmodified glutamate side chain. Because the water concentration is much higher than the amine concentration, the modified ester must be sequestered from solvent. Such a mechanism is congruent with previous EPR studies, which have obtained evidence for a post-translationally modified tyrosine side chain, M^{ox}, in site-directed mutants of PSII (59–61). In the previous analysis, it was shown that M^{ox} is generated by light-induced oxidation reactions in PSII.

Amine binding is observed in oxygen evolving spinach PSII preparations, which have been depleted of the 18- and 24-kDa subunits, in spinach PSII, which has been depleted of MSP and manganese, and in oxygen-evolving cyanobacterial PSII (23). Therefore, the activating post-translational modification is likely to be present in PSII before purification. Note that the
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FIGURE 5. Mechanism involving tyrosine, Tyr315, as the activator of E303D2. In this model, Tyr315 and Glu303 are covalently associated to form an aromatic ester. The ester is cleaved by light-induced oxidation of Tyr315 to generate a carboxonium ion and a neutral tyrosyl radical. In the presence of amines, this reaction leads to the formation of the amine-tagged glutamate. Alternatively, the unmodified glutamate can be formed by reaction of the carboxonium ion with water.

putative aromatic ester may be formed in PSII enzymatically or may be a consequence of light-induced electron transfer reactions, which lead to cross-linking of amino acid side chains.

Analysis of the E302V mutation provides evidence for the functional significance of the modified glutamate. For example, a reduction in the rate of steady state oxygen evolution by 50% is similar to the rate reduction observed in tyrosine D (Tyr160 D2) mutants (62). A possible role for the modified glutamate may be in stabilization of the intersubunit interaction between D2 and MSP. The MSP subunit plays an important role in the optimization of photosynthetic oxygen evolution (63) and may function as a proton donor/acceptor during parts of the oxygen-evolving cycle (53).

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