Site-directed Mutations at Residue 251 of the Photosystem II D1 Protein of Chlamydomonas That Result in a Nonphotosynthetic Phenotype and Impair D1 Synthesis and Accumulation*

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In Cyanobacteria and Chlamydomonas reinhardtii, substitution of valine for alanine at position 251 of the photosystem II D1 protein in the loop between transmembrane helices IV and V confers resistance to herbicides that reduce photosystem II function and increases sensitivity to photoinhibition. Using site-directed mutagenesis and chloroplast transformation in Chlamydomonas we have examined further the role of residue 251 in relation to D1 structure, function, and photosynthetic performance. Of the 12 different amino acid substitutions for Ala251 introduced at this position, five (Arg, Asp, Gln, Glu, and His) resulted in a nonphotosynthetic phenotype. Transformants with the Arg251 substitution synthesize a normal sized 32-kDa D1 protein with greatly reduced stability. The Gln, Glu, His, and Asp transformants make a 33–34-kDa form of the D1 protein of varying stability as well as an immunologically related polypeptide of 24–25 kDa corresponding to the N-terminal portion of D1 that is unstable and appears to be an aborted D1 translation product. All mutant forms of the D1 protein are intrinsic to the thylakoids. In contrast to previous studies in Cyanobacteria showing that residues in the IV–V loop can be mutated or deleted without loss of photosynthetic competence, our results suggest that Ala251 has a key role in the structure and function of the IV–V loop region.

In all oxygen-evolving organisms, the reaction center of the photosystem II (PSII) complex consists of the D1 protein, the structurally related D2 protein, cytochrome b559, and at least two small proteins of unknown function (1). The rapidly turning over D1 protein continuously undergoes a cycle of damage, degradation, and replacement in response to photodamage from normal PSII photochemistry (2). The D1 protein, encoded by the chloroplast psbA gene, is synthesized as a membrane associated 33.5-kDa precursor with a C-terminal extension. After processing to the 32-kDa mature form, D1 undergoes several posttranslational modifications postulated to facilitate translocation and proper assembly into PSII centers in the lipid bilayer (3), where it binds chlorophyll, pheophytin, quinone, carotenoid, iron, and manganese (4). D1 is thought to have five hydrophobic membrane-spanning helices, with its N terminus facing the chloroplast stroma and its C terminus projecting into the thylakoid lumen (5, 6). One stromally exposed region of D1, extending from the C terminus of helix IV through the N terminus of helix V (IV–V loop), participates in binding both Qa, the second stable quinone acceptor in PSII, and several classes of herbicides that inhibit photosynthetic electron transport (6). The IV–V loop region includes astromal helix thought to lie parallel to the membrane surface (6) that divides this loop into two parts, one thought to be involved in D1 degradation in vivo and the other functioning in herbicide and quinone binding (7, 8).

Phylogenetic conservation of the IV–V loop among Cyanobacteria, algae, and higher plants (9) suggests that most of these amino acid residues should be essential. However, numerous deletions and amino acid substitutions in this region of D1 in Cyanobacteria do not abolish photosynthetic competence (10–15). Substitution of Val for Ala251, in the putative membrane parallel helix between transmembrane helices IV and V, is reported to confer resistance to certain PSII herbicides and to increase sensitivity to photoinhibition in Cyanobacteria and in Chlamydomonas (8, 16). This modification also affects the electron transfer reactions on both the acceptor and donor sides of PSII, resulting in a reduced electron transfer rate between QA and QB (17), a decrease of the affinity binding constant of QB for its site (7), and modification of the normal oscillatory pattern of oxygen evolution (8).

Since these results pointed to a relationship between photosensitivity and an alanine-to-valine substitution at position 251 of the D1 protein, we have isolated and characterized homoplastic chloroplast transformants of Chlamydomonas reinhardtii with 12 of the 19 possible alterations at Ala251 of D1. Our results show that the Cys, Gly, Ile, Leu, Pro, Ser, and Val mutants retain photosynthetic function to different levels and will be described in a forthcoming article, whereas the Arg, Asp, Gln, Glu, and His mutants are nonphotosynthetic. In this article, we describe the capacity of these nonphotosynthetic mutants with respect to D1 synthesis, accumulation, and degradation. The Arg mutant makes a mature sized 32-kDa D1 protein with greatly reduced stability, whereas the other four mutants synthesize and accumulate a 33–34-kDa form of D1 of varying stability as well as a very unstable 24–25-kDa peptide that appears to be an aborted...
D1 translation product. Our results suggest that Ala251 plays a key role in the overall tertiary structure of D1 and consequently in the function of PSII.

MATERIALS AND METHODS

In Vitro Site-directed Mutagenesis, Molecular Cloning Strategies, and Plasmids—The 900-bp \( KpnI \)-EcoRI chloroplast DNA fragment from wild-type \( psbA \) containing the fourth exon (18) with adjacent intron sequences was subcloned into Bluescript KS\(^+\) vector (Stratagene) from P-528 (19). Single-stranded DNA prepared according to the method of Sambrook et al. (20) was mutated with the 32-nucleotide-long oligodeoxynucleotides psbAArg (5'-CTTCAAATTGTATGGCTTTCTAGATGAACCAATGGGGCA-3') and psbAGlu (5'-CTTCAATATTGTATGACTGC/TCTAATGATCTGACTGC/TACATAATGTAACAC-3') or psbAHisAasp (5'-CTTCAATATTGTATGC/TCTAGATCTGACTGC/TACATAATGTAACAC-3') using an oligodeoxynucleotide-directed in vitro mutagenesis system (version 2.1; Amersham Corp.). In addition to the mutations at position 251 (bold), a new \( SpfI \) restriction site (underlined) was created by substituting a \( T \) for a \( G \) (bold) at position 247, which does not lead to an amino acid substitution (Fig. 1A). After transfection of XL1-Blue cells (Stratagene), bacterial colonies were screened by hybridization with the aforementioned radiolabeled oligodeoxynucleotides (20). Alterations at D1 positions 247 and 251 were verified by sequencing (Life Technologies, Inc., double-stranded DNA cycle sequencing system, and \( [\gamma^{32}P]ATP, DuPont NEN). Mutagenized 250-bp \( BstEII-EcoRI \) fragments covering the end of exon 4 and the beginning of intron 4 (Fig. 1A) were then inserted into P-528 to replace the corresponding wild-type sequence. The resulting plasmids, P-529 (Ala \( \rightarrow \) Arg, As251R*), P-598 (Ala \( \rightarrow \) Gin, A251Q*), P-599 (Ala \( \rightarrow \) Glu, A251E*), P-601 (Ala \( \rightarrow \) His, A251H*), and P-802 (Ala \( \rightarrow \) Asp, A251D*) have amino acid alterations at position 251 and the \( SpfI \) restriction fragment marker at position 247 (designated by the asterisk). The codons for the introduced amino acids at position 251 were chosen according to the codon usage of the D1 protein in Chlamydomonas and are therefore expected to be properly translated.

The \( Np \) strain (CC-2835) of Lers et al. (21) was used to create the mutant A251D*::NP by exchanging the last 180 bp of exon 5 from \( psbA \) of the \( A. \) reinhardtii parental sequence (CC-125) with the amplified fragment generated from P-388 (21), thus introducing the stop codon at Ser245* and the new A\( II \) restriction fragment marker into the mutated \( psbA \) gene. The \( Bsm1-BamHI \) fragment of P-602 was replaced by the \( Bsm1-KpnI \) fragment of P-388, as explained below (see Fig. 1A). P-388 (21) was digested with \( KpnI \), and the overlapping ends were filled in using the Klenow reaction (20). P-602 (see above) was digested with \( BsmI \) and filled in using Klenow and T4 DNA polymerase. Both linearized plasmids P-388 and P-602 were then digested at the unique \( BsmI \) restriction site located in the middle of the fifth exon of \( psbA \) (Fig. 1A). The 11-kilobase fragment obtained from P-602 was then ligated to the 500-bp fragment derived from P-388. The chimeric plasmid was transformed into XL1-Blue cells to generate P-653. The Ala251* to Asp and the Ser245* to stop codon changes (21) were verified by sequencing.

Integumentation of the Mutant \( psbA \) Gene into the \( C. \) reinhardtii Chloroplast Genome, Selection of Transformants, and Determination of Homoplasmy—Each of the above plasmids carrying \( psbA \) mutations was introduced into wild-type strain CC-125 by cotransformation (22) together with P-228 (21), which possesses a point mutation in the 16 S rRNA gene conferring spectinomycin resistance (Fig. 1A). A wild-type control strain carrying both the spectinomycin resistance marker and the restriction fragment marker at position 247 of \( psbA \) was also created by transforming CC-125 (19) with P-528 and P-228. After transformation, cells were resuspended on Tris acetate-phosphate (TAP) medium—

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that their PSII centers are inactive even after dark adaptation and exhibit the high fluorescence phenotype typical of strains impaired in PSII function (27). All five mutants show an elevated level of dark adapted chlorophyll a fluorescence ($F_v$) as well as a reduction in $F_v/F_m$ (Table I), an indicator of the photochemical efficiency of PSII. Damage to PSII centers is known to result in the rise of the initial $F_v$ level (27), whereas differences in $F_0$ and $F_v/F_m$ between the mutants suggest that their PSII function is altered to various extents.

**Accumulation of D1 and D1-related Polypeptides—Immunoblot analysis of total cell extracts reveals that D1 accumulation in the mutants is perturbed to varying degrees with respect to wild type (Fig. 2, A and B). Antisera against the tubulin b subunit and OEE1, one of the three extrinsic proteins of the oxygen-evolving complex, were used as controls for equal loading. A251R* is the only mutant that accumulates nearly normal amounts (75%) of the 32-kDa mature form of the D1 protein (Fig. 2C). In contrast, the A251Q*, A251E*, A251H*, and A251D* mutants accumulate a slower migrating 33–34-kDa form of D1 (P33–34) (Fig. 2B) that is distinct from the D1 precursor. Indeed, a A251D* transformatant that carries the NP preprocessed mutation (A251D*-NP mutant) lacking the C-terminal D1 extension (21) still synthesizes P33–34 (Fig. 2D). Variations in D1 size were confirmed in a comparative pulse-labeling experiment (see below and Fig. 4B). Our inability to detect the P33–34 peptides by immunoblot analysis of deletion mutants in the psbA gene encoding D1 (CC-1078 and FUD 7; Ref. 19) proved that these peptides are not contaminant proteins recognized by the D1 antibody (data not shown) but, rather, variant forms of the D1 protein.

The A251Q*, A251E*, A251H*, and A251D* mutants also accumulate varying amounts of a 24–25-kDa peptide (P24–25), which is recognized (Figs. 2 and 3) and immunoprecipitated (data not shown) by the D1 antibody. When 1% LDS (final concentration) is used for protein solubilization, P24–25 is barely detectable, and a band migrating at the same position as the mature D1 protein can be seen (Fig. 2A). This latter band disappears, and P24–25 is intensified when the solubilization buffer contains 2% LDS, suggesting that accumulated P24–25 might be tightly associated with another small peptide of about 8–9 kDa (Fig. 2, compare A and B). Since we do not observe this association during pulse-chase labeling experiments, it must occur at a later time (see “Discussion”). The similar upshift of both P33–34 and P24–25 in the Glu and Asp mutants compared with the respective peptides in the Gln and His mutants is likely due to the negative charge of the amino acid introduced in the former mutants.

P33–34 accumulates up to ~45–50% of the wild-type D1 level in the A251H* and A251D* mutants but only reaches 9 and

### Table I

**Analysis of growth, O$_2$ evolution ($P_{max}$) and chlorophyll a fluorescence parameters $F_v$ and $F_v/F_m$ of transformants homoplasmic for mutations at the Ala$^{251}$ residue**

| Genotype | HS | TAP | MET | $P_{max}$ | $F_0$ | $F_v/F_m$ |
|----------|---|----|----|--------|-----|---------|
| WT       | + | + | - | 120    | 25.0 | 0.77    |
| A251R*   | - | + | + | 96.0   | 51.5 | 0.24    |
| A251Q*   | - | - | + | 53.0   | 65.0 | 0.23    |
| A251H*   | + | - | + | 32.0   | 61.0 | 0.46    |
| A251D*   | + | - | + | 68.0   | 60.1 | 0.41    |

*a* Cells were either grown on minimal HS medium (autotrophic growth), on acetate-containing TAP medium (mixotrophic growth), or on TAP medium containing 20 mM metronidazole (MET) at different light intensities from 70 to 600 μmol/m$^2$/s.

*b* Cells were pregrown in TAP medium at LL (15 μmol/m$^2$/s).

+; growth; −, no growth.
15% of the wild-type D1 level in the A251Q* and A251E* mutants, respectively (Fig. 2C). P24–25 as well as P33–34 is found in purified thylakoids of these mutants (shown for the A251D* mutant in Fig. 3), demonstrating that it can assemble into the photosynthetic membrane. Furthermore, extraction of polypeptides from thylakoids by various treatments (see "Materials and Methods") showed that P24–25 is intrinsic and as resistant to extraction as mature wild-type D1 and P33–34 (Fig. 4A). This latter band is a more rapidly migrating conformer of the mature 32-kDa protein (28). The band above P24–25 (●) of unknown origin is recognized by the D1 antisera in both mutant and wild type. C, quantification of D1 (the mature form or the slower migrating P33–34 form plus the conformer and P24–25 when present) in the different mutants from 2% LDS gels. These results are representative of those observed in six separate experiments. D, immunoblot of total cell proteins from the WT control and the A251D*, A251D*:NP, and NP mutants probed with D1 antisera. The band above P24–25 (●) is a polypeptide of unknown origin recognized by D1 antisera in both mutant and wild type. The band above P24–25 (●) is a polypeptide of unknown origin recognized by D1 antisera in both mutant and wild type. We hypothesize that the band in the A251D* mutant at the position of the wild-type D1:D2 heterodimer is a P33–34:D2 heterodimer. These heterodimers are artifacts arising during subcellular fractionation and thylakoid purification and are never detected in total cell extract immunoblots or in in vivo pulse-chase labeling experiments.

**FIG. 2.** Accumulation of D1 and immunologically related proteins in the nonphotosynthetic D1 residue 251 mutants and WT grown at low light. A, immunoblot of total cell proteins solubilized with 1% LDS, resolved onto 10–17.5% LDS-PAGE, electroblotted to nitrocellulose, and probed with a mixture of antisera specific to D1, β-tubulin and the 29-kDa oxygen-evolving protein OEE1 (40). B, immunoblot of total cell proteins solubilized with 2% LDS and probed with the D1 antibody. In these conditions, D1 in wild-type and the A251R* mutant migrates as a doublet, one band migrating at 32 kDa and another at 29 kDa (●). This latter band is a more rapidly migrating conformer of the mature 32-kDa protein (28). The band above P24–25 (●) of unknown origin is recognized by the D1 antisera in both mutant and wild type. C, quantification of D1 (the mature form or the slower migrating P33–34 form plus the conformer and P24–25 when present) in the different mutants from 2% LDS gels. These results are representative of those observed in six separate experiments. D, immunoblot of total cell proteins from the WT control and the A251D*, A251D*:NP, and NP mutants probed with D1 antisera.

**FIG. 3.** Immunoblot of total cell (C) and thylakoid (T) proteins from the A251D* mutant and WT grown at LL. Proteins were run on LDS-PAGE, electroblotted to nitrocellulose, and probed with D1 antisera. The band above P24–25 (●) is a polypeptide of unknown origin recognized by D1 antisera in both mutant and wild type. We hypothesize that the band in the A251D* mutant at the position of the wild-type D1:D2 heterodimer is a P33–34:D2 heterodimer. These heterodimers are artifacts arising during subcellular fractionation and thylakoid purification and are never detected in total cell extract immunoblots or in in vivo pulse-chase labeling experiments.

**FIG. 4.** Synthesis of D1 and D1 related proteins from the D1 residue 251 mutants and WT. Log phase cells grown in reduced sulfate- and acetate-containing medium at LL were equilibrated and preincubated in anisomycin to block cytoplasmic protein translation, and 5-ml aliquots were pulse labeled with 625 Ci of [35S]H2SO4, as described under "Materials and Methods." Labeled proteins separated by 10–17.5% gradient 1% LDS-PAGE were visualized by autoradiography, and D1 and D1-related peptides were identified by their migration relative to immunoprecipitated D1 and D1-related proteins on LDS-PAGE. A, pulse labeling of D1 and D1-related proteins in mutant and WT cells over a 20-min time course. The gels shown are representative of four independent experiments. B, longer term (1 h) labeling of D1 and related proteins in the A251* mutants and wild type compared with the NP mutant, which synthesizes a preprocessed D1 protein.
Changes at Ala^{251} That Impair D1 Synthesis and Accumulation

TABLE II

Effect of the D1 Ala^{251} mutations on the synthesis of the D1 and D1-related proteins

| Normalized rates of synthesis | WT | A251R* | A251Q* | A251H* | A251E* | A251D* |
|------------------------------|----|--------|--------|--------|--------|--------|
| D1 (% of WT)                | 100 | 51.1   | NA     | NA     | NA     | NA     |
| P33–34 (% of WT D1)         | NA  | NA     | 10.8   | 6.0    | 15.0   | 7.1    |
| P24–25 (% of WT D1)         | NA  | NA     | 7.0    | 34.6   | 107.2  | 53.2   |
| P33–34 + P24–25 (% of WT D1)| NA  | NA     | 17.8   | 40.6   | 122.2  | 60.4   |
| Ratio of rates of synthesis | 1   | NA     | 0.7    | 5.8    | 7.2    | 7.5    |

*NA, not applicable.

whereas the A251E* and A251D* mutants make a 25-kDa peptide. P24–25 is detected very early during the pulse-labeling experiment, before any P33–34 is seen, suggesting that it is probably not an N-terminal D1 degradation product. Furthermore, we never observed labeling of the smaller C-terminal peptide of 7–8 kDa, which would be expected if P33–34 was cleaved to yield P24–25.

D1 synthesis in the A251R* mutant occurs at ~50% of the wild-type rate (Table II). In the A251Q*, A251H*, A251E*, and A251D* mutants, synthesis of P33–34 is greatly reduced (6–15% of the wild-type D1 rate), and synthesis of P24–25 ranges from 7 to 10% of the wild-type rate of D1 synthesis. The A251H*, A251E*, and A251D* mutants synthesize P24–25 at a much higher rate than P33–34 (Table II and Fig. 4A). If the rates of synthesis of P24–25 and P33–34 are summed for each mutant, they amount to 18–122% of the wild-type rate of D1 synthesis.

Stability of D1 and D1-related peptides (P33–34 and P24–25) was determined by pulse-chase experiments (Fig. 5). In the A251Q* and A251H* mutants P33–34 is as stable as wild-type D1 (a greater than 6-h half-life), whereas its stability in the A251D* and A251E* mutants is only one-third to one-half of that of wild-type D1. In the A251R* mutant, D1 has only a 45-min half-life. Where present, most of the P24–25 is very unstable (half-life, 10–20 min) and disappeared extremely rapidly without any concomitant increase of label in P33–34. A small fraction of the P24–25 synthesized seems to be stable over the 3-h chase and might explain the accumulation seen on immunoblots (Fig. 2B). Since P24–25 appears before any detectable P33–34 during the pulse (Fig. 4A) and does not chase into P33–34 or vice versa (Fig. 5), this polypeptide is neither a D1 breakdown product nor a translation intermediate that will eventually give rise to full-length D1. Instead it appears to be an aborted D1 translation product, which terminates at a particular domain (see “Discussion”).

DISCUSSION

Although the loop between transmembrane helices IV and V of the D1 protein is highly conserved among Cyanobacteria, algae, and plants (9), most amino acid substitutions and several deletions created in this region, alone or in combination, still retain a certain level of photosynthetic capacity (Fig. 6). The ratio of rates of synthesis P24–25 to P33–34 is also given for each mutant.

Physiological Characterization of the Nonphotosynthetic A^{251} Mutants—Chloroplast transformants of Chlamydomonas having Arg, Glu, His, or Asp substituted for Ala at residue 251 in the D1 protein are incapable of photosynthetic O₂ evolution and defective in photosynthetic electron transport, as indicated by their resistance to metronidazole. Furthermore, their high F₅ₐ values are consistent with the other photosynthetic parameters in suggesting that the mutant PSII centers are much less efficient as energy traps than in the wild type (27).

Synthesis and Accumulation of an Electrophoretic Variant of the D1 Protein in the A251Q*, A251H*, A251E*, and A251D* Mutants—Immunoblot analysis (Fig. 2A) as well as pulse-la-
beling experiments (Fig. 4) clearly show that the Gln, His, Glu, and Asp mutants accumulate a modified form of the D1 protein (P33–34) that has a slightly reduced electrophoretic mobility but still localizes to the thylakoid membranes (Fig. 3). P33–34 was shown not to be the 33.5-kDa D1 precursor accumulated as a consequence of impaired C-terminal processing (Fig. 2C). A slower migrating form of D1 (D1*) resulting from N-terminal phosphorylation occurs in higher plants after photoinhibitory treatments (29, 30) but is never the exclusive form accumulated in vivo or in vitro, as is the case for P33–34 in these mutants under nonphotoinhibitory conditions. Phosphorylation of D1 has never been observed in C. reinhardtii (31) despite a conserved Thr residue present at its N terminus (18). For these reasons, P33–34 is likely to result from structural modifications of D1 other than phosphorylation that might protect it from proteolytic attack in the same manner as the N-terminal phosphorylation of D1 does in higher plants (32).

The discrepancy between P33–34 turnover and steady state levels in some of the mutants suggests that different forms of P33–34 with different half-lives might coexist in the thylakoid membrane. For example, in the Gln and His mutants, P33–34 is poorly synthesized but seems to be stable over 3 h in the pulse-chase labeling experiment. Although almost no P33–34 accumulates in the Gln mutant, a substantial amount is observed in the His mutant. Similar differences between the half-life of D1 determined by pulse-chase labeling and its accumulation analyzed by immunoblot (Fig. 2, A and B). Most of the P24–25 accumulated appears to be associated with another small protein of 8–9 kDa, giving rise to a dimer product running at about 32 kDa. This 8–9-kDa protein might be a PSI component that promotes assembly of P24–25 into the reaction center and protects it from degradation. Cross-linking of the α subunit of cytochrome b559 and the D1 protein has been shown to occur in isolated PSI reaction centers after a light treatment (35). Integration of the chlorophyll α and b-binding protein CAB-7p into photosystem I proceeds through a membrane intermediate that is originally cleavable and becomes protease-resistant during assembly (36). Thus stability of many photosynthetic membrane proteins seems to be promoted by their integration into photosynthetic complexes.

In barley, D1 is synthesized by thylakoid-bound polysomes, and translation intermediates of 15–25 kDa can be chased into full-length D1 (37). Six ribosome pausing sites, A–F, which correlate with D1 translation intermediates, are thought to be important for cotranslational binding of cofactors to D1 and for the insertion of the five transmembrane helices of this protein into the chloroplast thylakoids (38). Because the D1 proteins of C. reinhardtii and barley are highly similar in structure (>94% amino acid identity; Ref. 9), the P24–25 peptide we detect in the Gln, Glu, His, and Asp mutants might originate from an abortive translation in the C-terminal portion of the D1 IV-V loop at the last (pause F) or next to last (pause E) ribosome pausing site. Since these mutants also synthesize varying amounts of P33–34, we suggest that the nascent D1 polypeptide either: (i) is cleaved proteolytically during ribosome pausing at the E or F sites, yielding the unstable P24–25; or (ii) undergoes a conformational change or posttranslational modi-
fixation making it resistant to cleavage in the protease-sensitive loop connecting helices IV and V and yielding P33–34. Since D1 residue 251 influences Qb binding and herbicide resistance (8), the inability of the mutants to bind quinone during D1 synthesis might render the protein sensitive to proteolytic cleavage downstream of Ala251. Based on the molecular weight of P24–25, the predicted cleavage points would be between residues 258 and 269 near the C-terminal end of the IV-V loop.

During acceptor side photoinhibition, a D1 N-terminal peptide of 23 kDa is observed both in vitro (39) and in vivo (28) under certain physiological conditions. This D1 breakdown product should not be equated with the P24–25 D1 translation product we observe in the Gln, Glu, His, and Asp mutants for several reasons. First, the mutant cells were not subjected to any photoinhibitory treatment. Second, the 24–25 kDa peptide appears before we can detect P33–34 during a pulse-labeling experiment (Fig. 4A). Third, the synthesis rate of this peptide is greater than the synthesis rate of P33–34, and the ratio of P24–25 to P33–34 synthesis rates remains constant throughout the time course of a pulse experiment (data not shown). In the case of a degradation product, this ratio would have been expected to increase as a function of time (28).

The A251R* Mutant—The nonphotosynthetic Arg mutant synthesizes a mature sized 32-kDa D1 protein but neither P24–25 nor P33–34 (Figs. 2 and 4). If the D1 protein in this mutant is properly incorporated into PSII complexes of the thylakoid membrane with normal cofactor interactions, its capacity for photosynthetic electron transfer must be blocked for other reasons. Pereswoka et al. (8) showed that the Ala251 to Val change in Cyanobacteria has a long range side effect on the donor side of the protein in destabilizing the oscillatory pattern of oxygen evolution (O states). The A251R* mutation could have severe detrimental effects on the photochemistry of PSII without destroying the structure of the IV-V loop. Surprisingly, no breakdown products resulting from either acceptor side or donor side photoinhibition (39) were detected in a pulse-chase experiment, although the D1 protein in the Arg mutant was found to be very unstable (half-life, ~45 min) under these LL conditions (Fig. 5). As discussed earlier, forms of D1 with different half-lives coexisting in the thylakoids of this mutant could explain the high level of D1 accumulation seen on immunoblots, since synthesis is only 50% of that of wild type, and newly synthesized D1 appears rather unstable. Although the predominant form has a short half-life, a small fraction could be stable and accumulate in the membranes.

In conclusion, we find that substitution of amino acids that are charged and/or have a very long side chain for D1 residue Ala251 results in a nonphotosynthetic phenotype unlike most other amino acid substitutions and many deletions created in the IV-V loop of the D1 protein. The Gln, Glu, His, and Asp mutants represent the first instances in which a single amino acid substitution in D1 has been observed to result in premature termination of its translation. In contrast, the Arg mutant synthesizes nearly normal amounts of a mature sized D1, which is nonfunctional and appears rather unstable. Although all the mutant forms of the D1 protein were shown to be intrinsic in the thylakoid membrane, we do not know whether they assemble into PSII complexes. Collectively, these results suggest that the Ala251 residue of D1, in the putative parallel helix between transmembrane helices IV and V where the quinone binds, plays a critical role in the structural conformation of the IV-V loop and therefore in the proper electron flow between Qa and Qb. Etienne and Kirilovsky (7) have suggested in Cyanobacteria that D1 amino acids 248–251 at one end of this parallel helix may be buried in the thylakoid membrane, thus dividing the IV-V loop into two parts, one involved in Qb binding and the other in D1 degradation. Our findings support this hypothesis, since substitution of charged or very polar amino acids for Ala251 is likely to modify this association severely due to interactions with polar head groups of the lipid bilayer.
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