Biophysical, Biochemical, and Physiological Characterization of Chlamydomonas reinhardtii Mutants with Amino Acid Substitutions at the Ala251 Residue in the D1 Protein That Result in Varying Levels of Photosynthetic Competence

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The Q_b binding site of the D1 reaction center protein, located within a stromal loop between transmembrane helices IV and V formed by residues Ile219 to Leu272, is essential for photosynthetic electron transport through photosystem II (PSII). We have examined the function of the highly conserved Ala251 D1 residue in this domain in chloroplast transformants of Chlamydomonas reinhardtii and found that Arg, Asp, Gln, Glu, and His substitutions are nonphotosynthetic, whereas Cys, Ser, Pro, Gly, Ile, Val, and Leu substitutions show various alterations in D1 turnover, photosynthesis, and photoautotrophic growth. The latter mutations reduce the rate of Q_A to Q_B electron transfer, but this is not necessarily rate-limiting for photoautotrophic growth. The Cys mutant divides and evolves O_2 at wild type rates, although it has slightly higher rates of D1 synthesis and turnover and reduced electron transfer between Q_A and Q_B. O_2 evolution, D1 synthesis, and accumulation in the Ser, Pro, and Gly mutants in high light is reduced, but photoautotrophic growth rate is not affected. In contrast, the Ile, Val, and Leu mutants are impaired in photoautotrophic growth and photosynthesis in both low and high light and have elevated rates of D1 synthesis and degradation, but D1 accumulation is normal. While rates of synthesis/degradation of the D1 protein are not necessarily correlated with alterations in specific parameters of PSII function in these mutants, bulkiness of the substituted amino acids is highly correlated with the dissociation constant for Q_A in the seven mutants examined. These observations imply that the Ala251 residue plays a key role in D1 protein.

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Photosystem II (PSII)

The QB binding site of the D1 reaction center protein, located within a stromal loop between transmembrane helices IV and V formed by residues Ile219 to Leu272, is essential for photosynthetic electron transport through photosystem II (PSII). We have examined the function of the highly conserved Ala251 D1 residue in this domain in chloroplast transformants of Chlamydomonas reinhardtii and found that Arg, Asp, Gln, Glu, and His substitutions are nonphotosynthetic, whereas Cys, Ser, Pro, Gly, Ile, Val, and Leu substitutions show various alterations in D1 turnover, photosynthesis, and photoautotrophic growth. The latter mutations reduce the rate of Q_A to Q_B electron transfer, but this is not necessarily rate-limiting for photoautotrophic growth. The Cys mutant divides and evolves O_2 at wild type rates, although it has slightly higher rates of D1 synthesis and turnover and reduced electron transfer between Q_A and Q_B. O_2 evolution, D1 synthesis, and accumulation in the Ser, Pro, and Gly mutants in high light is reduced, but photoautotrophic growth rate is not affected. In contrast, the Ile, Val, and Leu mutants are impaired in photoautotrophic growth and photosynthesis in both low and high light and have elevated rates of D1 synthesis and degradation, but D1 accumulation is normal. While rates of synthesis/degradation of the D1 protein are not necessarily correlated with alterations in specific parameters of PSII function in these mutants, bulkiness of the substituted amino acids is highly correlated with the dissociation constant for Q_A in the seven mutants examined. These observations imply that the Ala251 residue plays a key role in D1 protein.

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Photosystem II (PSII) complexes of all oxygen-evolving organisms convert light energy into chemical free energy by transferring electrons from water to plastocyanin, associated with the release of molecular oxygen (1, 2). The D1 protein of the PSII reaction center turns over in a light-dependent manner more rapidly than any other chloroplast protein and is a primary target for photoinhibitory damage (3, 4). Based on homology with the crystal structure of the L protein from Rhodopseudomonas viridis (5), D1 is predicted to have five hydrophobic membrane-spanning helices (6). One of the stromally exposed regions of D1, extending from the C terminus of transmembrane helix IV through the N terminus of transmembrane helix V (IV–V loop), includes a stromal helix thought to lie parallel to the membrane surface (6). This loop, whose amino acid sequence is highly conserved among cyanobacteria, algae, and higher plants (7), is involved in binding both Q_B, the second stable quinone acceptor in PSII, and several classes of herbicides that inhibit photosynthetic electron transport at the Q_B docking site (8). Only a few of the many amino acid substitutions made in this region result in the loss of D1 function and photosynthetic capability (9, 10), suggesting that most positions may tolerate considerable variation in R group conformation or charge and still permit D1 function. However, particular residues may be necessary for optimal PSII activity or may provide functional advantages under certain environmental conditions (11, 12). Analysis of site-directed mutations in the region between Ile248 and Ala251 at one end of the parallel helix has led to the hypothesis that these residues may be buried in the thylakoid membrane (11), thus dividing the IV–V loop of D1 into two segments. One segment is postulated to be involved in Q_B binding and herbicide resistance (from the parallel helix to the helix V) and the other possibly in D1 degradation (from helix IV to the parallel helix).

Substitution of Val for Ala251 in the D1 protein in cyanobacteria and Chlamydomonas results in herbicide resistance and increased sensitivity to photoinhibition (13, 14), reduced photosynthetic yield associated with slower photoautotrophic growth (11, 15), and perturbation of the pattern of oxygen evolution in single turnover flashes (16). To examine further the role of the D1 Ala251 residue in PSII function and light sensitivity, we generated 12 of the 19 possible amino acid substitutions at this position in Chlamydomonas reinhardtii.

The abbreviations used are: PSII, photosystem II; LL, low light; HL, high light; LHCP, light-harvesting chlorophyll a/b-binding protein; LSU, large subunit of ribulose-bisphosphate carboxylase oxygenase; TL, thermoluminescence; DL, delayed luminescence; ST, single turnover; FRR, fast repetition rate; FRRF, FRR fluorometry, MTF, multiple turnover flash; STF, single turnover flash; LDS, lithiumdodecyl sulfate.
Five of these substitutions (Arg, Asp, Gln, Glu, and His) were shown to lead to a nonphotosynthetic phenotype and to impaired D1 synthesis and accumulation (10). Seven (Cys, Gly, Ile, Leu, Pro, Ser, and Val), which retain photosynthetic function to various degrees, affect herbicide resistance and photoautotrophic growth rates to various degrees (17). In this paper, we examine D1 synthesis and turnover, photosynthetic rate, quantum efficiency, and electron transfer between QA and QB in these seven mutants. We also show that the bulkiness of the R group of the amino acid substituted in each of 12 AlA251 mutants is correlated with the dissociation constant (Kd) for plastoquinone in the QA pocket, consistent with the hypothesis that the AlA251 residue of D1 is structurally involved in QA binding and is likely to be buried in the thylakoid membrane (11).

MATERIALS AND METHODS

Strains and Culture Conditions—Wild type and mutant strains of C. reinhardtii designated "CC" are available from the Chlamydomonas Genetics Center at Duke University (Dr. E. H. Harris, Box 91000, Duke University, Durham, NC 27708). Stocks were maintained on plates of solid TAP or HSHAYA media (18) under dim light (10 μmol m⁻² s⁻¹). For physiological, biochemical, and biophysical analysis, cells were grown photoautotrophically in liquid minimal HS medium (18) bubbled with 5% CO₂ in air under low light (LL; 70 μmol m⁻² s⁻¹) or high light (HL; 600 μmol m⁻² s⁻¹) at 25 °C. Cells were harvested in early exponential to midexponential growth (up to 2 × 10⁶ cells ml⁻¹), but sufficient doublings were allowed for full adaptation to each environmental condition. In HL, these conditions are saturating for maximal rates of efficient doublings were allowed for full adaptation to each environmental condition. In HL, these conditions are saturating for maximal rates of

Photophysical, Biochemical, and Biophysical Analysis of A251 mutations—The A251A* (wild type control, CC-3394, GCT), and A251D* (CC-3387, GTT), A251R* (CC-3376, CGT), A251Q* (CC-3374, CAA), and A251F* (CC-3370, CCG) mutants are correlated with the dissociation constant (Kd) for plastoquinone in the QA pocket, consistent with the hypothesis that the A251 residue of D1 is structurally involved in QA binding and is likely to be buried in the thylakoid membrane (11).

In Vitro Site-directed Mutagenesis and Chloroplast Transformation of C. reinhardtii—The A251A* (wild type control, CC-3394, GCT), and D1 mutants A251G* (CC-3393, GCT), A251T* (CC-3388, ATT), and A251C* (CC-3387, GTT), A251R* (CC-3376, CGT), A251Q* (CC-3374, CAA), A251D* (CC-3394, GCT), A251L* (CC-3389, CTT) in addition to A251G*, which was already obtained by site-directed mutagenesis. All A251A* transformants (10) are kept under conditions of acetosyringone (10, 17). Each amino acid alteration at position 251 is correlated with the dissociation constant (Kd) for plastoquinone in the QA pocket, consistent with the hypothesis that the A251 residue of D1 is structurally involved in QA binding and is likely to be buried in the thylakoid membrane (11).

Recovery of Some Site Suppressors—Photosynthetically competent same site suppressors arise spontaneously when cells of the nonphotosynthetic A251E* (CC-3377, GAA), A251H* (CC-3378, CAC), and A251D* (CC-3387, GTT) mutants are grown under conditions of acetosyringone (10, 17). Each amino acid alteration at position 251 is correlated with the dissociation constant (Kd) for plastoquinone in the QA pocket, consistent with the hypothesis that the A251 residue of D1 is structurally involved in QA binding and is likely to be buried in the thylakoid membrane (11).

Pulse labeling of HL-grown cells was carried out as described in Refs. 10, 20, and 21. The antisera were prepared as described in Refs. 10, 20, and 21. The antisera were prepared as described in Refs. 10, 20, and 21.

Fluorescence Measurements—Fluorescence measurements were done as described in Refs. 10, 20, and 21. Fluorescence measurements were done as described in Refs. 10, 20, and 21.

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perimenterially as the ratio of amplitudes of the first two monoexponential components (one ~300 μs and two ~2 ms in wild type) of Q_{B'} reoxidation in dark-adapted samples, following a single turnover (ST) flash. The semiquinone equilibrium constant \( K_a \) was determined from fluorescence decay kinetics by calculating the fraction of reduced \( Q_a \) 25 ms following the ST flash (29). Thus, \( K_a = \frac{q}{(Q_a)} \) 25 ms, and \( K_{op} \) was calculated as \( K_{op} = K_a (1 + K_b) \). Alternatively, \( K_{op} \) was determined experimentally from analysis of the decay of DL in the 0.1–100 s time interval as the ratio of the number of centers recombining from \( Q_b \) (slow component, \( 1 = -6 s \)) to the number of centers recombining from \( Q_a \) (fast component, \( 1 = -0.7 s \)) or as a ratio of the half-times of these two decay components (15). Although each of these methods provided slightly different absolute values of \( K_{op} \), \( K_{op} \) was highest when determined from \( Q_a \) 25 ms, the relative changes of \( K_{op} \) between wild type and each of the mutant strains were independent of the method used.

The remaining kinetic parameters of the two-electron gate model \( (k_{ea}, k_{ha}, k_{bh}, k_{br}) \) were calculated by solving the set of equations in Ref. 15.

**Estimation of the Number of PSII Centers in the Thylakoid**—The numbers of PSII reaction centers were determined from \( O_2 \) flash yields (30, 31). In wild type (CC-125), 2 chlorophyll \( a/O_2 \) averages 2014 mol/mol for cells grown at 70 μmol quanta m \(^{-2} \) s \(^{-1} \). Since each \( O_2 \) is generated from four sequential electron transfers through PSII, the numbers of PSII reaction centers can be calculated as 1 per 503 chlorophyll \( a \) (25).

We extended this approach to all mutants by calculating the functional PSII reaction centers can be calculated as 1 per 503 chlorophyll \( a \) (25).

**Estimation of Carbon Assimilation**—Cell carbon and nitrogen were determined by filtering replicate aliquots of known cell densities on precombusted Gelman A/E glass fiber filters. The filters were rinsed with distilled water, dried, and combusted in a Perkin-Elmer 241 Elemental Analyzer. Carbon-specific growth rates were calculated as described by Falkowski et al. (32).

**Estimation of Amino Acid Bulkiness**—In the absence of a robust, high resolution structural model for D1, molecular dynamic calculations of the predicted effects of amino acid substitutions at position 251 on \( Q_b \) binding are highly speculative. For this reason, we calculated the bulkiness of the substituted amino acids at position 251 as the volume of space occupied by their van der Waals surface (33). These calculations do not account for changes in binding properties associated with side chain charge distributions or tertiary structural interactions between amino acid 251 and other residues in the binding domain.

**RESULTS**

**Isolation of Site-directed D1 Mutants and Recovery of Same Site Suppressors with Varying Levels of Photosynthetic Competence**—Three site-directed D1 mutants resulting from amino acid substitutions at residue Ala\(^{251}\) (A251G\(^{a}\), A251I\(^{a}\), and A251V\(^{a}\)) retain some capacity for photoautotrophic growth. Four additional mutants at this position (A251C\(^{a}\), A251S\(^{a}\), A251P\(^{a}\), and A251L\(^{a}\)) plus A251G\(^{a}\), were recovered from the nonphotosynthetic A251R\(^{a}\) transformant (10) as spontaneous same site suppressors capable of photoautotrophic growth. Each of the codon changes in these suppressors required only a single base pair alteration, shown by site-directed mutagenesis to result in a Lys (AAA) revertant of the A251Q\(^{a}\) and A251H\(^{a}\) mutants. All of the Ala\(^{251}\) revertants expressed the Asn, Lys, and Tyr mutations predicted were all recovered among the suppressors. In contrast, the Arg and His mutants, which were shown previously to be nonphotoautotrophic when created by site-directed mutagenesis (10), were not recovered among the suppressors.

In vitro mutagenesis was used to change the wild type GCT Ala codon to the mutant CTG Arg codon. All possible mutations resulting from single base pair changes at the first, second, and third position of the mutant GCT Arg codon are indicated. The Ser, Gly, Cys, Pro, and Leu mutations predicted were all recovered as photoautotrophically competent suppressors. In contrast, the Arg and His mutants, which were shown previously to be nonphotoautotrophic when created by site-directed mutagenesis (10), were not recovered among the suppressors.

**Levels of D1 and LHCP Proteins**—Levels of the D1 and LHCP proteins of PSII have been examined in LL- and HL-grown cells by immunoblotting (Fig. 2, A and B), with the tubulin \( \beta \)-subunit used as a control for equal loading. Variations seen in the proportions of the 32- and 29-kDa D1 conformers might result from the specific amino acid amino acid substitutions introduced. Therefore, both bands were summed to give the total amount of D1 present, and these values were normalized to the amount of \( \beta \)-tubulin protein. When grown under LL, all mutants, with the exception of A251C\(^{a}\), showed a slight reduction (10–20%) in D1 level, expressed as the sum of the 32- and 29-kDa conformers, compared with wild type (Fig. 2B). In contrast, the D1 level under HL growth varied considerably between mutants. Thus, although A251C\(^{a}\) and A251I\(^{a}\) did not differ markedly from wild type, A251G\(^{a}\), A251S\(^{a}\), and A251P\(^{a}\) showed 16–28% reduction from the wild type levels, and A251L\(^{a}\) and A251V\(^{a}\) appeared to contain elevated amounts of D1 (Fig. 2B). The 24–25-kDa D1 polypeptides, thought to represent N-terminal aborted translation products in the A251Q\(^{a}\), A251I\(^{a}\), A251H\(^{a}\), and A251D\(^{a}\) D1 mutants with nonphotosynthetic phenotypes (10), were absent in all seven D1 mutants capable of photoautotrophic growth. The level of D1 in wild type, A251C\(^{a}\), A251G\(^{a}\), A251S\(^{a}\), and A251P\(^{a}\) under HL was 57–59% of that in the same genotypes under LL. In contrast, under HL A251I\(^{a}\), A251V\(^{a}\), and A251L\(^{a}\) contained 68–80% of the LL amount of D1.

HL-grown cells of wild type, A251C\(^{a}\), A251I\(^{a}\), A251L\(^{a}\), A251P\(^{a}\), and A251V\(^{a}\) contain 29–35% less LHCP compared with cells of the same genotypes grown under LL (Fig. 2B). This reduction in LHCP in the A251A\(^{a}\) wild type and in A251C\(^{a}\)

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2. B. Förster, unpublished observations.
D1 Mutations with Substitutions at Ala$^{251}$ in Chlamydomonas

A

WT

Mutants

D1

LL

HL

A251A* 

A251C*

A251G*

A251L*

A251P*

A251S*

A251V*

LL

32 kDa

32

32

30

30

30

30

HL

32 kDa

32

32

30

30

30

30

LL/LH

3

3

3

3

3

3

3

B

Levels of accumulation (% A251A*)

D1

LL

HL

A251A* 100 100

A251C* 98 95

A251G* 96 92

A251L* 91 87

A251P* 87 84

A251S* 89 81

A251V* 91 85

LHCP

LL

100 100

HL

100 100

Relative accumulation (HL/LL, %)

D1

LL

100 100 100 100 100 100

HL

100 100 100 100 100 100

LHCP

LL

100 100 100 100 100 100

HL

100 100 100 100 100 100

FIG. 2. Accumulation of the D1 and LHCP proteins in the A251A* wild type and the seven D1 mutants at residue 251 capable of photoautotrophic growth. Cells were grown in liquid minimal medium in LL (70 μmol m$^{-2}$ s$^{-1}$) or HL (600 μmol m$^{-2}$ s$^{-1}$), total cell protein was isolated, cells were solubilized with LDS, and equal total protein amounts were resolved on 10–17.5% LDS-polyacrylamide gel electrophoresis. A, total cell proteins electroblotted to nitrocellulose membranes, probed with antibodies raised against D1 or LHCP, the β-subunit of tubulin, and the OEE1 oxygen-evolving protein. B, equal total cell protein was isolated, cells were solubilized with LDS, and total cell proteins electroblotted to nitrocellulose membranes, probed with antibodies raised against D1 or LHCP, the β-subunit of tubulin, and the OEE1 oxygen-evolving protein. The photochemical competence of each mutant grown under LL or HL, whereas Ile, Val, and Leu substitutions lead to Ser for Ala does not affect the photoautotrophic growth rates been described elsewhere (17). Substitution of Cys, Gly, Pro, or C-terminal mobility of D1 slightly, whereas the other substitutions were reduced by about one-third compared with wild type (Fig. 3 legend; 1.9 and 2.0 × 10$^3$ versus 3.0 × 10$^3$). Degradation of the newly synthesized D1 during the 2-h chase was elevated in A251G*, A251L*, and A251V* compared with wild type and A251C*. In the case of A251L*, the high D1 degradation rate was correlated with an increased D1 synthesis rate. A251P* had the lowest rate of D1 synthesis and the highest rate of D1 degradation of all of the mutants examined. Rates of LSU degradation were comparable with wild type in A251G* and A251P* (Fig. 3 legend) and lower than wild type in A251C* and A251V*. No labeling of 24–25 kDa bands typical of the aborted D1 translation products found in the nonphotosynthetic D1 mutants (10) was observed.

Photosynthetic $O_2$ Evolution and Whole Chain Electron Transport—The relative apparent quantum yield ($\frac{\Phi_{\text{app}}}{\Phi_{\text{max}}}$), maximum quantum yield ($\frac{\Phi_{\text{max}}}{\Phi_{\text{max}}}$), and maximum rates of light-saturated photosynthetic $O_2$ evolution (P$_{\text{sat}}$ of the aforementioned strains are compared in Table I. Mutants A251C*, A251G*, A251P*, and A251S* grown under LL have P$_{\text{sat}}$ values somewhat lower than wild type, whereas P$_{\text{sat}}$ values for A251I*, A251L*, and A251V* are lower than wild type. All mutants grown in HL except A251C* have lower $\frac{\Phi_{\text{app}}}{\Phi_{\text{max}}}$ and $\frac{\Phi_{\text{max}}}{\Phi_{\text{max}}}$ values than wild type, with A251I*, A251V*, and A251L* lowest of all. With the exception of the A251C* mutant, which has rates of $O_2$ evolution equal to or higher than A251A*, all of the other photoautotrophic mutants appear to be impaired when grown under HL (Table I) and failed to acclimate to HL on a chlorophyll basis. Whereas wild type and A251C* show a 50% reduction in chlorophyll per unit biomass, and higher chlorophyll a/b ratios in HL, other mutants retain chlorophyll per unit biomass ratios and chlorophyll a/b ratios similar to those in LL.

Photoautotrophic Growth and Carbon Accumulation—LL and HL photoautotrophic growth rates for the seven Ala$^{251}$ mutants with varying levels of photosynthetic competence have been described elsewhere (17). Substitution of Cys, Gly, Pro, or Ser for Ala does not affect the photoautotrophic growth rates under LL or HL, whereas Ile, Val, and Leu substitutions lead to reduced growth rates under both conditions (17). This slower growth is not correlated either with decreases in whole chain (ΨWC) or PSII (ΨPSII) electron transfer (Table II) or with the total carbon per cell and the quantum requirement per carbon fixed (Table I). Therefore, the rates of these processes per se in the mutants do not appear to be limiting growth.

Efficiency of Primary Photochemical Charge Separation—The photochemical competence of each mutant grown under LL and HL was assessed as variable fluorescence measured as the single turnover of PSII using FRRF and by saturating, multiple turnover flashes with the PAM system. In general, the two Fv/Fm values obtained for each genotype grown under either LL or HL were in reasonable agreement (Table II), suggesting that potential quenching problems inherent to the PAM method (22) did not occur during the initial 1-s flash used for determination of dark-adapted Fm. The PAM Fv/Fm values for the wild type control A251A* were comparable with previously published values for wild type Chlamydomonas (19, 21). Mu-

correlates with a reduction in total chlorophyll. In contrast, A251G* and A251S* appear to be strongly impaired in their ability to down-regulate levels of both LHCP and total chlorophyll when grown in HL. The relationship between down-regulation of chlorophyll and LHCP in the A251I*, A251L*, A251P*, and A251V* mutants is less clear. Down-regulation of chlorophyll content occurs rapidly when LL-grown wild type cells of C. reinhardtii are shifted to HL (20), and down-regulation of LHCP in the related alga Dunaliella shifted from LL to HL depends on the redox state of plastquinone (34).

Synthesis and Degradation of the D1 Protein—Wild type and the four mutants tested (A251C*, A251G*, A251L*, and A251P*) synthesized D1 at a substantially higher rate than LSU under HL during the 17-min labeling period and degraded D1 faster than LSU during the 2-h chase. Synthesis of D1 in A251C* and A251L* occurred at rates equal to or greater than wild type (Fig. 3), whereas D1 labeling in A251G* and A251P* was reduced by about one-third compared with wild type (Fig. 3 legend; 1.9 and 2.0 × 10$^3$ versus 3.0 × 10$^3$). Degradation of the newly synthesized D1 during the 2-h chase was elevated in A251G*, A251L*, and A251V* compared with wild type and A251C*. In the case of A251L*, the high D1 degradation rate was correlated with an increased D1 synthesis rate. A251P* had the lowest rate of D1 synthesis and the highest rate of D1 degradation of all of the mutants examined. Rates of LSU degradation were comparable with wild type in A251G* and A251P* (Fig. 3 legend) and lower than wild type in A251C* and A251V*. No labeling of 24–25 kDa bands typical of the aborted D1 translation products found in the nonphotosynthetic D1 mutants (10) was observed.
D1 Mutations with Substitutions at Ala$^{251}$ in Chlamydomonas

The reduction in photochemical efficiency in HL suggests a loss of functional PSII reaction centers. Indeed, calculations show that A251G*, A251P*, and A251S* have a lower quantum yield of photochemistry ($\Theta_{\text{max}} = Fv/Fm$) than wild type and the other four mutants when grown under LL or HL and measured with either technique (Table II). $Fv/Fm$ values obtained for the mutants A251G*, A251P*, and A251S* grown under HL approach those measured for the nonphotosynthetic Ala$^{251}$ mutants that range from 0.13 to 0.46 (10).

![Image 246x388 to 554x729](image)

**Table I**

Maximum rate ($P_{\text{max}}$) and efficiency (quantum yield) of photosynthetic $O_2$ evolution at CO$_2$ saturation, chlorophyll (Chl) content, chlorophyll a/b ratio, carbon accumulation, and quantum requirement of carbon fixation (1/Ø) in D1 mutants of C. reinhardtii grown under LL and HL.

Values estimating the efficiency of photosynthetic $O_2$ evolution ($\Theta$) were calculated as a function of absorbed irradiance. The relative apparent quantum yield ($\Theta_{\text{rel}}$) represents the initial slope of the photosynthetic $O_2$ evolution curve, whereas the maximum quantum yield ($\Theta_{\text{max}}$) was calculated based on the optical absorption cross-sections normalized to chlorophyll a ($a$) (25).

| Genotype | $P_{\text{max}}$ | Quantum yield$^a$ | $\Theta_{\text{rel}}$ | $\Theta_{\text{max}}$ | $a$ | Total Chl | Chl a/b | Carbon | 1/Ø$_{\mu}$ |
|----------|-----------------|------------------|------------------|------------------|-----|-----------|--------|--------|-----------|
| LL       |                 |                  |                  |                  |     |           |        |        |           |
| A251A*   | 122 ± 5         | 1.44 ± 0.06      | 0.039            | 0.0096           |     | 39 ± 3    | 2.4    | 36.9   | 13        |
| A251C*   | 146 ± 0         | 1.39 ± 0.01      | 0.037            | 0.0098           |     | 39 ± 1    | 2.3    | 36.3   | 16        |
| A251G*   | 158 ± 4         | 1.33 ± 0.00      | 0.030            | 0.0086           |     | 45 ± 2    | 2.4    | 40.1   | 16        |
| A251P*   | 138 ± 8         | 1.15 ± 0.03      | 0.033            | 0.0091           |     | 38 ± 1    | 2.3    | 35.3   | 17        |
| A251S*   | 142 ± 6         | 1.25 ± 0.03      | 0.032            | 0.0085           |     | 42 ± 1    | 2.4    | 40.1   | 12        |
| A251I*   | 93 ± 3          | 0.95 ± 0.00      | 0.028            | 0.0098           |     | 39 ± 2    | 2.3    | 25.6   | 30        |
| A251V*   | 92 ± 5          | 0.96 ± 0.03      | 0.024            | 0.0093           |     | 38 ± 1    | 2.3    | 30.8   | 29        |
| A251L*   | 76 ± 3          | 0.90 ± 0.00      | 0.021            | 0.0084           |     | 37 ± 2    | 2.3    | 36.3   | 20        |
| HL       |                 |                  |                  |                  |     |           |        |        |           |
| A251A*   | 244 ± 6         | 2.84 ± 0.02      | 0.042            | 0.0097           |     | 18 ± 2    | 2.6    | 39.4   | 29        |
| A251C*   | 282 ± 16        | 2.80 ± 0.01      | 0.049            | 0.0098           |     | 18 ± 1    | 2.6    | 36.9   | 22        |
| A251G*   | 147 ± 2         | 1.03 ± 0.01      | 0.022            | 0.0097           |     | 36 ± 1    | 2.4    | 41.8   | 27        |
| A251P*   | 127 ± 1         | 1.03 ± 0.00      | 0.022            | 0.0099           |     | 38 ± 3    | 2.3    | 38.0   | 36        |
| A251S*   | 117 ± 3         | 0.86 ± 0.01      | 0.018            | 0.0091           |     | 36 ± 1    | 2.4    | 47.0   | 25        |
| A251I*   | 158 ± 7         | 1.26 ± 0.03      | 0.023            | 0.0106           |     | 32 ± 1    | 2.4    | 42.2   | 41        |
| A251V*   | 179 ± 12        | 1.22 ± 0.02      | 0.027            | 0.0094           |     | 29 ± 1    | 2.3    | 34.6   | 57        |
| A251L*   | 112 ± 4         | 0.56 ± 0.03      | 0.018            | 0.0096           |     | 36 ± 1    | 2.5    | 37.1   | 139       |

$^a$ Values for $\Theta_{\text{rel}}$ are shown as ($\mu$mol of $O_2$ mg of Chl$^{-1}$ h$^{-1}$) ($\mu$mol of photons absorbed m$^{-2}$ s$^{-1}$). Values for $\Theta_{\text{max}}$ are $\mu$mol of O$_2$/$\mu$mol of photons absorbed.

![Image](image)
excitation transfer between PSII units (percentage of connectivity, Table II). With the exception of the A251C* mutant, which behaves like wild type, the connectivity is reduced 1.6–1.7-fold in A251S*, A251I*, and A251V* and is virtually absent in A251P*, A251G*, and A251L*. Furthermore, a decrease in the effective absorption cross-section of PSII (functional size of PSII antenna) of 13% can be seen in A251C*, A251I*, A251L*, and A251V* (Table II). In contrast, $\sigma_{PSII}(D1)$ of A251G* and A251P* is slightly elevated and is unaffected in A251S*.

**Photochemical Fluorescence Quenching—**Photochemical fluorescence quenching can be related to the level of reduction of QA, the first stable acceptor of PSII, at a given irradiance. The irradiance dependence of $q_P$ derived during the photosynthetic light response curves of selected mutants is shown in Fig. 4. As observed for photosynthetic $O_2$ evolution, A251C* is the only mutant that has $q_P$ values similar to wild type when grown under both LL and HL. In contrast, A251G* (and A251P*, A251I*, and especially A251L* (as well as A251I* and A251V*)) exhibit a dramatic reduction in $q_P$, particularly when measured at low incident irradiances. The low values of $q_P$ in dark-adapted mutants other than A251C* and the A251A* wild type presumably reflect the higher proportion of nonfunctional (photochemically incompetent) PSII centers in these strains. The coefficient $1 - q_P$ is directly proportional to the average reduction state of QA. At steady-state irradiance, $q_P$ is related to the cross-section of PSII and the rate of oxidation of QA by the equation $(1 - q_P(t)) = (\sigma_{PSII} \times I(t) / \sigma_{PSII} \times I + 1 / \tau_{PSII})$ for any given light intensity ($I$). From Fig. 4 we can deduce that A251A* wild type and A251C* show much lower reduction states of QA than any of the other mutants at both growth irradiances. The more reduced QA pools in these genotypes might potentiate photoinhibitory damage and lead to loss of functional PSII reaction centers.

**Ability of the Reaction Center to Transfer Electrons from QA to $Q_B$ and Stability of $Q_B$ in the D1 Binding Pocket—**The effect of each amino acid substitution on the rate of electron transfer from QA to the plastocyanin pool and the stability of QA in the quinone binding pocket of the D1 protein can be assessed from a combination of variable fluorescence and luminescence measurements. The ratio of the maximum fluorescence yield induced by a multiple turnover flash (MTF) to that observed following a single turnover flash (STF) is indicative of the rate of reduction of secondary plastocyanin acceptors in PSII (i.e. $Q_B$ and the mobile plastocyanin pool) by QA (Table III). MTF/STF ratios ranged from 1.45 in the A251A* wild type control to 1.10 in A251L*, which appears to be most impaired in transferring electrons from QA to $Q_B$. Among the photosynthetically competent mutants, the correlations between MTF/STF ratios and the percentage of PSII centers containing reduced QA measured 25 ms following a saturating flash (Table III) fall into two groups. The A251A* wild type and mutants unimpaired in photosynthetic $O_2$ evolution show a range of MTF/STF from 1.19 to 1.45 with only 11–21% in [QA] 25 ms, whereas the
impaired mutants (A251I*, A251V*, and A251L*) show values of 24–42%. A251V*, which is strongly reduced in $P_{\text{max}}$ like A251I* and A251L*, shows a smaller reduction in MTF/STF and $[Q_A]_2$ 25 ms than the other two mutants. The proportion of the PSII reaction centers with $Q_A$ in the nonphotosynthetic mutants (10) is especially high (89–96%), which suggests that almost no electrons are transferred to $Q_B$ in these mutants at physiologically significant rates.

Fluorescence decay measurements by FRRF estimate the rate of electron transfer to $Q_B$. All mutants have slower electron transfer at PSII ($\tau_{\text{PSII}}$) than A251A*, and the most strongly impaired mutant is A251L* (Table II). Times for whole chain electron transport ($\tau_{\text{WC}}$) can be deduced for wild type and each of the photoautotrophic mutants from their $Q_B$ evolution required for recombination of $S_{2/3}$ states (donor side of PSII) and $Q_B$ (acceptor side of PSII) after trapping the sample in the state of charge separation at PSII. $K_{\text{off}}$ is the apparent equilibrium constant, indicative of the distribution of an electron between $Q_A$ and $Q_B$ at equilibrium. $k_{\text{off}}$ is the rate of $Q_B$ dissociation from its binding site on the D1 subunit. $K_{\text{on}}$ $Q_B$ dissociation constant, $k_{\text{on}}$ is the rate of $Q_B$ binding to its site. $k_{\text{on}}$ and $k_{\text{off}}$ indicate accessibility of the pocket and/or energy of bonds formed to ligate $Q_B$.

The accessibility of the $Q_B$ pocket and/or the energy of bonds formed to ligate $Q_B$ were estimated by the calculation of the kinetic parameters $k_{\text{on}}$ and $k_{\text{off}}$, which reflect the rates of association and dissociation of $Q_B$ with its binding site on the D1 protein, respectively (Table III). The wild type A251A* has $k_{\text{on}}$ and $k_{\text{off}}$ values of 0.48 and 0.23, respectively, indicating stable and efficient binding of $Q_B$ (15). Although A251S* and A251G* have $k_{\text{off}}$ reduced relative to $k_{\text{on}}$ as in wild type, suggesting that $Q_B$ binding in these strains is stable, their lower $k_{\text{on}}$ values indicate a reduced efficiency of $Q_B$ binding to its D1 site compared with wild type. Values of $k_{\text{off}}$ for the other five mutants capable of photoautotrophic growth are at least as high as their $k_{\text{on}}$ values, again suggesting a high instability of $Q_B$ in its binding pocket. Furthermore, the $k_{\text{on}}$ values indicate that the accessibility of $Q_B$ to its site is significantly reduced in all mutants except A251C*. No binding of $Q_B$ to its site on the D1 subunit could be detected in the five nonphotosynthetic mutants (10).

Retention of $Q_A$ reoxidation is correlated with the calculated bulkiness of the substituted amino acids, based on their increased side chain volume length. For example, when the plastoquinone dissociation constant ($K_{\text{Q}}$) was plotted against residue 251 bulkiness, a linear regression coefficient of 0.91 was obtained (Fig. 5), consistent with this residue being directly involved in plastoquinone binding. Indeed, residue 251 is predicted to be a member of the minimum set of amino acids forming the $Q_B$ pocket of D1 (35). Data in Table III show that the $K_{\text{Q}}$ dissociation constants for wild type and the seven mutants with varying photosynthetic capacity do not differ by more than a factor of 5. The accuracy of current modeling procedures does not allow a more quantitative interpretation of the effect of amino acid bulkiness without a detailed molecular structure of the protein complex.

### Table III

| Genotype | MTF/STF | $[Q_A]_2$ 25 ms | TL B | DL $t_{1/2}$ | $K_{\text{app}}$ | $K_{\text{on}}$ | $k_{\text{on}}$ | $k_{\text{off}}$ |
|----------|---------|----------------|------|-------------|----------------|--------------|-------------|-------------|
| A251A*   | 1.45    | 15             | 30   | 4.60        | 5.2            | 0.48         | 0.23        | 0.48        |
| A251C*   | 1.37    | 19             | 11   | 1.11        | 3.3            | 0.91         | 0.42        | 0.48        |
| A251G*   | 1.33    | 21             | 13   | 1.00        | 3.8            | 0.52         | 0.11        | 0.21        |
| A251I*   | 1.27    | 11             | 27   | 2.51        | 5.0            | 1.02         | 0.35        | 0.34        |
| A251S*   | 1.19    | 17             | 14   | 1.30        | 5.2            | 0.33         | 0.08        | 0.24        |
| A251H*   | 1.12    | 37             | 10   | 0.83        | 1.6            | 1.27         | 0.14        | 0.11        |
| A251V*   | 1.22    | 24             | 10   | 0.89        | 3.3            | 0.95         | 0.48        | 0.27        |
| A251L*   | 1.10    | 42             | 9    | 0.85        | 1.4            | 1.49         | 0.22        | 0.15        |
| A251D*   | ND      | 92             | ND   | 0.70        | <0.1          | >3          | ND          | ND          |
| A251N*   | ND      | 96             | ND   | 0.70        | <0.1          | >3          | ND          | ND          |
| A251P*   | ND      | 89             | ND   | 0.70        | <0.1          | >3          | ND          | ND          |
| A251A*   | ND      | 94             | ND   | 0.70        | <0.1          | >3          | ND          | ND          |
| A251R*   | ND      | 95             | ND   | 0.70        | <0.1          | >3          | ND          | ND          |

* ND, none detected.

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D1 Mutations with Substitutions at Ala251 in Chlamydomonas
Ala251 residue led to a nonphotosynthetic phenotype, and we showed that these five amino acid substitutions at the Ala251 residue 251 plays a critical role in conformation and insertion of a bulky, charged amino acid at position 251. These unable to compensate for the structural modification caused by mutations in the D1 protein or in other chloroplast proteins are unable to grow and evolve O2 as well as wild type under LL and HL (Table I), photoacclimates properly during growth in HL by down-regulating its LHCP and chlorophyll levels (Fig. 2B and Table I), and does not show resistance to PSII herbicides (17).

Analysis of the Nonphotosynthetic A251R* Mutant—

The Cys Substitution—For most of the parameters analyzed, the A251C* mutant appears indistinguishable from the wild type A251A* control (this paper and Ref. 17). However, detailed analysis of the electron flow between water and the plastoquinone pool in the thylakoid membranes indicated a noticeable reduction in the rate of Qr reoxidation and a destabilized S3Q2 (S3Q2) state, which can be attributed to a shift of the apparent equilibrium constant (Kapp) between Qr, Qb, and Q3Qb (Table III). Although the Cys substitution has some impact on the electron transfer between QA and Qb, this mutant is able to grow and evolve O2 as well as wild type under HL (Table I), photoacclimates properly during growth in HL by down-regulating its LHCP and chlorophyll levels (Fig. 2B and Table I), and does not show resistance to PSII herbicides (17). Based on the MTF/STF ratio in A251C* (Table III), the ability of this mutant to reduce fully its plastoquinone pool, as well as its overall rate of electron transport from water to the terminal acceptors, is similar to wild type. Cysteine possesses a reversibly reducible SH group, which might help mediate electron flow between QA and Qb. This could explain why the overall photosynthetic output and biomass production in this mutant are indistinguishable from those of wild type.

The A251G*, A251S*, and to a lesser extent A251P* mutants are not only markedly impaired in the electron transfer between QA and QB but also and to a lesser extent A251P* mutants are not only markedly impaired in the electron transfer between QA and QB but also and to a lesser extent A251P* mutants are not only markedly impaired in the electron transfer between QA and QB but also and to a lesser extent A251P* mutants are not only markedly impaired in the electron transfer between QA and QB but also impairment in photoinduced PSII damage have been detected under our experimental conditions (Tables I–III), the slight increase in the rates of D1 synthesis (3.5 × 10^3 versus 3.0 × 10^3) and turnover (−13.9 × 10^3 versus −13.5 × 10^3) observed in this mutant compared with wild type (Fig. 3) might nevertheless indicate an elevated repair of photodamaged PSII centers. Alternatively, the Cys substitution might affect the susceptibility of D1 to normal degradative processes. In either case, if the rate of D1 repair is sufficient to match the rate of D1 degradation caused by the Cys substitution, the number of functional PSII centers would be expected to remain unaffected.

DISCUSSION

We have used a combination of biophysical, biochemical, and physiological assays to characterize the effects of seven amino acid substitutions at the Ala251 residue of the D1 protein that result in varying levels of photosynthetic competence. These seven mutants and the five nonphotosynthetic Ala251 D1 mutants we characterized previously (10) can be grouped into four phenotypic classes with respect to D1 synthesis and steady state level, photosynthetic capacity, photoautotrophic growth rate, and herbicide resistance (Fig. 6): 1) similar to wild type (A251C*), 2) impaired in photosynthesis but not in photoautotrophic growth (A251G*, A251P*, and A251S*), 3) markedly impaired in photoautotrophic growth and photosynthesis and herbicide-resistant (A251I*, A251L*, and A251V*), and 4) not photosynthetically competent (A251R*, A251D*, A251Q*, A251E*, and A251H*). The effects of the individual amino acid substitutions on D1 synthesis/accumulation, growth, and carbon accumulation, O2 evolution, composition of the PSII reaction centers, and QA to QB electron transfer will be considered below.

The Arg, Asp, Gln, Glu, and His Substitutions—Previously, we showed that these five amino acid substitutions at the Ala251 D1 residue led to a nonphotosynthetic phenotype, markedly impaired D1 syntheses and accumulation, and severely affected primary photochemical charge separation efficiency (10). Here, we report that electron transfer between QA and QB is almost completely blocked in these five mutants (Table III). No binding of QB in its niche on the D1 protein could be detected in these strains. Electrons that can be extracted from water oxidation are blocked at the primary quinone acceptor Qb, which has been suggested to lead to a doubly reduced Qb (36). This could increase the possibility for recombination of the radical pair P700/Phe+ and generate highly toxic O2 via the 3Pb state (37). The conformation of the IV–V loop of the D1 protein in these mutants is presumably structurally altered compared with wild type (10).

Importance of the Ala251 Residue Deduced from Suppressor Analysis of the Nonphotosynthetic A251R* Mutant—Among about 40 isolates recovered after acetate starvation of the nonphotosynthetic A251R* mutant and sequenced, only seven suppressors were identified. This strongly suggests that other mutations in the D1 protein or in other chloroplast proteins are unable to compensate for the structural modification caused by insertion of a bulky, charged amino acid at position 251. These results reinforce the hypothesis that the amino acid residue present at position 251 plays a critical role in conformation and function of the IV–V loop (10, 16, 17, 35).

The Cys Substitution—For most of the parameters analyzed,
vesting light ameliorates the potentially destructive redox chemistry occurring in chloroplasts exposed to bright light, the question arises as to why the A251G*, A251P*, and A251S* mutants with their large antennae grow as well as wild type under HL (17). Decreases in PSII centers per cell seen in these three mutants (Table II) might be a consequence of photodamage or might reflect another adaptation of the cell to the HL environment. Photoinhibition has been defined as a light-dependent decrease in maximum photosynthetic efficiency (39), which results in damage and degradation of the D1 protein. For example, in A251G* and A251P*, D1 synthesis in short term pulse-chase experiments is reduced by about one-third, whereas D1 turnover is increased by 27 and 73%, respectively, compared with wild type (Fig. 3). These mutants also tend to accumulate less D1 than wild type (Fig. 2). Alternatively, photoinhibition may be considered a mechanism that matches overall photosynthetic electron flow to the photon flux rate through the down-regulation of PSII photochemistry (40–42). In fact, the decrease in the number of PSII reaction centers per cell observed in A251G*, A251P*, and A251S* (Table II) might reflect an adjustment of the photosynthetic electron transport system to high PSII excitation pressure.

The 3.3-h photoautotrophic doubling times obtained with wild type C. reinhardtii, A251C*, and the A251G*, A251P*, and A251S* mutants in microtiter plates under optimum conditions (saturating light and CO2) (17) are the shortest known for this alga. Hence, our observation that the growth rate of the latter three mutants is not photosensitive under these conditions suggests that photosynthetic electron transfer from QA to the plastoquinone pool is not rate-limiting for overall photosynthetic electron flow at light saturation. The calculated time for whole chain electron transport (rWC, Table II) shows no correlation with Kapp (Table III) in these mutants. Sukenik et al. (43) have shown that 1/rWC is correlated with carboxylation in the Calvin cycle rather than electron transport reactions within the thylakoid membranes. A similar discrepancy between photoautotrophic growth and photosynthetic efficiency has been observed in certain cyanobacterial mutants altering the CP47 polypeptide of PSII (44). The Ile, Leu, and Val Substitutions—Replacement of Val for Ala251, the only substitution at this position previously characterized in cyanobacteria and in C. reinhardtii, has been reported to result in resistance to certain PSII herbicides and increased the sensitivity to photoinhibition (13, 14). The cyanobacterial mutation was subsequently shown to affect the apparent equilibrium constant on the acceptor side (11) and the properties of the oxygen-evolving complex on the donor side (16). The phenotype of the new A251V* mutant of C. reinhardtii characterized in this paper is similar to that of the two aforementioned Val mutants. In addition, we created and characterized Ile and Leu substitutions that fall into the same phenotypic category (this paper and Ref. 17). Based on herbicide resistance, we find the A251I* and A251V* mutants to be virtually identical, while the A251L* mutant results in a more extreme herbicide resistant and photosynthetic defective phenotype.

A251I* and A251L*, and to a lesser extent A251V*, are severely impaired in the QA → QB electron transfer as indicated by a very slow reoxidation rate of QA, a high instability of QB, and a large shift in the equilibrium constant Kapp (Table III) but do not show any impairment in the primary charge separation (Table II). In contrast to the Gly, Pro, and Ser substitutions, the reduced photosynthetic yield in A251I*, A251L*, and A251V* is associated with slower photoautotrophic growth and increased herbicide resistance (17). Furthermore, these defects are observed at both LL and HL, and they differ from those in the herbicide-resistant Ser264 to Ala D1 mutant, which shows reduced growth only in HL (17).

Electron transfer on the acceptor side of the D1 protein is greatly impaired in A251I*, A251V*, and A251L* (Table III), leading to high PSII steady state levels of reduced QA as indicated by reduced qP values (Fig. 4). Given our finding that A251L* accumulates at least as much D1 as wild type (Fig. 2), the 30% increase in the rate of D1 synthesis seen in the pulse experiments appears to offset the 47% increase in the rate of degradation measured in chase experiments compared with wild type (Fig. 3).

Properties of the Amino Acid at Position 251 in Relation to D1 Structure and Function—In cyanobacteria, amino acids 248–251 of the IV–V loop of the D1 protein are thought to be buried in the thylakoid membrane, dividing the loop into two functional domains (11, 16). Our grouping of the 12 Ala251 mutants of C. reinhardtii into four phenotypic classes in terms of their effects on photosynthetic efficiency, photoautotrophic growth, and herbicide resistance (Fig. 6) relative to the nature of the substituted R group is consistent with the present structural models of the IV–V loop. Insertion of a charged or polar and relatively bulky amino acid at this position (e.g. Arg, Asp, Gln, Glu, or His) would be predicted to destroy the conformation of the D1 IV–V loop because of interactions of the R groups of these amino acids with polar heads of the lipid bilayer of the thylakoid (10). This is consistent with the total blockade of the electron flow between QA and QB and the nonphotosynthetic phenotype observed in these five mutants.

Substitution of Ala251 with an uncharged amino acid having a bulky side chain (Fig. 5, e.g. Ile, Leu, or Val) does not disrupt
D1 function completely but impairs photosynthetic electron transfer to various extents that appear to correlate with the position of the methyl group on the R group. Leu, whose methyl group is attached at C-3, has a more severe phenotype than Ile and Val, which have methyl groups attached at C-2. In contrast, substitution of small uncharged amino acids for Ala$^{251}$ (e.g., Cys, Gly, Pro, or Ser) has no effect on the mutants' photoautotrophic growth or herbicide sensitivity (17) and photosynthetic O$_2$ evolution, but all four mutations affect the function of the Q$_B$ binding niche to varying degrees. In the case of the Gly, Pro, and Ser substitutions, the acceptor side of PSII is affected. The effect of the Ser and Pro substitutions may be related to the polarizable hydroxyl group and five-ring structure, respectively, in the R groups of these two amino acids. That Gly, which has a hydrogen attached to the carbon backbone instead of the methyl group in the normal Ala residue, perturbs function suggests that steric interactions on the side chain are critical for plastoquinone binding. Interestingly, substitution of Cys with a sulfhydryl group, results in the fewest mutations examined. The fact that Cys can exist in either the oxidized or reduced form might help mediate proton and/or electron transfer to plastoquinone in the Q$_B$ pocket.

In the case of the seven Ala$^{251}$ mutants that retain the capacity for photoautotrophic growth, one has difficulty in correlating growth rate, carbon accumulation, and photosynthetic efficiency under LL and HL with the capacity for electron transfer between Q$_A$ and Q$_B$. Furthermore, no correlation has been found between Q$_B$ stability and the rate of acceleration in D1 turnover. Hence, Chlamydomonas may possess mechanisms for ameliorating the effects of reduced photosynthetic performance caused by certain of the Ala$^{251}$ mutations in the D1 protein and thus optimizing their fitness under particular environmental conditions. To identify other proteins and pathways involved in these processes, nuclear suppressors of the photosensitive A251L$^+$ and A251L$^-$ mutants have been isolated that have an elevated tolerance to very high light (2000 μmol m$^{-2}$ s$^{-1}$), which is photoinhibitory both to these mutants and to wild type. Detailed characterization of these suppressors is now in progress.

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