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Loss of inhibition by formate in newly constructed photosystem II D1 mutants, D1-R257E and D1-R257M, of *Chlamydomonas reinhardtii*

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Abstract

Formate is known to cause significant inhibition in the electron and proton transfers in photosystem II (PSII); this inhibition is uniquely reversed by bicarbonate. It has been suggested that bicarbonate functions by providing ligands to the non-heme iron and by facilitating protonation of the secondary plastoquinone Q_B. Numerous lines of evidence indicate an intimate relationship of bicarbonate and formate binding of PSII. To investigate the potential amino acid binding environment of bicarbonate/formate in the Q_B niche, arginine 257 of the PSII D1 polypeptide in the unicellular green alga *Chlamydomonas reinhardtii* was mutated into a glutamate (D1-R257E) and a methionine (DQ-R257M). The two mutants share the following characteristics. (1) Both have a drastically reduced sensitivity to formate. (2) A larger fraction of Q_A^- persists after flash illumination, which indicates an altered equilibrium constant of the reaction $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$, in the direction of $[Q_A^-]$, or a larger fraction of non-Q_B centers. However, there appears to be no significant difference in the rate of electron transfer from Q_A^- to Q_B. (3) The overall rate of oxygen evolution is significantly reduced, most likely due to changes in the equilibrium constant on the electron acceptor side of PSII or due to a larger fraction in non-Q_B centers. Additional effects on the donor side cannot yet be excluded. (4) The binding affinity for the herbicide DCMU is unaltered. (5) The mutants grow photosynthetically, but at a decreased ($\sim$70% of the wild type) level. (6) The F_o level was elevated ($\sim$40–50%) which could be due to a decrease in the excitation energy transfer from the antenna to the PSII reaction center, and/or to an increased level of $[Q_A^-]$ in the dark. (7) A decreased ($\sim$10%) ratio of F685 (mainly from CP43) and F695 (mainly from CP47) to F715 (mainly from PSI) emission bands at 77 K suggests a change in the antenna complex. Taken together these results lead to the conclusion that D1-R257 with the positively charged side chain is important for the fully normal functioning of

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; EDTA, ethylenediaminetetraacetic acid; F_o, initial level of chlorophyll *a* fluorescence; F_v ($=F_m-F_o$), variable level of chlorophyll *a* fluorescence, where F_m is maximum level of chlorophyll fluorescence; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HS, high salt culture medium; P680 and $P680^+$, the reduced and oxidized forms of the primary electron donor of photosystem II; PCR, polymerase chain reaction; PSII, photosystem II; Q_A, primary plastoquinone electron acceptor of photosystem II; Q_B, secondary plastoquinone electron acceptor of photosystem II; TAP, Tris-acetate-phosphate culture medium

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PSII and of growth, and is specially critical for the in vivo binding of formate. Several alternatives are discussed to explain the almost normal functioning of the D1-R257E and D1-R257M mutants.

Keywords: Arginine; Bicarbonate effect; D1 protein; Formate inhibition; Photosystem II reaction center; Site-directed mutant; (*Chlamydomonas reinhardtii*)

1. Introduction

Electron transfer in photosystem II (PSII) has been shown by numerous studies to be regulated by bicarbonate anion in higher plants, algae and cyanobacteria (see reviews [1–3]). Although there exists a donor side effect of bicarbonate (see e.g. [4]), depletion of bicarbonate with its analogue formate causes significant inhibition of the electron transfer on the acceptor side of PSII, particularly from Q_A^- to Q_B^- (see [5–7]). According to a suggestion of Michel and Deisenhofer [8], bicarbonate may be a functional homologue of the amino acid residue E232 of the M subunit of the *Rhodopseudomonas viridis* reaction center, and may play an important role in liganding to the non-heme iron in PSII. It may provide the fifth and/or the sixth ligand to the non-heme iron. A close association of bicarbonate and formate with the non-heme iron in PSII was suggested from EPR spectroscopic studies [9,10] as well as by a Fourier transform infrared difference spectroscopy study [11]. In addition to liganding to the iron, many experiments have suggested that bicarbonate may also function in promoting the protonation of Q_B^- or Q_B^{2-} [1,5–7,12]. Kinetic studies [13] suggested the possibility of two bicarbonate binding sites in the PSII reaction center. This second binding site is likely to exist in the Q_B niche and is considered to be related to the protonation of plastoquinone. Characterization of a number of Q_B region mutants, some of which are also herbicide-resistant, have implicated the Q_B binding niche in the bicarbonate/formate effect in PSII [14–19].

The aim of this study was to investigate the involvement of particular amino acid residues in binding to formate/bicarbonate in the PSII reaction center. Since anionic bicarbonate/formate may well be the active species functioning in the PSII reaction center [20], it is expected that the binding would be electrostatic in nature and therefore positively charged amino acid residues are likely to participate in anion binding. Only three positively charged D1 residues, D1-H252, D1-R257, and D1-R269, are found near the putative Q_B and the non-heme iron sites based on homology modeling (see [3,21]). D1-H252 near the Q_B binding niche has not yet been studied in relation to the bicarbonate effect. However, the two arginine residues, having a high pK_a value, should have a higher probability of anion binding. To investigate whether these two arginine residues are involved in the formate/bicarbonate binding and functioning in vivo, we have carried out site-directed mutagenesis on these two residues using a unicellular green alga *Chlamydomonas reinhardtii* as a model system. The study of a site-directed mutant on D1-R269 (D1-R269G) has revealed that this residue is important for the structure and function of the PSII complex affecting both the donor and acceptor sides of PSII [22,23]. However, this non-conservative mutation did not abolish the in vivo bicarbonate/formate binding and functionality. In this paper, we focus on the role of D1-R257 residue and its relation to the formate/bicarbonate effect. Sequence analysis of the D1 protein indicates the D1-R257 is closer to the Q_B niche making it a more likely residue to be involved in Q_B protonation. This residue is thought to be located on the stromal side between the putative transmembrane helices D and E of D1 and may be located within or close to the D1-*de* helix (according to three-dimensional models of the PSII reaction center, see e.g. [21,24]).

The association of arginine with bicarbonate (or carbonate) and its analogue formate has been shown in many protein systems. The X-ray crystal structure of human lactoferrin has a carbonate at the active site binding to an iron while being stabilized by hydrogen bonding interactions with an arginine and several other adjacent amino acid residues [25]. A high resolution X-ray structure of a similar protein, duck ovotransferrin shows a bicarbonate anion

bound to an arginine residue [26]. An arginine-formate binding interaction is observed in the X-ray crystal structure of hemoglobin and myoglobin in which formate is bound to the heme iron and directly interacts with an arginine [27]. Site-directed mutagenesis on phosphoenolpyruvate carboxylase from *Escherichia coli* has also indicated an arginine residue participating in bicarbonate binding [28]. Thus, it is possible that a similar binding motif may exist in the Q_B site of the PSII reaction center.

In this study, we describe the construction and characterization of two site-directed mutants of D1-R257 using a recently developed D1 mutagenesis system in the unicellular green alga *C. reinhardtii* [29]. The arginyl residue was mutated into a glutamate (E) and a methionine (M), which have similar sizes in side chains but different electrostatic properties. Biochemical and biophysical characterization of these two mutants lead to the conclusion that D1-R257 with the positively charged side chain is important for the fully normal functioning of PSII and of growth, and is specially critical for the in vivo binding of formate.

2. Materials and methods

2.1. Site-directed mutagenesis

Site-directed mutagenesis of *C. reinhardtii* D1 gene (*psbA*) was carried out using an engineered plasmid vector (pBA157) containing the intron-free *psbA* gene and the *E. coli aadA* gene conferring spectinomycin resistance in tandem [29] (Fig. 1). The codon for D1-R257 is located on exon 5 of *psbA* and contains a *Sal*I restriction site. Site-directed mutagenesis on D1-R257 using PCR was done as described earlier [29]. The D1-R257E mutation was made using a synthetic oligonucleotide primer that alters the arginine-257 codon CGA to GAA (antisense sequence: TTGGAAGATTAGTTCACCAAAGTAACC). The D1-R257M mutation was made with a primer (antisense sequence: TTGGAAGATTAGCATACCAAAGTAACC) changing CGA to ATG. The mutagenized plasmid was purified using the Magic Minipreps DNA purification system (Promega, Madison, WI). The mutations on the plasmid DNA were confirmed by automated DNA sequencing performed at the Genetics (Biotechnology) Facility of University of Illinois, using the primer TTAATCCGTGAAACAACTGAA (for residues 223–229).

Mutagenized plasmid DNA was used to transform a *psbA* deletion *C. reinhardtii* strain (ac-u-ε). Transformation was performed using a laboratory-built helium particle inflow gun which bombarded DNA-coated tungsten particles (1.1 μm) into *C. reinhardtii* cells. Cells were then plated on Tris-acetate-phosphate (TAP) medium [30] containing 100 μg/ml spectinomycin and incubated at 22°C under dim light. Two weeks after the bombardment with DNA, several colonies were obtained on the plates. As the engineered construct contains the intron-free *psbA* and the spectinomycin-resistant genes in tandem and the transformation was done with a *psbA* deletion host strain, the isolated colonies on the spectinomycin-containing plates are thus presumably homoplasmic for the newly introduced *psbA* gene. For further experimental details, see reference [29].

Total DNA of the transformants was isolated for further sequence confirmation (see legend of Fig. 2). The putative mutant and intron-free wild type cells

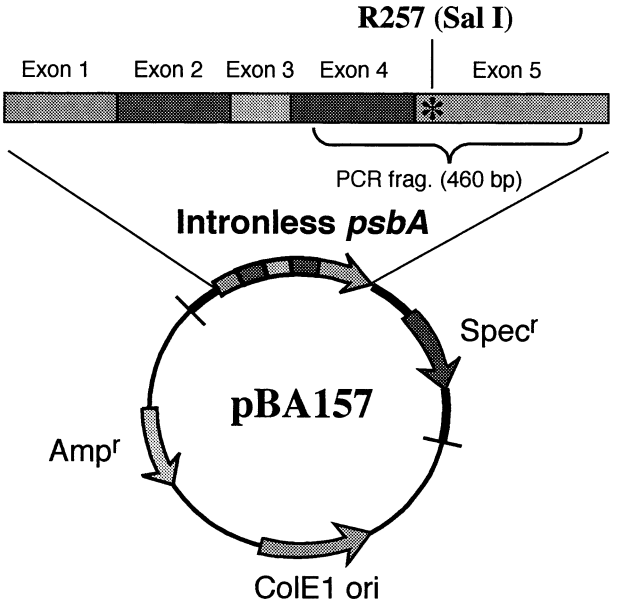

Fig. 1. Engineered plasmid vector (pBA157) containing the intron-free *psbA* gene and the spectinomycin resistance gene (Spec^r, *aadA* gene from *E. coli*) [29]. D1-R257, the site of mutagenesis, located at the exon 5, contains a *Sal*I restriction site. The relative position of the PCR product (460 bp) of the chloroplast DNA after transformation is also shown on the plasmid map.

grown in liquid TAP without spectinomycin and were harvested in the late logarithmic phase (OD at 750 nm, ~0.65; ~6×10^6 cells/ml). Harvested cells were concentrated to ~1 mg Chl/ml by centrifugation (14 000×*g*, 10 min). Chlorophyll concentration was calculated according to the equations in Porra et al. [31]. For isolating the DNA, concentrated cells from 100 ml culture were disrupted for 3 min by a minibead beater in a 2 ml tube containing 0.5 ml sand (0.1 mm), 1 ml extraction buffer (100 mM LiCl, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS) and phenol:chloroform (1:1). Extracted DNA was further purified with phenol:chloroform (1:1) and precipitated with sodium acetate:ethanol (1:25). The DNA was resuspended in 150 μl of water, of which 1 μl was used for PCR amplification for the fragment from residues 186–339 using the oligonucleotide primers (1 μM) CCAAGCAGAACACAACATCC and GAAGTTGTGAGCGTTACG (synthesized by the Biotechnology Facility of the University of Illinois at Urbana). The PCR reaction in a volume of 100 μl was carried out in a PCR buffer containing 1 mM $MgCl_2$, 50 mM KCl, 50 mM Tris-HCl (pH 8.3), and 0.2 mM nucleotide triphosphates (dNTPs). Taq polymerase (5 units, Gibco BRL, Gaithersburg, MD) was added to the above reaction mixture at 80°C after the reaction mixture was incubated at 96°C for 5 min. The thermal cycling reaction was carried out with 30 cycles of denaturation at 95°C (30 s), annealing at 58°C (30 s), and polymerization at 72°C (1 min), after which a final incubation at 72°C was carried out for 10 min. The amplified DNA fragment (460 bp) was subjected to restriction analysis with *Sal*I and *Hph*I (Gibco BRL), at 37°C for 4 h. The full length amplified PCR product was also purified from agarose gel electrophoresis using the Magic PCR Preps DNA purification system (Promega). The purified DNA was sequenced using the automated DNA sequencing facility at the University of Illinois at Urbana.

2.2. Growth of C. reinhardtii cells

C. reinhardtii wild type control with intron-free *psbA* and the confirmed mutant cells were grown at 22°C in a liquid TAP medium under ~10 μE/m^2/s white light. The intron-free wild type and mutant strains were maintained in TAP agar plates with 200 μg/ml spectinomycin and 100 μg/ml ampicillin to prevent bacterial contamination. For the liquid culture, the cells were grown in TAP in the absence of spectinomycin. The cell culture reaching the late logarithmic phase (OD at 750 nm, ~0.65; ~6×10^6 cells/ml) was harvested and used for the subsequent measurements. In order to determine the photosynthetic growth rate, the wild type and mutant cells were grown in a liquid high salt (HS) medium [30], bubbled with 5% CO_2 and illuminated with 70 μE/m^2/s white light with constant shaking. The inocula used were obtained from mature TAP-grown algal cultures which were washed twice with the HS medium. They were used to inoculate a flask of HS medium to reach an optical density of 0.04 (path length, 1 cm). The growth rate was determined by measuring the optical density of the cells in original culture media at 750 nm at 12 h intervals using a

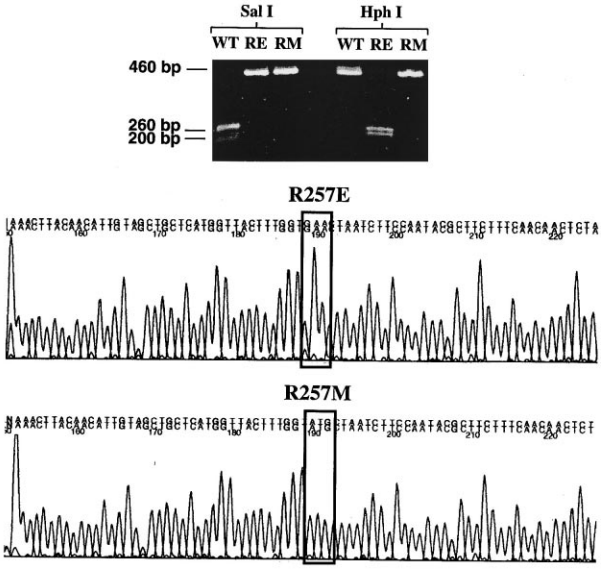

Fig. 2. Analyses confirming the presence of the introduced D1-R257E and D1-R257M mutations. The top panel shows a fragment (460 bp) of the intron-free *psbA* isolated from *C. reinhardtii* thylakoids of wild type and putative mutants was PCR amplified and treated with *Sal*I restriction enzyme. Wild type DNA was cut by *Sal*I producing 200 and 260 bp fragments. However, D1-R257E (RE), M (RM) mutants have this site removed. Furthermore, the D1-R257E mutation introduces a new *Hph*I site generating 200 and 260 bp fragments. The bottom two panels show that 460 bp PCR fragments were further sequenced and the presence of the two introduced mutations were confirmed (highlighted by box); no other mutations were found.

commercial spectrophotometer (Shimadzu UV160U, Shimadzu Co., Kyoto, Japan). Similar measurements were also made for the heterotrophically grown cultures when all the cells were kept in darkness. Cells were also grown mixotrophically in TAP medium under low light conditions (< 10 μE/m^2/s).

In all experiments in which a wild type control is referred to, the material used was from a recombinant strain in which the ac-u-ε was transformed with intron-free psbA containing the wild type gene.

2.3. Chlorophyll a fluorescence induction kinetics and measurements of F_o

Chl *a* fluorescence induction (transient; see [32]) of the wild type and mutant cells was measured with a commercial pulse-modulated fluorimeter (Walz PAM-2000, Effeltrich, Germany). Actinic and measuring beams were provided by the built-in red-light-emitting diodes. The intensity of the measuring light was 0.7 μE/m^2/s and the intensity of the actinic light was 470 μE/m^2/s. Before the measurements, the cells were resuspended in TAP medium with a Chl concentration of 5 μg/ml. All sample manipulations were done in the presence of a weak (< 0.3 μE/m^2/s) background green light. Chl *a* fluorescence transient measurements were made in the presence or absence of DCMU (10 μM) with samples dark-adapted for 5 min while being stirred.

Since conclusions regarding the photochemical activity are obtained from a knowledge of the variable Chl *a* fluorescence (F_v) whose value is dependent upon the precise value of F_o (see, e.g., [32]), special efforts were made to measure the true F_o. This was done separately using a different pulse-modulated fluorimeter (Walz PAM-103, Effeltrich, Germany).

2.4. Bicarbonate depletion and recovery treatments

Bicarbonate depletion of cells by formate was carried out with a formate treatment procedure as described by El-Shintinawy et al. [33], but with minor modifications (see [23]). The pH of the measurements was 6.5 unless otherwise indicated. Samples were treated with sodium formate at various concentrations and pHs as indicated. To overcome the inhibition of electron flow by formate, these samples were incubated with 10 mM sodium bicarbonate for 10 min.

2.5. Low temperature fluorescence spectra

Low temperature (77 K) Chl *a* fluorescence emission spectra of the wild type and mutant cells suspended in TAP medium containing 20% glycerol were obtained as described earlier [23]. Chlorophyll concentration was 30 μg/ml. Front surface fluorescence measurements were made using a Perkin Elmer LS-5 fluorescence spectrophotometer (Perkin Elmer, Oak Brook, IL). Emission spectra were corrected for the wavelength dependence of the photomultiplier sensitivity, but not the monochromator. The emission spectra for different samples were normalized at the 715 nm band.

2.6. Flash induced chlorophyll a fluorescence decay

Chl *a* fluorescence yield decay in darkness after single-turnover actinic flashes was measured with a laboratory-made multiflash fluorimeter [34]. All sample manipulations were done under a weak background green light (< 0.3 μE/m^2/s). Measurements were made as described elsewhere [7,23,34]. Chlorophyll concentration of the samples was 1 μg/ml. Chlorophyll *a* fluorescence yield decay traces were deconvoluted into three exponential components with the KaleidaGraph program. The fitting equation used was: F_v/F_o (also see [5–7,35]) $= A_1 \exp(t/\tau_1) + A_2 \exp(t/\tau_2) + A_3 \exp(t/\tau_3)$, where A represents the amplitude and τ the lifetime of the components. The τ_1, which is in sub-ms range, reflects the lifetime of the component involved in reoxidation of Q_A^- by Q_B (and/or by Q_B^-). The τ_2, which is in ms range, is affected by the equilibrium reaction between $Q_A^- Q_B$ and $Q_A Q_B^-$, and is also controlled by the movement of plastoquinone to PSII without bound Q_B. The τ_3, which is in the seconds range, reflects non-B centers (see [36]) and the characteristics of the component involved in the back-reaction between Q_B^- and the S states of the oxygen evolving complex (see [34,37], and references therein). As τ_3 is in the range of seconds, it was assumed to be $\gg t$; thus, $A_3 \exp(t/\tau_3)$ was considered to equal A_3.

To investigate differences in the herbicide DCMU binding in the wild type and the two mutants, vari-

ous concentrations of DCMU were added to the sample in complete darkness, and Chl *a* fluorescence decay measurements were made as described above.

2.7. Steady-state oxygen evolution

Steady-state oxygen evolution in intact *C. reinhardtii* cells was measured polarographically, using saturating white light (4600 μE/m^2/s) filtered through a 4% $CuSO_4$ solution in a round-bottom flask, with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH). A combination of two electron acceptors, 2,6-dichloro-*p*-benzoquinone (DCBQ) (0.1 mM) and potassium ferricyanide ($K_3Fe(CN)_6$) (1 mM), was used. DCBQ acts as the electron acceptor and the non-penetrating ferricyanide keeps DCBQ in the oxidized state. The measurement was done in the presence of 20 μM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) in the reaction medium to block electron flow between the plastoquinone pool and PSI (see [38]). Thus, the effect of CO_2 due to CO_2 fixing steps could be avoided. Samples were maintained at 25°C with a temperature-regulated water bath. The Chl concentration used for oxygen evolution measurements was 10 μg/ml. The reaction medium contained 100 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 6.5), and 2 μM nigericin.

3. Results

3.1. Site-directed mutagenesis of psbA and mutant confirmation

Using a recently developed protocol for site-directed mutagenesis of the D1 protein in *C. reinhardtii* [29] that involves the use of the intron-free *psbA* gene, we have mutated D1-R257 into a glutamate (E) and a methionine (M). These mutants, with these two amino acids of similar sizes in their side chains but of different electrostatic properties compared to arginine, were used to test the role of the positively charged guanido group in formate/bicarbonate binding in PSII of *C. reinhardtii*.

Prior to transformation, the mutagenized plasmid DNAs were sequenced to confirm the presence of both the introduced mutations (data not shown). After transformation, isolated algal transformants were further tested for the presence of these mutations. As shown in Fig. 2 (top panel), the PCR products (460 bp) of the chloroplast DNA of the transformants (putative mutants and intron-less wild type) were treated with *Sal*I restriction enzyme. The intrinsic *Sal*I restriction site at R257 position is confirmed in the wild type (intron-free), resulting in 200 and 260 bp digestion fragments. In the putative D1-R257E and D1-R257M mutants, the site was removed, as expected, as shown by their inability to be cut by *Sal*I. Furthermore, DNA sequence analysis indicated that D1-R257E mutation should have introduced a new *Hph*I restriction site. This has also been confirmed in the putative D1-R257E mutant when its PCR product was treated with *Hph*I restriction enzyme, generating 200 and 260 bp fragments. The wild type and the D1-R257M mutant could not be cut by *Hph*I. The absence of contaminating bands (i) at the 460 bp for the wild type, (ii) at 200 and 260 bp for the two putative mutants treated with *Sal*I, and (iii) at 460 bp for D1-R257E treated with *Hph*I verified the homoplasmy of the *psbA* transformants. No new restriction sites were generated for the D1-R257M mutant. The 460 bp PCR fragments of all three samples were further sequenced and the presence of the two introduced mutations were confirmed (Fig. 2, bottom panel, shown are only the mutant sequences); no other mutations were found.

3.2. Growth characteristics

Photoautotrophic growth of the wild type and the isolated D1-R257E and D1-R257M mutants was assayed by the optical density of the culture at 750 nm. Fig. 3 shows the growth curves of the wild type, the D1-R257E and the D1-R257M mutants in photoautotrophic conditions (in HS medium). During the logarithmic growth phase, the doubling time for the wild type culture is ~12 h, 18 h for D1-R257E, and 19 h for D1-R257M cultures. Thus, the mutations caused ~32–37% inhibition of the photosynthetic growth. However, under heterotrophic growth conditions (in TAP), the wild type and the two mutants have essentially the same rate of growth as the wild type with a doubling time of ~23 h.

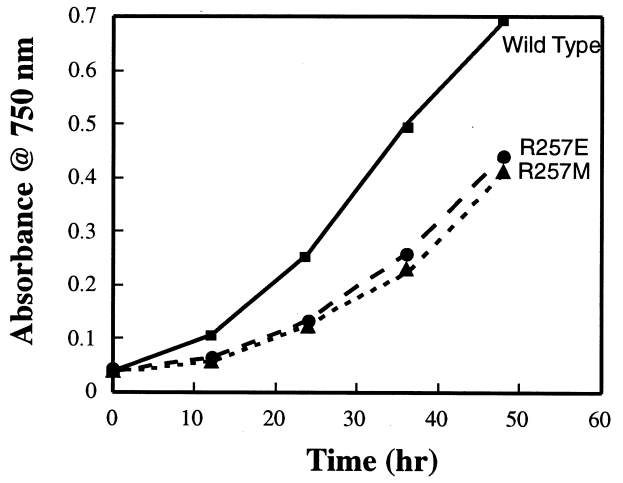

Fig. 3. Photosynthetic growth determination of the *C. reinhardtii* wild type, and D1-R257E and D1-R257M mutant cells. The algal cells were cultured in a liquid high salt (HS, autotrophic) medium in light (70 μE/m^2/s) and supplied with 5% CO_2. The growth curve was determined by measuring the optical density of the cell culture at 750 nm. The doubling time for the wild type in the autotrophic condition is ~12 h, and for the mutants 18–19 h.

3.3. Initial fluorescence level (F_o) and chlorophyll a fluorescence induction

Initial F_o measurements at various low light intensities in the presence and absence of DCMU (10 μM) are shown in Fig. 4. At very low light intensities of the measuring beam, fluorescence (F_o) intensity is a linear function of light intensities (I) (Fig. 4A). The quantum yield (F_o/I) remains almost constant at these low intensities, as it should for true F_o which should be independent of photochemistry (Fig. 4B). The significant result is that the true F_o values (i.e., those at the lowest intensities) of both the mutants were consistently higher than that in the wild type (~50%). This will be taken into consideration when interpreting fluorescence data in this paper.

To obtain general information on the status of PSII photochemistry of the wild type, and the mutants D1-R257E, and D1-R257M, chlorophyll *a* fluorescence induction (up to 1 s) (see, e.g., [32,39–41]) was measured in the mixotrophically grown (see Section 2) wild type and mutant cells with a pulse-modulated fluorimeter. The induction kinetics of Chl *a* fluorescence of these samples were measured in both the absence and the presence of herbicide DCMU, known to block electron flow by displacing Q_B, and in the presence of the bicarbonate analogue, formate, known to inhibit PSII electron transfer, and in the presence of both formate and bicarbonate (Fig. 5). For easy comparison of the kinetics of fluorescence induction, the F_o values of all the samples in Fig. 5 were normalized to the same level as the control in the wild type. Fluorescence transient was plotted on the logarithmic time scale, so that different rise components can be made visible. As reviewed earlier [18,32,41], the first rise (photochemical phase) reflects the net reduction of Q_A to Q_A^- but it also includes the influence of the S states. The later rise to the P level is due to the filling up of the plastoquinone pool.

Fig. 5A,B shows the fluorescence induction kinetics of the wild type cells. The control curve shows the normal O to P rise. Under the experimental con-

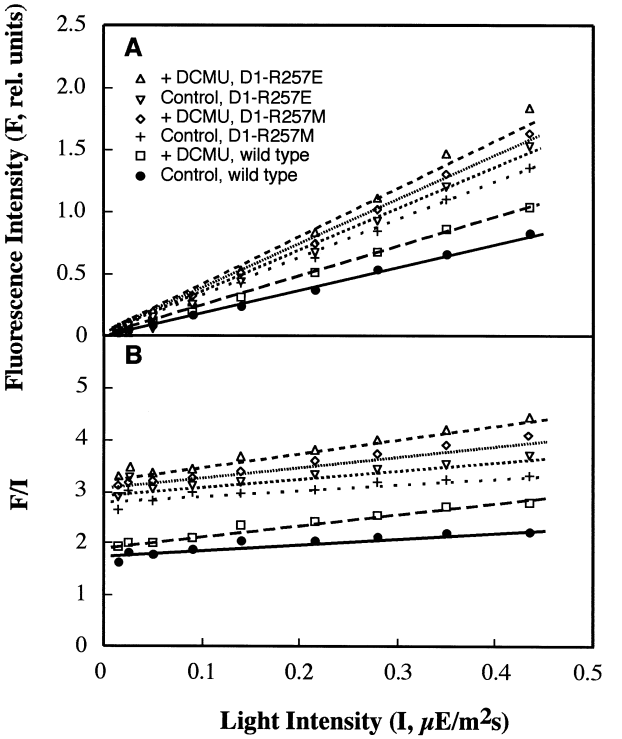

Fig. 4. Baseline Chl *a* fluorescence (F_o) (top panel, A) and the relative quantum yield of F_o, measured as the ratio of fluorescence intensity (F) to the light intensity (I) of the measuring beam (bottom panel, B), as a function of light intensities in low intensity range, in the presence and the absence of 10 μM DCMU. Measurements on the wild type *C. reinhardtii* and D1-R257E, and D1-R257M mutant cells were made as in Section 2.

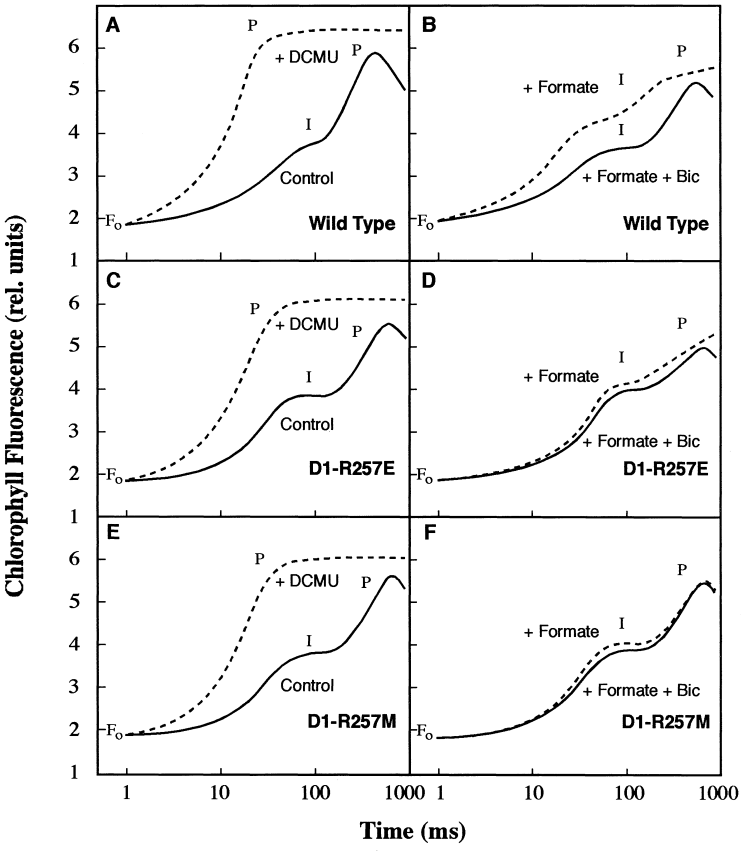

Fig. 5. Chlorophyll *a* fluorescence transients (as a function of time of illumination) of the mixotrophically grown *C. reinhardtii* wild type, and D1-R257E and D1-R257M mutant cells in the absence and the presence of 10 μM DCMU, and in the presence of 25 mM formate, and formate plus 10 mM bicarbonate, all at pH 6.5. Measurements were made with a PAM-2000 fluorimeter. The F_os of the mutants and the wild type were normalized to the wild type level. The data are plotted on logarithmic time scale. (A) The transient of the wild type in the absence and presence of DCMU. (B) The transients of wild type treated with formate and formate plus bicarbonate. (C) The transient of D1-R257E mutant in the absence and presence of DCMU. (D) The transients of D1-R257E mutant treated with formate and formate plus bicarbonate. (E) The transient of D1-R257M mutant in the absence of DCMU. (F) The transients of D1-R257M mutant treated with formate and formate plus bicarbonate. In all measurements, the [Chl] of the samples was 5 μg/ml, and the actinic illumination was 470 μE/m^2/s.

ditions used here, the rise to I occurs in about 50 ms, while the total time for reaching the final peak (F_p) is ~400 ms. The addition of DCMU (10 μM), blocking the reoxidation of Q_A^-, caused the Chl fluorescence to rise from F_o to P (also, the F_m, the maximal fluorescence) in only 30 ms, merging the various phases into one. The ratio of F_v/F_m, which represents the maximum yield of PSII photochemistry, in the wild type is 0.83 in agreement with previous results for the wild type *C. reinhardtii* [18].

Inhibition of the PSII electron transfer by formate and its subsequent reversal by the addition of bicarbonate have been studied extensively in the past (for reviews, see [1,3]). Data show that formate treatment (25 mM) of the wild type *C. reinhardtii* cells (Fig. 5B) results in a longer time to reach F_p (>1 s) compared to the control suggesting that the process of filling up the plastoquinone pool is inhibited. The addition of bicarbonate (10 mM) restored only partially the fluorescence kinetics to approach that of the control level.

Fig. 5C–F shows the Chl *a* fluorescence induction measurements of the D1-R257E and D1-R257M mutants in the absence and presence of DCMU and in the presence of formate plus bicarbonate. Data show that the I phase is slightly higher in the mutants than in the wild type cells. This result is consistent with a change in the equilibrium of $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ to be

shifted towards Q_A^-, and/or an increased amount of non-Q_B centers in the mutants. Unpublished observations of Govindjee (in the laboratory of J.J.S. van Rensen) and of J. Minagawa, Govindjee and Y. Inoue on thermoluminescence confirm that the redox potential of Q_B/Q_B^- is shifted to higher redox potentials supporting the conclusion that at least some of the residual Q_A^- is attributable to a change in equilibrium constant. The total time for reaching the F_p is also slightly longer (~500 ms) compared to the wild type (~400 ms) indicating a slight modification in the filling up of the plastoquinone pool step. However, the addition of DCMU caused the two mutant samples to reach a value of F_m similar to that in the wild type.

We note that since significantly higher (~40%) F_o (see Fig. 4) was observed in the mutant samples, the F_v/F_m levels (related to photochemistry) were lowered in the two mutants: 0.77 for D1-R257M and D1-R257E, as compared to that in the wild type (~0.83).

The largest differences observed between the mutants and the wild type are in the different sensitivity to the formate and formate plus bicarbonate treatments (compare Fig. 5B with Fig. 5D,F). The addition of bicarbonate to the mutants does not cause significant changes from that in the formate-treated samples. The results indicate a significantly lowered sensitivity of the mutant PSIIs to formate addition suggesting a modified binding capacity of mutant PSIIs to formate/bicarbonate. We emphasize that the fluorescence transient data were plotted on a log time scale for resolving the rise components. Data on a linear time scale would visually look different.

3.4. Low temperature fluorescence emission spectra

Lowered quantum yield of photochemistry and the increased F_o show that mutations of D1-257 from R to E and to M have caused alterations in the structure and function of the PSII complex. A possible effect on the antenna was examined by measurements of low temperature (77 K) fluorescence emission spectra of the wild type and the D1-R257E and D1-R257M mutants. At 435 nm excitation, PSII has two distinct emission bands at 685 nm (F685) and at 695 nm (F695). F685 is thought to originate

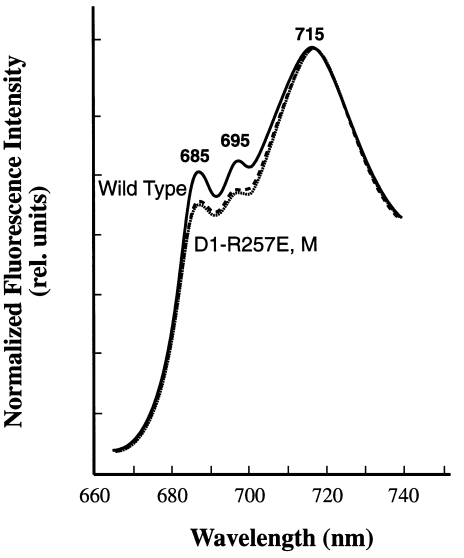

Fig. 6. 77 K chlorophyll *a* fluorescence emission spectra of the *C. reinhardtii* wild type and D1-R257E and D1-R257M mutant cells. Emission spectra of the samples (30 μg Chl/ml with 20% glycerol) were measured with front-surface optics (excitation at 435 nm; corrected for the wavelength dependence of the sensitivity of the photodetector) and normalized to the 715 nm peak. Both F685 (from CP43) and F695 (from CP47) bands were lowered in the mutant with respect to F715 band (from PSI).

mainly from the CP43 polypeptide, and F695 mainly from CP47 polypeptide, both located in PSII holochrome (see [42–44]). Haag et al. [45] showed correlation of the intensity of these two bands, especially F695, with the level of the PSII core proteins in the mutants they examined and used it as an indicator for the concentration of PSII. However, the intensities of F685 and F695 may also be influenced by the efficiency of excitation energy transfer from peripheral antenna chlorophylls to core antennae and from the core antennae to the PSII reaction center. Thus, caution must be exercised in the interpretation of the intensities of these 77 K emission bands.

Assuming that no changes have occurred in PSI, analysis of the undeconvoluted F685 and F695 bands (Fig. 6) shows that the PSII/PSI ratios in both mutants were 0.65 compared to 0.72 in the wild type, corresponding to an approximately 10% reduction. This result may indicate a reduction in these PSII antenna complexes provided the mutations had not caused changes in excitation energy transfer among these complexes and the PSII reaction center. Since

we assume that there were no changes in the CP43 and CP47 genes, this reduction could be partly attributed to the changes in the stability of the D1/D2 complexes with which these inner antenna proteins are associated [22,23,46], and partly to the changes in the excitation energy transfer to and away from the PSII reaction center.

3.5. Characterization of kinetics of electron transfer from reduced Q_A to Q_B, and of DCMU binding

To further determine the effect of mutation of D1-257 from R to E and to M on the PSII electron transport from Q_A^- to the plastoquinone pool, Chl *a* fluorescence decay in the microsecond to millisecond time scale, after single turnover flashes, was measured with the wild type, D1-R257E, and D1-R257M mutant cells in the absence or the presence of DCMU (1 μM). Samples contained 1 μg Chl/ml. The second flash kinetic traces are shown in Fig. 7, which would have corresponded only to the electron flow from Q_A^- to Q_B^-, if all PSIIs started in dark with 100% oxidized Q_B. However, in unmodified intact cells, the ratio of Q_B to Q_B^- in darkness is close to 1 (see, e.g., [35,47]). As shown in Fig. 7, the F_v/F_o ratios in ms time range (>2.5 ms) are significantly higher in both the mutants than the wild type suggesting that the equilibrium of the reaction $Q_A \leftrightarrow Q_B$ is towards Q_A^-, though the Chl *a* fluorescence decay curves for the two mutant samples appear, otherwise, to be similar to that of the wild type. Although deconvolution of the fluorescence yield decay curves suggests possible differences between the wild type and the two mutants (calculated lifetime of the fast component τ_1 being in the range of 80–120 μs, whereas τ_1s of the D1-R257E and D1-R257M mutants are in the range of 140–200 μs), they are not considered significant differences because of limited data points in the fast time scale. There appears to be a slight tendency for the electron flow from the reduced Q_A to Q_B to be slower in both the mutants. However, the simplest interpretation is that the mutants and the wild type are not significantly different in the rates of electron flow from Q_A^- to Q_B.

For the second fluorescence component, the lifetime τ_2 of the wild type is in the range of 2 ms, about 3–4 ms for the mutants; these differences are also not considered significant. However, the *amplitude* of this middle component was clearly higher in the mutants than in the wild type (see Fig. 7), which could be accounted for by the centers in the mutants either with smaller equilibrium constant for sharing an electron among Q_A and Q_B, or with larger fraction of vacant Q_B site in the dark, the former being more likely because of the unmodified Q_B niche in the mutants, which was shown by measuring DCMU binding affinity. The shift of the equilibrium constant for these mutants was further confirmed by thermoluminescence measurements (J. Minagawa, Govindjee and Y. Inoue, unpublished results).

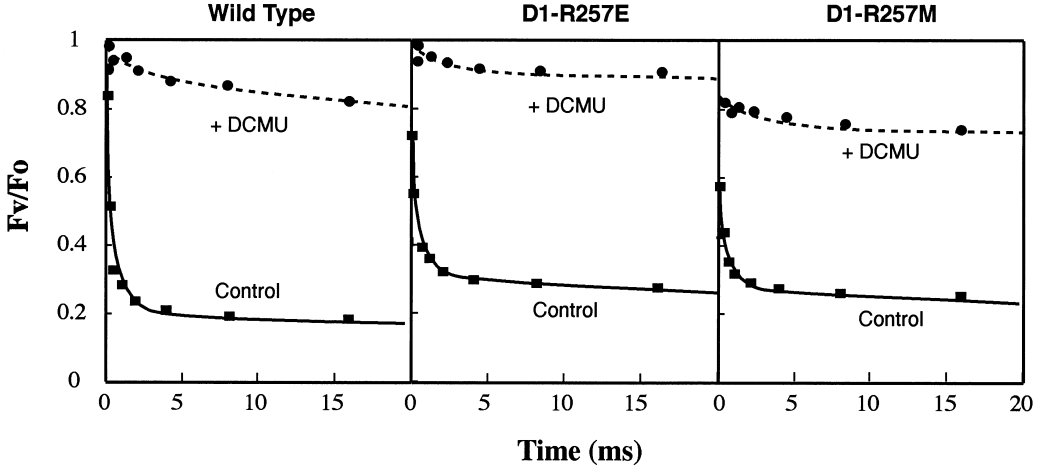

Fig. 7. Flash-induced chlorophyll *a* fluorescence decay kinetics of the *C. reinhardtii* wild type and the D1-R257E and D1-R257M mutant cells, with or without 1 μM DCMU, after the second actinic flash. Dark time between the first and the second flash was 1.5 s. Measurements were made with 1 μg Chl/ml samples. The F_o of the mutants was normalized to the wild type level.

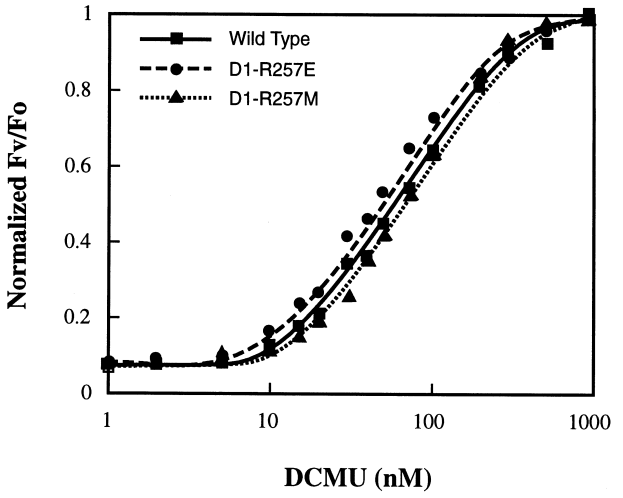

Fig. 8. Determination of DCMU binding in the *C. reinhardtii* wild type and D1-R257E and D1-R257M mutants using normalized variable chlorophyll *a* fluorescence yield (F_v/F_o) as a function of DCMU concentration. The Chl *a* fluorescence yield was measured at 1 ms after five actinic flashes spaced 1.5 s apart. No significant differences are found for the dissociation constant values of DCMU (47–62 nM) for these three samples.

The addition of 1 μM DCMU almost fully inhibits the electron transfer in the wild type and in both the mutant cells (see Fig. 7). To further quantify the sensitivity of the wild type and the mutants to DCMU, the increase in variable Chl *a* fluorescence yield was determined as a function of DCMU concentration (Fig. 8). The fluorescence yield at 1 ms after the fifth saturating flash was plotted as a function of DCMU concentration. The ordinate of the graph is normalized by the measured fluorescence yield at the fully bound state with the value of F_v/F_o at 1 μM set to be 1. For equal initial concentrations of binding site, DCMU concentration at the midpoint of the normalized F_v titration (I_{50}) was considered to provide a value for the relative dissociation-constant [16]. The calculated DCMU (I_{50}) value for the wild type is 54 ± 6 nM, for D1-R257E 47 ± 5 nM, and for D1-R257M 62 ± 6 nM. These three values are not statistically different from each other. The results suggest that the DCMU binding niche is not significantly altered by the mutation of R to E and to M at D1-257. This was surprising as the residue is considered to be near the Q_B niche (see, e.g., [24]) and, as presented in this paper, the mutants have somewhat inhibited photosynthetic growth, net electron transfer and PSII photochemical yield. Thus, the cause of the phenotypic changes cannot be simply due to the modification of the DCMU binding pocket.

3.6. *Effects of bicarbonate depletion by formate on the kinetics of Q_A:Q_B electron transfer*

Using Chl *a* fluorescence yield decay, we further characterized the effect of bicarbonate depletion by formate in the wild type and the two mutants. As shown in Fig. 9, Chl *a* fluorescence yield decay kinetics, measured as F_v/F_o after the second flash, in the wild type cells is significantly inhibited, as expected from the data in the literature [23], by the addition of 25 mM formate at pH 6.5. The addition of 10 mM bicarbonate is able to fully reverse the inhibition (data overlapped with that of the control). The lifetime for the fast component of the decay (τ_1) of the wild type is about 100 μs in the control and about 1400 μs in the presence of 25 mM formate, about a 14-fold effect. This shows a significant slowing down of the Q_A^- to Q_B^- (and, perhaps, to Q_B) electron transfer by formate treatment. However, both the E and M mutants show high resistance to inhibition by formate. Although the lifetimes, τ_1s, of both the mutants are about 2-fold longer in the mutants, it is considered minuscule as compared to the 14-fold effect in the wild type cells. It is important to note that the F_v/F_o in the ms time scale (e.g., from 2 ms to 20 ms) is much higher in both the mutants than that in the wild type, confirming the earlier observation and the conclusion that the equilibrium of $Q_A^-Q_B$ and $Q_AQ_B^-$ is towards Q_A^-. The most dramatic observation is that in the two D1 mutants, examined here, formate does not effectively block the Q_A^- to Q_B or to Q_B^- electron flow.

The inhibition of the Q_A^- to Q_B^- (and to Q_B) electron transfer by formate, titrated at various concentrations, and measured as the relative lifetimes of the first component (τ_1) after two flashes, are shown in Fig. 10A; for convenience, τ_1 of the control was set at an arbitrary value of 1. Chlorophyll *a* fluorescence yield decay data were deconvoluted with simple linear regression to provide the lifetime values. The regression equation for the wild type is $y = 2.54+0.45x$; for D1-R257E $y = 1.80+0.044x$; and for D1-R257M $y = 1.88+0.048x$. If we assume that the plastoquinone reduction reaction measured in

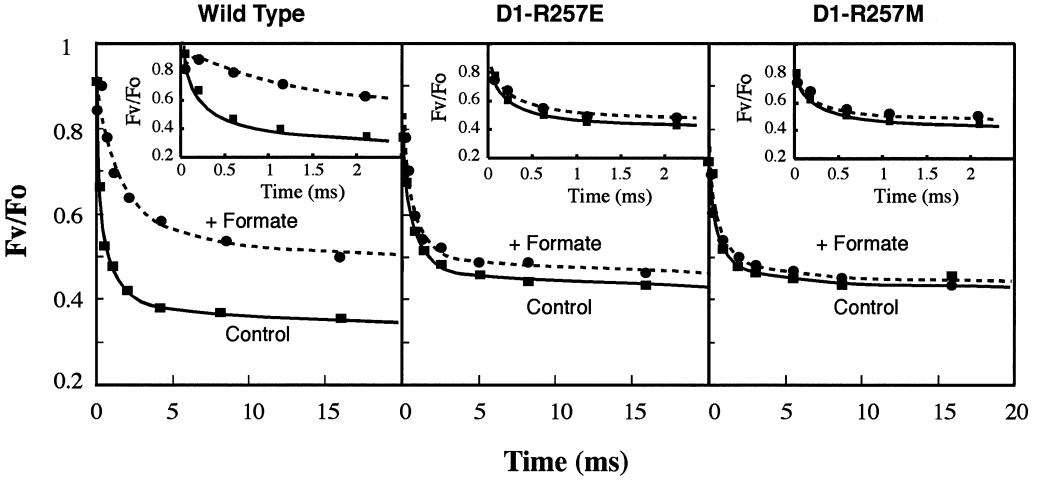

Fig. 9. Chlorophyll *a* fluorescence decay kinetics of the *C. reinhardtii* wild type and D1-R257E and D1-R257M mutants without formate (control) or treated with formate (25 mM). Kinetic traces after the second actinic flash are shown. The F_o of the mutants and the wild type was normalized to the wild type level. Insets, fluorescence decay kinetics plotted on an expanded scale (up to 2 ms). Formate blocks electron flow from Q_A^- to Q_B^- in the wild type, but the two mutants are nearly insensitive to it.

this assay is a quasi-equilibrium enzymatic reaction inhibited by formate, Fig. 10A can be regarded as a Dixon plot with the lifetimes equivalent to the reciprocals of the rates of the reaction. The apparent (equilibrium) dissociation constant (K_i) of formate can be calculated from the negative intercept of the regression lines on the *x*-axis (see [48]). In this way, the calculated K_i for the wild type is 5.6 mM, for D1-R257E 40.9 mM, and for D1-R257M 39.5 mM. Clearly, there is a large decrease in formate binding, when calculated from the extrapolated dissociation constants, in these two mutants compared to the wild type.

To study the effect of pH on the bicarbonate depletion, formate inhibition reaction was titrated at various pHs (from 6.0 to 8.0) after the addition of 25 mM formate. The resulting chlorophyll *a* fluorescence decay curves after the second flash were deconvoluted as described earlier. The lifetimes of the first component (τ_1) of the control at specific pHs are set to arbitrarily read 1. No significant variations of the τ_1 values of the control group of each sample were observed for different pHs used here (data not shown). The normalized τ_1s after the formate treatments are plotted as a function of pH (Fig. 10B). For the wild type, the optimum formate effect was at a pH range of 6–6.5, where an approximately 15-fold inhibition results. At above the neutral pH, formate inhibits the electron transfer reaction at a much lower efficiency in the wild type. However, the D1-R257E, and D1-R257M mutants show a drastically lowered inhibition to bicarbonate depletion compared to the wild type at all the pHs investigated (max. inhibition was $\sim$2.5-fold or less).

Table 1
Steady-state oxygen evolution of mixotrophically grown wild type and D1-R257E and D1-R257M mutant *C. reinhardtii* cells

	Control	+Formate	+Formate,+Bicarbonate
Wild type	189.7 ± 6.5	27.7 ± 1.5	160.9 ± 8.5
D1-R257E	121.0 ± 3.3	91.6 ± 3.1	117.7 ± 0
D1-R257M	117.8 ± 6.6	101.4 ± 3.3	114.5 ± 3.3

Reaction mixture contained 100 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 6.5), 2 μM nigericin, 0.1 mM DCBQ, 1 mM $K_3Fe(CN)_6$ and 20 μM DBMIB. Measurements were at 25°C. Chl *a* concentration was 10 μg/ml. 25 mM formate and 25 mM formate plus 10 mM bicarbonate treatments were as described in Section 2. Average values for rates of oxygen evolution (μmol O_2/mg Chl/h) of three separate experiments, along with standard errors, are presented.

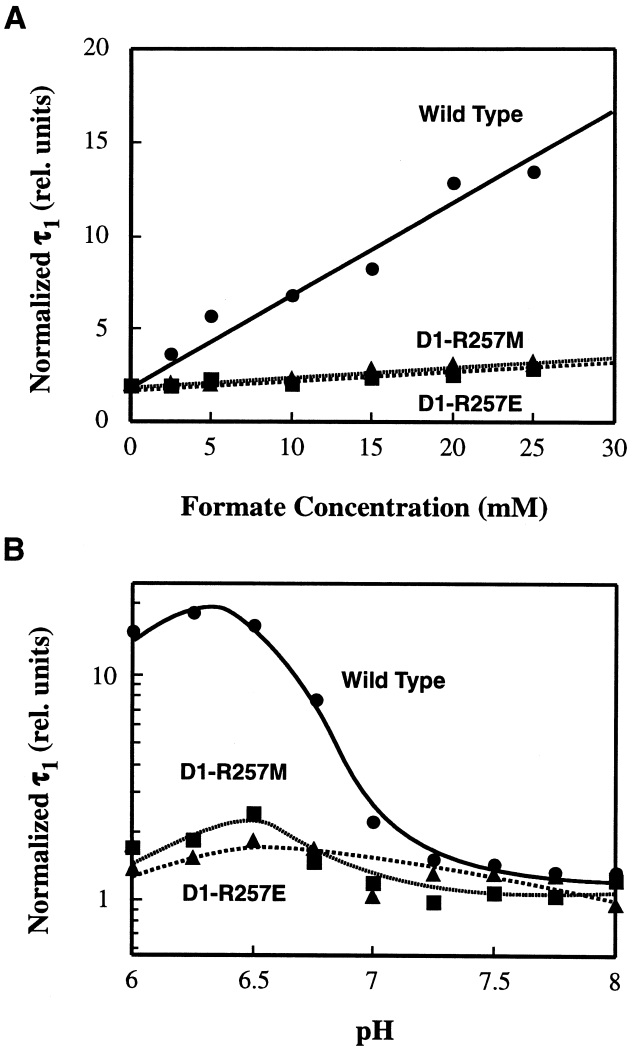

Fig. 10. (Top panel, A) Normalized lifetimes, τ_1s, of the fast Chl *a* fluorescence decay component after two actinic flashes in the *C. reinhardtii* wild type and D1-R257E and D1-R257M mutant cells treated with various concentrations of formate. For convenience of presentation, the lifetime of the fast components (τ_1) of all the three untreated (control) samples was set at a value of 1. (Bottom panel, B) Normalized lifetimes, τ_1s, of the fast Chl *a* fluorescence decay component for the wild type and D1-R257E, and D1-R257M mutants, after two actinic flashes, at various pHs after the addition of formate (25 mM). The lifetime (τ_1) of the fluorescence decay curves of the untreated controls was set at a value of 1. The optimum pH for formate inhibition for the wild type is 6–6.5. The formate inhibitory effect in the wild type is only effective at pH below 7. The D1-R257E and D1-R257M mutants did not show significant sensitivity to formate treatment (bicarbonate depletion) at all the pHs investigated.

3.7. *Bicarbonate-reversible formate inhibition of the PSII electron transfer*

Rate of steady-state oxygen evolution, from the wild type and mutant cells, with electron acceptors DCBQ and ferricyanide and the inhibitor DBMIB that acts beyond PSII, measures specifically PSII electron transfer (see data in Table 1). The rate of the PSII electron transfer in the wild type cells was inhibited by ~85% of the control level after treatment with 25 mM formate, but most of the inhibition was reversed by the addition of bicarbonate (to ~85% of the control level). The rate of electron transfer in the formate treated wild type cells is 17% of that with bicarbonate. However, the rates of electron transfer in the D1-R257E and D1-R257M mutants are ~60% of the wild type level, which parallels our measurements on the photosyn-

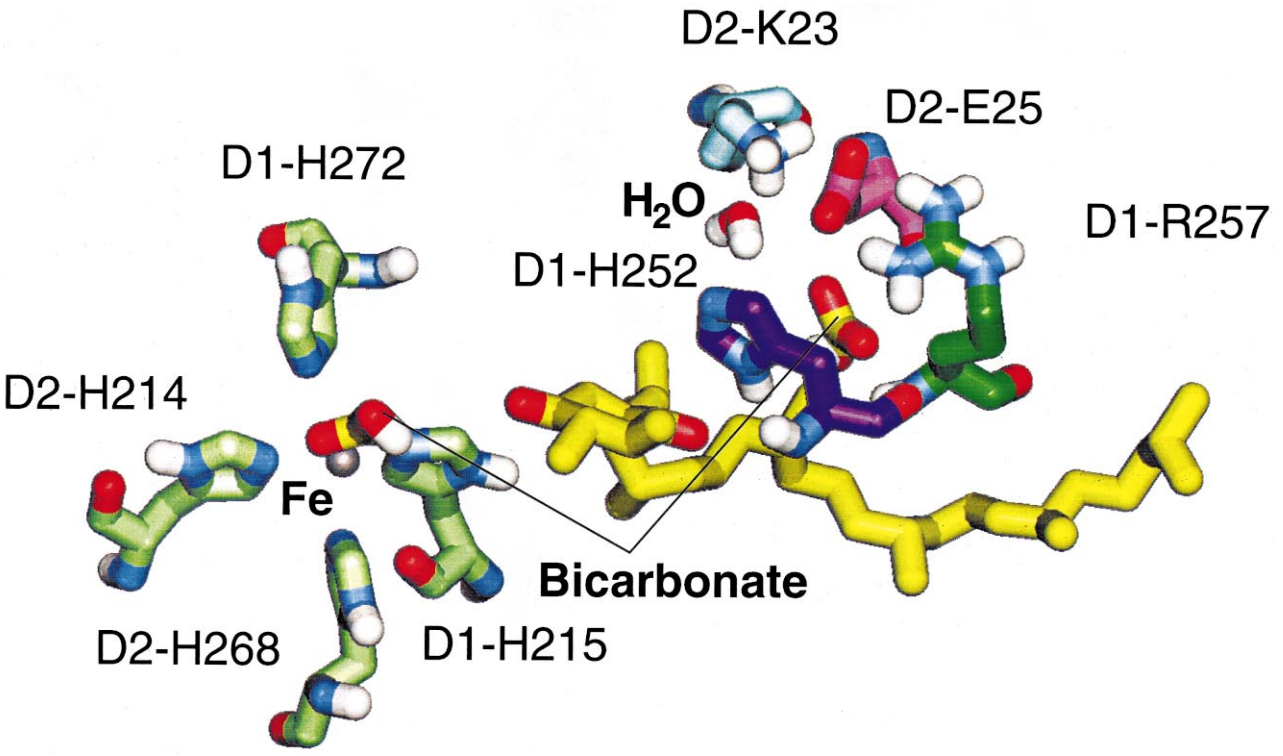

Fig. 11. A photosystem II reaction center model based on [24] for *Synechocystis* sp. PCC 6803 showing the possible arrangement of certain amino acids in the D1/D2 interface near D1-R257. One bicarbonate ion is near non-heme iron and the other is suggested to be near the arginine. It is suggested that a water molecule may also be present there. The yellow molecule is plastoquinone Q_B. Note that D2-K23 and D2-E25 in *Synechocystis* are D2-R23 and D2-D25 in *C. reinhardtii*, respectively; all others are the same as shown here.

thetic growth (Fig. 3) and fluorescence decay measurements (Figs. 7 and 9). The lowered rate of overall electron transport in the mutants, in all likelihood, is due to the changes in the equilibration on the acceptor side and/or the increased concentration of non-Q_B centers, although effects on the donor side of PSII cannot yet be excluded (cf. [22,23]). The much lowered sensitivity to formate inhibition in the D1-R257E and D1-R257M mutants is further confirmed here. In D1-R257E and D1-R257M mutants, formate treatment slowed electron transfer by only about 15% of the control level, while the bicarbonate treatment restored the level to 86–97% of the control level. The rate of electron transfer in formate treated cells is 78–89% of that with bicarbonate addition. Thus, there is only a marginal decrease after the formate treatment in both the mutant samples.

4. Discussion

We first constructed two site-directed mutants on D1-R257 (D1-R257E and D1-R257M) and then investigated the role of this arginine residue in bicarbonate/formate binding in PSII. Our results indicate that the mutation of R to E and to M in D1-257 leads to a phenotype that has: somewhat lowered photosynthetic growth (Fig. 3); somewhat lowered rates of oxygen evolution (Table 1); slightly lowered maximal yield of photochemistry (Fig. 5); raised minimal Chl *a* fluorescence F_o level (Fig. 4); an altered equilibrium of the $Q_A^- Q_B \leftrightarrow Q_A Q_B^-$ reaction, tilted towards Q_A^-; somewhat higher concentration of non-Q_B PSII centers; and, most importantly, a near absence of the bicarbonate/formate effect on the PSII electron transfer (Figs. 5, 9 and 10; Table 1).

The slower rate of photosynthetic growth and a bit

slower rate of net electron transfer in the D1-R257E and D1-R257M mutants of *C. reinhardtii* agree well with the characterization of a mutant in which R was changed to V on D1-R257, constructed in cyanobacteria [49]. This phenotype was attributed to the possible role of arginine to balance the negative charge formed by the dipole moment of the D1-*de* α-helix. However, the characterization of the cyanobacterial D1-R257V mutant showed an approximately 40-fold increase in diuron (DCMU) tolerance judged by measurements of Hill reaction, with DCPIP as the electron acceptor. In contrast, the D1-R257M and D1-R257E mutants of *C. reinhardtii* showed no apparent changes in DCMU sensitivity to that in the wild type as judged by effects on Chl *a* fluorescence measurements. Though the mutant and species used in the earlier work [49] are different from what was used in this study, the question of whether mutations on D1-R257 can induce significant herbicide resistance is certainly worth further investigation.

We have observed slightly lowered yield of photochemistry in the two mutants (Figs. 5, 7 and 9). Our low temperature Chl fluorescence spectra (Fig. 6) show a slight but consistent decrease in the F685 (mainly from CP43) and F695 (mainly from CP47) emission bands after the spectra are normalized at 715 nm (F715, mainly from PSI). This decrease could be interpreted either by changes in excitation energy transfer of by decreased PSII core stability in the mutants. The severe damage observed in PSII in the mutant thylakoid preparations (data not shown) may partially support the possible destabilization of the PSII complex. In the cyanobacterial D1-R257V mutant [48], a marked change in electrophoretic mobility of the D1 protein was observed suggesting significant conformational changes caused by the mutation. It is likely, however, that the changed electrophoretic ability itself was due to a change in the charge. Computer modeling of the PSII reaction center [24] indicated that D1-R257 is a contact residue located in between D1 and D2. Although D1-R257 is close to several amino acids in D2 (see Fig. 11), the details of the specific interaction may be model dependent (cf. [24] with e.g. [50]). We suggest that the mutations (R to E or to M) disrupt at least part of the putative protein-protein interactions destabilizing the conformation of reaction center core complex. Site-directed mutations of other proposed contact residues (see [24]) have induced severe structural changes in PSII. These residues include D1-R269, D2-H214, D2-G215, and D2-H268 (see [22,23,51,52]).

The most striking phenotype effect observed between the two mutants (D1-R257M and D1-R257E) and the wild type is the near absence or significantly lowered sensitivity to formate inhibition (Figs. 5, 9 and 10; Table 1). The results strongly implicate D1-R257 in formate binding. The question remains whether this residue is involved in bicarbonate binding as well since it is known that bicarbonate (HCO_3^-) and formate (HCO_2^-) share a similar structure and have similar charge delocalization (see review, [1]). In addition to many lines of evidence that indicate that formate treatment removes bicarbonate effects in PSII and the addition of bicarbonate readily reverses the inhibitory effect of formate (see reviews [1–3]), there are further experiments that show that formate is able to physically replace bicarbonate in thylakoid membranes and induce the release of CO_2 (see e.g. [53]), the interaction of which indicates an overlapping of binding niches of the two species in the PSII reaction center. The only structural difference between bicarbonate and formate is that the hydroxyl group, which is thought to be the crucial functional moiety for the bicarbonate stimulatory effects in PSII electron flow, is absent in formate. However, the common carboxyl group is thought to be involved in their binding (see [1]). On the other hand, certain kinetic analyses involving various PSII reaction center mutants have indicated that the binding (or dissociation) constants of the two anions may be independent of each other (see [16]). Thus, changes in the binding parameters of one anion may only imply but may not necessarily indicate similar changes in another.

The possibility exists that more than one arginine residue in the PSII reaction center may be involved in anion binding. Site-directed mutagenesis on the D2 protein in cyanobacteria (cited in Diner et al. [2]) has shown that D2-R265 near the non-heme iron may play a role in associating with formate/bicarbonate. In addition, unpublished study from B. Diner's research group seems to favor D2-K264 as a better candidate for bicarbonate binding in the non-heme iron site. The involvement of several other positively charged residues in bicarbonate binding

near the non-heme iron have been investigated in cyanobacteria and green algae [22,54,55]. D2-R233 and D2-R251 have been shown to increase the PSII susceptibility to formate inhibition of the PSII electron transfer by 10-fold, relative to the wild type, and are suggested to function in stabilizing bicarbonate binding in vivo [54]. We suggest that R129 of D2, modeled in [50] to be close to bound plastoquinone, should also be mutated not only to test its ability to bind formate, but to test the model itself. However, a mutation on D2-R139 (D2-R139H) did not show any effect in bicarbonate-reversible formate inhibition [55]. Thus, there is a clear specificity for certain arginines. Recently, a non-conservative mutation was made on D1-R269 (D1-R269G) [22,23]. Despite the wide ranging significant structural and functional perturbations in the mutant, its reaction from Q_A^- to the plastoquinone pool was $\sim$4-fold less sensitive to formate inhibition compared to the wild type. However, taken together with other available experimental evidence, conclusion was made that the residue is less likely to be a bicarbonate ligand in vivo.

Using a recently constructed three-dimensional PSII reaction center model[24] and earlier ideas [1], a hypothesis was proposed for the function of D1-R257 in bicarbonate-mediated Q_B^{2-} protonation [24]. Our current observations show clearly the importance of D1-R257 for the formate effect. Unfortunately, because the bicarbonate effect is characterized through the reversal of formate inhibition, in the absence of such inhibition, the experiments provide no direct estimate of the effects of these mutations on bicarbonate binding. There was apparently little effect of these mutations on DCMU binding compared to the wild type. This may suggest that the mutational effect is not localized at the Q_B site, but rather reflects an indirect interaction. Different models of photosystem II show R257 close to the interface with D2, but pointing away from the site, suggesting that the indirect effect might be related to the interaction of D1 with D2. Disruption of this interaction might lead to the observed slower rate of photosynthetic growth and slightly decreased rates of electron transfer. If R257 is involved in protonation, it is clearly not essential, and the fact that the mutant cells can still grow photoautotrophically at a considerable rate suggests that the algal cells have alternative mechanisms for protonating Q_B. In the bacterial reaction center where there is no formate/bicarbonate effect (see, e.g., [56]) and no conserved counterpart for D1-R257, the Q_B protonation relies on specific interaction of a few highly conserved residues with water molecules which provide a H-bonded network leading to the aqueous exterior [57]. We have suggested [24] the possibility that water molecules may also form a conduit for protonation even in PSII. However, such an elaborate pathway needs to be shown to exist in PSII, since there is no known equivalent of the H subunit, and most models show fairly direct access to the aqueous phase. Indeed, the pH dependence of the equilibria of the two-electron gate could be modeled assuming a pK change on a single residue, tentatively identified as His-252 [21]. In the present work, we changed a positively charged arginine to either the negatively charged glutamate, or the neutral, weakly polar methionine, both of similar length. It is clear from the lack of significant effect on electron transfer that the properties of the side chain do not contribute important determinants in the mechanism. A possible explanation for the effect on formate binding might be found in terms of the surface charge in the vicinity of the site. The models show R257 as exposed to the aqueous phase. We speculate that the charge on the arginine might serve to concentrate the formate anion locally, and thereby increase its effectiveness on competing with bicarbonate/CO_2. Such an explanation would make sense only if the competing species were differently charged, or if the local environment provided a specific site of access for formate which was not available to bicarbonate/CO_2.

4.1. Concluding remarks

In the above discussion, the idea that there may be two formate/bicarbonate binding sites on the electron acceptor side of PSII is of critical interest. The site associated with bicarbonate binding close to the non-heme iron seems well established; our present experiments suggest that a positively charged binding niche that involves D1-R257 in the wild type might also be important for formate binding. We cannot tell from our experiments if formate was able to displace bicarbonate in these mutants, and cannot therefore conclude if this second site is also a bicarbonate binding site. The possibilities are: (1) there is only

one bicarbonate binding site, but formate can enter that site preferentially from a volume close to R257. The positive charge concentrates formate locally, and it can therefore compete in the wild type centers, but not in the mutant strains used here; (2) there are two bicarbonate/formate sites, but in the absence of D1-R257, some other positively charged amino acid (or, perhaps, specifically bound water molecules) replace the role of D1-R257; the bicarbonate is still bound, but not displaced by formate; and (3) D1-R257 does not play any role in bicarbonate binding, but it is only a formate binding site. Formate binding at this site has an inhibitory effect that is reserved by bicarbonate through a non competitive binding at the other site. (4) The 'bicarbonate effect' reflects a reversal of formate inhibition rather than a requirement for bicarbonate, and neither binds if R257 is replaced. Only further research, perhaps with other D1-R257 mutants, will allow us to answer the question of the possible bicarbonate binding site in its vicinity.

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