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Modification of the photosystem II acceptor side function in a D1 mutant (arginine-269-glycine) of *Chlamydomonas reinhardti*

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Abstract

Bicarbonate anions have a strong positive influence on the electron and proton transfers in photosystem II (PS II). It has been suggested that bicarbonate binds to the non-heme iron and the Q_B binding niche of the PS II reaction center. To investigate the potential amino acid binding environment of bicarbonate, an arginine residue (R269) of the D1 protein of PS II of *Chlamydomonas reinhardtii* was mutated into a glycine; our characterization of the resultant mutant (D1-R269G) shows that both the Tyr_D^+ and $Q_A^- Fe^{2+}$ EPR signals are substantially reduced and assembly of the tetranuclear Mn is lost (R.S. Hutchison, J. Xiong, R.T. Sayre, Govindjee, Biochim. Biophys. Acta 1277 (1996) 83–92). In order to understand the molecular implications of this mutation on the electron acceptor side of PS II, we used chlorophyll (Chl) *a* fluorescence as a probe of PS II structure and function, and herbicide binding as a probe for changes in the Q_B binding niche of PS II. Chl fluorescence measurements with the heterotrophically grown D1-R269G mutant cells (or thylakoids), as compared to that of the wild type, show that: rate of electron transfer from Q_A^- to the plastoquinone pool, measured by flash-induced Chl *a* fluorescence decay kinetics, is reduced by $\sim$17 fold; the minimum Chl *a* fluorescence yield when all Q_A^- is oxidized, is elevated by 2 fold; the level of stable charge separation as inferred from variable Chl fluorescence is reduced by 44%; binary oscillation pattern of variable Chl *a* fluorescence obtained after a series of light flashes is absent, indicative of the loss of functioning of the two-electron gate on the PS II acceptor side; 77 K PS II Chl *a* fluorescence emission bands (F685 and F695) are reduced by 20–30% (assuming no change in the PS I emission band). Thermoluminescence data with thylakoids show the absence of the $S_2Q_A^-$ and $S_2Q_B^-$ bands in the mutant. Herbicide ^{14}C-terbutryn binding measurements,

Abbreviations: Chl, chlorophyll; D1-R269G or R269G, mutant of *Chlamydomonas reinhardtii* with a glycine substitution at residue 269 in the D1 protein of photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediaminetetraaccetic acid; EPR, electron paramagnetic resonance spectroscopy; F_m, maximal level of chlorophyll fluorescence; F_0, initial measured level of chlorophyll fluorescence in dark-adapted cells and thylakoids; F_v, variable level of chlorophyll fluorescence; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethane sulfonic acid); LHCIIb, light harvesting complex IIb; P680 and $P680^+$, the reduced and oxidized forms of the primary electron donor of photosystem II; PCC, Pasteur culture collection; PS II, 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; Tyr_D, a slow donor to $P680^+$, tyrosine 160 of the D2 protein of photosystem II

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also with thylakoids, show that the Q_B niche of the mutant is significantly modified, at least 7–8 fold increased terbutryn dissociation constant is shown (220 nM in the mutant versus 29 nM in the wild type); the PS II sensitivity to bicarbonate-reversible formate inhibition is reduced by 5 fold in the mutant, although the formate/bicarbonate binding site still exists in the mutant. This suggests that D1-R269 must play some role in the binding niche of bicarbonate. On the basis of the above observations, we conclude that the D1-R269G mutation has not only altered the structure and function of PS II (Q_B niche being abnormal), but may also have a decreased net excitation energy transfer from the PS II core to the reaction center and/or an increased number of inactivated reaction center II. We also discuss a possible scenario for these effects using a recently constructed three dimensional model of the PS II reaction center.

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

1. Introduction

Electron transfer in photosystem II (PS II) has been shown by numerous studies to be regulated by bicarbonate anions in higher plants, algae and cyanobacteria (see reviews [2–5]). There are several studies which show a donor side effect of bicarbonate (see e.g., Refs. [6–10]) on PS II. Furthermore, depletion of bicarbonate causes a significant inhibition of the electron transfer on the acceptor side of PS II, particularly on the Q_A^- to Q_B^- step (references in [2–5,10]).

Michel and Deisenhofer [11] suggested that bicarbonate may be a functional homologue to 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 PS II; bicarbonate may provide the fifth and/or the sixth ligand to the non-heme iron. A close association of bicarbonate with the non-heme iron in PS II was already known from EPR spectroscopic studies [12,13]. Fourier transform infrared difference spectroscopy study using ^{13}C-labeled bicarbonate has confirmed that bicarbonate is a ligand of the non-heme iron in PS II like M-E232 [14]. However, this suggested equivalence of E232 on the M subunit and bicarbonate has its limitations since site-directed mutagenesis of M-E232 to several amino acid residues (R, V, A, Q, etc.) in bacterial reaction centers did not modify the Q_A^- to Q_B (or to Q_B^-) electron flow [15]. Since anionic bicarbonate may be the active species functioning in the PS II reaction center [16], it is expected that the binding would be electrostatic in nature and therefore positively charged amino acid residues are likely to participate in bicarbonate binding. Further, bicarbonate has been suggested to aid in protonation of Q_B^{2-} [2,4,17,18]. Only a few positively charged D1 and D2 residues are found near the putative non-heme iron based on homology studies (see [4]). Some of these positively charged residues including D1-R139, D2-R233, D2-R251, D2-K264 and D2-R265 have been studied through site-directed mutagenesis in relation to the bicarbonate effect [3,5,19]. The resultant mutants show significantly varied degrees of bicarbonate/formate binding affinity compared with the wild type. However, D1-R139H displays wild type characteristics.

Sequence analyses of the D1 and D2 proteins indicate that D1-R269, D2-K264 and D2-R265 are the basic residues near the putative non-heme iron site. However, D2-K264 and D2-R265 are located roughly in between Q_A and the non-heme iron, whereas D1-R269 is the only basic residue between the non-heme iron and the Q_B according to our recent PS II model [20]. This residue is thought to be located on the stromal side of the putative transmembrane helix E and may be separated from D1-H272, one of the four putative non-heme iron ligands, by approximately 3/4 of a helical turn (according to a three dimensional model of the PS II reaction center [20]). Thus, a hypothesis that a close interaction between the arginine and the iron-liganding bicarbonate may exist has emerged. This hypothesis is partially supported by the analogy found in the X-ray crystal structure of human lactoferrin which has a (bi)carbonate binding to an iron at the active site [21]. In this protein, the (bi)carbonate is stabilized by hydrogen bonding interactions with an arginine and several other adjacent amino acid residues. X-ray crystal structure of hemoglobin and myoglobin with a

formate (a bicarbonate analog) bound to the heme iron also indicates the involvement of an arginine residue interacting with the formate [22]. Thus, it is possible that a similar binding motif may exist in the HCO_3^-/Fe site of the PS II reaction center.

To investigate whether D1-R269 is a bicarbonate liganding residue, we have constructed and partially characterized a site-directed mutant on this residue from a unicellular green alga *Chlamydomonas* (*C.*) *reinhardtii*, in which the arginine has been converted to a non-conservative glycine (D1-R269G) [1]. The mutant was found to be defective on the donor side of PS II even when the mutation was on the acceptor side. To understand the full implication of this mutation, we have focused here on the acceptor side of PS II. In this study, we have characterized the PS II electron transfer between Q_A and Q_B of the mutant and their relation to bicarbonate-reversible formate inhibition; and the binding niche of a PS II herbicide terbutryn. We show that (1) the heterotrophically grown mutant has a significantly reduced rate of electron transfer from Q_A^- to the plastoquinone pool; (2) the binary oscillation pattern of variable Chl *a* fluorescence, after a series of single-turnover flashes, indicative of the functioning of the two-electron gate of the PS II acceptor side, is lost in the mutant; (3) the mutant has a significantly elevated true F_0 level suggesting either a decrease in the excitation energy transfer from the antenna to the PS II reaction center or an increase in the back energy transfer from the reaction center to antenna, the latter is possible if the reaction centers are photochemically inactive (see Section 3); (4) thermoluminescence bands due to recombination of S_2 with Q_A^- and of S_2 with Q_B^- are absent in the mutant; (5) 77 K Chl *a* fluorescence emission spectra has a slightly decreased ratio ($\sim$ 20–30%) of F685 (from CP43) and F695 (from CP47) to F715 (from PS I); (6) the Q_B binding niche of the mutant was drastically altered as there is a 7–8 fold decreased affinity of the herbicide ^{14}C-terbutryn in the mutant; and (7) the sensitivity to the formate inhibition is reduced by $\sim$5 fold compared to that of the wild type. However, the bicarbonate binding still exists since bicarbonate can readily recover the formate inhibition. Results presented here and in reference [1] show that a mutation on the acceptor side significantly alters the structure and function of the PS II complex on both the donor and acceptor sides, and may indirectly perturb the bicarbonate/formate binding and functionality in vivo.

2. Materials and methods

2.1. Mutagenesis of psbA gene

The plasmid (pWT) which contains exons 4 and 5 of the *psbA* gene cloned onto a phagemid vector pBS(+) was used for site-directed mutagenesis of the D1 protein. The mutagenesis on the R269 located on the exon 5 of *psbA* DNA was according to Kunkel et al. [23] and Eggenberger et al. [24]. Arginine 269 was changed into a non-conservative residue glycine in an attempt to create a deletion-like mutation. A silent mutation at valine 307 was made, introducing a new *Sal*I restriction site, which was designed to facilitate the subsequent screening of the mutant DNA from *C. reinhardtii* transformants. The resulting mutagenized *psbA* DNA was used to transform the *C. reinhardtii* chloroplasts. The algal colonies containing the homoplasmic mutations were selected and the introduced mutations were further verified with DNA sequencing and Southern blot analyses. For details, see Hutchison et al. [1].

2.2. Growth of C. reinhardtii cells

The *C. reinhardtii* wild type (CC-125) and the D1-R269G mutant cells were grown at 22°C in total darkness in a liquid tris-acetate-phosphate (TAP) medium [25]. The wild type strain was maintained in TAP agar plates with 100 μg/ml ampicillin and the mutant strain was maintained in TAP plates with 200 μg/ml spectinomycin and 100 μg/ml ampicillin. The addition of ampicillin was to inhibit the potential bacterial contamination. The growth of the green algae which are eukaryotic is not affected by the antibiotic. The cell culture reaching the late logarithmic phase (750 nm O.D., $\sim$0.65; $\sim 6 \times 10^6$ cells/ml) was harvested and used for the subsequent measurements and preparations for thylakoids. At this stage, Chl concentration of the culture was $\sim$5 μg/ml. Chl concentration was determined by suspending the cells in 80% acetone at 40°C for 20 min. The samples were centrifuged at $14\,000 \times g$ for 1 min, and the resulting pellet was discarded. The

absorbance of the supernatant was measured at 663.6 and 646.6 nm using a dual-beam spectrophotometer (Shimadzu UV160U, Shimadzu, Kyoto, Japan). Chl concentrations were calculated according to the equations of Porra et al. [26]. The growth rate of wild type and the mutant was determined by measuring the optical density of the cells in the TAP culture medium [25] at 750 nm.

In the heterotrophic growth condition, both the wild type and the mutant had near identical growth rate (22 h).

2.3. Thylakoid preparation

The thylakoid preparation was as described earlier [27] with slight modifications. The late log-phase cells were centrifuged at $2000 \times g$ for 4 min at 4°C. The pellet was washed twice with a buffer containing 350 mM sucrose, 20 mM HEPES (pH 7.5), 2.0 mM $MgCl_2$. The cells were resuspended with the above buffer to ~ 0.5 mg Chl/ml and passed through a French press once at 14 000 lbs/in.^{2}. The broken cells (thylakoids) were centrifuged at $100\,000 \times g$ for 20 min at 4°C. The pellet was resuspended in a buffer containing 400 mM sucrose, 20 mM HEPES (pH 7.5), 5.0 mM $MgCl_2$, 5.0 mM EDTA, 1.0 mg/ml bovine serum albumin, and 20% (v/v) glycerol. It was further homogenized with a tissue grinder and briefly centrifuged ($1000 \times g$, 10 s) to remove the unbroken cells. The supernatant containing the thylakoids was re-centrifuged at $14\,000 \times g$ for 1 min. The pellet was resuspended with the above described buffer to a concentration of ~ 1 mg Chl/ml in 1.5 ml microcentrifuge tubes. The aliquots of resuspension was quickly frozen in liquid nitrogen and stored at 77 K.

2.4. Chlorophyll a fluorescence induction kinetics and measurements of F_0

The Chl *a* fluorescence induction of cell or thylakoid samples was measured with a commercial pulse-amplitude-modulated fluorimeter (Walz PAM-2000, Effeltrich, Germany). Actinic and measuring beams were provided by the built-in red (650 nm)-light-emitting diodes. The intensity of the measuring light was 0.7 $\mu E/m^2$ s and the intensity of the actinic light was 470 $\mu E/m^2$ s. Before the measurements, the cells were resuspended in TAP medium, and the thylakoids in a buffer containing 20 mM HEPES (pH 7.0), 100 mM sorbitol, 10 mM KCl, 10 mM $MgCl_2$, 0.1 mM NH_4Cl. Chl concentration of the samples was 5 μg/ml. All samples were prepared in the presence of weak (< 0.3 $\mu E/m^2$ s) background green light; and the measurements were done with samples dark-adapted for 5 min. When a light treatment of cells was needed, the dark-grown cells were illuminated with 70 $\mu E/m^2$ s white light for 1 h in the TAP medium while being stirred and bubbled with air.

In view of the fact that conclusions regarding the photochemical activity are obtained from a knowledge of the variable fluorescence (F_v) whose value is dependent upon the precise value of true F_0 (see, e.g., Ref. [28]), it is necessary to understand the complexity of the measured F_0, when the true F_0 of PS II is defined as F_0 of PS II with all $[Q_A]$ in the oxidized state. The cell or thylakoid samples are all dark-adapted for at least 5 min. before F_0 measurements to assure that all the Q_A are in the oxidized state. We, however, understand that this measured F_0 in cells is due to true $F_0 + F(\text{PS I}) + F$ (due to the prescence of some Q_A^- in dark due either to PS II inactive in transferring electrons from Q_A^- to Q_B or to the presence of Q_B^- in the dark which leads to some $Q_A^- Q_B$ from $Q_A Q_B^-$). Oxidants such as quinones do not work in *Chlamydomonas* cells to easily oxidize Q_B^- (see Vernotte et al. [29]) without drastically decreasing the F_v. In the fluorescence transient measurements of intact cells obtained with actinic light, involving F_0 to F_p rise, the measured F_0 may have all of the above mentioned components, and, thus its yield (F/I) will not be constant with increasing light intensity (I). In view of the above, the measured F_0 may be labeled as F_i (or $F_{(\text{initial})}$). However, this does not allow any better understanding and it has the serious potential of causing confusion since F_I (or F_i) is used already for the fluorescence intensity at 'I' in the 'O-I-P' fluorescence transient (see, e.g. [28]).

True PS II F_0 is the yield of fluorescence when $[Q_A]$ is maximal, i.e., when the yield of photochemistry is maximal; it reflects the PS II antenna fluorescence that is obtained in competition with excitation energy transfer to the reaction center. Since we were interested in knowing if the mutant had a different true F_0 than the WT, special efforts were made to

measure F_0 at the lowest possible intensities. At the very low light intensities, the quantum yield of true PS II F_0 (i.e., F_0/I, where I is the intensity of excitation) must be independent of I. The PS I F_0, that does not vary with light intensity at all, has a constant, but small (10% of total F_0) value (see, e.g., Refs. [30,31]). Further, the addition of herbicide DCMU in darkness is not expected to increase the value of true F_0 since DCMU is not expected to affect the excitation energy transfer process. Thus, F_0 was measured as a function of light intensities in the low range (0.01–0.45 $\mu E/m^2$ s) with and without 10 μM DCMU to evaluate how close we were in measuring the true F_0. This was done using a different pulse-modulated fluorimeter (Walz PAM-103, Effeltrich, Germany) than used for complete fluorescence transient measurements. Light intensity was varied with Oriel neutral density filters.

2.5. Flash induced chlorophyll a fluorescence changes

The kinetics of Chl *a* fluorescence changes in darkness after single-turnover actinic flashes were measured with a laboratory-made multiflash fluorimeter [32]. All sample manipulations were done in the dark with weak background green light (< 0.3 $\mu E/m^2$ s). Measurements were made with cells suspended in the TAP medium, or with thylakoids suspended in the buffer and conditions described in Section 2.4. Chl concentration of the measured samples was 5 $\mu g/ml$. When necessary, the cells and thylakoids were treated with 5 mM NH_2OH, or 5 mM hydroquinone, and/or 10 μM DCMU in total darkness. In spite of known difficulties in fully reoxidizing all Q_B^-, treatment of 5 min dark-adapted cells with 100 μM *p*-benzoquinone was made, and samples with partially reoxidized Q_B^- showed binary, albeit shallow, oscillation pattern.

Chl fluorescence changes with time after the flashes were deconvoluted into three exponential components with the KaleidaGraph™ program. The fitting equation used was:

$$\frac{F_v}{F_0} = A_1 \exp(t/\tau_1) + A_2 \exp(t/\tau_2) + A_3 \exp(t/\tau_3)$$

where 'A' represents the amplitude and 'τ' the lifetime of the components. We have assumed here (see, e.g., Ref. [33]) that the fast component (A_1, τ_1) in sub-ms range reflects combination of the kinetics of direct reoxidation of Q_A^- by Q_B, and, by Q_B^- ; the intermediate component (A_2, τ_2) in ms range reflects the equilibrium $[Q_A^-]$, partially controlled by the movement of plastoquinone to PS II without bound Q_B; and the slow component (A_3, τ_3) in seconds range reflects both the back-reaction between Q_A^- and the S-states and between Q_B^- and the S-states. Since there are no S-states in the mutant sample due to the lack of Mn cluster [1], the slow component may reflect the back reaction of Q_A^- to donor Z (D1-Y161).

2.6. Thermoluminescence

Thermoluminescence that probes both the donor and acceptor sides of PS II (see reviews by Inoue [34] and Vass and Govindjee [35]) was measured as described by Kramer et al. [36]. Thylakoid samples (~ 1 mg Chl/ml, pH 7.6) were isolated from the heterotrophically grown cells that were briefly adapted by light (20 min, 70 $\mu E/m^2$ s white light). The measurement was done by illuminating with one saturating flash in the presence and absence of 10 μM DCMU. The sample cooling rate was $> 5°C/s$. The thermoluminescence curves were recorded from -55 to $+55°C$ at a heating rate of $1°C/s$. The flashes were given at $-10°C$. For details see Ref. [36].

2.7. Bicarbonate depletion and recovery treatments

Bicarbonate depletion of cells by formate was carried out with a formate treatment procedure described in El-Shintinawy et al. [6] with modifications. The harvested dark-grown cells were resuspended to 100 μg Chl/ml in a buffer containing 100 mM HEPES (pH 5.83), 40 mM NaCl, and 5 mM $MgCl_2$. The resuspended samples were treated with various concentrations of sodium formate for 5 min under gentle vacuum, after which they were diluted to 5 μg Chl/ml in the same buffer at pH 6.5. The samples were immediately used for the Chl fluorescence yield measurements. Sodium bicarbonate (10 mM) was added to the formate-treated samples to check the reversibility of the formate inhibition. The control

samples were subject to similar treatments except without formate and bicarbonate in the buffers.

2.8. Low temperature fluorescence spectra

Measurements of the low temperature fluorescence emission spectra of the heterotrophically grown cells and the thylakoids isolated from these cells were performed at 77 K in TAP medium containing 20% glycerol (for cells) or in the thylakoid buffer (for thylakoids) as described in Sections 2.3 and 2.4 with the addition of 20% glycerol. Chl concentration was 30 μg/ml. Measurements were made using a Perkin Elmer LS-5 fluorescence spectrophotometer (Perkin Elmer, Oak Brook, IL) which was equipped with a red-sensitive photomultiplier (R928, Hamamatsu, Shizuoka-ken, Japan). The samples were placed in a Dewar flask with an optical clear region through which the fluorescence was excited and measured. The samples were frozen in liquid nitrogen prior to measurement. The excitation wavelength was set at 435 nm and the monochromator bandwidth 10 nm for excitation and 3 nm for emission. The fluorescence emission was collected from the front surface of the sample. The obtained 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.

2.9. Herbicide binding assay

The herbicide ^{14}C-terbutryn binding assay was done according to Vermaas et al. [37]. Thylakoid samples (25 μg Chl/ml) were incubated with various concentrations of ^{14}C-terbutryn (24 μCi/mg, kindly provided by Dr. Donald Ort), known to bind at the Q_B site, in the thylakoid buffer described above (Sections 2.3 and 2.4) at 25°C in darkness for 15 min with occasional shaking. The thylakoids were then centrifuged for 10 min at 14 000 × g, and the supernatant was mixed with a scintillation cocktail (Scintiverse II, Fisher Scientific, Fair Lawn, NJ). The radioactivity of the liquid mixture was counted in a scintillation counter (LS1701, Beckman Instruments, Fullerton, CA) to 1% error. To eliminate the contribution of the unspecific binding, the samples were measured in the presence and the absence of another herbicide, atrazine (20 μM), that also binds at the Q_B site. The specific binding was obtained by subtracting the atrazine-replaceable terbutryn binding from the total terbutryn binding.

3. Results and discussion

3.1. Chlorophyll a fluorescence induction

To obtain information on the electron transfer reactions on the PS II acceptor side in the D1-R269G mutant, Chl *a* fluorescence induction (up to 0.6 s) (see, e.g., Refs. [38,39]) was measured in the dark-grown wild type and mutant cells and thylakoids with a pulse-amplitude-modulated fluorimeter. The induction kinetics of Chl *a* fluorescence of these samples were measured in the absence and the presence of the herbicide DCMU, known to block electron flow by displacing Q_B, and are shown in Fig. 1. The rise kinetics of the fluorescence reflects the net balance between the rate of reduction of Q_A and reoxidation of Q_A^-. DCMU blocks the electron transfer beyond Q_A, by displacing Q_B [40], and thus Chl *a* fluorescence rapidly reaches the maximum level (F_m). The difference between F_m and the true minimal fluorescence level, F_0, is the maximal variable fluorescence (F_v) which is related to the maximal yield of photochemistry of PS II (Φ_p) as follows:

$$\Phi_p \equiv (F_m - F_0)/F_m = F_v/F_m$$

(see, e.g., Ref. [28]).

Fig. 1A and B show the measured fluorescence transients of dark-grown wild type and D1-R269G cells. Since F_m is approximately the same in the two cases, a 50% reduction in F_v may be interpreted to mean that the mutant has lowered photochemistry, provided the differences in measured F_0 are differences in true F_0. This conclusion is totally dependent upon the differences in the measured F_0 being differences in true F_0, as is shown later in Fig. 2. If we assume that changes in F_v reflect charge separation in PS II ($Z\ P680^*\ Q_A \rightarrow Z^+\ P680\ Q_A^-$), then we can conclude that the mutant has lowered yield of charge separation. The simplest interpretation, however, is that the D1-R269G mutant has decreased the number of active PS IIs/Chl (see [1]). Further, the shape of the transient in both the wild type and the mutant

shows that dark-grown cells are not normal, as is well known (see, e.g., [41]): the water oxidation machinery is not functional and one observes, perhaps, only one turnover of PS II following slow oxidation of Q_A^-.

Fig. 1C and D show the Chl *a* fluorescence transient of light adapted cells (dark grown cells exposed to 1 h of 70 $\mu E/m^2$ s white light). The light treatment causes the wild type kinetics to display a normal fluorescence transient, i.e., a slower rise phase, labeled as O-I-D-P phase [42]. F_p is shifted to a longer time (400 ms versus 25 ms after actinic light illumination) since both the donor and acceptor sides of PS II are now functional: the F_0 to F_p rise is biphasic with an intermediate F_i, or F_I (not to be confused with F_i, used by some authors to indicate 'initial' fluorescence); the rise reflects the filling up of the plastoquinone pool with electrons from the donor side of PS II. The phenomenon of photoadaptation which is a slower rise of transient kinetic curve to F_p due to the recovery of the PS II donor side was observed earlier in *C. reinhardtii* by Guenther et al. [43]. However, this transition is not observed in the R269G mutant, indicating that the mutation has inhibited the necessary photoadaptation process. In our earlier paper [1], we have shown that Mn centers which constitute the S-state complex for the donor side function are missing in the mutant. Further, after a light treatment, the measured F_v of the mutant cells becomes further reduced (only 35% of that of the wild type), and F_v/F_m reflecting photochemistry is reduced by 60%. This significant lowering of the F_v after light treatment indicates an increased suscepti-

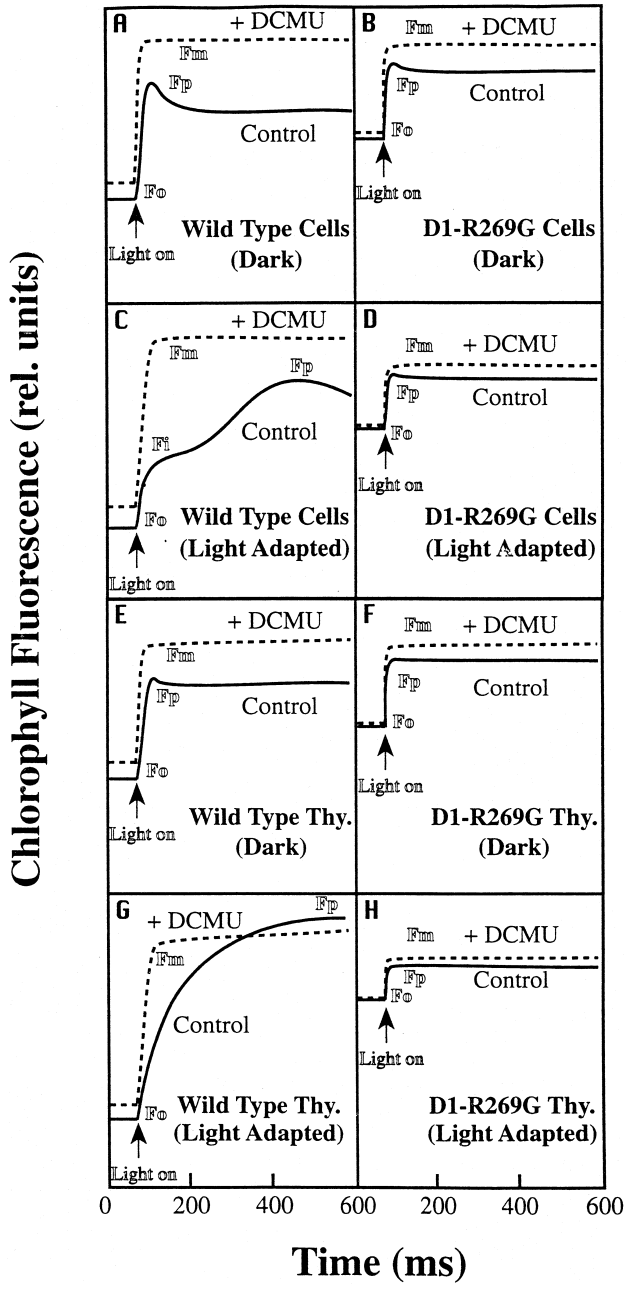

Fig. 1. Chl *a* fluorescence transients (as a function of time of illumination) of the dark-grown *C. reinhardtii* wild type and D1-R269G in the absence and the presence of 10 μM DCMU measured with a PAM-2000 fluorimeter. The full scale is 200 mV. F_0 is the measured F_0 of this instrument, and F_m is the $F_{maximum}$. (A) The transient of the wild type cells. (B) The transient of the mutant cells. (C) The transient of the light-adapted wild type cells (treated with 70 $\mu E/m^2$ s white light for 1 h). Note the transformation of the kinetics from the dark phase to the light phase indicated by the shift of the time of F_p. The F_i (denoting fluorescence intensity at the 'I' step of O-I-D-P transient) should not be confused with the F_i ($F_{initial}$) of other authors. (D) The transient of the light-treated (70 $\mu E/m^2$ s) mutant cells. Note the absence of the kinetic transformation and a more elevated measured F_0 and a decreased F_v, suggesting a possible photo-damage to the mutant PS II. (E) The transient of the wild type thylakoid isolated from the dark-grown cells. (F) The transient of the mutant thylakoid isolated from the dark-grown cells. (G) The transient of the wild type thylakoids isolated from the light-adapted cells. Note the F_v is basically unchanged compared with the cells. (H) The transient of the mutant thylakoids isolated from the light-adapted cells. Note the even lowered F_v in this sample. In all measurements, the [Chl] of the samples was 5 μg/ml, and the actinic illumination was 470 $\mu E/m^2$ s. Thy = thylakoids.

bility of the mutant PS II to photoinhibition, confirming conclusions in our earlier paper [1]. Fig. 1E and F are the Chl *a* fluorescence transient of the thylakoids isolated from the dark-grown cells. The measured F_v of the mutant thylakoids is 60% of the wild type level. After the light treatment of the cells, the measured F_v of the isolated mutant thylakoids is reduced to only ~20% of the wild type level (Fig. 1G and H), indicating that the combination of the steps involved in biochemical preparations and the light treatment may cause further lesions to the mutant PS II. It is noted that the maximal fluorescence level after DCMU treatment (F_m) is slightly lower than the F_p in the control (Fig. 1G). This lowering is thought to be due to the quenching of fluorescence by the plastoquinone (see review [28], and Vernotte et al. [44]).

3.2. The measured F_0 and the true F_0

D1-R269G mutant samples have a consistently elevated measured F_0 compared to that of the wild type. If this information can be confirmed by measuring the true F_0, that is the minimal level of Chl fluorescence originating from antenna, in competition with energy transfer to the PS II reaction center, an increase in F_0 would indicate a decreased excitation energy transfer to the active PS II reaction center, possibly due to the disconnection between antennae and the reaction center II, provided we can assume that the PS I component of F_0 is small and remains constant. A second possibility is the existence of inactive PS II [45] incompetent in trapping excitons arriving from antennae, which then can return to antennae and fluoresce; an increased number of inactive PS II reaction centers would lead to an apparent increase in antennae/active PS II reaction center. Our data do not allow us to distinguish between the possibilities. However, as shown in Fig. 1, the wild type cells or thylakoids display a small increase in F_0 when measured in the presence of DCMU which blocks the electron transfer beyond Q_A^-; this may be due to back reaction of electrons from reduced Q_B and Q_A. The slight increase in measured F_0 in the presence of DCMU could also include a contribution due to the small actinic effect of the measuring beam of the fluorimeter. Thus, the measured F_0 required further investigation according to the rationale given under Section 2.

Independent measurement of F_0 for the wild type and D1-R269G mutant cells at very low light intensities of the measuring beam indicate that F_0 intensity is a linear function of light intensities (I) (Fig. 2A). The quantum yield (F_0/I) remains constant as it should for true F_0 which should be independent of photochemistry (Fig. 2B) (see, e.g., Ref. [46]). Further, addition of DCMU did not cause significant increases in the measured F_0 levels, especially at the lowest intensities used, as it should since all [Q_A] remains essentially unchanged. Mutant F_0 and F_0/I were consistently double that in the wild type (~200%). Thus, true F_0 of the D1-R269G mutant is higher than the wild type, and F_v is, therefore, reduced in the mutant, as interpreted in Fig. 1.

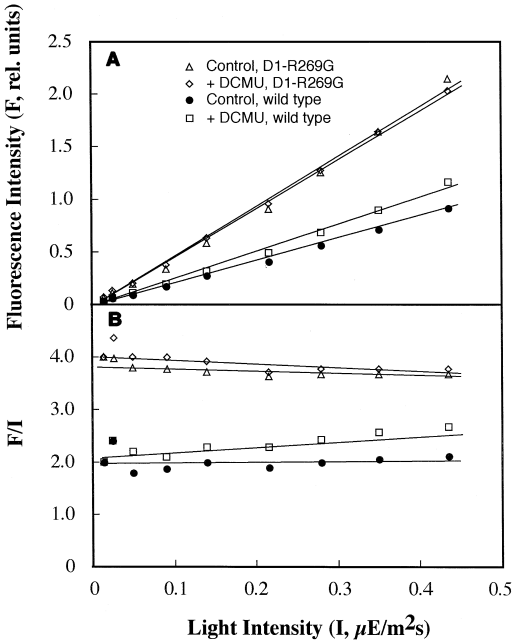

Fig. 2. (A) Baseline Chl *a* fluorescence (F_0) measured as a function of light intensities in the low intensity range, in the presence and the absence of 10 μM DCMU. Measurements of the heterotrophically grown wild type *C. reinhardtii* and D1-R269G mutant cells were made as in Section 2.4. (B) The quantum yield of measured F_0, or the ratio of fluorescence (F) to the light intensity (I) of the measuring beam, basically does not change with the light intensities indicating that the measured measured F_0 in this experiment is very close to the true F_0. It is further confirmed by the absence of any significant effect of DCMU. Data suggest that D1-R269G mutant has a two times true F_0 than the wild type cells.

3.3. Chlorophyll a fluorescence changes after a flash

To locate the effect of the mutation on the specific reaction of the acceptor side of PS II, we measured Chl *a* fluorescence changes on the microsecond time scale after a series of single turnover flashes. The kinetics of these changes correspond mainly to the electron transfer from Q_A^- to Q_B or Q_B^- (see, e.g., Refs. [17,47]); however, the fluorescence yield at F_m and in the sub-ms range also include effects of changes in electron flow on the donor side of PS II, i.e. changes in [$P680^+$], a quencher of Chl fluorescence [48]. A slowed equilibrium between $S_n \Leftrightarrow Z \Leftrightarrow$ P680 results in a lowering of F_m and a rise phase of fluorescence (see, e.g., Refs. [32,49,50]). Fig. 3 shows Chl *a* fluorescence yield changes for the dark-grown, non-oxygen evolving cells and thylakoids in the presence and absence of DCMU (10 μM) and of hydroxylamine (NH_2OH, 5 mM) and hydroquinone (5 mM), donors to $P680^+$. (For a rationale of using these chemicals, see Metz et al. [51].)

We will first discuss the donor side effects. When compared to the wild type, cells and thylakoids of D1-R269G mutant show (cf. Fig. 3A with B) significant decreases in the F_v/F_0 ratio. Further, the mutant thylakoids have even lowered F_v/F_0 level compared to not only the wild type thylakoids, but also mutant cells. These results may suggest, in addition to the existence of inactive PS IIs in the mutant, inhibitions on the donor side of PS II in the mutant (also see Section 3.5).

The addition of both 5 mM NH_2OH and 10 μM

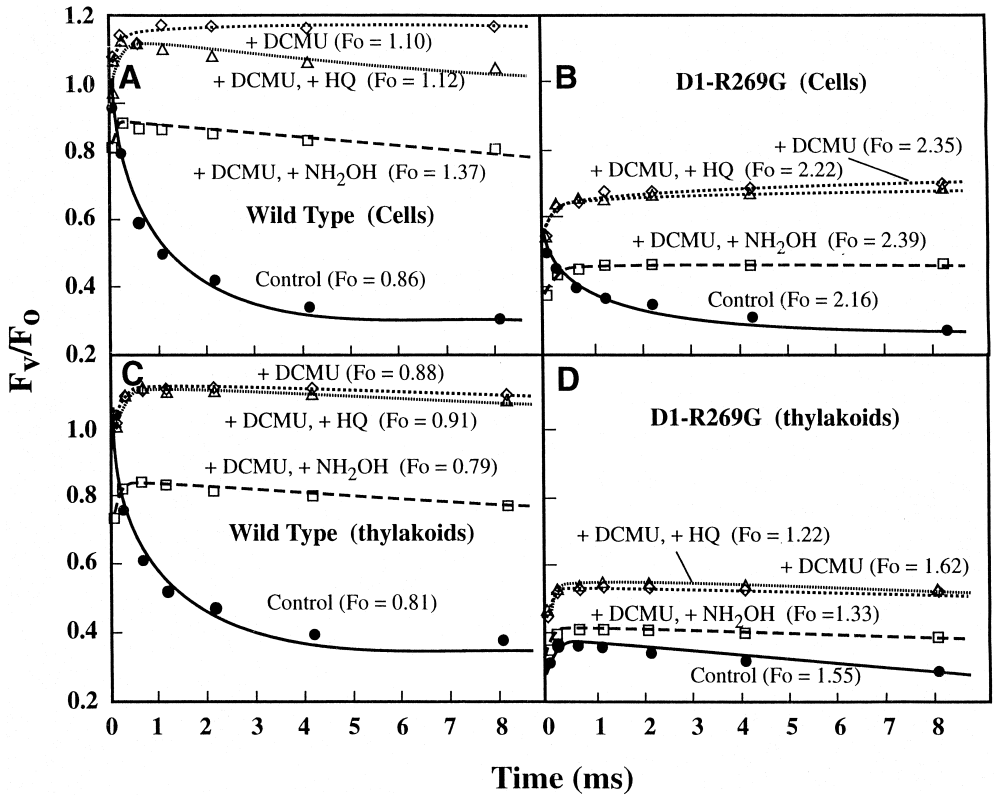

Fig. 3. Flash-induced Chl *a* fluorescence yield kinetics of the dark-grown wild type and the dark-grown D1-R269G mutant of *C. reinhardtii*, treated with or without DCMU (10 μM), or with DCMU (10 μM) and NH_2OH (5 mM), or with DCMU (10 μM) and hydroquinone (HQ, 5 mM). The kinetic measurements were done with 5 μg Chl/ml samples. Only the second-flash kinetic traces are shown. (A) Kinetics of fluorescence change from the wild type cells. (B) Kinetics of fluorescence change from the D1-R269G mutant cells. Data show a lowered yield of photochemistry (calculated from F_v/F_m) and a slowed rate of electron flow from Q_A^- to the plastoquinone pool in the mutant. (C) Kinetics of fluorescence change from the wild type thylakoids. (D) Kinetics of fluorescence change from the D1-R269G thylakoids. Note the slowed decay kinetics and the lowered F_v/F_m in the mutant samples suggesting an inhibition on the PS II acceptor side and the presence of high proportion of inactive PS II reaction centers. The addition of the good donor hydroquinone is unable to restore the Chl *a* fluorescence level in the mutant (cells or thylakoids) to the wild type level.

DCMU is unable to restore the maximum level of F_v/F_0 in the mutant sample (cells or thylakoids) to that of the wild type level. However, the addition of hydroquinone (5 mM) results in a variable fluorescence level equivalent to the DCMU treatment without added donors. This is simply because hydroquinone is a much better electron donor to $P680^+$ than hydroxylamine [51,52]. The consistent lower level of variable fluorescence in the D1-R269G mutant, even when DCMU and hydroquinone are present, indicates that the functional PS IIs may be much lower than those in the wild type similar to our observations of the mutant using EPR spectroscopy [1]. Fig. 3 also lists the increased measured F_0 in the mutant cells and thylakoids relative to the wild type: this confirms the observation in the above fluorescence induction measurements (Fig. 1).

We now discuss the acceptor side effects. The decay of fluorescence is slower in the mutant than in the wild type cells. The lifetime of the first component (τ_1) after the first flash is 90 μs in the wild type vs. 1.6 ms in the mutant; τ_1 after the second flash is 120 μs in the wild type vs. 2.0 ms in the mutant. There is ~17 fold decrease in the rate of fluorescence decay. The first component of the wild type constitutes ~66% of the total fluorescence decay process and that of the mutant only constitutes ~30% of the decay. In spite of the slight difference between flash 1 and 2 results, they do not represent the true Q_A^- to Q_B (or Q_B^-) steps, since Q_B to Q_B^- ratio in dark-adapted untreated intact cells is not too far from 1 (see Section 3.6). The decay rate for the mutant thylakoid samples was even slower (~50 fold) compared to that of the wild type (Fig. 3C and D), consistent with other lines of evidence that the mutant PS II has much less structural stability in the thylakoid preparations. The addition of high concentration (10 μM) of DCMU inhibits the electron transfer in both the wild type and the mutant cells and thylakoids.

3.4. Functioning of the two-electron gate

PS II variable Chl *a* fluorescence is controlled by the redox state of the primary plastoquinone, Q_A, a one electron carrier, which is oxidized by Q_B (or Q_B^-), a two electron carrier ('the two electron gate', [53,54]). Since the electron flow from Q_A^- to Q_B is faster than from Q_A^- to Q_B^-, it is reflected in a 'deep' period two oscillation pattern in the Chl *a* fluorescence decays (see [55]) in samples that start with $Q_B:Q_B^-$ ratio of 1:0. It is generally known that the $Q_B:Q_B^-$ ratio is close to 1:1 in intact photosynthetic cells [56,57]. Although, as noted earlier, it is not easy to fully oxidize Q_B^- in cells [29], partial effects are obtained if we pretreat the dark-grown wild type cells with *p*-benzoquinone (100 μM) and NH_2OH (5 mM), and illuminate the samples with a series of single turnover flashes. In this assay, the flash frequency was 0.67 Hz, and the variable fluorescence values at 200 μs are shown. NH_2OH was added to eliminate most of the period four oscillations due to the donor side activities and to serve as an electron donor, albeit poorer than hydroquinone, since the cells were unable to oxidize water. An obvious, although not very deep, binary oscillation pattern of the Chl variable fluorescence is observed for the wild type (Fig. 4). Crofts et al. [58] have used a benzoquinone treatment method that yields deep period two oscillations, but, then the F_v is drastically reduced. In the mutant sample, however, this period two oscillation pattern was eliminated suggesting a defective two-electron gate mechanism caused by the mutation. The data indicate that the PS II acceptor side reactions are significantly modified due to the R269G mutation.

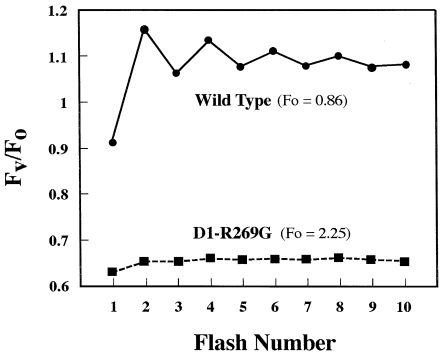

Fig. 4. Flash oscillation pattern for the variable Chl *a* fluorescence of the dark-grown wild type and D1-R269G cells of *C. reinhardtii* measured at 200 μs after an actinic flash. The samples were treated, by vacuum infiltration, with *p*-benzoquinone (100 μM) to convert some of the Q_B^- to Q_B and NH_2OH (5 mM) to block the S-state transitions, if any, prior to measurement. The [Chl] of the samples was 5 μg/ml, the flash frequency during the measurement was 0.67 Hz. The data indicate a loss of the period two oscillation pattern in the mutant.

3.5. Thermoluminescence

Since thermoluminescence (see [34,35]) measures the recombination of charges between S_2 and Q_A^- (the D or the Q band) and between S_2 (or S_3) and Q_B^- (the B band) (see, e.g., [36]), it can be used to check if the mutant is blocked in the S-state transition. Fig. 5 shows that the wild type thylakoids have the normal B band (a broad band in the 30–35°C region). Upon treatment with DCMU (10 μM), the B band is abolished, and the Q band (a broad band at 15°C) appears due to inhibition of electron transfer from $Q_A^- \rightarrow Q_B$ enabling stabilization of Q_A^-. The mutant, however, lacks both the B and Q bands confirming that it is unable to store charges on the S-states. However, a difference thermoluminescence curve (for DCMU-treated minus untreated mutant thylakoids) showed a slight negative band in the B band region, and a slight positive band in the Q band region, but it was within the noise level of our measurements. A high temperature band that includes a 'C' band, suggested to arise from recombination of Y_D^+ and Q_A^- [59], is however, present. A good part of the high temperature band, observed here, was unrelated to photosynthesis (data not shown); but this does not affect the clear conclusions noted above. Thermoluminescence results confirm the defective nature of the mutant on the donor side of its PS II [1].

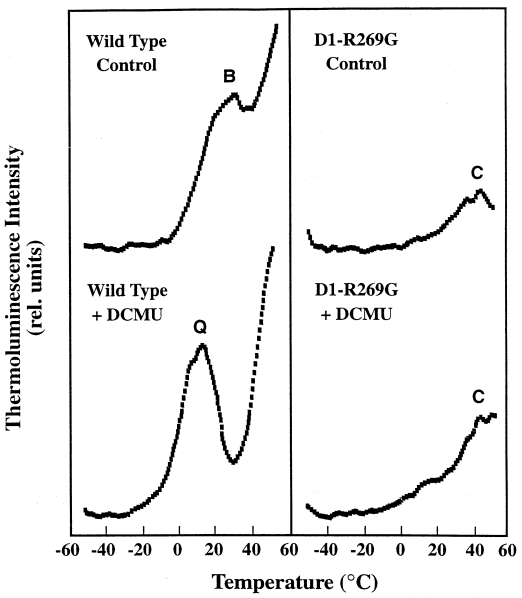

Fig. 5. Thermoluminescence of wild type *C. reinhardtii* and D1-R269G mutant thylakoid ([Chl] is ~1 mg/ml) in the presence and absence of DCMU (10 μM). The samples were measured after one saturating, single-turnover, flash. The heating rate was 1°C/s. Data obtained by David Kramer.

3.6. Bicarbonate depletion and recovery

Since our hypothesis was that D1-R269 is involved in HCO_3^- binding, we determined the effect of formate (analog of bicarbonate) inhibition on the Q_A^- to Q_B (or Q_B^-) electron transfer. Flash-induced Chl fluorescence decay of the dark-grown wild type and the D1-R269G cells with or without formate or formate plus bicarbonate after the second flash is shown in Fig. 6 (data after the first flash are not shown). Since in intact untreated cells, the ratio of Q_B to Q_B^- in darkness is close to 1 [56,57], Q_A^- to Q_B and Q_A^- to Q_B^- reactions are not easily separable. The addition of 25 mM (pH 6.5) formate slows down the electron flow from Q_A^- to Q_B (or Q_B^-) in the wild type (Fig. 6A). Formate inhibition is almost fully reversed by the addition of bicarbonate (10 mM). The Q_A^- to Q_B (or Q_B^-) reaction in the mutant samples are also inhibited by formate; bicarbonate readily reverses this effect (Fig. 6B). It is established [6–10] that formate has additional effects on the donor side; the insets in Fig. 6 show that formate causes decrease in F_v/F_0 at < 250 μs before an increase can be observed (also see Refs. [6,10]). This confirms the dual effect of bicarbonate-reversible formate effect in thylakoids of both wild type and D1-R269G *Chlamydomonas* cells.

A quantitative assay of the formate inhibition and bicarbonate recovery for both the wild type and the mutant samples is shown in Fig. 7. The dark-grown cells were treated with various concentrations of formate in the absence or presence of bicarbonate and assayed for Chl *a* fluorescence decay as above. The resulting decay curves were deconvoluted with three exponential components (see, e.g., Ref. [33]). The fast decay component represents the kinetics of Q_A^- to Q_B (or Q_B^-) electron transfer. Thus, we plotted the normalized lifetime of the first component (τ_1) as a function of formate concentration with the τ_1 of the control (without formate treatment) as 100 in arbitrary units. For the second flash, the τ_1 of the wild type in the control is 120 μs and that of the mutant is 2.0 ms, an ~17 fold effect. As shown in Fig. 7, at increasing formate concentration, the τ_1 of the wild

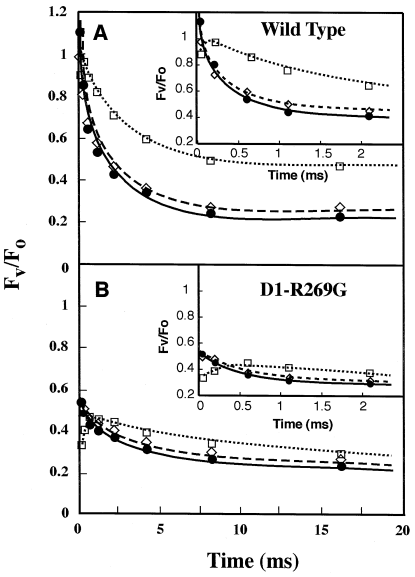

Fig. 6. Flash-induced Chl *a* fluorescence changes of the dark-grown *C. reinhardtii* wild type (A) and the D1-R269G (B) cells treated with (□) or without (●) sodium formate (25 mM, pH 6.5) and with a subsequent addition of sodium bicarbonate (10 mM) (◇). Insets show data plotted on an expanded scale up to 2 ms. Formate treatment was as in Ref. [6]. [Chl], 5 μg/ml. Second flash kinetics are shown.

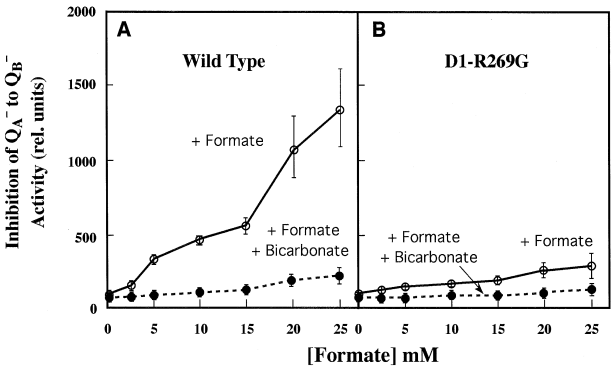

Fig. 7. (A) Dark-grown *C. reinhardtii* wild type cells treated with various concentrations of formate (pH 6.5) in the absence or the presence of bicarbonate (10 mM) and assayed for Chl *a* fluorescence decay after the second actinic flash. Formate treatment and bicarbonate recovery was as in Ref. [6]. The resulting Chl fluorescence decay curves were deconvoluted into three exponential components. The lifetime of the first component (τ_1) of the decay curve after the second flash, corresponding to a mixed kinetics of Q_A^- to Q_B, and to Q_B^- electron transfers, is depicted as a function of [formate] when the τ_1 of the control is set to read 100. (B) The same treatment and analysis for the D1-R269G mutant. The mutant is shown to be ~5 times less sensitive to formate inhibition than the wild type.

type increases up to 1.6 ms at the highest concentration (25 mM) corresponding to an 13 fold increase. However, τ_1 of mutant sample only increases from 2.0 ms (control) to 5.5 ms (25 mM formate) corresponding to only an 2.5 fold increase. The addition of bicarbonate is able to fully recover the inhibition in both the mutant and the wild type. Thus, there appears to be roughly a 5 fold difference in the sensitivity to formate suggesting that D1-R269 may play some role in the binding niche of bicarbonate. However, we note that since the maximum τ_1 for the wild type cells in 25 mM formate treatment is 1.6 ms, whereas the τ_1 of the mutant without formate treatment is 2.0 ms, it may be possible that any formate effect may be obscured by the intrinsic effect of the mutation on τ_1.

3.7. Low temperature fluorescence emission spectra

Based on the measurements, discussed in Sections 3.1 and 3.2, that indicate a significantly lowered quantum yield of photochemistry (indicating a lowered yield of stable charge separation) and elevated F_0 (indicating a lowered net excitation energy transfer from antennae to the PS II reaction center), it is reasonable to conclude that the D1-R269G mutation has altered the structure and function of PS II complex. This conclusion is fully consistent with our recent EPR measurement on Tyr_D^+ and Q_A^- Fe^{+2} [1]. To investigate possible physical changes in the PS II complexes, we used low temperature (77 K) fluorescence emission spectra as an indirect means to probe the state of PS II. At 77 K, PS II has two distinct emission bands at 685 nm (F685) and 695 nm (F695). F685 is thought to originate mostly from the CP43 polypeptide and F695 from CP47 polypeptide (see Refs. [60–62]). Haag et al. [63] suggest that the intensity of these two bands, especially F695, correlates well with the level of the PS II core proteins and can be used as an indicator for the concentration of PS II. Thus, whether we have an inactive or active PS II reaction center [PS II core] would influence the F685 and F695 fluorescence intensities. However, we also realize that the intensities of F685 and F695 must also be influenced by the efficiency of excitation energy transfer from peripheral (or distal) antenna Chls to core antennae and from the core antennae to the PS II reaction center.

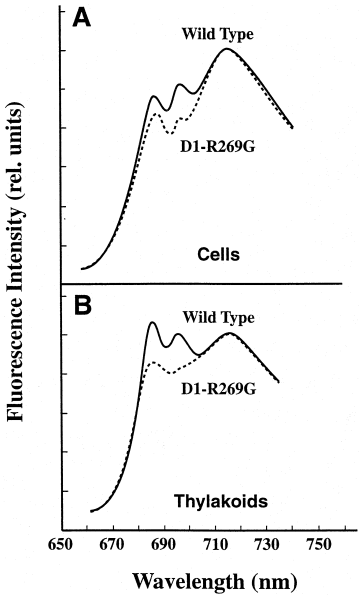

Fig. 8. Emission spectra at 77 K of *C. reinhardtii*. Emission spectra of the samples (30 μg Chl/ml with 20% glycerol) were measured with a front-surface optics (excitation at 435 nm; corrected for the wavelength dependence of the sensitivity of the photodetector) and normalized to the 715-nm peak. (A) The 77 K Chl *a* fluorescence emission spectra of the cells of the dark-grown *C. reinhardtii* wild type and the D1-R269G mutant. (B) The 77 K Chl *a* fluorescence emission spectrum of the thylakoids (pH 7.0) of the dark-grown *C. reinhardtii* wild type and the D1-R269G mutant. Both F685 (from CP43) and F695 (from CP47) bands were lowered in the mutant with respect to F715 band. Further, data indicate a differential reduction of the 685-nm (F685, CP43) and 695-nm (F695, CP47) emission peaks for the D1-R269G mutant cells and thylakoids.

The fluorescence emission spectra of the wild type and mutant cells and thylakoids were measured upon excitation by 435 nm light (absorption mainly by Chl *a*). The intensities of the emission bands of the obtained spectra (Fig. 8) were further deconvoluted into three individual peaks (data not shown) for F685, F695, and F715 (for PS I). Assuming that no changes occur in PS I, both F685 and F695 bands of the mutant cells and thylakoids show a reduction of 20–30% compared to the wild type. However, the F695 band appears to be reduced to a slightly greater extent than F685. The ratio of F695/F685 in the mutant cells was reduced by 36%, and in the mutant thylakoids by 22% compared to the wild type. This result may indicate a differential reduction in these PS II antenna complexes provided the mutation had not caused changes in excitation energy transfer among these complexes and the PS II reaction center. Since we assume that there were no changes in the CP43 and CP47 genes, this reduction was partly attributed to the changes in the stability of the D1/D2 complexes to which the antenna proteins are associated, and partly to the changes in the excitation energy transfer to and away from the PS II reaction center.

3.8. Herbicide binding assay

To test possible structural changes in the Q_B binding niche caused by the R269G mutation, a radioactive herbicide binding assay was performed. ^{14}C-terbutryn binding was measured with the thylakoids of the wild type and D1-R269G according to Vermaas et al. [37]. A double reciprocal plot for the ^{14}C-terbutryn binding is shown in Fig. 9. Mutant thylakoids have a significantly lowered ^{14}C-terbutryn binding affinity compared to the wild type. The dissociation constant for the wild type is 29 (±11) nM, and for the mutant 220 (±70) nM, about 8 fold effect. This suggests a drastically altered Q_B binding niche in the mutant. A variability is observed among thylakoid samples, especially with time of storage, and a difference of up to 30 fold with the wild type

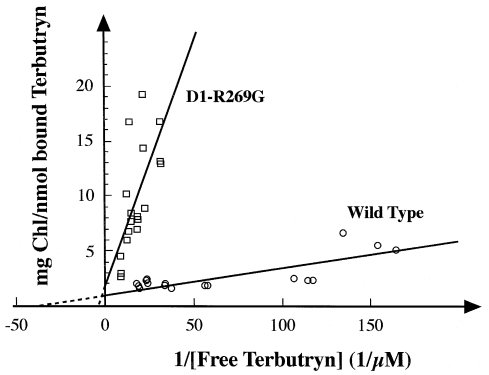

Fig. 9. Double reciprocal plot for the ^{14}C-terbutryn binding to the thylakoids of the dark-grown wild type and the D1-R269G mutant of *C. reinhardtii*. Terbutryn specific binding was obtained by subtracting the atrazine-replaceable terbutryn binding from the total terbutryn binding according to Vermaas et al. [37]. The reactions were performed as in Section 2.9. The calculated terbutryn dissociation constant for the wild type is 28.9 (± 11.1) nM and that of the mutant is 223.3 (±71.1) nM.

was observed in certain trials. This reflects the instability of the mutant thylakoids after they are prepared from the cells. The notion that the PS II stability is affected in the mutant is supported from assays such as the 77 K fluorescence emission spectra (Fig. 8), fluorescence kinetics measurements (Fig. 3), as well as the EPR, and western blot analyses published elsewhere [1]. Since mutant thylakoids are unstable, we speculate that the differences between herbicide binding in wild type and mutant cells would be much less than those observed in thylakoids.

3.9. Modeling: bicarbonate binding sites

A recently constructed three dimensional PS II reaction center model [20] suggests that D1-R269 is not a direct binding residue for bicarbonate at the non-heme iron site. The geometric position of D1-R269 is modeled 8–11 Å from bicarbonate/iron center, which does not support a direct interaction. D1-R269 is located near the N-terminal region of the transmembrane α-helix E of D1. According to the model, D1-R269 is separated from D1-H272, one of the non-heme iron ligands, by nearly 3/4 of a helical turn ($\sim$ 5 Å). This close vicinity to D1-H272 which is located approximately equally in between Q_A and Q_B, may help explain a structural perturbation of D1-R269G mutation on the functionality of the iron [1] and the liganding of bicarbonate and formate (Figs. 6 and 7).

In addition to D1-R269, several other positively charged residues on the D2 protein of cyanobacteria near the non-heme iron have also been investigated for involvement in bicarbonate/formate effect [3,5,19]. D2-R233 and D2-R251 have been shown to increase the PS II susceptibility to formate inhibition of full chain electron transfer by 10 fold relative to the wild type and are suggested to function in stabilizing bicarbonate binding in vivo [19]. However, a mutation on D2-R139 (D1-R139H) showed no effect on bicarbonate-reversible formate inhibition [5] suggesting specificity of other arginines. However, the

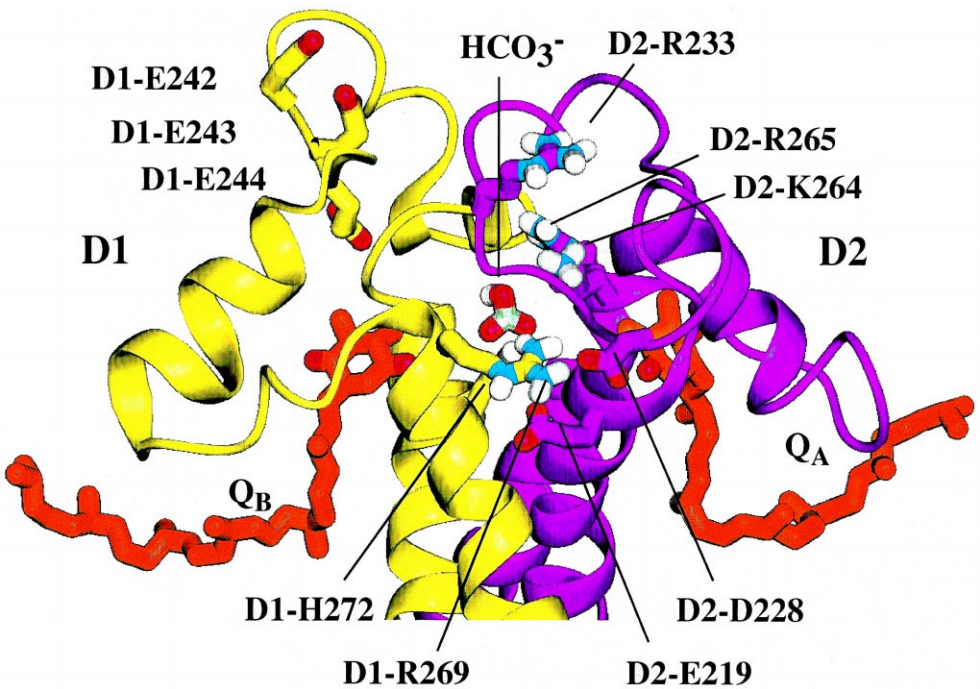

Fig. 10. A partial three dimensional model of the PS II reaction center according to Xiong et al. [20]. Shown are certain residues from the membrane helix D to helix E of both D1 and D2 proteins in their secondary structure representation. The D1-R269 residue is shown to be one of the contact residues located in the interface of D1 and D2 polypeptides and is also close to the putative non-heme iron and the liganding bicarbonate. The D1-R269G mutation is thought to abolish the putative interaction between the D1-R269 and several D2 residues, such as D2-E219 and D2-D228, affecting the non-heme iron and the Q_B sites, and even the stability of the PS II reaction center. Several other residues demonstrated in literature to be important for bicarbonate binding and function are also labeled.

lack of difference in phenotype compared to the wild type may possibly be a result of the potentially conservative mutation. D2-K264 was suggested to be a strong candidate for bicarbonate binding at the non-heme iron site, as its site-directed mutants were considerably slower in electron transfer from Q_A^- to Q_B^- compared to the wild type, and were very resistant to formate and NO treatment (unpublished data, see Ref. [3]). This mutant required much higher bicarbonate concentrations than the wild type to accelerate Q_A/Q_B electron transfer (unpublished data, see Ref. [3]), suggesting that the residue may be intimately involved in binding of bicarbonate. Mutations at a nearby residue D2-R265 also showed similar effects, though to a lesser extent.

In addition to liganding to the iron, bicarbonate may also function in promoting the protonation of Q_B^{2-} (see, e.g., Refs. [2,4,17,47]). Kinetic studies by Blubaugh and Govindjee [64] suggested the possibility of two tight bicarbonate binding sites in PS II. One of these binding sites may well be at the non-iron site, while the other may be related to the protonation of plastoquinone and thus likely to be near the Q_B site in the D1 protein. Mäenpää et al. [65] demonstrated that a strain of *Synechocystis PCC* 6803 with deletion of residues D1-E242 to D1-E244 near the Q_B site exhibits a 7 fold higher resistance to formate inhibition than the wild type. Our recent site-directed mutagenesis experiments on D1-R257 of *C. reinhardtii* have indicated that this residue is intimately involved in affecting the formate binding (J. Xiong, J. Minagawa, A.R. Crofts and Govindjee, unpublished data).

In the three dimensional PS II model [20], based on the analogy with a water transport channel in the bacterial reaction center [66], Xiong et al. [20] proposed a channel for transporting bicarbonate anions and water molecules for protonating Q_B and providing ligands to the non-heme iron in PS II reaction center. This channel or a large binding niche involves a series of charged residues of the D1 and D2 proteins, including D1-R269. Substitution on D1-R269 is thus expected to cause perturbations on formate effects, which is supported by the data in Figs. 6 and 7.

Our model [20] indicates that D1-R269 may also be involved in D1/D2 interaction. The correct assembly and stability of the D1/D2 complex of the PS II reaction center rely partly on the interactions of the contact residues located on the transmembrane spans. As shown in Fig. 10, D1-R269 may be a contact residue located in the interface of D1 and D2 polypeptides and may provide interactions important for maintaining the conformation of D1 and D2 polypeptides. Our model shows that D1-R269 may have electrostatic or hydrogen bonding interactions with certain D2 residues, such as D2-E219 and D2-D228 (Fig. 10). The glycine mutation may thus abolish such interactions affecting the stability of the PS II reaction center, resulting in a series of primary and secondary effects such as lowered charge separation, slowed PS II electron transfer, decreased binding of Q_B/herbicide, perturbed bicarbonate/formate functioning at the non-heme iron and/or Q_B site, etc. Therefore, it may be interesting to test this hypothesis by mutagenizing the D2-E220 or D2-D228 residue and see whether a similar structural instability effect on PS II may exist in these mutants.

In the model, D1-R269 is located on the acceptor side, the transduction of the mutational effect from the acceptor to the donor side can be due to D1-R269 being a contact residue affecting the assembly of the PS II altering the structure and function of both the donor and acceptor sides, as observed here and in [1]. Similar transduction of mutational effects between the donor side and acceptor side has previously been observed. Etienne and Kirilovsky [67] and Constant et al. [68] showed that several herbicide-resistant mutations at the Q_B site affect the S-state function. Similar effects have previously been observed for mutants lacking the PS II 43 kDa Chl binding protein and in site-directed mutants of the D1 protein [69,70]. Carpenter et al. [71] have observed that the S_2 and S_3 states (on the D1/D2) are affected by changes in CP-43 protein, and Kless et al. [72] have shown that alterations in the Q_A region of the D2 protein affects DCMU affinity in the D1 protein.

4. Conclusions

D1 residue R269 is critically important for the structure and function of PS II complex. D1-R269G mutation drastically alters the PS II photosynthetic apparatus and has profound impacts on both the donor and acceptor sides of PS II chemistry. The

functional impacts include: (1) significant modifications on the Q_B binding niche of PS II, affecting the electron transfer process from Q_A^- to the plastoquinone pool; (2) possible destabilization or inhibition of assembly of the PS II complex and/or a blockage of excitation energy transfer to the PS II reaction center, affecting the level of stable charge separation, the PS II susceptibility to photodamage, and the donor side functions. Though the in vivo bicarbonate/formate activity is clearly perturbed by the mutation, the bicarbonate binding site is still present. We propose that the residue may not be a direct liganding residue to the bicarbonate anion but may affect the bicarbonate/formate binding indirectly through a general conformational change and/or through its involvement in bicarbonate/water transport (for details, see Ref. [20]). This residue is also considered to play a structural role for maintaining the proper D1/D2 conformation.

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