ELSEVIER

Biochimica et Biophysica Acta 1277 (1996) 83–92

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Construction and characterization of a Photosystem II D1 mutant (arginine-269-glycine) of *Chlamydomonas reinhardtii*

Ronald S. Hutchison [a], Jin Xiong [b], Richard T. Sayre [a], Govindjee [b,*]

[a] *Departments of Biochemistry and Plant Biology, Ohio State University, Columbus, OH 43210, USA*
[b] *Department of Plant Biology, 265 Morrill Hall, 505 S. Goodwin Ave., University of Illinois, Urbana, IL 61801, USA*

Received 2 April 1996; revised 29 May 1996; accepted 26 June 1996

Abstract

Numerous lines of evidence indicate that bicarbonate anion regulates electron and proton transfer processes in the photosystem II (PSII) complex of chloroplasts and cyanobacteria. On the reducing side of PSII, the addition of bicarbonate to bicarbonate-depleted (or formate-treated) membranes accelerates, especially, $Q_A^- \rightarrow Q_B^-$ electron transfer kinetics. The site(s) at which bicarbonate binds is unknown. It is evident, however, from several spectroscopic studies that the bicarbonate binding site on the reducing side of PSII includes the non-heme iron located between the Q_A and Q_B sites. Since small anions may displace bicarbonate (Good, N.E. (1963) Plant Physiol. 38, 298–304) [1], it is apparent that the bicarbonate binding site is electrostatic in nature, presumably also involving positively charged amino acid residues. Previously, it had been predicted that residue arginine 269 of the PSII D1 protein may participate in bicarbonate binding. To test this hypothesis, we have generated a non-conservative mutation in the *psbA* gene of *Chlamydomonas reinhardtii* which converts residue R269 to a glycine (R269G). The R269G mutant was unable to grow photosynthetically or evolve oxygen. This phenotype is associated with a lack of the tetra-manganese water splitting complex and a reduced capacity to form a stabilized charge separated state (defined as Tyr_D^+/Q_A^- under the experimental conditions measured). In addition, the mutant cells have a less stable PSII complex than wild-type cells, particularly when grown in the light. It is apparent from analyses of the effect of formate on the magnitude of the $Q_A^-Fe^{+2}$ EPR signal, however, that the bicarbonate or formate binding site is not substantially affected by the R269G mutation. Although our results do not substantiate that residue R269 is the site at which bicarbonate is bound, they demonstrate the importance of R269 in the structure and function of PSII. It is apparent

Abbreviations: Bp, base pair; Chl, chlorophyll; D1-R269G or R269G, mutant of *Chlamydomonas reinhardtii* with a glycine substitution at residue 269 in the D1 protein of photosystem II; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCPIP, 2,6-dichlorophenolindophenol; DMBQ, 2,5-dimethyl-*p*-benzoquinone; DPC, diphenyl carbazide; EPR, electron paramagnetic resonance spectroscopy; F_O, initial level of chlorophyll fluorescence; F_V, variable level of chlorophyll fluorescence; FUDR, 5-fluoro deoxyuridine; HEPES, *N*-(2-hydroxyethyl)-1-piperazine-*N'*-2 ethane sulfonic acid; MES, 2-(N-morpholino)ethane sulfonic acid; P680 and $P680^+$, the reduced and oxidized forms of the primary electron donor of photosystem II; PBQ, phenyl-p-benzoquinone; 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; Tyr_D and Tyr_Z, redox active tyrosines 160 and 161, respectively, of the D2 and D1 proteins of photosystem II.

* Corresponding author. Fax: +1 (217) 2447246; e-mail: gov@uiuc.edu.

PII S0005-2728(96)00085-0

from analysis of the photosynthetic phenotype, that the structural perturbations on the stromal side of the D1 protein are transduced to the lumenal side of the membrane altering charge accumulating processes on the electron donor side of PSII.

Keywords: Photosynthesis; Photosystem II; Chloroplast; Site-directed mutagenesis; Membrane protein; Bicarbonate; Formate; (*Chlamydomonas reinhardtii*)

1. Introduction

Electron transfer kinetics on both the electron acceptor ($Q_A \rightarrow Q_B$) and donor (water oxidizing complex $\rightarrow$ P680) sides of the photosystem II (PSII) complex have been shown to be regulated by bicarbonate anions (see reviews [2–4] and recent papers [5–7] on this effect). As demonstrated by measurements of electron transfer rates and chlorophyll fluorescence decay kinetics, depletion of bicarbonate or its displacement by formate or NO results in significant inhibition of electron transfer from Q_A^- to Q_B^-, suggesting its involvement in protonation of Q_B^{2-} (see e.g., Refs. [8–10]). EPR spectroscopic investigations of the $Q_A^- Fe^{+2}$ signal indicate that bicarbonate is probably bound at the non-heme iron between Q_A and Q_B [11–14]. Since the non-heme iron is also coordinated by four histidine residues (located on the stromal side of transmembrane spans D and E of the D1 and D2 proteins), the function of bicarbonate may be analogous to residue M-E234 (*Rhodobacter sphaeroides* numbering) in the bacterial photosynthetic reaction center, i.e., bicarbonate may provide a fifth or both fifth and sixth ligands to the non-heme iron (see e.g., Ref. [15]). However, this suggested equivalence of E234 on the M subunit and bicarbonate has its limitations since site-directed mutagenesis of M-E234 to several amino acids (R, V, A, Q, etc.) in bacterial reaction centers did not modify the Q_A^- to Q_B (or to Q_B^-) electron flow [16].

Using a homology (*Rb. sphaeroides* photosynthetic reaction center) based structural model of the PSII reaction center complex, the environment near the non-heme iron binding site has been examined for positively charged residues which could bind bicarbonate and/or allow for bicarbonate coordination to the non-heme iron (see e.g., Ref. [4] and J. Xiong, S. Subramaniam and Govindjee, 1996, personal communication). One residue which meets these criteria for selection is a conserved arginine residue, R269, on the D1 protein, an idea that was suggested, based on the projected structure of the Q_B pocket and on intuitive grounds [2]. This residue is located near the stromal end of transmembrane spanning helix E and is separated from residue D1-H272, one of the four non-heme iron ligands, by approx. 3/4 of a helical turn.

A site-directed mutant in the unicellular green alga *Chlamydomonas reinhardtii* has been generated and characterized to test whether the D1-R269 residue binds bicarbonate. Residue R269 has been replaced with a non-conservative residue, glycine (D1-R269G). A preliminary report has been published in a conference proceedings [17]. In this paper we describe the construction and structural properties of this mutant, including the stability of the mutant D1 protein, the presence or absence of the manganese cofactors of the water oxidizing complex, and its ability to carry out partial electron transfer reactions, including electron acceptor side processes affected by bicarbonate. We are unable to confirm that D1-R269G is the direct binding ligand to bicarbonate, but the work shows the importance of R269 in the structure and function of PSII.

2. Materials and methods

2.1. Growth conditions

Wild-type (CC-2137) and mutant *C. reinhardtii* cultures were grown at 25°C in Tris-Acetate-Phosphate (TAP) media under continuous low light (8–12 μmol photon $\cdot m^{-2} \cdot s^{-1}$) exposure, continuous darkness, or continuous darkness plus 20 min low light exposure [18–20]. Cultures were inoculated from a starter culture at 1/100 volume and harvested 4 days later in log phase at a cell density of $\sim 5 \times 10^6$ cells $\cdot ml^{-1}$.

2.2. Mutagenesis of C. reinhardtii

A mutation changing residue R269 of the D1 protein into a glycine (R269G) was constructed using

the plasmid pWT [18], a 3.0 kb *Xba*I fragment containing exons 4 and 5 of the *psbA* gene cloned into pBS + (Stratagene Cloning Systems, La Jolla, CA). Two site-directed mutations were introduced into pWT [17] according to the procedures of Kunkel et al. [21]. Two separate oligonucleotides were used to create a glycine substitution at residue R269 (GAAGTGTAATGA *ACC*AGAGTTGTTGAA converts a CGT to a *GGT* codon) and a silent and diagnostic *Sal*I restriction endonuclease recognition site at residueV307 (ACCTTGTGAGTC*GAC*-TACTGATGGTT converts a GTA codon to a *GTC* codon). The resulting plasmid (pR269G/V301) was sequenced using a Sequenase 2.0 kit (U.S. Biochemicals, Cleveland, OH) to confirm the presence of the mutations.

Plasmid pR269G/V301 and plasmid p228, containing the chloroplast 16S rRNA gene conferring spectinomycin resistance [22], were co-transformed into wild-type cells using a helium particle inflow gun with 1 micron tungsten particles [19]. *C. reinhardtii* cells were grown in the presence of 0.5 mM FUDR for 2 days (~ 8 generations) in the dark to reduce the chloroplast DNA copy number prior to being shot [18]. Cells transformed with plasmid p228 were selected on TAP plates containing 85 μg/ml spectinomycin to identify chloroplast DNA transformants. Spectinomycin-resistant colonies were visible after 2–3 weeks.

To isolate the p228-pR269G/V301 chloroplast DNA co-transformants, spectinomycin-resistant colonies (transformed with p228) were screened for the presence of the diagnostic restriction endonuclease recognition site introduced into the *psbA* gene with plasmid pR269G/V301. Single colony isolates were transferred to 3 ml of TAP containing 85 μg/ml spectinomycin and grown for 1 week and an aliquot (100 μl) of the cell suspension was plated on TAP plates (containing 100 μg/ml spectinomycin) to begin the secondary screen for chloroplast transformants. Total DNA was isolated from 1.5 ml of the liquid culture. The cells were concentrated by centrifugation, frozen in liquid nitrogen, and the DNA extracted using an S&S Elu-Quik DNA purification kit (chromosomal DNA isolation protocol). The isolated DNA was then subjected to PCR amplification of the *psbA* region spanning the entire exon 5. The 5′ oligonucleotide (GGTTACTTTGGTCGTCTA) used for the PCR amplification corresponds to amino acid residues G253-L258 in the D1 protein, and the 3′ oligonucleotide (GAAGTTGTCAGCGTTACG) corresponds to amino acid residues R344-F339. These primers generate a 260 bp fragment which includes the R269G mutation site plus the diagnostic *Sal*I restriction site located at residue V307. Digestion of the PCR product with *Sal*I generates two diagnostic 160 and 100 bp fragments assuming complete exchange of the donor (transforming) with the recipient (chloroplast) DNA. Using this PCR screening procedure, heteroplasmic mutant colonies were identified which contained wild-type and mutant PCR products. Single colony isolates from the secondary screen were then subjected to a second round of screening by analysis of the PCR products digested with *Sal*I in order to isolate homoplasmic R269G mutant strains. In order to confirm that the mutants were homoplasmic, chloroplast DNA was isolated from putative homoplasmic mutants and wild-type strains [18], digested with *Sal*I, and probed on Southern blots using radiolabelled pWT DNA according to the Genescreen (DuPont, Wilmington, DE) protocol. DNA sequence analysis of the mutagenized region of the chloroplast DNA from homoplasmic isolates was carried out according to the U.S. Biochemicals cycle sequencing kit using the aforementioned PCR primers. It is important to note that the sequence of the transformed DNA fragment showed no other mutation except R269G and the silent V307.

2.3. Biochemical analyses of R269G

Chloroplast thylakoids were isolated as described by Roffey et al. [18,19]. PS II particles were prepared according to the procedure of Shim et al. [23]. Photosynthetic oxygen evolution was measured using a Hansatech CB1 electrode with thylakoids (5 μg Chl/ml) suspended in 0.3 M sucrose, 20 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 20 mM methylamine, 2 mM $K_3Fe(CN)_6$ and 200 μM of one of the three different electron acceptors: DCBQ, DMBQ, or PBQ. DCPIP photoreduction was measured spectrophotometrically at 600 nm with thylakoids having a Chl concentration of 5.0 μg/ml in 0.3 M sucrose, 20 mM HEPES (pH 7.5), 1 mM $MgCl_2$, and 25 μM DCPIP containing either 1 mM DPC or 5 mM NH_2OH as artificial electron donors. For wild-type thylakoids, the man-

ganese was extracted by treatment with 2 mM NH_2OH prior to assaying artificial electron donor activity. D1 protein levels (per unit Chl) were determined by Western blot analysis (according to the Immu-blot assay kit procedures, Bio-Rad, Richmond, CA) using an antibody directed against the C-terminus of the D1 protein [19,24]. Elemental analysis of the manganese content of EDTA-washed PSII particles was completed at the Ohio State University REAL labs using PSII particles containing 1 mg Chl [19].

Electron paramagnetic resonance (EPR) studies were performed in collaboration with Dr. C. Russ Hille using a Bruker ESP-300. The following instrument settings were used for the $Q_A^-Fe^{+2}$ measurements: modulation amplitude, 28 G; temperature, 5 K; microwave power, 32 mW. PSII particles having a Chl concentration of 5 mg/ml were illuminated at 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ in the presence of 100 mM formate for 10 min at 77 K. Each sample was scanned 15 times. For measurement of the Tyr_D^+ signal, dark-adapted samples were scanned at 150 K, warmed to room temperature, illuminated for 1 min, and scanned again at 150K in the dark. Instrument settings were as described in Roffey et al. [18].

3. Results

3.1. Mutant isolation

Approx. 200 spectinomycin-resistant colonies were obtained from the initial chloroplast DNA transformation attempt. This number corresponds to a chloroplast transformation frequency of 10^{-6}/cell. No spectinomycin-resistant colonies were recovered from a mock (without DNA) transformation of *C. reinhardtii* cells. Forty of the spectinomycin-resistant colonies were then screened for the presence of the R269G/V307 *psbA* mutation. DNA was amplified from a region of the *psbA* gene encoding the R269G and V307 silent mutations (*Sal*I recognition site) followed by digestion with the diagnostic restriction endonuclease *Sal*I. While it is preferable to directly tag the introduced site-directed mutation with a unique diagnostic restriction endonuclease recognition sequence, this was not possible with the R269G mutant. Therefore, the silent mutation which introduced the diagnostic *Sal*I restriction endonuclease recognition

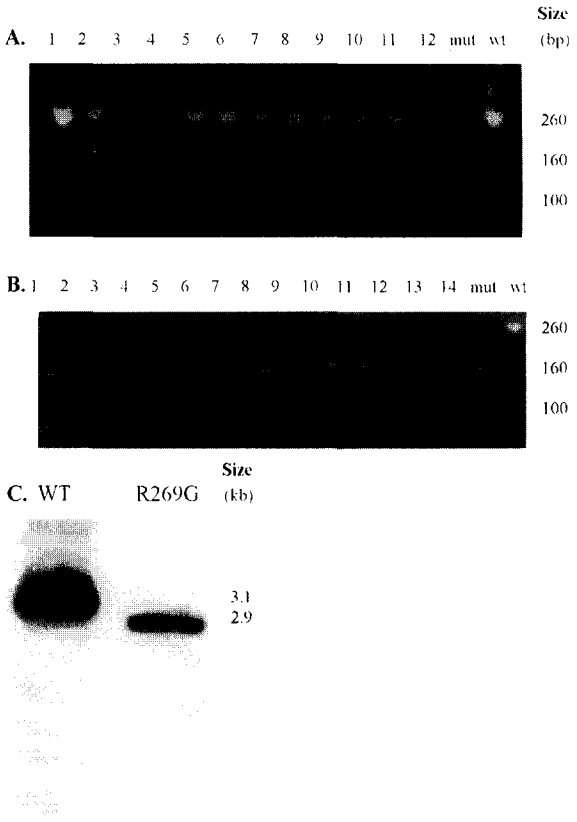

Fig. 1. Genetic verification of the R269G/V307 mutant in *Chlamydomonas reinhardtii*. Panel A shows the *Sal*I digested PCR products amplified from the *psbA* region spanning the V307 and R269G mutations. Lanes 1–12 are *Sal*I digested PCR products obtained from separate spectinomycin resistant colonies. Mut and WT correspond to the *Sal*I digested PCR products amplified from plasmids pR269G/V307 and pWT respectively. Panel B shows the *Sal*I digested PCR products obtained from single colony isolates derived from colony 2. Panel C is a Southern blot analysis of chloroplast DNA isolated from colony 12 (panel B) digested with *Sal*I and probed with pWT. The band migrating at 2.9 kb represents the expected size for the R269G/V307 mutant.

site was created at residue V307. Fig. 1 shows the pattern of DNA restriction fragments generated by digestion of the *psbA* PCR products with *Sal*I. PCR products containing the diagnostic *Sal*I site are cut into a 160 bp and a 100 bp fragment unlike wild-type DNA. As indicated in the figure, most of the spectinomycin resistant colonies had a wild-type *Sal*I restriction fragment pattern (i.e., undigested); however, two isolates (lanes 2 and 9) contained both undigested DNA (wild-type) and the 160 and 100 bp *Sal*I fragments diagnostic for the transforming *psbA* gene fragment. The presence of both the wild-type and

mutant DNA restriction patterns indicated that the cells were heteroplasmic for the R269G/V307 mutation [18]. Segregation of the mutant and wild-type genomes was subsequently achieved by streaking the heteroplasmic colonies (Fig. 1, lanes 2 and 9) to obtain single colony isolates. During cell division the mutant and wild-type genomes segregate giving rise to homoplasmic colonies. Subsequent analysis (*Sal*I digestion of the *psbA* PCR product) of the PCR products from the secondary screen indicated that most colonies had > 95% mutant DNA (Fig. 1B). In order to confirm that the colonies were homoplasmic for the mutation, Southern blot analyses were carried out using *Sal*I digested chloroplast DNA (4 μg, or approx. 10^6 *psbA* copies) [18]. As shown in Fig. 1C, one of the colonies obtained from the secondary screen had no apparent copies of the wild-type DNA, i.e., it was homoplasmic for the V307 mutation (Fig. 1C). DNA sequence analysis of the *psbA* gene from this isolate indicated that both the V307 and R269G mutations were present and that it was homoplasmic (data not shown). Furthermore, independent homoplasmic R269G transformants were also unable to grow photosynthetically, indicating that it is unlikely that a second site mutation resulted in obligate heterotrophic growth.

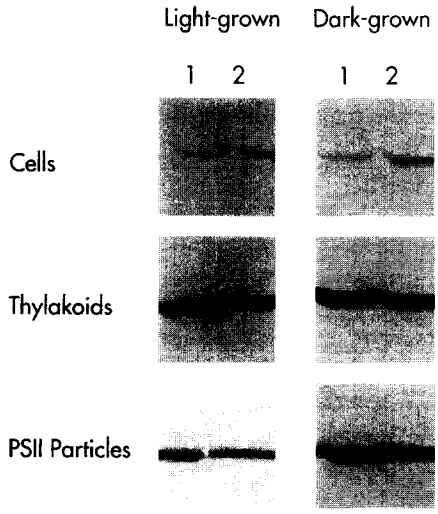

Fig. 2. Western blot analysis of the D1 protein levels in wild-type and R269G cells, thylakoids and PSII particles isolated from cells grown in the dark or light. Lane 1, wild-type; Lane 2, R269G. 10 μg of total chlorophyll was loaded for each lane.

Table 1
D1 protein content in D1-R269G cell and thylakoid membrane fractions isolated from dark- or light-grown cells

Fraction	% Wild-type	
	Low light grown	Dark grown
Whole cells	160	113
Thylakoids	62	83
PSII particles	60	88

D1 protein levels are expressed as a percentage of wild-type amount for each treatment and membrane fraction type. Values were determined by densitometry of Western blots (Section 2) and are the average of two measurements. The values varied by no more than 10% of the total.

3.2. D1 protein stability in the D1-R269G mutant

Our first priority was to determine whether the D1-R269G mutation affected the stability of the PSII complex. One measure of the stability of the PSII complex is the D1 protein content per unit chlorophyll [19]. Using Western blots, we compared the D1 content of cells, isolated thylakoids and PSII enriched particles from dark and continuous low light-grown, wild-type and R269G mutant cells. Sub-saturating amounts of the D1 protein, i.e., samples with < 10 μg Chl/lane, were used for the Western blot analysis in order to obtain a linear response from the antigen detection system [19]. As shown in Fig. 2 and Table 1, the D1 protein levels in R269G mutant cells were higher than in wild-type cells regardless of whether they were grown in the dark (13% > than wild-type) or continuous low light (60% > wild-type). It is noted that the D1 protein content of whole cells includes D1 protein present in intact as well as partially assembled PSII complexes [19]. In contrast to whole cells, the D1 protein content of thylakoids and PSII particles prepared from dark grown R269G cells was less than that of dark grown wild-type. The D1 protein content of thylakoids and PSII particles isolated from dark grown R269G cells was 17% and 12% respectively, less than in wild-type (dark-grown) membrane fractions (Fig. 2, Table 1). These results suggest that the D1 protein in the R269G membranes is less stable than in the membranes of wild type. The R269G D1 protein content was further reduced in mutant thylakoids relative to wild-type when the cells

were grown in continuous low light. The D1 protein content of thylakoids and PSII particles isolated from light-grown R269G cells was 62% and 60% of the corresponding wild-type fractions. Chlorophyll *a* fluorescence decay and induction kinetics measurements showed that Fv/Fm, an indicator of PSII activity, was drastically reduced in the D1-R269G mutant (J. Xiong, R.S. Hutchison, R.T. Sayre and Govindjee, unpublished data). These results suggest that the R269G mutant D1 protein is more susceptible to light-dependent (photoinhibitory) turnover than wild-type. Interestingly, detergent treatments associated with preparation of R269G PSII particles did not decrease the R269G D1 protein relative to wild-type PSII particles.

3.3. Biochemical properties of the R269G mutant

In order to determine what effects the R269G mutation had on photosynthetic electron transport, oxygen evolution was measured in the presence of various PSII (Q_B site) electron acceptors. As shown in Table 2, the D1-R269G mutant was unable to evolve oxygen regardless of the PSII acceptors used (DMBQ, DCBQ or PBQ). The absence of oxygen evolution could be due to inhibition anywhere between electron donation by water to electron acceptance by the artificial acceptors. In order to determine if the inability to evolve oxygen was due to a donor side effect, we measured the manganese content of wild-type and R269G PSII particles. Since the R269G PSII complex was shown to be sensitive to photoinhibition, we grew cells in the dark followed by a brief (20 min) exposure to low light to facilitate assembly of the water oxidizing complex with minimal photoinhibitory damage. Cells were also grown in complete darkness for comparison. As expected, the manganese content of PSII particles isolated from dark grown plus dim light exposed wild-type cells was 4.2 Mn/250 Chl, while the manganese content of totally dark grown wild-type cells was < 1 Mn/250 Chl (Table 2). The latter value is indicative of the light requirement for assembly of the functional manganese complex [25]. In contrast to the dim light exposed wild-type cells, the manganese content of dim light exposed R269G cells was < 1 Mn/250 Chl. These results indicate that the R269G mutant was unable to assemble or stabilize a tetra-manganese, water oxidizing complex.

The inability to form a manganese water oxidizing complex could be due to structural perturbations on the lumenal (donor) side of the complex or result from a disruption in electron transfer which would prevent photoligation of the manganese complex [25]. One measure of the ability to transfer electrons in PSII is the light-dependent reduction of DCPIP using artificial PSII electron donors which circumvent the water-oxidizing complex (see e.g., Ref. [19]). As

Table 2
Photosynthetic oxygen evolution, photosystem II artificial donor oxidation and manganese content of isolated membrane fractions from dark and dark plus 20 min dim light-grown cells

Strain	Growth conditions	Oxygen evolution [a]	DCPIP reduction [b]		Mn/250 Chl [c]
			DPC (1 mM)	NH_2OH (5 mM)	
μmol/mg Chl/h					
Wild-type	Plus Light *	265 ± 49	34 ± 4	43 ± 4	4.1 ± 0.2
Wild-type	Dark	ND	ND	ND	< 1.0
D1-R269G	Plus Light	0	0	0	< 1.0
D1-R269G	Dark	0	0	0	< 1.0

Values are the average of three separate measurements for electron transfer assays and two separate measurements for Mn determinations. See Section 2 for details.

[a] DMBQ used as an acceptor with wild-type thylakoids. Neither DCBQ, DMBQ or PBQ supported oxygen evolution in D1-R269G thylakoids; μmol O_2/mg Chl/h.

[b] Rates were determined with isolated thylakoids; μmol reduced DCPIP/mg Chl/h.

[c] Measurements were made with PSII particles.

ND, not determined.

* Dark plus 20 min dim (12 μE m^{-2} s^{-1}) white light-grown cells.

shown in Table 2, the R269G mutant was unable to reduce DCPIP using either DPC or hydroxylamine as a PSII donor. These results indicate several possible reasons for the lesion on the donor side as well as the acceptor side of PSII. Either the R269G mutant is unable to carry out a light-dependent charge separation, unable to oxidize artificial donors, or unable to reduce DCPIP. Experiments using DPC as a donor and silicomolybdate as an acceptor have not yet been conclusive and are, thus, not presented here. It is, however, obvious from measurements of chlorophyll fluorescence decay, after single turnover flashes, that the mutation has led to a significant block in electron transfer from Q_A^- to Q_B (or Q_B^-) ([17], J. Xiong, R. Hutchison, R.T. Sayre and Govindjee, 1996, unpublished data). Thus a block on both the electron acceptor and donor sides of PSII has occurred as a consequence of the R269G mutation.

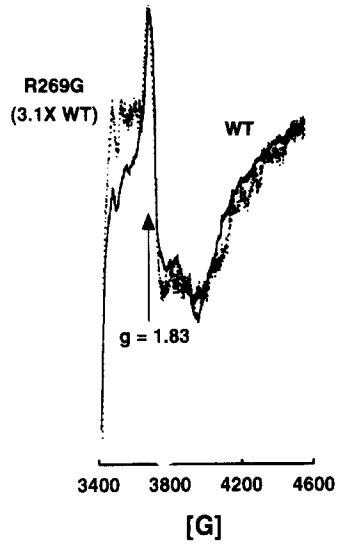

Fig. 4. The formate enhanced $Q_A^- Fe^{+2}$ EPR signal in PSII particles isolated from dark grown wild-type and R269G cells. Solid line, wild-type; dotted line, R269G mutant.

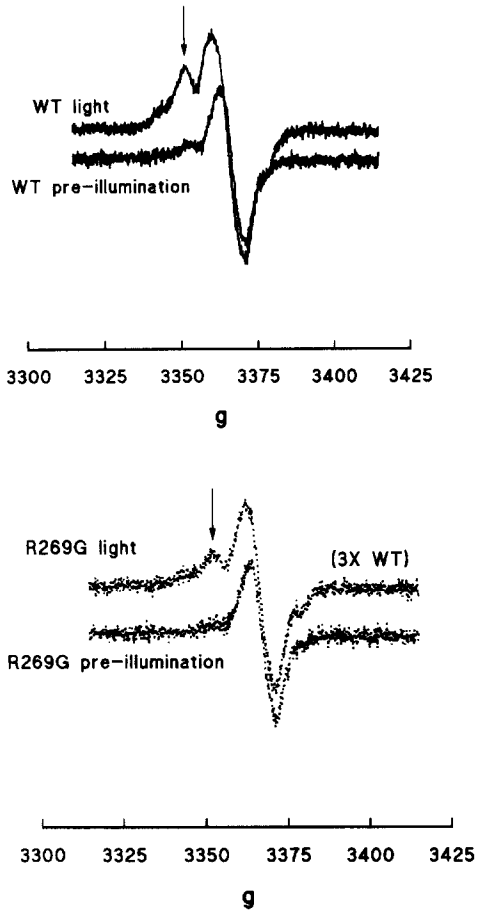

Fig. 3. Photo-accumulation of the Tyr_D^+ EPR signal in PSII particles isolated from dark grown, wild-type (top) and R269G (bottom) cells. The symmetrical signal centered around $g = 2.0$ is attributed to contamination from P700 [28]. The arrow indicates the location of the downfield hyperfine signal attributed to Tyr_D. Pre-illumination refers to dark samples.

In order to determine whether the R269G mutant could accumulate a stable charge-separated state (Tyr_D^+/Q_A^-), the formation of the dark stable radical Tyr_D^+ was measured in PSII particles from dark grown cells. Due to its long half-life, Tyr_D^+ accumulates in illuminated non-oxygen evolving PSII particles which are unable to oxidize water [26]. Tyr_Z signal amplitudes were not measured due to the sensitivity of Tyr_Z to photoinhibitory damage [27]. As shown in Fig. 3, the extent of Tyr_D^+ accumulation in D1-R269G PSII particles (isolated from dark grown cells) was approx. one-third that of wild-type. These results indicate that at least some portion of the mutant reaction centers were capable of accumulating Tyr_D^+, but not all. The inability to accumulate wild-type levels of Tyr_D^+ could result from either: (1) a reduced ability to form the charge separated state $P680^+/Q_A^-$, (2) a reduced ability of $P680^+$ to oxidize Tyr_D, or (3) an increased back reaction from $Q_A^- \rightarrow P680^+$.

In order to further characterize the ability of the R269G PSII particles to form a stabilized charge separated state and to probe the effect of the mutation on bicarbonate binding (acceptor side), we measured

the accumulation and hyperfine structure of the formate enhanced $Q_A^-Fe^{+2}$ EPR signal at $g = 1.83$ [11–13] (a probe of the HCO_3-Fe ligand). This signal can be trapped during illumination at 77 K and is greatly enhanced by formate pre-treatment [11]. The formate enhancement is due to the displacement of bicarbonate from its binding site which inhibits Q_A^- to Q_B (or Q_B^-) electron transfer. In addition to the extent of formation of the $Q_A^-Fe^{+2}$ signal, information on the coordination environment of the non-heme iron can be obtained from the g value and the line-width of the $Q_A^-Fe^{+2}$ EPR signal [11–13]. As shown in Fig. 4, wild-type PSII particles exhibit a typical formate (100 mM) enhanced $Q_A^-Fe^{+2}$ EPR signal at $g = 1.83$. Similarly, a $Q_A^-Fe^{+2}$ EPR signal was generated from illuminated R269G PSII particles; however, the yield of the signal was reduced by 68% relative to wild-type (Fig. 4). In fact, in the absence of formate it was not possible to detect the $Q_A^-Fe^{+2}$ EPR signal (unlike wild-type) in R269G PSII particles. Significantly, the line shape and g value of the $Q_A^-Fe^{+2}$ EPR signal were similar to wild-type. These results suggest that although the formate enhanced $Q_A^-Fe^{+2}$ EPR signal is not structurally perturbed in the R269G mutant, it is reduced to the same extent as the Tyr_D^+ signal.

4. Discussion

It is apparent from measurements of manganese content and partial electron transfer reactions reported in this paper (Table 2) that the R269G mutation leads to inhibition of both electron donor and acceptor side processes in PSII. The R269G mutant was unable to transfer electrons from DPC or NH_2OH to DCPIP, was unable to evolve oxygen, lacked Mn function in water oxidation, and had a reduced ability to form a stabilized charge separated state (Tyr_D^+/Q_A^-) (Fig. 3). In the present paper we have focused on the donor side processes and the stability of the R269G PSII reaction center complex. Three lines of evidence indicate that the R269G mutant has a reduced ability to accumulate a stabilized charge separated state relative to wild-type. First of all, the Tyr_D^+ and $Q_A^-Fe^{+2}$ EPR signals accumulate at reduced levels (30% of the wild-type signal) in PSII particles isolated from dark grown R269G cells. Importantly, neither the g value nor the line shape of the remaining Tyr_D^+ or $Q_A^-Fe^{+2}$ EPR signals was significantly altered. These results suggest that the environments near each species were not sufficiently perturbed to alter the electronic properties of Tyr_D^+, and the $Q_A^-Fe^{+2}$ couple [11–13,27]. Further evidence to indicate that the R269G mutant is capable of generating reduced steady-state levels of the stabilized charge separated state in PSII is its reduced sensitivity to photoinhibition relative to other non-oxygen evolving PSII mutants. D1 protein levels in thylakoids isolated from light-grown R269G cells were substantially greater (40% of wild-type) than those observed in other non-oxygen evolving PSII mutants (light-grown D1-H190F and H190Y mutants had 5% the D1 protein content of wild-type; Robin Roffey, unpublished observation) which were capable of reducing DCPIP and had near wild-type chlorophyll F_V/F_O fluorescence ratios (following repetitive flashes) ([17,19], J. Xiong, R. Hutchison, R.T. Sayre and Govindjee, 1996, unpublished data). These results show that the turnover of the D1 protein in light is altered in the R269G mutant. The similarity in the extent of accumulation of the dark stable Tyr_D^+ (30% of wild-type) and the freeze trapped $Q_A^-Fe^{+2}$ EPR signals (30% of wild-type) suggests that only 30–40% of the D1 protein is associated with competent charge accumulating PSII complexes in dark-grown cells. Since the D1 protein content of dark grown R269G thylakoids was 83% of wild-type, it is suggested that approx. half (wild-type level) of the D1 protein is associated with traps that may be impaired in electron flow from Q_A^- to Q_B or non-functional PSII centers [28]. Qualitatively, this interpretation is consistent with the Chl *a* fluorescence data: the F_O level of R269G thylakoid membranes (dark grown) was higher (1.5-fold) than wild-type (J. Xiong, R. Hutchison, R.T. Sayre and Govindjee, 1996, unpublished data). Experiments are in progress to test the effect of mutation on charge recombination in PSII by thermoluminescence method and the data will be published elsewhere.

Our overall objective was to determine whether substitution of the D1-R269 residue would preclude bicarbonate binding and alter PSII electron transfer processes in a pattern similar to bicarbonate depletion. Bicarbonate can be competitively displaced by formate which alters the coordination to the non-heme iron. Formate treatment has been shown to reduce Q_A^- to Q_B, but more so from Q_A^- to Q_B^-, electron

transfer and lead to the accumulation of the $Q_A^- Fe^{+2}$ EPR signal. Further, the structure of the $Q_A^- Fe^{+2}$ EPR signal is sensitive to the nearby protein and coordination (non-heme iron) environment [13,14]. We observed a formate enhancement of the $Q_A^- Fe^{+2}$ EPR signal, but at reduced levels relative to wild-type (30% of wild-type). These results indicate that formate displaces bicarbonate from its binding site in the R269G mutant although at a reduced efficiency. However, we cannot, as yet, eliminate the possibility of additional alterations due to the methods used in preparing PSII particles.

A formate effect on chlorophyll fluorescence decay (Q_A^- to Q_B (or Q_B^-) electron transfer) is also observed in the mutant, indicating that the bicarbonate binding site may not be residue D1-R269 (J. Xiong, R. Hutchison, R.T. Sayre and Govindjee, 1996, unpublished data). However, the sensitivity of the electron flow from Q_A to Q_B (or Q_B^-) to formate treatment is about four times lower in intact mutant cells than in the wild-type cells, suggesting that the formate and/or bicarbonate binding is different in the mutant cells. Thus, we cannot yet rule out the possibility of the involvement of D1-arginine 269 in the bicarbonate effect in wild-type cells.

In summary, the D1-R269G mutation alters the acceptor as well as the donor side electron transfer processes in PSII and destabilizes the PSII complex. This is intriguing since the R269G residue is predicted to be located at the stromal end of transmembrane span E (see e.g., Ref. [4]). Glycine residues are frequently found at the end of transmembrane spanning domains. Due to the free rotation permitted around the alpha carbon, the regular alpha helical structure of the transmembrane spanning domain can be broken [29]. This may lead to a shortening of the transmembrane span and/or disrupt interactions between transmembrane spans leading to structural perturbations on the lumenal or donor side of the complex.

It is noteworthy that lumenal side mutations (e.g., D1-H190F), as well as treatments which extract manganese from PSII, slow acceptor (Q_A^- to Q_B) side electron transfer processes [19]. These observations suggest that structural perturbations of extrinsic domains at the margins of transmembrane spans can be transduced from one side of the membrane protein to the other altering charge transfer processes. The perturbations do not, however, prevent assembly of the PSII complex. It is apparent that only a fraction of the mutant PSII centers are capable of forming a stabilized charge separated state. Further, long ranging, but apparently indirect, effects on the donor side activity following amino acid substitution in the D1 protein on the acceptor side have been reported in cyanobacteria [30,31]. Similar effects have previously been observed for mutants lacking the PSII 43 kDa chlorophyll binding protein and in site-directed mutants of the D1 protein [32,33]. Carpenter et al. [34] have observed that properties of S_2 and S_3 (on the D1/D2) are affected by changes in CP-43 protein, and Kless et al. [35] have shown that alterations in Q_A region in the D2 affects DCMU affinity in the D1 protein. It is not clear, however, whether the effect of the R269G mutation is to directly impair assembly of functional PSII complexes, destabilize intact complexes, or alter electron donor-acceptor interactions which in turn prevent assembly of the water-oxidizing complex of PSII.

Acknowledgements

We thank Dr. C. Russ Hille for assistance with the EPR experiments. This work was supported by the U.S. Dept. of Energy (DOE ER20076) to R.T.S. and by the N.S.F. (91-16838; 1994 supplement) to G. J.X. was supported by a training grant (DOE 92ERR20095) from the Triagency (DOE/NSF/USDA) program for Collaborative Research in Plant Biology.

References

[1] Good, N.E. (1963) Plant Physiol. 38, 298–304.
[2] Blubaugh, D. and Govindjee (1988) Photosyn. Res. 19, 85–128.
[3] Diner, B.A., Petrouleas, V.F. and Wendoloski, J.J. (1991) Physiol. Plant. 81, 423–436.
[4] Govindjee and Van Rensen, J.J.S. (1993) in The Photosynthetic Reaction Center (Deisenhofer, J. and Norris, J., eds.), vol. 1, pp. 357–389. Academic Press, San Diego.
[5] Cinco, R.M., MacInnis, J.M. and Greenbaum, E. (1993) Photosyn. Res. 38, 27–33.
[6] Demeter, S., Janda, T., Kovacs, L., Mende, D. and Wiessner, W. (1995) Biochim. Biophys. Acta 1229, 166–174.
[7] Klimov, V.V., Allakhverdiev, S.I., Feyziev, Y.M. and Baranov, S.V. (1995) FEBS Lett. 363, 251–255.

[8] Eaton-Rye, J.J. and Govindjee (1988) Biochim. Biophys. Acta 935, 237–257.
[9] Diner, B.A. and Petrouleas, V. (1990) Biochim. Biophys. Acta 1015, 141–149.
[10] Xu, C., Taoka, S., Crofts, A.R. and Govindjee (1991) Biochim. Biophys. Acta 1098, 32–40.
[11] Vermaas, W.F.J. and Rutherford, A.W. (1984) FEBS Lett. 175, 243–248.
[12] Petrouleas, V. and Diner, B.A. (1990) Biochim. Biophys. Acta 1015, 131–140.
[13] Petrouleas, V., Deligiannakis, Y. and Diner, B.A. (1994) Biochim. Biophys. Acta 1188, 260–270.
[14] Hallahan, B.J., Ruffle, S.V., Bowden, S.J. and Nugent, J.H.A. (1991) Biochim. Biophys. Acta 1059, 181–188.
[15] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[16] Wang, X., Cao, J., Maroti, P., Stilz, W.V., Finkele, U., Lauterwasse, C., Zinth, W., Oesterhelt, D., Govindjee and Wraight, C.A. (1992) Biochim. Biophys. Acta 1100, 1–8.
[17] Xiong, J., Hutchison, R., Sayre, R.T. and Govindjee (1995). in Photosynthesis: from Light to Biosphere (Mathis, P., ed.), Vol. I, pp. 575–578. Kluwer, Dordrecht.
[18] Roffey, R.A., Golbeck, J.H., Hille, C.R. and Sayre, R.T. (1991) Proc. Natl. Acad. Sci. USA 88, 9122–9126.
[19] Roffey, R.A., Kramer, D.M., Govindjee and Sayre, R.T. (1994) Biochim. Biophys. Acta 1185, 257–270.
[20] Kramer, D.M., Roffey, R.A., Govindjee and Sayre, R.T. (1994) Biochim. Biophys. Acta 1185, 228–237.
[21] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods. Enzymol. 154, 367–382.
[22] Harris, E.H., Burkhart, B.D., Gillham, N.W. and Boynton, J.E. (1989) Genetics 123, 281–292.
[23] Shim, H., Cao, J., Govindjee and Debrunner, P. (1990) Photosyn. Res. 26, 223–228.
[24] Mei, R., Green, J.P., Sayre, R.T. and Frasch, W. (1989) Biochemistry 28, 5560–5567.
[25] Radmer, R. and Cheniae, G.M. (1977) in Topics in Photosynthesis (Barber, J., ed.), Vol. 2, pp. 303–348, Elsevier, Amsterdam.
[26] Barry, B. A. and Babcock, G.T. (1987) Proc. Natl. Acad. Sci. USA 84, 7099–7103.
[27] Roffey, R., Van Wijk, K., Sayre, R.T. and Styring, S. (1994) J. Biol. Chem. 269, 5115–5121.
[28] Lavergne, J. and Briantais, J.-M. (1996) in Oxygenic Photosynthesis: The Light Reactions (Ort, D.R. and Yocum, C.F., eds.), Advances in Photosynthesis, Vol. 4, Kluwer, Dordrecht, in press.
[29] Aurora, R., Srinivasan R. and Rose, G.D. (1994) Science 264, 1126–1130.
[30] Etienne, A.-L. and Kirilovsky, D. (1993) Photosyn. Res. 38, 387–394.
[31] Constant, S., Perewoska, I., Nedbal, L., Miranda, T., Etienne, A.-L. and Kirilovsky, D. (1996) Plant Sci. 115, 165–174.
[32] de Vitry C., Olive, J., Drapier, D., Recouvreur, M. and Wollman F.-A. (1989) J. Cell Biol. 109, 991–1006.
[33] Hutchison, R.H. and Sayre, R.T. (1995) in Photosynthesis: from Light to Biosphere (Mathis, P., ed.), Vol. I, pp. 471–474. Kluwer, Dordrecht.
[34] Carpenter, S.D., Ohad, I. and Vermaas, W.F.J. (1993) Biochim. Biophys. Acta 1144, 204–212.
[35] Kless, H., Oren-Shamir, M., Ohad, I., Edelman, M. and Vermaas, W.F.J. (1993) Z. Naturforsch. 48c, 185–190.