CHARACTERIZATION OF A SITE-DIRECTED MUTANT (D1-ARGININE 269-GLYCINE) OF *Chlamydomonas reinhardtii*

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Introduction

Numerous studies have demonstrated that bicarbonate has a strong positive influence on the electron and proton transfers on the acceptor side (see review 1) of photosystem II (PSII) as well as on the electron transfer on its donor side (2, 3). Depletion of bicarbonate or its displacement with other small anionic molecules (*e.g.* formate) substantially slows the kinetics of electron transfer from Q_A^- to Q_B. Although the identity of the bicarbonate binding sites remains unknown, one of its sites is expected to be near the non-heme Fe based on EPR studies of bicarbonate-depleted membranes. In fact, bicarbonate may provide a fifth ligand for the non-heme iron. Since bicarbonate is anionic, 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. One likely candidate for the bicarbonate binding site is arginine 269 (R269) of the D1 protein. Based on the homology studies with the bacterial photosynthetic reaction center (1), this residue is expected to be located on the stromal side of the putative transmembrane helix E and in the close vicinity of the non-heme iron binding site. In this report, we describe the phenotype of the R269G mutant, in which arginine 269 has been converted to a non-conservative glycine. This mutant is characterized by its inability to evolve oxygen, slowed Q_A^- to Q_B electron transfer, a reduction of the formate enhanced Q_A^-Fe EPR signal, and structural instability of the PSII complex. These results suggest that the mutation somewhat destabilizes the PSII complex resulting in reduced numbers of PSII centers capable of charge separation and may indirectly affect the bicarbonate binding.

Methods

Site-directed mutations were generated in the chloroplast encoded *psbA* gene of *Chlamydomonas reinhardtii* by conventional Kunkel's method. Co-transformation of the mutagenized plasmid was done with a plasmid containing the 16S rRNA gene conferring spectinomycin resistance, according to the procedures of Roffey et al. (4). A diagnostic SalI restriction site was engineered into the *psbA* gene fragment containing the R269G mutation to identify transformants. Mutants were judged as homoplasmic for the R269G mutation on the basis of Southern blot analysis of chloroplast DNA and sequencing of the mutagenized region. For biochemical and biophysical studies all cells were grown in a heterotrophic Tris-Acetate-Phosphate (TAP) medium in total darkness. Thylakoid membranes and PSII particles were isolated according to (5). Electron transfer, Mn quantification, and fluorescence measurements were done as described earlier (4 and 5).

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Herbicide binding studies were conducted using ^{14}C Terbutryn according to established procedures (6). A long-term formate treatment of the intact cells was done at pH 6.5 as described by El-Shintinawy et al. (7). Low temperature (77 K) chlorophyll fluorescence emission spectra were measured using a Perkin Elmer LS-5 fluorescence spectrophotometer with a red-sensitive photomultiplier and with a chlorophyll concentration of 30 µg/ml in the sample. The excitation wavelength was set at 435 nm and the monochromator bandwidths 10 nm for excitation and 3 nm for emission. The spectra were corrected for the wavelength dependence of the photomultiplier sensitivity and normalized at the PSI emission band at 715-nm (F715). EPR studies were performed on a Bruker ESP-300. The following instrument settings were used for detection of the Q_A^-Fe EPR signal: modulation amplitude, 28G; temperature, 5 K; and microwave power, 32 mW. PSII particles were illuminated at 1,000 $\mu E/m^2/s$ in the presence of 100 mM formate for 10 min at 77 K at a chlorophyll concentration of 5 mg/ml. For Tyr_D EPR measurements, dark-adapted PSII particles were first scanned at 150 K, warmed to room temperature, illuminated for 1 min and scanned at 150 K.

Results and Discussion

A description of the isolation of the homoplasmic R269G mutant is presented elsewhere by the authors (R. Hutchison et al., in preparation). Since R269 may be located near the stromal end of the fifth transmembrane span of the D1 protein, its conversion to a glycine residue may disrupt the alpha-helical structure, prematurely terminating the transmembrane span and destabilizing the PSII complex. In order to determine whether the mutation destabilized the complex, we measured D1 protein levels and quantified the low temperature fluorescence emission intensity in cells, thylakoids and PSII particles. Western blot analysis indicated that the levels of D1 protein in dark grown whole cells, thylakoids and PSII particles were 113, 83 and 88% of the wild type levels, respectively. Low temperature fluorescence emission spectra of the wild type and the mutant were measured in an attempt to characterize the PSII assembly (Fig. 1). Our deconvolution analysis (with the help of Vladimir Shinkarev) of the spectra revealed that there is a reduction in both 685-nm (F685, CP43) and 695-nm (F695, CP47) bands for the mutant cells and the thylakoids with the F695 reduced to a greater extent. The ratio of F695/F685 of the mutant samples is reduced by 20-30% compared with the wild type. This indicates a differential reduction in these PSII antenna complexes *provided* the mutation had not caused changes in excitation energy transfers among these complexes and the PSII reaction center. Since there were no changes made in the CP43 and CP47 genes, the proposed reduction, especially of CP47, could well be related to the changes in the D1/D2 complexes to which the antenna complexes are associated. As was shown for several *Synecocystis* 6803 mutants, the reduction in CP47 correlates well with the reduction of the PSII core proteins (8). Therefore a lowered F695 band may indicate a lowered PSII assembly in this mutant.

In order to determine whether the mutation affected rates of electron transfer, we measured rates of oxygen evolution in the presence of various quinone acceptors. Under no conditions were we able to detect oxygen evolution. Furthermore, the inability to evolve oxygen was associated with a reduced Mn content in PSII particles. Wild type PSII particles typically have 4.2 Mn/250 Chl while R269G PSII particles had 1.0 Mn/250 Chl. In addition, the mutant was unable to effectively (5% of the wild type rate) oxidize artificial donors (hydroxylamine, diphenylcarbazide) to PSII using DPIP as an electron acceptor. In order to further characterize the ability to form a charge separated state and to characterize the bicarbonate effect on the electron transfer of Q_A^- to the plastoquinone pool, we measured the flash induced chlorophyll fluorescence (PSII) rise and decay in dark-grown

whole cells in the presence and absence of formate and/or bicarbonate (pH 6.5). The decay kinetics of the first flash is shown in Fig. 2, in which the ratio of Fv/Fo for R269G was approximately 44% of that of the wild type, indicative of a loss of functional centers (to be discussed by J. Xiong et al., in preparation). In addition, the rate of fluorescence decay was substantially slower than for wild type, indicating that electron transfer from Q_A^- to Q_B was slowed. Addition of formate (25 mM) to the wild type cells reduced not only the rate of fluorescence decay consistent with a reduction in the electron transfer rate from Q_A^- to Q_B, but the level of the Fv/Fo suggesting an additional effect on the donor side of PSII. The formate effects were partially reversed by bicarbonate (2.5 mM). Similarly, the addition of formate to R269G produced a small but detectable reduction in the rate of fluorescence decay which was partially reversed by addition of bicarbonate. Herbicide binding experiments (6), using ^{14}C-Terbutryn, showed that the mutant had dramatically reduced Terbutryn affinity relative to the wild type. This suggests a large change in the Q_B pocket.

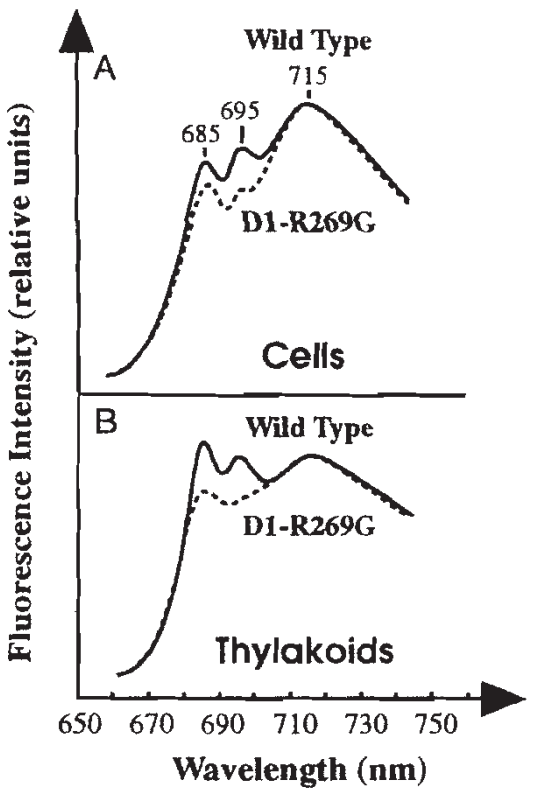

Fig. 1: 77 K chlorophyll fluorescence emission spectra.

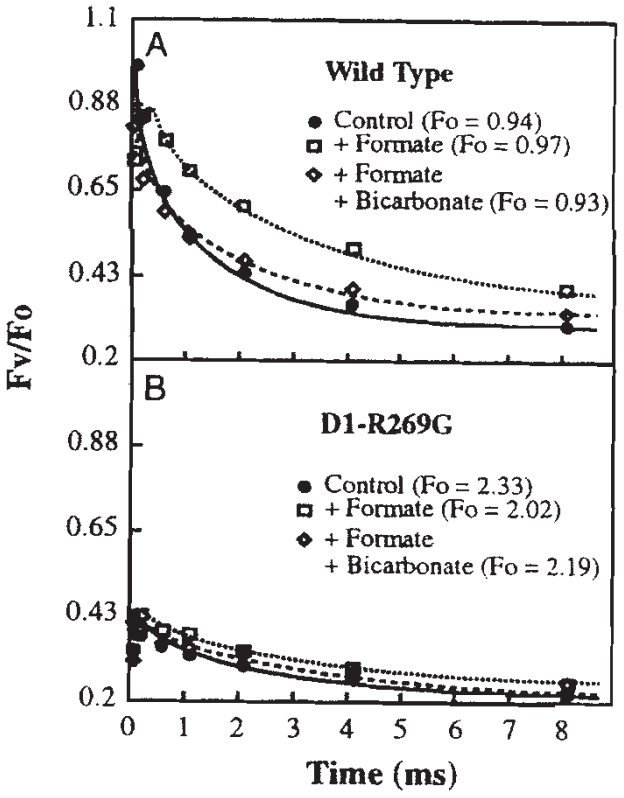

Fig. 2: Fluorescence decay kinetics (first flash) ± formate and bicarbonate.

Since western blot analyses indicated that there may be some reduction in the number of functional PSII complexes in R269G thylakoids and PSII particles, further studies were undertaken to quantify the number of functional PSII centers. Low temperature fluorescence measurements may be taken to support this conclusion if we suggest that the predicted reduction in CP43 and CP47 reflect reduction in PSII reaction centers. Since Tyr_D is dark stable and accumulates in the light (assuming no rate limitation in oxidation of Tyr_D), an estimation of the number of functional PSII complexes can be made by quantification of the Tyr_D. EPR signal in non-oxygen evolving PSII particles. There was a 68% reduction in the level of the Tyr_D EPR signal in R269G compared to the wild type (data not shown).

Since R269G does not evolve oxygen and since the formate effect on the fluorescence decay was difficult to quantify in R269G, we measured the effect of formate on the g = 1.83 Q_A^-Fe EPR signal. In the presence of formate, the g = 1.83 signal is substantially enhanced in PSII particles. Furthermore, the effects of structural perturbations on the bicarbonate binding site can be ascertained on the basis of the g value and line-width of the Q_A^-Fe EPR signal (9). As shown in Fig. 3, the addition of formate to R269G generated approximately 1/3 the Q_A^-Fe EPR signal produced in wild type. Furthermore, the signal had the same g value and line width as WT.

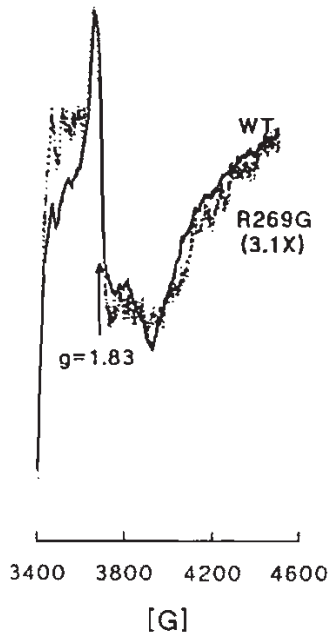

Fig. 3: Formate-enhanced Q_A^-Fe EPR signal.

The results of this study suggest that the D1 residue R269 is critically important in maintaining the structural integrity of the PSII complex and in facilitating electron transfer between Q_A^- and Q_B. The introduction of a helix breaking residue destabilizes the complex. Based on quantification of the Tyr_D, Q_A^-Fe EPR signals, and measurements of the variable chlorophyll fluorescence level, it is estimated that the steady state level of functional R269G PSII complexes is about 1/3 of that of the wild type. It is unlikely, however, that R269 is directly involved in bicarbonate binding since formate induces a structurally unperturbed Q_A^-Fe EPR signal, although at reduced levels. In addition, the residue, which is suggested to be located in the interface of D1 and D2 proteins, is considered to play a delicate but important structural role for maintaining the proper D1-D2 conformation (unpublished homology model of J. Xiong, S. Subramaniam and Govindjee).

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