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Arginine residues in the D2 polypeptide may stabilize bicarbonate binding in photosystem II of *Synechocystis* sp. PCC 6803

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Bicarbonate (HCO_3^-) causes a significant and reversible stimulation of anion-inhibited electron flow in photosystem II of higher plants and cyanobacteria. To test if selected arginine (Arg) residues are involved in the binding of HCO_3^-, we utilized oligonucleotide-directed mutagenesis to construct *Synechocystis* sp. PCC 6803 mutants carrying mutations in Arg residues in the D2 protein. Measurements of oxygen evolution showed that the D2 mutants R233Q (arginine-233 → glutamine) and R251S (arginine-251 → serine) were 10-fold more sensitive to formate than the wild type. The formate concentration giving half-maximal inhibition of the steady-state oxygen evolution rate was 48 mM, 4.5 mM and 4 mM for the wild type, R233Q and R251S, respectively. Measurements of oxygen evolution in single-turnover flashes confirm that the mutants are more sensitive to formate than the wild type. Measurements of chlorophyll *a* fluorescence decay kinetics after the second saturating actinic flash indicated that, after formate treatment, the halftime of Q_A^- oxidation was decreased by approximately a factor of 2, 4 and 6 in the wild type, R251S and R233Q, respectively. The recombination rate between Q_A^- and S_2 was approx. 2-fold slower in R251S and R233Q than in the wild type. In the presence of 100 mM sodium formate, reactivation of the Hill reaction by bicarbonate showed that the wild type had an apparent K_m for bicarbonate of 0.5 mM, while the K_m values for R233Q and R251S were 1.4 and 1.5 mM, respectively. We suggest that Arg-233 and Arg-251 in the D2 polypeptide contribute to stabilization of HCO_3^- binding in Photosystem II.

Introduction

A stimulatory effect of CO_2 on the Hill reaction was first observed by Warburg and Krippahl [1] who hypothesized that this effect was on water oxidation. However, measurements of chlorophyll (Chl) fluorescence induction kinetics in chloroplasts indicated that the effect of bicarbonate was on the reducing side of Photosystem II (PS II) [2]. A major effect of bicarbonate was later shown to be on the electron flow from Q_A^- to the plastoquinone pool, where Q_A is the primary bound plastoquinone [3]. Bicarbonate acts near Q_B and/or the non-heme iron positioned between Q_A and Q_B, where Q_B is the secondary bound plastoquinone. Formate, which removes CO_2 from the thylakoid membranes [4], is thought to replace bicarbonate; it is shown to inhibit electron transfer from Q_A^- to $Q_B^{(-)}$ [5]. Formate treatment greatly enhances the g = 1.82 form of the Q_A^-Fe(II) EPR signal and the enhancement is reversed upon bicarbonate addition [6]. Furthermore, an Fe(II)-NO EPR signal at $g = 4$ is diminished by the addition of $NaHCO_3$ [7]. Formate decreases the quadrupole splitting and the chemical shift of the non-heme Fe(II) Mossbauer spectrum [8,52], supporting the concept of binding of HCO_3^-/CO_2 and formate near the non-home iron. Bicarbonate effects on protonation of Q_B have been inferred from the pH

Abbreviations: Chl *a*, chlorophyll *a*; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethyl-*p*-benzoquinone; PS I, Photosystem I; PS II, Photosystem II; Q_A, bound plastoquinone, a one-electron acceptor in photosystem II; Q_B, another bound plastoquinone, a two-electron acceptor in Photosystem II.

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dependence of the Q_A^- to Q_B^- electron transport measurements in bicarbonate-depleted thylakoid membranes [5,9] and from the increased inhibition of electron transfer after multiple actinic flashes [3]. These results were explained by assuming that the large inhibition after second and subsequent flashes is due to an inhibition of protonation of Q_B^- and/or Q_B^{2-} after formate treatment and bicarbonate removal [5,9]. An arginine residue has been proposed to be a plausible ligand to bicarbonate [10].

The bicarbonate effect on electron transport between Q_A and Q_B in plants (see Refs. 10, 11 for reviews) and cyanobacteria [12,13] has been well established. However, no effect of bicarbonate on electron transport between the two quinones has been observed in purple photosynthetic bacteria [14] or in the green bacterium *Chloroflexus aurantiacus* (Govindjee, J. Trost and R. Blankenship, 1990, unpublished observations). This suggests a fundamental difference between bacterial and PS II reaction centers. The isolation, crystallization, and the X-ray structure of the reaction center complex from the photosynthetic bacterium *Rhodopseudomonas viridis* [15] provided a stimulus for the understanding of the PS II reaction center because the primary structures of D1 and D2 polypeptides of PS II show similarities to those of L and M subunits of photosynthetic bacteria, respectively [16]. In purple bacteria, a glutamate (Glu) residue in the M subunit provides two ligands to the non-heme iron. However, this Glu residue is not conserved in the D2 protein. Thus, Michel and Deisenhofer [16] suggested that bicarbonate may act as a bidentate ligand to the iron in PS II.

Indications for the involvement of the D1 protein in the bicarbonate effect have come from the studies of herbicide and bicarbonate interaction, and of herbicide-resistant mutants. Urea, triazine and phenol-type herbicides (known to interact with the Q_B site) decrease the apparent affinity of the thylakoid membrane for bicarbonate [17,18]. On the other hand, bicarbonate depletion affects the binding of herbicides [19]. Furthermore, a triazine-resistant mutant of *Amaranthus hybridus* (in which Ser-264 of D1 was changed to Gly) showed a 2-fold increase in the apparent dissociation constant of bicarbonate [19]. Recent studies on several herbicide-resistant D1 mutants of the cyanobacterium *Synechocystis* sp. PCC (Pasteur Culture Collection) 6714 [20] and of *Chlamydomonas reinhardtii* [21] demonstrate differential formate sensitivities.

On the basis of homologies with the reaction center from purple bacteria, the D2 protein, like D1, is thought to contribute to the binding of the reaction center P680 and the non-heme iron. It is suggested that D2 creates most of the binding niche for Q_A and a pheophytin, whereas D1 harbors Q_B and another pheophytin [16,22]. Site-directed mutagenesis has led to the identification of the Tyr-161 residue in the D1 protein as Z, the physiological electron donor to $P680^+$ [23], and the Tyr-160 residue in the D2 polypeptide of *Synechocystis* 6803 as the accessory slow PS II donor, D [24,25]. His-214 and Trp-253 in D2, which presumbly interact closely with Q_A, are essential to keep the stability of the entire reaction center [26]. Furthermore, the Glu-69 residue of D2 has been shown to be a potential ligand to Mn involved in photosynthetic oxygen evolution [27]. However, the role of the D2 protein in the bicarbonate effect had been obscure thus far.

In this paper, we report the differential sensitivity to formate in two site-directed mutants of *Synechocystis* sp. PCC 6803 (to be referred to as *Synechocystis* 6803) in which Arg-233 is changed into glutamine (R233Q) and Arg-251 into serine (R251S). Upon formate treatment, the mutants, in comparison with the wild type, showed more than 10-times greater inhibition by formate in the rate of oxygen evolution, and a 2–3-fold decrease in the rate of electron transfer between Q_A and Q_B. We suggest that Arg-233 and Arg-251 in the D2 polypeptide contribute to stabilization of HCO_3^- binding in PS II of *Synechocystis* 6803. Preliminary observations have been presented earlier, in abstract forms, at the 1989 and 1990 Midwest Photosynthesis Conferences in Indiana.

Materials and Methods

Growth conditions and transformation of Synechocystis. Wild type *Synechocystis* 6803 and the mutants were grown either in liquid media or on agar plates under constant illumination as described by Williams [28]. The mutants were maintained on media supplemented with appropriate antibiotics. Site-directed mutations were introduced into the *psb*DI/C$^-$, *psb*DII$^-$ double-deletion strain of *Synechocystis* 6803 as described by Vermaas et al. [29] by transformation with a plasmid containing a single-base change in the *psb*DI/C operon and a kanamycin-resistance cartridge downstream of *psb*C [29].

Site-directed mutagenesis. The procedure for site-directed mutagenesis in *Synechocystis* 6803 *psb*DI cloned into M13mp18 was as outlined earlier [29]. The oligonucleotides 5′ ATACTTTCCTTGGCATTTG3′ and 5′CCGCTAACAGTTTCTGG3′ were used for the replacement of Arg-233 with glutamine and Arg-251 with serine, respectively. The mutated base is underlined. Mutations were verified by sequencing using the dideoxynucleotide chain-termination method [30].

Growth curve assay. For measuring growth rates, the cyanobacterial cells were cultivated in BG-11 media and started at an absorbance of 0.08 at 730 nm as measured with a Shimadzu (Kyoto, Japan) UV-160 spectrometer. The absorbance at 730 nm represents scattering by cyanobacterial cells. For photohetero-

trophic growth, 5 mM glucose was included in the culture medium.

Herbicide binding assay. To quantify the diuron (also referred to as DCMU) affinity and the number of diuron binding sites on a Chl basis in whole cells of the wild type and mutants, cells were incubated with various concentrations of ^{14}C-labelled diuron (Amersham) in the dark at room temperature for 15 min. The Chl *a* concentration was 25 μg/ml. The amount of specifically bound diuron was calculated from the difference between the amount of free diuron found after centrifugation in the supernatant of the cell suspensions in the absence and presence of 20 μM unlabeled atrazine (for details, see Ref. 26). The diuron dissociation constant and the maximum number of diuron binding sites were calculated according to Tischer and Strotmann [31].

Steady-state oxygen evolution. The steady-state oxygen evolution in cyanobacterial cells was determined polarographically, under the saturating yellow light (3600 μE/m^2 per s) filtered with a Corning CS3-68 filter, using a Yellow Springs Instrument Clark-type electrode. The temperature was 25°C. A combination of two electron acceptors, 2,5-dimethyl-*p*-benzoquinone (DMQ) (0.5 mM) and $K_3Fe(CN)_6$ (1 mM) was used. DMQ acts as the electron acceptor and the non-penetrating ferricyanide keeps the DMQ in the oxidized state. 20 μM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was added in the reaction medium to block electron flow between the plastoquinone pool and PS I [32]. The Chl *a* concentration used for oxygen evolution measurements was 20 μg/ml. The suspension medium was the reaction medium containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 μM gramicidin D and 20 mM Hepes (pH 6.8). The reaction medium was adjusted to pH 6.8 after addition of 10 mM bicarbonate [12].

Flash dependence of oxygen evolution. Measurements of oxygen flash yield pattern of cyanobacterial cells upon excitation by Xenon flashes were made with a bare-platinum electrode, as described earlier [33]. The sample was applied directly on the platinum surface in a $1.5 \times 10 \times 0.18$ mm groove. This groove was covered by a piece of dialysis membrane, which kept the sample in its place. The sample was applied at 100 μg Chl *a*/ml. The sample was suspended in the following reaction medium (pH 6.8): 50 mM sodium phosphate, 20 mM Hepes, 70 mM KCl, and 30 mM $CaCl_2$. Above the dialysis membrane was a 4 ml chamber that was also filled with the same medium. A ring of silver, the Ag/AgCl electrode, was located in the upper chamber in a position shielded from the excitation light. The platinum electrode was biased at -450 mV relative to the standard hydrogen electrode. Signals were detected by a laboratory-built, DC-coupled transimpedance amplifier with a 0.5 ms rise time. Analog signals were digitized with a Biomation 805 waveform recorder and plotted with a chart recorder for signal analysis. The sample was incubated in the dark for 5 min on the electrode surface with the bias on. The platinum surface was scrubbed with a paste of $CaCO_3$ between each application of algal cells to the electrode. This was necessary to maintain maximum signal size and most rapid signal kinetics. Additions of formate or bicarbonate were made directly to the upper chamber of the electrode and incubation was allowed for 10 to 15 min. Flash excitation (full bandwidth at half height, 2.5 μs) was given with a 300 ms dark period and was provided by a General Radio Stroboslave 1539-A Xenon flash.

Chlorophyll a fluorescence decay. The kinetics of decay of variable Chl *a* fluorescence, after a saturating flash, were measured at 685 nm by a weak measuring light. The measuring light was fired at variable times after each actinic flash. The actinic (FX-124, EG and G) and the measuring flashes (Stroboslave 1593A, General Radio) were filtered with two Corning blue (CS 4-96) glass filters; both had a 2.5 μs duration at half-maximum peak [5]. Thylakoids were prepared by a procedure modified after Burnap et al. [34]. Thylakoid suspensions, at a Chl *a* concentration of 5 μg/ml, were dark adapted for 15 min. In order to calculate the rate constants of Q_A^- decay, the relative Q_A^- concentration was estimated from the variable Chl *a* fluorescence yield according to Joliot and Joliot [35] using the formula in Ref. 36. The connection parameter of 0.45 obtained from thylakoid membranes of the cyanobacterium *Phormidium laminosum* [37] was used.

Results

Growth characteristics

Cyanobacterial growth of the wild type and mutants in BG-11 liquid medium under photoautotrophic and photoheterotrophic conditions was assayed as the optical density of the culture at 730 nm as a function of time. Fig. 1 shows semilogarithmic growth curves of the wild type and the mutants R233Q and R251S. During the logarithmic growth phase, the doubling time of the wild type is 12 h, while the mutants showed slightly slower doubling time of 13 h (R233Q) and 15 h (R251S). However, under photoheterotrophic conditions (plus 5 mM glucose), when PS II is not required for growth, similar growth characteristics were observed in both the mutants and the wild type. This indicates that amino acid replacements in R233Q and R251S moderately affect the overall PS II activity.

Herbicide binding

A ^{14}C-labelled diuron binding assay was used to measure the total number of PS II centers in the mutants and the wild type. Plots of bound [^{14}C]diuron

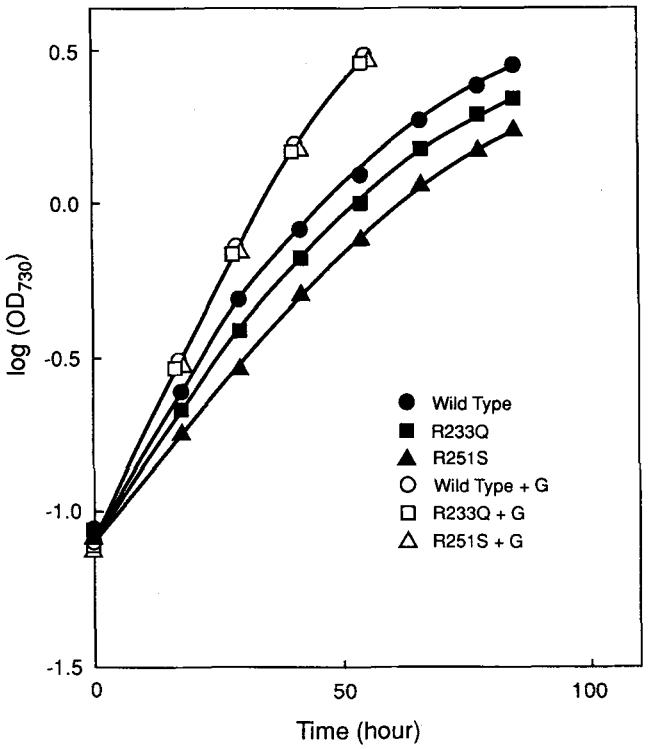

Fig. 1. Semilogarithmic growth curves of *Synechocystis* sp. PCC 6803 cells of the wild type (circles) and of R233Q (squares) and R251S mutants (triangles) under photoautotrophic (closed symbols) and photoheterotrophic (open symbols) growth conditions. Cyanobacterial cells were cultivated in BG-11 media, and for photoheterotrophic growth, 5 mM glucose (G) was included. Cyanobacterial growth was measured as the absorbance of the culture at 730 nm.

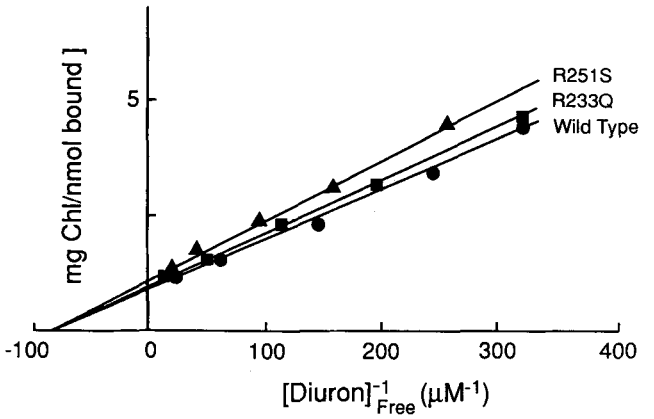

Fig. 2. Diuron binding in the wild type, R233Q and R251S mutant *Synechocystis* 6803 cells. Various concentrations of [^{14}C]diuron were added to 1 ml samples in 25 mM Hepes (pH 7.2). The chlorophyll concentration was 25 μg/ml. Circles: wild type; squares: R233Q; triangles: R251S.

against free diuron concentration show that the wild type as well as the mutants R233Q, and R251S have approximately the same diuron dissociation constant of 12.8 nM (Fig. 2). The maximum number of diuron binding sites for the wild type, R233Q, and R251S were calculated to be 1.14, 1.08 and 1.01 nmol bound diuron/mg Chl, respectively.

Steady-state oxygen evolution

It has been demonstrated that bicarbonate stimulates the Hill reaction in bicarbonate-depleted *Syne-*

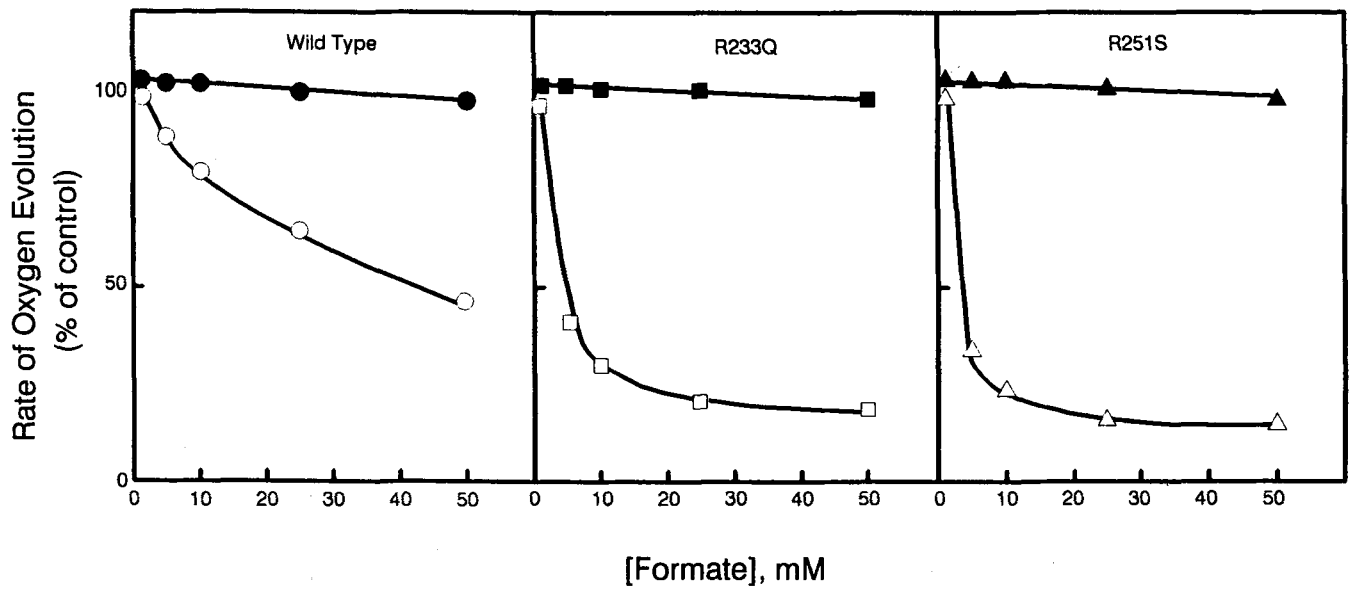

Fig. 3. Steady-state oxygen evolution rate of *Synechocystis* 6803 cells of the wild type, and R233Q and R251S mutants. The 2 ml reaction mixture contained 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4 (pH 6.8), 0.1 μM gramicidin D, 20 μM DBMIB and the indicated amount of sodium formate. The Chl *a* concentration was 20 μg/ml. A combination of DMQ (0.5 mM) and $K_3Fe(CN)_6$ (1 mM) was used as electron acceptor. The samples were incubated for one hour in medium containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM NaH_2PO_4 (pH 5.8), and the indicated amount of sodium formate, over which N_2 gas was passed (open symbols). To restore the Hill reaction, 10 mM bicarbonate was added (solid symbols).

chocystis 6803 cells and that this stimulatory effect is pH-dependent. The effect is maximal at a pH of about 6.8 [38]. Fig. 3 shows the Hill reaction activity (measured as O_2 evolution) of bicarbonate-depleted wild type and mutant cells in the presence of various formate concentrations. 100% in wild type was equivalent to 160 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, whereas 100% in mutants R233Q and R251S were 145 and 136 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, respectively. The slight decrease in the oxygen evolution rate in untreated R233Q and R251S can probably be accounted for partially by the loss of PS II centers, as calculated from herbicide binding data (see Fig. 2). The I_{50} value, the concentration of formate giving half-maximal inhibition of oxygen evolution rates, was calculated to be 48 mM for wild type, 4.5 mM for R233Q and 4.0 mM for R251S (open symbols). Note, however, that in none of the cases did formate totally inhibit electron transfer. The inhibitory effect of formate was fully reversed by the addition of 10 mM bicarbonate (closed symbols). Thus, mutants R233Q and R251S are an order of magnitude more sensitive to formate than the wild type is.

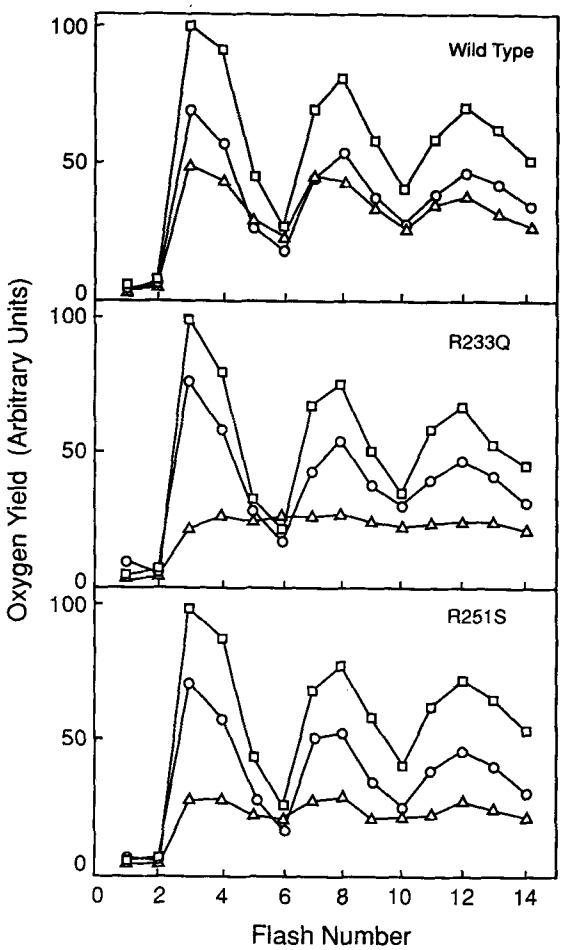

Fig. 4. Oxygen yield in a sequence of flashes in the *Synechocystis* 6803 cells of the wild type (top), R233Q (middle) and R251S (bottom). The Chl *a* concentration was 100 μg/ml. The suspension buffer contained 50 mM sodium phosphate and 20 mM Hepes at pH 6.8, 70 mM KCl, and 30 mM $CaCl_2$. 15 mM sodium formate was added and the cells were incubated in the dark for 10 min. To reverse the formate inhibition, 10 mM sodium bicarbonate was added to the formate-treated samples which were then dark-adapted for 10 min. Circles: control; triangles: 15 mM formate; squares: 15 mM formate + 10 mM bicarbonate.

Flash dependence of oxygen evolution

Bicarbonate depletion or formate addition is known to alter the oxygen yield in a sequence of flashes [20,39,40]. Fig. 4 shows the yield of oxygen per flash as a function of flash number in the wild type and in the mutants R233Q and R251S with and without the addition of 15 mM formate or the subsequent addition of 10 mM bicarbonate. The oxygen flash-yield pattern of cyanobacterial cells shows a peculiarity in comparison to higher plant chloroplasts: there is an electrochemical signal, but no oxygen yield on the first flash [41], whereas no such signal is observed at the first flash in dark-adapted thylakoids from higher plants [42]. In addition, the second maximum in the oxygen yield occurs at the eighth instead of the seventh flash. We observed a similar flash-yield pattern as reported in the cyanobacterium *Oscillatoria chalybea* [41]. The ratio of the sum of the oxygen yield in 14 flashes in the absence and presence of 15 mM formate is 1.4 in wild type cells, 2.1 in R233Q and 2.4 in R251S. Thus, in the case of R233Q and R251S mutants, 15 mM formate lowered the oxygen yield much more than in the wild type. Furthermore, the characteristic oscillation with a periodicity of four was very much damped in these mutants in the presence of formate; this oscillation could barely be seen in R233Q. Thus, mutants R233Q and R251S showed larger sensitivity to formate treatment than the wild type, but the difference in oxygen flash yield with and without formate is significantly less than what was measured in saturating continuous light. This will be discussed later.

Chlorophyll a fluorescence decay

In thylakoids, addition of bicarbonate to formate-treated samples increases the rate of electron flow from Q_A^- to the plastoquinone pool [5,9,33]. To measure the kinetics of Q_A^- oxidation by Q_B or Q_B^-, the decay of the Chl *a* fluorescence yield, which is related to the increase in Q_A concentration [43], was monitored (see, e.g., Refs. 5,9). Chl *a* fluorescence decays, monitoring the oxidation of Q_A^- following one ($Q_A^- \rightarrow Q_B$) or two ($Q_A^- \rightarrow Q_B^-$) actinic flashes, in thylakoids from wild type, R233Q and R251S, in the presence and absence of 25 mM formate are shown in Fig. 5. All fluorescence levels were normalized by the maximum variable fluorescence level in the presence of 10 μM DCMU. 20 μM quinhydrone was used to oxidize the acceptor quinones in the dark [44]. As mentioned in Materials and Methods, a nonlinear relation exists between F_v and $[Q_A^-]$. Thus, Q_A^- concentrations were

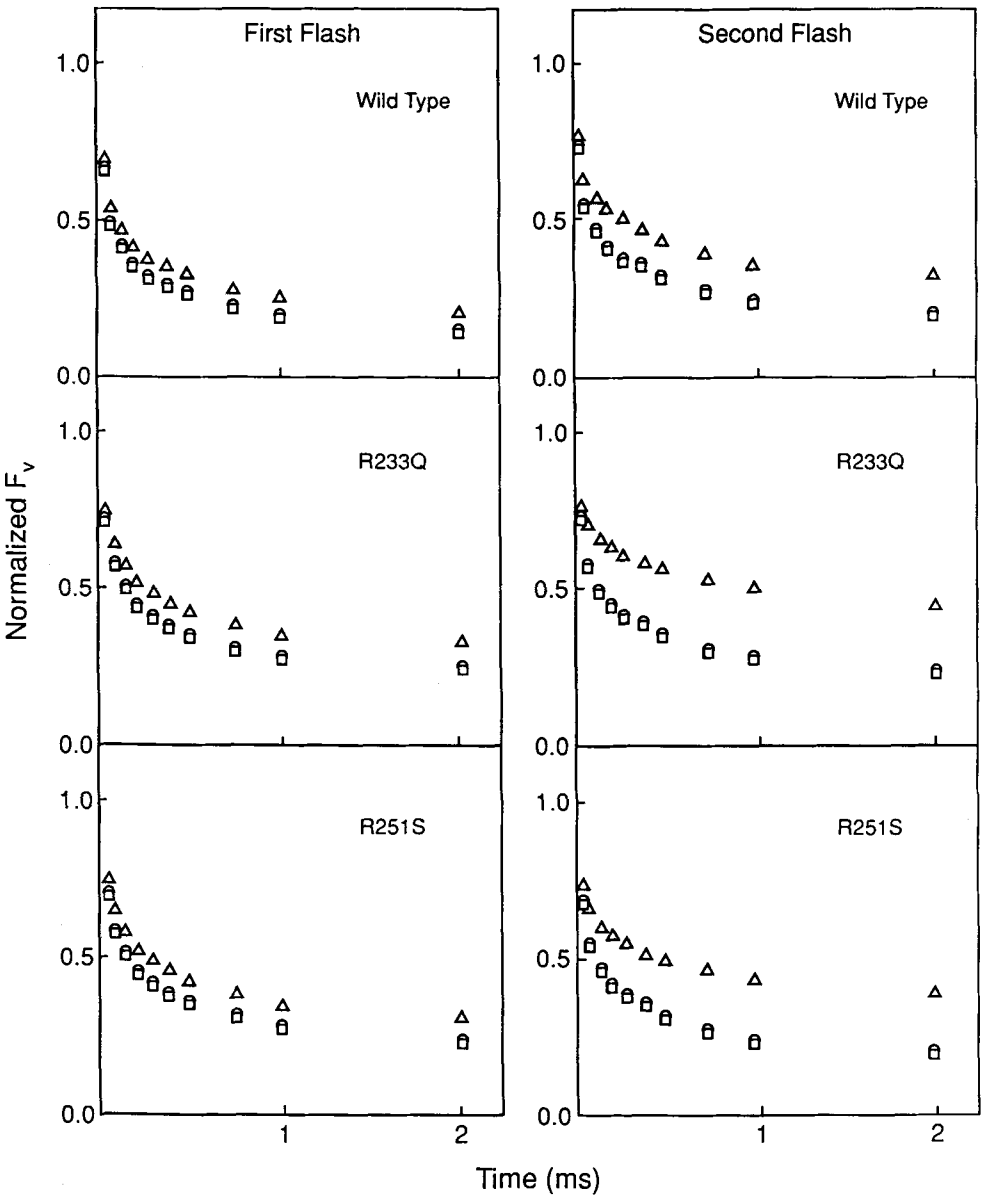

Fig. 5. Decay of variable chlorophyll *a* fluorescence following one and two saturating actinic flashes given to dark-adapted thylakoids from *Synechocystis* 6803 cells of the wild type, and of R233Q and R251S mutants. The circles and squares represent the control and bicarbonate-restored samples, respectively; the triangles represent the formate-treated samples. Thylakoids were suspended in a medium containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 μM gramicidin D, 20 mM Hepes (pH 6.8) and 25 mM sodium formate. The thylakoids were preincubated in the dark for 10 min in the presence of 20 μM quinhydrone. The formate treatment (25 mM) was done as described in the legend of Fig. 3. The variable chlorophyll *a* fluorescence (F_v) was normalized to the maximum fluorescence, which was the average of variable fluorescence measured after actinic flashes three through five with 10 μM DCMU present. The chlorophyll *a* concentration was 5 μg/ml. The flash frequency was 1 Hz.

calculated from F_v values. To simplify the comparison of the decay kinetics of Q_A^- between the wild type and mutants, only the fast first-order exponential decay process was considered, although the Q_A^- decay after one actinic flash contains two or more exponential components (see, e.g., Refs. 9, 45). The fast component of Q_A^- decay was resolved by subtracting the slow component. The half times of fast Q_A^- decay of the wild type are 225 μs and 361 μs following the first and the second flash, respectively, and are slightly faster than that of R233Q (half times of 327 μs and 499 μs) and R251S (270 μs and 451 μs). Also, in the mutants after the first flash the amount of remaining F_v at 2 ms after the flash is higher than in wild type, which could signify some change in the $Q_A^- \cdot Q_B \leftrightarrow Q_A \cdot Q_B^-$ equilibrium constant. More importantly, upon formate treatment the decay kinetics of R233Q and R251S after the second flash were slowed down more than in wild type. In the presence of 25 mM formate, the half time of fast Q_A^- decay after the second saturating actinic flash

increased by a factor of 4 (2.2 ms) and 6 (3.1 ms) in R251S and R233Q, respectively, while that of the wild type had only a 2-fold increase (to 0.8 ms). Furthermore, it appears that in the mutants not only the forward rate of electron flow from Q_A^- to Q_B^- has been slowed, but also the equilibrium constant of $Q_A^-Q_B^- \leftrightarrow Q_AQ_B^{2-}/Q_AQ_B$ H_2 has been altered since the slow component extrapolates to higher values at $t = 0$ in formate treated than in control and bicarbonate-restored samples. The ratio of the amplitude of slow component after formate treatment to that in the control is significantly higher for the mutants (1.77 for R233Q and 1.83 for R251S) than for the wild type (1.39). We note here that in view of the non-linearity of F_v and $[Q_A^-]$, the F_v of the slow component represents a significant fraction of Q_A^- [35]. The half times of Q_A^- decay in subsequent actinic flashes were similar to that after the second actinic flash (data not shown).

Chlorophyll a fluorescence decay in the presence of diuron

It is known that the reoxidation of the reduced acceptor Q_A^- after illumination in the presence of diuron is decreased in the presence of the artificial electron donor hydroxylamine (see, e.g., Refs. 46, 47). Thus, the oxidizing partner was suggested to be Z^+ or the S_2 state of the oxygen evolving complex. It has been observed that bicarbonate-depletion by formate treatment slows down the decay of Q_A^- even in the presence of diuron [9], suggesting that reoxidation of Q_A^- by Z^+/S_2 has been inhibited in the presence of formate. Reoxidation of Q_A^- in the presence of 10 μM diuron was measured for the wild type and mutant thylakoids. At pH 6.8, the half time of Q_A^- oxidation was 0.8 s, 1.2 s and 1.3 s in the wild type, R233Q and R251S, respectively. Upon formate treatment, the half time of Q_A^- oxidation increased to 6.6 s and 6.9 s in R233Q and R251S, respectively, while it increased to only 2.3 s in the wild type (Fig. 6).

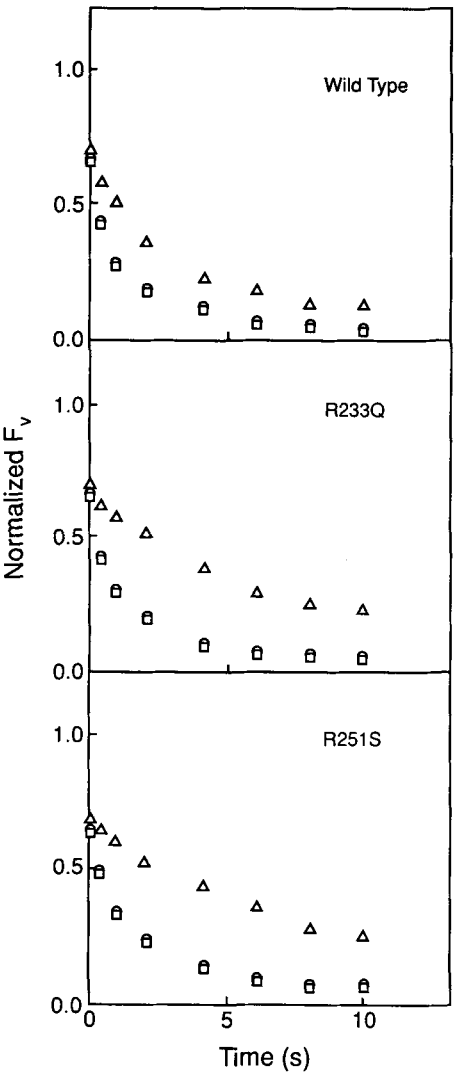

Fig. 6. Decay of variable chlorophyll *a* fluorescence after one saturating actinic flash in dark-adapted thylakoids from the wild type and from the mutants R233Q and R251S of *Synechocystis* 6803 in the presence of 10 μM DCMU. The other details were as in the legend of Fig. 5. Circles: control; triangles: 25 mM formate; squares: 25 mM formate + 10 mM bicarbonate.

Reactivation of the Hill reaction

One possible explanation for the higher formate sensitivity of the mutants is that Arg-233 and Arg-251 in the D2 polypeptide may function to stabilize bicarbonate binding in PS II of *Synechocystis* 6803. If this is the case, the apparent binding affinity of bicarbonate would be lowered in the mutants. A possible method to investigate bicarbonate binding affinity is to measure the Hill reaction rate after the addition of different concentrations of bicarbonate to bicarbonate-depleted samples [17,19] and compare the Michaelis-Menten constant K_m. The equilibration between bicarbonate and carbon dioxide is known to be completed within 1 min [48]. A time course of the reactivation of the Hill reaction by bicarbonate addition has indicated that the equilibrium is accomplished within 3 min [49], whereas carbonic anhydrase can further shorten the time to reach equilibrium between the two species. In this work we assured equilibration by measuring the restoration of the steady-state electron flow rate from water to plastoquinone by incubating samples in the dark for 10 min with various concentrations of bicarbonate. All the samples contained 100 mM formate. Fig. 7 shows the double-reciprocal plot of the oxygen evolution rate versus equilibrium bicarbonate concentration. The value of the intercept on the X-axis is Michaelis constant K_m, the bicarbonate dissociation constant in the presence of 100 mM formate. The apparent K_m values are 0.5 mM, 1.4 mM and 1.5 mM bicarbonate in the wild type, R233Q and R251S, respectively. This indicates that in the presence of 100

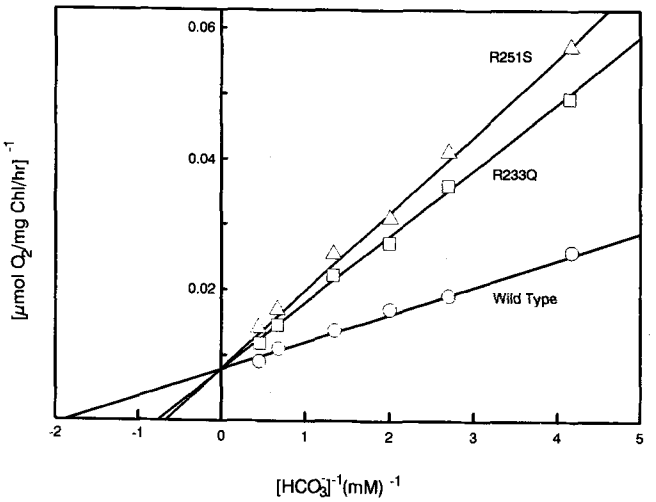

Fig. 7. Double reciprocal plots of the steady-state oxygen evolution rate as a function of the added sodium bicarbonate concentration in formate-treated *Synechocystis* 6803 cells of the wild type (circles) and of R233Q (squares) and R251S (triangles) mutants. The reaction medium included 100 mM formate. The experimental protocol was as described in the legend of Fig. 3.

mM formate the apparent K_m was increased by about 3-fold in the mutants as compared to the wild type.

Discussion

In this paper, we have investigated the role played by arginine residues in the D2 protein in the bicarbonate-reversible formate inhibition of Photosystem II reaction in *Synechocystis* 6803 cells. Mutation of Arg-233 to glutamine (R233Q) and of Arg-251 to serine (R251S) slowed down the photoautotrophic growth of cells only slightly (Fig. 1) and had an insignificant effect on characteristics of diuron binding (Fig. 2), but caused large differences in the sensitivity of formate to oxygen evolution in saturating continuous light (Fig. 3) and in flashing light (Fig. 4). The mutants were much more sensitive to formate than the wild type. This difference was accompanied by a significant change in the bicarbonate binding in the presence of 100 mM formate (Fig. 7). Data on decay of Chl *a* fluorescence yield showed that formate was more effective in slowing Q_A^- to Q_B^- electron transfer in the mutants than in the wild type (Fig. 5), and in slowing recombination of Q_A^- with S_2 in the presence of diuron (Fig. 6). All effects were reversed upon bicarbonate addition. On the basis of these results, we have concluded that the targeted arginine residues in the D2 protein play an important role in the bicarbonate effect on the electron acceptor side of Photosystem II.

Stemler et al. [39] had observed that in bicarbonate-depleted (formate-treated) higher plant thylakoids, oscillations in oxygen evolution as a function of flash number were reversibly damped; in addition, the relaxation of $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ was reversibly slowed. Furthermore, in higher plant thylakoids, Jursinic and Stemler [40] noted that formate treatment causes an increase in the ratio of S_0 to S_1 as well as an increase in the probability of the double hits. A damped oscillation was observed in R233Q and R251S mutants of Synechocystis 6803 with 15 mM formate (Fig. 4) as well as in wild type *Synechocystis* cells when high (50–100 mM) formate concentration was used (data not shown). Fitting of our experimental data on the oxygen flash yield to the S-state model, after removal of the signal observed at the first flash and 50% of the signal seen at the second flash [41], showed that formate treatment induced an increase of about 2.5-fold in the apparent S_0 to S_1 ratio and, surprisingly, an increase of about 5-fold in the probability of the double hits in the wild type and R251S. The oxygen evolution in R233Q was so much damped that it could not be fitted. These results can be best understood by a formate-induced shift in the redox equilibrium between Q_A and Q_B and/or a slowing down of electron transfer from Q_A^- to plastoquinone: relaxation of the system such that an S state transition can occur at a subsequent flash involves recovery of all the components in PS II reaction centers (see, e.g., discussions by Wydrzynski [42]). As noted above, we have observed a formate-induced damping of oscillations in oxygen evolution as a function of flash number in *Synechocystis* 6803. In contrast, however, Govindjee et al. [20] did not observe an obvious damped oscillation in oxygen yield upon formate treatment of *Synechocystis* 6714 cells. This difference could be due to the use of subsaturating, instead of saturating, formate concentration in the *Synechocystis* 6714 study. Furthermore, *Synechocystis* 6803 cells used in our study were grown in air (i.e., they were low CO_2 cells) whereas *Synechocystis* 6714 cells, used in the earlier study, were grown in CO_2-enriched atmosphere (high CO_2 cells).

Addition of 10 mM bicarbonate to formate-pretreated *Synechocystis* 6803 cells not only relieved inhibition caused by formate pretreatment and restored the normal flash-yield pattern but also enhanced oxygen yield to an extent larger than the control (Fig. 4). Furthermore, addition of 10 mM bicarbonate caused a similar enhancement of oxygen yield even in cells not pretreated with formate (data not shown). The larger oxygen yield enhancement than the control by bicarbonate addition (Fig. 4) might be related to so called 'Punnett effect' (see an earlier review [11]), in which addition of bicarbonate to untreated chloroplasts caused enhancement of phosphorylation and electron flow. In addition, this effect might be related to effects on the low affinity CO_2/HCO_3^- binding sites [10]. This effect was not studied further here since the focus of our work is on the differential sensitivity to formate inhibition in the mutants and wild type.

Bicarbonate molecules are considered necessary for a normal Hill reaction in PS II (electron transfer from water to the plastoquinone pool) [10,11]. Formate, an analog of bicarbonate, inhibits electron transfer in PS II, and this inhibition is fully reversed upon bicarbonate addition. Furthermore, formate releases CO_2 from spinach thylakoids as measured by both a mass spectrometer and a differential infra-red gas analyzer [4]. Formate has been suggested to be a competitive inhibitor of bicarbonate [10]. A 10-fold decreased I_{50} in the rate of steady-state oxygen evolution and a 3-fold increased K_m for bicarbonate in reactivation of the Hill reaction in the presence of formate indicate that a lower formate concentration is needed to reduce oxygen evolution activity and a somewhat higher bicarbonate concentration is required to overcome this formate inhibition in the mutants R233Q and R251S. We favor the interpretation that these mutations have altered binding of HCO_3^- and formate, so that bicarbonate molecules become more susceptible to displacement by the competitive formate anion. The inhibition of electron flow beyond Q_A^- upon formate treatment causing the slowing down of Chl *a* fluorescence decay after single-turnover flashes in *Synechocystis* 6803 mutants is in agreement with earlier studies on isolated higher plant thylakoids [5,9]. An increased inhibition of electron transfer after the second and subsequent actinic flashes ([3,5,9]; C. Xu, personal communication; also see Fig. 4) may be due to an impairment of protonation of Q_B^- and/or Q_B^{2-}. Upon formate treatment, the observed 2–3-fold larger increase in the half time of fast Q_A^- decay in the mutants compared to that in the wild type (Fig. 5) can be attributed either to a more loosely bound bicarbonate or a higher formate affinity. However, the difference in Q_A^- decay half time with and without formate is considerably smaller than that in the yield of steady-state oxygen evolution in saturating continuous light. Unlike continuous illumination, light flashes with 1 s dark intervals, used in Chl *a* fluorescence decay measurements, allow recovery of the PS II reaction centers in the dark intervals between actinic flashes. For instance, in formate treated thylakoid membranes half time of Q_A^- decay at flash frequency of 5 Hz is more than 8-fold slower than that at 1 Hz [5]. At still higher flash frequency ($\sim$ 30 Hz), the Q_A^- decay is further slowed [3].

In *Synechocystis* 6803, R251 is in the vicinity of the aromatic residue Trp-253, which is between pheophytin and Q_A and potentially involved in facilitating electron transport from pheophytin to Q_A and/or in binding of Q_A. Vermaas et al. [26], using site-directed mutagenesis, recently altered W253 into Leu. The PS II complex in the mutant appeared to be highly unstable. It was suggested that impairment of Q_A binding causes instability in the PS II complex. R233 is thought to be near the middle of the loop spanning the helices IV and V in the D2 protein of *Synechocystis* 6803 [50]. Its counterpart in photosynthetic bacteria may be R239 in *R. viridis*. Little is known about this arginine. However, in this region between the helices IV and V, Glu-232 (*R. viridis*) provides a bidentate ligand to the non-heme iron through its carboxylate group [16]. As noted earlier, bicarbonate/formate has no effect on electron transport between the two quinones in purple photosynthetic bacteria [14] or in the green bacterium *Chloroflexus aurantiacus* (Govindjee, J. Trost and R. Blankenship, 1990, unpublished observations). Michel and Deisenhofer [16] suggested that bicarbonate may act as a bidentate ligand to the iron in PS II. Glu-234 in *R. sphaeroides*, a counterpart of Glu-232 in *R. viridis*, has been changed into valine, glutamine and glycine through site-directed mutagenesis. These mutants show no significant inhibitory effects of formate or NO (Wang, X., Cao, J., Maroti, P., Stilz, H.U., Oesterhelt, D., Govindjee and Wraight, C.A., 1991, unpublished observations). This implies a fundamental difference between the iron niche of bacterial reaction centers and PS II reaction centers.

The crystallographic structure of human lactoferrin, in which binding of Fe(III) requires synergistic (bi)carbonate (HCO_3^-/CO_3^{2-}) ion binding, shows that a (bi)carbonate ion binds to Fe(III); other ligands to Fe(III) are provided by a histidine, an aspartate and two tyrosine residues [51]. Furthermore, the two oxygens of HCO_3^-/CO_3^{2-} are complexed to Fe(III) forming a bidentate ligand and are hydrogen bonded to an arginine and alanine, respectively; the third oxygen forms two hydrogen bonds with a glycine and threonine. It is possible that one or more of the Arg of D2 may provide hydrogen bonds to stabilize the binding of HCO_3^-/CO_3^{2-}, which likely forms a bidentate ligand with Fe(II) in PS II.

B. Diner and coworkers (unpublished data cited in a review [53]) have shown that site-directed mutants at Lys-264 in the D2 protein of *Synechocystis* 6803 had slowed electron transfer from Q_A^- to Q_B even in the presence of 10 mM bicarbonate. This raises the question about the importance of Lys-264 in CO_2/HCO_3^- binding. However, our results presented in this paper emphasize that other arginine residues (Arg-251 and Arg-233) in D2 also appear to be important for the stabilization of HCO_3^- binding.

In conclusion, observations in this paper show that the D2 protein is involved in the bicarbonate effect in PS II. Mutations of R233 and R251 in the D2 polypeptide in *Synechocystis* 6803, that lead to only minor disturbances in PS II function, result in a significant susceptibility to the replacement of bicarbonate by the competitive anion formate. We suggest that the two arginine residues, although not necessarily vital to bicarbonate binding, are important for the stabilization of bicarbonate binding in PS II.

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