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Differential effects of formate in single and double mutants of D_1 in *Synechocystis sp.* PCC 6714

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Abstract

Using several single and double mutants in the d-e interhelical loop of D_1 protein in *Synechocystis sp.* PCC 6714, we have shown that a differential sensitivity of formate inhibition on the acceptor side of Photosystem II occurs in the following order: S264A-F255L > S264A = N266T > N266T-S264A > F255L = N266D = WT ≫ N266T-A251V = A251V. On the basis of the absence of additivity of the effects between single and double mutants, it is concluded that the couples S264 and N266, N266 and A251, and S264 and F255 interact with each other in formate binding. It is considered highly likely that these effects are due to the modification, to some extent, of the overall conformation of the D_1 protein which, in turn, modifies the formate/bicarbonate binding at its site in the same protein.

Keywords: Bicarbonate effect; D_1 protein; D_1 mutant; Amino acid interaction; Photosystem II; (*Synechocystis* 6714)

1. Introduction

In plants, algae and cyanobacteria, but not in photosynthetic bacteria, CO_2 (or HCO_3^-) is involved in photosynthetic electron transport at the acceptor side of Photosystem II (PS II) (see reviews in [1,2]). This effect known as the bicarbonate effect is located in the Q_A-Fe-Q_B pocket of the PS II reaction center [1–3]. Incubation of plant thylakoids and cyanobacteria in media containing high concentrations of anions such as formate or acetate inhibits electron flow, and this inhibition is fully reversed by the addition of bicarbonate. Several site-selected and site-directed mutants of cyanobacteria carrying changes of aminoacids in D1 or D2 proteins of PS II (e.g., D_1-F211S, D_1-F255Y, D_1-S264A, D_2-R233Q and D_2-R251S) show differential sensitivity to bicarbonate-reversible formate inhibition of electron flow, suggesting that bicarbonate binding involves specific regions of the D_1 and D_2 proteins [1–8]. Studies with double mutants (e.g., D_1-F255L/S264A, D_1-F255Y/S264A and D1-A251V/F211S) have suggested interactions between different amino acids for formate/bicarbonate binding [4,8]. In a earlier paper [4], we have shown, on several *Synechocystis* PCC 6714 mutants, that inhibition by formate produced a decrease of oxygen evolution, an increase of the stationary level of Chl *a* fluorescence measured in continuous light and an increase of the slow phase of Chl *a* decay of fluorescence after a saturating flash, and that all these effects are correlated, showing evidence that inhibition is a block of the electron transfer between Q_A and Q_B.

Comparisons of single and double mutants are specially interesting. Horowitz et al [9], measuring binding of herbicides on several single and double mutants in D1 protein, have proposed that when two amino acids contribute to the binding of herbicides in an additive manner, it can be concluded that there is an apparent lack of interaction between these two amino acids. As we have several double mutants and the corresponding single mutants, the interactions in herbicide binding between amino acids at positions 251–266, 255–264 and 264–266 were studied [9]. The results obtained have suggested an apparent lack of interaction between the amino acids 264 and 266 but some interactions for the couples 251–266 and 264–255 in herbicide binding.

In this paper, we have tested the importance of D_1-N266 and D_1-F255 and of their interaction with D_1-S264 and D_1-A251, for sensitivity to formate, by using several single

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and double mutants in D_1 protein (N266T, N266D, F255L, S264A, A251V, N266T-S264A, N266T-A251V, S264A-F255L) of *Synechocystis sp.* PCC 6714.

2. Material and methods

2.1. Growth conditions

Synechocystis sp. PCC 6714 wild-type and herbicide-resistant D_1 mutants [10] were grown photoautotrophically in mineral medium as defined by Herdman et al. [11], but with twice the concentration of nitrate. Cultures placed on a Gallenkamp rotatory shaker were grown at 34° C in a CO_2-enriched atmosphere, with 70 $\mu E\, m^{-2}\, s^{-1}$ light from fluorescent tubes. Chlorophyll *a* concentrations were determined from methanol extracts using an extinction coefficient of 75 $ml\, mg^{-1}\, cm^{-1}$ as described in [12]. Growing rates (generation time of 6 h), Chl *a* contents per cell (0.8 μg per 10^7 cells) and PSII and PS I contents measured by fluorescence at 77K are similar in the wild-type and all the mutants. The binding of several herbicides by the mutants have been measured [10,13] and the values are summarized in Table 1.

2.2. Chlorophyll a fluorescence transients in continuous light

Chlorophyll a fluorescence transients were measured in cyanobacterial suspensions containing 1 μg Chl per mL. The suspension medium contained 100 mM Hepes (pH 6.5), 40 mM NaCl, 5 mM $MgCl_2$ and 20 μM *p*-benzoquinone. Samples were incubated for 10–15 min in darkness after the addition of formate or herbicides before the fluorescence measurements began. After this, bicarbonate (10 mM) was added and a second measurement was made after 0.5 h of incubation. Fluorescence was measured orthogonal to the exciting beam through red filters (Corning 2-64 and Schott RG 665) complementary to the blue exciting beam (passed through Corning CS 4-72 and 5-59 filters). The signal was recorded using a multichannel analyser, Interzoom (SEIN, France) [12].

Table 1
The herbicide resistances (*R/S*) of *Synechocystis* 6714 mutants carrying mutation in *psb*A1 gene encoding D_1 protein of PS II

Mutation(s)	DCMU	Atrazine	Metribuzin	Ioxynil
S264A	1000	100	1000	0.8
F255L	10	0.5	0.5	7.5
N266T	0.8	1	6.5	10
N266D	0.7	1		4
A251V	3	25	200	10
S264A-F255L	1000	1.5	1000	3
N266T-S264A	1000	100	~ 4000	4.5
N266T-A251V	2	100	1500	15

R/S is the ratio of the I_{50} of the mutant to that of the wild-type. I_{50} values were determined in whole cells as the inhibitor concentration which blocks half of the variable fluorescence (data from Refs. [10,11]).

2.3. Chlorophyll a fluorescence decay kinetics

Suspensions of *Synechocystis* wild-type or mutant cells, containing 100 μg chlorophyll per mL in the suspension medium described above, were placed in a thin cuvette (0.5 mm thick) equipped with a sample renewal system. Fluorescence yield decay kinetics, reflecting the rate of Q_A^- reoxidation, were measured after a single turnover saturating xenon flash (lamp XST 103, Walz Effeltrich, Germany) with Pulse Amplitude Modulation (PAM) fluorometer (Walz Effeltrich, Germany), as described by Schreiber et al. [14]. The flash light was collected by one arm of the light pipe. A CS 4-96 Corning blue filter was placed between the flash lamp and the light pipe input.

Kinetics were recorded with a digital storage oscilloscope (Thurlby 542, England). Deconvolution of the kinetics of fluorescence and of Q_A^- decays (after conversion of fluorescence to $[Q_A^-]$) was performed utilizing a Marquardt search algorithm program. The program calculated the maximum fluorescence yield by extrapolation of the analysis of the data obtained during 5 ms, the recording of which started 120 μs after the flash was given.

Prior to flash illumination, the samples were preincubated in the dark at 20° C for 10 min in the presence or the absence of 20 μM *p*-benzoquinone and with various concentrations of formate when used. Longer times of incubation in formate did not change the results.

We performed two types of fluorescence decay measurement: (1) on the same sample, we measured the average of a train of flashes with a frequency of 0.5 or 0.125 Hz (the four first flashes being omitted in the averaging), and (2) we renewed the sample before each flash and averaged 20 fluorescence decays.

$[Q_A^-]$ was calculated from Chl *a* fluorescence using the formula of Joliot and Joliot [15]

$$[Q_A^-] = F_v/p.F_v + 1 - p$$

where F_v is the variable fluorescence normalized to $F_{v\,max}$ and p the probability of PSII-PSII exciton transfer. The value of p is 0.5 in *Synechocystis* wild-type cells [16].

Under these above-described conditions, the deconvolution of Q_A^- decays could be fitted by three exponential components with lifetimes (T_1, T_2 and T_3) of about 0.4 ms, 2 ms and 1 s. The slowest component may correspond to reoxidation of Q_A^- in non-Q_B-transferring centers [17] or in "slow centers". The two fast components with lifetimes T_1 and T_2 can be interpreted in the model used by Crofts et al. [18]:

$$Q_A^- + (Q) \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} Q_A^- Q_B \underset{k_{ba}}{\overset{k_{ab}}{\rightleftarrows}} Q_A Q_B^-$$

Here Q_A is the primary plastoquinone acceptor bound on D_2 protein, (Q) is the diffusible plastoquinone and Q_B is

the secondary plastoquinone acceptor bound on D_1. The fastest component (0.4 ms) may reflect the $Q_A^- Q_B \leftrightarrow Q_A Q_B^-$ step, and the middle component (2 ms) the $Q_A^- + (Q) \leftrightarrow Q_A Q_B^-$ step.

3. Results and discussion

3.1. Chlorophyll a fluorescence in continuous light

The inhibition of Photosystem II electron transport between Q_A and Q_B by formate was measured by the changes in Chl *a* fluorescence. Most of the Chl fluorescence at room temperature is from PS II, not PS I. It is known that in intact photosynthesizing cells Chl fluorescence yield is controlled by both the redox state of Q_A, and by the so-called state I–state II transitions. To maintain the cells in the more fluorescent state (state I), we added 20 μM *p*-benzoquinone, which oxidizes the plastoquinone pool and maintains the cells in state I [19]. In conditions where light preferentially excites the Photosystem I and that reaching PS II is weak enough to keep the photochemical rate of active centers slow with respect to their recovery rate, there are very few PS II centers in the Q_A^- state and the variable fluorescence (F_v) is low. Addition of formate blocking or slowing electron transfer from Q_A^- to Q_B (or to Q_B^-) produced an increase of the variable fluorescence that is proportional to the number of PS II centers blocked in the Q_A^- state. In order to obtain precise information on the differential sensitivity of the D_1 mutants to formate, we calculated the inhibition produced by different concentrations of formate by measuring F_v in samples incubated in the presence and absence of various concentrations of formate. 100% and 0% of inhibition were obtained by the F_v levels with and without 10 μM DCMU (or 500 μM ioxynil, in the case of the mutants resistant to DCMU).

Figs. 1a and 1b show the differential sensitivity of the various mutants to the inhibition of electron flow responsible for the increased variable Chl fluorescence. The order of sensitivity, when we compared (Fig. 1a) the cells possessing the D_1-N266D mutation and the D_1-N266T mutation alone or associated either with D_1-S264A or D_1-A251V, is the following: S264A = N266T > N266T-S264A > N266D = WT ≫ N266T-A251V = A251V. The change of the asparagine (N) on residue 266 to threonine (T) produces an increased sensitivity to formate compared to the WT, but not when it is changed to aspartic acid (D). The two mutants D_1-N266D and D_1-N266T have been selected on ioxynil, a phenol-type herbicide, and have a relative resistance of 3 and 10 respectively. Molecular models have been constructed [20] and the hydrogen-bonding energies between the hydroxyl group of ioxynil and the respective amino acids at position 266 have been calculated. The values obtained decreased when the resistance levels increased. The order of sensitivity to formate is

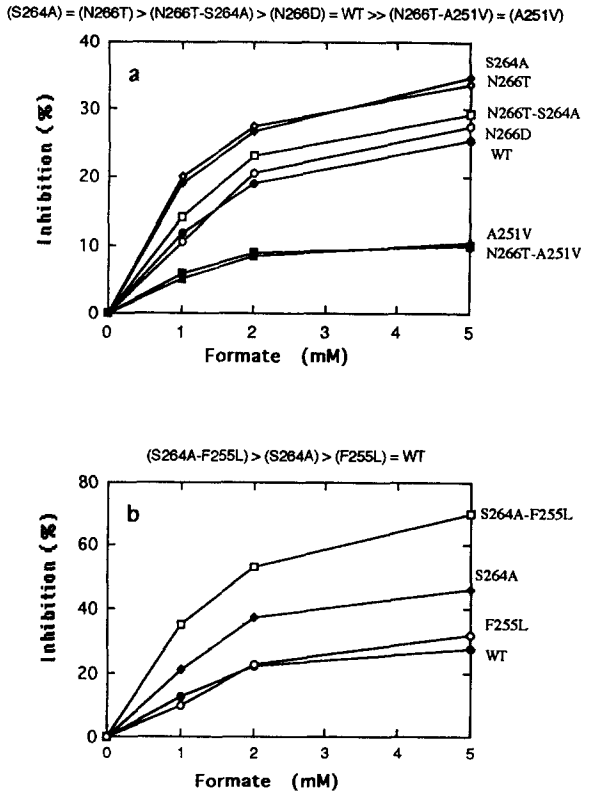

Fig. 1. Inhibition by formate addition of wild-type and mutant cells of *Synechocystis* PCC 6714, as measured by chlorophyll *a* fluorescence intensity in continuous light (see Material and methods and Results).

comparable to the order of resistance to ioxynil. This is probably because the nature of the various substitutions at residue 266 modify the electrostatic properties of the site. However, we cannot exclude a steric effect, since the more bulky of the amino acids concerned would be Asn in the wild-type. Steric hindrance of Asn and Asp are quite similar and Thr has the shortest side chain.

The association of the two mutations S264A and N266T does not produce additivity of the effect of each mutation on the sensitivity to formate but rather an opposite effect. According to the hypothesis of Horovitz et al. [9], this would suggest that these two residues are interacting amino acids for binding to formate. In contrast, we obtained the opposite conclusion concerning contribution of these two amino acids for binding to several herbicides [10].

The association of the mutation N266T (giving hypersensitivity to formate) and of the mutation A251V (which confers resistance to formate) does not produce an additive effect, the double mutant showing the same resistance to formate as the single mutant A251V. This can also be taken as evidence for the interacting nature of A251 with N266 concerning binding to formate as it was also the case concerning the binding of several herbicides.

Furthermore, the single mutation F255L does not produce (Fig. 1b) modification of the sensitivity compared to the wild-type, but its association with mutation S264A strongly enhances the sensitivity as compared to the single

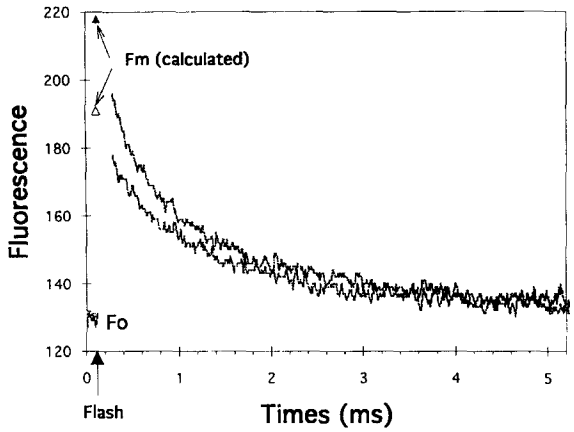

Fig. 2. Effect of 20 mM benzoquinone addition on the fluorescence decay of wild-type cells of *Synechocystis* PCC 6714. (20 flashes were given to the same sample with Δt of 8 s between flashes; for other conditions, see Material and methods). Top curve: with benzoquinone.

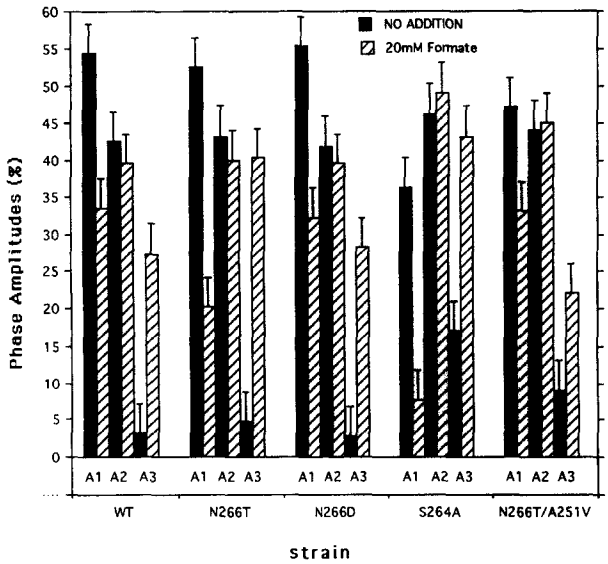

Fig. 3. Effect of formate on the relative amplitudes of the three phases of chlorophyll *a* fluorescence decay after repetitive flashes in wild-type and various mutant cells of *Synechocystis* PCC 6714. (32 or 64 repetitive flashes with $\Delta t = 8$ s were averaged; for each strain 2 to 8 experiments were performed). T_1 and T_2 were respectively 0.4 and 2 ms without formate and 0.6 and 3.0 with 20 mM formate (values $\pm 20\%$). T_3 was fixed at 500 ms in all cases.

mutant S264A. Here also it could be interpreted as some interaction of the two amino acids concerned as for the herbicides [10].

3.2. Chlorophyll fluorescence decays after flashes

To be sure that 20 μM benzoquinone is sufficient to oxidize the plastoquinone pool and to put the cells in state I when the cell suspensions are concentrated at 100 μg chlorophyll/mL, the fluorescence decays after flashes, with and without benzoquinone were measured. Fig. 2 shows that the variable Chl fluorescence is higher in the presence of 20 μM benzoquinone. Higher concentrations of benzoquinone did not increase it further (not shown). Thus, the samples used in these experiments were also in state I as required for our studies

Fig. 3 shows the amplitudes of the three phases of fluorescence decays of four (N266T, N266D, S264A and N266T-A251V) mutants in the presence and the absence of 20 mM formate. Several flashes (32 or 64) were given to the samples with a Δt between flashes of 8 s to allow the full recovery of F_0 level after each flash. Smaller Δt produced an increase of the initial level of fluorescence in the samples incubated with formate. Whereas WT, N266T and N266D mutants had the same type of decay in the absence of formate (i.e., very few slow centers) as previously observed [21], addition of 20 mM formate decreased the amplitude A_1 of the component with T_1 lifetime and increased A_3 more in the N266T mutant than in WT and in the N266D mutant. This corresponds to what we observed with fluorescence in continuous light (Fig. 1a). The two other mutants (S264A and A251V-N266T) had slower fluorescence decay kinetics in the absence of formate as compared to the WT [22], but the first (S264A) is more sensitive to formate and the second (A251V-N266T) is more resistant to formate than the WT. Thus, the differential sensitivity of the single and double D1 mutants to formate, observed with fluorescence transient measurements (Figs. 1a and b), seems to be located in the Q_A–Q_B reactions.

In all cases, what is obvious is that the amplitude of the rapid phase (A_1) is decreased, and the amplitude of the slow phase (A_3) is increased, whereas the amplitude of the medium phase A_2 is almost unchanged. Both T_1 and T_2 were increased by a factor of about 1.5.

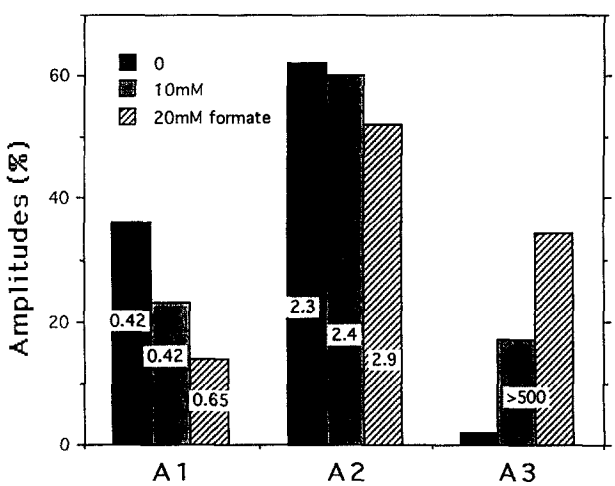

Fig. 4. Effect of various concentrations of formate on the three phase amplitudes of Q_A^- reoxidation after flashes in wild-type cells of *Synechocystis* PCC 6714. Same conditions as Fig. 3. Values indicated on the histogram correspond to the lifetimes in ms.

As shown in Fig. 4, transformation of Chl fluorescence to concentration of Q_A^-, according to the formula of Joliot and Joliot [15] with $p = 0.5$, did not modify these conclusions. The values of the amplitudes and lifetimes of the three phases of Q_A^- reoxidation measured in the absence and the presence of 10 and 20 mM formate show that it was the rapid phase which was more decreased by formate.

It was previously proposed that formate inhibits protonation of Q_B^- and $Q_B^=$ [1,23]. This conclusion was based primarily on experiments with isolated thylakoids where formate inhibited more the Q_A^- reoxidation after the second and subsequent flashes than after the first one. Before the first flash, the thylakoids are incubated in darkness and Q_B is essentially in the oxidized state, whereas it is in the semireduced state Q_B^- before the second flash. The test of this hypothesis in whole cells is difficult because intact cells contain equal proportions of Q_B and Q_B^- even after a long time of darkness [24]. Benzoquinone has been shown to oxidize the plastoquinone pool (Fig. 2). To test whether benzoquinone is able to also oxidize Q_B^-, the fluorescence decays were measured either after 20 flashes on the same sample (the four first flashes being omitted) or after the first flash, the sample being renewed before each flash. No differences can be observed between these two types of measurement (not shown).

In all cases, we did not observe a great modification of the middle phase, corresponding to centers without Q_B in the pocket, but we did observe a preferential decrease of the rapid phase of Q_A^- reoxidation, corresponding to the centers with Q_B or Q_B^- in the site. This observation might indicate that to obtain formate inhibition, it is necessary to have Q_B or Q_B^- attached to D_1. When there is no plastoquinone in the niche, formate might not have access to this site.

4. Concluding remarks

The order of sensitivity to formate of the various single and double mutants of *Synechocystis* PCC 6714 is as follows: S264A-F255L > S264A = N266T > N266T-S264A > F255L = N266D = WT ≫ N266T-A251V = A251V. Practically all the mutations of the D1 protein that have been shown to give resistance to various herbicides modify the sensitivity to formate as compared to the wild-type. Studies with *Chlamydomonas* [5,6] and *Synechococcus* 7942 [8] herbicide-resistant mutants indicated comparable effects of the mutations on the sensitivity to formate. Horovitz et al. [9] have proposed that when two amino acids contribute to the binding of herbicides in an additive manner, there is an apparent lack of interaction between these two amino acids. The results we have obtained with herbicides on our double mutants and the corresponding single mutants [10] have suggested an apparent lack of interaction between the amino acids 264 and 266 but some interactions for the couples 251–266 and 264–255 in herbicide binding. Here, data of Figs. 1a and b did not allow the calculation of binding constants of formate to D1, but they clearly show that there was no additivity of the effects of the mutations in the double mutants. This allows us to postulate that all the amino acids of the couples studied interact in formate binding, probably because all of them modify to some extent the overall conformation of the D_1 protein and consequently either modify the binding of bicarbonate or that of formate or both of them. In all the strains examined here, the electron transfer between Q_A and Q_B seems to be modified in the same manner but with different amplitude (see Fig. 3). There are quantitative, but no qualitative, differences of the effect of formate in the various mutants, and this independently of their characteristics of electron transfer or herbicide binding.

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