Volume 267, number 2, 273–276 FEBS 08615 July 1990

Differential sensitivity of bicarbonate-reversible formate effects on herbicide-resistant mutants of *Synechocystis* 6714

Govindjee[1], Claudie Vernotte[2], Brigitta Peteri[2], Chantal Astier[2] and Anne-Lise Etienne[2]

[1] *Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA* and [2] *UPR407 Photosynthèse, Bâtiment 24, CNRS, 91198 Gif-sur-Yvette, France*

Received 26 April 1990

Herbicide-resistant mutants of the cyanobacterium *Synechocystis* 6714, that are altered in specific amino acids in their D-1 protein, show differential sensitivity to formate treatment. Measurements on oxygen yield in a sequence of flashes, chlorophyll (Chl) *a* fluorescence transients and Chl *a* fluorescence yield decay after a flash reveal that the resistance of cells to formate treatment is in the following (highest to lowest) order: [double mutant] A251V/F211S (Az V) > [single mutant] F211S (Az I) ≃ wild type > [single mutant] S264A (DCMU II-A). Significance of these results in terms of overlapping between the herbicide and bicarbonate binding niches on the D-1 protein is discussed.

Photosystem II; Herbicide; Cyanobacterial mutant; D-1 protein; Bicarbonate; *Synechocystis* 6714

1. INTRODUCTION

Warburg and Krippahl [1] discovered that removal of CO_2 from a suspension of a green alga *Chlorella*, that was performing Hill reaction with benzoquinone as electron acceptor, led to a CO_2-reversible decrease in O_2 evolution. On this basis, Warburg [2] argued that the source of O_2 was CO_2, not H_2O. Such a role of CO_2 has not been confirmed, but instead, it has been established that a major effect of CO_2 lies between the primary plastoquinone electron acceptor, Q_A, of photosystem II (PS II) and the plastoquinone, PQ, pool [3–7]. This phenomenon is most easily studied by removing CO_2 by the addition of formate [8], nitrite [9] or nitric oxide [10]. Although it has been difficult to observe CO_2-release at pH 6.0 [11], formate treatment at pH 6.5 has been shown to release, both by a differential infra-red gas analysis and a sensitive membrane-inlet mass spectrometer, >1 CO_2/reaction center II (Govindjee, H.G. Weger, D.J. Turpin, J.J.S. van Rensen, O.J. deVos and J.F.H. Snel, unpublished, personal communication, 1989). Addition of CO_2 causes a recovery of electron flow in the anion-inhibited samples, and the phenomenon is referred to as the 'bicarbonate effect'. This is appropriate in view of the observation that the active species may be HCO_3^-, not CO_2 [12]. CO_2, however, is the diffusing species [13], and we cannot yet rule out that it may also be an active species [14]. Thus, CO_2 or HCO_3^- is interchangeably used in this paper. An interaction of HCO_3^- with the herbicide-binding protein, D-1, is suggested from studies on the effects of several herbicides on HCO_3^--depleted thylakoid membranes [15,16] since the herbicides used are known to bind to D-1 [17]. Two different, but non-exclusive, binding sites have been suggested for HCO_3^-: one on the Fe between Q_A and Q_B (the secondary plastoquinone electron acceptor) (see [7,10,18] and references therein), and the other on positively-charged amino acids, such as arginine [18]. Furthermore, two different, non-exclusive, functions have been suggested for HCO_3^-: one involves the stabilization of the D-1 and the D-2 proteins of the reaction center II, i.e. an allosteric role [12,15], and the other involves protonation of Q_B^- and, perhaps, Q_B^{2-} [7,18,19]. In addition, any hypothesis for the molecular mechanism of the role of bicarbonate will have to take into account a bicarbonate-reversible formate effect, recently observed in *Chlamydomonas* cells [20] and in spinach leaf discs [21], between the electron donor to PS II reaction center and Q_A.

Cyanobacteria are excellent model systems to study the role of HCO_3^- in PS II since (i) they are prokaryotes and, thus, amenable to established genetic manipulations; (ii) unlike photosynthetic bacteria [22], they clearly show the existence of the HCO_3^- effect [23]; and (iii) they have PS II that is similar in all major respects

Correspondence address: Govindjee, Department of Plant Biology, University of Illinois at Urbana-Champaign, 505 South Goodwin Avenue-289 Morrill Hall, Urbana, IL 61801-3793, USA

Abbreviations: Chl, chlorophyll; D-1, a 32 kDa intrinsic protein of the reaction center of PS II; D-2, another 32 kDa intrinsic protein of the reaction center of PS II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS II, photosystem II; Q_A, primary plastoquinone electron acceptor of PS II, a one-electron acceptor; Q_B, secondary plastoquinone electron acceptor of PS II, a two-electron acceptor

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

to that of higher plants. The availability of several herbicide-resistant mutants [24] of the cyanobacterium *Synechocystis* 6714, with known amino acid changes in the D-1 protein [24,25] and known changes in the kinetics of electron flow between Q_A and the PQ pool [25], allowed us to test the hypothesis if, indeed, the CO_2-displacing anion (e.g. formate) has overlapping niches with the herbicides, and, thus, a probable binding on the D-1 protein.

2. MATERIALS AND METHODS

2.1. *Growth conditions*

Synechocystis sp. Pasteur culture collection (PCC) 6714 was grown in minimal medium defined in [26] for 24 h at 34°C in a CO_2-enriched atmosphere (light intensity, 4000 lux). Cells were constantly shaken, the generation time of the cells was about 6 h, and the chlorophyll concentration was $\leq 10\ \mu g$/ml of the suspension.

2.2. *Oxygen measurements*

The amount of O_2 produced by each flash in a sequence of short (5 μs) saturating flashes (dark time between flashes, 500 ms) was measured in whole cells as described elsewhere [27]. Samples contained 500 μg Chl/ml of suspension. The suspension medium contained 20 mM Hepes, 70 mM KCl and 30 mM $CaCl_2$ (pH 6.8). The dark time between sequence of measurements was 5 min. Formate and bicarbonate were added separately to the circulation medium and allowed to incubate for 15 min in a closed cuvette before measurements. Damping and deactivation of the S-state were measured and calculated, as described in [28,29]. Deactivation of S_2 was obtained by measuring O_2 yield in the 3rd flash as a function of dark time between flashes 1 and 2.

2.3. *Chlorophyll a fluorescence transients in continuous light*

Chlorophyll *a* fluorescence transients were measured in cyanobacterial cell suspensions that contained 1 μg Chl/ml and were kept stirred in a closed 1 cm cuvette. Fifteen minutes darkness was given after the addition of formate, bicarbonate or herbicides, but before the measurements began. Fluorescence was measured orthogonal to the exciting beam through red filters (Corning C.S. 2-64 and Schott RG 665) complementary to the blue exciting beam (passed through Corning C.S. 4-72 and 5-59 filters). The intensity of exciting light was 65 W/m^2. The signal was recorded using a multichannel analyzer (Interzoom).

2.4. *Chlorophyll a fluorescence decay*

Chlorophyll *a* fluorescence decay, after a short saturating flash, was measured as described elsewhere [25]. The S-20 photomultiplier was protected by a KV 550 (Schott), a RG5 and a 685 nm interference filter; the actinic light had a full width at half-height of 5 μs, and was blue (a pair of Corning C.S. 4-96 filters). Cyanobacterial cell suspensions, at a [Chl] of 10 μg/ml, were dark-adapted for 10 min and the sample was renewed before each measurement. Data after both flashes 1 and 2 (dark time between flashes, 0.5 s) were obtained and were averaged 20 times. Fluorescence decay data were deconvoluted into three exponentials, the fitting was done between 150 μs and 25 ms with a point every 56 μs.

3. RESULTS AND DISCUSSION

3.1. *Oxygen yield in a sequence of flashes*

In order to obtain information on the activity of the PS II centers, O_2 yield per flash in a sequence of flashes was measured with and without addition of subsaturating amounts of formate (20 mM), and then after addition of bicarbonate (10 mM). The relative yield of O_2 was calculated as sum of the yields of the 9 flashes. In all cases, formate induced inhibition of O_2 evolution, but the amount and percentage of inhibition was dependent upon the mutant used. Fig. 1 shows results for S264A (DCMU-IIA) mutant. Here 20 mM formate caused 40% inhibition which was totally reversed upon 10 mM bicarbonate addition. There was no significant difference in the damping of the O_2 sequence (cf. e.g. [28]).

Table I shows data on the differential sensitivity of the various mutants. S264A (DCMU-IIA), that is resistant to metribuzin, DCMU and atrazine, was most sensitive to formate, whereas the double mutant F211S/A251V (Az V), that is quite resistant to metribuzin and atrazine, but relatively sensitive to DCMU, was most resistant to formate. Wild-type and the F211S, that are intermediate in their resistance to these herbicides, were intermediate in their resistance to formate. In all cases, the half-time of the S_2 deactivation was independent of the presence of formate and bicarbonate (Table I). These results suggest that formate-inhibited centers stay inhibited for times $\gg$ the dark time between flashes and thus, produce no acceleration of the S_2 deactivation kinetics. This leads us to conclude that the turnover of formate is very slow, even slower than that of DCMU [29].

3.2. *Chlorophyll a fluorescence in continuous light*

In order to obtain precise information on the differential sensitivity of the mutants to formate, we measured the effect of different concentrations of formate on the variable Chl *a* fluorescence, i.e. fluorescence above the 'F_o' level. 100 on the ordinate indicates the level of maximum fluorescence obtained with 10 μM DCMU or 200 μM ioxynil (in the case of DCMU II-A). Fig. 2 shows the differential sensitivity of the various mutants to the inhibition of electron flow responsible for increased variable Chl *a* fluorescence. The order of sensitivity (S264A > wild-type F211S > F211S, A251V), obtained here, is the same as with O_2 sequence data (Table I).

The CO_2-depletion procedure, as used earlier [7,23] (anaerobiosis under nitrogen bubbling for 1 h), led to an increase in the initial fluorescence (F_i) immediately upon turning on the continuous light: this increase is due to the formation of Q_A^- in darkness. This increase was eliminated by the addition of 200 μM benzoquinone after the depletion procedure. This suggests that the electrons arrive at Q_A from a back-flow of electrons from Q_B^- (intact cells in anaerobiosis have a high ratio of Q_B^-/Q_B in darkness (cf. e.g. [30]) and benzoquinone abolishes the effect as it reoxidizes Q_B^- in darkness. Thus, the formate effect may be similar to that of DCMU: formate may bind when the electron, shared between Q_A and Q_B, is on Q_A. Then, when formate is bound, electron transfer between Q_A and Q_B no

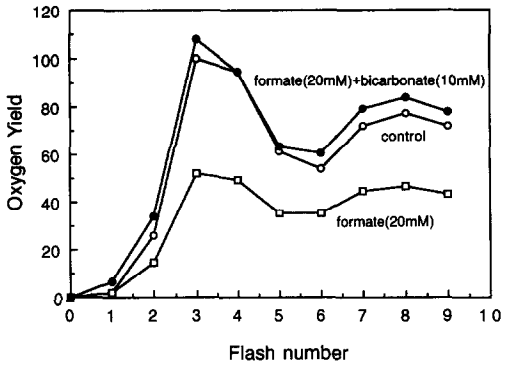

Fig. 1. Oxygen yield per flash as a function of flash number in cells of a *Synechocystis* 6714 mutant (S264A: DCMU II-A) treated with formate and bicarbonate. See section 2 for details of experimental conditions.

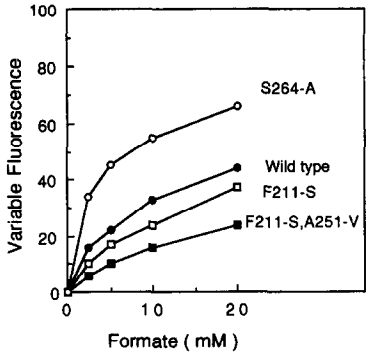

Fig. 2. Chlorophyll *a* fluorescence as a function of formate concentration in cells of *Synechocystis* 6714 (wild-type and mutants S264A (DCMU II-A), F211S (Az I), and F211S, A251V (Az V)). See section 2 for details of experimental conditions.

longer occurs. This effect of formate on F_i also showed the same differential sensitivity on the mutants as in Table I (data not shown).

3.3. *Chlorophyll a fluorescence decay measurements*

In agreement with the conclusions reached on the differential sensitivity of the mutants to formate addition (Fig. 2, Table I), chlorophyll *a* fluorescence decay measurements, that monitor the decay of $[Q_A^-]$, showed that the bicarbonate-reversible formate-induced increase in the slow ($t_{1/2} \geqslant 7$ ms) phase of $[Q_A^-]$ was largest in the S264A mutant followed by wild-type and the double mutant F211S/A251V (Table I). In formate-treated samples, the fluorescence decay could be analyzed as a sum of an unmodified normal decay and a slow fluorescence decay (DCMU-type) due to inhibited (blocked) centers.

Table I

Inhibitor effects on various D-1 mutants of *Synechocystis* 6714

Mutation (Name of the mutant)	F211S/A251V (Az V)	F211 (Az I)	Wild-type	S264A (DCMU II-A)
Resistance to herbicides (arbitrary units [24]				
DCMU	2	1	1	500
Atrazine	100	10	1	70
Metribuzin	400	8	1	>3000
Percent inhibition to 20 mM formate addition				
Σ O_2 in 9 flashes	11 ± 5	23 ± 5	18 ± 5	40 ± 5
Deactivation of the S_2 state, $t_{1/2}$				
− Formate	13 ± 2 s	26 ± 2 s	22 ± 2 s	13 ± 2 s
+ 20 mM formate	13 ± 2 s	26 ± 2 s	22 ± 2 s	13 ± 2 s
Variable chlorophyll *a* fluorescence intensity with 20 mM formate (100 = when all centers are closed)	20 ± 5	35 ± 5	40 ± 5	70 ± 5
Formate-induced increase in the amplitude of the slow components (>7 ms) of fluorescence decay				
%ΔF	17	–	21	43

3.4. *Concluding remarks*

Our data (O_2 yield in a series of flashes and chlorophyll *a* fluorescence decay after a flash) with *Synechocystis* 6714 cells show that in the presence of formate there are two types of centers: inhibited and non-inhibited. Formate acts as an inhibitor with a slow turnover. Bicarbonate is a good competitor to formate and when bicarbonate is added the electron transfer between Q_A and Q_B is restored.

The novel phenomenon, reported in this paper, is the differential sensitivity in the D-1 mutants of *Synechocystis* 6714 of the HCO_3^--reversible formate effect on PS II reactions. Since the double mutant (A251V/F211S) is highly resistant to formate as compared to the single mutant F211S, A251 seems to play a significant role in formate binding to the D-1 protein. The binding niches of DCMU and atrazine are somewhat different since some of the mutants that are resistant to atrazine are still sensitive to DCMU. Since S264A mutant is most sensitive to formate, either the change causes a stronger affinity to formate or a weaker affinity to bicarbonate. Khanna et al. [15] had also observed that it was easier to obtain bicarbonate depletion in the atrazine resistant mutant of *Amaranthus hybridus* that is also mutated at serine-264 to glycine (S264G). Our results in this paper with *Synechocystis* 6714 mutants clearly establish the involvement of the D-1 protein in the HCO_3^--reversible formate effect and provide a clue as to the possible

overlapping niches of formate binding with other herbicides.

Acknowledgement: Govindjee thanks the University of Illinois Center for Advanced Studies for Associate Membership during the Fall of 1989.

REFERENCES

[1] Warburg, O. and Krippahl, G. (1960) Z. Naturforsch. 15b, 367–369.
[2] Warburg, O. (1964) Annu. Rev. Biochem. 33, 1–18.
[3] Wydrzynski, T. and Govindjee (1975) Biochim. Biophys. Acta 387, 403–408.
[4] Govindjee, Pulles, M.P.J., Govindjee, R., Van Gorkom, H.J. and Duysens, L.N.M. (1976) Biochim. Biophys. Acta 449, 602–605.
[5] Jursinic, P., Warden, J. and Govindjee (1976) Biochim. Biophys. Acta 440, 322–330.
[6] Siggel, U., Khanna, R., Renger, G. and Govindjee (1977) Biochim. Biophys. Acta 462, 196–207.
[7] Eaton-Rye, J.J. and Govindjee (1988) Biochim. Biophys. Acta 935, 248–257.
[8] Khanna, R., Wydrzynski, T. and Govindjee (1977) Biochim. Biophys. Acta 462, 208–214.
[9] Eaton-Rye, J.J., Blubaugh, D.J. and Govindjee (1986) in: Ion Interaction in Energy Transfer Membranes (Papageorgiou, G.C., Barber, J. and Papa, S., eds) pp. 263–278, Plenum, New York.
[10] Diner, B.A. and Petrouleas, V. (1990) Biochim. Biophys. Acta 1015, 141–149.
[11] Stemler, A. (1989) Plant Physiol. 91, 287–290.
[12] Blubaugh, D. and Govindjee (1986) Biochim. Biophys. Acta 848, 147–151.
[13] Sarojini, G. and Govindjee (1981) Biochim. Biophys. Acta 634, 340–343.
[14] Stemler, A. (1980) Plant Physiol. 65, 1160–1165.
[15] Khanna, R., Pfister, K., Keresztes, A., Van Rensen, J.J.S. and Govindjee (1981) Biochim. Biophys. Acta 634, 105–116.
[16] Vermaas, W.F.J., Van Rensen, J.J.S. and Govindjee (1982) Biochim. Biophys. Acta 681, 242–247.
[17] Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
[18] Blubaugh, D. and Govindjee (1988) Photosynth. Res. 19, 85–128.
[19] Van Rensen, J.J.S., Tonk, W.J.M. and DeBruijn, S.M. (1988) FEBS Lett. 226, 347–351.
[20] El-Shintinawy, F., Xu, C. and Govindjee (1990) J. Plant Physiol. (in press).
[21] El-Shintinawy, F. and Govindjee (1990) Photosynth. Res. 24, 189–200.
[22] Shopes, R.J., Blubaugh, D.J., Wraight, C.A. and Govindjee (1989) Biochim. Biophys. Acta 974, 114–118.
[23] Cao, J. and Govindjee (1988) Photosynth. Res. 19, 277–285.
[24] Ajlani, G., Kirilovsky, D., Picaud, M. and Astier, C. (1989) Plant Mol. Biol. 13, 469–480.
[25] Etienne, A.-L., Ducruet, J.-M., Ajlani, G.K. and Vernotte, C. (1990) Biochim. Biophys. Acta 1015, 435–440.
[26] Joset-Espardellier, F., Astier, C., Evans, E.H. and Carr, N.G. (1978) FEMS Microbiol. Lett. 4, 261–264.
[27] Lemasson, C. and Etienne, A.-L. (1975) Biochim. Biophys. Acta 408, 135–142.
[28] Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
[29] Bouges-Bocquet, B., Bennoun, P. and Taboury, J. (1973) Biochim. Biophys. Acta 325, 247–257.
[30] Xu, C., Rogers, S.M.D., Goldstein, C., Widholm, J.M. and Govindjee (1989) Photosynth. Res. 21, 93–106.
[31] Kirilovsky, D., Ajlani, G., Picaud, M. and Etienne, A.-L. (1989) Plant Mol. Biol. 13, 355–363.