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

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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 1) > 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 CO2 from a suspension of a green alga Chlorella, that was performing Hill reaction with benzoinone as electron acceptor, led to a CO2-reversible decrease in O2 evolution. On this basis, Warburg [2] argued that the source of O2 was CO2, not H2O. Such a role of CO2 has not been confirmed, but instead, it has been established that a major effect of CO2 lies between the primary plastoquinone electron acceptor, QA, of photosystem II (PS II) and the plastoquinone, PQ, pool [3–7]. This phenomenon is most easily studied by removing CO2 by the addition of formate [8], nitrite [9] or nitric oxide [10]. Although it has been difficult to observe CO2-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 1 CO2/reaction center II (Govindjee, H.G. Weger, D.J. Turpin, J.J.S. van Rensen, O.J. deVos and J.F.H. Snel, unpublished, personal communication, 1989).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 CO2 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 HCO3–, not CO2 [12]. CO2, however, is the diffusing species [13], and we cannot yet rule out that it may also be an active species [14]. Thus, CO2 or HCO3– is interchangeably used in this paper. An interaction of HCO3– with the herbicide-binding protein, D-1, is suggested from studies on the effects of several herbicides on HCO3–-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 HCO3–: one on the Fe between QA and QB (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 HCO3–: 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 QB and, perhaps, QA[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 QA.

Cyanobacteria are excellent model systems to study the role of HCO3– 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 HCO3– effect [23]; and (iii) they have PS II that is similar in all major respects

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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 QA and the PQ pool [25], allowed us to test the hypothesis if, indeed, the CO₂-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 CO₂-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 ≤10 µg/ml of the suspension.

2.2. Oxygen measurements

The amount of O₂ 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 CaCl₂ (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 S₂ was obtained by measuring O₂ 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/m². The signal was recorded using a multichannel analyzer (Interscan).

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, O₂ yield per flash in a sequence of flashes was measured with and without addition of sub-saturating amounts of formate (20 mM), and then after addition of bicarbonate (10 mM). The relative yield of O₂ was calculated as sum of the yields of the 9 flashes. In all cases, formate induced inhibition of O₂ 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 O₂ 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 S₂ deactivation was independent of the presence of formate and bicarbonate (Table I). These results suggest that formate-inhibited centers stay inhibited for times >> the dark time between flashes and thus, produce no acceleration of the S₂ 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 ‘F₀’ level. 100 on the ordinate indicates the level of maximum fluorescence obtained with 10 µM DCMU or 200 µM loxynil (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 O₂ sequence data (Table I).

The CO₂-depletion procedure, as used earlier [7,23] (anaerobiosis under nitrogen bubbling for 1 h), led to an increase in the initial fluorescence (Fᵢ) immediately upon turning on the continuous light: this increase is due to the formation of QA in darkness. This increase was eliminated by the addition of 200 µM benzoquinone after the depletion procedure. This suggests that the electrons arrive at QA from a back-flow of electrons from Q₅ (intact cells in anaerobiosis have a high ratio of Q₅/Q₆ in darkness (cf. e.g. [30]) and benzoquinone abolishes the effect as it reoxidizes Q₅ in darkness. Thus, the formate effect may be similar to that of DCMU: formate may bind when the electron, shared between QA and QB, is on QA. Then, when formate is bound, electron transfer between QA and QB no
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 [QA], showed that the bicarbonate-reversible formate-induced increase in the slow (t1/2 > 7 ms) phase of [QA] 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.

3.4. Concluding remarks

Our data (O2 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 QA and QB is restored.

The novel phenomenon, reported in this paper, is the differential sensitivity in the D-1 mutants of Synechocystis 6714 of the HCOT-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 HCO3-reversible formate effect and provide a clue as to the possible
overlapping niches of formate binding with other herbicides.

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REFERENCES