

A UNIQUE ROLE OF CARBON DIOXIDE IN PHOTOSYSTEM II

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1. ABSTRACT

An evaluation of the effect of predicted global increases in CO₂ on photosynthesis should include not only an examination of the effects on CO₂ fixation, but also on the so-called bicarbonate effect in photosystem II. This bicarbonate effect involves a unique stimulatory role of CO₂ (or HCO₃⁻) in the conversion of plastoquinone to plastoquinol at the reaction center of photosystem II. After providing an introduction to the reaction centers of photosynthetic bacteria and photosystem II and to the bicarbonate effect, we will review here recent results that establish the following: (1) the bicarbonate effect is absent from the reaction centers of the green photosynthetic bacterium *Chloroflexus aurantiacus* and mutants of purple photosynthetic bacterium *Rhodobacter sphaeroides*; (2) inhibitors, such as formate, indeed release, and, thus, deplete CO₂ from thylakoid membranes; and (3) both the reaction center proteins D1 and D2 are involved in the bicarbonate effect since certain D1 and D2 mutants, altered in single amino acids, show differential sensitivity to bicarbonate-reversible formate effects. We will then discuss a current hypothesis, based on an iron-(bi)carbonate containing protein; it envisions CO₂/HCO₃⁻ binding on the Fe and certain specific amino acids, including arginines, in the PSII reaction center proteins D1 and D2. This binding is suggested to provide stability to the reaction center and stimulate electron flow and protonation required for plastoquinol formation. Finally, a possible relation of low affinity HCO₃⁻ binding sites to the phenomenon of photoinhibition will also be discussed.

2. INTRODUCTION

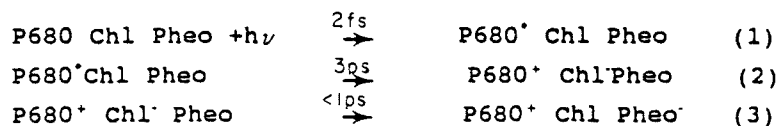
All life on earth has been divided into three domains: archaea; bacteria; and eucarya [1]. True photosynthesis involving redox components occurs only in the latter two. Photosynthetic bacteria, such as the well-known purple bacteria *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*, the green bacteria *Chlorobium thiosulfatophilum* and *Chloroflexus aurantiacus*, and the bacteriochlorophyll-g-containing

bacterium *Helibacterium chlorum*, are all anoxygenic, i.e., they do not evolve O₂. On the other hand, all cyanobacteria (example, *Synechocystis* sp.), algae (example, *Chlamydomonas reinhardtii*) and other photosynthesizing plants are all oxygenic, i.e., they evolve O₂.

Cyanobacteria and plants contain two types of reaction centers (I and II), that operate in series, to transfer electrons from H₂O to NADP⁺ [2-4]. Purple photosynthetic bacteria and *Chloroflexus* type green bacteria contain reaction centers that are similar to reaction center II [5] although a major difference is that they do not oxidize H₂O and do not contain Mn (cf. [6]). A cyclic reaction produces high-energy intermediate that is used to reduce NAD⁺ by reversed electron flow using external hydrogen donors. On the other hand, *Chlorobium* type green bacteria and *Helibacterium chlorum* contain reaction centers that appear to be similar to reaction center I: NAD is reduced by externally added hydrogen donors. A possible scenario for the evolution of photosynthetic reaction centers has been discussed by Olson and Pierson [7].

2A. Reaction Centers

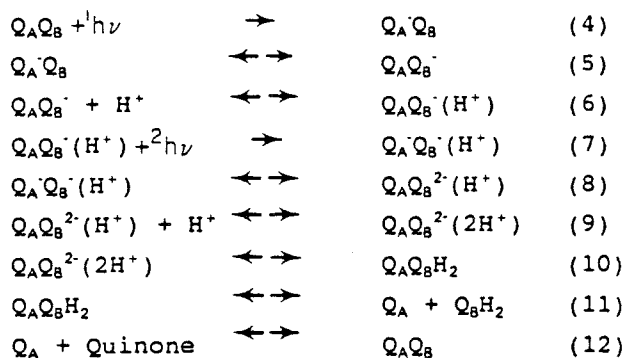
A major breakthrough in photosynthesis research has been the crystallization and X-ray structure of reaction centers of photosynthetic bacteria *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides* [8-10]. The reaction center proteins L and M, that are similar in many respects to the reaction center proteins D1 and D2 of photosystem II of plants and cyanobacteria, harbor 4 bacteriochlorophyll, a or b, 2 bacteriopheophytin a or b, two bound ubi- or mena-quinones Q_A and Q_B and an iron atom. These reaction centers transfer electrons from their primary electron donor special pair bacteriochlorophyll P870 (*R. sphaeroides*) or P960 (*R. viridis*) to their, respective, electron acceptor bacteriopheophytin a or b within about 3 ps [11]. Recently, Holzappel et al. [12] have established that a bacteriochlorophyll monomer is an intermediate between the primary electron donor and the bacteriopheophytin. However, the other bacteriochlorophyll monomer and the other bacteriopheophytin molecule, situated on the "M" side of the reaction center, do not participate in this photochemistry. The photosystem II reaction center has been shown to contain 6 chlorophyll a molecules and 2 bacteriopheophytin molecules [13]; the Q_A and Q_B are bound plastoquinone molecules. Wasielewski et al. [14] have shown that, like in photosynthetic bacteria, the charge separation, i.e., creation of oxidized reaction center chlorophyll a molecule, P680⁺, and the reduced pheophytin molecule, Pheo⁻, occurs in 3 ps. Furthermore, this charge separation, like that in photosynthetic bacteria [15], becomes faster (1.4 ps) at low temperatures (such as 15K) as compared to that at the ambient temperature [16]. As in photosynthetic bacteria, the primary electron donor, P680, also appears to be a dimer [17]. Whether chlorophyll a monomer is an intermediate between P680 and pheophytin is not yet known. By analogy to photosynthetic bacteria, we speculate that photosystem II primary photochemistry may occur as follows:



The primary reactants return to their original state after Pheo[•] transfers its electron to the primary quinone Q_A within 200 ps [18,19], and P680⁺ transfers its positive charge (hole) to the electron donor Z within 20-200 ns [20,21] depending upon the flash number that determines the state(s) of the charge accumulator (M) on the water oxidation side. Recently, it has been shown that Z is a tyrosine residue (Tyrosine-161) in the D1 protein [22]. (Since chlorophyll a fluorescence yield is extensively used to monitor photosystem II chemistry, we remind the readers that, in addition to the well-known quencher Q_A [23], P680⁺ and Pheo[•] also act as quenchers of antenna chlorophyll a fluorescence.)

The major difference between photosynthetic bacteria and photosystem II lies in the inability of the former to oxidize water to O₂. P680/P680⁺ has a very high (E_{m,7} = +1.1V) redox potential [24] and is, thus, capable of water oxidation (E_{m,7} for H₂O/O₂ = + 0.9V) whereas P870/P870⁺ has too low a redox potential (E_{m,7} = +0.4V). In addition, the amino acid sequences of D1 and D2 on the lumen side of photosystem II are such that they can easily bind Mn atoms [6,25,26] needed for O₂ evolution, whereas L and M bind, instead, cytochrome of the c type [10].

In addition to the similarity in the arrangement and the chemistry of the primary reactants of photochemistry, discussed earlier, the reduction of the secondary quinone (Q_B) to quinol (Q_BH₂) also appears quite similar [27,28]. A possible common scheme is:



The above scheme includes the concept of (a) the "two electron gate" (i.e., Q_B has to be doubly reduced before the quinol, Q_BH₂, will be released from the reaction center) [29,30]; and (b) protonation involves initial binding of H⁺ to an amino acid, rather than directly to Q_B[•] [31]. The involvement of aspartic acid-213 on the L-subunit of *R. sphaeroides* in protonation steps has been elegantly shown by site-directed mutagenesis studies of Takahashi and Wraight [32]. In spite of the suggested similarity of photosystem II and photosynthetic bacteria in reducing quinone to quinol, a remarkable difference has been observed. Neither the reaction centers of the purple photosynthetic bacteria [33,34,35], nor that of the green bacterium *Chloroflexus aurantiacus* [36] show inhibition of electron flow by formate [33-35] or nitric oxide

[35,36]. In contrast, both formate and nitric oxide cause remarkable bicarbonate-reversible inhibitory effects in plants [37,38] and cyanobacteria [39,40]. We refer the readers to reviews of Govindjee and Wasielewski [41] and of Hansson and Wydrzynski [42] for further details on photosystem II.

2B. The Bicarbonate Effect

Warburg and Krippahl [43] discovered that removal of CO₂ from photosynthetic samples inhibits Hill reaction. Readdition of CO₂/HCO₃⁻ restores the Hill reaction. This is called the bicarbonate effect. Warburg [44] argued that CO₂ must be the source of O₂ in photosynthesis contrary to the generally accepted idea that H₂O is the source of O₂ (see review [37]). Heavy oxygen-18 studies of Guy et al. [45] (J. Berry, personal communication, 1990) clearly show that H₂O is the source of O₂. Although a bicarbonate effect can be observed by removing CO₂ without the use of molecules that replace/displace CO₂ [46-49], a large reproducible effect is observed when formate [50,51] or nitric oxide [35,52] is used to replace/displace CO₂ or HCO₃⁻. Addition of formate to thylakoids at pH 5.5 indeed releases CO₂ [53] although Stemler [54] did not observe any CO₂ release upon formate addition to osmotically shocked maize thylakoids at pH 6.0.

The bicarbonate effect has been monitored, over the years, by measuring saturating rates of electron flow (O₂ evolution or reduction of electron acceptors); O₂ evolution, proton release or thermoluminescence per flash in a sequence of bright, brief light flashes; chlorophyll a fluorescence transients; decays of chlorophyll a fluorescence yield, delayed light emission, and absorbance changes due to the formation of Q_A⁻ or Q_B⁻ after light flashes; Mossbauer and ESR signals due to the non-haem iron; changes in photosystem II herbicide binding characteristics, etc. The phenomenon of the bicarbonate effect has been reviewed at regular intervals since 1978 [37,38,55-60].

No bicarbonate effect is observed in photosystem I [51,61]. No significant effect has been observed in O₂ evolution kinetics per se after a flash [58,62], electron flow between the intermediate "M" and "Z" [63] and between "Z" and P680 [63,64] on the electron donor side of PSII. A major site of the bicarbonate effect has been located between Q_A and the plastoquinone pool through measurements on partial electron flow segments of the Hill reaction [51,61,65,66] and through kinetic measurements of Q_A decay in repetitive [63,67] and after single flashes of light [35,68-73]. A much larger slowing down of electron flow occurs after second (or third) and subsequent flashes than after the first flash upon depletion of CO₂; this effect is fully restored upon the readdition of bicarbonate. A thorough depletion of CO₂ can easily lead to a 10-20 - fold inhibition of electron flow that can be fully restored to control values upon bicarbonate addition. In control membranes, the bottleneck reaction is the (transport and) reoxidation of plastoquinol: approximately 4 ms/e⁻ at room temperature. In CO₂-depleted thylakoids,

the decay of $[Q_A]$ after the 3rd and subsequent flashes is of the order of 150 ms [68]; a similar value was observed when the rereduction kinetics of $P700^+$ was measured [67]. In the first approximation, these slowed reactions, interpreted to be either in the production of quinol (Q_BH_2) and/or its release, seem sufficient to explain the inhibition of the Hill reaction. However, quantitation and precise explanation requires parallel measurements on the decay of various intermediates and the overall electron flow in a sequence of flashes with appropriate dark times between flashes in identically treated samples.

The location of the major effect between Q_A and the plastoquinone pool is supported by the following observations:

(1) Photosystem II herbicides, that are known to inhibit electron flow by displacing Q_B [74,75], affect the binding of herbicides [65,76] and vice versa [66,77].

(2) Nitric oxide, that binds to non-haem Fe[52] between Q_A and Q_B , shows bicarbonate-reversible effect in electron flow from Q_A to the plastoquinone pool [35]. Disulfiram, an iron chelator, is shown to cause effects similar to that of CO_2 -depletion [78] although this effect cannot be reversed by bicarbonate addition. Furthermore formate, used to deplete CO_2 , was shown to affect Q_A Fe ESR signal [40,79] and the Fe signal, as measured by Mossbauer spectroscopy [80,81].

(3) Herbicide-resistant D1 mutants, altered in single amino acids in the Q_B -binding region [82], show differential sensitivity to bicarbonate-reversible formate effects, both in *Synechocystis sp.* PCC 6803 [83] and in *Chlamydomonas reinhardtii* [84]. Furthermore, D2 mutants, that had been constructed in which arginine 233 was changed to glutamine (R233Q) or arginine 251 was changed to serine (R251S), were found to be ten times more sensitive to formate than the wild type *Synechocystis sp.* PCC 6803 [85].

Consistent with the effect on D1 and D2, but on a reaction prior to Q_A , Mende and Wiessner [87] have observed a parallel decrease in variable chlorophyll *a* fluorescence and O_2 evolution when CO_2 was removed from *Chlamydomonas reinhardtii* cells. This phenomenon was confirmed in our laboratory in spinach leaf discs [49] and in *Chlamydomonas reinhardtii* cells [88]. We have however established that the same phenomenon persists in mildly heated (that lose water oxidation activity) and hydroxylamine treated samples. Thus, this effect appears to be located somewhere between Z and Q_A . C. Xu (personal communication, 1990) has observed that in spinach thylakoids, at pH 6.0, an effect prior to Q_A is observed at short times after mixing of thylakoids with formate; at later mixing times the effect beyond Q_A predominates. It is necessary to test if this effect is between Z and P680, and/or between pheophytin and Q_A .

We shall now summarize our recent results on the absence of formate and NO effects on the reaction centers of the green bacterium *Chloroflexus aurantiacus* [36]; CO_2 release from thylakoid membranes upon formate addition [53]; differential sensitivity of formate in D1 mutants

of *Chlamydomonas reinhardtii* [84], and D1 [83] and D2 [85] mutants of *Synechocystis* PCC 6803. Finally, we will discuss a current hypothesis for the function of bicarbonate in Photosystem II.

3. ABSENCE OF FORMATE AND NITRIC OXIDE EFFECTS IN THE REACTION CENTERS FROM THE GREEN BACTERIUM *CHLOROFLEXUS AURANTIACUS* (COLLABORATION WITH J. TROST AND R. BLANKENSHIP) [36]

In spite of the known similarity of the D1 and D2 proteins of photosystem II and the L and M subunits of the purple photosynthetic bacteria [5], the latter do not show the (bicarbonate-reversible) formate effect (see e.g. [34]), as mentioned earlier. Nitric oxide, which binds to Fe [52] between Q_A and Q_B , produces effects similar to formate in photosystem II, but not in purple photosynthetic bacteria [35]. These differences must be due to differences in the amino acid sequence and subsequently the architecture of the D1/D2 proteins versus L/M subunits. The photosynthetic reaction center from the green bacterium *Chloroflexus aurantiacus* has several differences with the purple photosynthetic bacteria: one of the monomer bacteriochlorophyll molecule is replaced by bacteriopheophytin; Fe between Q_A and Q_B is replaced by Mn (see e.g. [89]); and there are several significant differences in their amino acid sequences [90,91]. *Chloroflexus* reaction centers also appear to lack an H-subunit [89,92]. Thus, we used formate and NO to test if these differences are of any importance in producing the "bicarbonate effect". We concluded, from our observations presented below, that *Chloroflexus aurantiacus* reaction centers do not possess formate and NO binding sites that are responsible for the bicarbonate effect.

Using reaction centers containing Q_B , prepared as described elsewhere [89], we monitored [93,94] the decay of the oxidized reaction center ($P865^+$), either after the first flash or after the fifth flash (dark time between flashes, 3s), by back reactions with Q_A^- ($t_{1/2} = 60$ ms) and with Q_B^- ($t_{1/2} = 0.5 - 3$ s; 2 components). If formate and NO were to inhibit electron flow between Q_A and $Q_B^{(1)}$, as observed in plants [35,71,72] and cyanobacteria [35, 39], the amplitude of the fast component would increase and that of the slow component(s) would decrease. This is similar to what occurs [89] when the inhibitor O-phenanthroline is added; this inhibitor blocks electron flow between Q_A and Q_B by displacing Q_B . In contrast to PSII, but as observed in other photosynthetic bacteria [34], our results (see abstract [36]) showed no significant change between the control reaction centers and those treated with 100 mM formate. In addition, no difference was observed between the 300 μ M NO treated (anaerobic) reaction centers and those treated with both NO and 10 mM bicarbonate. At pH 6.5, the lifetimes and (amplitudes) were 60 ms (50 + 5%) and 0.5 - 2 s (2 components; sum = 50 + 5%) for $P^+Q_A^-$ and $P^+Q_B^-$ recombinations, respectively. It thus appears that the differences in the amino acid sequence [90,91] in the L and M subunits of *Chloroflexus aurantiacus* from those of purple photosynthetic bacteria

are not a sufficient condition to bring about the binding of formate or NO.

4. ABSENCE OF FORMATE EFFECTS IN GLU^{M234} MUTANTS OF *RHODOBACTER SPHAEROIDES* (COLLABORATION WITH X. WANG, J. CAO, P. MAROTI, H.V. STILZ, D. OESTERHELT AND C. WRAIGHT) [95]

Michel and Deisenhofer [5] suggested a possible reason for differences in the "bicarbonate effect" in photosystem II and photosynthetic bacteria: the iron atom in photosystem II lacks the fifth and sixth ligands provided by glutamate (M234) in reaction centers of *Rhodobacter sphaeroides*. Wang et al. [95] have, by site-directed mutagenesis, altered GLU^{M234} with valine (M234EV), glutamine (M234EQ), and glycine (M234EG). These mutants grew well under photoautotrophic conditions and showed no detectable effects of bicarbonate depletion (formate addition) on: (1) cytochrome b_{681} or cytochrome C_2 reduction; (2) the recombination of $P^+Q_A^-$ or $P^+Q_B^-$; (3) kinetics of electron transfer from Q_A^- to Q_B^- ; and (4) flash dependent oscillation of semiquinone formation in the presence of exogenous donor to P^+ (Q_B^- turnover). Furthermore, addition of nitric oxide, instead of formate, also showed no effect on the electron acceptor complex of M234EV mutant (X. Wang and J. Cao, unpublished observations, 1990). The absence of a formate-bicarbonate effect in M234 mutants suggests that this amino acid may not be responsible for the absence of the phenomenon. These results are, however, interesting as they suggest that M234 may not be important for electron flow in the electron acceptor complex of *Rhodobacter sphaeroides*.

In conclusion, the existence of formate and NO effects in photosystem II lies strictly in the unique amino acid sequence of D1 and D2 proteins (see later sections). Now, I present data that show that formate indeed releases CO_2 from thylakoid membranes, and thus, depletes them of CO_2 when bound.

5. FORMATE RELEASES CARBON DIOXIDE/BICARBONATE FROM THYLAKOID MEMBRANES (COLLABORATION WITH H.G. WEGER, D.H. TURPIN, J.J.S. VAN RENSEN, O.J. DE VOS AND J.F.H. SNEL) [53]

There are two hypothesis for the action of formate: (1) Formate is an inhibitor of electron flow: it binds to empty sites, and bicarbonate displaces it and removes the inhibition. (2) Formate displaces the native bound bicarbonate and, thus, causes inhibition by the removal of the ion required for electron flow; readdition of bicarbonate displaces formate and restores electron flow. A third hypothesis may, however, invoke bicarbonate to be necessary for the most efficient electron flow, but in its absence a marginal or less efficient electron flow may proceed. Supportive evidence for hypothesis #2 was discussed earlier [37, 50]. However, absence of CO_2 release by formate addition to maize thylakoids at pH 6.0 [54] supported hypothesis #1. Using a sensitive membrane inlet

mass spectrometer and a differential infra-red gas analyzer, we showed [53] that 100mM formate treatment released about 1 CO₂ per reaction center II at pH 6.5 from spinach and pea thylakoids within minutes of formate treatment; at pH 6.0, CO₂ release was reduced to a value of 0.4 CO₂ per reaction center (Fig. 1). Since pH of the stroma matrix is alkaline, it is reasonable to assume that bicarbonate is bound in native thylakoid membranes and functions to provide efficient electron flow [37]. However, it is possible that some inefficient electron flow may take place in the absence of bicarbonate in thylakoid membranes.

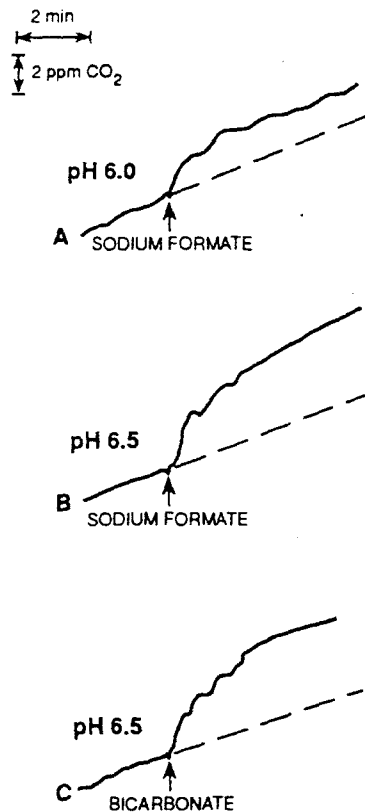


Figure 1: Formate-induced release of CO₂ from pea thylakoids as measured by a differential infra-red gas analyzer. Trace A is an illustration of CO₂ release at pH 6.0 upon addition of 100 mM formate to a thylakoid suspension containing 4 mg Chl/ml, recorded as the difference with the simultaneous addition of the same amount of formate to the second vessel containing the same medium without thylakoids. Trace B: Same as A, but pH 6.5. Trace C: recording of the difference of CO₂ in the gas phase upon addition of 10mM HCO₃⁻ to one of the vessels and water to the other, while both vessels contained 4 ml reaction medium without thylakoids at pH 6.5. At pH 6.5, 100 mM formate injection led to an estimated release of 1.2 HCO₃⁻/CO₂ per PS II reaction center and about 0.4 HCO₃⁻ at pH 6.0 (After ref. [53]).

6. DIFFERENTIAL SENSITIVITY OF FORMATE IN D1 MUTANTS OF CHLAMYDOMONAS

REINHARDTII (COLLABORATION WITH R. STRASSER, B. SCHWARZ AND J-D. ROCHAIX) [84]

The secondary plastoquinone, Q_B , is in a pocket between the helices IV and V of the D1 protein; this pocket is also lined by the amino acids that are found to be altered in the herbicide-resistant mutants [96-98]. If bicarbonate is bound to Fe and/or any of the amino acids in the Q_B region, we expect to see differential sensitivity of the bicarbonate-reversible formate effects in the different mutants. The nature of the differences may ultimately provide clues as to the nature of the bicarbonate binding. Chlorophyll *a* fluorescence transients indeed revealed differential sensitivity to 25 mM formate treatment; these effects were fully reversed by 10 mM bicarbonate [84]. The most sensitive mutant was S264A (DCMU-4) and the most resistant mutant was L275F (Br-202). The order of resistance (highest to lowest) was L275F (Br-202) > A251V (Mz-2) >> wild type = F255Y (Ar-207) = V219I (Dr-18) >> S264A (DCMU-4) (Figure 2). These results clearly show the involvement of D1 protein in bicarbonate-reversible formate effects *in vivo*.

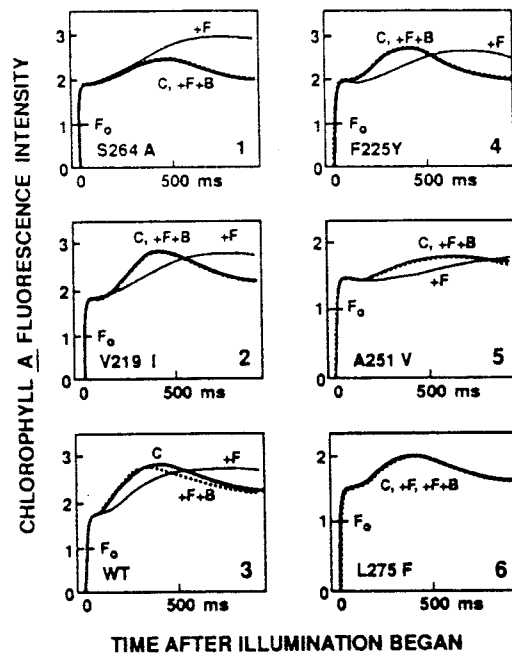


Figure 2: Chlorophyll *a* fluorescence transients of some *Clamydomonas reinhardtii* cells grown photoautotrophically and suspended in tris-phosphate medium at pH 6.5. C: control, +F for 25mM formate treated cells, and +F+B for 25mM formate followed by 10mM bicarbonate treated cells. Dark adaptation was 4 minutes. The ratio of variable fluorescence ($F_{\text{second}} - F_0$) after illumination with and without formate was 2.0 (for S264A [DCMU-4]), 1.48 (V291I [Dr-18]), 1.47 (for F255Y [Ar-207]), 1.4 (WT), 1.10 (A251V [MZ-2]) and 1.00 (L275F[Br-202]). Formate had no effect on PSII^r (Fud-7) cells (data not shown) (After ref. [84]).

7. DIFFERENTIAL SENSITIVITY OF FORMATE IN D1 MUTANTS OF *SYNECHOCYSTIS* PCC 6714 (COLLABORATION WITH A-L. ETIENNE, C. VERNOTTE, B. PETERI AND C. ASTIER) [83]

The concept that bicarbonate binding may involve the region of helices IV and V and the loop connecting the two was supported further by measurements of formate sensitivity in D1 mutants of *Synechocystis* PCC 6714. Measurements, not only on chlorophyll *a* fluorescence transients, but on oxygen yield in a sequence of flashes and chlorophyll *a* fluorescence yield decay after a flash revealed differential sensitivity of herbicide resistant mutant cells to formate treatment [83]. The order of resistance (highest to lowest) was : A251V/F211S (Az-V) > F211S (Az-I) = wild type > S264A (DCMU-IIA) (Figure 3). The extreme sensitivity of S264A mutant was confirmed. Bicarbonate addition restored all formate effects.

An interesting observation is that Az-V mutant, that was most resistant to formate, is the one most sensitive to exposure to excessive light [99]. Excess light, like formate treatment, not only produces photosystem II centers in which electron transfer is inhibited between Q_A and Q_B , but also centers in which Q_A^- formation is also inhibited [99]. We consider it a likely hypothesis that this mutant is resistant to formate because bicarbonate is poorly bound in it, and, the decreased bicarbonate binding makes it more susceptible to inactivation by excess light. Sundby et al. [100] and Sundby [101] have recently shown that bicarbonate protects against photoinhibition.

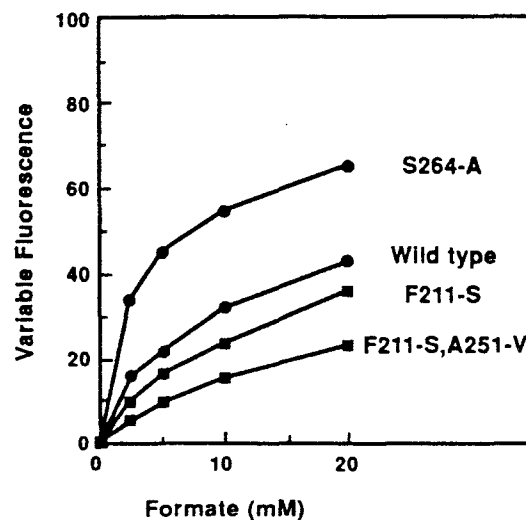


Figure 3. Chlorophyll *a* fluorescence as a function of formate concentration in cells of *Synechocystis* 6714 (wild type and mutants S264A (DCMU-IIA) & F211S (AZ-I) and a double mutant F211S/A251V (AZ-V). Fifteen minutes darkness was given after the addition of formate, but before the measurements began. 100 indicates the value obtained with the

herbicide 10 μ M diuron or 200 μ M ioxynil (for DCMU II-A). (After ref. [83].)

8. DIFFERENTIAL SENSITIVITY OF FORMATE IN D2 MUTANTS OF *SYNECHOCYSTIS* PCC 6803 (COLLABORATION WITH J. CAO AND W. F. J. VERMAAS) [85]

Blubaugh and Govindjee [37] had discussed a role of specific arginine residues in the binding of bicarbonate on the D1 protein. Recently crystal structure of human serum lactoferrin at 2.8 Å resolution has revealed that in this protein (bi)carbonate is bound to Fe^{III} as a bidentate ligand and the oxygens of (bi)carbonate form H-bonds with several amino acids including arginine. Thus, we considered it likely that one or more arginine residues in D2 may form H-bonds with (bi)carbonate. Cao et al. [102] constructed two arginine mutants in the D2 polypeptide of *Synechocystis* PCC 6803: R233Q and R251S. Measurements of steady-state rate of oxygen Hill reaction (H₂O to parabenzoquinone), as a function of increasing formate concentration showed that half maximal inhibition was at 50mM for the wild type, 4mM for R233Q and 3.5mM for R251S [85, 103] (Figure 4). Thus, the arginine mutants were several fold more sensitive to formate than the wild type.

Measurements of chlorophyll *a* fluorescence yield decay kinetics indicated that formate inhibited Q_A⁻ to Q_B reaction (flash 2) more than Q_A⁻ to Q_S reaction in the mutants as well as in the wild type as had been observed earlier in higher plant thylakoids [71, 72]. These results support the interpretation that CO₂ removal may interfere with the protonation at the reaction center II, that may not necessarily occur on D2. However, the halftime of the Q_A⁻ oxidation was increased by a factor of 2, 4 and 6 in the wild type, R251S and R233Q, respectively, confirming that the mutants were more sensitive to formate. Cao et al. [85] have suggested that arginine 233 and arginine 251 in the D2 polypeptide function to stabilize HCO₃⁻ binding in photosystem II of *Synechocystis* PCC 6803.

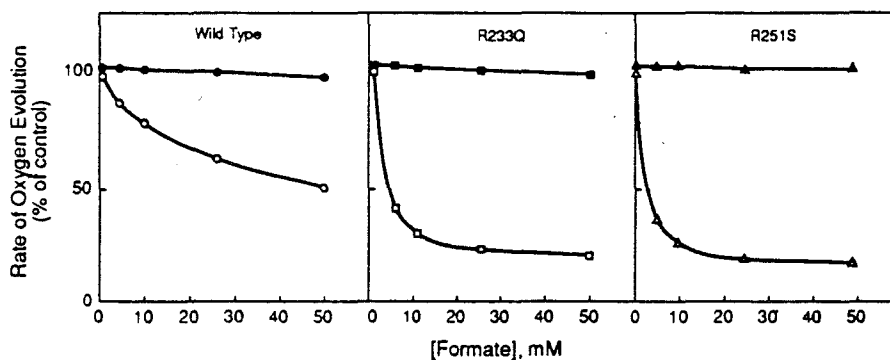


Figure 4. Steady-state oxygen evolution rate of *Synechocystis* 6803 cells of the wild type, and R233Q and R251S mutants. A combination of DMQ

(0.5 mM) and $K_3Fe(CN)_6$ (1 mM) was used as electron acceptor; measurements at pH 6.8. The samples were preincubated for one hour in the treatment medium (at pH 5.8) to which the indicated amount of sodium formate was added (open symbols). To restore the Hill reaction, 10 mM bicarbonate was added (solid symbols). (After [85]).

9. HYPOTHESIS

A working hypothesis for unique role(s) of HCO_3^-/CO_2 in photosystem II of plants and cyanobacteria is now emerging. It appears that, at physiological pH, HCO_3^-/CO_2 may H-bond to several amino acids on both D1 and D2, the reaction center proteins of photosystem II, and may form a ligand to iron that lies between Q_A (bound to D2) and Q_B (bound to D1). For a possible model, see binding of (bi)carbonate to Fe^{III} and to 4 amino acids including arginine in human serum lactoferrin [86] (Fig.5). HCO_3^-/CO_2 plays discrete and unique role(s) in photosystem II: its function may involve stabilization, by conformational means, of the reaction center protein that allows efficient electron flow and protonation of certain amino acids near Q_B [37]. Data on differential sensitivity of formate on the D1 mutants of *Chlamydomonas reinhardtii* (see section 6) and of *Synechocystis* 6714 (see section 7) clearly support binding domains of bicarbonate between helices IV and V and in the connecting loop of D1. Data on the extremely high sensitivity of R233Q and R251S mutants to formate suggest that arginines may stabilize bicarbonate on the reaction center II. Absence of the "bicarbonate effect" in purple photosynthetic bacteria [34, 95] (see section 4) and in the green bacteria (see section 3) shows that we should concentrate on the unique differences between the D1 and D2 and the L and M proteins to unravel the binding niche of bicarbonate and, thus, its function.

Unpublished results of C. Xu (personal communication, 1990) suggest that the inhibitory species in formate solutions added to remove CO_2 may be the formic acid, not formate ions. If this is confirmed, we can begin to look for domains that would bind formic acid, not formate ions. Diner et al. [38] suggest the importance of Lysine-264 on D2 in the bicarbonate effect. It appears that we have to explore several possibilities before a final picture will emerge.

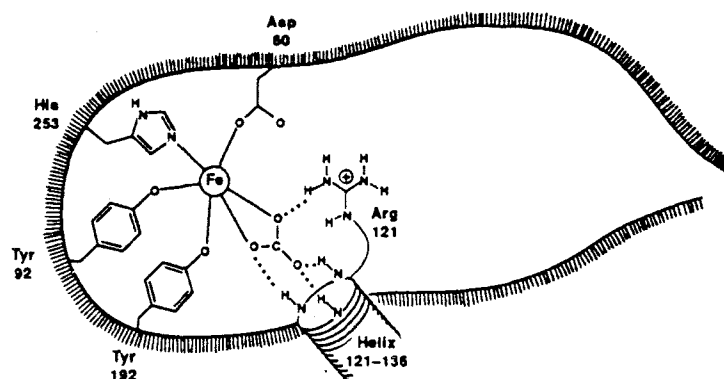


Figure 5. Schematic representation of the iron and anion-binding sites in lactoferrin. Numbering is for the N-lobe, but the same arrangement is found in the C-lobe. (After Anderson et al. [86].)

10. CONCLUDING REMARKS

It seems that binding of bicarbonate to both D1 and D2 affects the stability of the reaction center II and, thus, electron flow and protonation of the electron acceptor in a somewhat complicated manner, the nature of which still needs to be discovered. However, I suspect that specific roles of $\text{CO}_2/\text{HCO}_3^-$ will emerge that may even include protection against photoinhibition. Further site-directed mutagenesis of the electron acceptor side of both D1 and D2 proteins is expected to unravel the molecular bases of the "bicarbonate effect".

No evidence has been found to support Warburg's concept that the CO_2 effect is related to O_2 evolution per se. We have, however, been able to explore, over the years, the original conclusion of Wydrzynski and Govindjee [104] that the CO_2 effect is on the electron acceptor side of photosystem II. Although tremendous amount of research is still needed, we feel that the end of the tunnel is in sight.

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