

PHOTOSYSTEM II: FROM A FEMTOSECOND TO A MILLISECOND

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ABSTRACT

We present here a minireview on the time sequence of events in water-plastoquinone oxido-reductase (*i.e.*, photosystem II, PSII) from a femtosecond to a millisecond. The minimal components of PSII include: three intrinsic polypeptides D-1 and D-2, and cytochrome (Cyt) *b*-559, and an extrinsic polypeptide of 33 kilodalton (kDa) mass (labeled as Y-33). The first act of photosynthesis, light absorption, occurs in chlorophyll (Chl) *a* molecules in about 1 femosecond (fs). Then, the following reactions take place. (1) The primary charge separation, *i.e.*, the formation of the oxidized reaction center Chl *a*, $P680^+$, and the reduced pheophytin, $Pheo^-$, has been shown to occur in reaction center preparation (Nanba and Satoh, 1987) with a lifetime of 3 ± 0.6 picoseconds (ps) (Wasielewski *et al.*, 1989), using 500 fs laser flashes. (2) Electron flow from $Pheo^-$ to the first plastoquinone electron acceptor, Q_A , has been estimated to occur in several hundreds of ps. (3) The re-reduction of $P680^+$ by the electron donor Z [suggested recently to be a tyrosine molecule (Debus *et al.*, 1988; Vermaas *et al.*, 1988, in press)] occurs in 25 to 300 nanoseconds (ns) (Brettel *et al.*, 1984). (4) The electron flow from the reduced Q_A , Q_A^- , to the second plastoquinone electron acceptor, Q_B , occurs in several hundredths of a microsecond (μ s). Approximately, in the same time range, Z^+ is restored to Z by electron flow from the water oxidation complex (WOC) producing the stable charge-separated state $S_2Q_B^-$, where S_2 refers to the redox state of the WOC -- the dark-adapted state being S_1Q_B . Thus, after twelve orders of magnitude of time, a stable state is created. We shall not discuss here the steps of oxygen evolution, and the details of the production of plastoquinol. However, we shall review the possible role of bicarbonate (HCO_3^-) in the mechanism of plastoquinone reduction in plants and cyanobacteria (Blubaugh and Govindjee, 1988, in press). It is suggested

that HCO_3^- functions to provide (a) a proper conformation to the reaction center by binding to the iron in the $\text{Q}_A\text{-Fe-Q}_B$ complex, and (b) protons for plastoquinol formation by binding to an arginine/histidine pair on the D-1 protein of PSII.

INTRODUCTION

The function of Photosystem II (PSII) is to act as water-plastoquinone oxido-reductase (WPOR). Water is oxidized to O_2 , and plastoquinone (PQ) is reduced to plastoquinol (PQH_2): $2\text{H}_2\text{O} + 2\text{PQ} + 4\text{H}^+$ (outside) + $4h\nu \rightarrow \text{O}_2 + 2\text{PQH}_2 + 4\text{H}^+$ (inside). This uphill transfer of electrons requires 4 light quanta ($h\nu$), which are used via the excitation of the reaction center (RC) chlorophyll (Chl) *a* of PSII, P680; the latter leads to the primary charge separation: $\text{P680} \cdot \text{Pheo} + h\nu \rightarrow \text{P680}^* \cdot \text{Pheo} \rightarrow \text{P680}^+ \cdot \text{Pheo}^-$, where Pheo stands for pheophytin. This minireview is concerned solely with the light reaction and the electron transport in PSII. For earlier reviews, see Govindjee (1980, 1982, 1984), Velthuys (1980, 1987), van Gorkom (1985), Diner (1986), Mathis (1986), Ort (1986), and Andréasson and Vänngård (1988). The electron acceptor side of PSII has been discussed by Vermaas and Govindjee (1981), Crofts and Wright (1983), and Govindjee and Eaton-Rye (1986), and the electron donor side by Ghanotakis and Yocum (1985), Govindjee *et al.* (1985), Renger and Govindjee (1985), Andersson and Åkerlund (1987), Babcock (1987), and Renger (1987).

Figure 1 shows a self-explanatory generalized model of the thylakoid membrane with the four major complexes: PSII, cytochrome b_6/f complex, PSI and the ATP synthase, whereas Fig. 2 shows a detailed current picture of the electron flow in PSII, along with the measured or estimated times of reactions. Electron carrier redox pairs are placed vertically according to their approximate known or estimated redox midpoint potential (E_m ,7). The main path of electron flow is as follows (alternate names of the intermediates are in the figure): $\text{H}_2\text{O}/\text{O}_2$ (E_m ,7, +0.82V) \rightarrow $\text{Mn}^{3+}/\text{Mn}^{4+}$ \rightarrow Z/Z^+ (+1.0V; tyrosine/tyrosine⁺) \rightarrow $\text{P680}/\text{P680}^+$ (+1.15V) $\xrightarrow{h\nu}$ $\text{P680}/\text{P680}^*$ (-0.7V) \rightarrow $\text{Pheo}/\text{Pheo}^-$ (-0.6V) \rightarrow Q_A/Q_A^- (0 to -30mV) \rightarrow Q_B/Q_B^- \rightarrow PQ/PQH_2 (+90mV). Oxygen and protons are released to the inside, and protons are picked up from the outside for the production of PQH_2 . Here, the symbol "M" under $\text{Mn}^{3+}/\text{Mn}^{4+}$ represents more than the Mn atoms: it is the charge-accumulating entity necessary for water oxidation and it may, therefore, include the necessary (Y-33, the extrinsic polypeptide with 33 kDa molecular mass) and stimulatory (regulatory) polypeptides (Y-24 and Y-18), chloride (Cl^-), and entities (proteins?) producing absorbance changes at 320nm (Y-320), etc. The redox state of "M" is referred to as the S-states, where S_0 , S_1 , S_2 , S_3 , and S_4 represent the five different states

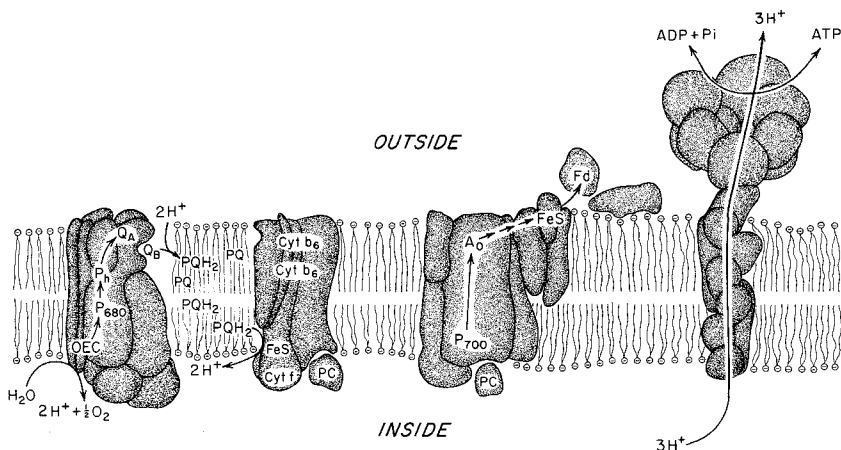


Figure 1. A schematic diagram of the four major protein complexes. From left to right: Photosystem II [OEC = oxygen evolving complex, P680 = reaction center chlorophyll a , Ph = functional pheophytin, Q_A = first plastoquinone electron acceptor (on D-2), Q_B = second plastoquinone electron acceptor (on D-1), PQ (PQH_2) = mobile plastoquinone (plastoquinol)]; Cytochrome b_6/f complex (cyt b_6 = cytochrome b_6 , Cyt f = cytochrome- f , FeS = Rieske Fe-S center, Pc = plastocyanin); Photosystem I (P700 = reaction center chlorophyll a , A_0 = chlorophyll a , first electron acceptor, FeS = iron sulfur centers, Fd = ferredoxin); and the ATP synthase (the membrane portion is called CF_0 ; and the hydrophilic portion is called CF_1). Simplified by Donald R. Ort (1988, personal communication) from Govindjee and Eaton-Rye (1986). Courtesy of D. Ort.

with increasing positive equivalents on them. "Z" represents the entities that donate electrons directly to $P680^+$; the oxidized form of Z, Z^+ , is described by its ESR signal labeled II very fast (IIvf); earlier, it was suggested to be an oxidized plastoquinol molecule. Now, new data suggest that it may be tyrosine-160 on the polypeptide D-1 of PSII (Debus *et al.*, 1988, Vermaas *et al.*, 1988). $P680$, which is most likely a dimer in its ground state, behaves as a monomer in its cationic and triplet form. In the current picture, it is attached to the α -helices of the D-1 and D-2 polypeptides of the reaction center, just as the reaction center bacteriorchlorophyll is attached to the "L" and "M" subunits of the bacterial reaction center (Michel and Deisenhofer, 1988). The photochemically active Pheo, one of the two pheophytin molecules in the reaction center complex, is a monomer and suggested to be located on the D-1 polypeptide (see Fig. 3). Q_A , the bound plastoquinone, may also be on D-2; by analogy, again, with photosynthetic bacteria, a non-heme iron (Fe^{2+}) sits in the middle of Q_A and the second bound plastoquinone Q_B , which is located on D-1. Q_B acts as a two-electron gate; it is very tightly

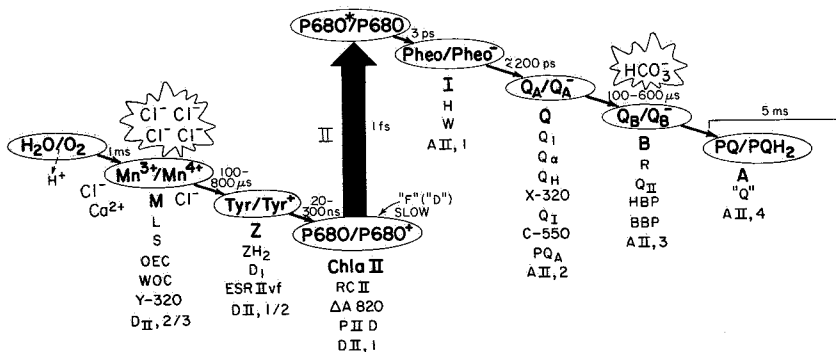


Figure 2. Electron transport scheme of Photosystem II α . Ovals show major components; measured or estimated times involved in each step are also shown. Location of sites of Cl^- and HCO_3^- actions are highlighted. Alternate names of intermediates, used by various authors for the major components, are also shown (see text). Modified after Govindjee (1984) and Govindjee *et al.* (1985).

bound in its Q_B^- form; and it exchanges with a plastoquinone molecule only after it is doubly reduced (Crofts and Wraight 1983; Velthuys, 1987). Both the D-1 and D-2 are suggested to span the membrane 5 times (Trebst and Draber, 1986; Trebst, 1987). Herbicides (Trebst 1986) and bicarbonate (Blubaugh and Govindjee 1988) are suggested to bind to this protein on the side facing the stroma, whereas manganese and chloride, involved in O_2 evolution, are suggested to bind on the side facing the lumen (Coleman and Govindjee, 1987).

A working model for the organization of PSII (excluding the light harvesting Chl *a*/Chl *b* complex) is shown in Fig. 4. In most higher plants, active PSII is located in the "appressed" regions of the membrane; this is, perhaps, the PSII α units (for a discussion of heterogeneity of PSII, see Vermaas and Govindjee, 1981; and Black *et al.*, 1986). We recognize a minimum of 7 polypeptides: (I) 47 kDa (CP-47), an internal Chl *a*-protein antenna complex; it fluoresces at 696nm at 77K; (II) 43 kDa (CP-43), a distal Chl *a*-protein antenna complex; it fluoresces at 685nm at 77K; (III) D-1: 32 kDa Q_B -binding lysine-rich protein to which herbicides can also bind (HBP) and that may also carry bicarbonate binding sites (BBP); (IV) D-2: 34 kDa Q_A -binding lysine-poor protein; (V) cytochrome *b*-559 having two polypeptides of 4 and 9 kDa molecular masses; (VI) Y-33: a 33 kDa extrinsic polypeptide, that can be released by high concentrations (1M) of CaCl_2 , is involved in O_2 evolution; (VII) Y-24: a 24 kDa extrinsic polypeptide that can be released by low

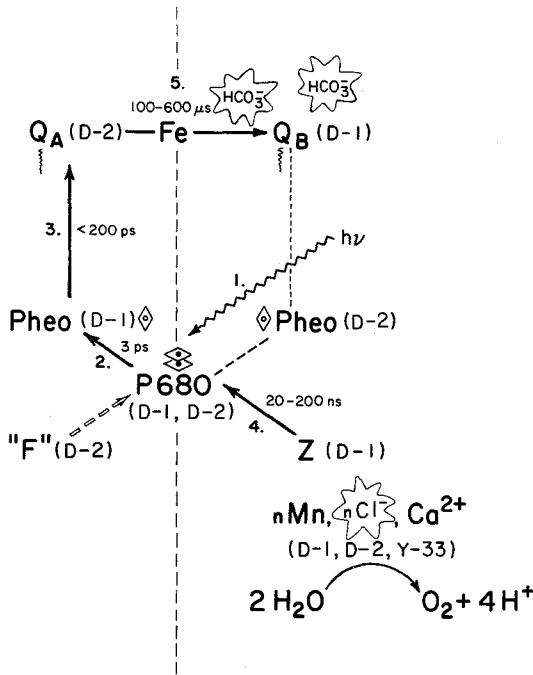


Figure 3. A simplified model of the arrangement of the various chromophores in the reaction center of photosystem II α . P680 = reaction center chlorophyll *a*, Pheo = pheophytin, Q_A = first plastoquinone electron acceptor, Fe = iron, Q_B = second plastoquinone electron acceptor, Z = suggested to be a tyrosine; D-1 = the 32 kilodalton intrinsic protein, D-2 = the 34 kilodalton intrinsic protein, Y-33 = the 33 kilodalton extrinsic protein (that is not included in the Nanba-Satoh preparations). Cytochrome *b*-559, included in the Nanba-Satoh preparation, is not shown in the diagram. A slow electron donor "F" (or "D") on the protein D-2, identified as tyrosine, is shown. Measured or estimated approximate times for reactions are shown over the arrows that indicate the direction of electron flow.

concentrations of salts; it stimulates O₂ evolution and is required for Ca²⁺ and Cl⁻ binding to the membrane; and (VIII) Y-18: a 18 kDa extrinsic polypeptide that can also be released by low concentrations of salts; its function is less clear than that of Y-24. Cyanobacteria do not contain Y-18 and Y-24. The minimal system required, thus far, for O₂ evolution contains, at least, the first 6 polypeptides (Ghanotakis *et al.*, 1987). However, the reaction center (RC) preparation, capable of the primary, charge separation, is simpler and was shown to contain only three polypeptides: D-1, D-2, and cytochrome *b*-559; it contained 4 chlorophyll

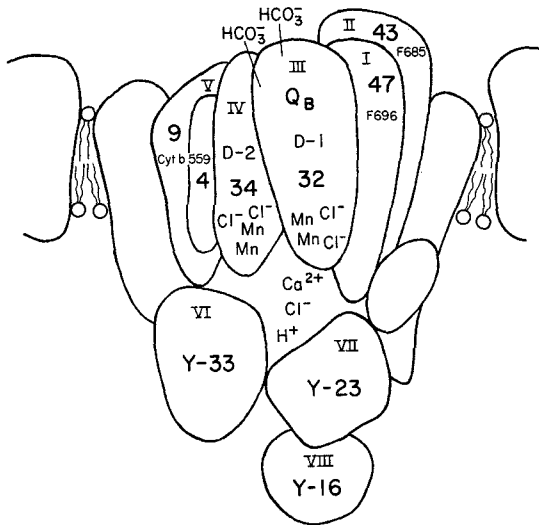


Figure 4. A possible architecture of the oxygen-evolving complex. Modified from Andersson and Åkerlund (1987). (See text for the symbols and the nomenclature.)

molecules (2 of which, presumably, form P680) and 2 pheophytin molecules (Nanba and Satoh, 1987). Unfortunately, the currently available RC preparations lack Q_A and Q_B . Although the role of cytochrome *b*-559 in the main electron flow has not been discovered, it has been shown to act as an electron donor at 77K or when normal-electron flow is blocked.

The major electrogenic event in PSII is suggested to be due to the electron flow from reduced pheophytin to Q_A . This is supported by electrically-induced reduction of Pheophytin by Q_A^- (Meiburg *et al.*, 1983); a direct kinetic measurement of membrane potential by a macroscopic electrode technique in PSII suggests that it is a fast reaction (<200 ps) (see Trissl and Kunze, 1985).

What follows is a discussion of the major components and their function in the electron transfer from the reaction center chlorophyll *a* P680 to pheophytin; pheophytin⁻ to Q_A ; and from Q_A^- to Q_B . We shall end with a discussion of the role of HCO_3^- in the reduction of plastoquinone.

PRIMARY REACTANTS AND REACTIONS: FEMTOSECONDS TO PICOSECONDS

Reactants.

P680. The existence of a reaction center Chl *a* in PSII, labeled as Chl *a* II, was first observed as an absorbance decrease at 690nm by Döring *et al.* (1967, 1969), and later by others (see reviews in Witt, 1975; Parson and Ke, 1982; Ke and Shuvalov, 1987). Floyd *et al.* (1971) measured these changes at 77K and labeled the responsible component as P680 by analogy with P700 of PSI. Since still earlier measurements in the 680-690nm region were shown to be artifacts of Chl *a* fluorescence (Rubinstein and Rabinowitch, 1963), it took many experiments and several years to accept this change to be due to an absorbance decrease (see *e.g.*, Govindjee *et al.*, 1970; Butler, 1972; Eckert *et al.*, 1984). Finally, measurements of absorbance increase in the 820nm region (van Gorkom *et al.*, 1975; Van Best and Mathis, 1978) established that the change was due to the oxidation of P680 to P680⁺, not due to Chl *a* fluorescence increase. Chl *a* cations (Chl_a⁺) show such a positive absorbance change (Fujita *et al.*, 1978). Although the 820nm change is not as specific to P680⁺, it is easier to measure. It is now generally believed that: (a) P680 is a dimer in its ground state, but the positive charge in the cation (P680⁺) and the triplet (³P680) states are localized on a monomer (see *e.g.*, Diner, 1986; Den Blanken *et al.*, 1983); and (b) the redox potential (Em,7) of P680/P680⁺ is 1.1V (Jursinic and Govindjee, 1977; Klimov *et al.*, 1980).

Recovery of P680 from P680⁺. The kinetics of the decay of P680⁺ to P680 are rather complex. Several components in the range of 200μs, 35μs, 5μs, 250 ns and 20-30 ns, have been observed. These may represent electron donation by electron donors and/or by reduced electron acceptors available for back reactions. The fastest (20-250 ns components representing 50-60% of the total signal) must be due to the main routes of electron flow from Z to P680⁺. These reactions have been measured by:

- (1) Absorbance changes at 820nm (see *e.g.* van Best and Mathis, 1978, who measured a 30 ns component in dark-adapted thylakoids; Brettel *et al.*, 1984; also see Schlödder *et al.*, 1985) show that the kinetics of the reduction of P680⁺ depend upon the redox state of WOC; it is fastest when the WOC is in S₀ and S₁ states (20 ns), and slowest (50 and 260 ns; two phases of equal amplitude) in S₂ or S₃ state. This suggests that the coulombic force of the positive charge on the WOC slows the electron donation from Z to P680⁺. Furthermore, existence of two donors Z₁ and Z₂ was suggested. (For an earlier discussion of Z₁ and Z₂, see Govindjee and Jursinic, 1979.)

- (2) Chlorophyll *a* fluorescence increase. Mauzerall (1972) observed a Chl *a* fluorescence rise time of 20-30 ns in dark-adapted algae; Butler (1972) explained these data to be due to the disappearance of P680⁺ which acts as a chlorophyll *a* quencher. Sonneveld *et al.* (1979), in an elegant analysis of Chl *a* fluorescence rise during a flash excitation at different light intensities, established that in dark-adapted thylakoids, this rise is best explained by a 20-40 ns reduction time of P680⁺ to P680. However, in preilluminated samples, this rise was 400 ns. Whether these differences are due simply to different charged states of WOC, or, also due to different electron donors, (*e.g.* Z₁ and Z₂, see *e.g.* Bouges-Bocquet, 1980) is not yet known. Just as in kinetic data on absorbance changes, fluorescence changes also show microsecond components (see *e.g.* Jursinic and Govindjee, 1977).

Pheophytin. Pheophytin is a chlorophyll molecule in which magnesium in the center has been replaced by two hydrogens. Klimov *et al.* (1977) showed that Pheo⁻ can be accumulated in PSII if P680⁺ is kept reduced by an external donor and Q_A is chemically reduced prior to illumination (see review by Klimov and Krasnovsky, 1981). Pheophytin *a* is different from chlorophyll *a* in several respects: (a) it absorbs more weakly in the red and more strongly at 505 and 535nm than Chl *a* (Fujita *et al.*, 1980); (b) *in vitro*, it can be reduced more easily compared to Chl *a* (Fajer *et al.*, 1980); (c) its midpoint redox potential is -0.6V, less negative than that of Chl *a* (Klimov *et al.*, 1979; Rutherford *et al.*, 1981); pheophytin exists in a monomer form (Klimov *et al.*, 1980) in PSII, but there are 2 pheophytin molecules per reaction center (Murata *et al.*, 1986; Omato *et al.*, 1984). As suggested in Fig. 2, Pheo must sit quite close to P680 as no membrane potential seems to be observed.

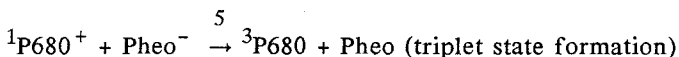
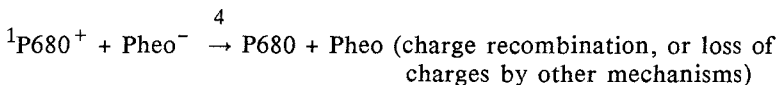
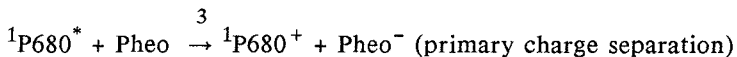
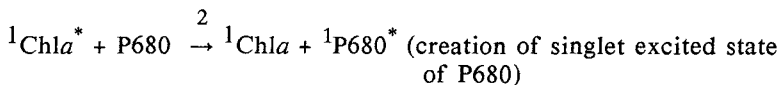
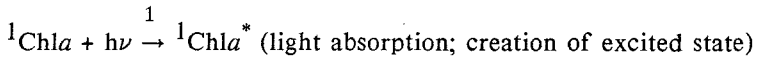
Reaction Center Preparation

In order to measure the primary photochemical reaction, *i.e.*, the charge separation, one needs to have a purified, stable reaction center preparation that is free of antenna Chl *a* molecules. Nanba and Satoh (1987) isolated a complex that contains only three proteins: D₁, D₂ and cytochrome *b*-559 (also see Satoh *et al.*, 1987; Barber *et al.*, 1987; Marder *et al.*, 1988; Seibert *et al.*, 1988). This preparation contains only 4 or 5 Chl *a*, 2 Pheo, and a single β -carotene. Since Pheo *a* could be reduced by illumination, it was suggested that this complex is analogous to the reaction center from photosynthetic bacteria [see *e.g.* that the latter contains 4 Bchl and 2 B Pheo molecules (Deisenhofer *et al.*, 1984)]. Unfortunately, the Nanba-Satoh preparation lacks the bound quinones Q_A and Q_B, and has not been crystallized for structural and functional

studies. However, herbicide binding sites are shown to exist (Giardi *et al.*, 1988). Akabori *et al.* (1988) have, however, prepared fractions that contain the bound quinones but their method is much more involved. Marder *et al.* (1987) have demonstrated that D-1 and D-2, in their preparation, are indeed the products of appropriate genes; and Chapman *et al.* (1988) have measured electron flow properties of these preparations. Although electron flow from Pheo⁻ to Q_A cannot be measured, these preparations are highly suitable for measurements on primary charge separation. We shall review below our results, obtained in collaboration with Doug Johnson (of Argonne) and Mike Seibert (of SERI) (Wasielewski *et al.*, 1989). A major reason for our success was the availability of a preparation that is stable and remains optically clear for an entire day at 4°C under intense illumination (10⁵ laser pulses). The stability and clarity were obtained (H. McTavish, R. Picorel and M. Seibert, 1988) through the following: (1) reaction center was concentrated with polyethylene glycol (a stabilizer); (2) addition of low concentration (final 0.04%) of Triton X-100 keeps the material from aggregating; (3) the material was kept under anaerobic conditions by the addition of glucose, catalase, and glucose oxidase.

Photochemistry

The primary reactions can be written as:



The act of light absorption is the most rapid reaction and is estimated from the interaction of light with Chl *a* molecule. For example, Chl *a* goes into first singlet excited state by absorbing red light (680nm). The 680nm light oscillates with a frequency of 4.41×10^{14} cycles s⁻¹. Thus,

one transition (or cycle) occurs in 2.5×10^{-15} s or 2.5 femtoseconds. This, then, is the approximate time of excitation (reaction 1). The excitation energy transfer in a reaction center must also be very rapid, considering the rise time for $^1\text{P680}^*$ production to be within the 500 fs instrument function (reaction 2). Wasielewski *et al.* (1989) have measured the lifetime of reaction 3 to be 3.0 ± 0.6 picoseconds in stable reaction center II preparations (to be discussed in details). The lifetime of $\text{P680}^+ - \text{Pheo}^-$ (reaction 4) had been measured in Nanba and Satoh preparations to be 32 nanoseconds (ns) by Takahashi *et al.* (1987) and Danielius *et al.* (1987). Hansson *et al.* (1988) have reported that the lifetime of $\text{P680}^+ - \text{Pheo}^-$ depends upon the number of antenna Chl *a* remaining attached to the reaction center. Finally Okamura *et al.* (1986) have shown the formation of the triplet state of P680, and have established the radical pair nature of its precursor through its spin polarization characteristics. Nuijs *et al.* (1986a,b) have studied primary reactions, using a 35ps laser, in photosystem II particles enriched in reaction centers (P680/80 Chl). This study, however, does not provide direct answers to the steps involved in reaction 3 above. We discuss below the newest finding, that is, the first direct measurement of the kinetics of charge separation in a stable reaction center of plant photosynthesis, using 500 fs laser flashes.

Charge Separation in Photosystem II Reaction Center

Wasielewski *et al.* (1989) have provided the first direct measurement of charge separation in stabilized PSII reaction centers with a 500 fs resolution. Using 610nm exciting flashes, they observed the following: (1) an instrument, limited (0.5 ± 0.4 ps) decrease in absorbance at 670nm and 485nm indicating the formation of the singlet excited state of P680 ($^1\text{P680}^*$); (2) an increase in absorbance at 820nm (due to the formation of P680^+) with a time constant ($1/e$) of 3.0 ± 0.6 ps (Table I); (3) a decrease in absorbance at 670nm and an increase in absorbance at 485nm or at 538nm reflecting the formation of $\text{P680}^+ \text{Pheophytin}^-$ with a time constant also of approximately 3 to 4 ps; and (4) a decrease in absorbance at 650nm (at the isosbestic point for the $\text{P680}^+ \text{Pheo}^- - \text{P680 Pheo}$ changes), reflecting the decay of $^1\text{P680}^*$ with a time constant of 2.6 ± 0.6 ps that matches, within experimental error, the formation of P680^+ (Fig. 5). Furthermore, it was shown that when Pheo was kept reduced by the addition of sodium dithionite, methylviologen and light, only the absorbance changes due to the formation of $^1\text{P680}^*$ were observed; the absorbance increase at 820nm was eliminated confirming that the observed changes are indeed due to the charge separation.

The above conclusion was further supported by quantitative comparisons of the absorbance changes measured at various wavelengths (Table II). For example, the expected ratio of absorbance at 800nm (due

Table I. Summary of Rise and Decay Components (From data of Wasielewski *et al.*, 1989)

λ , nm	absorbance	time	components
820	increase	3.0 ± 0.6 ps	$P680^+$ formation
670	decrease	0.5 ± 0.4 ps	Instrument-limited, $P680^*$ formation
		3.3 ± 0.4 ps	$P680^+$ and $Pheo^-$ formation
650	decrease	2.6 ± 0.6 ps	Decay of $P680^*$ (isosbestic for both $P680^+$ and $Pheo^-$)
538	increase	2.8 ± 0.6 ps	$P680^+$ and $Pheo^-$ formation
485	decrease	0.5 ± 0.4 ps	Instrument-limited, $P680^*$ formation
	increase	3.6 ± 0.8 ps	$P680^+$ and $Pheo^-$ formation

to $P680^+Pheo^-$) and that at 538nm trough (due to $Pheo^-$) of 5 ($2 \times 10^4 M^{-1} cm^{-1} / 4 \times 10^3 M^{-1} cm^{-1}$) was matched by the same observed ratio (ΔOD at 800nm = $0.035 / \Delta OD$ at 538nm trough = 0.007). The same conclusion was obtained from the ratio of absorbance changes at 674nm to that at 800nm, where the extinction coefficient ratio was 4.0 ($8 \times 10^4 M^{-1} cm^{-1}$ at 674nm / $2 \times 10^4 M^{-1} cm^{-1}$ at 800nm) and the measured absorbance ratio was 4.3 (OD 674nm = 0.15 / OD at 800nm = 0.035). Wasielewski *et al.* (1989) further concluded that: (1) no other intermediate exists between $P680$ and pheophytin at times > 500 fs, and (2) the quantum yield of charge separation is about 1.0.

ELECTRON FLOW FROM PHEOPHYTIN TO Q_A : PICOSECONDS TO A NANOSECOND

Since the back reaction between $Pheo^-$ and $P680^+$ has been measured to be 30ns (Takahashi *et al.*, 1987; Danielius *et al.*, 1987) in Satoh and Nanba's D-1, D-2, cyt *b*-559 preparations, and, is expected to be 2-4ns in intact PSII, the forward electron flow from $Pheo^-$ to Q_A is expected to be many orders of magnitude faster. Based on the measurements of the lifetime of chlorophyll *a* fluorescence of 180 to 300 ps from PSII in algae

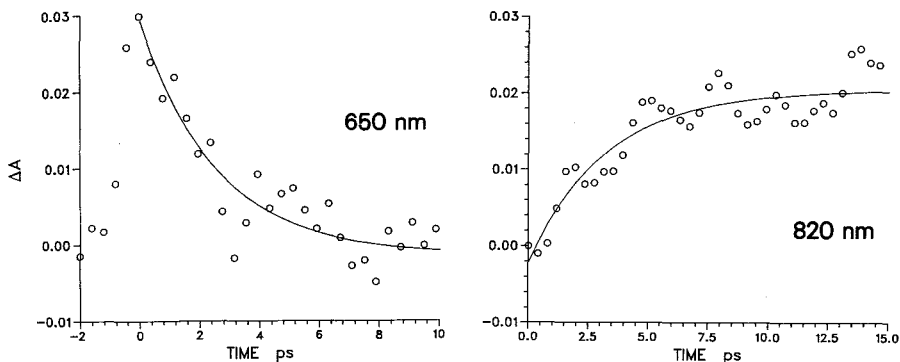


Figure 5. Transient absorption changes at 820nm (due to formation of $P680^+$) and at 650nm (due to decay of $^1Chla^*$) for photosystem II reaction centers following a 100 μJ , 500 fs laser flash at 610nm. Left: 650nm change; right: 820nm change. (After Wasielewski *et al.*, 1989.)

when the reaction centers are fully open, the reduction time of Q_A is suggested to be <300 ps (see Moya *et al.*, 1986; Holzwarth, 1987, for reviews on lifetime of fluorescence measurements). Our own (J. Fenton, N. S. Rao, E. Gratton, 1982, presented at the Midwest Photosynthesis Congress, Argonne, IL, USA) unpublished measurements of 200 ps in spinach thylakoids is in agreement with this conclusion. Since $P680^+$ to $P680$ reaction occurs in 20-30ns ($P680^+$ is a quencher of $Chl a$ fluorescence), the reduction time of Q_A to Q_A^- cannot be measured by fluorescence. However, the electron flow from $Pheo^-$ to Q_A can be measured by absorbance changes. Nuijs *et al.* (1986a), from measurements with 35ps resolution, concluded that the electron transfer from $Pheo^-$ to Q_A occurs with a time constant of 270ps in spinach PSII preparations enriched in $P680$ ($^1P680/80 Chl a$). On the other hand, Schatz *et al.* (1987), using *Synechococcus* PSII preparations enriched in $P680$ (also $^1P680/80 Chl a$), estimated that the electron transfer from $Pheo^-$ to a subsequent quinone electron acceptor takes place in ~ 500 ps. Furthermore, the quantum yield of photoreduction of Q_A has been estimated to be 0.9 (see Thielen and van Gorkom, 1981).

Since electron flow from $Pheo^-$ to Q_A is assumed to traverse a large portion of the thylakoid membrane, a membrane potential ($\Delta\Psi$) is expected to be produced. Measurements of Trissl and Kunze (1985) by macroelectrodes suggest that $\Delta\Psi$ in PSII occurs in the hundreds of picosecond range, and the flash-induced reduction of pheophytin is not

Table II. Magnitudes of Absorbance Changes (From Data of Wasielewski *et al.*, 1989)

λ , nm	Component	Extinction coefficient $M^{-1} cm^{-1}$	ΔOD , observed
800	P680 ⁺	10^4	
	Pheo ⁻	10^4	
	P680 ⁺ + Pheo ⁻	2×10^4	0.035
538 trough	Pheo ⁻	4×10^3	0.007
Ratio 800/538	--	5	5
674	P680 ⁺ + Pheo ⁻	8×10^4	0.15
Ratio 674/800	--	4	4.3

electrogenic. This confirms the picture that P680 and pheophytin are on the same side of the membrane and Q_A is on the other side.

ELECTRON FLOW OUT OF Q_A^- : THE Q_A TO Q_B REACTION: NANOSECONDS TO A MILLISECOND

Reactants.

Q_A . The major electron acceptor Q_A is a plastoquinone permanently bound on the D_2 protein. There is a tremendous heterogeneity among the quinone acceptors in PSII (see *e.g.* Vermaas and Govindjee, 1981; Black *et al.*, 1985; Diner, 1986; Rich and Moss, 1987). Since it is the second acceptor of PSII, the first being pheophytin, it is also referred to as AII₂. Originally, it was called "Q" for quencher of fluorescence (Duysens and Sweers, 1963; see a still earlier suggestion by Kautsky *et al.*, 1960), and is equivalent to Q_1 of P. Joliot, and, most likely, has a E_m of 0 mV (see *e.g.* Horton and Croze, 1979; Malkin, 1982). Its operating potential could be as low as -130mV. It is assigned to be equivalent to Q_H (a Q high potential). (For an earlier discussion of Q_1 and Q_2 , see R. Govindjee *et al.*, 1970.)

When Q_A is reduced to the semiquinone Q_A^- , a characteristic absorbance change at 320nm is observed, and is labeled as X-320 (discovered by Stiehl and Witt (1968). (See Schatz and van Gorkom, 1985, for a recent UV spectra.) Although the protein (or a group) near Q_A may become protonated (Rutherford and Zimmerman, 1984), no evidence of protonation of Q_A^- is observed even at pH4.0 (van Gorkom, 1974).

Q_A^- interacts with pheophytin $^-$ as well as a non-heme iron (see *e.g.* Klimov *et al.*, 1980). Although there is no apparent reason for believing that the antenna complexes CP-43 and CP-47 are involved in any chemistry, deVitry *et al.* (1984) have observed that both these complexes were necessary for Q_A reduction.

All the permanently bound acceptors in the so-called PSII α are also referred to as Q_α and those in PSII β as Q_β . There is the possibility that the low potential Q , Q_L , may be equivalent to Q_2 (Joliot and Joliot, 1981), or Xa (Eckert and Renger, 1980). Neither Q_2 nor Xa is a quinone. The so-called Q_{400} that has been shown to be Fe (see Pterouleas and Diner, 1986) of the Q_A -Fe- Q_B complex cannot be equivalent to Q_L . No membrane potential ($\Delta\Psi$; C-550) is associated with Q_2^- or X_a^- , but Q_A^- production shows $\Delta\Psi$, and, an associated C-550 change that involves pheophytin and carotene.

In view of the above, it is not clear whether there exists only one or two tightly bound iron-quinones per reaction center in PSII. Brettel *et al.* (1985) find evidence for only one, but Evans and Ford (1986) claim that there are two tightly-bound iron quinones. Relationship to the PSII α and PSII β centers must be examined before we can reach any conclusions.

Q_B . There has not been much discussion of heterogeneity of Q_B except that there are two types of centers: "B type" (*i.e.*, those that go through a two electron gate, see explanation below) and those that are "non-B type" (*i.e.* those that do not go through a two electron gate) (see Lavergne, 1982). PSII β centers also do not go through the two electron gate (Thielen and van Gorkom, 1981). It is assumed that some non-B type centers exist in PSII α centers also. Q_B is bound to the 32 kDa lysine-free protein; it is also a herbicide binding protein, as mentioned in the Introduction. We have suggested that it also binds two HCO_3^- ions per polypeptide (see Blubaugh and Govindjee, 1988). Binding of the herbicide atrazine has been shown by photoaffinity labeling of ^{14}C azido-atrazine (Gardner, 1981; Satoh *et al.*, 1983). Herbicides (inhibitors) compete with Q_B binding by binding on the apoprotein; they are suggested to displace Q_B (Velthuys, 1981; Wraight, 1981). Herbicide resistance has been traced to alterations in single amino acids in the Q_B apoprotein (see *e.g.* Erickson *et al.*, 1985; Johannngmeier *et al.*, 1987).

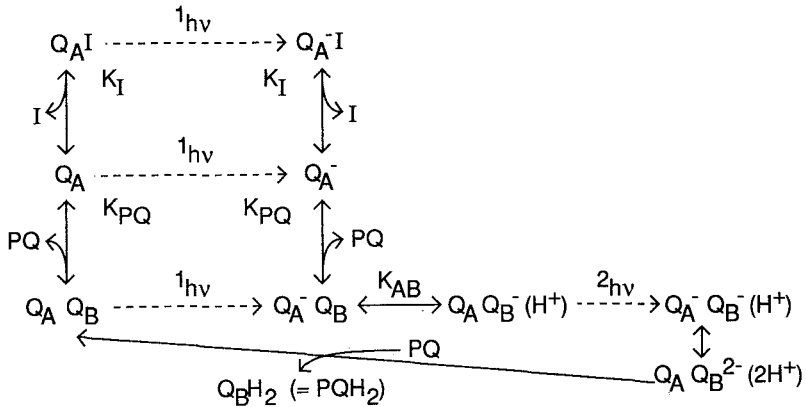
Oettmeier and Soll (1983) and Vermaas *et al.* (1984) have shown apparent competition of short-chain plastoquinone analogs with ^{14}C -DCMU and ^{14}C -atrazine, respectively. Lavergne (1982) has clearly shown that binding of DCMU to centers with Q_B^- is much lower than with Q_B ; furthermore, Lavergne (1982) has also shown competition of Q_B with benzoquinone. Recently, Wydrzynski and Inoue (1987) showed that artificial quinones can easily replace Q_B and modify the properties of the PSII acceptor site.

It is of interest to us to mention that effects on the D-1 protein, usually on the acceptor side by mutation or by inhibitors, can show effects on the donor side of PSII (*e.g.* Golbeck and Warden, 1985 for salicylaldoxime; Metz *et al.*, 1986 for herbicides; and Warden and Csatorday, 1987 for linolenic acid).

Electron Flow from Q_A^- to Q_B : Electrons are rapidly transferred out of Q_A^- with a halftime of 200 to 600 μs (Forbush and Kok, 1968; Mauzerall, 1972; Zankel, 1973). Bowes and Crofts (1980) measured this time as a function of flash number and have obtained evidence that $\text{Q}_\text{A}^- \text{Q}_\text{B}^- \rightarrow \text{Q}_\text{A} \text{Q}_\text{B}^-$ occurs in 200 to 400 μs and $\text{Q}_\text{A}^- \text{Q}_\text{B}^- \rightarrow \text{Q}_\text{A} \text{Q}_\text{B}^{2-}$ in 600-800 μs . The slowness of the second reaction may be due to electrostatic repulsion of the negative charge. In the presence of herbicides, that displace Q_B , electron flow out of Q_A^- is blocked and takes seconds.

As hinted above, Q_B is a two electron acceptor and acts as a two electron gate. Bouges-Bocquet (1973) and Velthuys and Amesz (1974), independently, reported evidence for the existence of what they have called "B" or "R", which they suggested accepts electrons from $\text{Q}^-(\text{Q}_\text{A}^-)$. Bouges-Bocquet (1973) measured the reduction of methylviologen (an electron acceptor for photosystem I only) in PSI light, and found that the number of electrons reaching methylviologen was higher after the 2 and 4 than after 1 and 3 preilluminating flashes in PSII; thus, it was suggested that a component B is reduced by the first PSII flash to B^- and then to B^{2-} by a second flash and only when B^{2-} was formed were electrons transferred to the PQ pool, and then to PSI. Velthuys and Amesz (1974) first blocked the electron donor side of PSII, added artificial electron donors to donate electrons to Z^+ or P680^+ , exposed the samples to a series of light flashes, and then measured DCMU-induced Chl *a* fluorescence increase (ΔF) by a weak measuring beam. They observed a binary oscillation in F with peaks after odd number of flashes. This was explained as follows: after flash 1, Q^-R is converted to QR^- ; DCMU blocks further electron flow, equilibrium is shifted to Q^-R and fluorescence is high as Q^- is a condition for high fluorescence. After flash 2, Q^-R^- is converted to QR^{2-} , electrons move out to the PQ pool, QR is produced; DCMU addition gives low fluorescence since the system

is in low fluorescent state as Q is a condition for low fluorescence. Thus, the concept of the "two electron gate" was born. "B" or "R" has now been renamed Q_B . The important concept as to how electrons are transferred from Q_B to the PQ pool was suggested by Velthuys (1981): In this concept, after Q_B^{2-} is formed, it exchanges with a PQ molecule on the Q_B protein. The reactions can now be written as (see *e.g.* Stein *et al.*, 1984):



Here, I = inhibitor, Ks stands for equilibrium constants and PQ for plastoquinone.

The ratio of $Q_B^-:Q_B$ in dark-adapted isolated thylakoids is approximately 7 : 3 (see *e.g.* Wollman, 1978). However, in intact chloroplasts (Govindjee, T. Ono, and Y. Inoue, unpublished, 1983), leaves (Rutherford *et al.*, 1984), and in higher plant cells grown in tissue culture (Xu *et al.*, 1988) it is 1:1. Figure 6 shows that the decay of Chl *a* fluorescence, reflecting Q_A^- to Q_A decay, is identical after both flash 1 and 2 (Xu *et al.*, 1988) suggesting 1:1 ratio for $Q_B^-:Q_B$ in dark-adapted soybean cells.

ROLE OF BICARBONATE IN PLASTOQUINONE REDUCTION

Warburg and Krippahl (1958, 1960) (also see Franck, 1945) discovered that CO_2 (or HCO_3^-) stimulates electron flow in the Hill reaction. Warburg (see *e.g.*, his paper in 1964) considered this phenomenon as evidence for CO_2 being the source of O_2 in photosynthesis. Although Metzner (1975) and Stemler (1982) have favored an important role of CO_2 (HCO_3^-) on the electron donor, *i.e.* the O_2 evolving side of PSII, we have

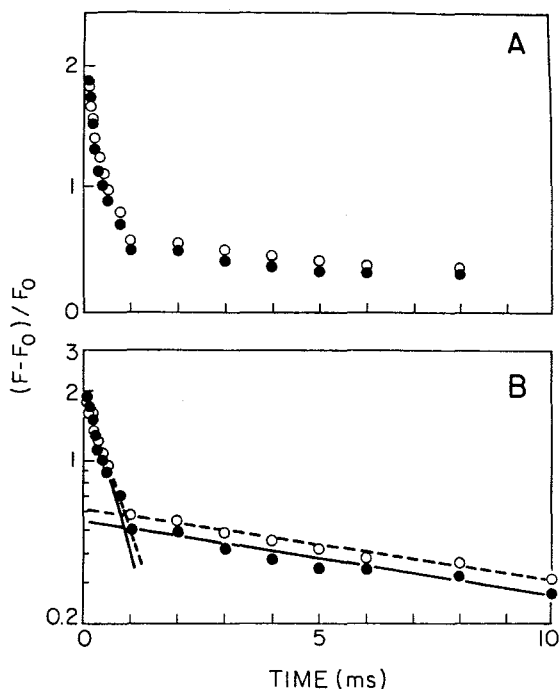


Figure 6. (A) Decay of variable chlorophyll *a* fluorescence $((F - F_0) / F_0)$ after the first (\bullet) and second (\circ) actinic flash, spaced 1 second apart, in soybean cells. (B) The same data on a semilogarithmic scale. F_0 is the fluorescence yield taken with the measuring light when all Q_A are oxidized and F is the fluorescence yield after the actinic flash. Absence of difference in data between flash 1 and 2 indicates that the ratio of $Q_B:Q_B^-$ is 1 in dark-adapted intact higher plant cells. (After Xu *et al.*, 1988.)

consistently obtained evidence for a major effect of CO_2 (HCO_3^-) on the electron acceptor, *i.e.*, the plastoquinone reduction side of PSII (see reviews by Govindjee and van Rensen, 1978; Vermaas and Govindjee, 1981, 1982; van Rensen and Snel, 1985; Govindjee and Eaton-Rye, 1986; Blubaugh and Govindjee, 1988). We believe that HCO_3^- is required for the electron flow and the protons needed for plastoquinone reduction in PSII.

Our current picture is: CO_2 is the diffusing and HCO_3^- is the active species (see *e.g.*, Sarojini and Govindjee^a, 1981; and Blubaugh and Govindjee, 1986). At the pH of the stroma, we expect enough ($220\mu M$) HCO_3^- , much higher than the estimated $35\text{--}60\mu M$ dissociation constant, to be present at ambient air levels. We consider that two tight HCO_3^- binding sites exist on the Q_B -apoprotein, discussed above: (1) on the Fe of the Q_A -Fe- Q_B complex, as suggested by Michel and Deisenhofer

^aInitially, the faster kinetics with CO_2 was misinterpreted for it being the active species, rather than the diffusing species.

(1988); and (2) on one of the arginines, near the Q_B binding, as suggested by Shipman (1981). Perhaps, the binding to the Fe in the Q_B protein places the PSII reaction in its proper conformation (allosteric effector) so that Q_B can efficiently accept electrons from Q_A^- , and Q_B^{2-} can efficiently exchange with the PQ pool. On the other hand, the function of the second HCO_3^- may be to bring protons needed for the reduction of the plastoquinone (for details, see Blubaugh and Govindjee, 1988).

A binding to Fe is supported by the experiments of Vermaas and Rutherford (1984), who observed a large formate-induced (*i.e.* HCO_3^- removal) increase in the $Q_A^-Fe^{2+}$ complex EPR signal at $g = 1.82$. A role of HCO_3^- in protonation, that may not involve the Fe site, was suggested first by experiments of Khanna *et al.* (1980) and later by Govindjee, J.J. van Rensen, J.F.H. Snel and W. Tonk (unpublished, 1983; see Govindjee and Eaton-Rye, 1986). The latter authors showed that a binary oscillation, superimposed on a period of 4 oscillation, of H^+ release to the lumen was abolished by HCO_3^- depletion (confirmed, reversibility of the effect shown, and extended by van Rensen *et al.*, 1988). These experiments may not provide any clue as to any direct effect of HCO_3^- on the protonation reactions *per se*, since the dips at uneven flashes may not be due to protonation of Q_B^{2-} but due to increased H^+ release during PQH_2 oxidation at even flashes. On the other hand, pH dependence of electron flow from Q_A^- to Q_B , as measured by Chl *a* fluorescence decay, shows remarkable differences with and without HCO_3^- present. In HCO_3^- -depleted samples, Eaton-Rye and Govindjee (1988) observed that at pH 7.5, two turnovers of the Q_B "two-electron gate" were necessary before the maximum slowing down of Q_A^- to Q_B electron flow could be seen on subsequent flashes. By contrast, at pH 6.5, the slowed time was maximum only after one turnover of Q_B (*i.e.*, after two flashes). However, the maximum slowed time was much greater at pH 7.5 than at pH 6.5. After 1 or 2 flashes, however, the situation was reversed, *i.e.*, the decay was greater at pH 6.5 than at pH 7.5 (Fig. 7). Although complicated, the phenomenon can be understood by considering that there are two effects of HCO_3^- depletion: one, a conformational change and the other a protonation event. Whether the second is a result of the first or whether they are two separate effects remains to be determined. As a result of conformational change, binding of plastoquinone that forms Q_B is suggested to be decreased leading to changes in rate of $Q_A^-Q_B \rightarrow Q_AQ_B^-$ reaction. The effect is pH dependent, being faster at alkaline pH. It is likely that this effect involves changes at the Fe site. However, after higher flash numbers, either the conformational effect exposes another protonation effect, or a new separate protonation effect becomes observable. At alkaline pH: absence of HCO_3^- leads to a larger lack of H^+ s for the protonation of plastoquinone sites, thus, slowing down the Q_A^- to $Q_B^{(-)}$ reactions. At

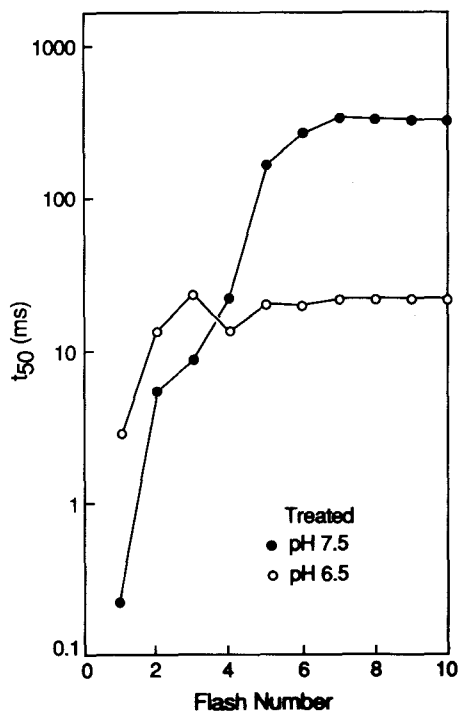


Figure 7. Plot of the times (t_{50}) at which (Q_A^-) is 50% of maximum [Q_A^-] in HCO_3^- -depleted (treated) membranes with measurements at pH 6.5 and pH 7.5. At pH 7.5, (Q_A^-) decay is much slower than at pH 6.5 after four flashes suggesting the role of HCO_3^- in protonation reactions. The flash frequency was 1 Hz. (After Eaton-Rye and Govindjee, 1988.)

acidic pH, however, HCO_3^- has a lesser role to play due to the greater availability of protons. This explains the larger slowing down at pH 7.5 than at pH 6.5.

The concept of two binding sites was further supported by the data of Blubaugh and Govindjee (1988b) who showed by kinetic analysis that the residual activity in HCO_3^- -depleted thylakoids was due to endogenously bound HCO_3^- : the amount of Hill activity restored to HCO_3^- -depleted thylakoids by a half-saturating [HCO_3^-] was non-linear with respect to [Chl]. Thus, the essentiality of HCO_3^- was suggested. After corrections were made for endogenous [HCO_3^-], the activity versus [HCO_3^-] no longer followed Michaelis-Menten kinetics, and suggested the existence of two cooperative binding sites ($n > 1.4$).

A detailed model of how HCO_3^- may function as a H^+ donor was discussed by Blubaugh and Govindjee (1988) (see Fig. 8). It is proposed that when Q_B (PQ) is reduced to Q_B^- , the pKa of a nearby histidine shifts

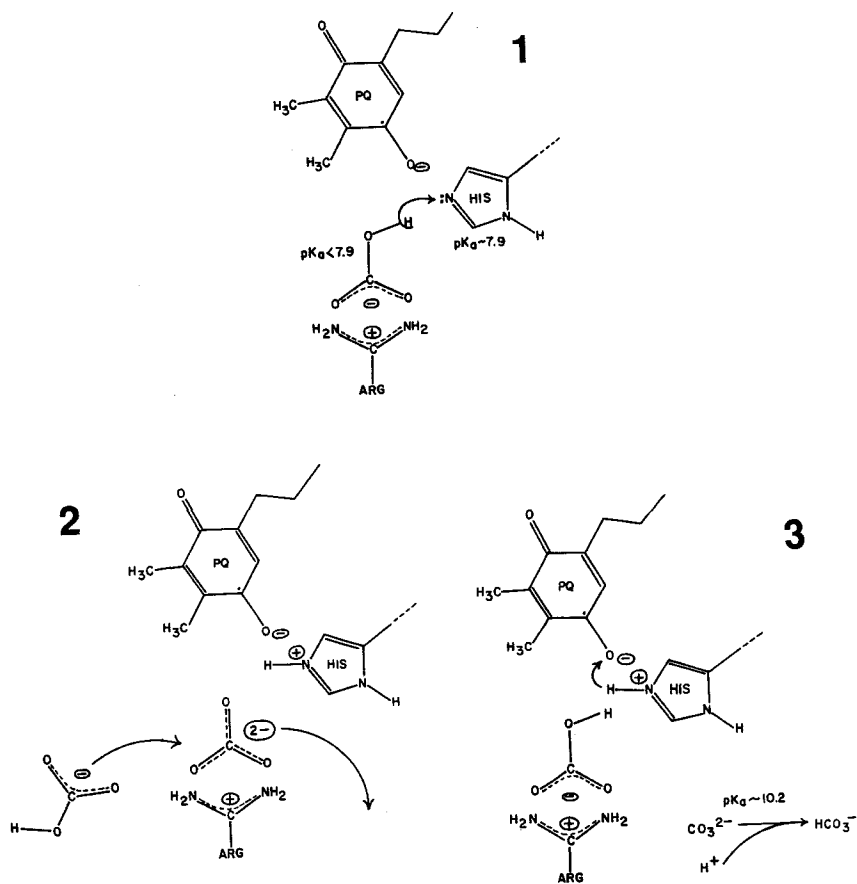


Figure 8. A working model for the involvement of HCO_3^- in the protonation of Q_B^- . 1: Bicarbonate sits on an arginine (ARG), and shares its proton with a nearby histidine (HIS); PQ stands for Q_B^- . 2: HCO_3^- replaces CO_3^{2-} , a H^+ has already been delivered to HIS. 3: Proton on HIS provides the positive charge needed to stabilize Q_B^- formed after a light reaction. The CO_3^{2-} is converted to HCO_3^- by the uptake of a H^+ from the surrounding (see text for details). (After Blubaugh and Govindjee, 1988.)

from 6.4 to 7.9 (see Crofts *et al.*, 1984) due to repulsive interaction between the negative charge on Q_B^- and the lone pair of electrons on histidine. The pK_a of the HCO_3^- bound to a neighboring arginine is presumed to change from 10.2 to 7.9 due to electron withdrawal by

arginine. Thus, a H^+ is shared between HCO_3^- and histidine. This H^+ ends up on histidine as another HCO_3^- replaces the CO_3^{2-} group holding the H^+ close to the histidine. This ensures irreversibility of H^+ transfer from HCO_3^- to histidine. Since the pKa of free CO_3^{2-} is 10.2, reprotonation is rapid. Finally, steric effects between the OH of HCO_3^- and H on the histidine favor the transfer of H^+ from histidine to near Q_B^- or Q_B^{2-} . We favor the possibility that arginine 257 and histidine 252 on D-1 protein may be the most likely pairs involved in protonation of Q_B^- or Q_B^{2-} . Thus, they are our targets of site-directed mutagenesis in order to test the above hypothesis.

At present, this test can be done on a transformable cyanobacterium *Synechocystis* PCC 6803. It, thus, became necessary to check for the existence of bicarbonate effect in cyanobacteria. The only published work on this effect on cyanobacteria showed a negative result (van Rensen and Vermaas, 1981). However, results of J. Cao and Govindjee

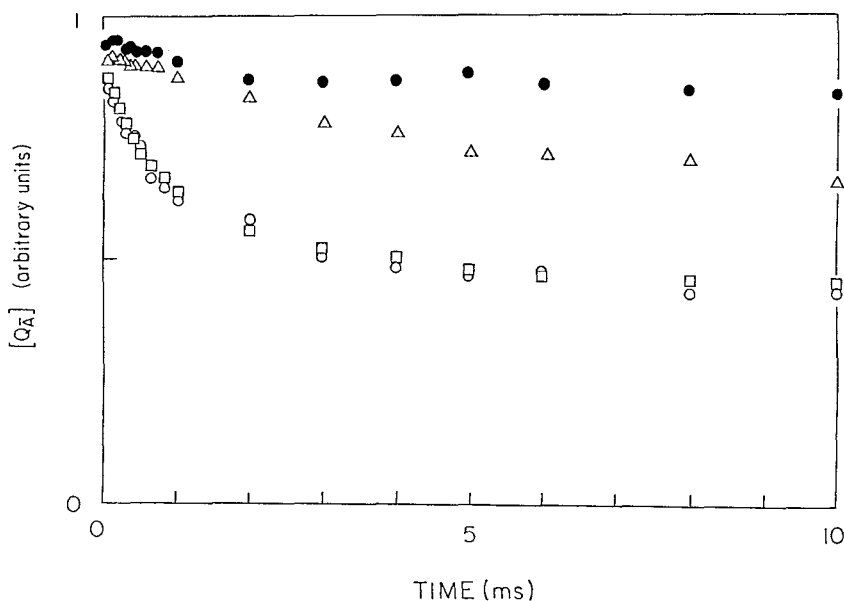


Figure 9. Decay of (Q_A^-), calculated from chlorophyll *a* fluorescence decay, in thylakoids from *Synechocystis* PCC 6803. Open circles: Control; triangles: depleted of HCO_3^- (25mM sodium formate); squares: restored with 5mM HCO_3^- addition; solid circles: control with 5µM diuron. Note that HCO_3^- depletion reversibly blocks the decay of Q_A^- just as diuron does. (Data of J. Cao and Govindjee, 1988, unpublished.)

(unpublished, 1988) show the existence of HCO_3^- effect in both thylakoids and intact cells of *Synechocystis* PCC 6803. Figure 9 shows that the decay of Q_A^- after the third flash is dramatically slowed down upon bicarbonate depletion approaching the rate of decay in herbicide treated samples.

Photosynthetic bacteria, that have quite a different amino acid sequence on their electron acceptor side, do not show any significant bicarbonate effect (R. J. Shopes, D. Blubaugh, Govindjee and C. Wraight, unpublished, 1987). As noted earlier, Michel and Deisenhofer (1988) have suggested that in PSII, HCO_3^- may replace the M-252 glutamate that provides the fifth ligand to the Fe. The possible relationship between the bacterial and plant systems as regards the second HCO_3^- binding site (an arginine) has not yet been explored.

A major question about the HCO_3^- effect is its role *in vivo*. A hint of this CO_2 effect was provided for wheat leaves by Gerbaud and André (1980). Govindjee, J. F. H. Snel and J. J. S. van Rensen (1983, unpublished; cited by Govindjee and Eaton-Rye, 1986) showed that bicarbonate depletion reversibly decreases the parabenzquinone Hill reaction by several fold and produces a Chl *a* fluorescence transient that mimics DCMU-treatment in *Scenedesmus* cells. Garab *et al.* (1983) showed similar changes in Chl *a* fluorescence transient in leaves when air or CO_2 was replaced with N_2 . Mende and Wiessner (1985) showed that in *Chlamydomonas stellata*, but not in *Chlorella fusca*, replacement of air with CO_2 -free-air first led to a decreased fluorescence suggestive of a blockage on the electron donor or water side, followed by an increase in fluorescence suggestive of a block on the electron acceptor side. Unfortunately, the parallel measurement on O_2 evolution was not done in identical conditions and perceived correlation between the two measurements was not established. Ireland *et al.* (1987), using wheat and maize leaves, showed that decreasing $[\text{CO}_2]$ from already a low level (when CO_2 fixation was negligible) to a very low level caused changes that reflected a block between Q_A^- and Q_B^- . Garab *et al.* (1988) showed that removal of CO_2 from the leaf atmosphere produced thermoluminescence bands indicating a preponderance of Q_A^- over Q_B^- clearly showing that the absence of CO_2 blocked electron flow from Q_A^- to Q_B^- . Ireland *et al.* (1987) working on wheat and maize, and Fatma El-Shintinawy and Govindjee (paper presented at the annual meeting of the American Society of Plant Physiology, 1988, Reno) working on spinach leaves showed that, when methylviologen is used as an electron acceptor (anaerobic conditions), absence of HCO_3^- caused a faster fluorescence rise indicating a block in electron flow from Q_A^- to Q_B^- . Addition of HCO_3^- reversed the effect. Interestingly, El-Shintinawy and Govindjee observed that if the spinach leaves were infiltrated for a very short time (*e.g.*, 10s) with 100 mM sodium formate, both O_2 evolution

and Chl *a* fluorescence levels decreased, apparently, suggesting a block on the electron donor side of PSII (Fig. 10). However, a longer infiltration (*e.g.*, minutes) showed a dramatic decrease in O₂ evolution and a dramatic increase in the rate of Chl *a* fluorescence rise indicating a block on the electron acceptor side. It appears that the HCO₃⁻ pool associated with the first effect must be more loosely bound than that associated with the second effect. Our data on leaves, although reminiscent of Mende and Wiessner's data on *Chlamydomobryx*, cannot be interpreted, however, as showing a block on the electron donor side because a block between pheophytin and Q_A (a site close to the Fe site) would also give the same result since reduced pheophytin is a quencher of Chl *a* fluorescence.

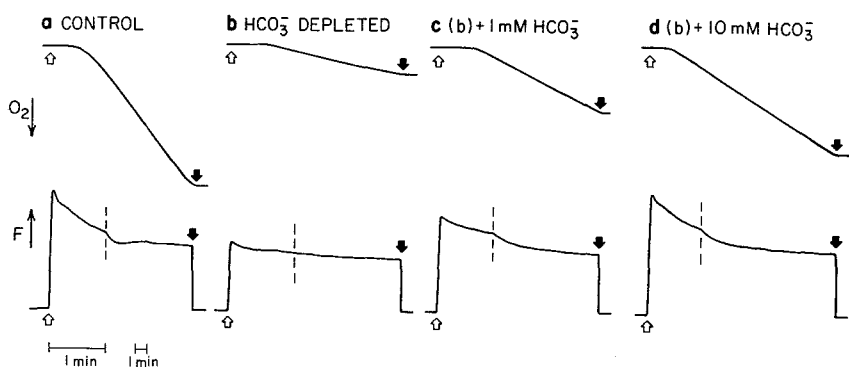


Figure 10. Parallel measurements on the rate of oxygen evolution (top curves) and chlorophyll *a* fluorescence changes in spinach leaf discs. (a): control; (b) infiltrated with 100mM sodium formate for 10s; (c) : (b) restored with 1mM HCO₃⁻; (d) : (b) restored with 10mM HCO₃⁻. Infiltration of leaf discs with 100mM sodium formate for longer time (> 1 min) shows elimination of O₂ evolution and a faster fluorescence rise to maximum fluorescence (data not shown). (After F. El-Shintinawy and Govindjee, 1988, presented at the 1988 Americal Society of Plant Physiology Meeting, Reno, Nevada.)

It appears to us that when HCO₃⁻ is completely removed, electron flow from Q_A⁻ to the plastoquinone pool is almost completely blocked after 1 or 2 turnovers of the two electron gate, (see Vermaas and Govindjee, 1982; Govindjee, 1984; Govindjee *et al.*, 1984; J. Cao and Govindjee, unpublished, 1988). Experiments are in progress to further understand the mechanism of bicarbonate effect *in vivo*.

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