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Effects of amino acid residue substitutions on bicarbonate function in the plastoquinone reductase in cyanobacteria

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EFFECTS OF AMINO ACID RESIDUE SUBSTITUTIONS ON BICARBONATE FUNCTION IN THE PLASTOQUINONE REDUCTASE IN CYANOBACTERIA

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THESIS

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EFFECTS OF AMINO ACID RESIDUE SUBSTITUTIONS ON BICARBONATE FUNCTION IN THE PLASTOQUINONE REDUCTASE IN CYANOBACTERIA

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The electron transfer at the Q_AFeQ_B complex is significantly and reversibly inhibited in chloroplasts of higher plants and algae depleted of bicarbonate while no such inhibition has been observed in photosynthetic bacteria. It was observed in this study that a more than four fold stimulation of the Hill reaction by 5 mM bicarbonate and a significant reversible slowing of oxidation of Q_A^- in bicarbonate-depleted cells and thylakoids of Synechocystis sp. PCC 6803, showing the existence of the bicarbonate effect in cyanobacteria. Thus, this effect is present in all Photosystem II (PSII) reaction centers.

Oligonucleotide-directed mutagenesis was used to construct Synechocystis 6803 mutants carrying mutations in arginine residues in the D2 protein. Measurements of oxygen evolution showed that the D2 mutants D2-R233Q (arginine-233 → glutamine) and D2-R251S (arginine-251 → serine) were ten-fold more sensitive to formate, which displaces bicarbonate, than the wild type. Measurements of oxygen evolution in single-turnover flashes and chlorophyll (Chl) a fluorescence decay kinetics confirmed that D2-R251S and D2-R233Q are more sensitive than the wild type. It is suggested that the D2

protein is involved in the bicarbonate effect in PSII and the two arginine residues are important for the stabilization of bicarbonate binding in PSII.

The D1 protein of PSII is known to be involved in the bicarbonate effect. To examine the changes in the binding affinity of bicarbonate and formate in site-selected herbicide-resistant D1 mutants in Synechococcus sp. PCC 7942, a rapid equilibrium model involving activator-inhibitor interactions was used to estimate the dissociation constant for bicarbonate and for formate. The data indicated that these D1 mutations increase the dissociation constants for bicarbonate while those for formate are virtually unchanged. A working hypothesis is proposed in which bicarbonate forms a bidentate ligand to Fe^{2+} and participates in the proton transfer pathway to the formation of plastoquinol Q_BH_2 at the plastoquinone reductase.

To my father

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ABBREVIATIONS

BSA: bovine serum albumin

Chl *a*: chlorophyll *a*

DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone

DCBQ: 2,6, dichloro-*p*-benzoquinone

DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea

DMQ: 2,5-dimethyl-*p*-benzoquinone

EDTA: ethylenediamine tetraacetic acid

FWHM: full width at half maximum

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

MES: 2-[N-morpholino]ethanesulfonic acid

P_{680}: primary electron donor of reaction center II

PCC: Pasteur culture collection

Pheo: pheophytin

PSI: photosystem I

PSII: photosystem II

Q_A: bound plastoquinone, a one-electron acceptor in photosystem II

Q_B: reversibly bound plastoquinone, a two-electron acceptor in photosystem II.

CHAPTER I. INTRODUCTION

A. Literature Review

It is well known that photosynthesis in plants fixes CO_2 and evolves O_2. CO_2 acts not only as a substrate for ribulose-1,5-bisphosphate carboxylase/oxygenase, but also serves a role in photosynthesis by enhancing the rate of electron transport between the primary quinone Q_A and the secondary quinone Q_B in Photosystem II (PSII). Warburg and Krippahl (1960) observed an effect of bicarbonate on the Hill reaction and reasoned that the effect was on water oxidation, currently referred to as the oxidizing side of PSII(see Warburg, 1964). However, later studies showed that the site of bicarbonate stimulation was on the reducing side of PSII (Wydrzynski and Govindjee, 1975). Recent comprehensive reviews of this effect, the " bicarbonate effect", are those by Blubaugh and Govindjee (1988) and Govindjee (1991). The following literature review is focused on the molecular mechanism of bicarbonate function in the Q_AFeQ_B complex of PSII, where Fe is a non-heme iron situated between Q_A and Q_B. This will be the subject of the present thesis.

1. Site(s) of bicarbonate effect

The notion that bicarbonate effect is at the Q_AFeQ_B complex of PSII is supported by the following experiments:

(A) The time course of chlorophyll (Chl) a fluorescence yield rise, during continuous illumination, is faster in the

formate-treated\CO_2-depleted thylakoids in which the oxygen evolution capacity has been inhibited than in the CO_2 restored samples in the presence of an external electron donor (Wydrzynski and Govindjee, 1975), indicating a slowing of electron transfer beyond Q_A. The kinetics of Chl *a* fluorescence yield decay, reflecting Q_A^- oxidation after single-turnover saturating actinic flashes, is slowed in formate-treated/CO_2-depleted thylakoids, with significant slowing after the second and subsequent flashes. It suggests an inhibition of protonation of Q_B^- and a requirement of bicarbonate to provide protons for the formation of plastoquinol (Q_BH_2) (Govindjee et al., 1976; Jursinic et al., 1976; Robinson et al., 1984; Eaton-Rye and Govindjee, 1988a,b; Diner and Petrouleas, 1990).

(B) Formate drastically alters EPR signal of $Q_A^-Fe^{2+}$ of PSII (Vermaas and Rutherford, 1984; Bowden et al., 1991; Hallahan et al., 1991); NO, carrying an unpaired electron (S= 1/2), ligates to non-heme iron and produces S=3/2 Fe(II)-NO EPR signal at g=4. Bicarbonate diminishes this signal (Diner and Petrouleas, 1990); formate decreases the quadrupole splitting and the chemical shift of the non-heme Fe^{2+} Mossbauer spectrum (Diner and Petrouleas, 1987; Semin et al., 1990). These observations indicate that bicarbonate interacts with the non-heme iron. A kinetic analysis suggests two cooperating binding sites of bicarbonate (Blubaugh and Govindjee, 1988). However, how the two events or two sites, structural

requirement at Fe, and the functional requirement for protonation at the Q_B site, are related is still an open question.

Bicarbonate is required to be present at its binding site in PSII reaction centers when a normal electron transfer at the Q_AFeQ_B complex occurs (Blubaugh and Govindjee, 1988; Govindjee et al., 1991c; Van Rensen, 1991). In this view, inhibitory anions like formate inhibit electron transfer at the Q_AFeQ_B complex by displacing bicarbonate from its binding site. However, another view of the bicarbonate effect is that a normal electron transfer can occur when the site is either filled with bicarbonate or free of any inhibitory anion (Stemler, 1989; Jursinic and Stemler, 1991). There are experimental data in support of the former view (Govindjee et al., 1991c; Van Rensen, 1991) and those in favor of the latter view (Stemler, 1989; Jursinic and Stemler, 1991). Further investigations are required to resolve the difference in the two theories.

2. Involvement of D1 and D2 proteins

D1 and D2 proteins are two reaction center proteins resembling the L and M subunits in photosynthetic purple bacteria. Indications for the involvement of the D1 protein in the bicarbonate effect first came from the studies of herbicide and bicarbonate interaction, and of herbicide-resistant D1 mutants. The apparent affinity of the thylakoid membranes for bicarbonate is reduced by the treatment with

urea, triazine and phenol-type herbicides (known to interact with the Q_B site) (Van Rensen and Vermaas, 1981; Vermaas et al., 1982; Snel and Van Rensen, 1983). The binding of herbicides is affected by bicarbonate depletion (Khanna et al., 1981). Furthermore, a triazine-resistant mutant of Amaranthus hybridus (in which a single residue Ser-264 of D1 protein was mutated to Gly) showed a 2-fold increase in the dissociation constant of bicarbonate compared to the wild type (Khanna et al., 1981). Recently several herbicide-resistant D1 mutants of the cyanobacterium Synechocystis 6714 (Govindjee et al., 1990) and of Chlamydomonas reinhardtii (Govindjee et al., 1991a) demonstrated differential sensitivities to formate.

Aspartic acid-213 and glutamic acid-212 on the L subunit of Rhodobacter sphaeroides in the Q_B-binding site play an essential role in the proton transfer pathway leading to the formation of Q_BH_2 (Takahashi and Wraight, 1990, 1991). Furthermore, weak acid anions such as nitrite, cyanate, formate, azide and acetate, which inhibit electron transfer reaction at the Q_AFeQ_B complex in PSII (Stemler and Murphy, 1985; Eaton-Rye et al., 1986; Cao and Govindjee, 1989), substantially stimulate proton transfer in site-directed mutants of the two ionizable residues Glu^{L212} and Asp^{L213} (Takahashi and Wraight, 1991).

On the basis of homologies with the reaction center from purple bacteria, the D2 protein like D1, is thought to contribute to the binding of the reaction center Chl *a* P_{680} and

the non-heme iron. It is suggested that D2 creates most of the binding niche for Q_A and a pheophytin, whereas D1 harbors Q_B and another pheophytin (Trebst, 1986; Michel and Deisenhofer, 1986; Rutherford, 1987). In this thesis I will show (see chapter III; Cao et al., 1991) that the replacement of D2-R233 and D2-R251 in Synechocystis sp. PCC 6803, by neutral residues caused a ten fold increase in the sensitivity to formate compared to the wild type in steady-state oxygen evolution rate, suggesting that the arginines may stabilize the bicarbonate binding on the D2 protein. Diner and coworkers (cited in Diner et al., 1991) mutated D2-R264 and D2-K265 also in Synechocystis sp. PCC 6803. These site-directed mutants showed slowed photoautotrophic growth rate and required much higher bicarbonate concentration than wild type to stimulate electron transfer from Q_A^- to $Q_B^{(-)}$, to be referred to in this thesis as Q_A-Q_B electron transfer. These results unequivocally show that D2 protein is involved in the bicarbonate effect and that cationic residues like arginine and lysine play a role in bicarbonate binding /stabilization in PSII.

3. Difference between Photosystem II and bacterial reaction centers

The bicarbonate effect has been well established in PSII of higher plants (see Blubaugh and Govindjee, 1988, for a review) and cyanobacteria (Chapter II, this thesis; Cao and Govindjee, 1988; Nugent et al., 1988). PSII reaction centers have a similar non-heme iron closely associated with the

primary (Q_A) and secondary (Q_B) quinones as in bacterial reaction centers. However, no formate treatment effect on electron transfer at Q_AFeQ_B complex has been observed in purple bacteria (Shopes et al., 1989). Neither does NO have any effect on the rate of electron transfer from Q_A^- to $Q_B^{(-)}$ in reaction centers of Rb. sphaeroides (Diner and Petrouleas, 1990). The electron transfer at the Q_AFeQ_B complex is also normal in the green bacterium Chloroflexus aurantiacus (Govindjee et al., 1991) in the presence of formate or NO. The different response to bicarbonate depletion might be a result of differences of amino acid sequence in the polypeptides of PSII and bacterial reaction centers. The fifth and sixth ligands of the iron atom are furnished by the carboxylate group of a glutamic acid residue in purple bacteria (Glu^{M232} in Rhodopseudomonas virdis) while this mechanism is absent in PSII. Therefore, bicarbonate was suggested to function to provide bidentate ligand to Fe in PSII (Michel and Deisenhofer, 1988). However, site-directed mutations at the site of M-E234 in Rb. sphaeroides into valine, glutamine or glycine showed no effect of formate-treatment/bicarbonate depletion on the kinetic of Q_A^- to Q_B electron transport (collaborative work of the author with the research group of Professor Colin Wraight, see Wang et al., 1991). Therefore, the glutamic acid residue is not essential to the normal functioning of the acceptor quinone complex in bacterial reaction centers and the role of bicarbonate in PSII is

distinct from the role of this residue in bacterial reaction centers.

In addition, the iron in bacteria is different from that in PSII in other respects. In PSII, but not in purple bacteria, Fe^{2+} can be oxidized to Fe^{3+} at redox potentials above about 400 mV and Fe^{3+} can be rapidly reduced by Q_A^- within several μs (Rutherford, 1987). In purple bacteria the redox midpoint potential of the Fe^{2+}/Fe^{3+} transition is significantly higher than in plants. Only in plants, Fe^{2+} can be oxidized by ferricyanide and quinones, while formate inhibits this oxidation (Zimmermann and Rutherford, 1986). The $Q_A^-Fe(II)$ EPR signal in PSII is pH-dependent (g=1.82 at low pH, g=1.9 at high pH) (Rutherford and Zimmermann, 1984). The g=1.82 signal is apparently correlated with the high-potential form of Fe(III)/Fe(II) redox couple (Petrouleas and Diner, 1987). Formate can greatly enhance the g=1.82 form of the Q_A^- Fe(III) EPR signal (Vermaas and Rutherford, 1984). No EPR signal arising from an Fe(II)-NO adduct was observed in Rhodospirillum rubrum chromatophores and Rb. sphaeroides reaction centers as in PSII, indicating that NO is not able to coordinate the iron in bacterial reaction centers. Non-heme iron of PSII is oxidizable. However, attempts at such oxidation in bacterial reaction centers have been unsuccessful, even in the presence of HCO_3^- (Beijer and Rutherford, 1987; Rutherford, 1987; Petrouleas and Diner, 1987).

B. Objectives of the Present Thesis

As described above, the D1 polypeptide is known to be involved in the bicarbonate effect, but the role of D2 polypeptide in this effect was unknown at the time I began my thesis work. My aim was to identify the amino acid ligands and structural requirements for bicarbonate binding in PSII. For instance, cationic arginine residues in both D1 and D2 proteins are likely candidates responsible for bicarbonate binding in PSII reaction centers. I planned to use a cyanobacterium Synechocystis sp. PCC 6803 as a model for the reason that it is highly transformable and genetic engineering techniques were available for pinpointing the structure/function relationship in this organism.

Though the presence of the bicarbonate effect had been established in higher plants, nothing was known about the bicarbonate effect in cyanobacteria. The first objective of this thesis was to investigate the bicarbonate effect in this photosynthetic prokaryote (Chapter II). Then the role of certain arginine residues in D2 polypeptide in the bicarbonate effect was examined through site-directed mutagenesis (Chapter III). Another aim of this thesis was to clone psbA gene from Synechocystis 6803 in an effort to set up a site-directed mutagenesis system for the D1 protein (see Chapter IV). I also planned to study the bicarbonate effect using the already available site-selected D1 mutants of cyanobacterial

Synechococcus sp. PCC 7409, altered in single amino acids in the Q_B binding niche, to study the changes in different anion affinity (Chapter V).

Appendices I and II are directed at the use of Chl a fluorescence as a probe of PSII reactions. These were attempts to set up systems with higher plants thylakoids to ultimately apply to cyanobacterial systems in our laboratory.

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CHAPTER II. BICARBONATE EFFECT ON ELECTRON TRANSFER IN PLASTOQUINONE REDUCTASE IN CYANOBACTERIA

A. Introduction

The Hill reaction is significantly and reversibly inhibited in chloroplasts of higher plants depleted of bicarbonate (HCO_3^-) in the presence of formate (see reviews by Vermaas and Govindjee, 1981; Blubaugh and Govindjee, 1988a). A recent hypothesis (see Blubaugh and Govindjee 1988a) is that there are two major sites of "bicarbonate effect" on the electron acceptor side of photosystem II (PSII): (1) as a ligand to Fe^{2+} in Q_A-Fe-Q_B complex that keeps the D1 and D2 proteins in their proper functional conformation (see supportive data and arguments in Vermaas and Rutherford, 1984; Michel and Deisenhofer, 1988; Chapter I, this thesis); (2) as a participant in the protonation of Q_B^- (Blubaugh and Govindjee, 1988a). Arginine has been proposed to be responsible for binding these HCO_3^- ions. A knowledge of the specific binding sites of HCO_3^- in PS II will help in understanding the role of bicarbonate and in clarifying the controversy regarding the site of bicarbonate action (electron acceptor versus electron donor side of PS II; see Stemler, 1982). The molecular genetic approach is a promising avenue to achieve this goal. However, no effective transformation system in higher plants is available that could enable native genes to be removed and replaced with modified genes. On the other

hand, in transformable photosynthetic bacteria, no "bicarbonate effect" has been observed (Shopes et al., 1989). Therefore, it was considered important to look at the "bicarbonate effect" in cyanobacteria, since these organisms have the relative simplicity of being prokaryotic cells, but are fundamentally similar to oxygenic photosynthetic system found in chloroplasts (Stanier and Cohen-Bazire, 1977; Curtis and Haselkorn, 1984; Williams and Chisholm, 1987). The information obtained from cyanobacteria will certainly shed light on the understanding of the photosynthetic mechanism in higher plants. In the present study Synechocystis PCC 6803 was selected as a cyanobaterium model, since molecular-genetic techniques have been developed for probing the structure/function relationships in D1 and D2 proteins in this organism (see e.g., Vermaas et al., 1988). In addition, this unicellular cyanobaterium offers an easier intact system to answer questions related to the "bicarbonate effect" in vivo. The ultimate goal of this study is to use the current molecular-genetic technique as a tool to test the possible sites of bicarbonate binding and to investigate the role of bicarbonate in PSII (see Chapter III).

Few studies on the "bicarbonate effect" in cyanobacteria have been performed. At the time of this research, the only work known was that of Van Rensen and Vermaas (1981). They found no bicarbonate dependence in Anacystis nidulans. However, the thylakoids from Anacystis nidulans used in their

study had low oxygen evolution activity, and thus no difference between bicarbonate depletion and addition could be observed. This may explain their negative results. In Synechocystis 6803 a remarkable reversible inhibition of Q_A^- decay (where Q_A is the first plastoquinone electron acceptor of PS II), after saturating actinic flashes, was observed in the thylakoids depleted of bicarbonate in the presence of formate. Furthermore, the rate of oxygen evolution increased to more than 4-fold when bicarbonate was added to the bicarbonate-depleted intact cells.

B. Materials and Methods

Synechocystis sp. PCC (Pasteur Culture Collection) 6803 was obtained from Dr. H.B. Parkrasi. It was grown in BG-11 medium (Rippka et al., 1979) at a temperature of 28 °C and under continuous illumination of 70 μmol photons m^{-2} s^{-1}. Light was provided by warm white fluorescent lamps (Westinghouse Electric Corporation) (65 μmol photons $m^{-2}s^{-1}$) and incandescent lamps (General Electric) (5 μmol photons m^{-2} s^{-1}). The liquid culture was constantly bubbled with air pre-humidified by bubbling through a 1.0% aqueous $CuSO_4$ solution. $CuSO_4$ was added to prevent the growth of contaminating organisms in the water flask. Cells in logarithmic growth phase were used throughout our studies.

Thylakoid isolation was essentially as described by Vermaas et al. (1986). Cells were first suspended in a medium

containing 20 mM HEPES (pH 7.5), 0.3 M sucrose, 2 mM $MgCl_2$, 0.1% BSA and 1 mM EDTA before they were broken. Thylakoids were suspended in a medium containing 20 mM HEPES (pH 7.5), 0.3 M sucrose, and 2 mM $MgCl_2$.

Bicarbonate-depleted and bicarbonate-recovered samples were prepared as described by Eaton-Rye and Govindjee (1988). Depletion medium contained 0.3 M sorbitol, 25 mM $NaHCO_2$, 10 mM NaCl, 5 mM $MgCl_2$ and 10 mM NaH_2PO_4 (pH 5.8). The bicarbonate depleted samples were incubated for 4 hours in the depletion medium (pH 5.8) over which N_2 gas was passed. Nitrite and azide treatments were done following the protocol of formate treatment at pH 6.5. The reaction medium contained 0.1 M sorbitol, 20 mM $NaHCO_2$, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 μM gramicidin D and 20 mM NaH_2PO_4 (pH 6.5) or 20 mM HEPES (pH 7.5).

The kinetics of decay of variable chlorophyll a fluorescence were measured at 685 nm (10 nm bandwidth) by a weak measuring flash. The measuring flash was fired at variable times after each actinic flash. The actinic flash (FX-124, EG and G) and the measuring flash (Stroboslave 1593A, General Radio) were filtered with Corning blue (CS 4-96) glass filters; both had a 2.5 μs duration at half-maximal peak. For further details, see Eaton-Rye (1987; see pp 35-38) and Eaton-Rye and Govindjee (1988). Chlorophyll a concentration was 10 μg/ml.

Oxygen evolution was determined polarographically using

a Yellow Spring Instrument Clark-type electrode in a saturating yellow light (for details, see Blubaugh, 1987; pp 43-44). A combination of two electron acceptors, DMQ and $K_3Fe(CN)_6$, was used in the present study. Here, DMQ acts as the electron acceptor and ferricyanide keeps the DMQ in the oxidized state. DBMIB was used to block electron flow beyond the plastoquinone pool, but before photosystem I (Trebst, 1980). Other details are given under Results and Discussion.

C. Results and Discussion

Figure 2.1 shows Q_A^- decay after 3 actinic flashes (dark time between flashes, 1s) at pH 6.5(A) and pH 7.5(B) in Synechocystis 6803 thylakoids. Q_A^- concentration was estimated from variable Chl a fluorescence according to Joliot and Joliot (1964), using the formula given by Mathis and Paillotin (1981). The connection parameter (p) was assumed to be 0.5 (for justification, see Bowes and Bendall, 1983). $[Q_A^-]$ is arbitrarily defined as 1 when fluorescence reaches the maximum. At both the pH values, the HCO_3^- depletion significantly slowed down the Q_A^- decay. By adding 2.5 mM HCO_3^- to the CO_2-depleted (formate-treated) samples, the inhibition of Q_A^- oxidation was relieved and the decay curve was fully restored to that of the control thylakoids. Govindjee et al. (1976) had found a very large slowing down of Q_A^- decay after three or more flashes in HCO_3^- depleted higher plant chloroplasts. Robinson et al. (1984) demonstrated that the Q_A^-

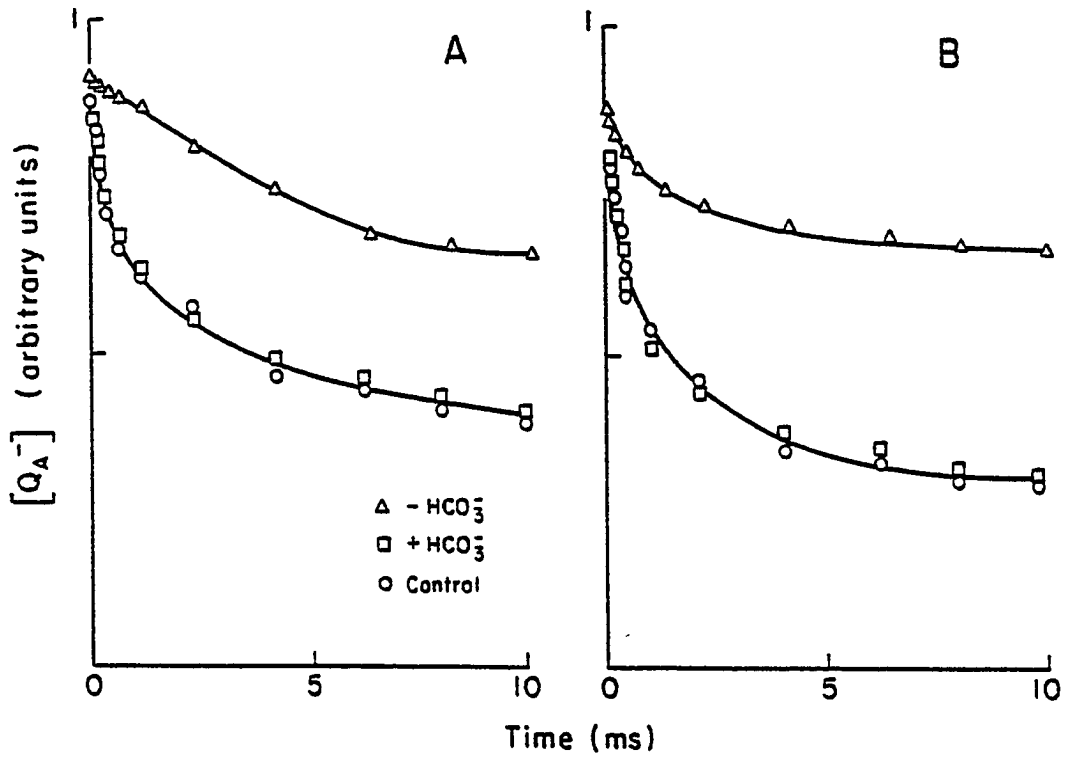


Figure 2.1. Decay of Q_A^- after 3 flashes in thylakoids of Synechocystis 6803 at pH 6.5 (A) and pH 7.5 (B). The flash frequency was 1 Hz. (O) control; (Δ) bicarbonate-depleted for four hours; (□) restored by adding 2.5 mM HCO_3^-. $[Q_A^-]$ is the concentration of the reduced Q_A.

decays after three or more flashes were 36-fold slower in the bicarbonate-depleted chloroplasts. The results obtained after 1 and 2 flashes in bicarbonate-depleted thylakoids of the cyanobaterium showed inhibition of Q_A^- decay consistent with that obtained with higher plant thylakoids (data not shown).

After an odd number of flashes, the following equilibrium exists: $Q_A^- Q_B \leftrightarrow Q_A Q_B^-$, but a biphasic decay is observed. This biphasic decay of Q_A^- in chloroplasts has been discussed by Robinson and Crofts (1983). After a flash, the fast phase is suggested to be due to the electron transport in centers that have Q_B bound before the actinic flash, while the slow phase is suggested to be a second order process involving the binding of Q_B from the PQ pool. Both the fast and the slow phases were observed in Q_A^- decay in the thylakoids of the cyanobacterium. The amplitudes and halftimes, from four experiments for data up to 1 s, are shown in Table 2.1. At pH 6.5, halftimes were 403±5 μs (fast) and 26±1 ms (slow). At pH 7.5, somewhat shorter halftimes (331±3 μs (fast) and 21±1 ms (slow)) were observed. Bicarbonate depletion did not lead to significant changes in the halftimes of Q_A^- decay suggesting that these may be from centers that still had bound HCO_3^- (see Blubaugh and Govindjee, 1988b). At pH 6.5, HCO_3^- depletion led to a decrease in the amplitude of the fast component from 42% to 14%, and an increase in the amplitude of the slow component from 58 % to 86 %. At pH 7.5, HCO_3^- depletion led to a decrease in the amplitude of the fast component from 56 % to 26 %, and

an increase of the slow component from 45 % to 74 %. After 2.5 mM HCO_3^- was added, the amplitudes were restored to those of the controls (Table 2.1) proving complete reversibility.

Five μM DCMU almost eliminated the decay of Q_A^- in thylakoids from Synechocystis (Figure 2.2). This inhibitor is known to block the reoxidation of Q_A^- by displacing Q_B from its binding site (Velthuys, 1981). Thus, by analogy, bicarbonate depletion must also impose a block on the electron transport from Q_A^- to the PQ pool.

The inhibition of electron transport was also observed when, instead of formate (Figure 2.3A), nitrite (Figure 2.3B) or azide (Figure 2.3C) anion was used. The inhibitory effect followed the order (most to least effective): nitrite > formate > azide according to the inhibition of the reoxidation of Q_A^- at 2 ms. The inhibitory effect of nitrite was fully reversed by bicarbonate ions. However, addition of 5 mM bicarbonate to 100 mM azide-treated samples partially restored Q_A^- oxidation; at 2 ms, only 50% of the inhibition of Q_A^- oxidation was reversed. Stemler and Murphy (1985) were able to obtain a 3.6-fold stimulation of the Hill reaction by the addition of 5 mM bicarbonate to thylakoids that had been incubated with 20 mM nitrite. Eaton-Rye et al. (1986) observed a 7 fold bicarbonate-induced increase in steady state oxygen evolution in nitrite-treated thylakoids. An irreversibility of the inhibition of Q_A-Q_B electron transfer has been observed with other inhibitors. For instance, disulfiram

Table 2.1. The amplitude (A) and the halftime ($t_{1/2}$) of the fast (f) and slow (s) Q_A^- decay components in control, HCO_3^- depleted and HCO_3^- restored thylakoids of Synechocystis 6803 at pH 6.5 and pH 7.5. The parameters were calculated from the data after 3 actinic flashes. The flash frequency was 1 Hz.

Treatment	pH of reaction medium	A_f (%)	A_s (%)	$t_{1/2}$ (f) (μs)	$t_{1/2}$ (s) (ms)
Control	6.5	42 ± 1[a]	58 ± 1	403 ± 5	26 ± 1
	7.5	56 ± 2	44 ± 2	331 ± 3	21 ± 1
HCO_3^- depleted	6.5	14 ± 1	86 ± 2	433 ± 6	26 ± 1
	7.5	26 ± 1	74 ± 2	340 ± 3	33 ± 2
Restored	6.5	42 ± 1	58 ± 1	395 ± 4	25 ± 1
	7.5	57 ± 2	43 ± 2	328 ± 2	22 ± 1

a: standard deviations; number of experiments averaged, 4.

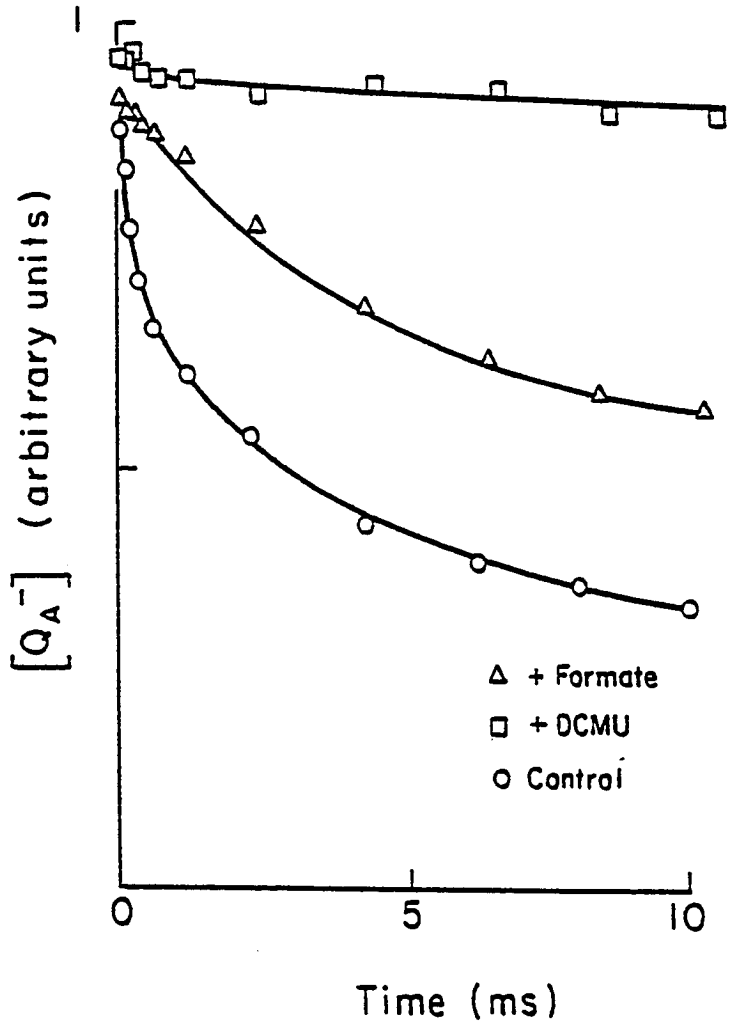
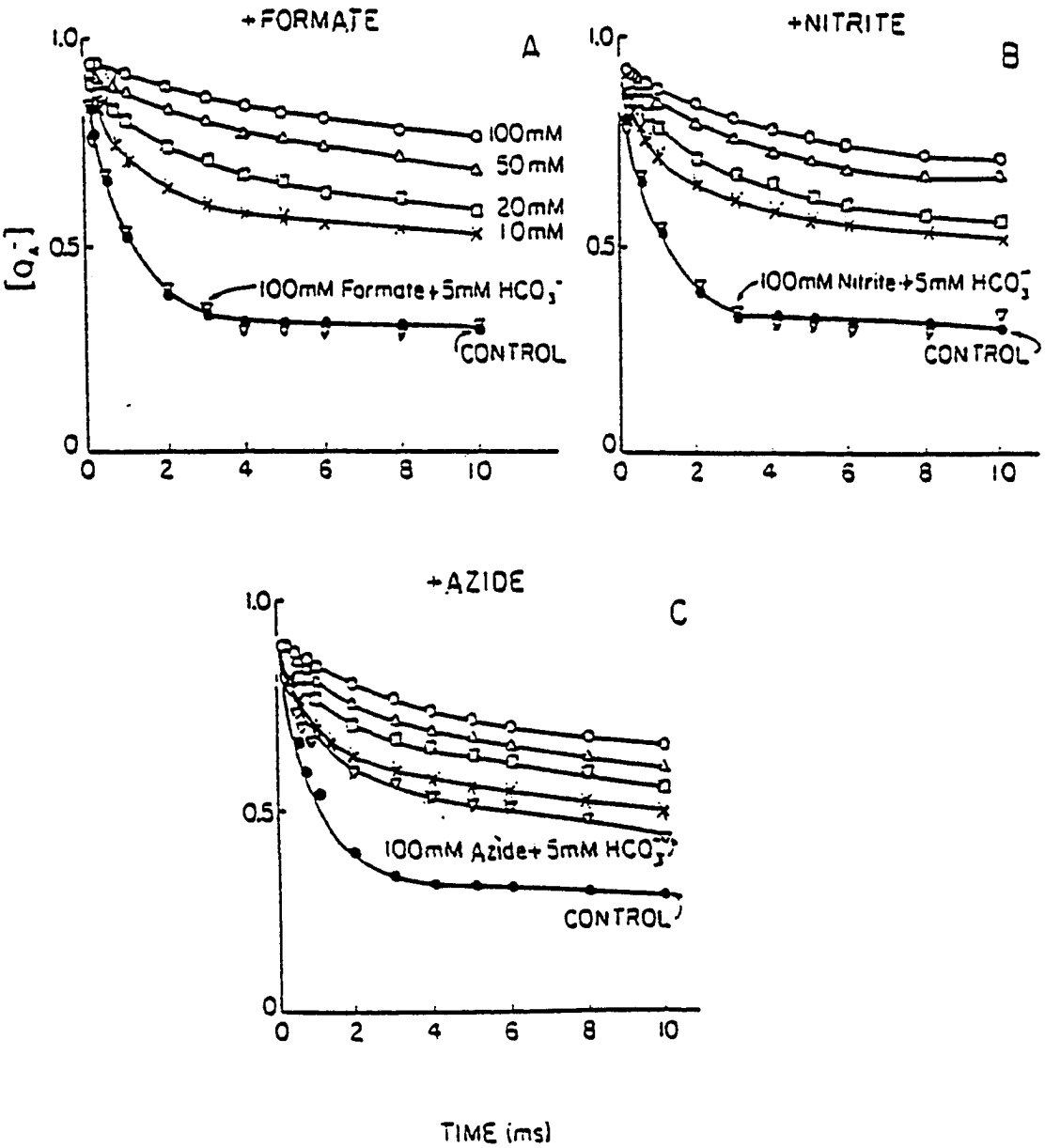


Figure 2.2. Decay of Q_A^- after 3 actinic flashes in control (○), and DCMU-treated (□) thylakoids of Synechocystis 6803 at pH 6.5. For comparison with the bicarbonate-depletion effect, Q_A^- decay in a HCO_3^- depleted sample from Fig 1A is also shown (Δ). The DCMU concentration was 5 μM. [Q_A^-] is the concentration of the reduced Q_A.

Figure 2.3. The inhibitory effects of different concentrations of formate, nitrite and azide on Q_A^- decay in Synechocystis cells. The measurement was made after the incubation of anions for 15 min.



(tetraethylthiuram disulfide), a potent metal chelator which may complex with Fe^{2+}, greatly slows down electron transfer from Q_A^- to Q_B following one or more flashes (Blubaugh and Govindjee, 1988c). CN^-, a possible ligand to Fe^{2+}, also slows Q_A to Q_B electron transfer similar to that observed for formate and NO (Diner and Petrouleas, 1990). In contrast to the reversibility by bicarbonate of the formate and NO effects, the effects of disulfiram and CN^- are irreversible. All of the above anionic or nonanionic agents, whether they produce reversible or irreversible bicarbonate effect have interactions or possible interactions with the non-heme iron. It has been known that in the PSII, the midpoint redox potential of Fe(II)/Fe(III) is significantly lower than in purple bacteria (Rutherford, 1987) which do not show the bicarbonate effect (Shopes et al., 1989). In PSII, but not in purple bacteria, Fe(II) can be oxidized to Fe(III) by the addition of ferricyanide or certain quinones (Zimmermann and Rutherford, 1986; Petrouleas and Diner, 1987; Diner and Petrouleas, 1987). However, oxidation of Fe^{2+} by ferricyanide is inhibited after bicarbonate depletion by formate treatment (Zimmermann and Rutherford, 1986), suggesting that removal of bicarbonate greatly increases the Fe(II)/Fe(III) midpoint potential.

The effect of bicarbonate on electron transport rates (O_2 evolution with DMQ as electron acceptor) in bicarbonate-depleted cells, following an addition of 20 mM HCO_3^-, is shown

in Figure 2.4 and Table 2.2. After 4 hours of bicarbonate depletion, the oxygen evolution rate was 37 μmol O_2 (mg Chl*a*)$^{-1}$ h^{-1}. The addition of 20 mM bicarbonate (pH adjusted to pH 6.5) stimulated the oxygen evolution rate to 263 μmol (mg Chla)$^{-1}$ h^{-1}. For cells of *Synechocystis* 6803, bicarbonate was also a source of CO_2 for carbon reduction since an oxygen evolution rate of 99 μmol (mg Chl*a*)$^{-1}$ h^{-1} was observed in bicarbonate-recovered samples in the absence of artificial electron acceptors. The net stimulation (corrected for CO_2 fixation) in electron transport rate from water to DMQ by the addition of bicarbonate was 4.4 fold. To be sure that our conclusion was not affected by CO_2 fixation, an experiment was done in which an inhibitor DBMIB, which blocks electron transport flow between plastoquinone pool and photosystem I (Trebst, 1980), was used. In *Synechocystis* cells, a minimum of 20 μM DBMIB in the reaction mixture was found necessary to cause a practically complete blockage of electron transport. In chloroplasts of higher plants, however, lower concentration of DBMIB (0.2-1 μM) can inhibit electron flow. The difference may lie in the different accessibility of DBMIB in the reaction medium to the thylakoids in the whole cells and in the chloroplasts. Here, there was no electron flow after the addition of bicarbonate when there was no electron acceptor. In the presence of DBMIB and the electron acceptors DMQ and ferricyanide, addition of 20 mM bicarbonate stimulated the electron transport rate by 4.2 fold. Similar stimulatory

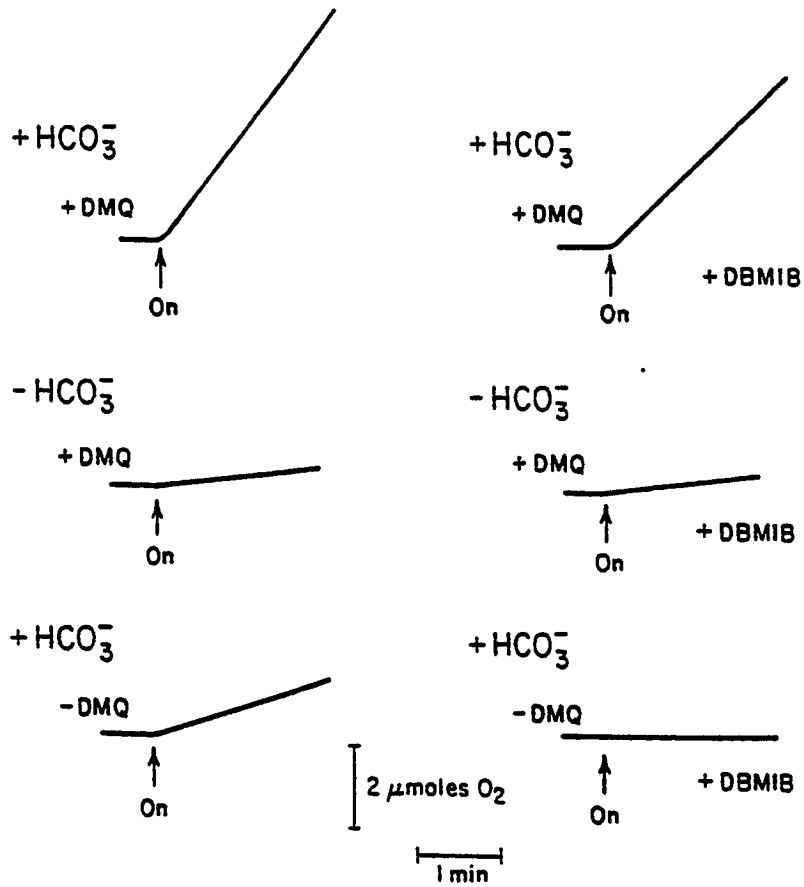


Figure 2.4. Effect of bicarbonate on oxygen evolution in formate-treated Synechocystis cells. Cells containing 20 μg Chl a ml^{-1} were used. The reaction medium was adjusted to pH 6.5 after the addition of 5 mM HCO_3^-. A combination of two electron acceptors (DMQ, 0.5 mM; $K_3Fe(CN)_6$, 1 mM) was used. Twenty μM DBMIB was included to block electron transfer beyond plastoquinone pool, but before photosystem I. **Left:** middle: with DMQ and formate; top: same as middle, with bicarbonate; bottom: without DMQ, but with bicarbonate. **Right:** middle: with DMQ, DBMIB and formate; top: same as middle, with bicarbonate; bottom: without DMQ, but with DBMIB and bicarbonate.

Table 2.2. Effect of bicarbonate on oxygen evolution in bicarbonate-depleted cells of Synechocystis 6803. The 2 ml reaction mixture contained 0.1 M sorbitol, 25 mM sodium formate, 10 mM Nacl, 5 mM MgCl, 20 mM NaH_2PO_4 (pH 6.5), and 0.1 μM gramicidin D. The reaction medium was adjusted to pH 6.5 after addition of 20 mM HCO_3^-. Depletion time was 4h. Concentration of the inhibitor DBMIB was 20 μM. Artificial electron acceptors were DMQ (0.5 mM) and $K_3Fe(CN)_6$. Cells of Synechocystis 6803 containing 20 μg Chl a ml^{-1} were used.

Rate of oxygen evolution (μmol O_2 (mg Chl a)$^{-1}$ h^{-1})			Ratio $+HCO_3^-/-HCO_3^-$	
A	B	C	C-B	C-B/A
$-HCO_3$ plus DMQ and $K_3Fe(CN)_6$	+20 mM HCO_3^- minus DMQ and $K_3Fe(CN)_6$	+ 20 mM HCO_3^- plus DMQ and $K_3Fe(CN)_6$		
37.2 ± 4[a]	99.4 ± 14	263.3 ± 32	163.9 ± 17	4.4
plus DBMIB				
35.7 ± 2	0.0 ± 1	150 ± 14	150.3 ± 7	4.2

a: standard deviations; number of experiments averaged, 4

effect of bicarbonate was also observed in the presence of an uncoupler S_{13}, a salicylanilide (data not shown), that also eliminates CO_2 fixation. Thus, the bicarbonate effect observed here is clearly unrelated to CO_2 fixation.

A maximum bicarbonate effect was found at approximately pH 6.7 (Figure 2.5). This result may be superimposed by the pH dependence of PSII oxygen evolution activity. Blubaugh and Govindjee (1988b) have shown that HCO_3^- is the active species involved in spinach thylakoids; we assume it to be the same here.

The data in Table 2.2 show that 4-hour bicarbonate depletion produces a significant "bicarbonate effect" in intact Synechocystis 6803 cells independent of CO_2 fixation. Results presented in Figures 2.1 and 2.2 show the existence of the "bicarbonate effect" in both intact cells and thylakoids of Synechocystis 6803. However, Van Rensen and Vermaas (1981), who had measured the Hill reactions in the thylakoids of a cyanobacterium Anacystis nidulans, found no such bicarbonate effect. The reason for this negative result is not known. Nevertheless, the data in the current study establishes the existence of bicarbonate effect in a cyanobacterium and, thus, makes it easier to unravel, in the future, the mechanism of the bicarbonate effect on the electron transport. A bicarbonate effect, through measurements on the EPR signal of Q_A^-Fe, in a cyanobacterium was independently reported by Nugent et al., (1988).

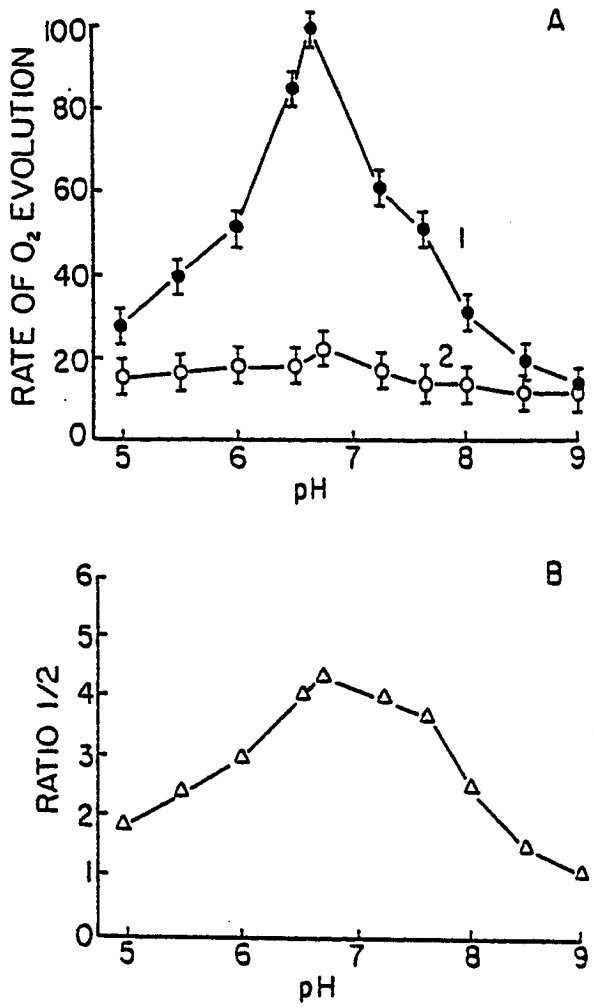


Figure 2.5. (A) rate of oxygen evolution as a function of pH in formate-treated (O) and bicarbonate-recovered (•) Synechocystis cells. 10 arbitrary units = 15 μ mol O2 $(mgChl\underline{a})^{-1}h^{-1}$. (B) the ratio of oxygen evolution in bicarbonate-recovered cells (1) and the formate-treated cells (2).

Data presented in this chapter are based on the published work of the author (Cao and Govindjee, 1988, 1990).

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CHAPTER III. CONSTRUCTION AND CHARACTERIZATION OF SITE-DIRECTED MUTANTS OF D2 POLYPEPTIDE IN *SYNECHOCYSTIS* SP. PCC 6803

A. Introduction

A stimulatory effect of CO_2 on Hill reaction was observed by Warburg and Krippahl (1960) who hypothesized that this effect was on oxygen evolution, currently known as the electron donor side of PSII (see Warburg, 1964). However, the measurement of Chl *a* fluorescence induction kinetics in chloroplasts indicated that the effect of bicarbonate was on the electron acceptor side of PSII (Wydrzynski and Govindjee, 1975). A major effect of bicarbonate-reversible formate inhibition was later shown to be on the electron transfer from Q_A^- to the plastoquinone (PQ) pool , where Q_A is the one-electron acceptor bound plastoquinone (Govindjee et al., 1976; Jursinic et al., 1976; Siggel et al., 1977; Eaton-Rye and Govindjee, 1988a). Two major bicarbonate action sites have been suggested to exist on the PSII reaction center: one on the reaction center non-heme iron between Q_A and Q_B, where Q_B is the two-electron acceptor bound plastoquinone, and the other on amino acid residues involved in protonation of Q_B^- (see review by Blubaugh and Govindjee, 1988). The identification of the former site was derived from the observations that treatment with formate enhances greatly the g=1.82 form of the $Q_A^-Fe(II)$ EPR signal and that this

enhancement is reversed after bicarbonate addition (Vermaas and Rutherford, 1984). Furthermore, Fe(II)-NO EPR signal at g=4 is diminished by the addition of $NaHCO_3$ (Diner and Petrouleas, 1987; 1990). Formate has now been shown to decrease the quadrupole splitting and the chemical shift of the non-heme Fe(II) Mossbauer spectrum (Petrouleas and Diner, 1987; Semin et al., 1990). Thus, binding of HCO_3^-/CO_2 to non-heme iron is supported. The possibility of a second site, dealing with protonation, was based on the observations that electron transport from Q_A^- to Q_B^- in bicarbonate depleted thylakoid membranes is pH dependent (Eaton-Rye and Govindjee, 1988a,b) and the maximum inhibition of electron transfer occurs after two or more actinic flashes (Govindjee et al., 1976; Robinson et al., 1984; Eaton-Rye and Govindjee, 1988a,b; Xu et al., 1991). This was explained by assuming that the large inhibition after second and subsequent flashes is due to an inhibition of protonation of Q_B^-, and an arginine residue in the D1 protein has been proposed to be a plausible ligand to bicarbonate anion (Shipman, 1981; Crofts et al., 1987; Blubaugh and Govindjee, 1988).

The bicarbonate effect on electron transport between Q_A and Q_B in plants (see Vermaas and Govindjee, 1981; Blubaugh and Govindjee, 1988 for reviews) and cyanobacteria (Cao and Govindjee, 1988; Nugent et al., 1988) has been well established. However, no effect of bicarbonate on electron transport between the two quinones has been observed in purple

photosynthetic bacteria (Shopes et al., 1989) or in the green bacterium Chloroflexus aurantiacus(Govindjee et al., 1991a). This shows a fundamental difference between bacterial and PSII reaction centers. The isolation, crystallization, and the X-ray structure of the reaction center complex from the photosynthetic bacterium Rhodopseudomonas viridis (Deisenhofer et al., 1985; Michel and Deisenhofer, 1986) provided a stimulus for the understanding of the PSII reaction center because the primary structures of PSII D1 and D2 polypeptides show similarities to those of bacterial L and M subunits, respectively. In purple bacteria, a glutamate (Glu) residue in the M subunit provides two ligands to the non-heme iron. However, this Glu residue is not conserved in the D2 protein. Thus, Michel and Deisenhofer (1988) suggested that bicarbonate may act as a bidentate ligand to the iron in PSII. The replacement of M-E234 in Rb. sphaeroides by valine, glutamine or glycine does not affect the Q_A-Q_B electron transfer, indicating that the glutamic acid is not crucial to the function of the $Q_A Fe Q_B$ complex in bacterial reaction centers (Wang et al., 1991).

Indications for the involvement of the D1 protein in the bicarbonate effect has come from the studies of herbicide and bicarbonate interaction, and of herbicide-resistance mutants. Urea, triazine and phenol type herbicides (known to bind to D1 protein) decrease the apparent affinity of the thylakoid membrane for bicarbonate (Van Rensen and Vermaas, 1981; Snel

and Van Rensen, 1983). On the other hand, bicarbonate depletion affects the binding of herbicides (Khanna et al., 1981). A herbicide resistant mutant, S264G on D1 protein, of Amaranthus hybridus showed a 2 fold increase in dissociation constant of bicarbonate binding (Khanna et al., 1981). Studies on several herbicide-resistant D1 mutants of the cyanobacterium Synechocystis 6714 (Govindjee et al., 1990) and of Chlamydomonas reinhardtii (Govindjee et al., 1991b) demonstrate differential formate sensitivities. Formate replaces bicarbonate in PSII.

In the absence of a PSII crystal structure, the most straightforward approach to verification of the function of amino acid residues that are presumed to bind components at the acceptor side, and to identification of residues that affect bicarbonate binding is to change these residues into amino acids that are expected to be unable to, or less able to, function appropriately. Subsequently, the effects of such replacements on the function and structure of the reaction center complex can be monitored. A specific amino acid replacement is possible through in vitro site-directed mutagenesis of the gene coding for the polypeptide into which the mutation should be made, followed by introduction of the modified gene into the organism of choice. As was reviewed earlier (Vermaas et al., 1989; Vermaas et al., 1990a), the cyanobacterium Synechocystis sp. PCC 6803 is eminently suitable for this approach since it can grow in the absence of

PSII activity when the medium is supplemented with glucose, and it is spontaneously transformable with exogenous recombination (reviewed by Williams, 1988). A number of prokaryotic antibiotic-resistance genes has been found to be useful as markers in Synechocystis a well, making simple selection of transformants possible.

On the basis of homologies with the reaction center from purple bacteria, D2 protein, like D1 protein, is thought to contribute to the binding of the reaction center Chl a P_{680} and the non-heme iron. It appears that D2 creates most of the binding niche for Q_A and a pheophytin, whereas D1 harbors Q_B and another pheophytin (Michel and Deisenhofer, 1986; Mathis and Rutherford, 1987; Rutherford, 1987). Site-directed mutagenesis has led to the identity of Tyr-161 residue in D1 protein as Z, the electron donor to P_{680}^+ (Debus et al., 1988a; Metz et al., 1989) and Tyr-160 residue in the D2 polypeptide of Synechocystis 6803 as the accessory slow PSII donor, D (Debus et al., 1988b; Vermaas et al., 1988). His-214 and Trp-253 in D2, which presumably closely interact with Q_A, are essential to keep the stability of the entire reaction center (Vermaas et al., 1990a). Furthermore, Glu-69 residue of the D2 is shown to be a potential ligand to Mn involved in photosynthetic oxygen evolution (Vermaas et al., 1990b). The role of the D2 protein in the bicarbonate effect had been obscure until my observation (this Chapter) that two D2 arginine (Arg-233 and Arg-251) mutants (see abstract by Cao et

al., 1989) were extremely sensitive to formate (also see abstract by Cao et al., 1990c), and that by D. Chisholm and B. Diner (unpublished, cited in Diner et al., 1991) on two other D2 mutants (at Lys-264 and Arg-265) having slowed Q_A-Q_B electron transfer even in the presence of 10 mM bicarbonate. Diner et al. (1991) suggested that D2-lys-264 and D2-arg-265 possibly play a role in binding HCO_3^-/CO_2 which in turn make ligands to the non-heme iron.

In this Chapter, I present results on the differential sensitivity to formate in two site-directed arginine mutants of Synechocystis sp. Pasteur Culture Collection 6803 (referred to as Synechocystis 6803) in which Arg-233 is changed into glutamine and Arg-251 into serine. In comparison with the wild type, the mutants showed a slightly slow photoautotrophic growth rate and more than ten times greater inhibition by formate treatment in the rate of oxygen evolution and twice to three times slowing of electron transfer between Q_A and Q_B. This study suggests that Arg-233 and Arg-251 in the D2 polypeptide function to stabilize HCO_3^- binding in PSII of Synechocystis 6803.

B. Materials and Methods

1. Site-directed mutagenesis

The recombinant phage and plasmid DNA and the double deletion mutant described in this chapter had been constructed in the laboratory of Professor Wim Vermaas (Vermaas et al.,

1990a). A procedure combining protocols developed by Kunkel and coworkers (Kunkel, 1985; Kunkel et al., 1987) and Vandeyar et al. (1988) was utilized for site-directed mutagenesis of psbDI in E. coli. A 2.9 kb EcoRV/EcoRI fragment carrying psbDI as well as 1 kb from upstream of the gene and 0.9 kb of the CP43-encoding gene psbC, the beginning of which overlaps with the end of psbDI, was cloned into the bacteriophage M13 mp19 (see Figure 3.1). The construct contains several restriction sites that can be used to cut certain portion of the psbDI after site-directed mutagenesis. The first part of the mutagenesis protocol was essentially identical to what is recommended by Biorad in its mutagenesis kit manual. The recombinant phage (Figure 3.1) was grown in E. coli. strain CJ236. This strain is $Dut^{-}Ung^{-}$ (dUTPase and Uracil-N-glycosylase deficient), and allows occasional stable incorporation of dU rather than dT into its DNA and into M13. Viral single-stranded DNA was isolated, and a 5'-phosphorylated mutagenic oligonucleotide (15-18 nucleotide long) having 1-3 internal mismatches (see Table 3.1) with the wild-type sequence at a specific region of the gene was hybridized to the viral DNA. The complementary strand was synthesized (with the oligonucleotide as a primer) using T4 polymerase, dATP, dGTP and dTTP; in the reaction mixture, dCTP was replaced by methyl-dCTP for reasons explained in the next paragraph. The newly synthesized strand was circularized with T4 ligase.

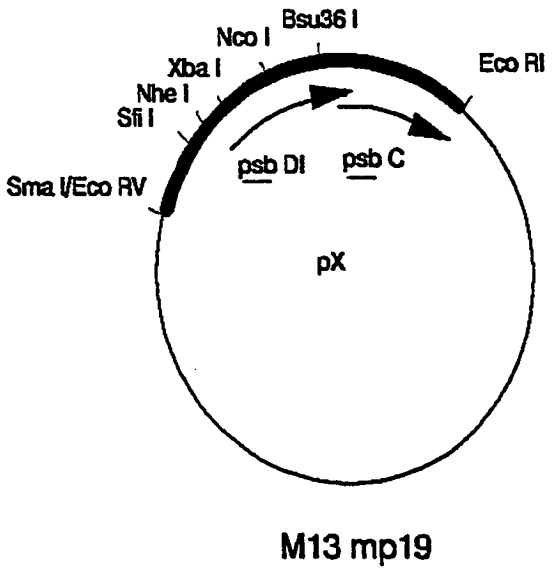


Figure 3.1. The recombinant M13 phage containing psbDI gene. A 2.9 kb of EcoRV/EcoRI fragment carrying psb DI as well as 1 kb from upstream of the gene and 0.9 kb of the CP43 encoding gene psbC was cloned into the bacteriophage M13mp19 (Vermaas et al., 1990a).

Table 3.1. List of the oligonucleotides used in site-directed mutagenesis of *Synechocystis* 6803

Mutation	Codon change	Oligonucleotide
R233 → Q	CGG → CTG	AT ACT TTC CTG GCA TTT G
R251 → S	CGT → AGT	CC GCT AAC AGT TTC TGG
R251 → Q	CGT → TGT	CC GCT AAC TGT TTC TGG
R251 → C	CGT → CAA	CC GCT AAC CAA TTC TGG

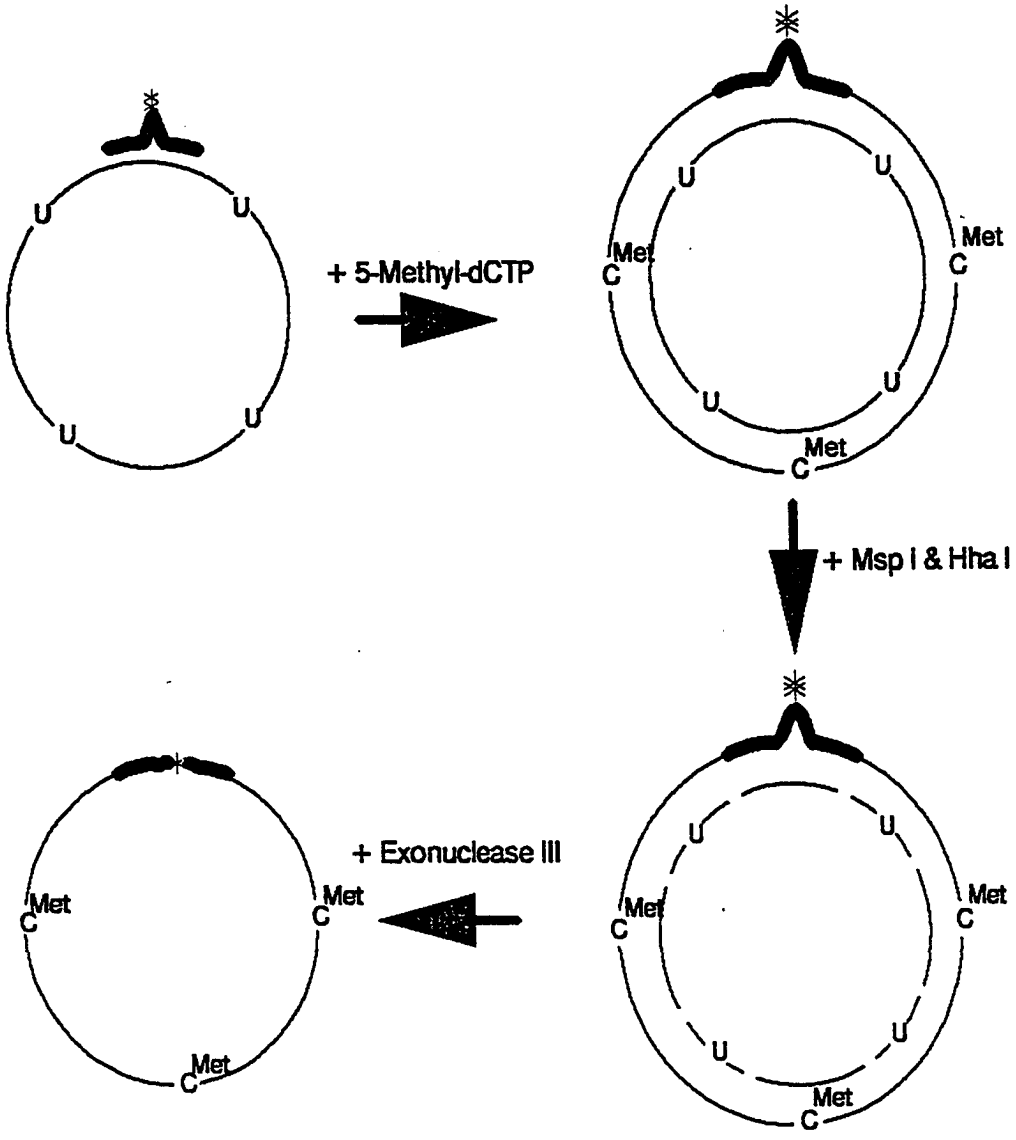
The resulting duplex DNA was transformed into an E. coli strain that was dut$^+$ung$^+$, in which the dU-containing wild-type strand has a selective disadvantage. Additionally, an independent mutant enrichment procedure developed by Vandeyar et al. (1988) was used: this method calls for the use of methyl-dCTP rather than normal dCTP when synthesizing the mutant M13 strand. After a circular double stranded DNA was closed by T4 ligase, the non-methylated wild type strand was nicked with restriction enzymes MspI and Hha I and treated with exonuclease II (Figure 3.2). Mutations were verified by sequencing the dideoxynucleotide chain-termination method (Sanger et al., 1977).

2. Growth conditions and transformation of Synechocystis

Wild type Synechocystis 6803 and the mutants were grown either in liquid media or in agar plates under constant illumination as described by Williams (1988). The mutants were maintained on media supplemented with appropriate antibiotics. A site-directed mutation was introduced into the double deletion strain of Synechocystis 6803 (Vermaas et al., 1990a). Transformation was carried out with a plasmid containing a single base change in the psbDI/C operon with a kanamycin-resistance cartridge down stream of psb C. Transformants were then identified by their resistance to kanamycin (Vermaas et al., 1990a).

A Synechocystis double-deletion mutant was created by two successive transformations of the wild type cyanobacterium

Figure 3.2. A combination of Kunkel's method with the method of Vanderyar et al. for site-directed mutagenesis (Vermaas et al., 1990a).



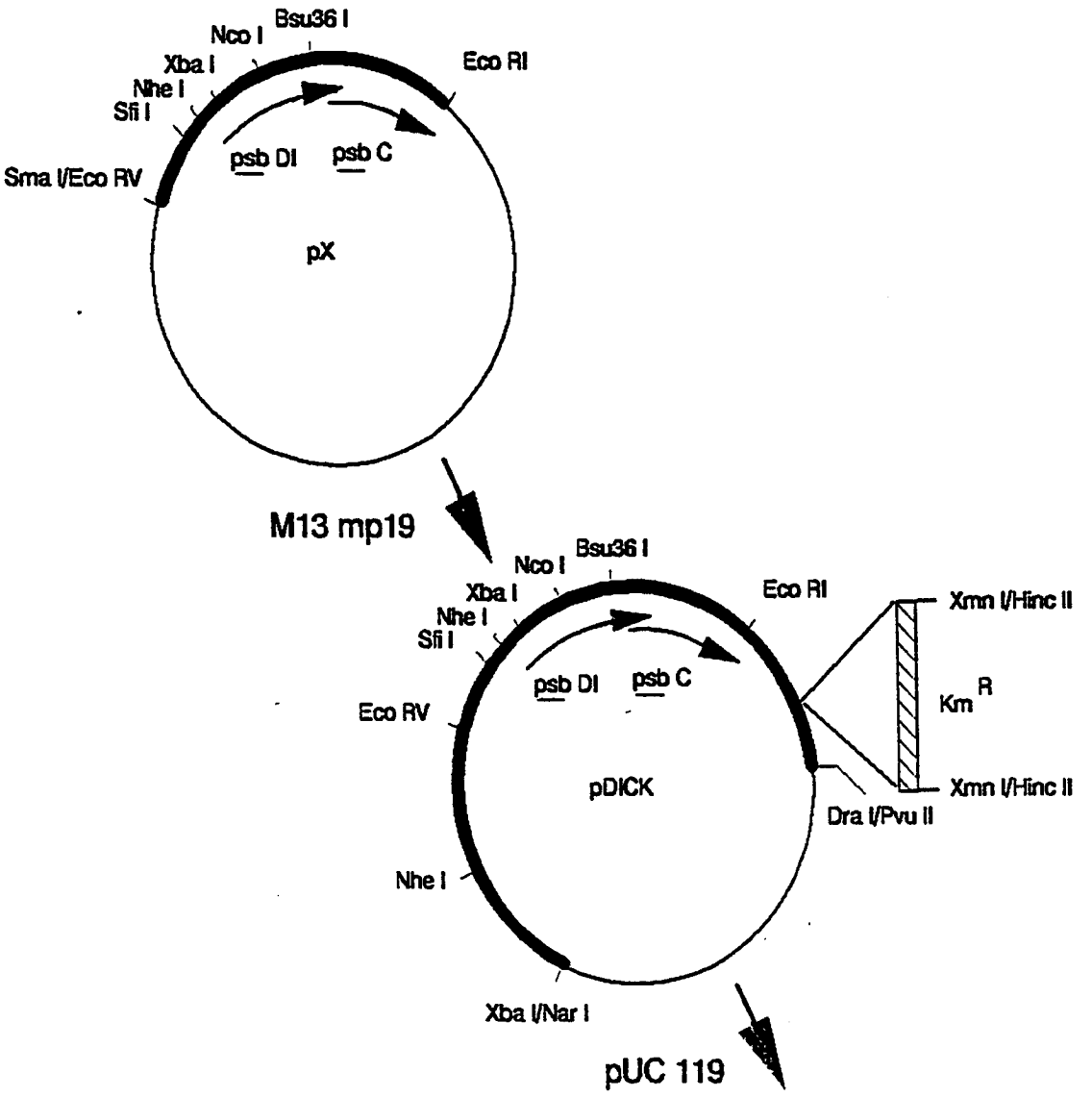
with a plasmid containing a chloramphenicol-resistance cartridge flanked on one side by psbDI and on the other side by psbC, and a plasmid containing a spectinomycin-resistance cartridge flanked by PsbDII (Vermaas et al., 1990a). The thus created double-deletion mutant lacks all of psbDI and all but 161 bp at the 5' end of psbDII. This mutant can be used as an acceptor strain for reintroduction of psbDI provided that the plasmid carrying psbDI has sufficient length of flanking sequencing regions on both sides to efficiently transform the double-deletion mutant. Figure 3.3 outlines the plasmid, pDICK, that was used for reintroduction of psbDI along with psbC. This plasmid has a 2.4 kbp XbaI/EcoRV fragment upstream of psbDI in common with the double-deletion cyanobacterial mutant, and a 0.4 kbp XmnI/DraI fragment downstream of psbC. A kanamycin-resistance cartridge had already been introduced at the XmnI site downstream of psbC to allow selection of transformants.

The XbaI/EcoRI fragment of the mutated psbDI gene in M13 mp19 phage vector was transferred to the pUC 119 vector containing psbDI gene and psbC gene with a K_m resistance cartridge at its downstream. The pUC 119 vector was then used to transform a psbDI/C/DII-deletion strain (Figure 3.3).

After transformation of the double-deletion mutant with the mutated psbDI-containing plasmid, the mutants can be functionally and structurally analyzed.

3. Growth curve assay

Figure 3.3. The strategy for site-directed mutagenesis. The M13mp 19 phage vector pX contains the mutated psbDI gene as well as psbC gene. Xba I/EcoR I fragment of pX was transferred to the pUC119 vector with a kanamycin resistant (K_m^R) cartridge inserted downstream of psbC gene. This new vector was labelled as pDICK (DI for psbDI gene, C for psbC gene and K for kanamycin). The pDICK was then used to transform a Synechocystis 6803 strain that lacked psbDI/C/DII genes (Vermaas et al., 1990a)



Transform the double-deletion mutant

For photoheterotrophic growth, 5 mM glucose was included in the culture medium. For measuring growth rates, the cyanobacterial cells were cultivated in BG-11 media and started at an optical density of 0.08 at 730 nm as measured with a Shimadzu (Kyoto, Japan) UV-160 spectrometer. The optical density at 730 nm represents scattering by cyanobacterial cells.

4. Herbicide binding assay

To quantify the diuron (also referred to as DCMU) affinity and the number of diuron binding sites on a Chl basis in whole cells of the wild type and mutants, cells were incubated with various concentrations of ^{14}C-labelled diuron (Amersham) in the dark at room temperature for 15 minutes. The Chl *a* concentration was 25 μg/ml. The amount of specifically bound diuron was calculated from the difference between the amount of free diuron found after centrifugation in the supernatant of the cell suspensions in the absence and presence of 20 μM unlabeled atrazine (for details, see Vermaas et al., 1990c). The diuron dissociation constant and the maximum number of diuron binding sites were calculated according to Tischer and Strotmann (1977).

5. Steady-state oxygen evolution

The steady-state oxygen evolution in cyanobacterial cells was determined polarographically, under the saturating light (3600 μmoles of photons/$m^2 \cdot s$) filtered with a Corning CS3-68 yellow filter, using a Yellow Springs Instrument Clark-type

electrode. The temperature was 25 ^{0}C. A combination of two electron acceptors, 2,5-dimethyl-p-benzoquinone (DMQ) (0.5 mM) and $K_3Fe(CN)_6$ (1 mM), was used. DMQ acts as the electron acceptor and the non-penetrating ferricyanide keeps the DMQ in the oxidized state. Twenty μM 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) was added in the reaction medium to block electron flow between the plastoquinone pool and PSI (Trebst, 1980). The Chl a concentration used for oxygen evolution measurements was 20 μg/ml. The suspension medium was the reaction medium containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 μM gramicidin D and 20 mM HEPES (pH 6.8). The reaction medium was adjusted to pH 6.8 after addition of 10 mM bicarbonate (Cao and Govindjee, 1988).

6. Flash dependence of oxygen evolution

Measurements of oxygen flash yield pattern of cyanobacterial cells upon excitation by Xenon flashes were made with a bare-platinum electrode, as described earlier (Jursinic and Stemler, 1984). The sample was applied directly on the platinum surface in a 1.5 x 10 x 0.18 mm groove. This groove was covered by a piece of dialysis membrane, which kept the sample in its place. The sample was applied at 100 μg Chl a/ml. The sample was suspended in the following reaction medium (pH 6.8): 50 mM sodium phosphate, 20 mM HEPES, 70 mM KCl, and 30 mM $CaCl_2$. Above the dialysis membrane was a 4 ml chamber that was also filled with the same medium. A ring of silver, the Ag/AgCl electrode, was located in the upper

chamber in a position shielded from the excitation light. The platinum electrode was biased at -450 mV relative to the standard hydrogen electrode. Signals were detected by a laboratory-built, DC-coupled transimpedance amplifier with a 0.5 ms rise time. Analog signals were digitized with a Biomation 805 waveform recorder and plotted with a chart recorder for signal analysis. The sample was incubated in the dark for 5 min on the electrode surface with the bias on. The platinum surface was scrubbed with a paste of $CaCO_3$ between each application of algal cells to the electrode. This was necessary to maintain maximum signal size and most rapid signal kinetics. Additions of formate or bicarbonate were made directly to the upper chamber of the electrode and incubation was allowed for 10 to 15 min. Flash excitation (full bandwidth at half height, 2.5 μs) was given with a 300 ms dark period and was provided by a General Radio Stroboslave 1539-A Xenon flash.

7. Chlorophyll a fluorescence decay

The kinetics of decay of variable Chl a fluorescence, after a saturating flash, were measured at 685 nm by a weak measuring light. The measuring light was fired at variable times after each actinic flash. The actinic (FX-124, EG and G) and the measuring flashes (Stroboslave 1593A, General Radio) were filtered with two Corning blue (CS 4-96) glass filters; both had a 2.5 μs duration at half-maximum peak (Eaton-Rye and Govindjee, 1988a). Thylakoids were prepared by a procedure

modified after Burnap et al. (1989). Thylakoid suspensions, at a Chl *a* concentration of 5 μg/ml, were dark adapted for 15 minutes. In order to calculate the rate constants of Q_A^- decay, the relative Q_A^- concentration was estimated from the variable Chl *a* fluorescence yield according to Joliot and Joliot (1964) using the formula provided by Mathis and Paillotin (1981). The connection parameter, p, of 0.45 obtained from thylakoid membranes of the cyanobacterium *Phormidium laminosum* (Bowes and Bendall, 1983) was used.

C. Results

1. Growth characteristics

Cyanobacterial growth of the wild type and mutants in BG-11 liquid medium under photoautotrophic and photoheterotrophic conditions was assayed as the optical density of the culture at 730 nm as a function of time. Figure 3.4 shows semilogarithmic growth curves of the wild type and the mutants D2-R233Q and D2-R251S. During the logarithmic growth phase, the doubling time of the wild type is 12 hours, while the mutants showed slightly slower doubling time of 13 hours (D2-R233Q) and 15 hours (D2-R251S). However, under photoheterotrophic conditions (plus 5 mM glucose), when PSII is not required for growth, similar growth characteristics were observed in both the mutants and the wild type. This indicates that amino acid replacements in D2-R233Q and D2-R251S only moderately affect the overall PSII activity.

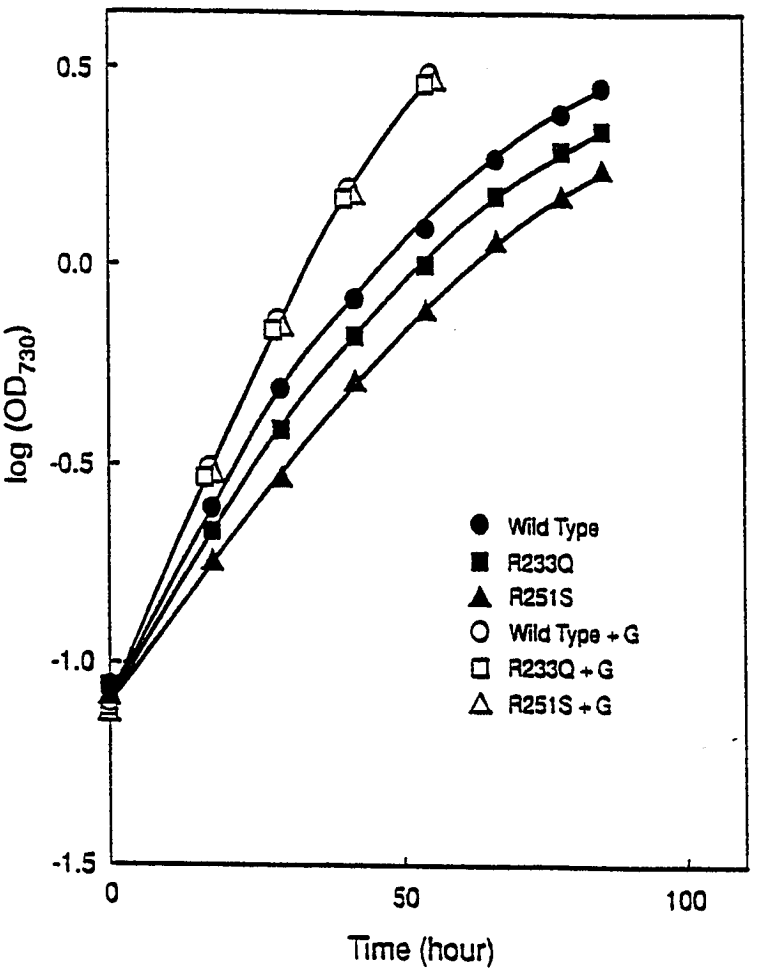


Figure 3.4. Semilogarithmic growth curves of Synechocystis 6803 cells of the wild type (• , O) and of D2-R233Q (■ , □) and D2-R251S mutants (▵ , ▲) under photoautotrophic (open symbols) and photoheterotrophic (solid symbols) growth conditions. Cyanobacterial cells were cultivated in BG-11 media, and during photoheterotrophic growth, 5 mM glucose (G) was included. Cyanobacterial growth was measured as the optical density of the culture at 730 nm (OD_{730}).

2. Herbicide binding

A ^{14}C-labelled diuron binding assay was used to measure the total number of PSII centers in the mutants and the wild type. Plots of bound ^{14}C-diuron against free diuron concentration show that the wild type as well as the mutants D2-R233Q, and D2-R251S have approximately the same diuron dissociation constant of 12.8 nM (Figure 3.5). The maximum number of diuron binding sites for the wild type, D2-R233Q, and D2-R251S were calculated to be 1.14, 1.08 and 1.01 nmoles bound diuron/mg Chl, respectively.

3. Steady-state oxygen evolution

It has been demonstrated that bicarbonate stimulates the Hill reaction in bicarbonate-depleted Synechocystis 6803 cells and that this stimulatory effect is pH-dependent (Chapter II). The effect is maximal at a pH of about 6.8 (Cao and Govindjee, 1990a). Figure 3.6 shows the Hill reaction activity (measured as O_2 evolution) of bicarbonate-depleted wild type and mutant cells in the presence of different formate concentrations. 100 % in wild type was equivalent to 160 μmoles $O_2 \cdot (mg\ Chl)^{-1} \cdot h^{-1}$, whereas 100 % in mutants D2-R233Q and D2-R251S were 145 and 136 μmoles $O_2 \cdot (mg\ Chl)^{-1} \cdot h^{-1}$, respectively. The slight decrease in the oxygen evolution rate in untreated D2-R233Q and D2-R251S can probably be accounted for partially by the loss of PSII centers, as calculated from herbicide binding data (see Figure 3.5). The I_{50} value, the concentration of formate giving half-maximal inhibition of oxygen evolution rates, was

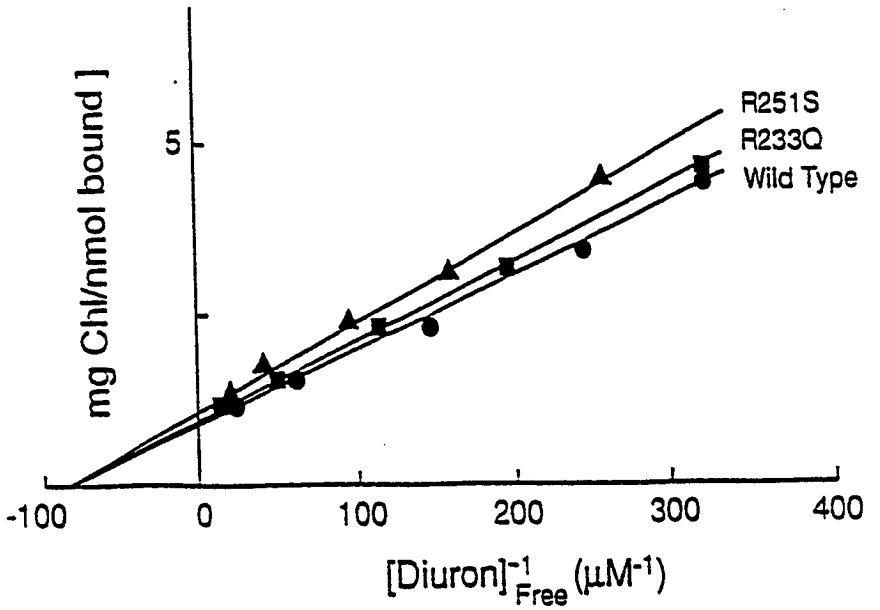
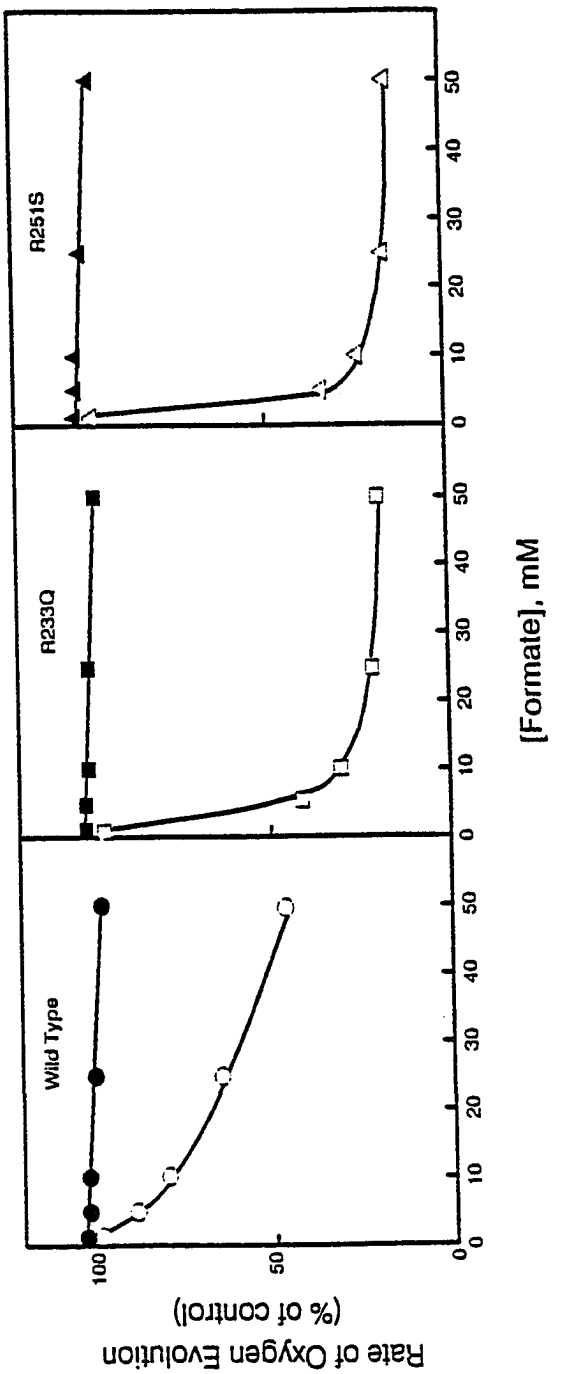


Figure 3.5. A double-reciprocal plot of bound diuron against free diuron concentration in the wild type, and D2-R233Q and D2-R251S mutant cells. Various concentrations of ^{14}C-diuron were added to 1 ml samples in 25 mM HEPES (pH 7.2). The bound diuron at saturating free diuron concentration is the calculated value. The chlorophyll concentration was 25 μg/ml.

Figure 3.6. Steady-state oxygen evolution rate of Synechocystis 6803 cells of the wild type, and D2-R233Q and D2-R251S mutants. The 2 ml reaction mixture contained 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4 (pH 6.8), 0.1 μM gramicidin D, 20 μM DBMIB and the indicated amount of sodium formate. The Chl a concentration was 20 μg/ml. A combination of DMQ (0.5 mM) and $K_3Fe(CN)_6$ (1 mM) was used as electron acceptor. The samples were incubated for one hour in the medium, containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM NaH_2PO_4 (pH 5.8), and the indicated amount of sodium formate, over which N_2 gas was passed (open symbols). To restore the Hill reaction, 10 mM bicarbonate was added (solid symbols).



calculated to be 48 mM for wild type, 4.5 mM for D2-R233Q and 4.0 mM for D2-R251S (open symbols). Note, however, that in none of the cases formate totally inhibited electron transfer. The inhibitory effect of formate was fully reversed by the addition of 10 mM bicarbonate (closed symbols). Thus, mutants D2-R233Q and D2-R251S are an order of magnitude more sensitive to formate than the wild type is.

4. Flash dependence of oxygen evolution

Bicarbonate depletion or formate addition is known to alter the oxygen yield in a sequence of flashes (Stemler et al., 1974; Jursinic and Stemler, 1982; Govindjee et al., 1990). Figure 3.7 shows the yield of oxygen per flash as a function of flash number in the wild type and in the mutants D2-R233Q and D2-R251S with and without the addition of 15 mM formate (for 10 min) and after the subsequent addition of 10 mM bicarbonate. The oxygen flash-yield pattern of cyanobacterial cells shows a peculiarity in comparison to higher plant chloroplasts. There is a small electrochemical signal, but no oxygen yield on the first flash (Bader et al., 1983; Bader, 1984), whereas no such signal is observed in dark-adapted thylakoids from higher plants (see review by Wydrzynski, 1982). In addition, the second maximum in the oxygen yield pattern occurs at the eighth instead of the seventh flash. I observed a similar flash-yield pattern in Synechocystis 6803 as reported in the cyanobacterium Oscillatoria chalybea (Bader et al., 1983). The ratio of the

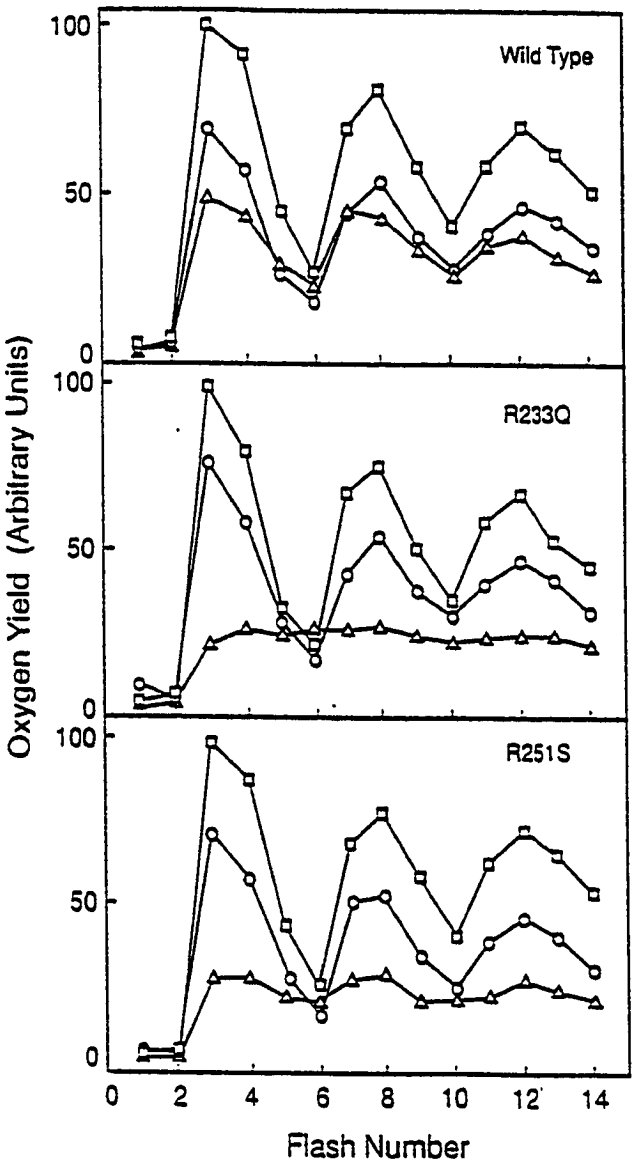


Figure 3.7. Oxygen yield in a sequence of flashes in the Synechocystis 6803 cells of the wild type (top), and D2-R233Q (middle) and D2-R251S (bottom) mutants. The Chl a concentration was 100 μg Chl a/ml. The suspension buffer contained 50 mM sodium phosphate and 20 mM Hepes at pH 6.8, 70 mM KCl, and 30 mM $CaCl_2$. Fifteen mM sodium formate was added and the cells were incubated in the dark for 10 minutes. To restore the formate inhibition, 10 mM sodium bicarbonate was added to the formate-treated samples with 10 minute dark adaptation. O : control; Δ : 15 mM formate; □ : 15 mM formate + 10 mM bicarbonate.

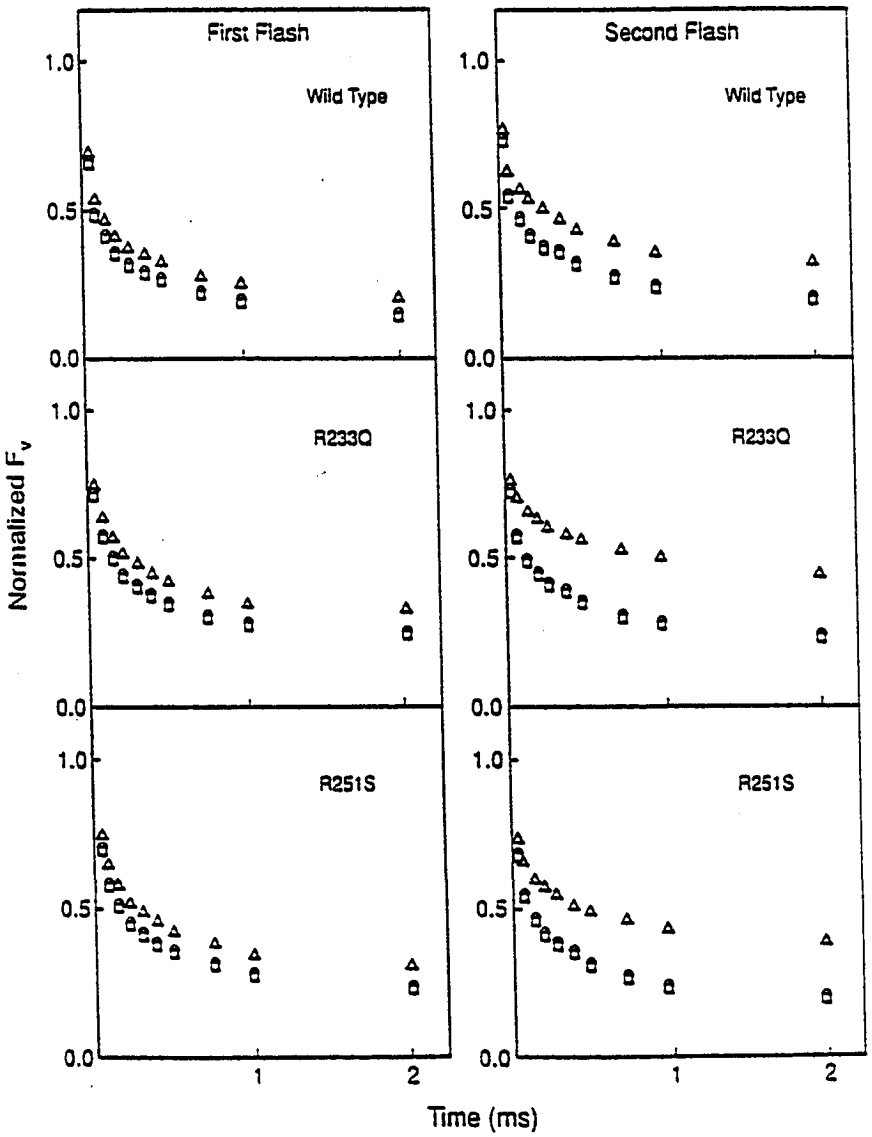
of the oxygen yield in 14 flashes in the absence and presence of 15 mM formate is 1.4 in wild type cells, 2.1 in D2-R233Q and 2.4 in D2-R251S. Thus, in the case of D2-R233Q and D2-R251S mutants, 15 mM formate lowered the sum oxygen yield much more than in the wild type. Furthermore, the characteristic oscillation with a periodicity of four was very much damped in these mutants in the presence of formate; this oscillation could barely be seen in D2-R233Q. Thus, mutants D2-R233Q and D2-R251S showed larger sensitivity to formate treatment than the wild type, but the difference in oxygen flash yield with and without formate is significantly less than what was measured in saturating continuous light. This will be discussed later.

5. Chlorophyll a fluorescence decay

In thylakoids, addition of bicarbonate to formate-treated samples increases the rate of electron flow from Q_A^- to the plastoquinone pool (Jursinic et al., 1976; Jursinic and Stemler, 1984; Eaton-Rye and Govindjee, 1988a, 1988b). To measure the kinetics of Q_A^- oxidation by Q_B or Q_B^-, the decay of the Chl a fluorescence yield, which is related to the increase in Q_A concentration (Duysens and Sweers, 1963), was monitored (see e.g., Eaton-Rye and Govindjee, 1988a, 1988b). Chl a fluorescence decays, monitoring the oxidation of Q_A^- following one ($Q_A^- \rightarrow Q_B$) or two ($Q_A^- \rightarrow Q_B^-$) actinic flashes, in thylakoids from wild type, D2-R233Q and D2-R251S, in the presence and absence of 25 mM formate are shown in Figure 3.8. All

fluorescence levels were normalized by the maximum variable fluorescence level in the presence of 10 μM DCMU. Twenty μM quinhydrone was used to oxidize the acceptor quinones in the dark (Bowes and Crofts, 1980). As mentioned in Materials and Methods, a nonlinear relation exists between F_v and $[Q_A^-]$. Thus Q_A^- concentrations were calculated from F_v values. To simplify the comparison of the decay kinetics of Q_A^- between the wild type and mutants, only the fast first-order exponential decay process was considered, although the Q_A^- decay after one actinic flash contains two or more exponential components (see e.g., Eaton-Rye and Govindjee, 1988a; Cao and Govindjee, 1990b). The fast component of Q_A^- decay was resolved by subtracting the slow component. The half times of fast Q_A^- decay of the wild type are 225 μs and 361 μs following the first and the second flash, respectively, and are slightly faster than that of D2-R233Q (halftimes of 327 μs and 499 μs) and D2-R251S (270 μs and 451 μs). Also, in the mutants after the first flash the amount of remaining F_v at 2 ms after the flash is higher than in wild type, which could signify some change in the $Q_A^-.Q_B \leftrightarrow Q_A.Q_B^-$ equilibrium constant. More importantly, upon formate treatment the decay kinetics of D2-R233Q and D2-R251S after the second flash were slowed down more than in wild type. In the presence of 25 mM formate, the half time of fast Q_A^- decay after the second saturating actinic flash increased by a factor of 4 (2.2 ms) and 6 (3.1 ms) in D2-R251S and D2-R233Q, respectively, while that of the wild

Figure 3.8. Decay of variable chlorophyll a fluorescence following one or two saturating actinic flashes given to dark-adapted thylakoids from Synechocystis 6803 cells of the wild type, and D2-R233Q and D2-R251S mutants. The circles and squares represent the control and bicarbonate-restored samples, respectively; the triangles represent the formate-treated samples. Thylakoids were suspended in the medium containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 μM gramicidin D, 20 mM HEPES (pH 6.8) and 25 mM sodium formate. The thylakoids were preincubated in the dark for 10 min in the presence of 20 μM quinhydrone. The formate treatment (25 mM) was done as described in the legend of Figure 3.6.



type had only a 2-fold increase (to 0.8 ms). Furthermore, it appears that in the mutants not only the forward rate of electron flow from Q_A^- to Q_B^- has been slowed, but also the equilibrium constant of $Q_A^-Q_B^- \leftrightarrow Q_AQ_B^{2-}/Q_AQ_B\ H_2$ has been altered since the slow component extrapolates to higher values at t=0 in formate treated than in control and bicarbonate-restored samples. The ratio of the amplitude of slow component after formate treatment to that in the control is significantly higher for the mutants (1.77 for D2-R233Q and 1.83 for D2-R251S) than for the wild type (1.39). In view of the non-linearity of F_v and $[Q_A^-]$, the F_v of the slow component represents a significant fraction of Q_A^- (Joliot and Joliot, 1964). The half times of Q_A^- decay in subsequent actinic flashes were similar to that after the second actinic flash (data not shown).

6. Chlorophyll a fluorescence decay in the presence of diuron

It is known that the reoxidation of the reduced acceptor Q_A^- after illumination in the presence of diuron is decreased in the presence of the artificial electron donor hydroxylamine (see e.g., Bennoun, 1970; Mohanty et al., 1971). Thus, the oxidizing partner was suggested to be Z^+ or the S_2 state of the oxygen evolving complex. It has been observed that bicarbonate-depletion, by formate treatment, slows down the decay of Q_A^- even in the presence of diuron (Eaton-Rye and Govindjee, 1988b), suggesting that reoxidation of Q_A^- by Z^+/S_2 has been inhibited in the presence of formate. Reoxidation of

Q_A^- in the presence of 10 μM diuron was measured for the wild type and mutant thylakoids. At pH 6.8, the half time of Q_A^- oxidation was 0.8 s, 1.2 s, and 1.3 s in the wild type, D2-R233Q, and D2-R251S, respectively. Upon formate treatment, the half time of Q_A^- oxidation increased to 6.6 s and 6.9 s in D2-R233Q and D2-R251S, respectively, while it increased to only 2.3 s in the wild type (Figure 3.9).

7. Reactivation of the Hill reaction

One possible explanation for the higher formate sensitivity of the mutants is that Arg-233 and Arg-251 in the D2 polypeptide may function to stabilize bicarbonate binding in PSII of Synechocystis 6803. If this is the case, the apparent binding affinity of bicarbonate would be lowered in the mutants. A possible method to investigate bicarbonate binding affinity is to measure the Hill reaction rate after the addition of different concentrations of bicarbonate to bicarbonate-depleted samples (Khanna et al., 1981; Van Rensen and Vermaas, 1981) and compare the Michaelis-Menten constant K_m. The equilibration between bicarbonate and carbon dioxide is known to be completed within 1 min (Cooper et al., 1968). A time course of the reactivation of the Hill reaction by bicarbonate addition has indicated that the equilibrium is accomplished within 3 min (Blubaugh and Govindjee, 1986), whereas carbonic anhydrase can further shorten the time to reach equilibrium between the two species. In this work, the equilibration was assured by measuring the restoration of the

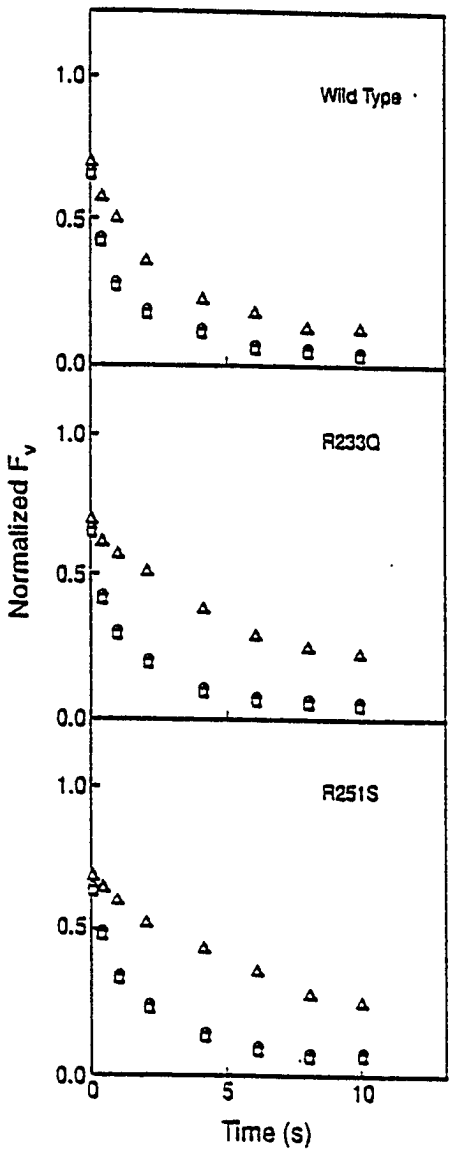


Figure 3.9. Decay of variable chlorophyll a fluorescence after one saturating actinic flash obtained from the dark adapted thylakoids from the wild type and from the mutants D2-R233Q and D2-R251S of Synechocystis 6803 in the presence of 10 μM DCMU. The other details of the protocol were as in the legend of Fig. 3.8 O : control; Δ : 25 mM formate; □ : 25 mM formate + 10 mM bicarbonate.

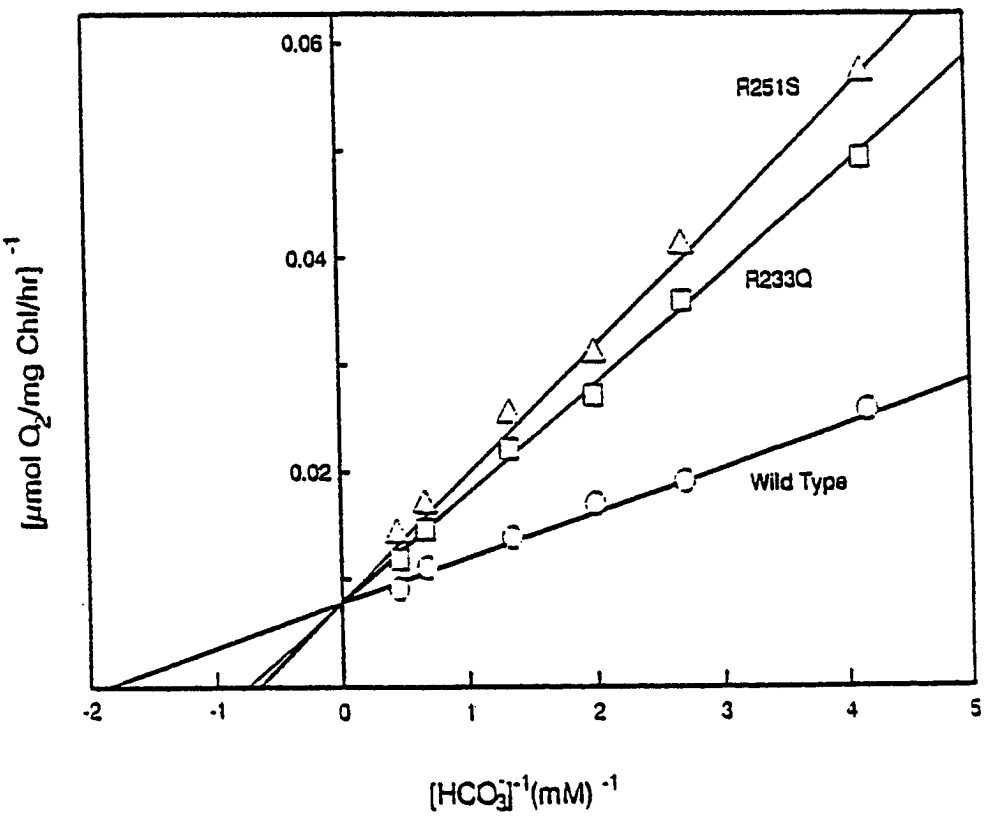


Figure 3.10. Double reciprocal plots of the steady-state oxygen evolution rate as a function of the added sodium bicarbonate concentration in formate treated Synechocystis 6803 cells of the wild type (O) and of D2-R233Q (□) and D2-R251S (△) mutants. The formate concentration was 100 mM. The experimental protocol was as described in the legend of Figure 3.6.

steady-state electron flow rate from water to plastoquinone by incubating samples in the dark for 10 min with various concentrations of bicarbonate. All the samples contained 100 mM formate. Figure 3.10 shows the double-reciprocal plot of the oxygen evolution rate versus equilibrium bicarbonate concentration. The value of the intercept on the X-axis is Michaelis constant K_m, the bicarbonate dissociation constant in the presence of 100 mM formate. The apparent K_m values are 0.5 mM, 1.4 mM and 1.5 mM bicarbonate in the wild type, D2-R233Q and D2-R251S, respectively, which indicates that in the presence of 100 mM formate the apparent K_m was increased by about 3-fold in the mutants as compared to the wild type.

D. Discussion

In this study, effort was made to examine the role played by arginine residues in the D2 protein in the bicarbonate-reversible formate inhibition of PSII reaction in Synechocystis 6803 cells. Mutation of Arg-233 to glutamine (D2-R233Q) and of Arg-251 to serine (D2-R251S) slowed down the photoautotrophic growth of cells only slightly (Figure 3.4) and had an insignificant effect on characteristics of diuron binding (Figure 3.5), but caused large differences in the sensitivity of formate to oxygen evolution in saturating continuous light (Figure 3.6) and in flashing light (Figure 3.7). The mutants were much more sensitive to formate than the wild type. This difference was accompanied by a significant

change in the bicarbonate binding in the presence of 100 mM formate (Figure 3.10). Data on decay of Chl a fluorescence yield showed that formate was more effective in slowing Q_A^- to Q_B^- electron transfer in the mutants than in the wild type (Figure 3.8), and in slowing recombination of Q_A^- with S_2 in the presence of diuron (Figure 3.9). All effects were reversed upon bicarbonate addition. On the basis of these results, it is concluded that the targeted arginine residues in the D2 protein play an important role in the bicarbonate effect on the electron acceptor side of photosystem II.

Stemler et al. (1974) observed that in bicarbonate-depleted (formate-treated) higher plant thylakoids, oscillations in oxygen evolution as a function of flash number were reversibly damped; in addition, the relaxation of $S_1' \rightarrow S_2$ and $S_2' \rightarrow S_3$ was reversibly slowed. In higher plant thylakoids, Jursinic and Stemler (1982) noted that formate treatment causes an increase in the ratio of S_0 to S_1 as well as an increase in the probability of the double hits. A damped oscillation was observed in D2-R233Q and D2-R251S mutants of Synechocystis 6803 with 15 mM formate (Figure 3.7) as well as in wild type Synechocystis cells when high (50-100mM) formate concentration was used (data not shown). Fitting of the experimental data on the oxygen flash yield to the S-state model, after removal of the signal observed at the first flash and 50% of the signal seen at the second flash (Bader et al., 1983), showed that formate treatment induced an increase of

about 2.5-fold in the apparent S_0 to S_1 ratio and, surprisingly, an increase of about 5-fold in the probability of the double hits in the wild type and D2-R251S. The oxygen evolution in D2-R233Q was damped to the extent that it could not be fitted. These results can be best understood by a formate-induced shift in the redox equilibrium between Q_A and Q_B and/or a slowing down of electron transfer from Q_A^- to plastoquinone: relaxation of the system such that an S state transition can occur at a subsequent flash involves recovery of all the components in PSII reaction centers (see e.g., discussions by Wydrzynski, 1982). As noted above, a formate-induced damping of oscillations in oxygen evolution was observed as a function of flash number in Synechocystis 6803. In contrast, however, Govindjee et al. (1990) did not observe an apparent damped oscillation in oxygen yield upon formate treatment of Synechocystis 6714 cells. This difference could be due to the use of subsaturating, instead of saturating, formate concentration in the Synechocystis 6714 study. Furthermore, Synechocystis 6803 cells used in this study were grown in air (i.e., they were low CO_2 cells) whereas Synechocystis 6714 cells used in the earlier study were grown in CO_2-enriched atmosphere (high CO_2 cells).

Addition of 10 mM bicarbonate to formate-pretreated Synechocystis 6803 cells, not only relieved inhibition caused by formate pretreatment and restored the normal flash-yield pattern but also enhanced oxygen yield to an extent larger

than the control (Figure 3.7). Furthermore, addition of 10 mM bicarbonate caused a similar enhancement of oxygen yield even in cells not pretreated with formate (data not shown). The oxygen yield enhancement over the control by bicarbonate addition (Figure 3.7) might be related to the so called "Punnett effect" (Punnett and Iyer, 1964; Punnett, 1965), i.e., addition of bicarbonate to untreated chloroplasts caused enhancement of phosphorylation and electron flow. In addition, this effect might also be related to effects on the low affinity CO_2/HCO_3^- binding sites (Blubaugh and Govindjee, 1988a). This effect was not further studied here since the focus of the present work is on the differential sensitivity to formate inhibition in the mutants and wild type.

Bicarbonate molecules are considered necessary for a normal Hill reaction in PSII (electron transfer from water to the plastoquinone pool) (Govindjee and Van Rensen, 1978; Blubaugh and Govindjee, 1988). Formate, an analog of bicarbonate, inhibits electron transfer in PSII, and this inhibition is fully reversed upon bicarbonate addition. Furthermore, formate releases CO_2 from spinach thylakoids as measured by both a mass spectrometer and a differential infra-red gas analyzer (Govindjee et al., 1991c). Formate has been suggested to be a competitive inhibitor of bicarbonate (Blubaugh and Govindjee, 1988). A ten-fold decreased I_{50} in the rate of steady-state oxygen evolution and a 3-fold increased K_m for bicarbonate in reactivation of the Hill reaction in the

presence of formate indicate that a lower formate concentration is needed to reduce oxygen evolution activity and a somewhat higher bicarbonate concentration is required to overcome this formate inhibition in the mutants D2-R233Q and D2-R251S. The interpretation is favored that these mutations have altered binding of HCO_3^- and formate, so that bicarbonate molecules become more susceptible to displacement by the competitive formate anion. The inhibition of electron flow beyond Q_A^- upon formate treatment causing the slowing down of Chl *a* fluorescence decay after single-turnover flashes in *Synechocystis* 6803 mutants is in agreement with earlier studies on isolated higher plant thylakoids (Eaton-Rye and Govindjee, 1988a,b). An increased inhibition of electron transfer after the second and subsequent actinic flashes (Govindjee et al., 1976; Eaton-Rye and Govindjee, 1988a,b; Xu et al., 1991; also see Figure 3.7, this thesis) may be due to an impairment of protonation of Q_B^- and/or Q_B^{2-}. Upon formate treatment, the observed 2-3 fold increase in the half time of fast Q_A^- decay in the mutants compared to that in the wild type (Figure 3.8), can be attributed either to a more loosely bound bicarbonate or a higher formate affinity. However, the difference in Q_A^- decay half time with and without formate is considerably smaller than that in the yield of steady-state oxygen evolution in saturating continuous light. Unlike continuous illumination, the light flashes with 1 s dark intervals, used in Chl *a* fluorescence decay measurements,

allow recovery of the PSII reaction centers in the dark intervals between actinic flashes. For instance, in formate treated thylakoid membranes half time of Q_A^- decay at flash frequency of 5 Hz is more than 8-fold slower than that at 1 Hz (Eaton-Rye and Govindjee, 1988a). At still higher flash frequency (about 30Hz), the Q_A^- decay is further slowed (Govindjee et al., 1976).

In Synechocystis 6803, D2-R251 is in the vicinity of the aromatic residue Trp-253 (see Figure 3.11), which is between pheophytin and Q_A and potentially involved in facilitating electron transport from pheophytin to Q_A and/or in binding of Q_A. Vermaas et al. (1990b), using site-directed mutagenesis, recently altered W253 into Leu. The PSII complex in the mutant appeared to be highly unstable. It was suggested that impairment of Q_A binding causes instability in the PSII complex. D2-R233 is thought to be near the middle of the loop spanning the helices IV and V in the D2 protein of Synechocystis 6803 (Williams and Chisholm, 1987). Its counterpart in photosynthetic bacteria may be M-R239 in R. viridis. Little is known about this arginine. However, in this region between the helices IV and V, M-Glu-232 (R. viridis) provides a bidentate ligand to the non-heme iron through its carboxylate group (Michel and Deisenhofer, 1988). As noted earlier, bicarbonate/formate has no effect on electron transport between the two quinones in purple photosynthetic bacteria (Shopes et al., 1989) or in the green bacterium

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S. 6803,1                    MTIAVG-RAPV-ERGWFDVLDDWLKRDRFVFIGWFGLL  36
S. 6803,2                    MTIAVG-RAPV-ERGWFDVLDDWLKRDRFVFIGWSGLL  36
S. 7002-1,2                  MTIAVG-RAPQ-ERGWFDVLDDWLKRDRFVFVGWSGIL  36
S. 7942-1,2                  MTIAVG-RAPA-ERGWFDVLDDWLKRDRFVFVGWSGLL  36
Chlamy                       MTIAIGTY-KD-KNTWFDDADDWLRQDRFVFVWSGGLL  36
Pea                          MTIALGKFTKD-QNDLFDIMDDWLRRDRFVFVGWSGLL  37
Spinach                      MTIAVGKFTKD-EKDLFDSMDDWLRRDRFVFVGWSGLL  37
MS          AEYQNIFSNVQVRGPADLGMTEDVNLANRSGVGPFSTL-LGWF-GNAQL-       47
MV          ADYQTIYTQIQARGPHITVSGEWGDNDRVGKPFYSYWL--GKI-GDAQI-       46

S. 6803,1   LFPCAFMALGGWLTGTTFVTSWYTHGLASSYLEGANFLTVAVSSP----A       82
S. 6803,2   LFPCAFMALGGWLTGTTFVTSWYTHGLASSYLEGANFLTVAVSSP----A       82
S.7002-1,2  LFPCAFMALGGWLTGTTFVTSWYTHGLASSYLEGCNFLTVAVSSP----A       82
S.7942-1,2  LFPCAYLALGGWLTGTSFVTSWYTHGIASSYLEGGNFLTVAVSTP----A       82
Chlamy      LFPCAYFALGGWLTGTTFVTSWYTHGLATSYLEGCNFLTAAVSTP----A       82
Pea         LFPCAYFAVGGWFTGTTFVTSWYTHGLASSYLEGCNFLTAAVSTP----A       83
Spinach     LFPCAYFALGGWFTGTTFVTSWYTHGLASSYLEGCNFLTAAVSTP----A       83
MS          -GPIYLGSLGVLSLFSGLMWFFTIGIWFWYQAGWNPAVFLRDLFFFSLEP      96
MV          -GPIYLGASGIAAFAFGSTAILIILFNMAAEVHFDPLQFFRQFFWLGLYP      95

S. 6803,1   DAFGHSLLFLWGPEAQGNLTRWFQIGGLWPFVALHGAFGLIGFMLRQFEI     132
S. 6803,2   DAFGHSLLFLWGPEAQGNLTRWFQIGGLWPFVALHGAFGLIGFMLRQFEI     132
S.7002-1,2  DSLGHSLLFLWGPEANWNFARWCQLGGLWSFVALHGAFGLIGFMLRQFEI     132
S.7942-1,2  DAFGHSLMLLWGPEAQGNFVRWCQLGGLWNFVALHGAFALIGFMLRQFEI     132
Chlamy      NSMAHSLLFVWGPEAQGDLTRWCQLGGLWAFVALHGAFGLIGFMLRQFEI     132
Pea         NSLAHSLLLLWGPEAQGDLTRWCQLGGLWTFVALHGAFALIGFMLRQFEL     133
Spinach     NSLAHSLLLLWGPEAQGDFTRWCQLGGLWAFVALHGAFALIGFMLRQFEL     133
MS          PAPEYGLSFAAP----------LKEGGLWLIASFFMFVAVWSWWGRTYLR     136
MV          PKAQYGM-GIPP----------LHDGGWWLMAGLFMTLSLGSWWIRVYSR     134
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Figure 3.11. Amino acid sequences of the D2 protein from Synechocystis sp. PCC 6803 (Williams and Chisholm, 1987), Synechococcus sp. PCC 7002 (Gingrich et al., 1990), Synechococcus sp. PCC 7942 (Golden and Stearns, 1988), Chlamydomonas reinhardtii (Erickson et al., 1986), pea (Rasmussen et al., 1984) and spinach (Alt et al., 1984; Holschuh et al., 1984), and of the M subunit from Rb sphaeroides (J.C. Williams et al., 1983) and Rps viridis (Michel et al., 1986). S.6803-1 and S.6803-2: Synechocystis sp. PCC 6803; S.7002-1,2: Synechococcus sp. PCC 7002; S.7942-1,2: Synechococcus sp. PCC 7942; Chlamy: Chlamydomonas reinhardtii; MS: Rps sphaeroides; MV: Rps viridis.

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S. 6803,1    SRLVGIRPYNAIAFSGPIAVFVSVFLIYPLGHSSWFFAPSFGVAGIFRFI 182
S. 6803,2    SRLVGIRPYNAIAFSGPIAVFVSVFLIYPLGQSSWFFAPSFGVAGIFRFI 182
S.7002-1,2   ARLVGIRPYNAIAFSGPIAVFVSVFLMYPLGQSSWFFAPSFGVAGIFRFI 182
S.7942-1,2   ARLVGVRPYNAIAFSGPIAVFVSVFLMYPLGQSSWFFAPSFGVAAIFRFL 182
Chlamy       ARSVNLRPYNAIAFSGAIAVFVSVFLIYPLGQSGWFFAPSFGVAAIFRFI 182
Pea          ARSVQLRPYNAIAFSGPIAVFVSVFLIYPLGQSGWFFAPSFGVAAIFRFI 183
Spinach      ARSVQLRPYNAIAFSGPIAVFVSVFLIYPLGQSGWFFAPSFGVAAIFRFI 183
MS           AQALGMGKHTAWAFLSAIWLWMVLGFIRPILMGSWSEAVPYGIFSHLDWT 186
MV           ARALGLGTHIAWNFAAAIFFVLCIGCIHPTLVGSWSEGVPFGIWPHIDWL 184

S. 6803,1    LFLQGFH-NWTLNPFHMMGVAGILGGALLCAIHGATVENTLF-EDGDGAN 230
S. 6803,2    LFLQGFH-NWTLNPFHMMGVAGILGGALLCAIHGATVENTLF-EDGDGAN 230
S.7002-1,2   LFLQGFH-NWTLNPFHMMGVAGILGGALLCAIHGATVENTLF-EDSDQAN 230
S.7942-1,2   LFLQGFH-NWTLNPFHMMGVAGILGGALLCAIHGATVENTLF-EDSEQSN 230
Chlamy       LFFQGFH-NWTLNPFHMMGVAGVLGAALLCAIHGATVENTLF-EDGDGAN 230
Pea          LFFQGFH-NWTLNPFHMMGVAGVLGAALLCAIHGATVENTLF-EDGDGAN 231
Spinach      LFFQGFH-NWTLNPFHMMGVAGVLGAALLCAIHGATVENTLF-EDGDGAN 231
MS           NNFQLVHGNLFYNPFHGLSIAFLYGSALLFAMHGATILAVSRFGGERELE 236
MV           TAFSIRYGNFYYCPWHGFSIGFAYGCGLLFAAHGATILAVARFGGDREIE 234

S. 6803,1    T--FRAFEPTQAEETYSMVTANRFWSQ-IFGIA-FSNKRWLHFFMLFVPV 276
S. 6803,2    N--FRAFEPTQAEETYSMVTANRFWSQ-IFGIA-FSNKRWLHFFMLFVPV 276
S.7002-1,2   T--FRAFEPTQAEETYSMVTANRFWSQ-IFGIA-FSNKRWLHFFMLFVPV 276
S.7942-1,2   T--FRAFEPTQAEETYSMVTANRFWSQ-IFGIA-FSNKRWLHFFMLFVPV 276
Chlamy       T--FRAFNPTQAEETYSMVTANRFWSQ-IFGVA-FSNKRWLHFFMLLVPV 276
Pea          T--FRAFNPTQAEETYSMVTANRFWSQ-IFGVA-FSNKRWLHFFMLFVPV 277
Spinach      T--FRAFNPTQAEETYSMVTANRFWSQ-IFGVA-FSNKRWLHFFMLFVPV 277
MS           QIADRGTAAER---------AALFWRW--TMGFN-ATMEGIHRWAIWMAV 274
MV           QITDRGTAVER---------AALFWRW--TIGFN-ATIESVHRWGWFFSL 272
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Figure 3.11. Continued.

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S. 6803,1    TGLWMSSVGIVGLALNLRAYDFVSQELRAAEDPEFETFYTKNILLNEGMR 326
S. 6803,2    TGLWMSSVGIVGLALNLRAYDFVSQELRAAEDPEFETFYTKNILLNEGMR 326
S.7002-1,2   TGLWMSSVGIVGLALNLRAYDFVSQEIRAAEDPEFETFYTKNILLNEGMR 326
S.7942-1,2   TGLWMSSIGIVGLALNLRAYDFVSQELRAAEDPEFETFYTKNILLNEGIR 326
Chlamy       TGLWMSAIGVVGLALNLRAYDFVSQEIRAAEDPEFETFYTKNILLNEGIR 326
Pea          TGLWMSALGVVGLALNLRAYDFVSQEIRAAEDPEFETFYTKNILLNEGIR 327
Spinach      TGLWMSALGVVGLALNLRAYDFVSQEIRAAEDPEFETFYTKNILLNEGIR 327
MS           LVTLTGGIGILLSGTVV-DNWYVWGQNHGMAPLN---------------- 307
MV           MVMVSASVGILLTGTFV-DNWYLWCVKHGAAPDYPAYLPATPDPASLPGA 321

S. 6803,1    AWMAPQDQPHEN-FIFPEEVLPRGNAL                        352
S. 6803,2    AWMAPQDQPHEN-FIFPEEVLPRGNAL                        352
S.7002-1,2   AWMAPQDQIHEQ-FVFPEEVLPRGNAL                        352
S.7942-1,2   AWMAPQDQPHEK-FVFPEEVLPRGNAL                        352
Chlamy       AWMATQDQPHER-LVFPEEVLPRGNAL                        352
Pea          AWMATQDQPHEN-LIFPEEVLPRGNAL                        353
Spinach      AWMAAQDQPHEN-LIFPEEVLPRGNAL                        353
MS
MV           PK-------------------------                        323
```

Figure 3.11. Continued.

Chloroflexus aurantiacus (Govindjee et al., 1991a). Michel and Deisenhofer (1988) suggested that bicarbonate may act as a bidentate ligand to the iron in PSII. M-Glu-234 in R. sphaeroides, a counterpart of M-Glu-232 in R. viridis, has been changed into valine, glutamine and glycine through site-directed mutagenesis. These mutants show no significant inhibitory effects by exogenous formate or NO (Wang et al., 1991), which implies a fundamental difference between the iron niche of bacterial reaction centers and PSII reaction centers.

The crystallographic structure of human lactoferrin, in which binding of Fe(III) requires synergistic (bi)carbonate (HCO_3^-/CO_3^{2-}) ion binding, shows that a (bi)carbonate ion binds to Fe(III); other ligands to Fe(III) are provided by a histidine, an aspartate and two tyrosine residues (Anderson et al., 1989). Furthermore, the two oxygens of HCO_3^-/CO_3^{2-} are complexed to Fe(III) forming a bidentate ligand and are hydrogen bonded to an arginine and alanine. The third oxygen forms two hydrogen bonds with a glycine and threonine. It is possible that one or more of the Arg of D2 may provide hydrogen bonds to stabilize the binding of HCO_3^-/CO_3^{2-}, which likely forms a bidentate ligand with Fe(II) in PSII.

D. Chisholm and B. Diner (cited in Diner et al., 1991) have shown that site-directed mutants at Lys-264 in the D2 protein of Synechocystis 6803 had slowed electron transfer from Q_A^- to Q_B even in the presence of 10 mM bicarbonate, which

raises the question about the importance of Lys 264 in CO_2/HCO_3^- binding. However, results presented in this Chapter emphasize that other arginine residues (Arg-251 and Arg-233) in D2 also appear to be important for the stabilization of HCO_3^- binding.

In conclusion, observations in this Chapter show that the D2 protein is involved in the bicarbonate effect in PSII. Mutations of R233 and R251 in the D2 polypeptide in Synechocystis 6803, that lead to only minor disturbances in PSII function, result in a significant susceptibility to the replacement of bicarbonate by the competitive anion formate. The present study suggests that the two arginine residues, although not necessarily vital to bicarbonate binding, are important for the stabilization of bicarbonate binding in PSII.

The mutant D2-R239H (constructed by Govindjee in the laboratory of Wim Vermaas in 1990) and a mutant with an extra arginine inserted between D2-F235 and D2-E236 showed similar steady-state oxygen evolution in the presence of various formate concentrations as compared with the wild type (data to be published elsewhere by Govindjee and coworkers). Another mutant D2-W253F mutant had very low Hill activity (Vermaas et al., 1990b) and bicarbonate failed to recover the impaired activity (data not shown). These negative results indicate the specificity for the involvement in the bicarbonate effect at sites of D2-Arg251 and D2-Arg233 as well as at D2-Lys264.

Data presented in this chapter are based on the published work of the author (Cao et al., 1991).

E. References

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CHAPTER IV. CLONING OF PSBA GENE FROM SYNECHOCYSTIS SP. PCC 6803

A. Introduction

Indications for the involvement of the D1 protein in the bicarbonate effect have come from the studies of herbicide and bicarbonate interaction, and of herbicide-resistance mutants. Urea-, triazine- and phenol-type herbicides (known to bind to D1 protein) decrease the apparent affinity of the thylakoid membrane for bicarbonate (Van Rensen and Vermaas, 1981; Van Rensen, 1982; Snel and Van Rensen, 1983). Bicarbonate depletion affects the binding of herbicides (Khanna et al., 1981). Khanna et al (1981) found that a herbicide-resistant mutant (S264G on D1 protein) of Amaranthus hybridus had a 2-fold increase in the dissociation constant of bicarbonate binding. Recent studies on several herbicide-resistant D1 mutants of the cyanobacterium Synechocystis 6714 (Govindjee et al., 1990a) and of Chlamydomonas reinhardtii (Govindjee et al., 1990b) indicated differential formate sensitivities. Formate replaces bicarbonate in PSII.

Cationic amino acid residues, such as arginines in D1 protein, have been proposed to be responsible for the binding of bicarbonate. D1-R269 will be one of the mutagenesis targets in the R269/H272 pair (Blubaugh and Govindjee, 1988). Since the R257/H252 pair, like the R269/H272 pair, is also separated by a single helical turn, D1-R257 is selected as another

target. Both of these two mutagenesis targets are candidates for protonating Q_B^- and/or Q_B^{-2} (H^+). Results obtained from the mutants at both sites are expected to provide insight into bicarbonate effect. Synechocystis sp. PCC 6803 has three psbA genes, psbA1, psbAA2 and psbA3, which encode the D1 protein. The strategy of site-directed mutagenesis for D1 mutation in Synechocystis 6803 is to create the mutation in psb A2 and transform the double deletion mutant that lacks both psbA1 and psbA3 with the mutated psbA2. In this chapter, I discussed my attempts to initiate this research.

B. Materials and Methods

1. Bacterial and cyanobacterial materials

Escherichia (E.) coli strains (DH5α, HB101, NM522) were grown at 37^{0}C in LB medium (Ausubel, et al., 1989). Plasmids pUC18, pUC19, pUC 119, pBR322, etc. and their derivatives were maintained in the presence of ampicillin (50 μg/ml) or tetracycline (12 μg/ml). When appropriate, selective media were supplemented with 50 μg/ml X-gal and 50 μg/ml IPTG.

Our original source of Synechocystis sp. PCC 6803 was from Dr. H. Pakrasi at Washington University at St. Louis and Dr. W. Vermaas at Arizona State University at Tempe. Synechocystis 6803 were grown on BG-11 medium (Rippka et al., 1979) in liquid culture or on agar plates. Growth conditions were: 30 ^{0}C and 65 μmol photons $m^{-2}s^{-1}$ fluorescent light. The liquid culture was constantly bubbled with air through an in-

line bacterial filter (Gelman #4210). Five mM glucose was included in the growth medium when appropriate (J.G.K. Williams, 1988).

2. Cyanobacterial DNA isolation

To prepare DNA from cyanobacteria, cells from 300 ml of a thick culture were harvested by centrifugation at 4,500 g for 6 min. The wet weight of the cell pellet ranged from 1 g. For each gram of cells, 2 ml of saturated NaI was added. Cells were suspended by vortexing, incubated at 37 ^{0}C for 20 min, diluted with water to a final volume of 25 ml, and centrifuged at 10,000 g for 10 min to remove the NaI. Then the cell pellet was suspended by vortexing in 10 ml of 50 mM Tris-HCl, pH 8.5, 50 mM NaCl, 5 mM EDTA, followed by addition of 1.5 ml of lysozyme (50 mg/ml), incubation at 37 ^{0}C for 20 min and addition of 1 ml of 10% N-lauryl sarcosine, and the mixture was incubated at 37 ^{0}C for 20 min. Finally, the pellet was extracted once with an equal volume of phenol and then with 5 volumes of chloroform. The DNA was precipitated with ethanol.

3. Construction and screening of a plasmid library

The library was constructed by digesting genomic DNA with BamH I and Pst I and then ligating these fragments with the vector pUC18 that had been cut with BamH I and Pst I. The original library was diluted 10^6 times and then used of 100 μl per plate for screening. Total 25-50,000 colonies were screened in order to isolate the desired gene.

Recombinant plasmid library was screened as described by

Hanahan and Meselson (1983). The 10^6 dilution was obtained by successively diluting 50 µl into 5 ml LB medium. A hundred µl of the dilution was spread on each 100 mm petri dish and incubated for 4-5 hours at 30 ^{0}C. Millipore HATF filters were laid on the plates and incubated overnight. Then the filters with colony growth were peeled off and transferred to chloramphenicol plate (150 µg/ml) for additional 4 hour amplification. The filters were placed on Whatman filters for 5 min that had been soaked in 0.5 M NaOH and 1.5 M NaCl, and then on Whatman filters soaked with neutralized solution (0.5 M Tris-HCl, pH 7.4, 1.5 M HCl) for 5 min. The filter was transferred to 2 X SSC-soaked Whatman filter and treated for 5 min. After air dry at room temperature for 30 min, the filters were dried at 80 °C in a vacuum oven for 2 hrs. The filters were ready for hybridization.

Probing replicas with radioactive nucleic acids can identify a colony of cells carrying sequences homologous to the probe. Keying back from an autoradiogram to the master plate localizes the colony. Colonies were removed from the regions of hybridization and dispersed in LB medium, and an appropriate dilution was spread on a fresh nitrocellulose filter to give 100-200 colonies. This enriched population was replicated and probed, allowing isolation of pure clones of the hybridizing species.

4. Polymerase chain reaction

Oligonucleotides 5' AGGAATTATAACCAAATGACAACG 3' (15 bp

upstream of psbA2) AND 5' ACCAAGGAATTAACCGTTGACAGC 3'(9 bp downstream of psbA2) were synthesized to produce 1.1 kb PCR (Polymerase Chain Reaction) fragment by referring to the published psbA2 sequence (Ravnikar et al., 1989). In screw-capped tubes, 100 µl reaction medium contained 10 ng cyanobacterial genomic DNA, 200 µM dNTP, 200 ng oligonucleotides, PCR buffer (250 mM KCl, 50 mM Tris-HCl, pH 8.0, and 7.5 mM $MgCl_2$), 0.05 % Tween-20 and NP40, 2.5 U Taq DNA polymerase. Mineral oil was overlaid to prevent evaporation. With the Perkin-Elmer Cetus Instruments Thermal cycler, the following temperature profiles were used: 1 min at 95 ^{0}C, 1 min at 50 ^{0}C, and 1 min at 72 ^{0}C. After 30 cycles, the temperature was cooled down to 4 ^{0}C (J.F. Williams, 1989; Ausubel et al., 1989).

5. Southern blot analysis

The DNA in the agarose gel was depurinated by soaking the gel in 0.25 M HCl for 10 min. Then the DNA was denatured by placing the gel in a bath of 0.5 N NaOH, 1 M NaCl for 30 min. The gel was neutralized by bathing it in 0.5 M Tris-HCl (pH 7.4), 3 M NaCl for 30 min. Transfer of DNA to nitrocellulose ZETA-PROBE blotting membranes was accomplished via capillary action in 20 X SSC (1X SSC is 0.15 M NaCl, 0.015 M Na citrate). The 0.7 kb Pst I/Xba I fragment of pBS/DI.7 vector containing a majority of ps2B-1 (ps2B-1 is a gene for the herbicide binding protein) of cyanobacterium Fremyella diplosiphon coding sequence, which was subcloned from plasmid

pBM5 (gift from Professor L. Bogorad at Harvard University at Cambridge), was used to label radioactive probes in this study. The 0.7 kb fragment was labelled with [α-^{32}P]dCTP using the Random Labelling kit supplied by Bethesda Research Laboratories (BRL), and was purified through a column of Sephadex G-50 before use. The filters were prehybridized for 5-6 hours at 45^{0}C in 50% formamide, 1.5 X SSPE (1 X SSPE is 0.18 M NaCl, 10 mM Na_2HPO_4 and 1 mM EDTA), 0.5% Blotto (0.5 g nonfat powdered milk, 0.01 g sodium azide) solution, 1% SDS and 100 μg/ml denatured Salmon sperm DNA. Hybridization was carried out overnight at 42 ^{0}C in the same solution. The filters were washed twice for 5 min at room temperature in 2X SSC, and then twice for 15 min at 55^{0}C in 2 X SSC/0.1% SDS before exposure to X-ray film at -70^0 with an intensifying screen.

C. Results and Discussion

Figure 4.1 shows the restriction analysis of Synechocystis 6803 genomic DNA. Two μg genomic DNA was digested with various restriction endonucleases and electrophoresed on a 0.8% agarose del. The left panel in Figure 4.1 shows the ethidium bromide stained restriction fragments of the genomic DNA. The right panel is a autoradiogram obtained from the Southern blot hybridization of the same samples with the ^{32}P-labeled probe derived from thee psb2B-1 gene. On the autoradiogram (right panel) three bands

Figure 4.1. Restriction digestions of the genomic DNA from Synechocystis 6803. Left panel shows the ethidium bromide stained agarose gel containing genomic DNA digested with combinations of five restriction enzymes in the polylinker region of pUC18. Right panel shows Southern blot of the same sample probed with ps2B-1 coding sequence of cyanobacterium Fremyella diplosiphon.

were detected with respect to three copies of the psbA gene of Synechocystis 6803. In many cyanobacteria three copies of psbA gene per genome have been observed (Debus et al., 1988; Jansson et al., 1987). The hybridization strength was higher in two of three bands, indicating that two of the three psbA genes had higher homology with the psbB-1 gene of Fremyella diplosiphon than the third copy. The most information on the regulation of expression of the three copies of psbA in cyanobacteria is available for Synechococcus sp. PCC 7942. The three copies express differently in response to different light intensity (Golden et al., 1986; Schaefer and Golden, 1989).

A total of seven positive regions of hybridization containing about 10 colonies were removed from the primary screening out of 25,000 colonies screened and dispersed in LB medium, and an appropriate dilution was spread on a fresh nitrocellulose filter to give 100-200 colonies. This enriched population was replicated and probed, allowing isolation of pure clones of the hybridizing species.

Restriction digestion and Southern blot analysis showed that four of the colonies were identical, and contained an approximately 6 kb BamHI/PstI restriction fragment which contained psbA1 gene and were hybridized to ps2B-1 gene probe of Fremyella diplosiphon (Mulligan et al, 1984). No psbA2 or psbA3 were found during the screening. It is likely that the library made was not complete (Figure 4.2).

Figure 4.2. Screening of a genomic DNA library of Synechocystis 6803. (A) Primary screening of positive clones from the library of Synechocystis 6803; (B) Second screening of positive clones out of the clones identified in the primary screening. (C) The ethidium bromide stained agarose gel containing recombinant pUC plasmid digested with BamH I and Pst I. The 2.8 kb band is the pUC plasmid, while the 6 kb band is the BamH I/Pst I fragment containing the psb A1 gene. (D) Southern blot shows a strong positive 6 kb band.

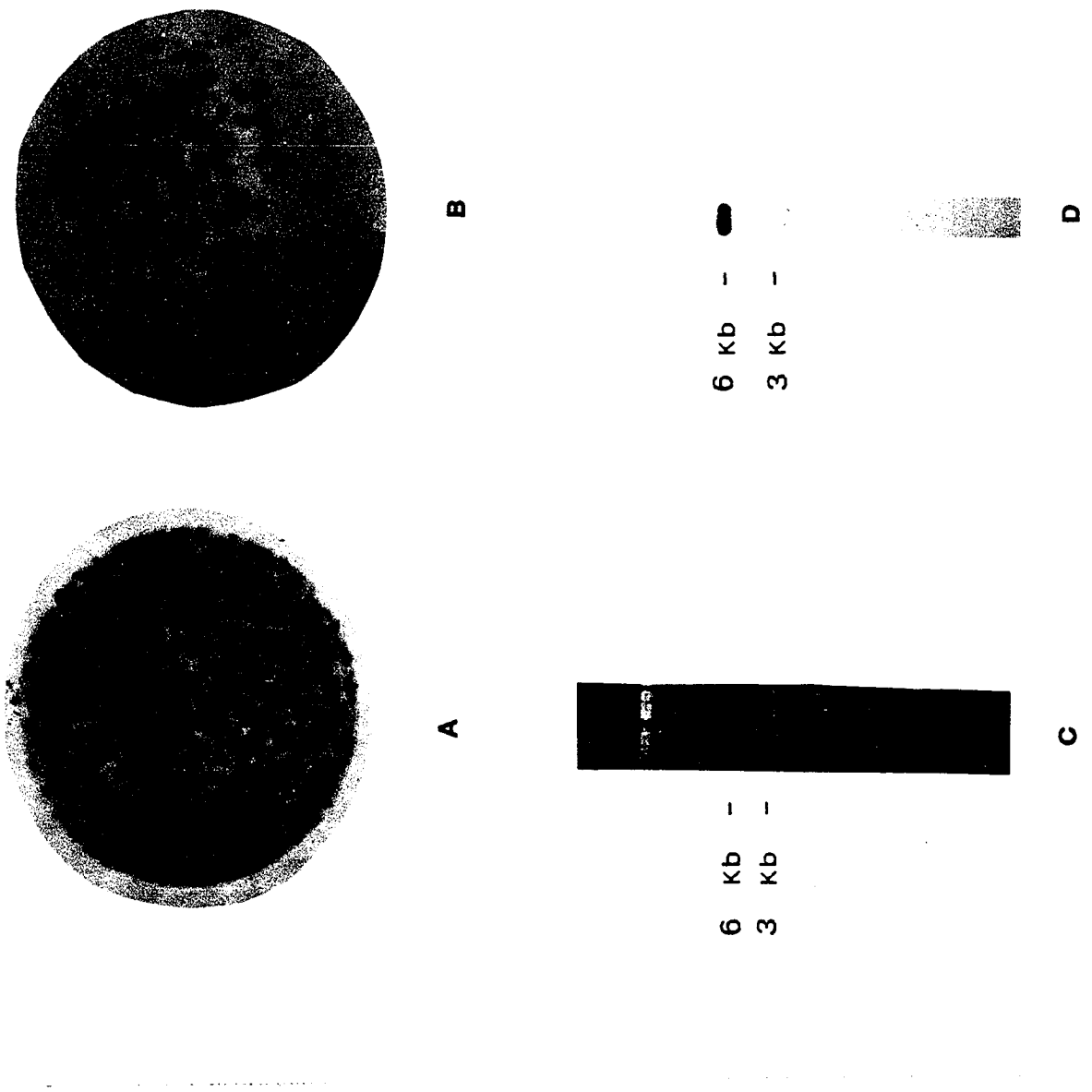
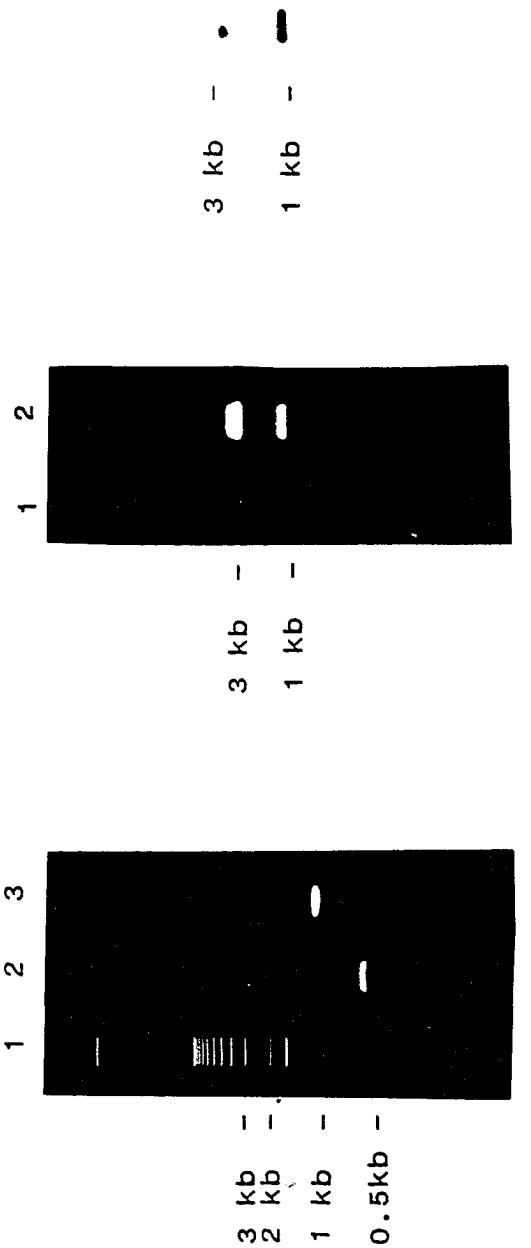


Figure 4.3. PCR amplified product carrying 1.1 kb psbA2 (left panel, lane 3) and three fragments produced by digesting at two Kpn I sites: 0.12, 0.45 and 0.55 kb (lane 2). Middle panel shows the ethidium bromide stained agarose gel of recombinant pUC plasmid containing 1.1kb psbA2. The 2.8 kb band is the pUC plasmid digested with Kpn I. Right panel shows Southern blot with the 1.1 kb band hybridization with the ^{32}P-labeled probe.



PCR method was used to clone psbA2 gene. Figure 4.3 (left panel) shows PCR amplified product carrying 1.1 kb psbA2 and three fragments produced by digesting at two known Kpn I sites (Ravnikar et al., 1989): 0.12, 0.45 and 0.55 kb. After phenol and chloroform extraction and ethanol precipitation of the PCR product, the 1.1 kb fragment was extracted and isolated from the agarose gel with Prep-A-Gene™ DNA Purification Kit. Klenow fragment was used to blunt the PCR fragment. Then the 1.1 kb fragment was cloned into pUC18 by using blunt-end cloning and confirmed by Southern blot as shown in Figure 4.3.

So far psbA1 and psbA2 had been cloned, and psbA3 also needed to be cloned before we could delete two psbA genes and mutate the third psbA gene. However, the effort of cloning of psbA genes from Synechocystis described above was stopped when the necessary deletion mutant, lacking psbA1 and psbA3, as well as the vectors containing the psbA2 for site-directed mutagenesis were made available by the laboratories of Professor Lee McIntosh and Professor A. Crofts.

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CHAPTER V. THE BINDING AFFINITY OF BICARBONATE AND FORMATE IN SITE-SELECTED D1 MUTANTS OF SYNECHOCOCCUS SP. PCC 7942

A. Introduction

The herbicides that inhibit photosynthesis have been developed during the last century (Brian, 1976). These inhibitors block electron transfer between Q_A and Q_B, the primary and secondary plastoquinone electron acceptors of PSII, by displacing plastoquinone from the Q_B binding site (Velthuys, 1981; Wraight, 1981). Experiments with the water soluble enzyme trypsin indicated that the binding sites of these herbicides are of a proteinaceous nature. The sensitivity of the Hill reaction to herbicides could be removed by treatment of chloroplasts with trypsin (Regitz and Ohad, 1975; Renger, 1976; Van Rensen and Krammer, 1979). The use of photoaffinity labeling technique allowed the identification of a 32 kDa polypeptide subunit of PSII as the herbicide or Q_B-binding protein (Pfister et al., 1981).

Hirshberg and McIntosh (1983) found a single mutation in the chloroplast gene psb A in an atrazine-resistant mutant of Amaranthus hybridus. This mutation leads to an amino acid substitution of residue 264 in the D1 protein (also called herbicide binding protein) from serine to glycine. The same mutation in psb A has also been found in other atrazine-resistant mutants of higher plants. Eleven different

structural PSII herbicide-resistant mutants have now been identified, some of which are homologous to mutations identified in photosynthetic bacteria.

In several herbicide-resistant D1 mutants in higher plants, the damping of variations in flash number dependent oxygen evolution yields was increased (Holt et al., 1983), the deactivation of the S_2 state of the oxygen evolving complex was faster (Holt et al, 1983), and the thermoluminescence peak corresponding to back reaction of S_2 with Q_B^- was replaced by a peak at lower temperatures due to back reaction of S_2 with Q_A^- (Demeter et al., 1985). These changes are consistent with an increased quasi-steady-state Q_A^- concentration due to a decreased equilibrium constant for the reaction $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$. In several cyanobacterial herbicide-resistant mutants, the initial phase of the electron transfer from Q_A^- to Q_B is unaltered but the electron transfer equilibrium between these two acceptors is also displaced towards Q_A^- (Etienne et al., 1990).

Indications for the involvement of the D1 protein in the bicarbonate effect has come from the studies on the interaction of herbicides and bicarbonate, and on herbicide-resistance mutants. The presence of urea, triazine and phenol-type herbicides (known to interact with the Q_B site) increase the apparent dissociation constant Kd for bicarbonate by at least 2-fold (Van Rensen and Vermaas, 1981); in other words, these inhibitors decrease the apparent affinity of the

thylakoid membrane for bicarbonate, indicating the closeness of the action sites of bicarbonate and PSII herbicides (Van Rensen and Vermaas, 1981; Van Rensen, 1982; Vermaas and Govindjee, 1982; Snel and Van Rensen, 1983). On the other hand, studies using ^{14}C-labelled atrazine revealed that bicarbonate depletion reduced the binding affinity of the herbicide to thylakoid membranes more than two fold, and trypsin treatment, known to modify the D1 protein and reduce Q_B binding, significantly decreased the stimulatory effect of bicarbonate on the electron transfer at the PSII acceptor complex (Khanna et al., 1981). Furthermore, a triazine-resistant mutant of Amaranthus hybridus (in which Ser-264 of D1 was changed to Gly) showed a 2-fold increase in the dissociation constant of bicarbonate (Khanna et al., 1981). Recent studies on several herbicide-resistant D1 mutants of the cyanobacterium Synechocystis 6714 (Govindjee et al., 1990) and of Chlamydomonas reinhardtii (Govindjee et al., 1991a) demonstrate differential formate sensitivities. Formate replaces bicarbonate at the Q_AFeQ_B complex. Measurements on Chl a fluorescence transients in herbicide-resistant mutants of Chlamydomonas reinhardtii revealed differential sensitivity to 25 mM formate treatment: the most sensitive mutant is S264A and the most resistant mutant is L257F. The order of sensitivity is S264A >> V219I =F255Y > wild type >> A251V > L275F (Govindjee et al., 1991b). Based on the data of oxygen evolution, Chl a fluorescence decay and Chl a fluorescence

transient kinetics, the order of sensitivity of Synechocystis 6714 mutants to formate is: S264A > wild type > F211S >> F211S/A251V (Govindjee et al., 1990).

In this Chapter, I examine the effect of mutations in specific amino acids in the D1 protein on the binding affinity of the inhibitory and stimulatory anions formate and bicarbonate, respectively. The mutants used were D1-F255Y (Tyr5), D1-S264A (Dil), D1-F255Y/S264A (D5) and D1-F255L/S264A (Di22) from Synechococcus sp. PCC 7942. Measurements on oxygen evolution with different concentrations of formate in the presence of fixed bicarbonate concentration and vice versa, analyzed in terms of an equilibrium activator-inhibitor model (Seggel, 1975), led to the conclusion that it is the dissociation constant (K_A) of bicarbonate binding that is increased in S264A, and that this increase is maximum when F255 is co-mutated to L, than when F255 is co-mutated to Y. In the absence of this co-mutation, K_A is still larger in S264A than that in the wild type and F255Y mutant, suggesting the importance of D1-S264 to bicarbonate binding.

B. Materials and Methods

1. Growth of cyanobacteria

Synechococcus PCC 7942 and mutants of this strain (see Table 5.1) were kindly provided by Dr. J. Hirschberg at The Hebrew University in Jerusalem. The cyanobacteria were grown in BG-11 liquid medium at 30 °C.

Table 5.1. D1 mutants of Synechococcus sp. PCC 7942 used in this study[a].

Name	Mutation	Designation
Tyr5	Phe-255 → Tyr	D1-F255Y
Dil	Ser-264 → Ala	D1-S264A
D5	Phe-255 → Tyr; Ser-264 → Ala	D1-F255Y/S264A
Di22	Phe-255 → Leu; Ser-264 → Ala	D1-F255L/S264A

a: Hirshberg et al., 1987; Horovitz et al., 1989.

2. Steady-state oxygen evolution

The steady-state oxygen evolution in cyanobacterial cells was determined polarographically under saturating light (3600 μmoles of photons/$m^2 \cdot s$) filtered with a Corning CS3-68 yellow filter, using a Yellow Springs Instrument Clark-type electrode. A combination of two electron acceptors, 2,5-dimethyl-p-benzoquinone (DMQ) (0.5 mM) and $K_3Fe(CN)_6$ (1 mM), was used. DMQ acts as the electron acceptor and the non-penetrating ferricyanide keeps the DMQ in the oxidized state. Twenty μM 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) was added in the reaction medium to block electron flow between the plastoquinone pool and PSI (Trebst, 1980). The Chl a concentration used for oxygen evolution measurements was 20 μg/ml. The suspension medium was the reaction medium containing 0.1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 μM gramicidin D and 20 mM HEPES (pH 6.8) (see Cao and Govindjee, 1988).

3. Chlorophyll a fluorescence decay

The kinetics of decay of variable Chl a fluorescence, after a single-turnover saturating flash, were measured at 685 nm by a weak measuring light. The measuring light was fired at variable times after each actinic flash. The actinic (FX-124, EG and G) and the measuring flashes (Stroboslave 1593A, General Radio) were filtered with two Corning blue (CS 4-96) glass filters; both had a 2.5 μs duration at half-maximum peak (see details in Eaton-Rye and Govindjee, 1988a). Thylakoids

were prepared by a procedure modified after Burnap et al. (1989). Thylakoid suspensions, at a Chl a concentration of 5 μg/ml, were dark adapted for 15 min. In order to calculate the rate constants of Q_A^- decay, the relative Q_A^- concentration was estimated from the variable Chl a fluorescence yield according to Joliot and Joliot (1964) using the formula given by Mathis and Paillotin (1981). The connection parameter, p, of 0.45 obtained from thylakoid membranes of a cyanobacterium Phormidium laminosum (Bowes and Bendall, 1983) was used.

C. Results

1. Sensitivity to herbicides: Chl a fluorescence yield measurements

Figure 5.1 shows the rise of variable Chl a fluorescence yield as a function of DCMU concentration. The fluorescence yield is measured at 1 ms after a saturating flash. The ordinate of the graph is normalized by the measured fluorescence yields at fully bound states at saturating DCMU concentrations. The dissociation constants for DCMU calculated from the $[Q_A^-]$ vs. [DCMU] plots are 17.8 nM, 31.6 nM, 3.2 μM, 4.0 μM and 177.8 μM for wild type, D1-F255Y, D1-S264A, D1-F255Y/S264A and D1-F255L/S264A mutants, respectively. These data are in agreement with the I_{50} values of the mutants as measured from the reduction of DCPIP (Hischberg et al., 1987; Ohad et al., 1990; Horovitz et al., 1989).

2. Sensitivity to formate: Hill reaction measurements

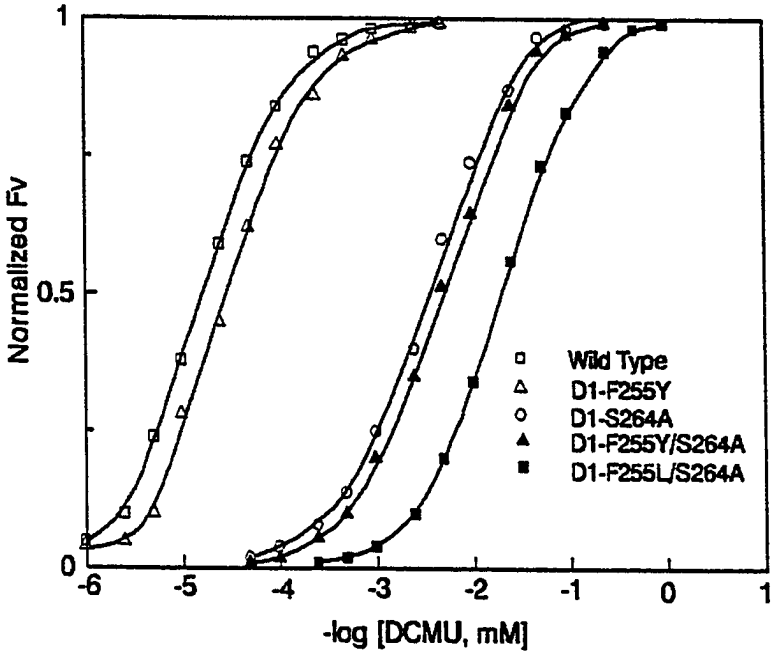


Figure 5.1 The rise of variable Chl a fluorescence yield (F_v) as a function of DCMU concentration in D1 mutants of Synechococcus 7942. The fluorescence yield is measured at 1 ms after a saturating actinic flash.

It has been demonstrated that bicarbonate stimulates the Hill reaction in bicarbonate-depleted Synechocystis 6803 cells (Cao and Govindjee, 1988, 1990). Figure 5.2 shows the Hill reaction activity (measured as oxygen evolution) of bicarbonate-depleted wild type and mutant cells in the presence of various formate concentrations. 100% in wild type was equivalent to 185 μmoles O_2/mgChl.h, whereas 100% in mutants D1-F255Y, D1-S264A, D1-F255Y/S264A and D1-F255L/S264A were 146, 144, 131 and 120 μmoles/mgChl.hr, respectively. The I_{50} value, the concentration of formate at which half-maximal inhibition of oxygen evolution rate occurs, was calculated to be 46, 37, 30, 27 and 20 mM, respectively, for wild type, D1-F255Y, D1-S264A, D1-F255Y/S264A and D1-F255Y/S264L. The inhibited reaction was fully recovered by the addition of 10 mM bicarbonate in all cases. Thus, the order of sensitivity (lowest to highest) to formate inhibition is wild type < D1-F255Y < D1-S264A < D1-F255Y/S264A < D1-F255L/S264A.

3. Analysis of Hill reaction measurements: activator-inhibitor model

In the previous studies of the reactivation by bicarbonate of Hill reaction in bicarbonate-depleted samples, the apparent dissociation constant of bicarbonate in the presence of formate was estimated by taking bicarbonate assubstrate and formate as the inhibitor that competes with the substrate and blocks the enzymatic activity (Stemler and Murphy, 1983; Snel and Van Rensen, 1983,1984; Blubaugh and

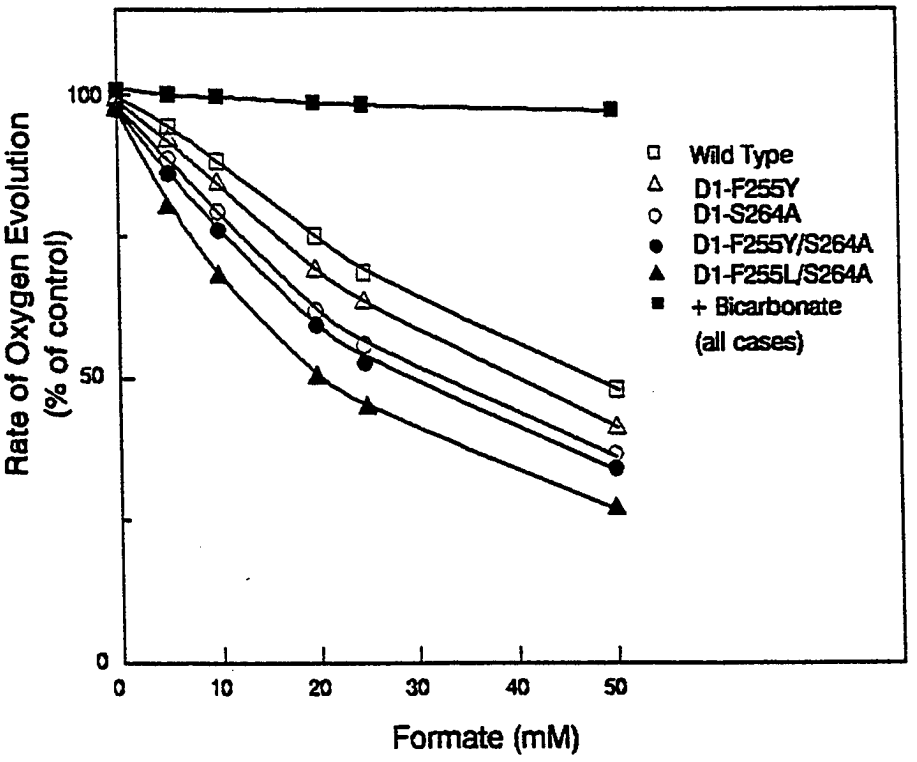
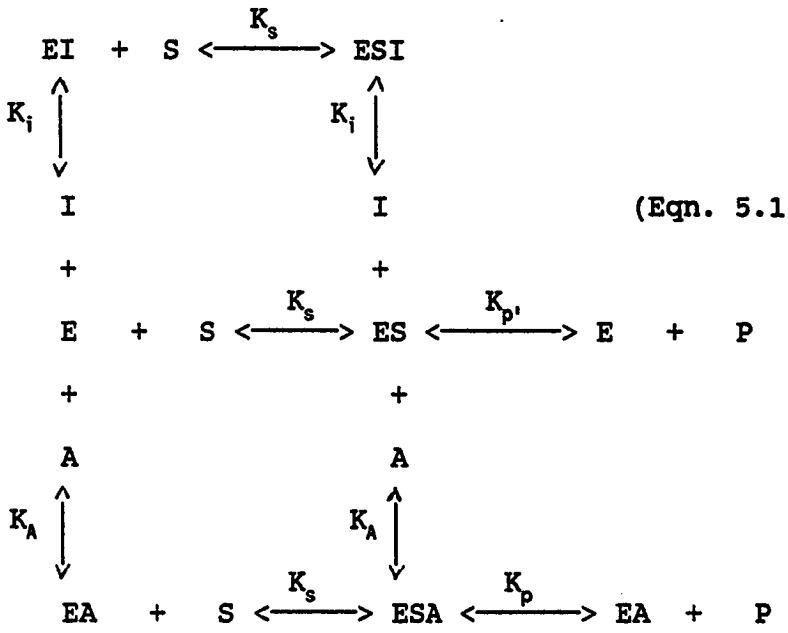


Figure 5.2. The Hill reaction activity (measured as oxygen evolution) of bicarbonate-depleted wild type and mutant cells of Synechococcus 7942 in the presence of different concentrations of formate. 100% in wild type was equivalent to 185 μmoles O_2/mgChl.h. The I_{50} value, the concentration of formate at which half-maximal inhibition of oxygen evolution rate occurs, was calculated to be 46, 37, 30, 27 and 20 mM, respectively, for wild type, D1-F255Y, D1-S264A, D1-255Y/S264A and D1-F255L/S264A. The inhibited reaction was fully recovered by the addition of 10 mM bicarbonate in all cases.

Govindjee, 1988). However, the catalytic activity of PSII is to reduce plastoquinone to plastoquinol and to oxidize water to oxygen. It has not been well established that bicarbonate directly participates as a catalytic substrate in PSII electron transfer activity. It is more appropriate to consider its function as an activator that stimulates the PSII activity, and accordingly formate acts to replace the activator inhibiting the enzymatic function of PSII. Here, I use a rapid equilibrium enzymatic model to describe the oxygenic activity of PSII. In this model, the binding of activator (bicarbonate) stimulates the enzymatic activity of PSII. When the activator (A) is not bound to the enzyme (E) the enzymatic activity is low. The inhibitor (e.g. formate) (I) competes with the binding of the activator at its binding site that is distinct from that of the substrate; however, the inhibitor itself does not interfere with the binding of the substrate. As a result, the ESI complex can form but it is inactive. The activator competes with the inhibitor for the inhibitor site. When the activator occupies the inhibitor site, the ESA complex forms and the PSII activity is stimulated. I is a noncompetitive inhibitor with respect to S; I and A are competitive. If the inhibitor acts noncompetitively with respect to the substrate but the activator and inhibitor are mutually exclusive, then the equilibria of the system can be depicted as:



(Eqn. 5.1)

where E is the enzyme, S substrate, A activator, and I inhibitor. The various equilibrium constants are:

$$K_s = \frac{[E][S]}{[ES]} = \frac{[EI][S]}{[EIS]} = \frac{[EA][S]}{[ESA]} \qquad \text{(Eqn. 5.2)}$$

$$K_i = \frac{[E][I]}{[EI]} \qquad \text{(Eqn. 5.3)}$$

$$K_A = \frac{[E][A]}{[EA]} \qquad \text{(Eqn. 5.4)}$$

$K_{p'}$ = rate for the breakdown of ES to P

K_p = rate for the breakdown of ESA to P

The velocity equation for the system is:

$$\frac{1}{v} = \frac{(1 + \frac{Ks}{[S]})}{Ki\ Vmax\ (1 + \frac{[A]}{K_A})}[I] + \frac{1}{Vmax}(1 + \frac{Ks}{[S]}) \quad \text{(Eqn. 5.5)}$$

where v is the initial velocity and Vmax the maximum velocity.

In the system above, the K_A and K_i values can be determined by measuring the effect of different concentrations of A on the inhibition by I. This is most conveniently accomplished by Dixon plots of 1/v versus [I] at a constant [S] and different fixed concentrations of A (Segel, 1975). Figure 5.3 shows several Dixon plots, the reciprocal of oxygen evolution rate versus formate concentration at different concentrations of bicarbonate. The intercept on the [I]-axis gives $-K_i(1 + K_s/[S])(1 + [A]/K_A)/(1 + K_s/[S])$. A series of the above values can be obtained at various [A] values and the K_A and K_i values solved. Furthermore, the horizontal-axis intercept gives $-K_A$ in the slope plot. In this study, the steady-state oxygen evolution rate was measured after incubating samples in the dark for 10 min with various concentrations of bicarbonate and formate. Since HCO_3^- is considered the active species for the stimulation of electron transfer in PSII complex (Blubaugh and Govindjee, 1986), the equilibrium concentration of HCO_3^- at the experimental pH value (pH 6.8) was used in the calculation; the equilibrium between bicarbonate and carbon dioxide is known to be completed within

Figure 5.3. Dixon plots for the wild type and the D1 mutants of Synechococcus 7942. The reciprocal of oxygen evolution rate versus formate concentration at different concentrations of bicarbonate: □: control; △: 0.5 mM bicarbonate; ○: 0.75 mM bicarbonate; ■: 1.0 mM bicarbonate. The oxygen evolution rate for the wild type (185 μmoles O_2/mgChl.h) was normalized to 100 %.

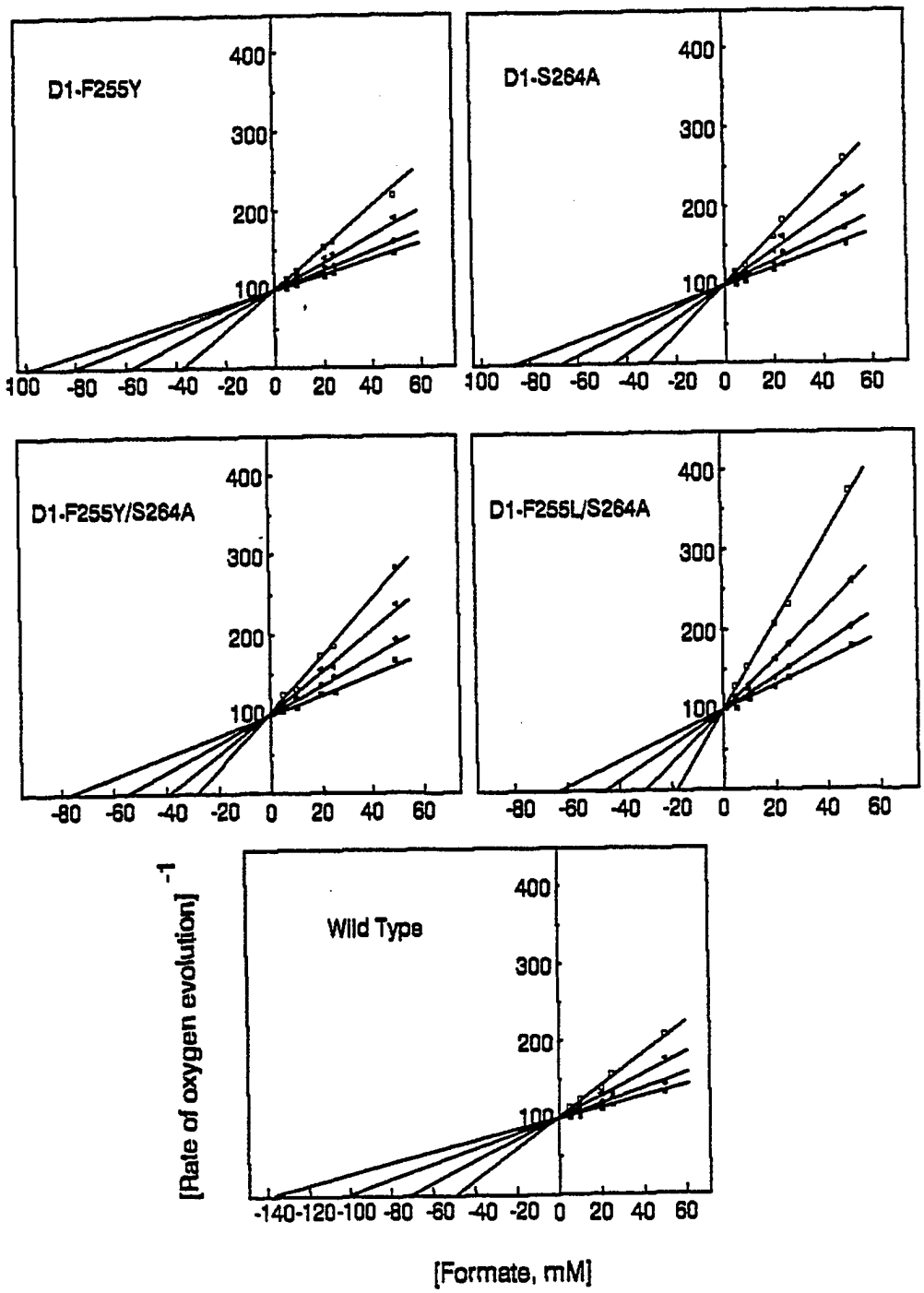


Table 5.2. I_{50} values for formate and dissociation constants for formate (K_i) and bicarbonate (K_A) of the wild type and D1 mutants.

	I_{50}	K_i	K_A
Wild type	46 mM	3.5 mM	21 μM
D1-F255Y	40 mM	3.3 mM	23 μM
D1-S264A	32 mM	3.2 mM	26 μM
D1-F255Y/S264A	28 mM	3.2 mM	32 μM
D1-F255L/S264A	20 mM	3.3 mM	44 μM

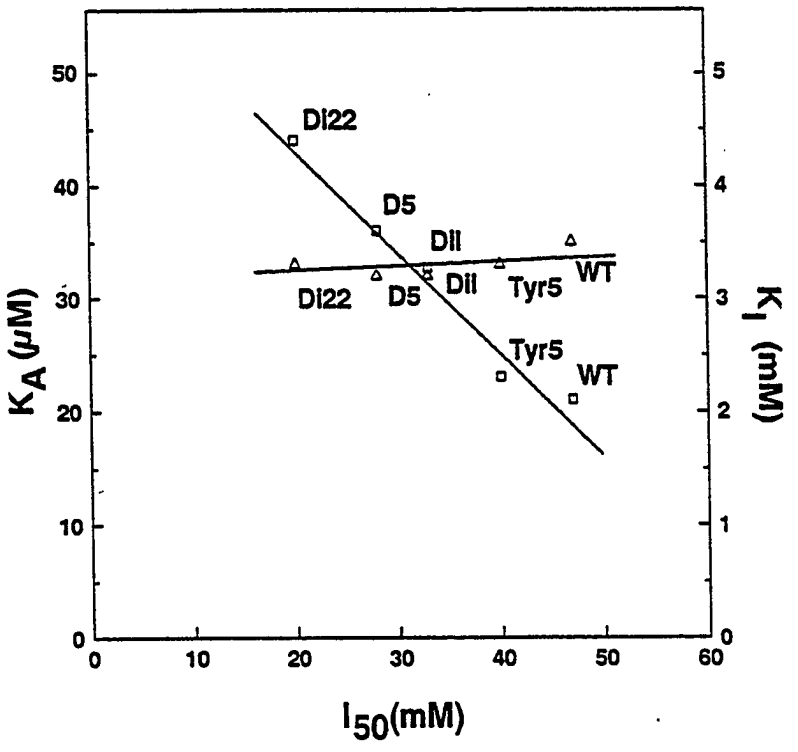


Figure 5.4. Plots of K_A, dissociation constant for bicarbonate (□) and K_i, dissociation constant for formate (△) in the wild type and mutants of Synechococcus 7942 against the I_{50}.

1-3 min (Cooper et al., 1968; Blubaugh and Govindjee, 1986).

Table 5.2 lists dissociation constants for bicarbonate and formate (K_A and K_i) in the wild type as well as in the mutants. It indicates that D1-S264A and D1-F255L/S264A have increased dissociation constants for bicarbonate while the dissociation constants for formate are nearly unaltered in all the cases. Figure 5.4 shows the relationship between K_A (and K_i) and I_{50} for the four mutants. There exists a correlation between K_A and I_{50} values. The data fit on a roughly linear regression. However, K_i remains unchanged. The correlation between K_A and I_{50} indicates that the I_{50} decreases in the mutants is caused by the increase in K_A value and not by a change in K_i. It suggests the involvement of D1-S264 in bicarbonate binding.

D. Discussion

A single amino acid mutation in the D1 protein can lead to herbicide resistance in chloroplasts (Hirshberg and McIntosh, 1983). Among other amino acids at positions 211, 219, 251 and 275, Phe-255 and Ser-264 are frequently exchanged in D1 protein (Brusslan and Haselkorn, 1987, 1988), and have turbutryn-resistant homologues in the L subunit of R. sphaeroides and/or R. viridis at positions L-F215 and L-S223 (Paddock et al., 1987; Sinning and Michel, 1987). In bacteria the amino acid changes are different: L-F216 → S instead of D1-F255 → Y and L-S233 → P instead of D1-S264 → A or G.

D1-S264 interacts with herbicides diuron and atrazine as well as the triazinones, while D1-S264 → A is more resistant to diuron than to atrazine (Brusslan and Haselkorn, 1988). D1-F255 interacts with atrazine (Trebst, 1986). D1-F255Y has nearly wild type character regarding diuron resistance, D1-F255Y/S264A and D1-S264A are highly resistant to diuron. Substitution of Phe-255 with Leu instead of the aromatic amino acid Tyr in mutant D1-S264A leads to a double mutant D1-F255L/S264A with an extremely high tolerance to diuron. It is in this mutant that shows the highest sensitivity to formate.

Among other amino acids, S264 and F255 are suggested to be in contact with Q_B binding domain (Trebst, 1986). X-ray crystallographic studies on the reaction centers of R. viridis suggest that in contrast to the environment of Q_A, Q_B is surrounded by polar and charged amino acid residues. It is believed that hydrogen bonding between an acceptor molecule, or an inhibitor molecule, and amino acid residues in the Q_B binding site play a crucial role in the binding and release of plastoquinones and inhibitors in PSII (Brusslan and Haselkorn, 1988). In addition to their role in sterical arrangement, H-bonds are assumed to dominate the binding affinity.

Several of the mutations in the Q_B domain not only affect herbicide affinity, but also Q_B properties. For example, in Ser-264-Gly mutants, the equilibrium between $Q_A^-.Q_B$ and $Q_B.Q_B^-$ shifts considerably to the left as compared to the wild type (Ort et al., 1983; Vermaas and Arntzen, 1983; Vermaas et al.,

1984; Govindjee et al., 1991b). This causes a decrease in the quantum yield of charge separation because of an increase in the steady-state Q_A^- concentration, which leads to a lower maximum rate of photosynthesis. It should be emphasized that the primary physiological effect of triazine resistance on Q_B function is not a slowing down of electron transport between Q_A and Q_B, as often implied in the literature (for example, Pfister and Arntzen, 1979), but a decrease of the midpoint potential of the Q_B/Q_B^- redox couple, causing a shift in the semiquinone equilibrium between Q_A and Q_B (Etienne et al., 1990; Ohad et al., 1990).

Double mutants in Synechoccocus 7942 showed that the effect of some mutations are additive for herbicide resistance. For example, the resistance of D1-S264A and D1-F255Y to atrazine. For other herbicides, this pattern is not observed; for example, the resistance to monuron is increased 570 X in the D1-264 mutant and 1.5 X in the D1-255 mutant, yet only 250 X in the double mutant. In the case of diuron, Tyr has little effect while Leu has a drastic effect. It is likely that the aromatic group plays an essential role in diuron binding (Hirshberg et al., 1987).

Measurements in several laboratories of a dissociation constant (K_d) for the bicarbonate in PSII complex of 80 μM (Stemler and Murphy, 1983; Snel and Van Rensen, 1984; Jursinic and Stemler, 1986) must have been overestimated since nearly half of the added HCO_3^- was converted into CO_2. Considering the

equilibrium concentration of HCO_3^- in the reaction medium, the Kd is about 40 μM (Blubaugh and Govindjee, 1988) in chloroplasts of higher plants. Results of the present study show that Kd for HCO_3^- in cyanobacterial cells is 21 μM at pH 6.8 (see Table 5.2).

The dissociation constant for formate is calculated to be about 3 mM, about 100 times higher than that of bicarbonate. In the kinetics of formate binding and HCO_3^--recovery studies, through measurements on F_v decay, the half time of F_v rise by formate binding is in the minute range, whereas that of F_v decline by HCO_3^--recovery is in second range (Diner and Petrouleas, 1990). This is consistent with the results of K_A and K_i. Xu et al. (1991) show that formic acid, but not formate, is the effective inhibitory species. As a result, the K_i for formic acid would be 30 μM. Nitride oxide (NO), another PSII inhibitor liganding to non-heme iron, has a Kd of about 30 μM (Diner and Petrouleas, 1990). Therefore, HCO_3^- has K_d similar to that of formic acid and NO. However, HCO_2^- has similar structure and degree of charge delocalization as HCO_3^-. Both HCO_2^- and HCO_3^- have a carboxyl group, with the same degree of charge delocalization, but only HCO_3^- stimulates the Hill reaction. The main structural difference between the two is the presence of a hydroxyl group in HCO_3^- that is absent in HCO_2^-. It appears likely that the hydroxyl group is the functional moiety, whereas the carboxyl group is involved in binding (Blubaugh and Govindjee, 1988).

Upon reduction of Q_B by Q_A^-, Q_B^- is formed, and it stays at the Q_B site in D1 until Q_A is reduced again by a second turnover of the reaction center. Subsequent oxidation of Q_A^- and protonation of Q_B^{2-} will lead to the formation of Q_BH_2. This fully reduced Q_B can leave the Q_B site. A plastoquinone molecule from the pool in the thylakoid can then bind to the Q_B site and accept the next pair of electrons (see a review by Crofts and Wraight, 1983). The possibility of a second site of the bicarbonate effect, dealing with protonation, was based on the observations that the maximum inhibition of electron transfer occurs only after two or more actinic flashes (Govindjee et al., 1976; Robinson et al., 1984; Eaton-Rye and Govindjee, 1988a,b; Xu et al., 1991) and that electron transport from Q_A^- to Q_B^- in bicarbonate depleted thylakoid membranes is pH dependent (Eaton-Rye and Govindjee, 1988a,b). As implied above, the large inhibition after the second and subsequent flashes was explained to be due to a result of an inhibition of protonation of Q_B^-. An arginine residue in the D1 protein has been proposed to be a plausible ligand to bicarbonate anion (Shipman, 1981; Crofts et al., 1987; Blubaugh and Govindjee, 1988). In photosynthetic bacteria, Glu^{L212} and Asp^{L213} are involved in protonation of Q_B, and replacement of these ionizable residues with nonionizable amino acids results in lesions at Q_B protonation steps (Takahashi and Wraight, 1990, 1991), which resembles the bicarbonate-depletion in PSII. It is likely that the

carboxylate group of bicarbonate forms ligands to Fe^{+2}, and the cationic side chains of Arg and/or Lys residues in the vicinity form hydrogen bonds to bicarbonate as in human lactoferrin (Anderson et al., 1989). The hydroxyl group of bicarbonate may be hydrogen bonded to other ionizable residues to participate in a proton translocation pathway to Q_B. The double mutation (S264 → A; F255 → L) may have the strongest effect on carboxylate group linkage with Fe^{2+} and/or neighboring cationic side chains, and result in the lowest K_A for HCO_3^- among the mutants, while the single mutation (F 255 → Y) may have little effect, having a single K_A as the wild type.

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CHAPTER VI. SUMMARY AND A HYPOTHESIS

The conclusions obtained in the previous chapters are summarized here. Based upon the present studies in this thesis and recent researches that have been done in other laboratories, a working hypothesis is proposed regarding the bicarbonate binding and function in PSII reaction centers.

A. The bicarbonate effect is present in cyanobacteria

The Hill reaction is significantly and reversibly inhibited in chloroplasts of higher plants and algae depleted of bicarbonate in the presence of formate (Blubaugh and Govindjee, 1988; Govindjee, 1991a). However, in photosynthetic bacteria, no "bicarbonate effect" has been observed (Shopes et al., 1988; Govindjee et al., 1991; Wang et al., 1991). Therefore, it was considered important to look at the "bicarbonate effect" in cyanobacteria, since these prokaryotes are fundamentally similar to oxygenic photosynthetic system found in the chloroplasts, and are genetically transformable with foreign DNA (Stanier and Cohen-Bazire 1977, Curtis and Haselkorn 1984, Williams and Chisholm 1987).

I have discussed the results on the bicarbonate effect in the cyanobacterium Synechocystis sp. PCC 6803 in Chapter II. Treatment of Synechocystis 6803 thylakoids with formate, nitrite or azide caused a slowing of the oxidation of Q_A^-, as

calculated from Chl a fluorescence decay after single turnover saturating flashes. Addition of 5mM bicarbonate fully reversed this inhibition in formate- and nitrite-treated samples. However, in 100 mM azide-treated samples only 50% of the inhibition at 2 ms after the actinic flash was reversed. The inhibitory anion treatment affects the Q_A-Q_B electron transfer. The Hill reaction in bicarbonate-depleted Synechocystis cells was stimulated more than 4 fold by 5 mM bicarbonate. The pH range for the optimum stimulatory effect was about pH 6.8. These data show the existence of the bicarbonate effect in cyanobacteria, suggesting its existence in all PSII reaction centers while absent in reaction centers of anoxygenic photosynthetic bacteria. These results made it possible to use the highly transformable cyanobacterium Synechocystis sp. PCC 6803 to probe the structure/function relationships in the D2 by genetic engineering techniques.

B. Cationic amino acids in the D2 protein may involve bicarbonate binding/stabilization

Cationic amino acid residues such as arginine have been proposed to be responsible for the binding of bicarbonate in PSII (Shipman, 1987; Blubaugh and Govindjee, 1988). To test if selected arginine (Arg) residues are involved in the binding of HCO_3^-, oligonucleotide-directed mutagenesis was used to construct Synechocystis sp. PCC 6803 mutants carrying mutations in Arg residues of the D2 protein. I have discussed

my results in Chapter III. Measurements of oxygen evolution showed that the D2 mutants D2-R233Q (arginine-233->glutamine) and D2-R251S (arginine-251->serine) were ten-fold more sensitive to formate than the wild type. The formate concentration giving half-maximal inhibition of the steady-state oxygen evolution rate was 48 mM, 4.5 mM and 4 mM for the wild type, D2-R233Q and D2-R251S, respectively. The equivalent formic acid concentrations are 48 μM, 4.5 μM and 4μM, respectively. Measurements of oxygen evolution in single-turnover flashes confirm that mutants are more sensitive to formate than the wild type. Measurements of Chl a fluorescence decay kinetics after the second saturating actinic flash indicated that, after formate treatment, the halftime of Q_A^- oxidation was decreased by approximately a factor of 2, 4 and 6 in the wild type, D2-R251S and D2-R233Q, respectively. The recombination rate between Q_A^- and S_2 was approximately two-fold slower in D2-R251S and D2-R233Q than in the wild type. In the presence of 100 mM sodium formate, reactivation of the Hill reaction by bicarbonate showed that the wild type had an apparent Km for bicarbonate of 0.5 mM, while the K_m's for D2-R233Q and D2-R251S were 1.4 and 1.5 mM, respectively.

Observations in this study (Chapter III) clearly show that the D2 protein is involved in the bicarbonate effect in PSII. Mutations of R233 and R251 in the D2 polypeptide in Synechocystis 6803, that lead to only minor disturbances in PSII function, result in a significant susceptibility to the

replacement of bicarbonate by the competitive anion formate (or formic acid). The present study suggests that the two arginine residues (233 and 251), although not necessarily vital to bicarbonate binding, are important for the stabilization of bicarbonate binding in PSII, whereas arginine 139 is not.

C. Amino acid substitutions in Q_B binding pocket leading to herbicide resistance affect the bicarbonate binding, but not formate binding, in PSII

The involvement of the D1 protein in the bicarbonate effect has been known from the studies of herbicide and bicarbonate interaction, and of herbicide-resistance mutants. (Khanna et al., 1981; Vermaas and Govindjee, 1982; Snel and Van Rensen, 1983). Studies on several herbicide-resistant D1 mutants of the cyanobacterium Synechocystis 6714 (Govindjee et al., 1990) and of Chlamydomonas reinhardtii (Govindjee et al., 1990b) confirm the involvement of the D1 protein in the bicarbonate effect.

In Chapter V, I used an equilibrium model involving an activator and inhibitor (Seggel, 1975) to fit the oxygen evolution data obtained under conditions of competition between bicarbonate and formate in four site-selected D1 mutants. The dissociation constants for the anions was estimated in order to examine the effect of mutations on the binding affinities of formate and bicarbonate. The I_{50} value,

the concentration of formate at which half-maximal inhibition of oxygen evolution rate occurs, was calculated to be 46, 37, 30, 27 and 20 mM, respectively, for wild type, D1-F255Y, D1-S264A, D1-F255Y/S264A and D1-F255L/S264A. The inhibited reaction was fully recovered by the addition of 10 mM bicarbonate. Thus, the order of sensitivity (lowest to highest) to formate inhibition is wild type < D1-F255Y < D1-S264A < D1-F255Y/S264A < D1-F255L/S264A. The dissociation constant K_A for bicarbonate was calculated to be 21 μM, 23 μM, 32 μM, 26μM and 44μM, respectively, and the dissociation constant K_i for formate was 3.5 mM, 3.3mM, 3.2 mM, 3.2 mM and 3.3 mM, respectively, for wild type, D1-F255Y, D1-S264A, D1-F255Y/S264A and D1-F255L/S264A. These results indicate that the mutations especialy at D1-S264 increase the dissociation constants for bicarbonate while the dissociation constants for formate are nearly unaltered. This is particularly so when it is accompanied by co-mutation in F255 to L, but not to Y.

It is likely that the carboxylate group of bicarbonate forms ligands to Fe^{+2}, and the cationic side chains of Arg and/or Lys residues in the vicinity form hydrogen bonds to bicarbonate as in human lactoferrin (Anderson et al., 1989). The hydroxyl group of bicarbonate may be hydrogen bonded to other ionizable residues to participate in a proton translocation pathway to Q_B. As noted above, the double mutation (S264 → A; F255 → L) has the strongest effect on carboxylate group linkage with Fe^{2+} and/or neighboring cationic

side chains, and result in the lowest K_A for HCO_3^- among the mutants, while the single mutation (F 255 → Y) may have the least effect, having a similar K_A as the wild type.

D. A hypothesis

The notion that bicarbonate interacts with non-heme iron in PSII came from the observation that formate drastically increases the amplitude of g=1.82 EPR signal of $Q_A^-Fe^{2+}$ of PSII (Vermaas and Rutherford, 1984). Formate also decreases the quardrupole splitting and the chemical shift of the non-heme Fe^{2+} Mossbauer spectrum (Diner and Petrouleas, 1987; Semin et al., 1990). Furthermore, NO, carrying an unpaired electron, forms ligation to non-heme iron and produces S=3/2 Fe(II)-NO SPR signal at g=4, and NO inhibits Q_A-Q_B electron transfer in a manner similar to that by formate. Bicarbonate diminishes this signal and recovers the inhibited electron transfer (Diner and Petrouleas, 1990). This indicates that bicarbonate binds to Fe^{2+}.

The fifth and sixth ligands of the non-heme iron are furnished by the carboxylate group of Glu^{M232} in the reaction center of Rb. viridis while this glutamic acid residue is absent in PSII. Bicarbonate is suggested to provide bidentate ligand to iron in PSII (Michel and Deisenhofer, 1988). The crystallographic structure of human lactoferrin reveals that a carbonate ion forms a bidentate ligand to Fe^{3+} (Anderson et al., 1989). The oxygens of carbonate are hydrogen bonded to

four neighboring residues including an arginine.

No Mossbauer spectral or EPR Fe(II)-NO adduct signals are observed with bacterial RCs. This is consistent with the examination of the X-ray structure showing that M-glu-232 forms ligand to Fe^{2+}. The removal of such a ligand in mutants at the site of M-E234 in Rb. sphaeroides by site-directed mutagenesis shows no effect on the kinetics of Q_A^- to Q_B electron transfer with or without formate treatment (Wang et al., 1991). Thus, the ligand between Glu^{M234} and Fe^{2+} is not essential for electron transfer at the Q_AFeQ_B complex. This implies that the hydroxyl group of bicarbonate may be crucial for bicarbonate function in PSII while its carboxylate group ligands to iron.

The crystallographic structure of human lactoferrin, in which binding of Fe(III) requires synergistic (bi)carbonate (HCO_3^-/CO_3^{2-}) ion binding, shows that a (bi)carbonate ion binds to Fe(III) (Anderson et al., 1989). The oxygens of HCO_3^-/CO_3^{2-} are hydrogen bonded to four residues in the vicinity, including an arginine. It is possible that one or more of the Arg of D2 provide hydrogen bonds to stabilize the binding of HCO_3^-/CO_3^{2-}, which in turn forms a bidentate ligand with Fe(II) in PSII.

Mutagenesis studies show a severe lesion in electron transfer from Q_A to Q_B in D2-lys-264 mutant (see Diner et al., 1991) and a high sensitivity to formate treatment in D2-Arg 251 and Arg-233 mutants (Cao et al., 1991; Chapter III) in

Synechocystis 6803 (see Figure 6.1). These observations indicate the importance of these cationic residues in bicarbonate binding in PSII.

Bicarbonate has been suggested to be involved in the protonation of Q_B (Blubaugh and Govindjee, 1988; Eaton-Rye and Govindjee, 1988; Van Rensen et al., 1988). The kinetics of Chl a fluorescence yield decay, reflecting Q_A^- oxidation after saturating actinic flashes, is slowed in formate-treated/bicarbonate-depleted thylakoids, and significant slowing occurs only after the second and subsequent flashes. To best interpret the data, an inhibition of protonation of Q_B^- by bicarbonate depletion is suggested. Several mutants at D1-S264 (see Figure 6.2) are most sensitive to bicarbonate-depletion, implicating the role of bicarbonate in the protonation of Q_B since D1-S264 interacts with Q_B.

Protonation of Q_B in bacterial reaction centers is likely to involve a number of ptotonatable amino acid residues (Maroti and Wraight, 1988 a,b). They cooperate to transfer protons from the aqueous phase to the Q_B site. Allen et al. (1988) and Deisenhofer and Michel (1989) have indicated some residues that may be involved in such a pathway.

Site-directed mutagenesis studies show that Glu^{L212}, Asp^{L213} and Ser^{L223} in close vicinity to Q_B are involved in terminal proton delivery to Q_B (Paddock et al., 1989; Takahashi and Wraight, 1990, 1991), and replacement of the ionizable Glu^{L212}

Figure 6.1. Diagrammatic representation of the region of the fourth and fifth putative transmembrane helices of the D2 protein in spinach. The residues indicated by arrows have been mutated to examine the effects on bicarbonate function in PSII. The D2 residue numbering of cyanobacteria is one residue less than that of higher plants (Based on models by Trebst, 1986, and Vermaas and Ikeuchi, 1991).

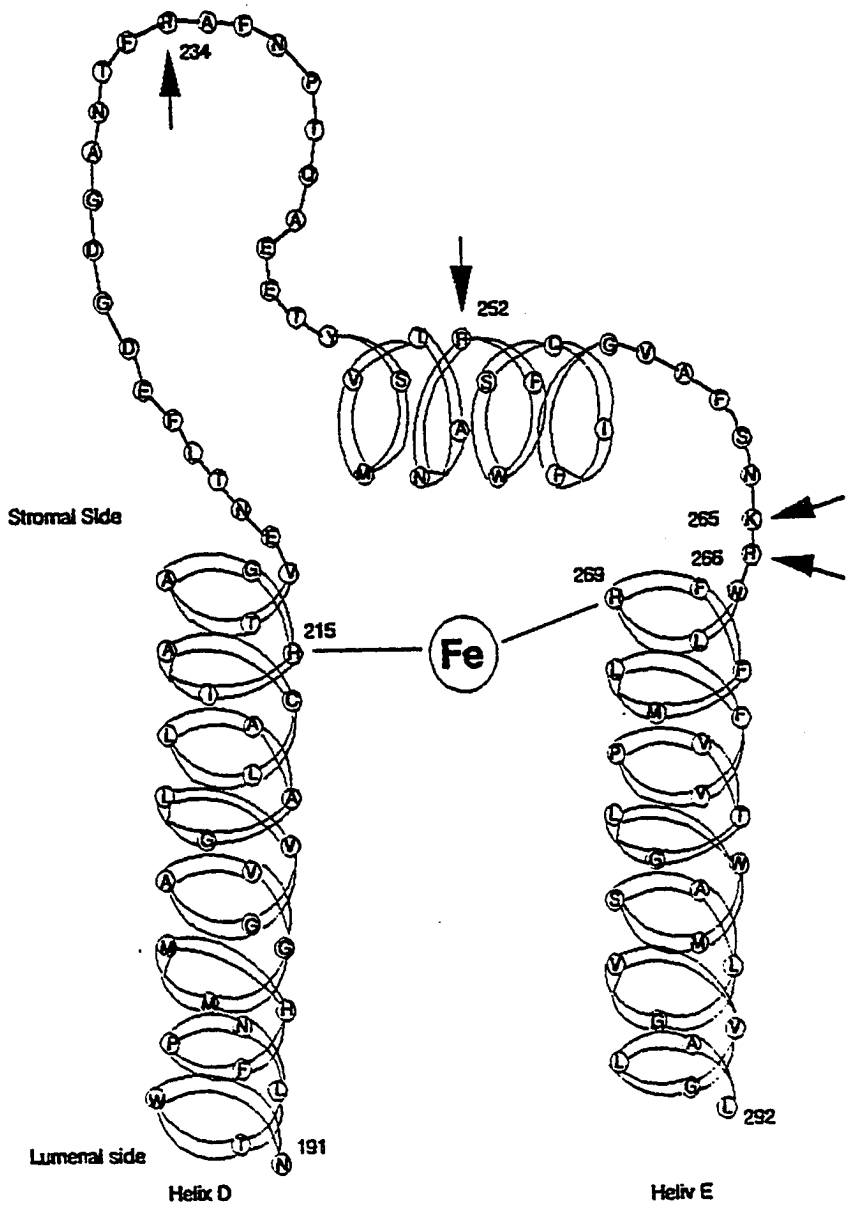
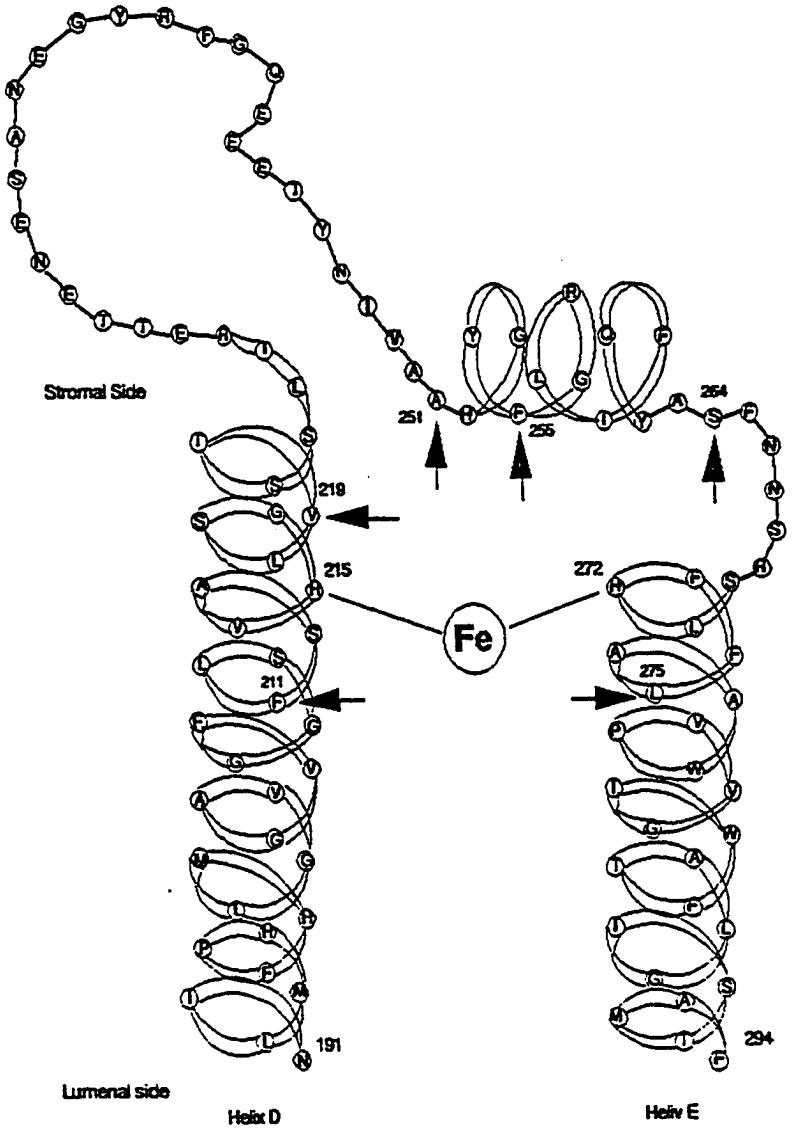


Figure 6.2. Diagrammatic representation of the region of the fourth and fifth putative transmembrane helix of the D1 protein in spinach. The herbicide resistant mutants at the residues indicated by arrows have been examined regarding the effects on bicarbonate function in PSII (Based on models by Trebst, 1986, and Vermaas and Ikeuchi, 1991).

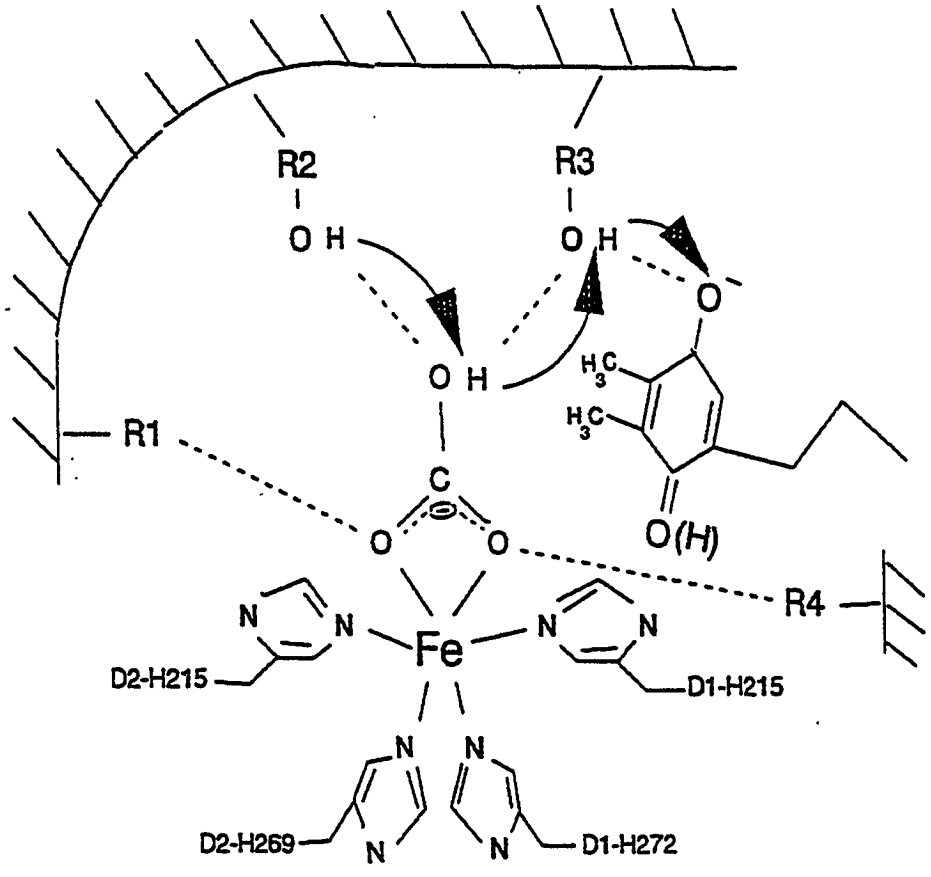


and Asp^{L213} residues with nonionizable amino acids results in lesions at Q_B protonation steps, which resemble the bicarbonate-depletion in PSII. A structural model of the Q_B binding pocket in PSII proposed by Robinson and Crofts and coworkers (see Crofts et al., 1987) provides no carboxylate residues analogous to Glu^{L212} and Asp^{L213}, but places a histidine residue in a similar position.

In light-driven or ATP-driven H^+ pump systems, proton translocation networks use hydrogen bonded chain made of the amino acid side chains of these proteins (Nagle and Morowitz, 1978; Nagle and Tristram-Nagle, 1983). The amino acid side chains that would be able to form a hydrogen bonded chain are probably those with pK values low enough that they will not bind protons too tightly. These protonatable amino acids are aspartic and glutamic acids, histidine, serine, threonine and tyrosine. Translocation of protons from the aqueous phase of stroma to Q_B must need some intraprotein transfer. The protein environment of the Q_B pocket contains a number of above-mentioned polar residues.

A proton transfer mechanism involving bicarbonate is proposed as shown in Figure 6.3. In the diagram of Figure 6.3, four histidines ligand to iron (Trebst, 1986), and the carboxylate group of a bicarbonate is bidendate liganded to Fe^{2+}. It is equally possible that bicarbonate forms a single ligand with Fe^{2+}. The two oxygens are hydrogen-bonded to two amino acid residues R1 and R4 in D2 and/or D1. The hydroxyl

Figure 6.3. A working hypothesis for the binding and function of bicarbonate in PSII.



group of bicarbonate is hydrogen-bonded to R2 and R3 residues, forming a proton translocation pathway to the secondary plastoquinone Q_B.

When $Q_B^-(H)$ further is reduced, a proton transfers from R3 to $Q_B^-(H)$. This leaves a hole-defect which will make the proton on the hydroxyl group of bicarbonate hop to R3. In a similar manner, R2 residue delivers a proton to bicarbonate that lacks a proton. As a result, the proton needed for the reduction of $Q_B^-(H)$ is transferred via this hydrogen-bonded chain involving amino acids and bicarbonate.

The bidendate ligand provided by the carboxylate group of bicarbonate to Fe^{2+} may be responsible for the lower midpoint potential of iron in PSII. It is proposed here that its hydroxyl group may participate in the proton delivery pathway to $Q_{B^-}(H)$. The pK (10.2) of the hydroxyl group would be significantly lowered by the influence of Fe^{2+} and neighboring cationic residues such as R1 and R4. The inhibition by formate, nitrite, NO etc. is due to the replacement of bicarbonate without the comparable hydroxyl group.

The candidates for R1 and R4 in D2 and D1 would be D2-R233, D2-R251 (Cao et al., 1991), D2-K264, or D2-R265 (Diner et al., 1991); D1-R269 may also be a candidate as it is not conserved in the bacterial M subunit. These cationic residues may not only provide hydrogen bonds to bicarbonate, as in human lactoferrin (Anderson et al., 1989), for the structural support of bicarbonate binding, but may also lower the pK of

the hydroxyl group of bicarbonate through the charge repulsion effect.

The residue of R3 is most likely D1-S264 which interacts with a carbonyl oxygen of Q_B (A.R. Crofts, personal communication). D1-S264A mutants are most sensitive to bicarbonate -depletion and D1-S264 has already been implicated in the functioning of bicarbonate in PSII (Govindjee et al., 1990; 1991b; Chapter V). When Q_B is reduced, the pK of a histidine in the vicinity shifts from 6.4 to 7.9 (Crofts et al., 1984). This histidine may also be involved in the proton transfer pathway (A.R. Crofts, personal communication). R2 could be an amino acid with a low pK such as Glu, Ser, Thr or Tyr.

I hope that this hypothesis will stimulate studies in the future to further understand the molecular mechanism of bicarbonate in PSII.

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APPENDIX I. CHLOROPHYLL A FLUORESCENCE TRANSIENT AS AN INDICATOR OF ACTIVE AND INACTIVE PHOTOSYSTEM II IN THYLAKOID MEMBRANES*

A. Introduction

Kautsky and Hirsch (1934) observed time-dependent changes in chlorophyll a (Chl a) fluorescence yield when a dark-adapted photosynthetic sample was illuminated with light. Since that time Chl a fluorescence transient (known as the Kautsky phenomenon) has been used as a non-destructive, sensitive tool for monitoring various processes in photosynthesis (see reviews by Govindjee and Papageorgiou, 1971; Papageorgiou, 1975; Lavorel and Etienne, 1977; and Govindjee et al., 1986). However, this application has been fraught with complexities. For example, even the nature and the meaning of the fast OIDP (see below for explanation of symbols) fluorescence changes has been debated, leading to contradictions in the literature (see e.g., Govindjee and Satoh, 1986).

The "O" (for origin) level (also called F_o), the so-called constant fluorescence, is the level obtained instantaneously (within 1 ns) of illumination, when all Q_A is in the oxidized state; it is the minimum fluorescence in PS II

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* Based on J. Cao and Govindjee (1990) in Biochim. Biophys. Acta 1050: 180-188.

membranes: in a model in which excitation can travel randomly among all the pigments ('lake model') (however, see equation 2)

$$F_o=F_{minimum}=\Phi_{f\ minimum}(I_{abs})=\frac{k_f}{k_f+k_h+k_t+k_p[Q_A]}(I_{abs}) \qquad (Eqn\ A1.1)$$

where, $\Phi_{f\ minimum}$= minimum quantum yield of fluorescence, I_{abs}= number of photons absorbed, k_f= rate constant of fluorescence, k_h= rate constant of radiationless (heat) loss, k_t= rate constant of excitation energy transfer, and k_p = rate constant of photochemistry, and $[Q_A]$, the concentration of the electron acceptor Q_A of PSII. Maximum $[Q_A]$ is taken as 1. In thylakoid membranes, the measured F_o at room temperature also includes a minor (about 20%) contribution from PSI Chl a fluorescence (Wong and Govindjee, 1979). The initial fluorescence level, measured by most instruments, is called F_i (for $F_{initial}$) unless it is proven to be F_o. The major criterion for F_i to be F_o is the independence of its yield ($\Phi_{f\ minimum}= F_o/I_{abs}$) on exciting light intensity and on the addition of electron acceptors (Briantais et al., 1986). Under these conditions, changes in F_o can be ascribed to changes in the heat loss (k_h) or in excitation energy transfer (k_t) provided k_f remains constant, i.e., the absorption spectra remain unchanged. The OID phase (where I or F_I stands for fluorescence intensity at the first inflection or intermediate peak, and D stands for dip or plateau; ID is alternatively called pl for plateau) was

suggested earlier by Munday and Govindjee (1969) and Schreiber and Vidaver (1974) to arise from the interplay of PSII and PSI in intact algal cells due to the transient changes in the [Q_A^-] in the main pathway of photosynthesis. Furthermore, the following D to P (P for peak) rise reflects the net accumulation of [Q_A^-] as the plastoquinone pool is reduced, and, there is a "traffic" jam in the flow of electrons all the way up to the electron acceptors of PSI (Munday and Govindjee, 1969). Addition of the electron acceptor methyl viologen, that accepts electrons from PSI, abolishes the D to P rise, but not the OID phase (Munday and Govindjee, 1969; also see Lavergne, 1974). Melis (1985) suggested, without proof, that the initial chloroplast fluorescence rise from F_o to F_{pl} was identified as the variable fluorescence yield controlled by a type of Photosystem II centers called PSIIß. Graan and Ort (1986) showed that the number of PS II centers capable of active water-oxidation was increased by a chloro-quinone 2,6 dichloro-p-bezoquinone and closely matched the number of herbicide binding sites. However, the number of PSII centers active in water oxidation was much lower when methyl-substituted benzoquinone, 2,5 dimethyl-p-benzoquinone (DMQ) was used as an electron acceptor. These experiments were explained by the hypothesis of the existence of two types of PSII centers, the inactive centers being the ones that are ineffective in electron flow to plastoquinone and, thus, inactive in net electron flow and water oxidation. The nature

of the active and inactive PSII centers has been examined by independent means (Chylla et al., 1987; Chylla and Whitmarsh, 1989). In spinach thylakoids, the majority of the photosystem II reaction centers (68%) recover in less than 50 ms. However, approximately 32 % of the photosystem II reaction centers require a half-time of 2 to 3 s to recover.

In this study, the effects of DCBQ and DMQ on the OID (or OP1) phase of fluorescence transient was investigated to assess their relationship to the active and inactive centers, as defined earlier (Graan and Ort, 1986). This has allowed us to obtain information on the nature of the OID phase that had remained controversial. The results shown here indicate that DCBQ is able to quench F_I much more effectively than DMQ while F_o remains unchanged. Since DCBQ has been found to intercept electrons from the "inactive" PSII centers (Graan and Ort, 1986), we suggest that the OID phase reflects the reduction of Q_A to Q_A^- in the inactive PS II centers.

Our study also showed that in spinach and soybean thylakoids high temperature (55°C) induced increase in fluorescence yield (e.g., F_{50ms}, fluorescence at 50 ms) is quenched differentially by the electron acceptor DCBQ and DMQ. The amplitude of F_i in 55 °C treated samples decreased proportionally by DCBQ addition, but it was much less affected by DMQ. Thus, we suggest that the increase in fluorescence at high temperature is mainly da result of the increase in Fi level due to the conversion of active PSII centers to inactive

PSII centers. In addition, decay kinetics of Q_A^-, which is calculated from the Chl a fluorescence decay data, with and without DCMU after one flash is analyzed in terms of a multiple exponential decay model. In the heated chloroplasts, a slower decay was noticed than the normal slow component, which is suggested to reflect the back reaction between Q_A^- and the S_2 state of the oxygen-evolving complex. It is attributed to an additional heat effect on an inhibition of the S-state turnover in the oxygen evolving system.

B. Materials and Methods

Chloroplast thylakoid membranes were isolated from commercial spinach and growth chamber soybean as described by Graan and Ort (1984; 1986). Soybean plants were grown at 30°C/20°C day/night temperatures and 10h/14h day/night photoperiod. The chlorophyll concentration of chloroplast suspension was determined by measuring absorbance at 664 nm and 647 nm (Ziegler and Egle, 1965; Graan and Ort, 1984).

Thylakoids were suspended in a reaction medium containing 0.4 M sorbitol, 5 mM Mes-KOH (pH 6.5), 20 mM KCl, 2 mM $MgCl_2$, and 1 μM nigericin for fluorescence measurements. The chlorophyll concentration was 40 μM. The suspension buffer for heat treatment contained 0.4 M sorbitol, 0.5 mM Mes-KOH (pH 6.5), 20 mM KCl, and 2 mM $MgCl_2$. The heat treatment (55°C) time was one minute for spinach and six minutes for soybean thylakoids. The samples were kept in a water bath in dark.

The Chl *a* fluorescence transient measurement was performed in a home-built spectrofluorometer (Blubaugh and Govindjee, 1988) with modifications. The light from the exciting lamp (Kodak Carousel 4200 projector) was filtered with Corning CS 5-56 and CS 4-76 blue filters. The fluorescence was filtered with a Corning CS 2-61 red filter before it entered a monochromator set at 685 nm (slit widths: 4 mm; band pass: 13.2 nm). A EMI (9958 B) photomultiplier (S-20 response) was used as a photo-detector. The output signal was digitized with 8 bit precision by a Biomation Model 805 waveform recorder and stored on an LSI 11 minicomputer (Digital Equipment Corporation) (Blubaugh and Govindjee, 1988).

The kinetics of decay of variable chlorophyll *a* fluorescence was measured at 685 nm (10 nm banbwidth) by a weak measuring light. The measuring light was fired at variable times after each actinic flash. The actinic flash (FX-124, EG and G) and the measuring flash (Stroboslave 1593A, General Radio) were filtered with Corning blue (CS 4-96) glass filters; both flashes had a 2.5 μs duration at half-maximal peak (Eaton-Rye and Govindjee, 1988).

Q_A^- concentration was calculated from variable Chl *a* fluorescence according to Joliot and Joliot (1964) using the formula given by Mathis and Paillotin (1981). There is a hyperbolic relationship between the fluorescence yield F(*t*)

and the fraction q(t) of closed reaction centers at time t:

$$\frac{F(t)-F_o}{F_{max}-F_o}=\frac{(1-p)\,q(t)}{1-pq(t)} \qquad (Eqn\ A1.2)$$

where F_{max} is the maximum fluorescence yield when all Q_A is in the reduced state. F_o is the minimum fluorescence yield when all Q_A is in the oxidized state, and p is the connection parameter which reflects the probability of the intersystem energy transfer from a closed unit to any other unit in the system. In this work, p was assumed to be 0.5, as measured earlier for chloroplasts (Forbush and Kok, 1968). Thus, q(t) could be represented explicitly as:

$$q(t)=\frac{2F_v(t)}{F_v^{max}+F_v(t)} \qquad (Eqn\ A1.3)$$

where $F_v(t)=F(t)-F_o$ and $F_v^{max}=F_{max}-F_o$.

Based on the multiple exponential decay model, the Q_A^- concentration at time t, i.e., q(t) is given by a sum of N exponential components:

$$q(t)=q(0)\sum \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \qquad (Eqn\ A1.4)$$

where q(0) is the Q_A^- concentration at time 0, α_i is the amplitude and τ_i the lifetime of the ith component. The fitting of equation (A1.4) with the experimental data was carried out by a least-squares program using iterative methods (Marquard search algorithm). Quality of fits was judged by the reduced χ^2 criterion and the plot of the weighted difference

between theoretical fit and the experimental data, normalized to the square root of the data points (Haehnel et al., 1983).

C. Results

Figure A1.1 shows the chlorophyll a (Chl a) fluorescence transients in spinach thylakoids in the presence of different concentrations of DMQ (top graph) and DCBQ (bottom graph). In the control without exogenous quinones, the curve shows clearly the various characteristic points, namely OIDP (Govindjee and Papageorgiou, 1971; Papageorgiou, 1975; Lavorel and Etienne, 1977; Govindjee and Satoh, 1986). I and P levels occur at 50 ms and 3 s after the start of illumination, respectively. In both spinach and soybean thylakoids (data not shown), increasing concentrations of DMQ and DCBQ (2.5 to 20 μM) affect Chl a transient differently. DMQ quenches only the DP rise, whereas DCBQ is able to quench, in addition, OI phase.

Figure A1.2 is a plot of the dependence of the fluorescence yields at 50 ms and 2 s after the start of illumination on quinone concentration. DMQ quenches F_p level greatly, but does not affect F_i level (Figures A1.1 and A1.2A). The quenching effect on F_p is saturated at 20 μM DMQ. On the other hand, DCBQ not only quenches F_p but F_i level also (Figures A1.1 and A1.2B). This quenching effect is saturated at 15 μM DCBQ. However, this concentration of DCBQ does not affect F_o level at all. In these calculations, the value of F_o

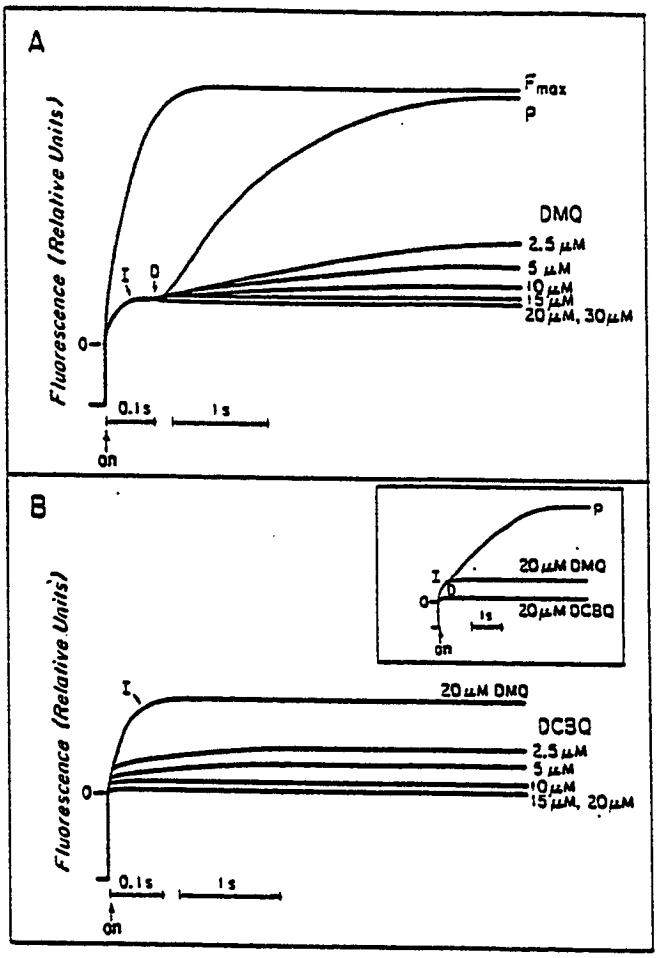

Figure A1.1. Chlorophyll a fluorescence transients of spinach thylakoids in the presence and absence of quinones at 20 °C. Thylakoids were suspended in a reaction medium containing 0.4 M sorbitol, 5 mM Mes-KOH (pH 6.5), 20 mM KCl, 2 mM $MgCl_2$, and 1 μM nigericin. Chlorophyll concentration was 39 μM. The inset in (B) shows the fluorescence transient of control and 20 μM quinone treated thylakoids. Thylakoids were dark-adapted for 10 minutes before the fluorescence measurement. O: constant fluorescence; I: intermediate peak fluorescence; D: fluorescence yield at the plateau or the dip; P: fluorescence yield at the peak (maximum). In (A), DMQ effects are shown; the fast rising curve is with 1 μM DCMU; F_{max} is the maximum fluorescence level. In (B), DCBQ effects are shown.

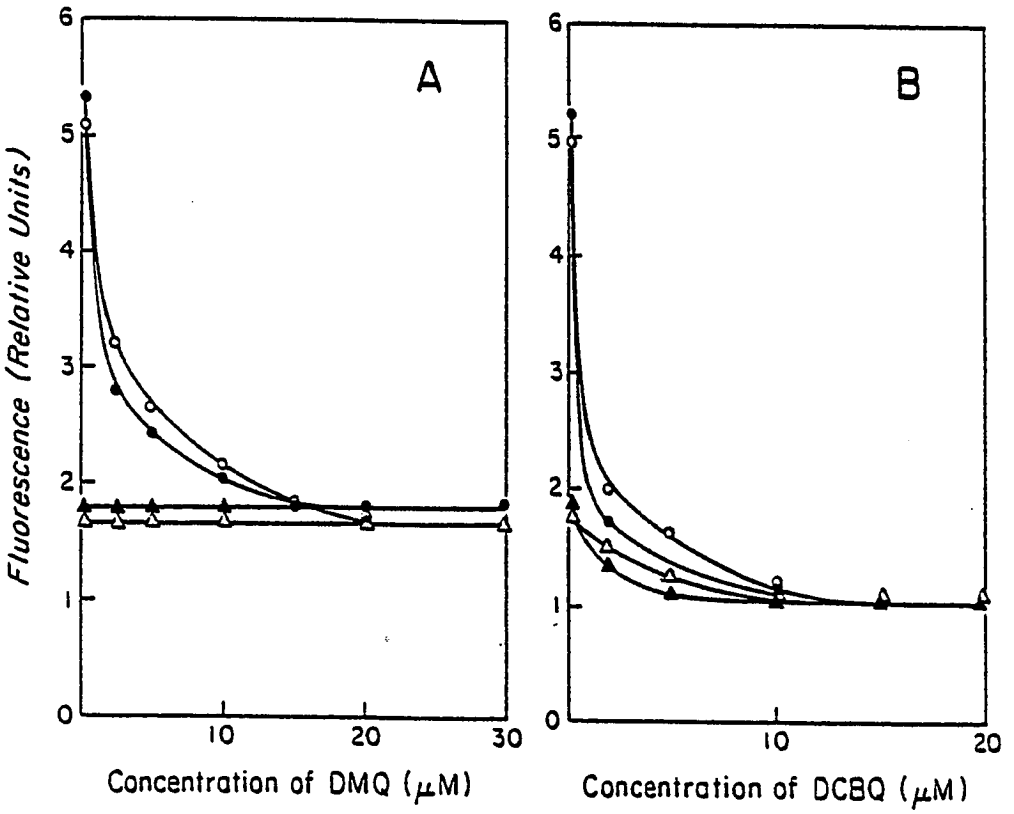

Figure A1.2. Effects of increasing concentrations of DMQ (A) and DCBQ (B) on chlorophyll a fluorescence yields at 50 ms and 3 s in thylakoid membranes. Fluorescence yield at 50 ms (▲,△) and 3 s (•,○) in spinach (▲,•) and soybean (△,○) thylakoid membranes. F_o level is normalized to 1.

was obtained by the value of F_i at 30 μM DCBQ; this value was confirmed by measurements of F_o mainly by extrapolation of true F_o measured at low light intensities, assuming independence of fluorescence yield at F_o with light intensity (data not shown).

In order to quantify the quenching effect of the quinones on fluorescence yield at 50 ms (F_{50ms}), the amplitude of (F_I-F_o) is normalized as 100 units and the quenching of fluorescence (F_{50ms}) by quinone is calculated (Figure A1.3). Quenching is absent when no exogenous quinone is added. In DCBQ treated thylakoids, the concentration of DCBQ at which F_I-F_o is half quenched is 2.5 μM for spinach; however, F_{50ms} does not decrease with increasing concentration of DMQ in the micromolar range. This concentration dependence of F_{50ms} on DCBQ is in quantitative agreement with the earlier concentration dependence data (Graan and Ort, 1986) on the increase in the number of PSII centers capable of water-oxidation in the presence of DCBQ in spinach thylakoids. In soybean thylakoids, 3.5 μM of DCBQ is needed in order to quench 50 % of the amplitude from F_o to F_I (data not shown).

Heat treatment caused a significant change in Chl a fluorescence transient in comparison to the control at 20 °C in spinach (Figure A1.4; measurements at 20 °C). The normal clear OID characteristic points disappeared and instead a fast rise of fluorescence yield occurred with treatment at 55 °C (see section on methods). Figures A1.5 shows the quenching

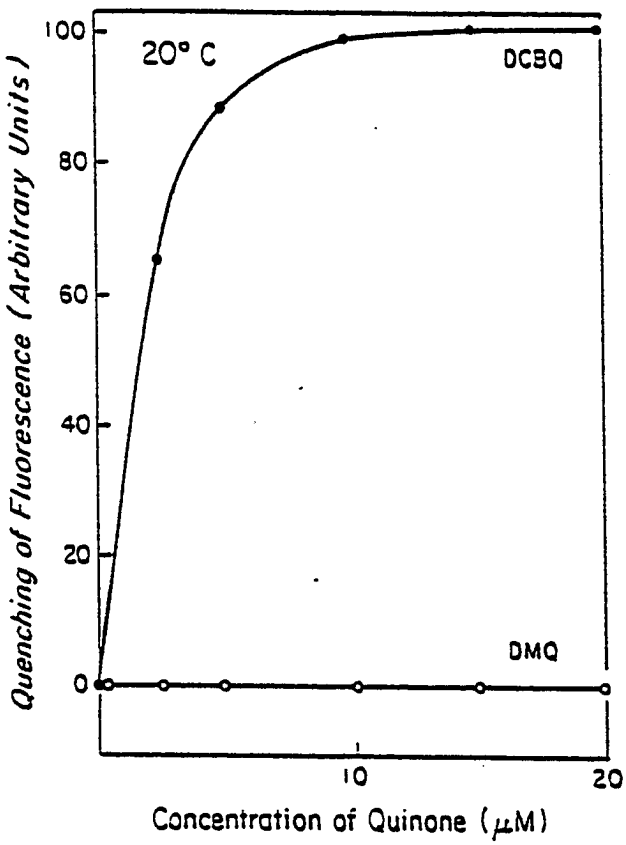

Figure A1.3. Quenching of chlorophyll a fluorescence at 50 ms by DCBQ (•) and DMQ (O) in spinach thylakoid membranes. The amplitude difference (F_I-F_o) is normalized to 100. The quenching of fluorescence yield is the decrease in fluorescence yield from the F_I level.

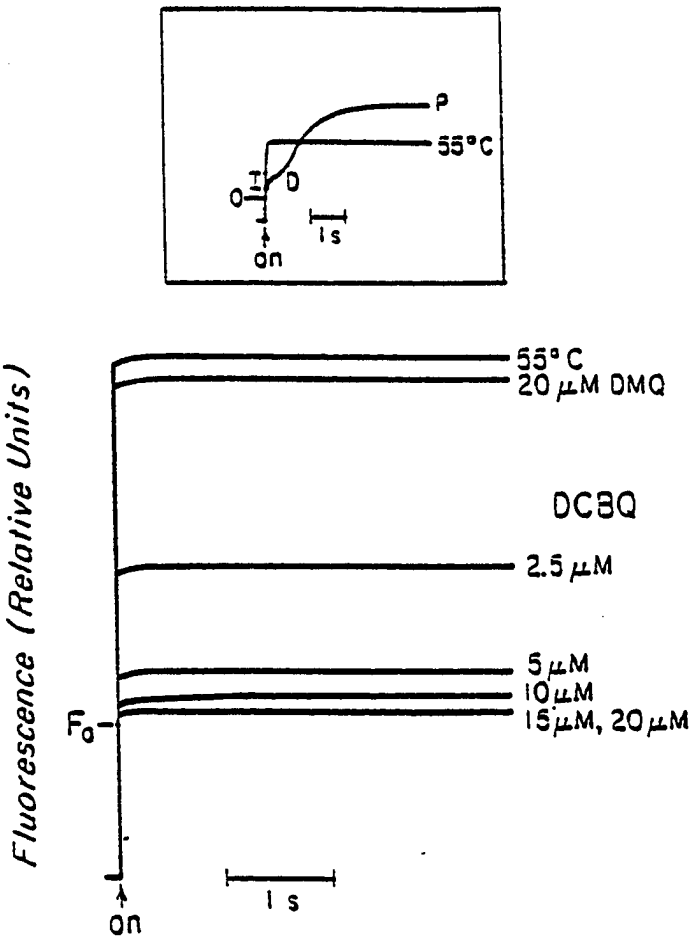

Figure A1.4. Chlorophyll a fluorescence transients of heat-treated thylakoid membranes of spinach and the effects of quinones on the fluorescence yield. Inset (at top) shows fluorescence transients at 20 °C and 55 °C. Thylakoid membranes were heated for 1 min in a medium consisting of 0.4 M sorbitol, 5 mM Mes-KOH (pH 6.5), 20 mM KCl, and 2 mM $MgCl_2$. Then the thylakoids were resuspended in the same medium with added 1 μM nigericin when the fluorescence transient was measured at 20 °C. F_o is the constant fluorescence yield. See the legend of Figure A1.1 for other abbreviations.

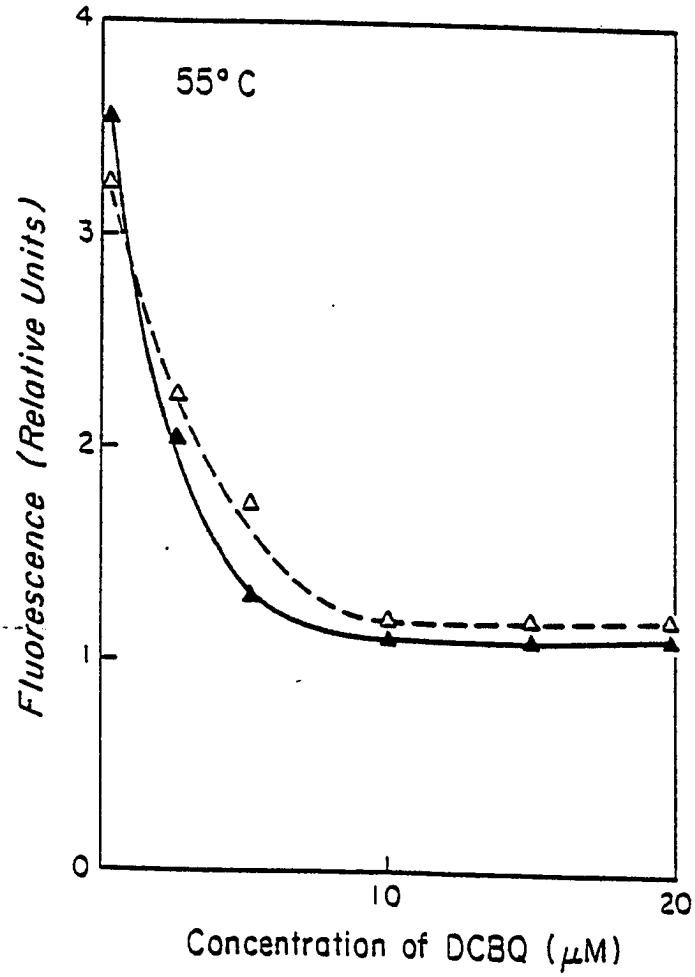

Figure A1.5. Fluorescence yield at 50 ms as affected by DCBQ in heat-treated spinach (▲) and soybean (△) thylakoid membranes. F_o level is normalized to 1.

effect of DCBQ on F_{50ms} in heat-treated chloroplasts. The heat-induced fluorescence yield declined as DCBQ concentration increased and the quenching effect of fluorescence by DCBQ was proportional to its concentration as in control thylakoids at 20 °C (cf. with Figure A1.3).

However, in heat-treated samples, even 15-20 μM DCBQ could not totally quench fluorescence yield to its F_o level as in the samples at 20 °C. These residual small increases in fluorescence yield may reflect heat-induced increase in F_o level. Again DMQ was an ineffective quencher here (Figure A1.4). Based on the present data on both DCBQ and DMQ and comparison with data at 20 °C, it is suggested that the increase in fluorescence yield due to heating is mainly a result of the rise in "I" level, 97% in spinach thylakoids. Similar results were found with soybean thylakoids (data not shown). This heat-induced rise in the "I" level is a result of an increase in the inactive PSII centers. Only 5% or less of heat-induced fluorescence rise is due to increase in the "O" level.

The top graph (A) in Figure A1.6 shows the data of decays of the variable Chl a fluorescence after one flash (flash 5) in spinach thylakoids. The first data point is at 70 μs. The F_o levels used in these calculations are true F_o levels, not the measured F_i levels. When 10 μM DCMU is added, the forward reaction of electron flow from Q_A^- to Q_B is known to be totally blocked and a slow decay of variable Chl a fluorescence is

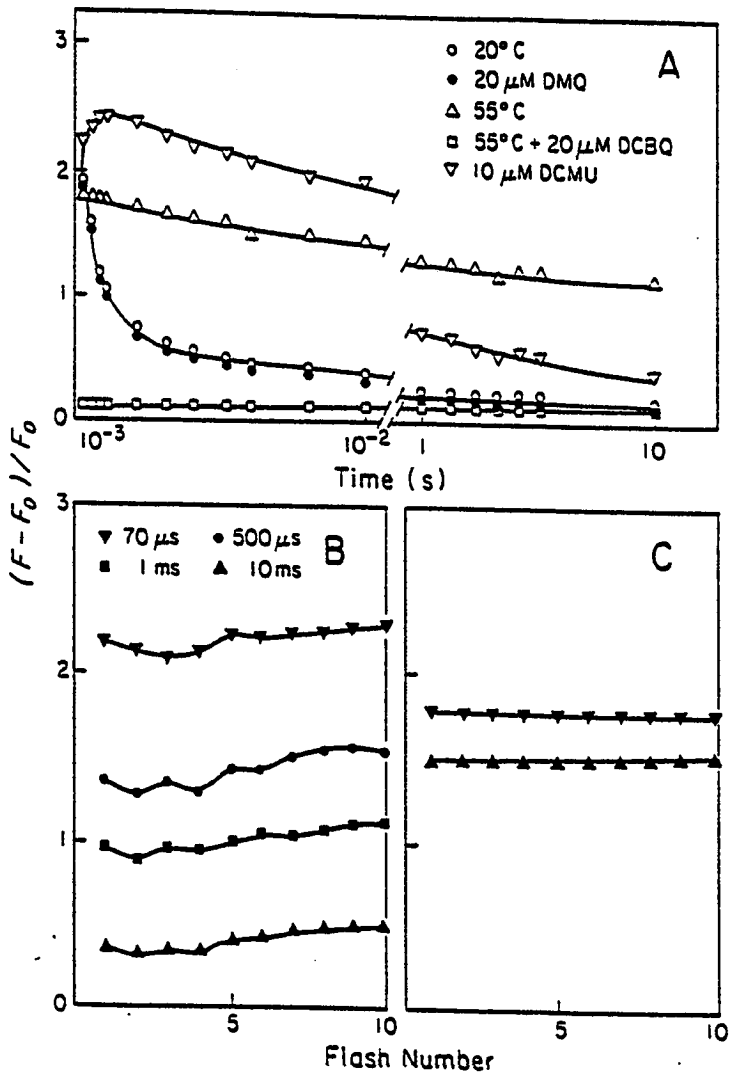

Figure A1.6 Variable Chl a fluorescence yield of spinach thylakoids as a function of time and flash number. Decay of variable Chl a fluorescence after the fifth actinic flash (A). F_o is the Chl a fluorescence yield from the measuring flash with all Q_A oxidized, and F is the fluorescence yield at the indicated time after the actinic flash. The actinic flash frequency was 1 Hz. (O): 20 °C; (•): 20 μM DMQ; (Δ): 55 °C; (□): 55 °C + 20 μM DCBQ; (▽): 10 μM DCMU. (B) Variable Chl a fluorescence as a function of flash number at 20 °C; (C) Variable Chl a fluorescence as a function of flash number at 55 °C. The times indicated are when the measuring flash was fired.

observed (see e.g., Bennoun, 1970; Robinson and Crofts, 1983). Heat treatment (55 °C, 1 minute) causes an even slower decay (Figure A1.6). Furthermore, in agreement with the conclusion that 55 °C treatment does not simply increase the 'O' level, a significant variable fluorescence was observed (curves with triangle). Twenty μM DMQ has little effect on variable fluorescence decay at 20 °C (cf. curves with open and closed circles) as is observed with the addition of electron acceptor methyl viologen (Eaton-Rye and Govindjee, 1988). Both abolish the DP fluorescence rise during fluorescence induction, but show no effect on fluorescence decay after an actinic flash. This is simply because the electron flow from Q_A^- to Q_B is faster than electron acceptance by these acceptors at more distance sites. However, the addition of 20 μM DCBQ quenches the fluorescence level ($t \leq 70\mu s$) to be close to the F_o of the control (20 °C). This suggests an efficient electron acceptance by DCBQ from close to the Q_A^- site.

The fluorescence, as a function of flash number, at various times after the flash is shown in the bottom graph of Figure A1.6 (B). In a control sample (20 °C), a complex oscillation pattern that is superimposition of a binary oscillation (due to the operation of the two electron gate on the electron acceptor side of PSII) and an oscillation of period four (due to the turnover of the S state in the oxygen-evolving system) is observed (see e.g., Eaton-Rye and Govindjee, 1988). Heat treatment, however, damps those

oscillations significantly (see Figure A1.6C).

Q_A^- decay involves multiple processes (see e.g., Crofts et al., 1984). Based on the exponential decays, the decay of Q_A^- observed in the current study was quantitated in terms of three decay components representing different pathways. In reality, these processes may not be necessarily independent of each other or even follow the first order reaction. The experimentally measured Chl a fluorescence data were converted to Q_A^- using equation (A1.4). Q_A^- decay data as a function of time were fitted by equation (A1.3) with N=3. In Figure A1.7, the best fit for the three exponential model is superimposed on the experimental decay curve in spinach thylakoids. A plot of the weighted difference between the experimental data and the best fit is also shown. The calculated amplitudes and halftimes are summarized in Table A1.1. The residual plot and χ^2 show that the best fit by a three exponential decay match the Q_A^- data very well and the derivations are due only to statistical noise. In both spinach and soybean thylakoids, large portion (60 to 62%) of Q_A^- decay is through the fast component (halftime of 365 to 395 μs). The amplitudes of the intermediate component (halftime of 6 to 7 ms) and the slow component (halftime of 1.4 to 1.7 s) are 18% and 20%, respectively. In DCMU-treated samples, reduced Q_A recombines with S_2 state after one actinic flash; the halftime of Q_A^- decay is 1.5 to 1.6 s. This value fits the slow component in the DCMU-free samples. In addition, an initial rise in

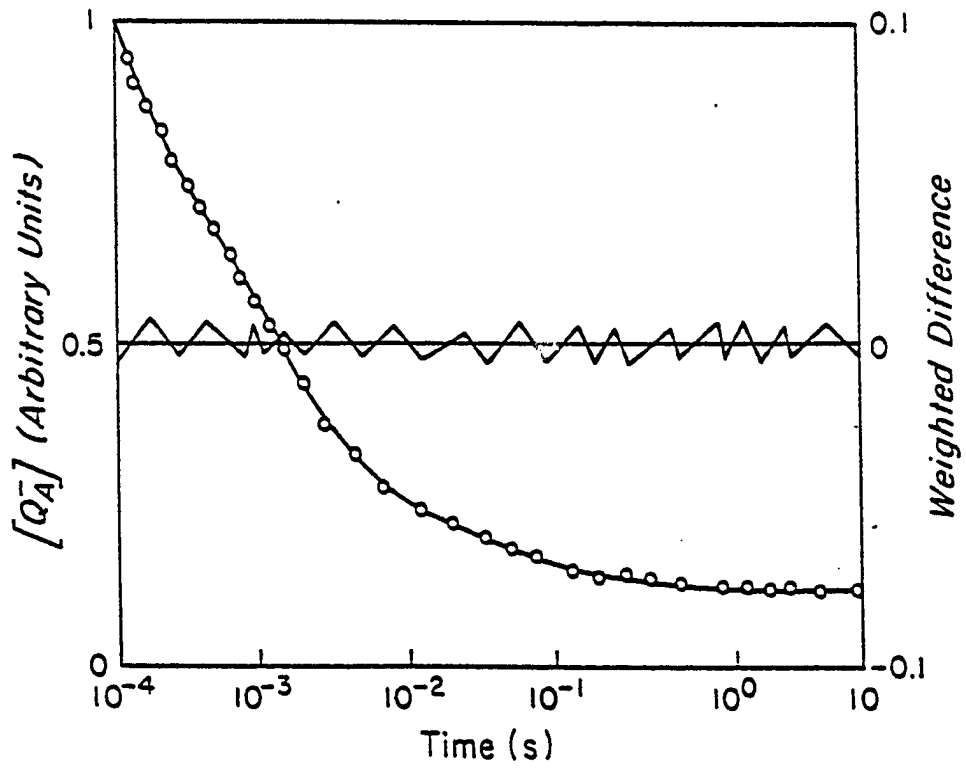

Figure A1.7. The calculated Q_A^- concentration (O) and the best fit (–) of a three exponential decay model in spinach thylakoids. The weighted difference is the difference (the saw-tooth curve) between experimental data and the best fit, weighted by the square of the data points.

Table A1.1. The calculated amplitude (α) and the lifetime (τ) of decay of Q_A^- with three independent exponential components.

	α	τ	$t_{1/2}$[a]	χ^2
Control				
spinach	0.62	527 μs	365 μs	
	0.18	10 ms	7 ms	1.1
	0.20	2 s	1.4 s	
soybean	0.60	570 μs	395 μs	
	0.19	9 ms	6 ms	1.2
	0.21	2.5 s	1.7 s	
+ 10 μM DCMU				
spinach	1.11	2.2 s	1.5 s	1.1
	-0.11	26 μs	18 μs	
soybean	1.08	2.3 s	1.6 s	1.2
	-0.08	28 μs	19 μs	

a: $t_{1/2}$ is the halftime: $t_{1/2} = \ln 2\ (\tau)$.

variable fluorescence is observed in the presence of 10 μM DCMU (Figure A1.6). In spinach and soybean thylakoids, the lifetimes for the rise component are 26 μs and 28 μs, respectively (see Table A1.1); its origin will be discussed later.

D. Discussion

A heterogeneity among PSII centers has been known for quite sometime (Black et al., 1986; Govindjee, 1990). Doschek and Kok (1972) found that single first order reaction did not fit fluorescence rise under continuous illumination in DCMU-treated chloroplasts. Melis and Homann (1975; 1976) observed a biphasic first-order growth of area curve and identified the existence of fast-rising PSII centers, labelled as PSIIα, and a slow-rising centers, labelled as PSIIß. The rate of reduction of electron acceptors in PS II is about 3-times slower in PSIIß than in PSIIα centers (Melis and Homann, 1976). The PSIIß centers are suggested to have smaller chlorophyll antenna (Melis and Homann, 1978; Melis and Duysens, 1979); electron transport in these centers does not proceed via the two-electron-accumulating plastoquinone (Thielen and Van Gorkom, 1981a). Although PSIIß centers may be able to oxidize water, it appears that they do so slowly (Melis, 1985; Thielen and Van Gorkom, 1981b); PSIIα centers are the dominant source of electrons for the reduction of NADP.

Lavergne (1982a) has found another heterogeneity; that of the non-Q_B (or B) centers; these are impaired at the Q_B site and they do not transfer electrons efficiently to the plastoquinone pool. These may be considered inactive PSII centers. Lavergne (1982b) showed that these non-Q_B centers can be induced to turnover by exogenous quinones, although neither di- nor tetramethyl-quinone seemed to fully relieve the acceptor side limitation. As noted earlier, Graan and Ort (1986) showed that micromolar concentrations of a chloroquinone DCBQ can support flash-induced water oxidation in what they called inactive PSII centers much more effectively than DMQ. From Chl a fluorescence transient experiments (Figures 1A.1 through 1A.3), conducted under similar experimental conditions, it was found that DCBQ is much more effective than DMQ in quenching the ID (F_I) fluorescence level. The absence of any effect of DCBQ (at the concentrations used) on true F_o indicates that the observed quenching is a result a diminished Q_A^- concentration, but not due to nonphotochemical quenching.

Based on the differential effects of micromolar concentrations of electron acceptors DMQ and DCBQ on F_I quenching (Figures 1A.1 and 1A.2), it is suggested that OID phase reflects the reduction of the electron acceptor Q_A to Q_A^- in the inactive PSII centers. An earlier study by Melis (1985) on the quenching of fluorescence rise (F_o to F_I) was performed by adding 100-200 μM DMQ and TMQ to spinach thylakoids. The

interpretation given was that the initial fluorescence rise from F_o to F_I is due to the prompt reduction of the primary electron acceptor Q_A of PSIIß and the consequence of the inability of PSIIß to donate electron, on a fast scale, to secondary electron acceptors. Although this interpretation is apparently similar to that given in this paper, it is based on the use of high concentrations of quinones that affect the F_o level and not on the differential effects of low (μM) quinones DCBQ and DMQ on inactive and active centers. The results in this study justify our conclusions, but those in the earlier paper (Melis, 1985) do not. Furthermore, it is not clear whether the inactive centers are identical to PSIIß centers (Melis and Homann, 1975; 1976). Inactive PSII centers are defined by the interaction between Q_A and the PQ pool, whereas PSIIß centers are defined by their antenna size. They represent two different criteria of classifying PSII centers (Chylla and Whitmarsh, 1989). A recent work (Guenther et al., 1988) suggests that PSIIß exist in both active and inactive forms. Therefore, it is equally likely that PSIIα centers also exist in inactive and active forms. Hsu and Lee (1991) have suggested that the inactive PSII centers, which give rise to the fluorescence rise from F_o to F_i, belong to the α-type PSII centers.

Schreiber and Armond (1978) measured heat-induced (up to 60 °C) changes of Chl fluorescence in Larrea divaricata chloroplasts and observed the F_o level rising greater than 3-

fold from that at 25 °C; a concomitant F_{max} decline was also observed. It was postulated that heat-induced increase in F_o reflects inhibition of energy conversion at system II centers, which may be caused by a functional separation of the electron acceptor Q_A from the primary electron donor P680. This implies that $k_p[Q_A]$ in equation (A1.1) that is maximum at F_o is no longer maximum; furthermore, this increase in fluorescence could also be a result of the decrease in k_t due to other physical changes. Sane et al. (1984) suggested that heating causes an increase in excitation energy transfer (k_t from PSII to PSI, or a change from the 'state I' to 'state II'; (see Fork and Satoh, 1986); this theory could be used to explain the decline of variable fluorescence. Weis (1982) observed almost parallel decline of variable fluorescence, F_v, and rise of F_o versus temperature by measuring Chl fluorescence transient in heat-pretreated spinach thylakoids. F_o rise at above 50 °C was 3- to 4-fold. Sundby et al. (1986) concluded that at 30 °C there were 30% PSIIß and 70% PSIIα centers, whereas at 45°C, as much as 84% of centers were PSIIß.

Based on the Chl a fluorescence transient in the absence of PSII inhibition (Figures A1.4 and A1.5), it is indicated that F_{50ms} rise in heat-treated thylakoids can be diminished by 15 to 20 μM of DCBQ, while only small quenching was obtained by 20 μM DMQ under the current experimental conditions. Twenty μM DMQ is able to quench F_p to F_I, while 20 μM DCBQ quenches F_p to F_o (Figures A1.1 and A1.2). Thus, the fluorescence yield

differences between the effects of 20 μM DMQ and 20 μM DCBQ could be attributed mainly to heat-induced rise in the amplitude of F_o to F_I. Only a minor change occurs in the F_o level upon heat treatment, in contrast to data reported by Schreiber and Armond (1978) and Weis (1982). This residual small effect, however, could be interpreted by changes in excitation energy transfer. The differential effect of quinones DCBQ and DMQ on heat-induced fluorescence rise leads us to suggest that the significant increase of fluorescence in heat-treated thylakoids is on F_I level, not F_o level. Thermal conversion from active PSII centers to inactive PSII centers increases F_I level, as expected from our interpretation of OID phase. DCBQ is able to draw electrons from inactive PSII centers and therefore is able to quench heat-induced fluorescence rise, while DMQ is less effective. The increase in F_I is accompanied by a dramatic decrease in the rate of O_2 evolution. The small quenching by 20 μM DMQ may be a result of the absence of most of the active PSII centers (Figure A1.4).

From freeze-fracture studies, Gounaris et al. (1984) found reorganization of the thylakoid membrane at moderately elevated temperatures. Sundby and Anderson (1985) found that at above 30 °C, there is a disassociation of peripheral light-harvesting Chl a/b complex (LHC II) from PSII and a migration of LHC II to the PS-I-rich non-appressed thylakoid regions. It could be speculated that the heat-induced reorganization of thylakoid membranes may also alter the reaction center II (D1

and D2 protein) conformation and, thus, the previously active PSII centers in which electron transfer to plastoquinone pool is impaired and a quick rise in initial fluorescence would occur as in the DCMU-inhibited PSII centers.

After one flash, the initial first order component of variable Chl a fluorescence reflects electron flow from Q_A^- to Q_B in reaction centers which have Q_B bound before the flash (Crofts et al., 1984). In spinach chloroplasts, the half-time of the fast component is 320 μs at pH 6.5 (Eaton-Rye and Govindjee, 1988). Similar halftimes of this component at the same pH level are obtained from our study: 365 μs for spinach and 395 μs for soybean. The intermediate component has a halftime of 6 to 7 ms. This value may reflect the process of movement of plastoquinone molecules from the plastoquinone pool to the reaction centers which have no Q_B bound in the dark (Crofts et al., 1984). The third component, i.e., the slow component, is suggested to reflect the back-reaction between Q_A^- and S_2 state of the oxygen evolving complex, since its halftime matches that of Q_A^- decay in the presence of DCMU (Table A1.1 and Eaton-Rye and Govindjee, 1988). It has been known that the reoxidation of the acceptor Q_A^- after an illumination in the presence of DCMU is a recombination between Q_A^- and S_2, one of the redox states of the oxygen evolving complex (Bennoun,1970). In the presence of 10 μM DCMU, Q_A^- is oxidized with a halftime of 1.5 s after one flash (Robinson and Crofts, 1983). This back-reaction is pH-

dependent. At pH 6.5, the time at which [Q_A] is 50% of the maximum Q_A^- for S_2 and Q_A^- back-reaction is 1.3 s and it is 2 s at pH 7.5 (Eaton-Rye and Govindjee, 1988). These values are in agreement with the slow component observed in the current study (Table A1.1).

In the DCMU-treated thylakoid membranes, an initial rise occurs in variable Chl a fluorescence before it declines (Figure A1.6). This rise component corresponds to a process with a lifetime of about 26 μs in spinach and can be compared with an increase of F_v with a halftime range of 50 μs (Robinson and Crofts, 1987) and 250 μs (Xu et al., 1989). It was suggested that the rise component reflects the reduction of the oxidized reaction center P_{680}^+; the observed times were related to the equilibration of electrons on the donor side of PSII (Robinson and Crofts, 1987; Xu et al., 1989). The reduction of P_{680}^+ follows multiphasic kinetics (see a review by Wydrzynski, 1982). Eckert and Renger (1988) measured the relaxation of absorption changes at 820 nm versus time, which reflects the P_{680}^+ reduction kinetics. They found three decay components of lifetimes 20 ns, 330 ns and >1.4 μs, after the first saturating flash. It has been known that the inhibition of Q_A^- reoxidation by Q_B at low temperature (Eckert and Renger, 1988), or in the presence of DCMU (Renger and Wolff, 1976) alters the kinetics of P_{680}^+ decay. In order to explain the F_v rise with 26 μs lifetime in the presence of DCMU in this work, the effect of blockage of electron transport between Q_A^- and

Q_B by DCMU is assumed to decrease the fast components in the nanosecond range and to increase the slow component in the microsecond range for P_{680}^+ decay. The halftime of the slow component(s) in the microsecond range varies in the literature (Renger and Wolff, 1976; Sonneveld et al., 1979; Eckert and Renger, 1988). It is also considered likely that the 26 to 28 μs rise component observed in the current experiment may originate in the recombination of Q_A^- with P_{680}^+; if so, the Chl a fluorescence yield in the presence of P_{680}^+ Q_A^- is postulated to be smaller than that in the presence of P_{680} Q_A.

Chylla and Whitmarsh (1989) showed that in spinach leaves, oxidation of Q_A^-, monitored by fluorescence induction measurement, displays a halftime of about 1.5 s, which matches the halftime of the recombination between Q_A^- and S_2 state. As a result, one could speculate that the major decay pathway of Q_A^- in inactive PSII centers may be through the recombination between Q_A^- and S_2 state. Since, as has been shown, heat alters the ratio of the active PSII centers to inactive centers, it is expected that a larger portion of Q_A^- may oxidize through this back reaction route in heated samples. A slow decay of Q_A^- is indeed observed (Figure A1.6). This reaction is further slowed in heat-treated samples, because of additional heat-induced effects (Weis, 1982; Coleman et al., 1988).

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APPENDIX II. CHLOROPHYLL A FLUORESCENCE LIFETIME STUDIES IN THE OPEN AND CLOSED REACTION CENTERS IN THYLAKOID MEMBRANES AND PSII-ENRICHED MEMBRANES

A. Introduction

Light energy is captured by antenna pigments which are composed of chlorophyll-protein complexes in chloroplasts. The excitation energy is transferred through a series of ultrafast energy transfer steps in a time of tens of picoseconds before it reaches the reaction centers for photochemical charge separation. The yield of PSII fluorescence is dependent on the redox state of the reaction center. In the open center, where the one electron acceptor Q_A is oxidized, the fluorescence yield is low (Fo). In the closed center, where Q_A is reduced, the fluorescence yield is maximum (Fmax). Along with the increase in fluorescence yield a parallel increase in the fluorescence lifetime is expected which gives a direct access to a measurement of the charge-separation processes. For reviews on fluorescence, see Govindjee et al. (1986) and Krause and Weis (1991).

Measurements of the lifetime of chlorophyll (Chl) a fluorescence after picosecond excitation of photosynthetic organisms containing light-harvesting Chl a/b protein complex have revealed multiexponential decay kinetics (see Karukstis and Sauer, 1983a; Holzwarth, 1987, 1990, 1991). The overall fluorescence decay can usually be statistically defined by

three major components: 30-300 ps; 500-800 ps; and 1.3-2.5 ns, which undergo complex changes in both their lifetimes and yields upon closure of Photosystem II (PSII) reaction centers to photochemistry (see e.g., Haehnel et al., 1983; Moya et al., 1986a,b; Hodges and Moya, 1987; Keuper and Sauer, 1989).

The origins of each of these components are still not fully understood. It has been shown, from time-resolved emission spectra (Gulotty, et al., 1985; Hodges and Moya, 1986) and from PSI and PSII mutant studies (Green et al. 1984), that a part of the rapid decay arises from PSI (30 ps; 100-150 ps) and another (100-350 ps) from PSII. Klimov et al. (1978) proposed that the slowest component (1.3-2.5 ns) originates from PSII radical pair recombination between the oxidized reaction center Chl a of PSII, P_{680}^{+}, and reduced primary acceptor (Pheophytin) of PSII, $Pheo^{-}$.

The idea that the complexity of the kinetic components of fluorescence decay could be interpreted in terms of the existence of two types of PSII centers PSIIα and PSIIß (see e.g. Melis and Homann, 1986) was first suggested by Butler and coworkers (Butler et al., 1983; Berens et al., 1985a,b). Based on time-resolved emission and excitation spectral analyses performed under conditions when reaction centers are open (F_o, Q_A is oxidized) and when they are closed (F_m, Q_A^{-} is reduced), Holzwarth et al. (1985) proposed that a 80 ps component arises from PSI, a 180 ps component from open PSIIα centers, the original 'middle' component (500 ps) from open, and slow (1.2

ns) from closed PSIIß centers, while the long-lived decay (2.2 ns) is emitted by closed PSIIα centers; the later may include contributions from "disconnected" Chl a, if any.

Using a simpler system, i.e., PSII particles and low intensity of excitation, Schatz and Holzwarth (1986) and Schatz et al. (1987) observed that upon closure of reaction centers (Q_A^--closed), lifetimes of fluorescence changed from about 80 ps and 520 ps to 220 ps and 1-3 ns. Schatz et al. (1988) suggested that Q_A^- controls primary charge separation; with Q_A^- present, the charge separation was slowed/decreased, as confirmed by Trissl et al. (1987): the 520 ps time was related to the time of electron transfer from reduced pheophytin to Q_A. Longer lifetimes (10-30 ns), observed by other investigations (e.g. Schlodder and Brettel, 1988), was suggested to be the result of the damage in their preparations.

The discrete component analysis of the fluorescence assumes that all the radiating fluorophores decay with a well defined set of lifetimes. However, in the case of heterogeneous systems containing proteins, membranes, etc., in which the electronic environments of the emitting molecules are far from being unique and can change during the excited state lifetime, such an approach becomes questionable (Alcala et al., 1987a). The simulation study of Alcala et al. (1985) showed that the discrete component analysis with one or two exponentials, when used to study distributions of lifetimes,

was very sensitive to the number and range of frequencies at which the data are collected. In general a two exponential fit to a symmetric distribution yielded a nonsymmetric result. The result of the fit was symmetric only with very particular sets of frequencies whose values depended on the distribution shape. To distinguish among the different factors involved in decay is impossible due to the limited resolvability of the data in lifetime components provided by current instrumentation. The observed signal may easily constitute a superposition of heterogenous decays comprising individual lifetime values that are very close to one another (Alcala et al., 1987b). As a result, the assignment of one or more exponentials to describe the overall decay process can hide the true physical origin of lifetime heterogeneity.

PSII is known to be a chlorophyll-protein-containing heterogenous membrane system (Kaplan and Arntzen, 1982). It is well established that protein structural fluctuations can occur in the nanosecond-picosecond time scale (Lakowicz and Weber, 1973; Careri et al., 1979; Karplus and McCammon, 1983). The concept of distribution of lifetime values has been introduced in fluorescence (James and Ware, 1985) and has now been used in lifetime analysis of Chl *a* fluorescence (Govindjee et al., 1990) of reaction center II preparations that lack Q_A (Nanba and Satoh, 1987).

I present here an analysis, obtained in collaboration with my advisor Govindjee, M. Van de Ven, C. Royer and E.

Gratton, of fluorescence decay data in thylakoid membranes and PSII-enriched membranes, using multifrequency phase fluorometry. These data were analyzed for both open and closed PSII centers in terms of distributions of lifetimes based on the principles outlined by Alcala et al. (1987a,b,c) and Govindjee et al. (1990). Analysis of the data with both multiexponential function and Lorentzian distribution function showed that upon closure of reaction centers (Q_A^--closed centers), the lifetime peak of the major Lorentzian distribution shifted to longer lifetimes: from 0.25 ns to 1.67 ns in thylakoid membranes; and from 0.23 ns to 1.31 ns in PSII membranes. This change in fluorescence properties of PSII may be caused by a transmembrane electric field and th charge of Q_A^- (Keuper and Sauer, 1989; Holzwarth, 1991). However, in Phe^--closed centers, the lifetime distribution (0.57 ns and for PSII 0.77 ns) had shorter lifetimes compared to that of Q_A^--closed centers and longer lifetimes compared to that of open centers. In this case, doulbly-reduced Q_A may be formed and lost from the Q_A site in some centers (Van Miegham et al., 1989), in which the electrostatic effect of Q_A^- vanishes, resembling the open centers.

B. Material and Methods

Thylakoid membranes and PSII preparations were prepared from appressed membrane fragments of chloroplasts from Spinacea oleracea (spinach), as described by Dunahay et al.

(1984). The Chl concentration was determined using extinction coefficients published by Ziegler and Egle (1965) for absorbance at 664 nm and 647 nm (see Graan and Ort, 1984).

Samples were suspended in a reaction medium containing 0.4 M sorbitol, 5 mM Mes-KOH (pH 6.5), 20 mM KCl, 2 mM $MgCl_2$, and 1 μM nigericin when fluorescence was measured. The Chl concentration was 5 μM. The F_o (open centers) condition was obtained by the addition of 15 μM DCBQ (Cao and Govindjee, 1990; Appendix I, this thesis) and the F_m by adding 5 μM DCMU (closed centers; to be referred as Q_A^- closed centers). In addition, reaction centers were closed by prereducing pheophytin to pheophytin$^-$ by adding 1.5 mM freshly prepared sodium dithionite and 15 μM methyl viologen, as described earlier (Wasielewski et al., 1989; Govindjee et al., 1990). Such closed centers are referred to as Pheo$^-$ closed centers.

To study the time-resolved fluorescence emission, a multifrequency cross-correlation phase fluorimeter was used. The light source consisted of a Coherent Antares 76-S Neodymium Yttrium-Aluminum-Garnet (Nd-YAG) laser, mode-locked at 76 MHz. The picosecond optical pulse train generated by this system synchronously pumped a cavity dumped, model 701-3 Rhodamine 6G dye laser (Coherent). The repetition rate of the Coherent model 7200 cavity dumper was set at 2 MHz. The sample was excited under 'magic angle' conditions at 580 nm with an attenuated, collimated 1 mW beam. The emission was observed at 680 nm through a UV/VIS F/3.5 monochromator (Instruments SA

model H10) equipped with a concave holographic grating with 1200 grooves/mm. Bandwidths of 8 nm FWHM were used throughout the experiments. Both reference and sample detectors consisted of highly sensitive, low-dark-noise-Hamamatsu R-928 photomultipliers operated at room temperature. Radio frequency signals were obtained from a Marconi model 2022A signal generator and subsequently amplified by a Electronic Navigation Instruments model 603L RF power amplifier. The cross-correlation signal was set at 40 Hz (see details in Gratton and Limkeman, 1983; Alcala et al., 1985; Govindjee et al., 1990).

When a fluorescence system is excited by a sinusoidally modulated light intensity at an angular frequency ω, we have:

$$E(t) = E_0(1+M_e\sin\omega t) \qquad (Eqn\ A2.1)$$

where E_0 is the average intensity and M_e is the modulation of the excitation. The fluorescence response of the system can be written in the form

$$F(t) = F_0[1+M_f\sin(\omega t-\phi)] \qquad (Eqn\ A2.2)$$

F_0 and M_f are the average fluorescence and its modulation, respectively. The fluorescence is phase shifted with respect to the excitation by a value ϕ and demodulated such that the ratio $M=M_f/M_e < 1$. At a given modulation frequency, the measurable quantities ϕ and M are related to the physical parameters of fluorescence population by the following equations (Alcala et al., 1987a):

$$\phi = \tan^{-1} S(\omega)/G(\omega) \qquad (Eqn\ A2.3)$$

$$M = [S^2(\omega) + G^2(\omega)]^{1/2} \qquad (Eqn\ A2.4)$$

where,

$$S(\omega) = 1/N \int_0^\infty I_F(t) \sin\omega t dt \qquad (Eqn\ A2.5)$$

$$G(\omega) = 1/N \int_0^\infty I_F(\omega t) \cos\omega t dt \qquad (Eqn\ A2.6)$$

The function I_F contains the information on the distribution of components in the time domain. S(ω) and G(ω) are the sine and cosine Fourier transformations of I_F and N, a normalization factor (Alcala et al., 1987a). Phase and modulation data were generated by using sets of components with amplitudes determined according to a given distribution function. Analysis of the data with multiexponential function assumed that the light emission by a fluorescence sample upon delta-function excitation can be described as a superposition of discrete exponential decays (Gratton and Limkeman, 1984; Alcala et al., 1985; Govindjee et al., 1990).

C. Results

1. Closure of PSII reaction centers: Q_A^- closed PSII

We measured lifetimes of fluorescence at the two extremes: ($F_{o)}$ when all Q_A was oxidized and (F_{max}) when all Q_A was reduced because the kinetics of decay are simplified and

the analyses are unaffected by the organization of the PSII units (i.e., they are independent of whether the matrix or separate package model is appropriate). Figure A2.1 shows the phase shift, Φ, and relative modulation, M, as a function of frequency in MHz in open and Q_A^- closed PSII centers in thylakoid and PSII-enriched membranes. The open (F_o) condition was ascertained by the addition of μM DCBQ (see Cao and Govindjee, 1990, for justification), whereas the Q_A^- closed condition was obtained by the addition of [μM] of DCMU in both thylakoid membranes and PSII particles. The samples were excited at 610 nm and the fluorescence was measured at 680 nm. Closure of PSII centers causes large changes in both demodulation M and phase shift Φ (compare data with open and closed samples). The results of multiexponential model fits are shown in Table A2.1. Three exponential decay components were required to fit the overall decay at both the minimum and maximum fluorescence levels (see Hodges and Moya, 1986; Keuper and Sauer, 1989), while four decay components led to only a slight lowering of the χ^2 and produced an additional component with an insignificant amplitude. In order to fit the data, a minimum of three exponential components are necessary as judged by the residuals (Figure A2.2) and the low χ^2 values (Table A2.1). A single- or double-exponential fit is not sufficient to describe the data (data not shown). When Q_A was oxidized in the presence of DCBQ, three resolved lifetime components in the open PSII centers in PSII preparations were

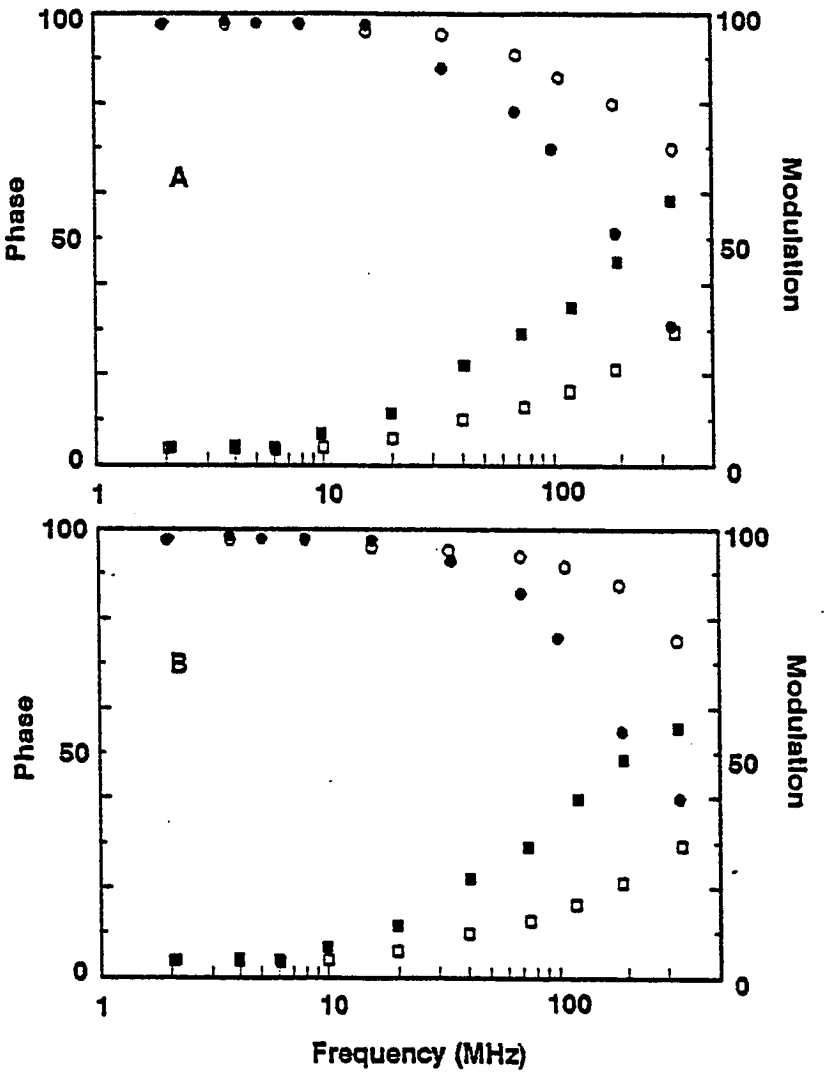

Figure A2.1. Phase and modulation as a function of frequency for the Chl a fluorescence decay in thylakoid membranes (A) and PSII preparations (B) in the presence of 5 μM DCMU (•,■) (Q_A^--closed PSII centers) or 15 μM DCBQ (O,□) (open PSII centers). Samples were suspended in a reaction medium containing 0.4 M sorbitol, 5 mM Mes-KOH (pH=6.5), 20 mM KCl, 2 mM $MgCl_2$ and 1 μM nigericin. The Chl concentration was 5 μM.

Table A2.1. Lifetimes (τ) and amplitudes (f) obtained by a triple exponential fit to Chl a fluorescence decay in thylakoid membranes and PSII preparations. The Q_A^--closed PSII centers in the samples were obtained by adding 5 μM DCMU while open PSII centers were obtained by adding 15 μM DCBQ.

	τ_1(ns)	τ_2(ns)	τ_3(ns)	f_1	f_2	f_3	χ^2
Open centers							
PSII	0.04	0.47	8.19	0.32	0.65	0.03	1.80
Thylakoid membranes	0.04	0.60	5.89	0.30	0.64	0.06	2.30
Q_A^--closed centers							
PSII	0.05	1.27	5.56	0.08	0.85	0.07	1.77
Thylakoid membranes	0.14	1.55	5.10	0.11	0.78	0.11	2.05

approximately 40 ps (32%, fractional intensity), 474 ps (65%) and 8.2 ns (3%), and those in thylakoid membranes were approximately 40 ps (30%), 596 ps (64%) and 5.9 ns (6%). Upon closure of the PSII centers (Q_A^- closed), the three lifetime components were approximately 47 ps (8%), 1.3 ns (85%) and 5.6 ns (7%) in PSII preparations; in thylakoid membranes these components were approximately 137 ps (11%), 1.6 ns (78%) and 5.1 ns (11%). Thus closure of the reaction centers leads to an about three fold increase of the lifetime of the middle component, but the fractional intensity increases only by a factor of 1.2 to 1.3. There is a minor decrease in the lifetime and a 2 fold increase in fractional intensity of the slow component. The amplitude of the fast component decreases by 3 to 4 fold upon closure of the reaction centers. This decrease is accompanied by the combined increase in the amplitudes of the middle and slow components. Untreated control samples give results similar to that of Q_A^- closed centers since high intensity of light, used here, also reduces all Q_A to Q_A^-.

Analysis with single or double Lorentzian function gives lifetime distributions shown in Figure A2.3; the parameters for Lorentzian fit functions are presented in Table A2.2. In open PSII centers, a single Lorentzian lifetime distribution with a center at about 230 ps (PSII) or 250 ps (thylakoid membranes) is obtained. In closed PSII centers, double Lorentzian lifetime distribution with a dominant distribution

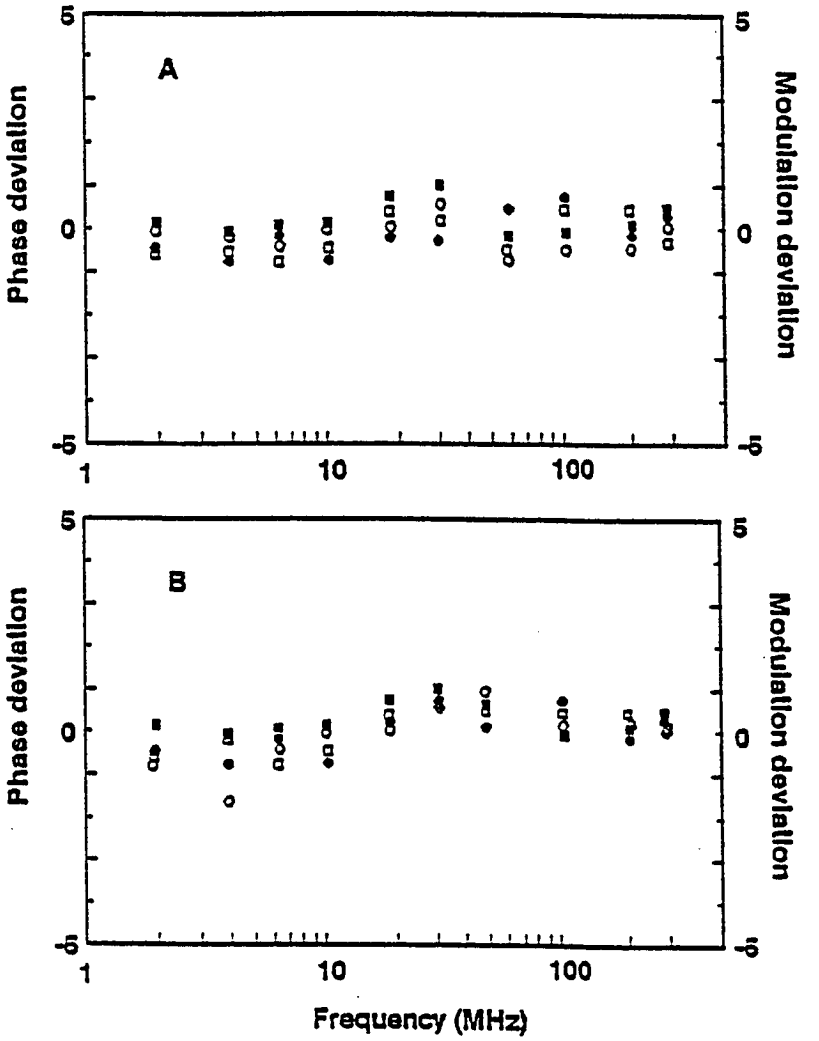

Figure A2.2. Phase and modulation deviations given as weighted residuals between the calculated and experimental data of Figure A2.1 are shown in the middle of the plot with scale +5 to -5. A: thylakoid membranes; B: PSII preparations. The non-linear least squares data analysis procedure was applied.

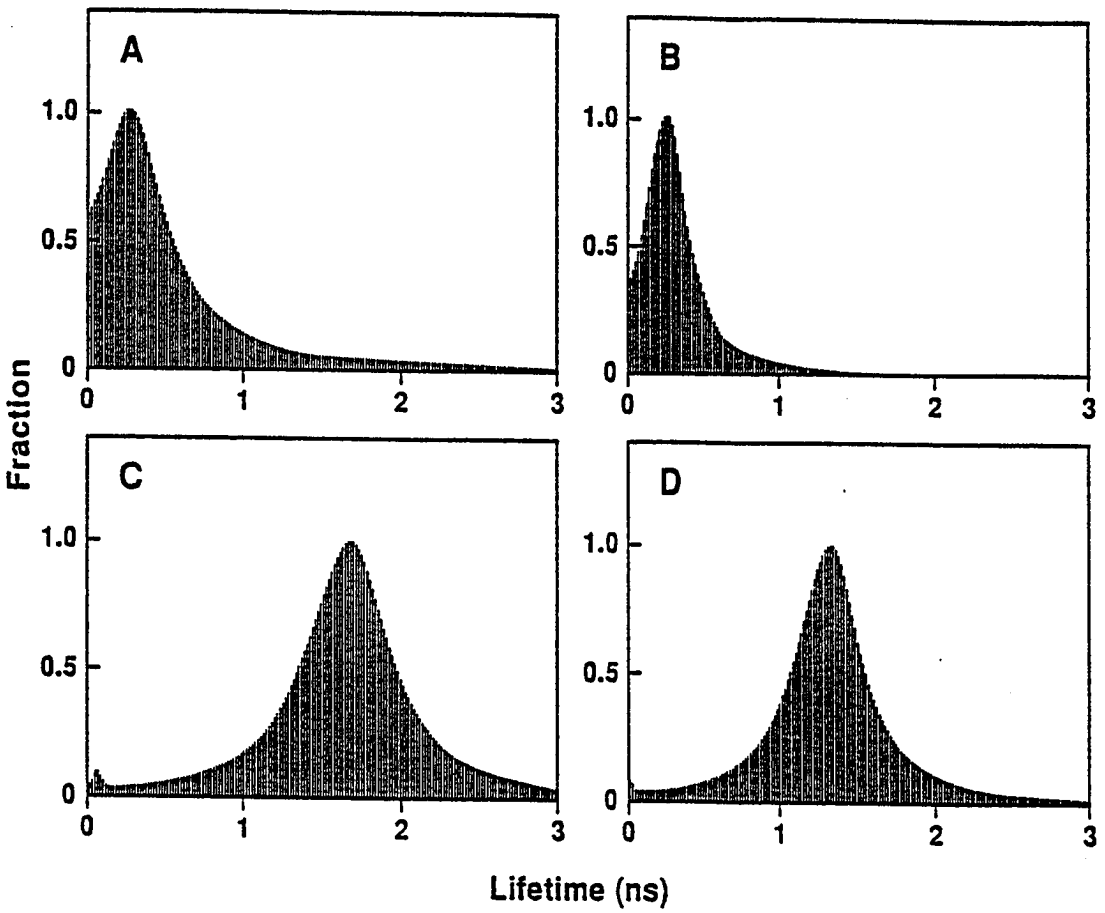

Figure A2.3. Lorentzian lifetime distributions for Chl a fluorescence decay in thylakoid membranes (A and C) and PSII preparations (B and D) in the presence of 5 μM DCMU (C and D: closed PSII centers) or 15 μM DCBQ (A and B: open PSII centers). The open center data were fitted with a double Lorentzian function while the Q_A^--closed center data were fitted with a single Lorentzian function. The other details are given in Figure A2.1.

Table A2.2. Center (c), width (w) and fractional intensity (f) obtained by a Lorentzian distribution model for Chl *a* fluorescence decay in thylakoid membranes and PSII preparations. The Q_A^--closed PSII centers in the samples were obtained by adding 5 μM DCMU while open PSII centers were obtained by adding 15 μM DCBQ.

	c_1 (ns)	c_2 (ns)	w_1 (ns)	w_2 (ns)	f_1	f_2	χ^2
Open centers							
PSII	0.23	0.35	1.00				3.88
Thylakoid membranes	0.25	0.59	1.00				3.02
Q_A^--closed centers							
PSII	1.31	0.50	0.95	0.01	0.05	0.05	1.33
Thylakoid membranes	1.66	0.62	0.93	0.06	0.05	0.07	1.04

narrower width was observed. A shift from a shorter lifetime peaked at 1.3 ns (PSII) or 1.6 ns (thylakoid membranes), respectively, was observed (see Figures A2.4A and A2.4B). The other minor peak has a small fractional intensity of 5% or 7% and is centered at 8 ps or 64 ps. In addition, a slightly distribution to a longer lifetime distribution occurred upon closure of PSII (Q_A^--closed) as expected.

It has been established that DCBQ opens all fast and slow PSII centers by oxidizing the primary quinone acceptor Q_A (see e.g. Cao and Govindjee, 1990). On the other hand, pretreatment with methylviologen and sodium dithionite and light leads to prereduction of pheophytin, and, thus, PSII centers are Pheo$^-$ closed centers (see e.g. Van Mieghem et al., 1989). Figure A2.4 shows the phase shift, Φ, and relative modulation, M, as a function of frequency in MHz in Q_A^--closed and Pheo$^-$ closed PSII centers in thylakoid membranes (A) and PSII membranes (B). As mentioned above, closing of PSII centers by prereducing pheophytin to pheophytin$^-$ also changes the demodulation M and phase shift Φ significantly. Parameters obtained by a triple exponential fit and double Lorentzian distribution fit for the above fluorescence data are presented in Tables A2.3 and A2.4. In Q_A^--closed centers, the fast decay component of open PSII centers is approximately 34 ps (9%, fractional intensity) in thylakoid membranes and 38 ps (21%) in PSII preparations; the middle decay component is approx. 1.4 ns (76%; PSII membranes) and 1.8 ns (76%; thylakoids); and

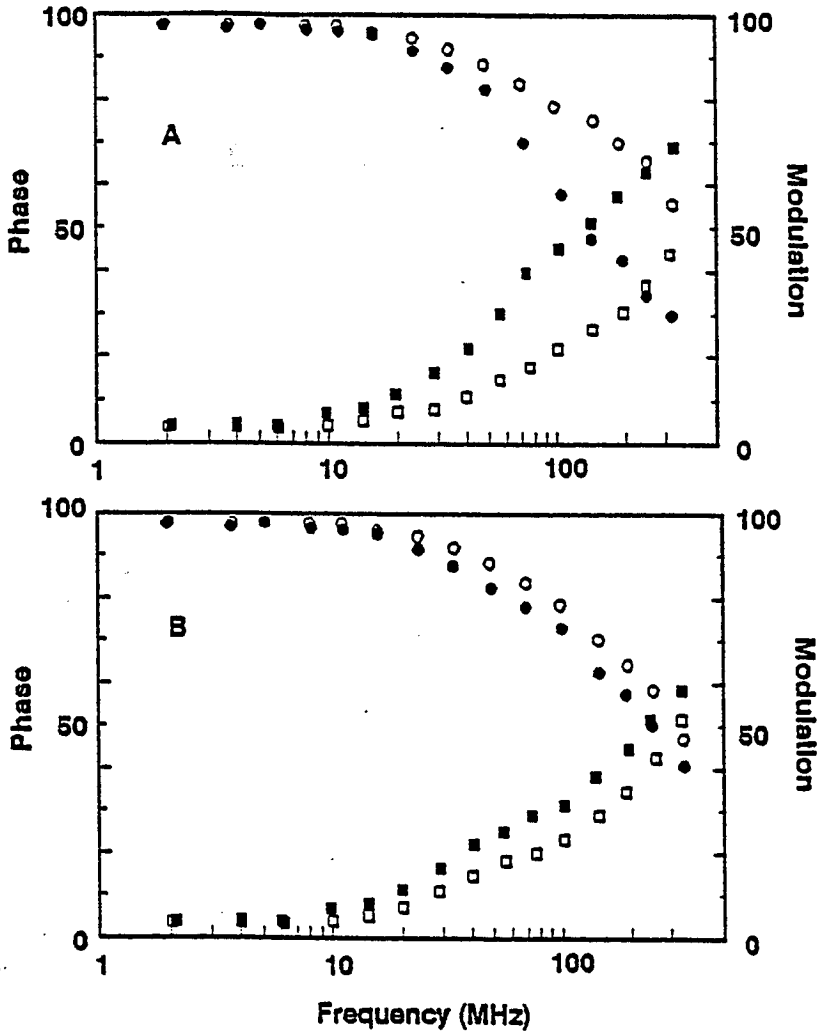

Figure A2.4. Phase and modulation as a function of frequency for Chl a fluorescence decay in thylakoid membranes (A) and PSII (B) of Q_A^--closed centers (•,■) and $Pheo^-$-closed centers (O,□). To produce $Pheo^-$-closed centers, 1.5 mM sodium dithionite and 15 μM methyl viologen were added in the resuspension buffer and the samples were illuminated first with a standard flashlight and subsequently with 1.5 W 532 nm laser light for 2 minutes. Q_A^--closed centers were generated by illumination with the laser.

Table A2.3. Lifetimes (τ) and amplitudes (f) obtained by a triple exponential fit to the Chl a fluorescence decay in thylakoid membranes and PSII preparations. The $Pheo^-$-closed PSII centers in the samples were obtained in the presence of sodium dithionite and methyl viologen plus laser illumination while Q_A^--closed PSII centers were obtained with laser light.

	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	f_1	f_2	f_3	χ^2
Q_A^- closed centers							
PSII	0.38	1.42	8.11	0.21	0.76	0.15	7.95
Thylakoid membranes	0.34	1.79	4.46	0.09	0.76	0.03	13.88
$Pheo^-$-closed centers							
PSII	0.41	1.33	24.4	0.45	0.49	0.06	5.95
Thylakoid membranes	0.21	0.79	5.63	0.27	0.62	0.11	3.91

Table A2.4. Center (c), width (w) and fractional intensity (f) obtained by a double Lorentzian distribution model for Chl a fluorescence decay in thylakoid membranes and PSII preparations. The $Pheo^-$-closed PSII centers in the samples were obtained in the presence of sodium dithionite and methyl viologen plus laser illumination while Q_A^--closed PSII centers were obtained with laser light.

	c_1 (ns)	c_2 (ns)	w_1 (ns)	w_2 (ns)	f_1	f_2	χ^2
Q_A^- closed centers							
PSII	0.37	1.37	0.05	0.36	0.16	0.84	8.51
Thylakoid membranes	0.34	2.00	0.05	0.54	0.07	0.93	14.56
$Pheo^-$-closed centers							
PSII	0.75	0.00	0.05	2.00	0.51	0.49	4.43
Thylakoid membranes	0.32	0.59	1.27	0.05	0.65	0.35	3.70

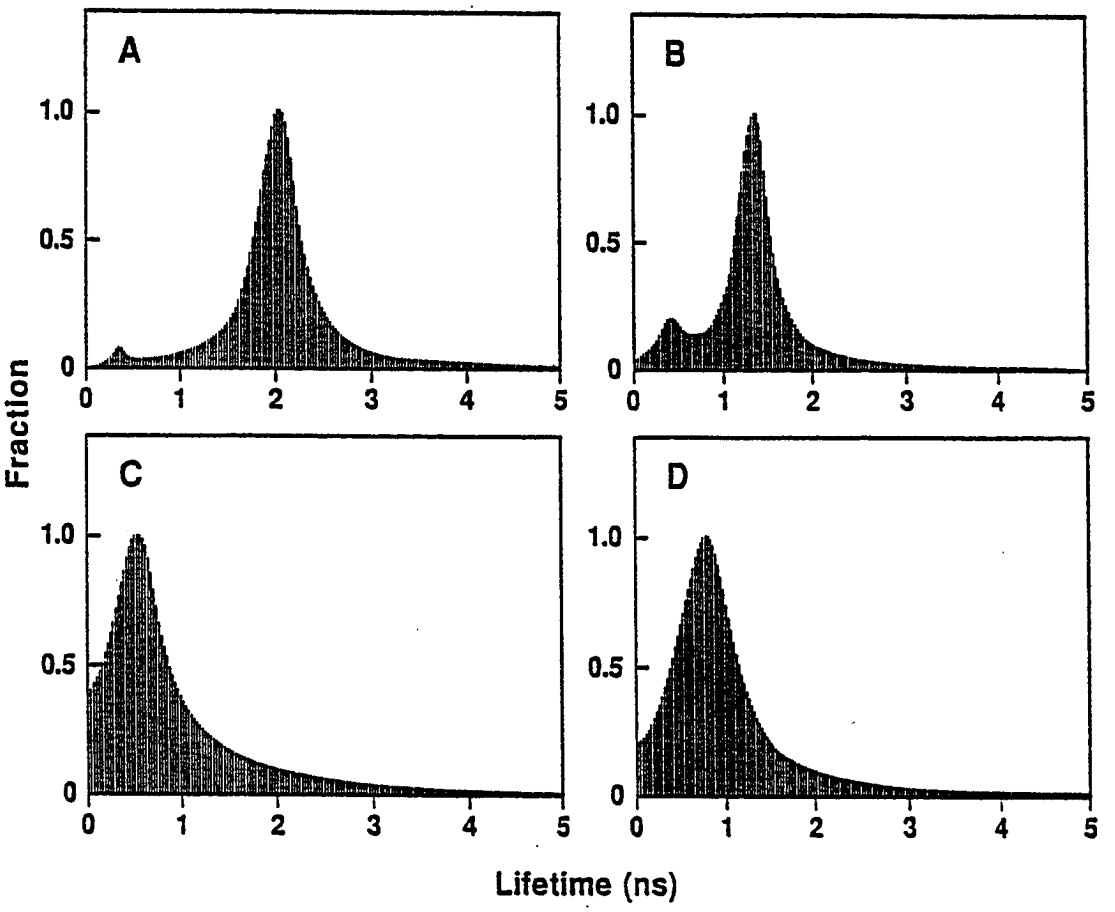

Figure A2.5. Lifetime distribution for Chl a fluorescence decay in thylakoid membranes (A and C) and PSII (B and D) in the presence of laser illumination for the Q_A^--closed centers (A,B) and sodium dithionite and methyl viologen plus laser light for the $Pheo^-$-closed centers (C,D). Data were fitted with double Lorentzian function. The other details are given in Figure A2.5.

the slow component is 4.5 ns (15%) and 8.1 ns (3%). Upon closure of centers (Pheo$^-$-closed) the lifetime of middle component increases two fold in thylakoid membranes and 1.5 fold in PSII centers, whilst lifetimes of fast and slow components both decrease. As mentioned above, the factional intensity of the middle component increases at the expense of that of the fast component upon closure of the centers.

Figure A2.5 shows double Lorentzian functions (lifetime distributions) of thylakoid membranes (A) and PSII membranes (B); the fit parameters for Lorentzian functions are presented in Table A2.4. In Q_A^--closed centers, the majority of fractional intensity localizes at a Lorentzian function center 2 ns (thylakoid membranes) or 1.37 ns (PSII-enriched membranes). In Pheo$^-$-closed centers, however, fractional intensity shifted to shorter lifetimes. The main centers of double Lorentzian function fits for thylakoid membranes and for PSII are 0.57 ns (97%, fractional intensity) and 0.77 ns (98%). In the open centers, these center lifetimes are 0.23 ns (thylakoid membranes) and 0.25 ns (PSII-enriched membranes).

D. Discussion

The present study measured chlorophyll a fluorescence lifetimes in the open and Q_A^-- and Pheo$^-$-closed PSII centers in thylakoid membranes and PSII-enriched membranes, using multifrequency phase fluorometry. The fluorescence decay data were analyzed in terms of multiexponential fluorescence decay

functions and Lorentzian distribution functions. Multiexponential analysis showed three lifetime components: fast (40-130 ps), middle (400-600 ps) and slow (5-8 ns). Upon closure of the PSII centers (Q_A^--closed), the middle component increased by nearly 3 fold in PSII preparations and thylakoid membranes; the fast component, however, stayed relatively unchanged, whereas the slow component dropped a little. The analysis with Lorentzian distribution functions showed that in Q_A^--closed centers, the lifetime peaks shifted (0.23-0.25 ns) of the major Lorenztian distribution shifted to longer lifetimes (1.31-1.67 ns), whereas in $Pheo^-$-closed centers, the lifetime peaks were shorter (0.57-0.77 ns) than those of Q_A^--closed centers.

The drop in the slow lifetime component disagrees with the lifetime increase in the slow component in the broken chloroplasts seen by Keuper and Sauer (1989). As far as the fractional intensity (amplitude) is concerned, a large decrease (3-4 fold) in the fast component, a slight increase in the middle component (1.3-1.4 fold), and a 2- to 3-fold increase were observed for the slow component. These results on thylakoids are in a qualitative agreement with the reports by Haehnel et al. (1983) and by Keuper and Sauer (1989) (see Table A2.5). The increase in the fractional intensity of the slow component, however, was smaller in the current experiment.

Here, we have confirmed that upon closure of the PSII

centers (Q_A^- closed), a large increase in the lifetime and a slight increase in fractional intensity of the middle (400-600 ps) component, a relatively small change in lifetime and significant decrease in fractional intensity of the fast (40-200 ps) component and an increase in the amplitude of the slow component (1-8 ns) occurs in PSII membranes as well as in thylakoids (Keuper and Sauer, 1989). These results are in line with the observation that the yield of the slow component is close to zero (0.04) (cf. Haehnel et al. 1983) in the presence of DCBQ, which keeps all PSII centers open (Cao and Govindjee, 1990). The lifetime of the slow component reflects the average transfer time of an excited state from a closed reaction center to a neighboring open center. The fast component showed a decrease in the yield from a high value (0.30 to 0.60) to a very low value (0.11-0.15) when PSII centers were closed. This provides strong evidence that the fast component is controlled by the process of energy conversion in the open PSII reaction centers.

The data shown in Figure A2.5 were also analyzed in terms of the global analysis for a 3-exponential and 4-exponential fit as given in Table 1 of Keuper and Sauer (1989). The χ^2 values are very high. Thus, the lifetimes obtained by Keuper and Sauer (1989) do not quantitatively fit our data (see Table A2.5) although a qualitative agreement was noted.

Schatz et al. (1987) showed that charge separation in PSII reaction centers is trap-limited. This is consistent with

Table A2.5. Multi-exponential analysis of thylakoid membranes and PSII preparations with fixed parameter values from table 1 of Keuper and Sauer (1989).

	Global 3-exponential fit, χ^2	Global 4-exponential fit, χ^2
Q_A^--closed centers		
PSII	1853	1862
Thylakoid membranes	167	169
$Pheo^-$-closed centers		
PSII	5274	5259
Thylakoid membranes	180	175

a linear relationship between the total charge separation time and the antenna size (Pearlstein, 1982). In open PSII centers the halftime of electron transfer from $Pheo^-$ to Q_A is 300-500 ps (Nuijs et al., 1986; Schatz et al., 1987). In closed centers the long-lived (larger than 2 ns) lifetime components for the radical pairs contained less than 10% of total fluorescence. The increase in fluorescence yield was considered to be caused by a lengthening of the excited state lifetime due to a decreased yield of charge separation. In contrast, Mauzerall (1985) concluded that the long-lived (about 2 ns) fluorescence from closed PSII centers is recombination luminescence as proposed by Klimov et al. (1978). However, recent experimental results have given more evidences against the hypothesis of recombination luminescence (Krause and Weis, 1991).

Fairly high yields (up to 67%) and long lifetimes of transient radical pairs (up to 32 ns) for closed PSII centers have been reported (Takahashi et al., 1987; Hansson et al., 1988; Schlodder and Brettle, 1988). The long lifetimes were suggested to be due to the formation of a relaxed radical pair from the primary radical pair. However, recent experimental results have given more evidences against the hypothesis of recombination luminescence (Krause and Weis, 1991).

In $Pheo^-$-closed centers in the thylakoid membranes and PSII samples, lifetime distribution shifted to shorter lifetimes as compared to those in Q_A^--closed centers, but had

somewhat longer lifetimes than those in open centers. In the centers treated with sodium dithionite and methyl viologen plus illumination, Q_A is reduced as well as Pheo. The effect of Q_A^- on the transmembrane field makes these centers resembling Q_A^--closed centers. A longer lifetime is observed than that of open centers. However, since $Pheo^-$ is a quencher of Chl a fluorescence, the lifetime is expected to shift to a shorter time as compared to that of Q_A^--closed centers. It is also likely that some so-called "jammed" centers (Van Miegham et al., 1989) in which Q_A is doubly-reduced and detached from the Q_A site are present. As a result, the electrostatic effect of Q_A^- on $P_{680}^+Pheo^-$ radical pair would be lost and these centers would resemble the open centers with short fluorescence lifetime.

The fluorescence decay is customarily resolved in terms of exponential components, and the values of the decay rates and preexponential factors of each component are associated with a particular conformation and with the relative population of each conformation. However, the preexponential factors cannot be related to the fraction of molecules in each conformation. The fluorescence lifetime distribution is determined by the multitude of conformational substates in a protein and by the dynamics of the protein (Gratton et al., 1986). The lifetime distribution method, as described here, provides a good approach for the study of conformational substates and of the energetics of such substates. In the

limiting case of the frozen protein (negligible dynamics), one may consider that the fluorescence is determined by a set of exponentials of which the lifetimes and amplitudes are characteristic of the set of environments of the excited residues in the protein. However, as the dynamic nature of the protein is allowed to play its role, the excited chlorophylls become exposed to electronic environments, the nature of which vary with time. Here in this study, the changes in the lifetime distribution of Chl a fluorescence decay in Q_A^--closed centers and $Pheo^-$-closed centers have provided a newer view on the changes that occur when PSII reaction centers are closed.

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