Characterization of the State Transition in the Cyanobacterium
Synechococcus sp. 7002 and a Phycobilisome-less Mutant

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ABSTRACT

Photosynthetic state transitions were investigated in the cyanobacterium Synechococcus sp. PCC 7002 in both wild-type cells and mutant cells lacking phycobilisomes. Preillumination in the presence of DCMU (3(3,4 dichlorophenyl) 1,1 dimethyl urea) induced state 1 and dark adaptation induced state 2 in both wild-type and mutant cells as determined by 77K fluorescence emission spectroscopy. Light-induced transitions were observed in the wild-type after preferential excitation of phycocyanin (state 2) or preferential excitation of chlorophyll a (state 1). The state 1 and 2 transitions in the wild-type had half-times of approximately 10 seconds. Cytochrome f and P-700 oxidation kinetics could not be correlated with any current state transition model as cells in state 1 showed faster oxidation kinetics regardless of excitation wavelength. Light-induced transitions were also observed in the phycobilisome-less mutant after preferential excitation of short wavelength chlorophyll a (state 2) or carotenoids and long wavelength chlorophyll a (state 1).

One-dimensional electrophoresis revealed no significant differences in phosphorylation patterns of resolved proteins between wild-type cells in state 1 and state 2. It is concluded that the mechanism of the light state transition in cyanobacteria does not require the presence of the phycobilisome. The results contradict proposed models for the state transition which require an active role for the phycobilisome.

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ACKNOWLEDGEMENTS

INTRODUCTION

A variety of photosynthetic organisms containing either chlorophyll (Chl) a/b light-harvesting complexes (LHC) or phycobilisomes (PBS) as antenna complexes have the ability to regulate the distribution of excitation energy between photosystem 2 (PS2) and photosystem 1 (PS1) by a process commonly known as the light state transition (Bonaventura and Myers, 1969; Murata, 1969). The state transition serves to balance the relative activities of PS2 and PS1 by adjusting the distribution of excitation energy between the photosystems in response to preferential excitation of either one. Upon overexcitation of PS1 cells undergo transition to state 1 whereas when PS2 becomes overexcited, cells change to a condition known as state 2. While the molecular basis for this regulatory response is believed to be understood in the LHC Chl a/b-containing organisms (for a review see Williams and Allen, 1987), the mechanism remains controversial in the PBS-containing red algae and cyanobacteria (Biggins *et al.*, 1984a; Allen *et al.*, 1985; Mullineaux and Allen, 1988). This is discussed in detail in the sections **State Transitions** and **Phosphorylation**.

The original model for the state transition in PBS-containing organisms, as proposed by Murata (1969), differs from that for LHC-containing organisms. The change in energy distribution between PS2 and PS1 was supposed to be controlled by "spillover" of excitation energy from PS2 Chl a to PS1 Chl a, the rate of spillover being high in state 2 and low in state 1. The role of spillover has been confirmed in a number of later studies (Ley and Butler, 1980;

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Bruce *et al.*, 1985; Bruce *et al.*, 1986) and is discussed in the section **State Transitions**. The kinetics (Biggins and Bruce, 1985) and energetic requirements (Bruce *et al.*, 1984b) of the state transition in PBS-containing organisms are also very different from those observed in LHC-containing organisms. These observations, and experiments to determine the role of phosphorylation in the mechanism, led to the development of a model for the state transition in PBS-containing organisms which did not invoke phosphorylation (Bruce *et al.*, 1984a).

Two other models have been postulated as discussed under **State Transitions** which suggest that phosphorylation of the PBS is an essential requirement for the state transition to occur. In both models it is supposed that upon transition to state 2 the PBS is phosphorylated and dissociates from PS2. In contrast, the PBS is not essential to the mechanism of the original spillover model, and its presence may not be required for the state transition to occur.

The objective of this study was to characterize the state transition in the cyanobacterium Synechococcus sp. 7002 by studying fluorescence emission and excitation spectra, kinetics, and protein phosphorylation patterns in these cells. The results show that this cyanobacterium could perform typical light-induced and redox-induced state transitions and did not appear to require a phosphorylation event to effect these transitions. In addition a PBS-less mutant of this cyanobacterium was investigated to determine if state transitions could occur in the absence of the PBS. Our results show that this mutant has the ability to perform redox-induced and light-induced state transitions (despite the difficulty in preferentially

exciting either photosystem). These results contradict the PBS/PS2 dissociation models for the state transition in PBS-containing organisms.

LITERATURE REVIEW

Photosynthetic Electron Transport

The ultimate function of oxygenic photosynthesis is to convert solar energy into stored chemical energy in the form of ATP and to provide reducing power through NADPH. This is done by a series of electron transfer steps initiating with water and ending at the terminal electron acceptor $NADP^+$. This represents a large potential difference of approximately 1.1 V and requires the cooperation of two photoreactions. These reactions also result in the movement of protons into the lumen of the thylakoids, driving the synthesis of ATP. The sequence of electron transfer steps as it is understood presently is shown in Figure 1. Each of these redox components are stabilized by specific polypeptides which, in turn, are housed by specialized complexes known as PS2, the cytochrome (cyt) b_6/f complex (b_6f), and PS1. These are discussed in detail below.

Photosystem 2

PS2 is defined functionally as the Chl-protein complex which catalyzes the light-driven transfer of electrons from water to plastoquinone (PQ), evolving oxygen in the process. Two protons are generated for every molecule of water which is oxidized while two additional protons are pumped across the thylakoid membrane in the full reduction and re-oxidation of PQ (Ort, 1986).

Until recently, little was understood of the structure of this reaction centre. However, in the past few years a wealth of information on the structure has been revealed, due primarily to the crystallization and X-ray structure determination of the reaction

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Figure 1. 'Z-scheme' of photosynthesis in oxygen-evolving organisms.

P-700+/P-700*　　(-1.2)

A0/A0-

(-1.0)
A1/A1-

(-0.73)
Fx/Fx-

(-0.55)
FAB/FAB-

(-0.4)
Fd

(-0.3)
NADP+/NADPH

Em(V)

-1.0

P-680+/P-680*

(-0.62)
Ph/Ph-

-0.5

(-0.05)
QA/QA-

0

(+0.117)
QB/QB-

(+0.29)
Rieske

(+0.34)
cyt f

(+0.37)
PC

(+0.48)
P-700+/P-700

+0.5

(+0.82)
O2/2H2O

S
s
t
a
t
e
s

+1.0

(+1.12) Z+/Z　(+1.14)P-680+/P-680
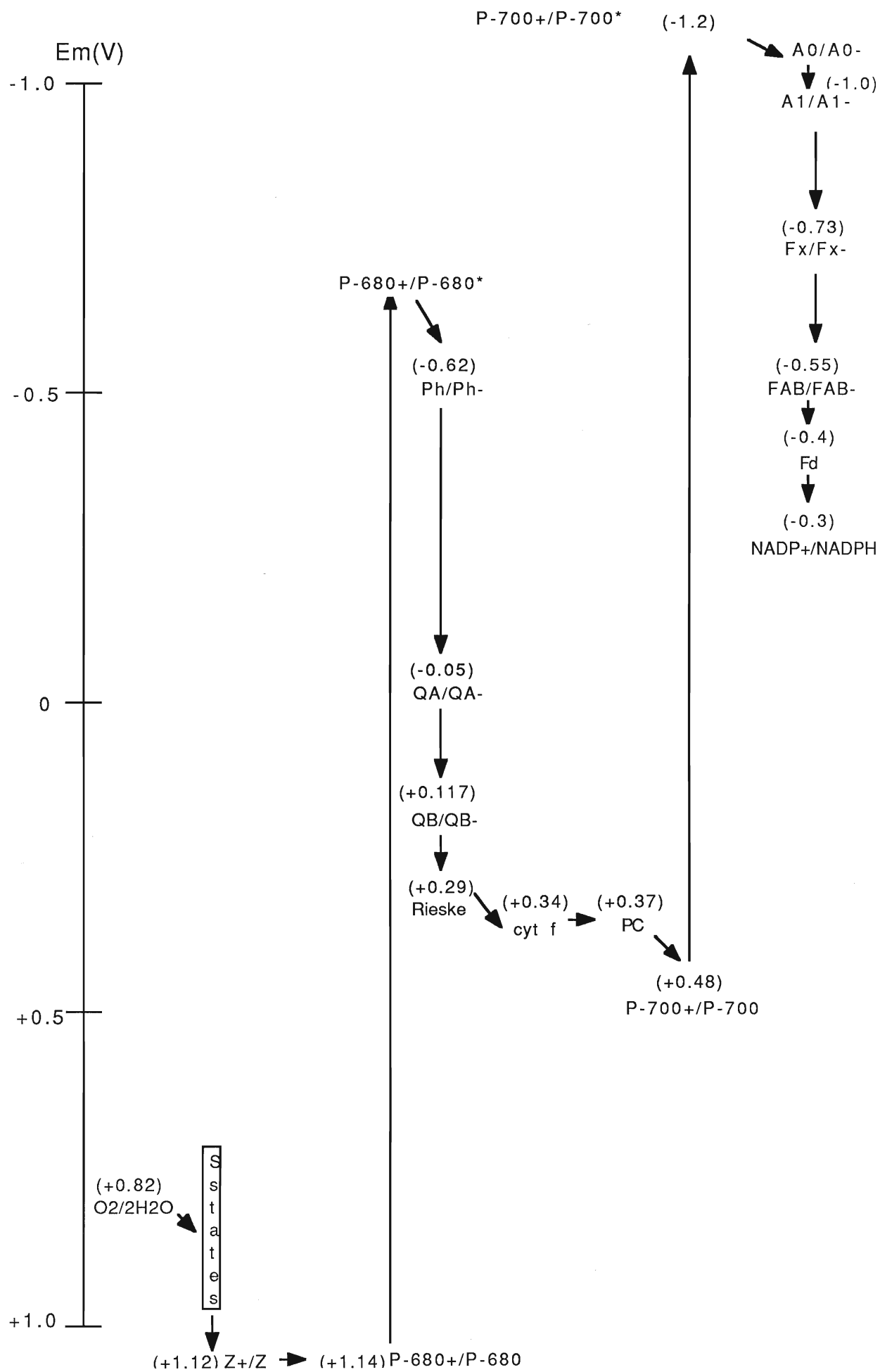
centre from the purple bacteria <u>Rhodopseudomonas viridis</u> (Deisenhofer *et al.*, 1985a). Sequencing data for genes encoding proteins comprising PS2 and the bacterial reaction centre make it clear that the purple bacterial reaction centre is an evolutionary precursor to PS2 (Deisenhofer *et al.*, 1985b). The redox centres in the two reaction centres are virtually identical if the oxygen-evolving complex is removed from PS2 and the sequence homologies of polypeptides between the two indicates that the residues have been conserved to preserve function (for reviews on this subject see Bryant, 1986 and Arntzen and Pakrasi, 1986).

The primary electron donor in PS2 is known as P-680 due to the absorbance change at 680 nm upon oxidation and its identity is now known to be a Chl <u>a</u> dimer. The purple bacteria instead have a bacteriochlorophyll (Bchl) dimer. Upon absorption of a photon by P-680, the dimer enters an excited state (P-680*), increasing the mid-point potential from approximately 1.1 V to -0.7 V. The low potential of P-680/P-680$^+$ allows it to be reduced by water (O_2/H_2O = 0.82 V) while the rather high redox potential of P-680$^+$/P-680* allows it to reduce the primary electron acceptor, a pheophytin (Ph). This transfer of an electron is known to occur on the order of a picosecond while the second step results in the partial reduction of a quinone and occurs in about 100 ps (for review see Glazer and Melis, 1987). This special PQ has been designated Q_A. Q_A then reduces another PQ, Q_B which requires two electrons before it can become protonated and move into the PQ pool. Another oxidized PQ can then take its place.

As Q_B requires two electrons before any further electron transfer steps are to take place and the redox centres prior to Q_B are one electron carriers, P-680 must be photo-oxidized twice before this can occur. Thus after the first photon is absorbed and Q_B is reduced to a semiquinone P-680$^+$ must be re-reduced before it can absorb another photon. The immediate donor to P-680$^+$, called Z, is thought to be reduced by manganese in the oxygen-evolving complex (Pecoraro *et al.*, 1987). Up until very recently, Z$^+$ was thought to be a plastosemiquinol cation on the basis of EPR and absorbance difference spectroscopy (Blankenship *et al.*, 1975). However, sequence homologies with the L and M subunits in bacterial reaction centres indicated that Z could be a tyrosyl residue in the D1 protein of PS2 (Rutherford and Styring, 1987). Strong support for this argument was given in an elegant series of experiments by Barry and Babcock (1987).

The sequence of electron acceptors associated with PS2 appear to be the same in both higher plants and cyanobacteria. The polypeptide arrangement of the core is also very similar, however large differences occur in the polypeptides which bind the antenna pigments outside the core. Higher plants contain membrane-embedded pigment-protein complexes known as LHC and contain Chl a and Chl b. Cyanobacteria (and red algae) do not contain any Chl b. Instead they contain complexes known as PBS which are bound to the membrane and are associated with PS2 (Gantt, 1981). This soluble complex contains 2 or 3 different bilin pigments, including erythrobilin and cyanobilin (Glazer, 1984). In addition, bound to PS2

in these organisms is a large polypeptide which links the PBS to PS2 (Redlinger and Gantt, 1982).

Table 1 shows the different proteins associated with PS2 which have been identified. The 47 and 43 kilodalton (kD) proteins function as antenna for P-680, binding Chl $\underline{a}$ and fluorescing at 695 and 685 nm respectively (Delepelaire and Chua, 1979; Satoh et $al.$, 1983). The 32 kD protein binds Q_B and is known as D1. D1 houses the tyrosyl residue Z and is homologous to D2, the 34 kD peptide (Chua and Gillman, 1977). These in turn are homologous to subunits L and M of the purple bacterial reaction centre, respectively. The presence of conserved residues in D1, D2 and L and M suggests the possibility that the electron transferring components Fe, Q_A, Q_B, P-680, Ph, and the monomeric Chl may be bound to D1 and D2. This has been recently verified by the isolation of a PS2 reaction centre containing only D1, D2, and cyt $\underline{b}$-559 (Nanba and Satoh, 1987). This complex carries out a light-induced charge separation, indicating the presence of these redox components. As well, from comparison to the purple bacterial reaction centre it is evident that there are two Chl monomers and Ph despite the fact that only one pathway is used (Won and Friesner, 1988). The precise roles of the Chl monomer, the Fe atom and the second pathway are not well understood.

The last electron transfer step in PS2 occurs between Q_A and Q_B. Upon full reduction of Q_B the plastoquinol moves to the mobile pool and can carry an electron to the second redox complex; the cyt b_6/f complex.

Table 1. Polypeptide composition of photosystem 2 in PBS-containing organisms.

Molecular weight (kD)	Description
70-95	PBS linker
47	chl-binding antenna protein fluoresces at 695nm
43	chl-binding antenna protein fluoresces at 685nm
36	chl-binding protein fluoresces at 685nm
34	D2 protein binds primary redox components + QB
33	extrinsic protein O2 evolution
32	D1 protein binds primary redox components
8-14	cytochrome b-559

Cytochrome b_6/f Complex

This complex is defined functionally as a PQ-plastocyanin oxidoreductase. Similar to the cyt bc_1 complex of mitochondrial and bacterial electron transport chains, it contains one cyt f, one 2Fe-2S "Rieske" centre and two cyt b's. These cyt b's have different redox potentials but are associated with the same peptide. A complex composed of five polypeptides has been isolated from spinach chloroplasts with molecular weights 37, 33.5, 22, 19, and 16.5 kD (Clark *et al.*, 1984). The 33.5 and 22 kD proteins exhibit heme-associated peroxidase activity and are presumed to represent cyt f and b_6. The other Fe protein is the 19 kD Rieske protein. Interestingly enough, the 37 kD protein has been identified as the ferredoxin-NADP$^+$ oxidoreductase (FNR). The fact that this protein copurifies with the cyt b_6/f complex has interesting implications for cyclic electron transport. FNR binds electrostatically to the cyt b_6/f complex and its removal does not impair PQ-plastocyanin oxidoreductase activity. A complex was isolated from the cyanobacterium Anabaena variabilis consisting of four polypeptides: cyt f (31 kD), cyt b_6 (22.5 kD), Rieske 2Fe-2S (22kD) and subunit IV (16 kD). FNR did not co-purify (Krinner *et al.*, 1982).

The immediate acceptor to the Rieske Fe-S protein is cyt f. The redox centre of the cyt is found in the lumen, providing easy access for the mobile electron carrier plastocyanin PC or cyt c-553. Presumably all higher plants contain only plastocyanin, however some cyanobacteria contain either one or both of these carriers, Cu and Fe concentrations dictating which is the more predominantly synthesized (Wood, 1978). Either of these carriers can transport an

electron from the b_6/f complex to PS1. It is also thought that in cyanobacteria the cyt b_6/f complex functions in a dual role, operating in the photosynthetic electron transport chain in the light and in the respiratory chain in the dark (Scherer *et al.*, 1988). This is discussed in the section **Respiratory Electron Transport**.

Photosystem 1

PS1 is defined functionally as the pigment-protein complex which catalyses the light-driven transport of electrons from reduced plastocyanin (or cyt c-553) to oxidize ferredoxin (Fd). Although the structure of PS1 is not as well understood as PS2 it is apparent that PS1 is similar to the reaction centre of green sulfur bacteria such as Chlorobium (Pierson and Olson, 1987). Chlorobium is an obligate anaerobic N_2-fixing photolithoautotroph, using H_2 or reduced sulphur compounds to reduce CO_2. In Chlorobium the primary electron donor P-840 is a Bchl a dimer (Wasielewski *et al.*, 1982) and is associated with a 65 kD polypeptide (Hurt and Hauska, 1984). The initial electron acceptor is a special lipophilic form of Bchl c (Braumann *et al.*, 1986) and the secondary acceptor is an Fe-S centre capable of reducing Fd directly (Knaff *et al.*, 1979). It is thought that electrons from Fd can go either to NAD^+ via FMN or to the menaquinone pool, the cyt bc_1 complex and to a cyt c-553, the immediate donor to P-840^+ (Pierson and Olson, 1987). This suggests an analogous path of cyclic flow in PS1.

Another organism which has been described is an N_2-fixing photosynthetic bacteria called Heliobacterium chlorom (Gest and Favinger, 1983). Evidence suggests that the primary donor P-798 is

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either a monomer or dimer of Bchl g, an isomer of Chl a (Prince *et al.*, 1985). The initial acceptor absorbs at 670 nm and may be a Chl a-like acceptor (Fuller *et al.*, 1985). There appears to be secondary acceptors, a quinone, and an Fe-S centre (Brok *et al.*, 1986). The electron donor to P-798 is a cyt c-553 (Fuller *et al.*, 1985).

As mentioned previously, the green sulphur bacteria and <u>Heliobacter</u> are thought to be evolutionary precursors to PS1. However, only recently has this become evident. The primary electron donor, P-700, was thought to be either a Chl a monomer or dimer. The EPR signal from the triplet state of P-700 is similar to that of monomeric Chl a triplet <u>in vitro</u> (Rutherford and Mullet, 1981), suggesting P-700 may be a monomer. However at the same time EPR spectra lend support to the dimer hypothesis. The P-700$^+$ band is narrowed compared to Chl a$^+$, with a half width of 7.1 G compared to 9.6 G (Mathis and Rutherford, 1987). Present notions are that P-700 is a dimer with the molecules close enough to provide an electronic interaction in the reduced state, but upon oxidation, such an interaction disappears and the electron hole is localized on only one of the Chl. A dimeric structure is consistent with circular dichroism spectra of P-700 (Philipson *et al.*, 1972) obtained more than 15 years ago. This model is further supported by recent experiments (Ikegami and Itoh, 1988) which indicate that the absorbance band width of the reduced P-700 is large, corresponding to a degeneration of two monomer absorbance bands. The oxidized P-700 has a narrow absorbance band, suggesting a monomeric structure of oxidized P-700. The obvious conclusion is that the charge is localized on only one of the two Chl.

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Another question concerning P-700 is the identity of the Chl. Some evidence has been presented suggesting that P-700 may be a Chl $\underline{a}'$ (the C10 epimer of Chl $\underline{a}$) as it was thought to be present in a molar ratio of 1:1 with P-700 in a P-700-enriched particle (Watanabe *et al.*, 1985). However, the results of Ikegami and Itoh (1988) clearly show that only one molecule per dimer is present and thus cannot be P-700. Another form proposed to be present in PS1 is a 13-hydroxy-20-chloro Chl $\underline{a}$ (Dornemann and Senger, 1986). Although supported in the literature (Glazer and Melis, 1987) more recent evidence suggests this molecule is not present in PS1 (Fajer *et al.*, 1987). The simplest explanation of the results to date is that P-700 is a Chl $\underline{a}$ dimer while the primary acceptor is a Chl $\underline{a}'$. This primary acceptor has been designated A_0 in the literature and is likely located close to the dimer P-700 (Golbeck, 1988). This proposed identity of A_0 is also consistent with results from $\underline{H.}$ $\underline{chlorum}$ which suggests that the primary acceptor in this organism is a Chl-like molecule (Fuller *et al.*, 1985).

The secondary acceptor, A_1, appears to be a phylloquinone (vitamin K-1). Extraction and reconstitution experiments (Itoh *et al.*, 1987; Setif *et al.*, 1987) indicate that the removal of vitamin K-1 from PS1 particles increased the charge recombination between P-700^+ and A_0^- by blocking the rapid oxidation of A_0^-. Itoh and Iwaki (1988) showed that vitamin K-1 extraction enhanced the delayed fluorescence in PS1, consistent with vitamin K-1's proposed role as a secondary electron acceptor.

It is thought that the primary photochemistry takes place in the 'core' of PS1. This consists of 2 polypeptides of 60-80 kD

(Vierling and Alberte, 1983), a situation analogous to that of PS2. Extending this analogy it is supposed that P-700 lies at the interface of the two polypeptides (Mathis and Rutherford, 1987). This core also contains A_0 and perhaps A_1 as well (Rutherford and Mullet, 1981). An analogous complex of 2 polypeptides are thought to form the outer core antenna complex of PS1, resulting in 1 P-700 for every four 70 kD polypeptides. Such a complex also binds about 130 Chl $\underline{a}$ and 16 carotenoid molecules (Glazer and Melis, 1987).

The next acceptor, F_x, is also thought to be associated with the core of PS1 (Golbeck and Cornelius, 1986). It is known to be a 2Fe-2S cluster and is quite unusual in comparison to other Fe-S centres due to its redox potential (700 mV) and its broad EPR signal in the reduced state (Bertrand *et al.*, 1988). Some results suggest this may be due to an interaction with Chl $\underline{a}$ pigments in the core (Ikegami and Itoh, 1987).

Two additional 4Fe-4S clusters are present in PS1 and are associated with smaller polypeptides. Lagoutte *et al.* (1984) tentatively identified an 8 kD peptide as an Fe-S protein in spinach by carboxymethylation of iodo-^{14}C-acetate to identify the available cysteines. The sequences of the genes encoding the PS1 polypeptides will likely shed considerable light on the possible binding sites of these various redox centres. It may be interesting to note that in PS1 preparations from Synechococcus 6301 a subunit ratio of $[70kD]_4[18-20kD]_1[17.7kD]_1[16]_1[10]_2$ was found (Lundell *et al.*, 1985). The two 10 kD polypeptides may represent F_A and F_B binding proteins, however, F_A and F_B are thought to be present on the same polypeptide in close proximity (Andreasson and Vanngard, 1988). It

is not known whether F_A and F_B act in parallel or in series as electron transfer has not been observed between the two (Chamorovsky and Cammack, 1982). It has even been suggested, although there is no evidence, that they act in parallel, one functioning in linear electron transfer and the other in cyclic (Mathis and Rutherford, 1987).

The next acceptor in PS1, Fd, is a stable, soluble Fe-S protein with a molecular weight of 10 kD. It also reacts with the thylakoid membrane at a second site, becoming reoxidized by the thylakoid bound FNR (Whatley *et al.*, 1963). FNR acts as a two electron gate, Fd being a one electron carrier and NADP a two electron acceptor.

It has been known for some time that Fd catalyses cyclic electron transport around PS1 (Arnon, 1977), although not everyone is in agreement with this (Myers, 1986). This generates a proton gradient which can be utilized to generate ATP without the production of NADPH. As mentioned previously, green sulphur bacteria are capable of cyclic electron transport, Fd presumably donates electrons to the MQ pool (Pierson and Olson, 1987). Similar ideas are proposed for oxygen-evolving organisms and the presence of FNR on the cyt b_6/f complex lends suppport to this idea. The presence of FNR on the cyt b_6/f complex also has implications for respiratory electron transfer in which NADH could donate electrons to the cyt b_6/f complex in the dark.

Respiratory Electron Transport

Cyanobacteria are rather interesting organisms to study for the bioenergeticist due to the lack of both mitochondria and chloroplasts. Instead, they have thylakoid membranes which apparently house both the photosynthetic and respiratory pathways (Peschek, 1987). In addition, these pathways have been shown conclusively to share certain regions of the chain (Aoki and Katoh, 1982). One possible model of this interaction between the photosynthetic and respiratory pathways is shown in Figure 2. It illustrates that the PQ pool (ubiquinone pool in mitochondria), the cyt b_6/f complex (cyt bc_1 complex in mitochondria) and cyt c-533 can be utilized to reduce either P-700 or O_2. Evidence for the involvement of the PQ pool has been provided by studies on a thermophilic Synechococcus sp., employing fluorescence induction experiments to monitor the redox state of PQ (Aoki and Katoh, 1982). Peschek (1983) concluded from results with Anacystis nidulans that the cyt b_6/f complex participates in both respiratory and photosynthetic electron transport. It was found that physiological oxidation of anaerobic membranes by either light or O_2 induced similar spectral changes in the cyt absorption spectrum.

Figure 2 also indicates that either NADH or NADPH can be used as the initial electron donor to the quinone pool. From different sensitivities to inhibitors it has been concluded that the sites of oxidation are different for the two molecules. PQ reduction by NADH was inhibited by antimycin A while the NADPH pathway remained open (Matthijs et al., 1984). The intersection of the respiratory and

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photosynthetic electron transport pathways has enabled researchers to manipulate the redox state of the PQ pool without changing the light quality. This has provided strong evidence that the redox state of PQ controls the light state transition (Dominy and Williams, 1987; Mullineaux and Allen, 1986) in cyanobacteria.

Figure 2. Interaction of the redox components of photosynthetic and respiratory electron transport in cyanobacteria.

NAD(P)H $\longrightarrow$ PQ $\longrightarrow$ cyt b6f $\longrightarrow$ cyt c-553 $\longrightarrow$ cyt oxidase $\longrightarrow$ O_2

P-680 $\longrightarrow$

$\longrightarrow$ P-700
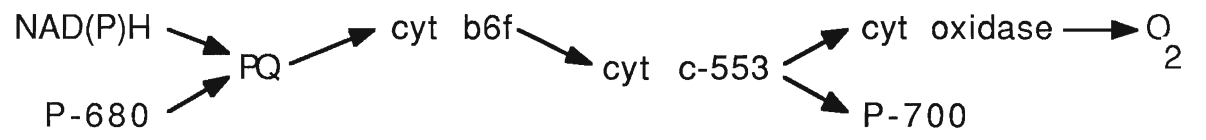
Applications of Fluorescence Spectroscopy to Photosynthesis

The measurement of chlorophyll fluorescence has been a very useful tool in the analysis of photosynthesis. The relationship between the yield of fluorescence and photosynthetic electron transport was first realized by Kautsky *et al.* (1960) in a kinetic analysis of room temperature fluorescence in cells of the green alga Chlorella. They were not only able to determine that two photochemical reactions were involved in oxygenic photosynthesis but that the yield of fluorescence was controlled by a molecule which quenched fluorescence in the oxidized state. This molecule has since been recognized as Q_A, a one electron acceptor immediate to the pheophytin (see section on **Photosynthetic Electron Transport**) (for review see Hipkins and Baker, 1986).

Room temperature fluorescence kinetic studies are routinely performed on both whole cells and chloroplasts. The cells or chloroplasts are DCMU-poisoned to prevent complicated photosynthetic induction effects; as a result electron transfer is stopped at the Q_A site. When dark-adapted cells are initially illuminated, fluorescence immediately rises to a minimal level known as F_o. In this situation all of the PS2 reaction centres are open, that is, Q_A is maximally oxidized. The fluorescence yield at F_o, $\emptyset_{Fo}$, is given by:

$$\emptyset_{Fo} = k_f/(k_f + k_h + k_{t(II->I)} + k_p) \qquad (1)$$

where k_f is the rate constant for fluorescence, k_h is the rate constant for radiationless decay to heat, $k_{t(II->I)}$ is the rate constant for energy

transfer from PS2 to PS1, and k_p is the rate constant for PS2 photochemistry (Hipkins and Baker, 1986). This fluorescence arises from PS2, PS1 being virtually non-fluorescent at room temperature.

After the initial rise to F_0 there is a considerably slower rise in fluorescence to a maximum level known as F_m. At F_m all of the PS2 centres become closed and Q_A is maximally reduced. The fluorescence yield at Fm is given by:

$$\emptyset Fm = k_f/(k_f + k_h + k_{t(II->I)}) \qquad (2)$$

The difference between F_0 and F_m is known as the variable fluorescence (F_v) and is given by:

$$\emptyset Fv = \emptyset Fm - \emptyset Fo \qquad (3)$$

A contentious issue for some time has been the origin of F_v (for review see Holzwarth, 1986). Some researchers have suggested that the variable fluorescence observed at 695 nm reflects a back reaction from the Ph to P-680 (Klimov *et al.*, 1978). This presumably occurs with increasing frequency as Q_A becomes more reduced. This assumes that charge separation can occur between P-680 and the Ph even when Q_A is already reduced. An alternative view suggests that the fluorescence arises directly from the pigments rather than charge recombination and time-resolved fluorescence studies seem to support this (Haehnel *et al.*, 1983).

While fluorescence studies are commonly done at room temperature these studies are limited to the analysis of PS2 Chl. PS1 fluorescence can be seen at room temperature, however spectrometers with extremely fast time resolution are required. The alternative is to freeze samples in liquid nitrogen (77K). This not only results in a huge increase in steady state PS1 fluorescence but is

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resolves PS2 fluorescence in cyanobacteria and red algae into three bands. These bands are seen at approximately 685, 695, and 750 nm while the broad PS1 band is seen at 715-720 nm. Room temperature studies are most often seen associated with kinetic studies whereas fluorescence emission and excitation spectra are usually done at 77K.

Obtaining a fluorescence emission spectrum usually involves exciting the sample with monochromatic light and passing the emitted radiation from the sample into a scanning monochromator to obtain the fluorescence emission in a selected wavelength range. Doing such an experiment at 77K allows one to not only observe PS2 and PS1 fluorescence but it also enables the researcher to observe energy transfer between pigments. Figure 4 shows typical fluorescence emission spectra from cyanobacterial cells excited at 590 nm, a wavelength chosen to excite PC (phycocyanin). Despite the fact that PS1 and PS2 Chl a absorb very little of this radiation fluorescence emission peaks are seen from these species. This is because energy is transferred from the PC to these other components.

Additional information can be obtained by examining fluorescence excitation spectra of samples. This involves transmitting the actinic light through a scanning monochromator over a specific wavelength range and monitoring the emitted fluorescence from the sample at a fixed wavelength, usually one chosen which corresponds to a fluorescence peak. This gives information on which pigments contribute to which photosystem and in effect is an absorption spectrum for the fluorescence emission peak which is monitored.

STATE TRANSITIONS

As mentioned in the introduction, a variety of photosynthetic organisms containing either LHC Chl a/b or PBS as antenna complexes have the ability to regulate the distribution of excitation energy between PS2 and PS1. This process is known as the light state transition (Bonaventura and Myers, 1969; Murata, 1969). This mechanism is thought to balance the relative activities of PS2 and PS1 by adjusting the distribution of excitation energy between the photosystems in response to preferential excitation of either one. The response to overexcitation of PS1 is known as state 1 while overexcitation of PS2 leads to state 2. A discussion of the theory behind the state transition and the opposing views held on the mechanism in PBS-containing organisms will be presented.

Theory

Two general types of mechanisms have been proposed to explain the changes in the energy pathway seen upon imbalanced excitation of either photosystem. These are **spillover** and **absorption cross-section** and are discussed below.

Absorption cross-section

The absorption cross-section is given as:

$$\sigma = (x/E) \qquad (4)$$

where x is the average number of hits to a photosynthetic unit and E is the fluence: the quanta per unit area (Mauzerall and Greenbaum, 1989). This measure is most often used in association with

photosynthetic activity and is known as ($\sigma(z)$). As defined by Mauzerall and Greenbaum (1989) the cross-section of a photosynthetic unit is measured by determining the relative yield of a product or intermediate z (ie P-700$^+$ or O$_2$) as a function of a single turnover flash of monochromatic light on an optically thin sample. This can be a very useful measurement as it does not require knowing the total amount of pigment associated with a particular reaction, only those pigments which when excited can give rise to the product or intermediate are taken into account. It has been used to measure the amount of energy transfer between PS2 reaction centres (Mauzerall, 1978), the effects of light adaptation (Ley and Mauzerall, 1982), and the photochemical yields of specific electron transfer steps (Cho *et al.*,1984). It can also be used to study the effects of state transitions which will be addressed later in this section. However, in this context it is important to realize that absorption cross-section is a measure of all those pigments which contribute to a specific reaction, usually PS1 or PS2 electron transport activity.

Spillover

One of the first mentions of spillover was by Myers (1963), not long after the realization that two photoreactions were involved in oxygenic photosynthesis. He envisioned that energy initially absorbed by PS2 antenna normally transferred the energy to the PS2 reaction centre, however, if PS2 was 'full', the energy could spillover into PS1. By 'full' he meant that the PS2 reaction centre is closed, that is, it cannot accept the energy until Q$_A$ is re-oxidized (see

previous section).   In the red algae <u>Porphyridium cruentum</u> it has been found that the yield of energy transfer from PS2 to PS1 (spillover) increased from 50% when the reaction centres were open to 90% when the centres were closed (Ley and Butler, 1976).

There are two factors which can affect the efficiency of spillover between PS2 and PS1, these are the distance between PS2-associated Chl <u>a</u> (the donor) and PS1-associated Chl <u>a</u> (the acceptor), and the orientation between the two.   An expression has been derived for the rate of this type of energy transfer, also known as Forster energy transfer (Forster,1967).   However, the mathematics of this mechanism is beyond the scope of this discussion.   What is important to know is that the rate of energy transfer from PS2 to PS1 is inversely proportional to the distance between the donor and acceptor molecules raised to the sixth power and is also proportional to an orientation constant known as k^2.   Thus, any changes in the distance between the photosystems (or the donor and acceptor pigments within them) and/or the orientation with respect to each other will lead to dramatic changes in the rate of spillover between PS2 and PS1.

A common error made when describing the state transition in the literature is the notion that changes in spillover and absorption cross-section are two mutually exclusive events (Fork and Satoh, 1986; Williams and Allen, 1987).   From the theory described above it should be apparent that a change in energy spillover between PS2 and PS1 will result in a change in the absorption cross-section of the two reaction centres.   If for example, on transition to state 2 one

observes an increase in energy spillover from PS2 to PS1 then one should see an increase in activity when using wavelengths which excite the PBS and PS2 Chl $\underline{a}$. This is because the energy initially absorbed by the PBS and PS2 Chl $\underline{a}$ is being diverted over to PS1 at the expense of PS2 activity. Thus one should also see a decrease in PS2 activity when using these same wavelengths. In other words, the absorption cross-section of PS2 is decreasing in state 2 while the absorption cross-section of PS1 increases. A change in spillover causes a change in absorption cross-section.

When researchers speak of an alternative to spillover as the mechanism of the state transition they are not in fact referring to a change in the absorption cross-section but a change in the distribution of antenna pigments between PS1 and PS2. The controversy is actually one of energy spillover between the photosystems versus antenna distribution. The specific models which have been proposed within the framework of these two opposing ideas will be addressed later in this section.

History

The redistribution of excitation energy between PS2 and PS1 was first observed in PBS-containing organisms by Murata (1969). Using Porphyridium cruentum, a red alga, he measured both room temperature and 77K fluorescence emission of Chl $\underline{a}$. He found that pre-illumination with light preferentially absorbed by the PBS resulted in changes in the distribution of energy between PS2 and PS1. Murata suggested that some conformational change in the thylakoid membrane, resulting in either a change in distance or

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orientation between the two photosystems could allow the cell to regulate the distribution of excitation energy.

Ley and Butler (1976, 1977, 1980) have done considerable work in an effort to determine the changes in the energy transfer rates as well as the amount of spillover observed in each state. When the yields of energy transfer in either state were calculated at the F_m level they were very similar (state 1 - 0.97, state 2 - 0.94). However if these values were calculated for each state at the F_0 level, that is, when all of the PS2 centres were open, very different values were obtained. These were 0.48 in state 1 and 0.61 in state 2. In all, they found that accompanying the transition from state 1 to state 2 was: a decrease in the yield of fluorescence from PS2, an increase in the minimum yield of energy transfer from PS2 to PS1, and no changes in the relative absorbance cross-section of the photosystems, that is, the absorbance cross-sections of PS2 and PS1 which have been corrected for spillover (α and β). However in a subsequent study Ley (1984) found in the same organism that accompanying a state 2 to 1 transition was a 50% increase in the effective absorption cross-section per PS2 as measured by O_2 production.

In two detailed studies (Ried and Reinhardt, 1977; Ried and Reinhardt, 1980) the kinetics and quantum requirements of the state transition were examined in red algae. The state 1 transition required a 200 ms medium intensity pulse at 443 nm to induce a complete transition, however the kinetic of the actual transition was considerably longer (10-20 seconds as measured by room temperature Chl <u>a</u> fluorescence). It was found that 2-4 photons absorbed in surplus by PS1 were required per electron transport

chain to induce a half maximum transition to state 1. The state 2 transition required a slightly higher number of photons.

A further study of the kinetics was performed on the red alga Porphyridium cruentum (Biggins and Bruce, 1985). State 1 transitions were investigated as a function of the intensity and exposure to light absorbed by PS1 (light 1). In a typical experiment where the intensity was increased from 7 to 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ the half-time decreased from 3.8 to 1.3s as measured by 77K fluorescence emission. It was also shown using single-turnover flashes that between 10 and 20 flashes were required at a frequency of 2.5 Hz. Frequencies either lower or higher than this value resulted in inhibition of the state 1 transition, suggesting that the turnover time for the mechanism triggering the transition is on the order of hundreds of milliseconds.

The transition to state 2 was also investigated as a function of light intensity and time of exposure to light absorbed by PS2 (light 2). The transition was found to be biphasic and, interestingly, the kinetics of the transition were dependent on the length of time in state 1 preceding the transition unlike the state 1 transition which was independent of the time in state 2. The slow phase was found to be associated with the kinetics of photosynthetic induction and could be almost eliminated if the time spent in state 1 was only a few seconds. The authors suggest that during long periods of time in state 1 deactivation of enzymes involved in CO_2 assimilation would occur. It is interesting to note the faster kinetics in this study compared to the previous studies, however the earlier papers used

room temperature fluorescence emission to assay the state transition whereas the more recent paper used low temperature fluorescence emission. It would be interesting to see a study in which the kinetics of the transition were monitored simultaneously using both assays.

Picosecond fluorescence spectroscopy was also used to study the state transition in <u>Porphyridium cruentum</u> (Bruce *et al.*, 1986). It was found that fluorescence emission from the PBS components was independent of the state transition whereas Chl a was not. Emission from Chl a in state 2 showed that PS2 Chl a fluorescence rose faster than the PS1 Chl a emission and decayed more rapidly. The converse of this was seen in state 1. These experiments confirmed earlier ones (Bruce *et al.*, 1985) that the PS2 Chl a decay accelerated on transition to state 2. The faster time resolution of these studies also allowed the authors to show that the PS2 Chl a emission rose more rapidly and the PS1 Chl a emission more slowly in state 1 . These results are consistent with the spillover model for the state transition in red algae.

State Transition Models

Structural differences between the antenna of LHC- and PBS-containing organisms are rather dramatic; instead of the membrane-embedded LHC chl a/b, PBS-containing organisms have antennae known as PBS which are attached on the stromal side of the thylakoid membrane and are associated with PS2 (Gantt, 1982). They consist of phycobiliproteins - proteins which bind the bilin

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pigments, and linker proteins which are required for both assembly and integrity of the PBS (Glazer, 1984). One complex can have a mass in excess of one million daltons. As well the ultrastructure of the membranes are different, membrane stacking and lateral inhomogeneities are not seen in the PBS-containing organisms. This could well be due to the difference in the antenna between the two types of organisms.

As Biggins and Bruce (1989) have pointed out, three questions must be answered to elucidate the mechanism of the state transition. These are: 1) The changes in the pathway of excitation energy transfer from the antenna to the two photosystems; 2) The physical changes in the photosynthetic apparatus responsible for controlling the pathway of excitation energy transfer; and 3) The mechanism responsible for sensing the imbalance of the activities of PS2 and PS1 to trigger the state transition. Three models which have been proposed for the state transition in the PBS-containing organisms will be discussed below including a critical examination of the way in which they describe these three criteria.

Structurally, all three models essentially agree on the photosystem and antenna configuration in state 1, this is shown in Figure 3. The PBS transfers energy directly to PS2 and the spillover of energy from PS2 to PS1 is at a minimum. However, in all three models a different situation is envisioned for state 2. The first model, the **spillover model**, originally proposed twenty years ago (Murata, 1969) and again more recently (Ley and Butler, 1977,1980; Biggins *et al.*, 1984a&b; Biggins and Bruce, 1989; Brimble and Bruce, 1989; Bruce *et al.*, 1989a,b), is shown in Figure 3a. In state 2 the

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Figure 3. Proposed models for the state transition in PBS-containing organisms.

STATE 1
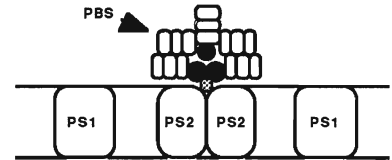
STATE 2

a) Spillover Model
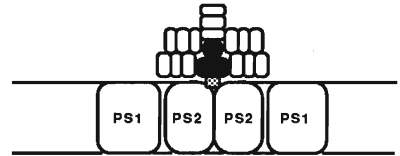
b) Mobile PBS Model
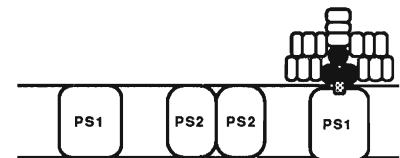
a) PBS Dissociation Model
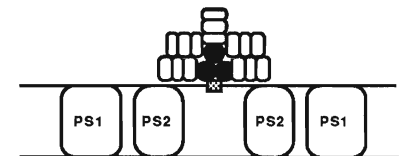
amount of energy which reaches PS1 via PS2 is increased through a change in the distance and/or orientation between PS2 and PS1. As the probability of energy transfer between the two is a function of the distance between the two pigments raised to the sixth power, small conformational changes can result in large changes in energy spillover from PS2 to PS1. This model predicts that there should be complementary changes in the PS2 and PS1 activity absorbance cross-sections with the state transition. In other words, on transition to state 2 when the PBS and chl a absorption contributions to PS2 decrease there should be a complementary increase in these contributions to PS1. In this model the PBS and PS2 do not move independently, thus there is no rerouting of energy between the PBS and PS2. The re-routing occurs at the level of the PS2 Chl a.

The second model, the **mobile antenna model**, is analogous to the model envisioned in higher plants and is shown in Figure 3b. In state 2, the PBS detaches from PS2, relocating on the thylakoid membrane to become associated with PS1 (Allen *et al.*, 1985). This means that energy initially absorbed by the PBS will now be directly transferred to PS1 whereas energy absorbed initially by PS2 Chl a will not have an increased probability of reaching PS1. This model also predicts complementary changes in the photosystem activity absorption cross-sections, however this will only be observed upon PBS excitation. This model has been retracted recently (Williams and Allen, 1987), primarily because the observed changes in the absorption cross-sections include Chl a as well as the PBS as assayed by both room temperature and low-temperature fluorescence

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emission (Murata, 1969; Dominy and Williams, 1987; Brimble and Bruce, 1989).

The third model, the **PBS dissociation model,** is a more complicated model and is in fact a photoprotective model. This is illustrated in Figure 3c. In state 2 the PBS detaches from PS2 (presumably remaining associated with the membrane) and allows PS2 to move closer to PS1, increasing the amount of energy spillover from PS2 to PS1 (Mullineaux and Allen, 1988). This model does not predict complementary changes in the absorption cross-sections of the two photosystems. Upon transition to state 2 the PBS contributions to PS2 and PS1 should both decrease due to the PBS decoupling. Complementary changes will occur however, if Chl $\underline{a}$ is excited. While fluorescence data may not yet discriminate conclusively between the spillover and dissociation models there is no evidence to indicate an increased yield of PBS fluorescence in state 2 as the dissociation model would predict. While the authors of the model have suggested the existence of a species which quenches the fluorescence, its existence or identity has not been demonstrated.

The third criterion, that is, what actually does trigger the state transition has also been outlined in all three models. In higher plants it is thought that the redox state of plastoquinone is the trigger. How this actually activates the kinase is not yet known. In both the PBS dissociation and mobile PBS models a similar situation in envisioned. A kinase is activated when the PQ pool becomes reduced, as a result two proteins become phosphorylated, a 15 kD protein associated with PS2 and an 18.5 kD protein from the PBS (Allen *et al.*, 1985; Sanders et al, 1986; Sanders and Allen, 1987). As

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a result of the negative charges introduced there is mutual repulsion between the two species, causing the PBS to relocate in one model and the PS2 to relocate in the other.

A sketchy picture of a possible trigger for the conformational change has been proposed in favour of the spillover model (Biggins *et al.*, 1984a; Biggins and Bruce, 1989). Based on the fact that transition to state 1 requires coupled PS1 electron transport and full conversion requires only 15 turnovers of PS1 corresponding to the formation of the transmembrane proton gradient (Graan and Ort, 1982; Biggins and Bruce, 1985) they suggested that the proton gradient may be localized, causing a charge differential between PS1 and PS2. This could then result in a change in the distance between the two photosystems via electrostatic interactions, changing the probability of energy spillover. No hard evidence exists however to support such a theory. It is interesting to note that Duysens (1972) had suggested that the redox state of some electron transport component may trigger the state transition phenomenon while Ley and Butler (1980) suggested a cooperative effect of both the redox state of electron transport components and ion gradients around either photosystem.

A question which has been raised (Biggins and Bruce, 1989; Bruce *et al.*, 1989) is the use of room temperature millisecond fluorescence induction kinetics to measure changes in spillover and direct absorption cross-section (Mullineaux *et al.*, 1986; Mullineaux and Allen, 1988). Butler (1978) assumed that changes in both F_o and F_m would indicate spillover from PS2 antenna Chl <u>a</u> to PS1 whereas changes in F_m only would indicate spillover from the PS2 reaction

centre to PS1, a less likely situation. F_o represents the condition when cells are first illuminated after being in the dark for some time. The PQ pool is completely oxidized due to the absence of PS2 activity, thus when the light is turned on the PQ pool becomes a sink for the energy absorbed by PS2. It acts as a fluorescence quencher because PS2 can use all of the energy it initially receives, thus the fluorescence is at a minimum. As the PQ pool fills up PS2 cannot maintain its high activity. As a result the energy is transferred back to the Chl $\underline{a}$ antenna or never visits the reaction centre at all. The energy is then emitted from the antenna as fluorescence. This fluorescence reaches a maximum level and is called F_m (see previous section).

As cells effect a transition from state 1 to 2, both F_o and F_m are observed to go down in PS2 (Mullineaux *et al.*, 1986). While it has been suggested that this in fact indicates a change in direct absorption cross-section it is apparent that the initial exciton density will change whether the energy is transferred from PS2 antenna chl $\underline{a}$ to PS1 or from the PBS to PS1. In either case the excitation energy has been re-routed before it can reach the PS2 reaction centre, decreasing both F_o and F_m. Changes in F_m only would indicate that spillover occurred from the reaction centre to PS1, which seems more unlikely than spillover from antenna Chl $\underline{a}$. Thus, analysis of these measurements are not sufficient to distinguish between changes in direct absorption cross-section and spillover. To do so requires kinetic analysis of PS1 as well; this can be done either at 77K or using picosecond resolution spectroscopy (Holzwarth, 1986).

Electron microscopic data (Olive *et al.*, 1986) indicates that in state 1 EF particles tend to be situated in rows whereas in state 2 they appear to be dispersed more randomly through the membrane. Morschel and Schatz (1987) have shown in fact that these EF particles are PS2 dimers. This suggests that the PS2 centres are further away from the PS1 centres in state 1 than in state 2. An important piece of information which is still lacking however is what happens to the PBS during these transitions. The spillover model would suggest that the PBS remain attached to PS2, thus in state 1 the PBS would be arranged in rows. The PBS dissociation model suggests that PS2 moves independently of the PBS, thus the PBS arrangement is not required to change significantly in either state. Such data would have to be obtained using the freeze-etching technique which allows one to look at the surface of the membranes where the PBS are located (Branton, 1966).

There is other evidence to indicate that structural changes do occur during the state transition. In a series of elegant experiments using the thermophilic cyanobacterium Synechococcus lividus (Fork and Satoh, 1983) cells were cooled to room temperature after the state transition had been induced at the higher growth temperature. As a result the state transition was inhibited in both directions, presumably because the lipid bilayer was in the gel phase, negating protein movement through the membrane which would accompany the state transition.

Other evidence has been presented suggesting that conformational changes in the thylakoid membrane accompany the state transition. Glutaraldehyde, the bifunctional cross-linker, was

found to literally fix the cells in one state or another depending upon the pre-illumination conditions (Biggins, 1983; Bruce *et al.*, 1985; Bruce and Biggins, 1985; Brimble and Bruce, 1989). This again suggests conformational changes in the protein arrangement of the thylakoid membrane are required for the state transition to occur.

A more critical examination of possible conformational changes accompanying the state transition was performed using the spectroscopic technique linear dichroism (Bruce and Biggins, 1985). Linear dichroism allows one to measure the orientation of specific pigments, pigments which are associated intimately with specific proteins. They found that upon transition from state 1 to 2 there was a single change at 656 nm shown to originate from the allophycocyanin (APC) core in the PBS. A subsequent study using both Synechococcus 6301 and Porphyridium cruentum (Brimble and Bruce, 1989) has shown that this change is a result of APC orientation with respect to the whole cell rather than the thylakoid membrane. They also showed a small shift in the Chl $\underline{a}$ Q_y transition which was not dependent upon the integrity of the cell. This was thought to reflect either a reorientation of Chl $\underline{a}$ or a state-dependent change in the light-scattering properties of the PBS/thylakoid membrane fragments.

Electron Transport Measurements

While fluorescence is the common assay for the light state transition, others are available. Perhaps the most important are the electron transport properties of PS1 and PS2. Increases in PS1 turnover of 30-100% have been shown to occur upon transition to

state 2 (Fork and Satoh, 1983; Satoh and Fork, 1983; Biggins, 1983; Biggins *et al.*, 1984b). These results are rather dramatic and are consistent with the changes in fluorescence emission observed upon the state transition.

A problem with many of the electron transport measurements are that they reflect steady-state conditions. In a typical experiment designed to measure P-700 redox changes, the sample is manipulated into either state 1 or 2 and then the sample is illuminated with PBS light. PS2 activity should result in reduction of the P-700 whereas PS1 turnover should result in the oxidation of P-700. Under constant illumination a competition occurs between PS1 and PS2, resulting in a steady state redox level for the P-700. Initially PS1 will oxidize P-700 but PS2 and respiratory activity will begin to reduce it. While P-700 will ultimately become more oxidized full oxidation will not be reached as long as there is PS2 or respiratory activity. While PS2 activity can be eliminated in the presence of DCMU respiratory electron transport will still reduce P-700. DCMU is a potent inhibitor of the light-induced transition to state 2. The solution to the problem is to use single turnover flashes which turn the centres over once and oxidize P-700 on a much faster time scale than the subsequent re-reduction. The extent of this oxidation using PBS light should be greatest in state 2 according to the spillover model as presumably more energy is being transferred from PS2 to PS1, the oxidizer. The detached model would predict P-700 turnover to go down in state 2 as less energy initially absorbed by the PBS is reaching both PS1 and PS2. Such work has yet to be done but is critical if the correct model is to be unequivocally proven.

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It would be interesting to design this experiment such that actual absorbance cross-section values could be calculated as has been done for PS2 (Ley, 1984).

Phosphorylation

Perhaps one of the most common post-translational protein modifications, phosphorylation is studied in a variety of different systems in both the animal and plant kingdoms. In the simplest reversible model, two steps are required. These are:

protein + nATP -> protein-Pn + nADP -> nPi + protein + ADP.
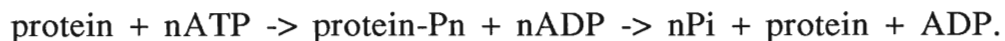
The first reaction requires a protein kinase as a catalyst while the second hydrolysis reaction requires a phosphoprotein phosphatase (Ranjeva and Boudet, 1987). This reversible system is the basis of a cascade system (Chock *et al.*, 1980) and is the foundation of many mechanisms including signal transmission and enzyme regulation. Such a mechanism has been studied by a great number of researchers recently with the discovery of phosphoproteins in thylakoid membranes of higher plants (Bennett, 1977). There appears to be more than one kinase involved and all are light-dependent.

The most widely studied of the phosphoproteins are the 25 kD components of the light-harvesting complex containing Chl $\underline{a}$ and $\underline{b}$. The majority of these complexes are associated with PS2 and are commonly referred to as LHCP2. The kinase involved appears to be light-dependent and is mediated by the redox state of the PQ pool which obviously depends on the light-driven electron transport

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through the pool. The kinase becomes activated when the pool becomes reduced (Horton *et al.*, 1981). The proposed kinase has been isolated and has an apparent molecular weight of 64 kD. The phosphatase responsible for dephosphorylating the LHCP2 is bound to the thylakoid membrane and is light-insensitive (Bennett *et al.*, 1980). When the complex is dephosphorylated it is associated with PS2 and thus transfers the harvested energy preferentially to PS2. Upon phosphorylation, induced by high PS2 activity which causes the PQ pool to become reduced, the LHCP2 move away from the appressed regions rich in PS2 to the stromal regions rich in PS1. This movement may result from the negative surface charges introduced by the phosphate group and presumably allows the LHCP2 to transfer energy more effectively to PS1, correcting the previous imbalance in photosystem activity (Barber, 1982).

This rather unique way in which to regulate the distribution of excitation energy between PS1 and PS2 is not universally accepted, primarily due to the lack of good evidence that phosphorylation increases the absorption cross-section of PS1 (see section on **State Transitions**). Structural support for this model is provided by subfractionation of the thylakoid membrane which shows that a special pool of LHCP2 dissociates from PS2 upon phosphorylation to the unappressed regions rich in PS1 (Kyle *et al.*, 1983). While it is agreed that upon phosphorylation the antenna size of PS2 decreases due to the migration of the LHCP2 there is little evidence of a concomitant increase in the antenna size of PS1. Larsson *et al.* (1986) showed a low efficiency of interaction between the LHCP2 and PS1 while Haworth and Melis (1983) could not detect any

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changes in the absorption cross-section of PS1 measured by kinetic measurements of P-700 photooxidation. This has been recently supported by Allen and Melis (1988) in green algae as well. Light-induced redox changes in cyt $\underline{f}$ in phosphorylated chloroplasts have also been studied (Horton and Black, 1981). Their results appeared to indicate that phosphorylation does not increase the amount of energy transferred from LHCP2 to PS1 but decreases the amount of energy getting from the LHCP2 to PS2. Another viewpoint which has been derived from time-resolved fluorescence spectroscopy suggests that the LHCP2 moves into the unappressed regions and becomes associated with the ß PS2 centres present there. These PS2 centres are in turn, closer to PS1, allowing more of the energy to reach PS1 (Wendler and Holzwarth, 1987). While the original mobile antenna hypothesis is based on a change in the direct absorption cross-section of the photosystems this model is based on a change in energy spillover.

There have been many reports of other effects phosphorylation has on thylakoid membranes. Packham (1988) has proposed that the 9 kD phosphoprotein (phosphorylated when PQ reduced) may affect electron transfer from Q_A to Q_B by altering the Q_B pocket. When PQ is reduced the protein becomes phosphorylated, introducing negative surface charge in such a way as to change the conformation of the site, impeding PQ binding. This may stimulate cyclic electron around PS2 (Horton and Lee, 1983). While this 9 kD protein has not been identified Packham suggests from sequencing data available that it may be related to the H subunit of photosynthetic bacteria. The obvious but unproven conclusion is that this phosphoprotein

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functions as protection from photoinhibition during times of high-light intensity.

A number of other effects of phosphorylation in the thylakoid membrane have also been characterized. Jursinic and Kyle (1983) have shown an enhanced stability of the bound Q_B^- at the two electron gate of PS2 upon phosphorylation. They suggest this is due either to a surface charge effect that increases the ability of an endogenous reductant to interact with Q_B, or to the promotion of cyclic electron transport around PS2 involving Q_B^-. This is supported by Horton and Lee (1983) who showed that phosphorylation stimulated an hydroxylamine-sensitive cyclic electron-transfer pathway around PS2. It has been shown that phosphorylation attenuates the rate of electron transfer from H_2O to DCPIP at saturating light intensities (Hodges et al., 1985). This may reflect an effect on electron transfer from Q_A to Q_B. The question in all of these studies that has not been clearly defined is the physiological significance of these effects.

Many detailed studies have been done on phosphorylation both in vivo and in vitro using green alga. Using the green alga Chlamydomonas reinhardtii Owens and Ohad (1982) found that thylakoids possessed protein kinase activity which was stimulated in the light. The membranes also had phosphatase activity as was found in vivo. What was interesting and in contradiction to experiments with higher plant chloroplasts was that kinase activity was present in the dark in vivo. In keeping with the idea that the redox state of the PQ pool was the trigger for the state transition

they suggested that in the dark whole cells can maintain the photosynthetic transport chain more reduced than in vitro due to the presence of an endogenous reductant which is lost in the thylakoid isolation procedure. They could not find any differences in phosphorylation patterns upon illumination of intact cells in far red light and dark-adapted cells. However, no fluorescence data was shown to indicate that the state transition did occur. In a subsequent study (Wollman and Delepelaire, 1984) it was shown that differences could be seen in vivo if the treatments to induce the state transition were more drastic. It must be kept in mind, however, that under normal physiological conditions the cell will not be subject to DCMU or glucose oxidase treatment.

Another series of experiments were performed on C. reinhardtii by Delepelaire and Wollman (1985). They examined the kinetics of the phosphorylation/dephosphorylation versus the fluorescence changes upon the state transition and found that the half-times for phosphorylation and dephosphorylation of two LHC components was 330 and 160 seconds while the corresponding fluorescence changes had half times of 320 and 120 seconds. In an attempt to determine what the rate-limiting step was for the state transition they proposed six steps in the state transition. These are: (1) change in external redox potential; (2) reduction/oxidation of the PQ pool; (3) activation/deactivation of the kinase; (4) phosphorylation/ dephosphorylation of the LHC; (5) migration of the LHC; and (6) connection of the LHC with PS2/PS1. While the first step is environmentally controlled the second step is the cell's first response. This occurs within a few seconds and cannot be the rate-

limiting step. Somewhat surprisingly, the rate-limiting step cannot be the migration of the LHC either as the kinetics of the state 2 transition are independent of where the state 1 transition is interrupted. In other words, regardless of the distance between the LHC and PS1 the kinetics are the same. Activation or deactivation of the kinase is presumed to involve a change in the redox state of PQ. This suggests that the kinetics of the state transition should be dependent on the amplitude of the redox change if this was the rate-limiting step. This is not the case. Since the changes in phosphorylation and fluorescence have the same kinetics the rate-limiting step is likely to be the catalytic activities of the enzymes themselves.

To further understand phosphorylation in green algae experiments were done on several mutants lacking PS2, the LHC, and the cyt b_6/f complex (de Vitry and Wollman, 1988; Wollman and Lemaire, 1988). Using mutants lacking PS2 reaction centres it was found that LHC phosphorylation did not require the presence of PS2. Phosphorylation of the PS2 polypeptides required the presence of the LHC. A mutant lacking Chl b yet still containing some LHC apoproteins showed characteristic changes in the phosphorylation patterns of these proteins consistent with a state transition. This is unlike the situation observed in higher plant Chl b mutants which did not do state transitions (Haworth *et al.*, 1982). One possible explanation for this is that two phosphoproteins of 33 and 35kD which bind Chl a in green algae are not found in higher plants. These proteins could function as the mobile antenna.

Another C. reinhardtii mutant used in this study, BF4 contained almost no LHC apoproteins and in fact showed almost no kinase activity and no state transitions could be induced. The absence of phosphoproteins in PS2 as well as the LHC suggests that the presence of the LHC-PS2 complex is necessary for phosphorylation of PS2 proteins. This is supported by the fact that a 64 kD kinase copurifies with the LHC (Coughlan and Hind, 1987).

Another study on mutants of C. reinhardtii attempted to determine a possible explanation for the proposed PQ redox control of phosphorylation (Wollman and Lemaire, 1988). The authors proposed that a possible association between the kinase and an integral membrane protein containing PQ-binding sites, with different affinities depending upon the redox state of the molecule would elicit an interaction between the kinase catalytic activity and the regulation site. It was found that mutants lacking the D1 and D2 proteins of PS2 had no affect on the LHC phosphorylation or state transition. A theory proposed by Allen et al. (1987) suggested that the 9-10 kD phosphoprotein of PS2 could function in introducing negative charges to PS2 which would induce repulsion between both the LHC and PS2. However Wollman and Lemaire (1988) showed that the absence of this polypeptide had no affect on the state transition.

Mutants which contained no cyt b_6/f complexes still contained PS2 phosphoproteins but the activity was not PQ-dependent. The absence of both LHC phosphorylation and the state transition suggests that either the absence of an LHC-kinase or the regulation site causes inhibition of the transition. This site may be the binding

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site of PQH_2 on the cyt b_6/f complex. This would provide an easy way in which to explain the apparent PQ redox control of kinase activity. The inhibitor DBMIB is known to induce state 2, it may do this by binding to the site PQH_2 normally would, activating the kinase.

Few investigations on protein phosphorylation in bilin-containing organisms have been carried out to this point, primarily due to the lack of an _in vitro_ preparation which undergoes state transitions. Biggins _et al._ (1984a) did not find any changes in the whole cell phosphorylation profile of P. cruentum in state 1 and state 2. They concluded from these results that protein phosphorylation did not play a role in the mechanism of the state transition in this organism. Schuster _et al._ (1984) could find no light-dependent phosphorylation in the cyanobacterium Fremyella diplosiphon. They found a light-independent phosphorylation of three thylakoid polypeptides of molecular weight 14.5kD and a doublet of 21kD.

In contrast to these studies, the light-dependent phosphorylation of two polypeptides were reported in the cyanobacterium Synechococcus 6301 (Allen _et al._, 1985). The two polypeptides were of molecular weights 15kD and 18.5kD and were identified as PS2 and PBS components respectively. While the proposed PS2 component was indeed shown to be a membrane protein (Allen _et al._, 1985) there is no evidence to indicate that it is a PS2 component. The other phosphoprotein, the 18.5kD species, was later shown to be a PBS component (Sanders and Allen, 1987). In the first study an _in vivo_ analysis indicated the phosphorylation of the two proteins in the light with considerably lower levels occurring

in the dark, indicating a light-dependent process. In this same study an <u>in vitro</u> analysis was also done with thylakoid membranes which, as might be expected, showed only the light-dependent phosphorylation of the 15kD species. Despite the absence of the 18.5kD polypeptide the state transition mechanism was still apparently intact as indicated by fluorescence measurements.

Based on these results a model was proposed in which the PBS was assumed to have the capacity to relocate on the membrane and become associated with PS2 in state 1 and PS1 in state 2. Upon transition to state 2 the PBS and PS2 components would become phosphorylated and the mutual repulsion caused by the negative charges on each of the two phosphorylated polypeptides would result in the PBS becoming detached from PS2 to become associated with PS1. Dephosphorylation would cause the PBS to return to associate with PS2. While there are several problems with such a model, some immediate inconsistencies within the model's authors' data are apparent. Firstly, as already mentioned, the mechanism is apparently intact in the absence of the PBS in the <u>in vitro</u> studies performed in this study (Allen *et al.*, 1985). The absence of the 18.5kD would mean that no mutual repulsion between the two phosphoproteins could occur. Secondly, the light-dependent phosphorylation would indicate that the phosphorylated species exist in state 1 and not state 2 as suggested in this study. As already mentioned it has been clearly shown that the dark state represents state 2 (Mullineaux and Allen, 1986; Olive *et al.*, 1986; Brimble and Bruce, 1989).

In the subsequent study (Sanders and Allen, 1987) it was shown that the 18.5kD polypeptide was phosphorylated in the presence of light 2 but not light 1. This shows a wavelength-dependent phosphorylation of this protein but, unfortunately, is not consistent with the earlier data which showed the protein to be unphosphorylated in the dark (state 2). This should indicate that the phenomenon observed, while interesting, is independent of the state transition. An alternate explanation does exist, however. The dark state induced in whole cells in the earlier study was carried out for 15 hours whereas the state transition is known to occur in considerably faster time than this (Ried and Reinhardt, 1980; Biggins and Bruce, 1985). Prolonged dark treatment may result in the turning off of specific kinases whose function may not be necessary after prolonged dark-adaption, perhaps an energy-conserving process. While fluorescence emission spectra of light and dark-adapted cells indicate the state transition still occurred, samples used for fluorescence measurements had only been light or dark-adapted for 10 minutes. It may be that the state transition would not be apparent in the cells which had been dark-adapted for 15 hours which were used in the phosphorylation analysis. It is clear however that this issue remains to be completely resolved. It is interesting in this context to note that after 21 hours of dark treatment followed by a brief period in the light that the subsequent transition to state 2 in the dark was inhibited (Mullineaux and Allen, 1986).

As previously mentioned, the major hindrance to these studies is the lack of an _in vitro_ preparation and the relatively fast kinetics of the state transition. It is interesting to note that Delepelaire and

Wollman (1985) found the rate limiting step in the state transition in green algae to be the actual enzymatic reactions themselves. The considerably faster state transitions in bilin-containing organisms suggests it is unlikely that phosphorylation could be responsible for the state transition in these organisms.

MATERIALS AND METHODS

Description of Wild-type and PBS-less Mutant

Synechococcus sp. PCC 7002 (formerly Agmenellum quadruplicatum strain PR-6) is a transformable, marine, unicellular cyanobacterium (Stevens and Porter, 1980; Rippka et al, 1979). The wild-type organism produces PBS comprised of phycocyanin (PC) and allophycocyanin (APC) (Bryant, 1988). The mutant was graciously provided by Don Bryant of Penn State. This mutant is devoid of PBS and detectable phycobiliproteins, as assayed by fluorescence, and was a spontaneous secondary mutant derived from a constructed mutation. The primary mutant carried a deletion of the apcA and apcB genes, which encode the $\propto$ and ß subunits of APC, respectively (ΔapcAB); these genes were replaced by a DNA fragment encoding the neo gene of Tn5 which confers resistance to neomycin/kanamycin (Bryant, 1988). The secondary mutant (ΔapcABcpc-) also does not express the cpc genes encoding the and ß subunits of PC.

Growth Conditions

The wild-type was grown autotrophically at 30 C on A$^+$ medium (composition shown in Appendix B) (Stevens and Porter, 1980) at a light intensity of 25 $\mu E \cdot m^{-2} \cdot s^{-1}$. The mutant was grown photoheterotrophically on A$^+$ medium supplemented with 5 mM glycerol under the same conditions as above; however the mutant was photosynthetically competent and could be grown

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autotrophically although growth was considerably slower than observed in the wild-type.

Harvesting

Both wild-type and mutant cells were harvested during exponential phase by centrifugation (3,000g), washed and resuspended in growth medium to an optical density of less than 0.1 to avoid self-absorption artifacts in the fluorescence determinations. Typically 25 ml of cells were centrifuged and the resuspended sample was kept at 30 C under room light until used for the experiments.

Induction of State Transitions

In all experiments cells were brought to state 1 or state 2 at their growth temperature of 30 C. The light-induced transitions were accomplished by a 2 minute illumination of a 75 μl algal sample in a capillary cuvette which was then quickly frozen in liquid nitrogen. The wild-type was brought to state 1 by illumination with blue light (Ditric optics 460 nm short pass filter) at 350 $\mu E \cdot m^{-2} \cdot s^{-1}$ and state 2 by illumination with orange light (combination of a Ditric optics 600 nm short pass filter and a 580 nm long pass glass colour filter) at 350 $\mu E \cdot m^{-2} \cdot s^{-1}$. The mutant was brought to state 1 with either 700 nm light at 100 $\mu E \ m^{-2} \ s^{-1}$ or 520 nm illumination at 50 $\mu E \cdot m^{-2} \cdot s^{-1}$; state 2 was induced with 435 nm at 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ or 670 nm illumination at 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. These illumination wavelengths were dispersed by a Jobin Yvon H20 monochromator (10 nm bandpass). Alternatively, state transitions were induced in cells treated with 1 μM DCMU. These experiments were conducted with

stirred 10 ml samples from which 75 μl aliquots were withdrawn into capillary tubes and quickly frozen. Samples were withdrawn sequentially after a 2 minute dark adaptation, 2 minutes after addition of DCMU in the dark, 2 minutes after illumination (Ditric optics 460 nm short pass filter, 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) in the presence of DCMU, and 2 minutes after a second dark adaptation. In a second experiment this protocol was repeated with an additional step, a 2 minute incubation of the dark-adapted cells with 1 mM KCN prior to the addition of DCMU.

In one control experiment a 10 ml sample of cells was divided into two 5 ml samples. To the first was added 100 μL of 100% ethanol while the other sample served as a control. This received 100 μL of A^+ medium. A 75 μL sample was taken from both for low temperature fluorescence emission.

In another control experiment, cells were subject to the following conditions. A 10 ml sample of cells was incubated in the dark for two minutes before glutaraldehyde (Sigma) was added to a final concentration of 0.5% as described previously (Biggins, 1983). Cells were diluted with 20 ml of A^+ medium, centrifuged and resuspended in A^+ medium to the original volume. A sample was then removed and frozen as described above. A 1 ml sample of this was also removed and treated with 10 μM DCMU in the light. A 75 μL sample was removed and frozen. A separate sample of cells (10 ml) was incubated in the light in the presence of 1 μM DCMU. After two minutes the cells were fixed with 0.5% glutaraldehyde and diluted, centrifuged and resuspended as described above. A sample of this was frozen for fluorescence emission measurements.

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Cytochrome f and P-700 Redox Kinetics

To measure cytochrome f redox kinetics a single beam spectrometer was designed and constructed. Light from a 50 W tungsten halogen lamp was passed through a Bentham optical chopper before being dispersed through a Jarrel Ash 1/4m spectrometer. This 420 nm light was brought at right angles to the sample via a fibre optic cable and picked up on the other side of the 1 cm cuvette with another fibre optic cable. This was placed in front of an Hammamatsu R928 end on photomultiplier tube in a mu metal shield with a 420 nm interference filter in front of it. The oscillating signal was measured with a Standard Research Systems lock-in amplifier and stored on a Norland IQ 400 digital storage signal averager. Excitation light was provided by a 100 W tungsten halogen lamp and passed through the appropriate filter or filter combination. The light passed through a Uniblitz electronic shutter before it was brought to the sample at right angles to the actinic beam via a fibre optic cable.

P-700 kinetics were measured with the same apparatus as described above with the following changes. Instead of the photomultiplier tube a UDT 555 photodiode was used and a 700 nm filter was placed in front of it.

State Transition Kinetics

State transitions were induced as described above. To measure the kinetics of the state 1 transition cells were placed in the dark in the presence of DCMU for three minutes. A 50 μL sample was quickly withdrawn and frozen. The cells were then illuminated with 680 nm light (see above) and 50 μL samples withdrawn at specific time intervals. After three minutes the light was then turned off and the experiment repeated. Low temperature fluorescence measurements were then taken and the ratio of the 695 nm peak to the 715 nm peak was measured for each time interval. The average of the two experiments was taken and the data averaged with three other experiments performed on different cultures of cells. State 2 transition kinetics were measured as above, however, the cells were first incubated in the presence of DCMU and 680 nm light for two minutes, a sample removed, and then the light turned off. Samples were again taken at specific intervals for three minutes. A total of five separate experiments were performed.

Low Temperature Fluorescence Measurements

Corrected 77K fluorescence excitation spectra were determined with a SPEX Industries Inc. (Metuchen, NJ) 1680 spectrofluorimeter using a 2.0 nm bandwidth as described previously (Biggins, 1983). Corrected 77K fluorescence emission spectra were obtained with a spectrofluorimeter based on an Jarrel Ash 1/4 meter spectrograph and EG&G diode array detector (1420R) controlled by an EG&G detector interface (1461) accessed by an IBM AT compatible computer as described previously (Brimble and Bruce, 1989; Bruce *et*

al., 1989a). Excitation light (10 nm bandwidth) was supplied by a 100 W tungsten halogen lamp dispersed by a Jobin Yvon H20 spectrometer.

Absorbance

Room temperature absorption measurements were made with an Aminco DW-2 spectrometer which was interfaced to an IBM AT compatible computer by OLIS Inc. (Georgia).

Solubilization Procedures

Whole Cells

A simple procedure was developed for the solubilization of whole cells which was an improvement on a previously described technique (Biggins *et al.*, 1984a). Initially 200 mL of the primary mutant ΔapcAB was harvested and resuspended into 100 mL of a buffer containing 10 mM Hepes (pH 7.4), 5 mM NaCl, and 2 mM EDTA. The sample was stirred while 300 mL of acetone was added to the suspension. After 5 minutes this suspension was centrifuged and resuspended into 2ml of the buffer after 2 washes of 80% acetone. The sample was then divided into two 1 mL samples. The first sample served as a control while the second was incubated at 30°C in 0.1% lysozyme for thirty minutes. The two samples were then centrifuged and resuspended again in the buffer to a final volume of 1 ml each. The two samples were again washed in 80% acetone, centrifuged and resuspended to the original volume. They were then

solubilized, centrifuged and equal amounts of the supernatant loaded onto a gel (see below). Examination of cells acetone treated and acetone/lysozyme treated under the microscope revealed that while the former cells were still intact the majority of the latter were broken. This suggests that simple acetone treatment does not cause the cell to release all of its protein.

Protein Solubilization for 1-d Electrophoresis

Samples were diluted 1:4 in Laemmli buffer (0.063 M Tris pH 6.8,10% glycerol (v/v), 2% SDS (w/v), 5% ß-mercaptoethanol (v/v), and 0.0025% bromophenol blue (w/v)) and heated at 95 C for 4 minutes after solubilizing for 1 hour (Laemmli, 1970).

Gel Electrophoresis

One dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (Laemmli, 1970) using a 0.75 mm width 4% stacking gel and an 11% separating gel using a Bio-Rad Mini-Protean II apparatus. Polymerization was carried out using an acrylamide/bis-acrylamide ratio of 36.5, an ammonia persulfate concentration of 0.05%, and a TEMED concentration of 0.1% in the stacking gel and 0.05% in the separating gel. Samples were loaded onto the stacking gel and electrophoresed in a running buffer (125 mM Tris, 960 mM glycine, 17 mM SDS, pH 8.3; diluted 1:4 with dH_2O) at 200V until the dye front reached the bottom of the gel.

Two-dimensional gel electrophoresis was performed essentially as described in Bulletin 1144 from Bio-Rad. Cells were treated as

described above, however the lysozyme concentration was increased to 0.2% and treatment was carried out for 15 minutes at 30C. Cells were solubilized in the following solution at a 4:1 dilution: 16.7% (v/v) of 10% (w/v) SDS, 6.7% (v/v) of ampholytes 6-8, 26.7% (v/v) of ampholytes 3.5-10, 16.7% 2-mercaptoethanol, and 33.3% Triton X-100. Gels were cast in 1.2 mm diameter tubes to a length of 6 cm. The gel mixture contained 7.35 M urea, 26% dH_2O (v/v), 3.2% acrylamide (w/v) (acrylamide/bis-acrylamide ratio of 36.5), 1.85% Triton X-100 (v/v), 3.6% 3.5-10 ampholytes, and 0.9% 5-7 ampholytes (v/v) (Bio-Rad or LKB). After polymerization the samples were placed on top of the gels and the tubes and upper chamber filled with 0.1N NaOH. The bottom chamber was filled up to the top of the gel with 0.06% H_3PO_4. The gels were then electrophoresed at 400V for fifteen hours and 800V for two hours. The tubes were then placed on ice for ten minutes before extruding the gels into a reducing buffer (12.5% 0.5 M Tris (v/v), pH 6.8, 5% B-mercaptoethanol (v/v), 30% (v/v) of 10% SDS (w/v), 0.01% bromophenol blue (w/v), and 52% dH_2O) for twenty minutes. The gels were then placed carefully on top of a 11% separating and 4% stacking 1.5 mm wide slab gel. Electrophoresis was then carried out as described above.

Radioactive Labelling

200 ml of cells were grown to log phase and 1 ml was removed and analyzed for the ability to perform state transitions in the

presence of DCMU as described above. If the cells were state transition competent the remaining cells were centrifuged and resuspended in 10 ml of phosphate depleted A+ medium. Cells were incubated for six hours at 30C before again being removed. 400 μCi of $KH_2{}^{32}PO_4$ was added to the cells and they were incubated at 30C for fifteen hours. Ten hours before harvesting a further 50 μL of 75 mM KH_2PO_4 was added to the cells. The cells were then centrifuged and resuspended in 10 ml of A+ medium containing 100 μL of 1 mM DCMU. The sample was then divided into two 5 ml volumes. After stirring the first sample under white light for 2 minutes a 50 μL sample was removed and rapidly frozen in liquid nitrogen. A 50 μL volume of 1 M NaF was then added to the remaining cells followed immediately by 20 ml of ice-cold 100% acetone. The suspension was allowed to stir for 2 minutes, centrifuged and resuspended in 5 ml of10 mM Tris buffer (pH 7.8) containing 10 μM PMSF, 10 mM NaCl, 5 mM $MgCl_2$, and 2 mM EDTA. This 'state 1' sample was kept on ice until further treatment. Essentially the same treatment was performed on the other 5 ml sample ('state 2' sample) with the exception that the cell was pre-treated for three minutes in the dark rather than the two minute light treatment.

The two samples were then incubated on a rotary shaker at 30C for thirty minutes in the presence of 0.1% lysozyme. The two samples were then centrifuged and resuspended in 10 ml of ice-cold acetone. The samples were homogenized and recentrifuged. The pellets were resuspended in 500 μL of Tris buffer and kept on ice until ready for electrophoresis. Both two-dimensional and one-dimensional gels were performed as described above, 440 μL of the

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sample was used for two-dimensional gels and 50 µL for one-dimensional gels..

Radioactive Determination

From each of the two samples 5 uL was removed and placed in separate Eppendorf tubes. To each of these was added 50 µL of 1 mg/ml BSA and 50 µL of 75 mM KH_2PO_4. To this was added 1 ml of 20% TCA, the tubes mixed by inversion and the samples kept on ice for thirty minutes. The samples were then transferred to a Whatman GF/C glass filter. The tubes were washed twice with ice-cold 8% TCA and the wash added to the respective filters. Another 10 ml of ice-cold 8% TCA was added directly to the filters followed by 10 ml of ice-cold 95% ethanol. After drying the filters were removed and further dried under an infrared lamp. The filter paper for each sample was then placed in the bottom of a scintillation vial and 8 ml of ICN Universol scintillation cocktail was added to each. As a control another 8 ml of the cocktail was added to a vial with no filter paper. This was considered to be background radiation. The vials were then placed in a Beckman LS 1800 liquid scintillation counter and the radioactivity counted for five minutes for each sample.

Protein Assay

Another 5 µL was removed from each of the two samples and placed in 2.5 ml of 20% Bio-Rad Bradford reagent. These were mixed

and allowed to sit for ten minutes before the optical density was measured for each sample at 595 nm. A standard curve was also produced by using successive dilutions of a 1 mg/ml BSA solution and performing the same description described above for each of the standards.

Gel Staining and Autoradiography

After the gels were run as described above, the gels were removed from the glass plates and stained with either Coomassie Blue stain or the silver stain method. The Coomassie Blue staining procedure was performed as follows: gels were placed in a container containing approximately 100 mls of 40% methanol, 10% acetic acid, and 0.1% Coomassie blue stain. Gels of 0.75 mm thickness were incubated on a rotary shaker for 45 minutes while 1.5 mm gels were incubated for 2-4 hours. The stain was then removed and the gels destained with 40% methanol and 10% acetic acid. Typically five 100 ml washes were performed for three hours for 0.75 mm gels and overnight for 1.5 mm gels. Gels were then scanned using a Helena Laboratories Auto-Scanner with a 595 nm filter.

Alternatively, gels were stained using the silver stain method which has greater sensitivity. Gels of 0.75 mm thickness were stained in the following manner. After electrophoresis gels were removed and placed in 200 ml of a 40% methanol/10% acetic acid (v/v) fixative for 30 minutes. Gels were then placed in a fixative of 200 ml of 10% ethanol/5% acetic acid for 15 minutes. This step was then repeated. The gels were then placed in 100 ml of 10% Bio-Rad

oxidizer concentrate for 5 minutes, followed by two 5 minute dH_2O washes. The gels were then placed in 100 ml of 10% Bio-Rad silver reagent concentrate for 20 minutes, followed by a 1 minute dH_2O wash. The gels were then placed in 200 ml of a 3.2% (w/v) solution of Bio-Rad developer concentrate for 30 seconds. The developer was replaced and changed every 5 minutes until sufficient development had occurred. The development was stopped with 200 ml of 5% acetic acid (v/v). Gels of 1.5 mm thickness were stained used the same procedure with the following modifications. All treatments up to the second wash (after the oxidizing step) were doubled in length. An additional 10 min dH_2O wash was performed and the silver reagent was used for 30 min. The remaining steps were the same.

Gels which were to be assayed for radioactivity were dried down on filter paper for one hour for 0.75 mm gels and two hours for 1.5 mm gels using a Hoefer Scientific Instruments SE 540 Slab Gel Dryer. The dried gels were then taped to a gel cassette and exposed to X-ray film for 3 to 7 days.

RESULTS

Fluorescence (Wild-type)

The low-temperature fluorescence emission spectra of the wild-type cells in states 1 and 2 are shown in Figure 4. This figure shows the fluorescence emission spectra of 75 μL aliquots taken from a 2 ml stirred sample as described in **Materials and Methods**; the actinic wavelength was chosen to excite PC (590 nm). The dotted line is for dark-adapted cells, the dashed line is for cells after the addition of DCMU in the dark and the solid line is after a 2 minute preillumination of the DCMU treated cells. There is a large change in the relative yield of PS2 Chl $\underline{a}$ and PS1 Chl $\underline{a}$ emission (F695/F720) indicative of a state transition between dark-adapted cells (state 2) and cells preilluminated in the presence of DCMU (state 1). The addition of DCMU in the dark slightly increased F695/F720 but the emission spectrum more closely resembles that of dark-adapted cells (state 2) than cells preilluminated in the presence of DCMU. The ratios of the F695 to the F720 nm peaks show this in Table 2. A 31% decrease in this ratio is seen when comparing cells illuminated in the presence of DCMU to cells in the dark (no DCMU). This decrease is only 20% when DCMU is present in the dark-adapted sample. A difference is also observed in the phycobilin region (645-665 nm), this is most pronounced when comparing the dark-treated sample to the two which were DCMU-poisoned. While the difference in the phycobilin region appears to be due to the DCMU a control experiment shown in Figure 5 shows that the change in fluorescence yield of the PC peak at 650 nm, seen upon addition of DCMU in the

Figure 4. 77K fluorescence emission spectra of wild-type cells of Synechococcus sp. PCC 7002. The light solid line is for DCMU-poisoned (1 μM) cells illuminated for 2 minutes, the dashed line for dark-adapted cells with DCMU and the dotted line is for dark-adapted cells. Excitation wavelength is 590 nm.
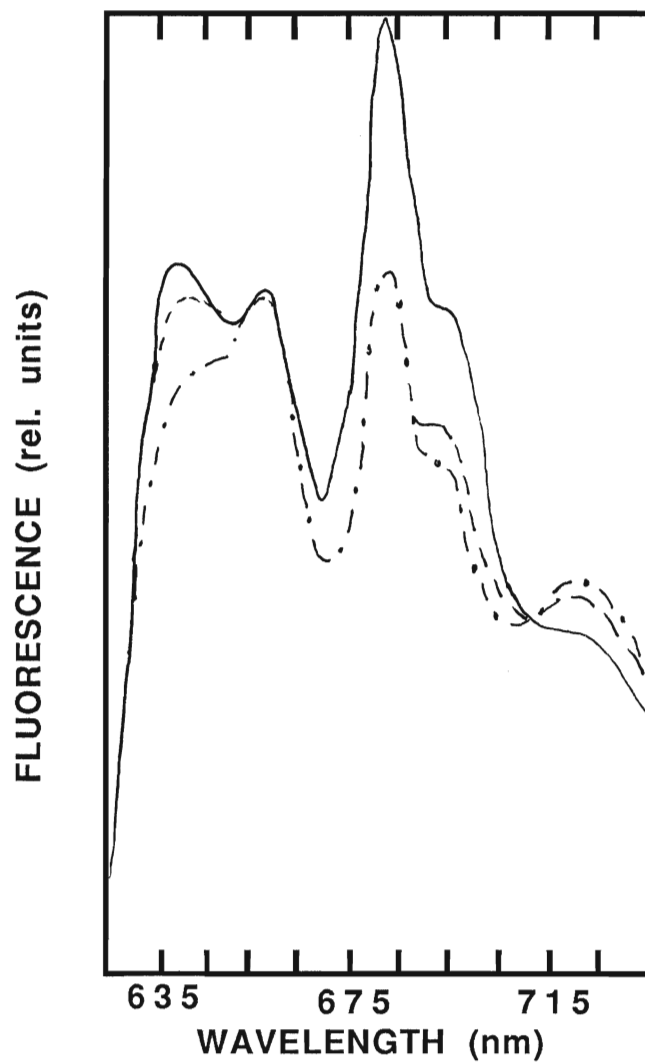
Table 2. The ratio of the F695 (PS2) to F720 (PS1) peaks in the wild-type of <u>Synechococcus sp. PCC 7002</u> is shown under the described conditions. Excitation wavelengths are shown in the brackets. The decrease in this ratio relative to cells in state 1 (light + DCMU or light 1) is also shown. The error in these ratios is 2%.

TREATMENT (excitation λ)	$\dfrac{F695}{F720}$	%DECREASE	ILLUMINATION WAVELENGTH	$\dfrac{F695}{F720}$	%DECREASE
			(590 nm exc.)		
DARK(590)	1.26	31	light 2,590 nm	1.35	30
DARK+DCMU(590)	1.45	20	light 1,435 nm	1.92	--
LIGHT+DCMU(590)	1.82	- -	(435 nm exc.)		
DARK(435)	0.39	39			
LIGHT+DCMU(435)	0.64	- -	light 2,590 nm	0.59	18
			light 1,435 nm	0.72	--

Figure 5. 77K fluorescence emission spectra of wild-type cells of Synechococcus sp. PCC 7002. The solid line is for cells treated with 1% ethanol (v/v) and the dashed line is for control cells treated with 1% (v/v) A+ medium. Excitation wavelength is 590 nm.

FLUORESCENCE (rel. units)

WAVELENGTH (nm)

dark, was due to the addition of 1% ethanol with the DCMU. However, the addition of ethanol alone did not dramatically affect the F695/F720 ratio. This is seen in Table 3.

The cells could be cross-linked with glutaraldehyde, as described previously (Biggins, 1983), into a high F695/F720 state (state 1) or low F695/F720 state (state 2) if glutaraldehyde was added during preillumination in the presence of DCMU or during dark adaptation of cells, respectively. The addition of DCMU and subsequent preillumination did not appreciably alter the F695/F720 ratio of dark-adapted cells which had been cross-linked with glutaraldehyde. This is shown in Figure 6 and Table 4. The glutaraldehyde cross-links proteins, thus presumably preventing changes in protein-protein interaction.

Figure 7 shows fluorescence emission spectra from the same cells as those used in Figure 5, however the excitation wavelength chosen was 435 nm which is absorbed by Chl _a_. As expected the only significant peaks are due to Chl _a_ fluorescence. As observed in Figure 5, cells in state1 had a higher PS2 fluorescence yield while cells in state 2 has a higher PS1 yield. The F695/F720 ratios shown in Table 2 indicate that the state transition can be induced whether the actinic wavelength used was chosen to excite PC or Chl _a_.

Figure 8 shows spectra from cells treated with both DCMU and KCN in the light (solid line) and dark (dotted line). The dashed line shows the spectrum for cells incubated in the presence of only DCMU in the dark. Cells in the presence of DCMU in the light had a virtually identical emission spectrum to cells in the presence of both DCMU and KCN in the light. As summarized in Table 5 the addition of KCN

Table 3. The ratio of the F695 (PS2) to F720 (PS1) peaks and F655 (PC) to F665 (APC) in the wild-type of <u>Synechococcus sp. PCC 7002</u> is shown in the presence and absence of 1% ethanol. The decrease in this ratio in cells without ethanol relative to cells in the presence of ethanol is also shown. The error in these ratios is 2%.

TREATMENT			F695/F720	%DECREASE
LIGHT	+	ETHANOL (F655/F665)	1.03	- -
LIGHT	-	ETHANOL (F655/F665)	0.85	1 7
LIGHT	+	ETHANOL (F695/F720)	3.03	- -
LIGHT	-	ETHANOL (F695/F720)	2.92	3.6

Figure 6. 77K fluorescence emission spectra of wild-type cells of Synechococcus sp. PCC 7002. The solid line is for cells illuminated in the presence of $2\mu M$ DCMU and chemically fixed in 0.5% glutaraldehyde, the dashed line is for dark-adapted cells chemically fixed in 0.5% glutaraldehyde, and the dotted line is for dark-adapted cells chemically fixed in 0.5% glutaraldehyde and subsequently treated with $10\mu M$ DCMU. Excitation wavelength is 590 nm.

Table 4. The ratio of the F695 (PS2) to F720 (PS1) peaks in the wild-type of <u>Synechococcus sp. PCC 7002</u> is shown under the described conditions. Cells were fixed with 0.5% glutaraldehyde: in the dark (dark/fixed); in the light with DCMU present (light+DCMU/fixed); or in the dark, DCMU added after fixation (dark/fixed+DCMU). The decrease in the ratio of cells fixed in the dark is shown relative to cells fixed in the light. The error in these ratios is 2%.

TREATMENT	F695/F720	%DECREASE
DARK/FIXED	1.81	30
LIGHT + DCMU/FIXED	2.57	- -
DARK/FIXED+DCMU	1.85	28

Figure 7. 77K fluorescence emission spectra of wild-type cells of Synechococcus sp. PCC 7002. The dashed line is for dark-adapted cells with DCMU (1 µM) and the solid line is for DCMU-poisoned cells illuminated for 2 minutes. Excitation wavelength is 435 nm.

Figure 8. 77K fluorescence emission spectra of wild-type cells of Synechococcus sp. PCC 7002. The solid line is for cells illuminated for 2 minutes and the dashed line is for dark-adapted cells. Both samples were in presence of 1 μM DCMU and 1 mM KCN. The dotted line is for dark-adapted cells in presence of 1 μM DCMU. Excitation wavelength is 590 nm.
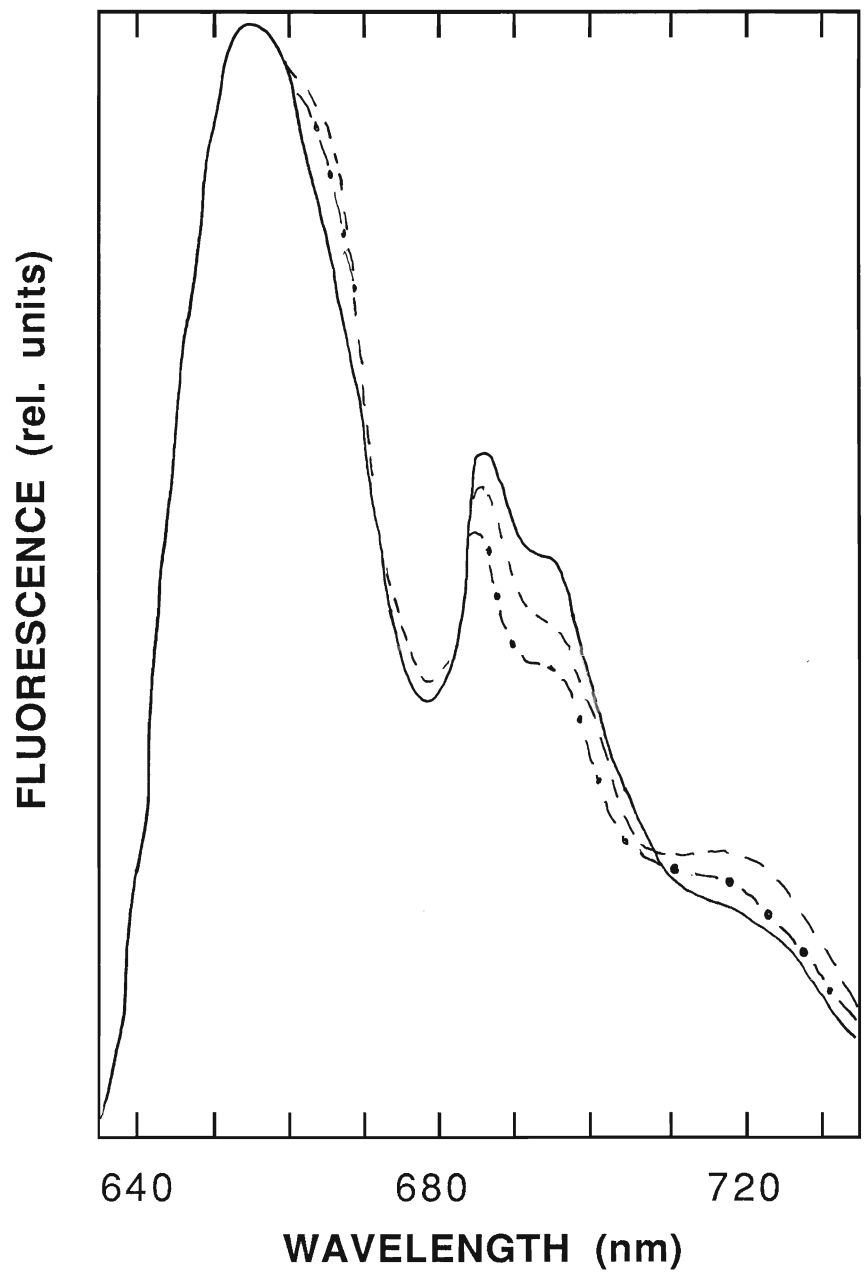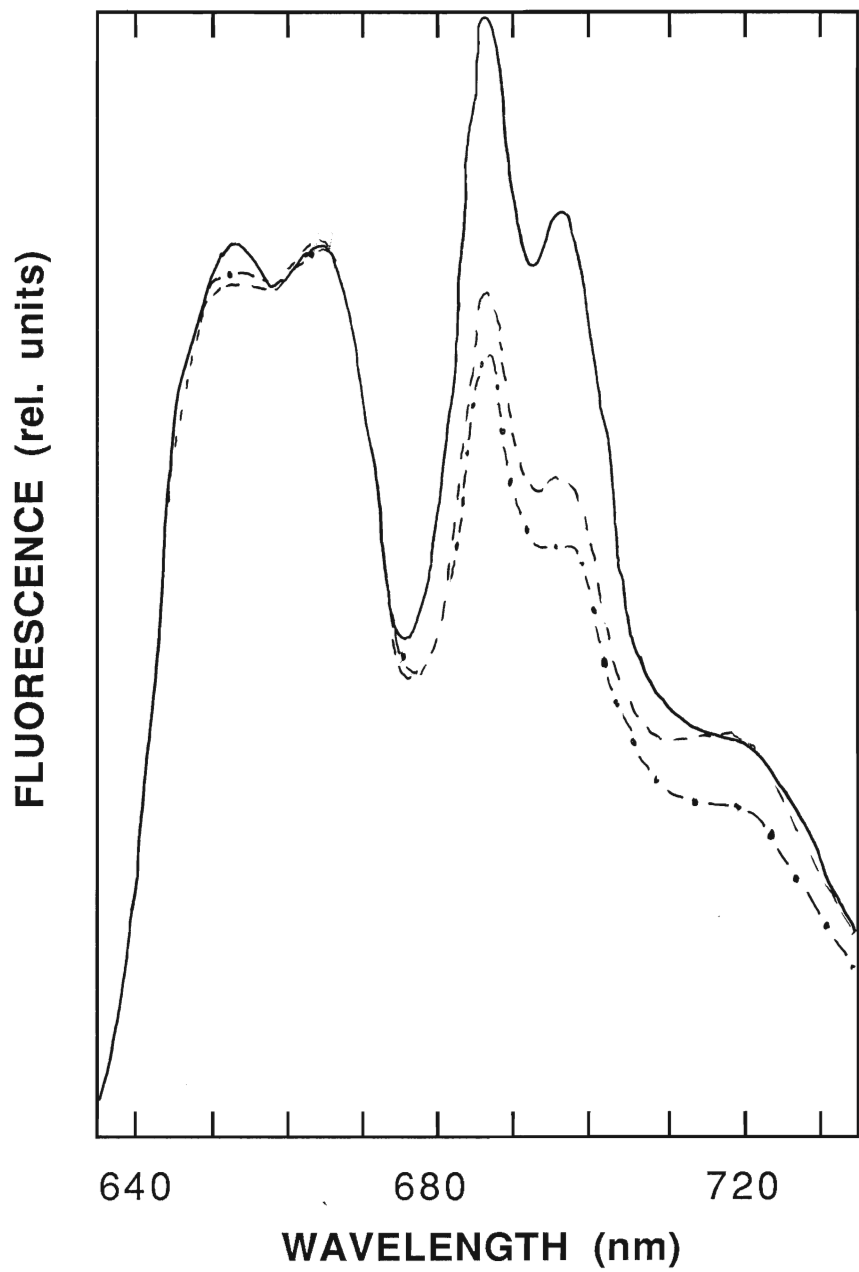
Table 5. The ratio of the F695 (PS2) to F720 (PS1) peaks in the wild-type of Synechococcus sp. PCC 7002 is shown under the described conditions. Cells were in the presence of 1μM DCMU and subject to either a light or dark treatment in the presence and absence of 1mM KCN. The decrease in the ratio of cells in the dark relative to the ratio of cells in the presence of cells in the light is shown. The error in these ratios is 2%.

TREATMENT	F695/F720	%DECREASE
LIGHT + DCMU	2.37	- -
LIGHT + DCMU + KCN	2.38	- -
DARK +DCMU	1.84	2 2
DARK + DCMU + KCN	1.63	31

in the dark results in an even lower F695/F720 ratio while not affecting the ratio in the light.

These cells are also capable of doing classical light-induced state transitions without chemical treatment. Figures 9 and 10 show this for 590 and 435 nm excitation. While no appreciable change is seen in the phycobilin fluorescence in either state it is clear that there are large changes in the fluorescence yields of PS2 and PS1 Chl a irrespective of the excitation wavelength. The respective ratios are shown in Table 2, indicating that similar but smaller changes in the energy distribution patterns are seen upon light-induced state transitions as compared to those which are redox-induced.

Experiments were performed to determine the kinetics of the state transition in the wild-type. Figures 11 and 12 show the time courses of the change in the F695/F720 ratio as described in **Materials and Methods** for the state 1->12 and 2->1 transitions respectively. These experiments represent the averaged data from 4 and 5 separate experiments respectively. Standard errors are shown as well. Under the conditions described in **Materials and Methods** it was found that these transitions both had half-times of approximately 10 s.

Cytochrome f

Kinetic experiments were performed to measure the changes in the redox state of cyt f as a function of the state of the cells in the wild-type. Figure 13 shows the oxidation of cyt f with 590 nm light at two different light intensities for cells in state 1 and 2. The half-times and extents for these experiments are shown in Table 6. The
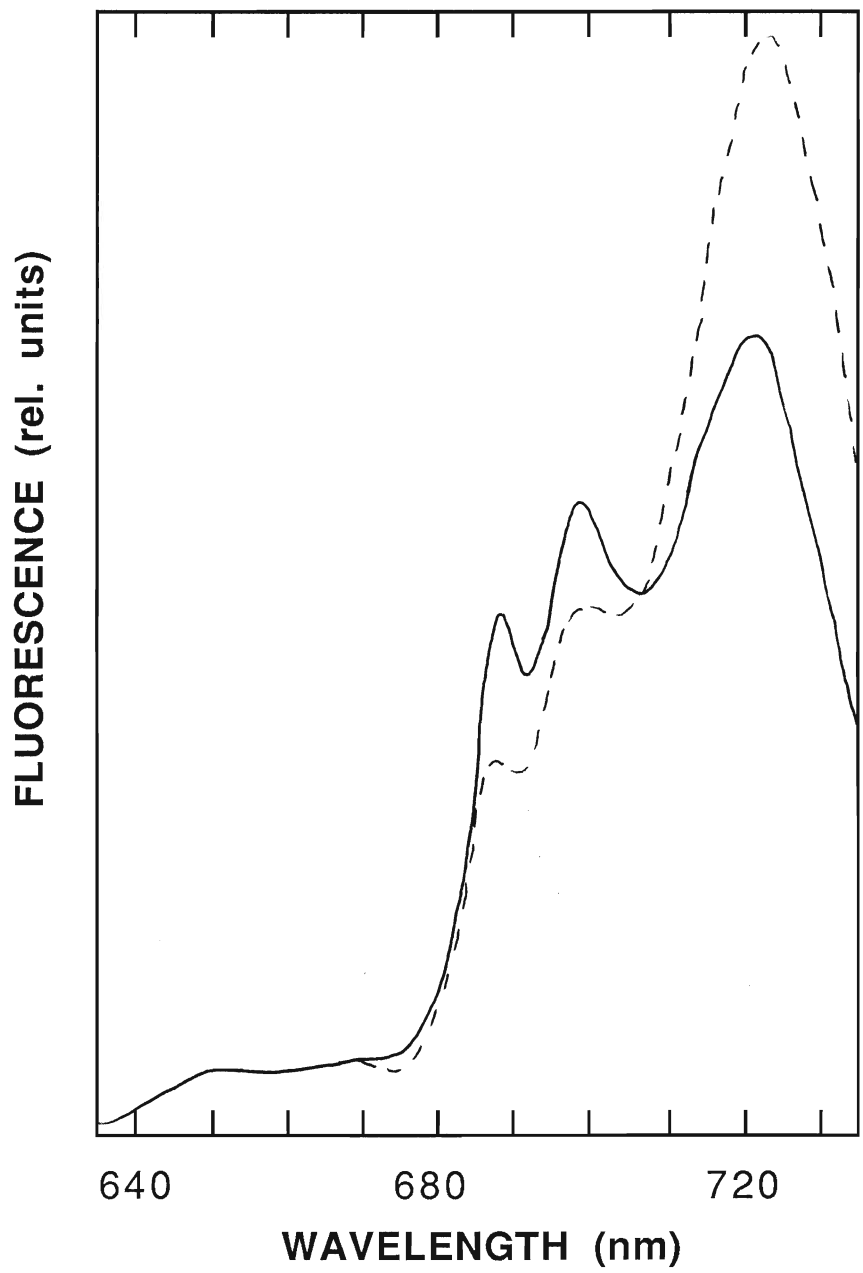
Figure 9. 77K fluorescence emission spectra of wild-type cells of Synechococcus sp. PCC 7002. The solid line is for cells preilluminated with blue light (see Materials and Methods) for two minutes (state 1) and the dashed line is for cells preilluminated with orange light (see Materials and Methods) for two minutes (state 2). Excitation wavelength is 590 nm.
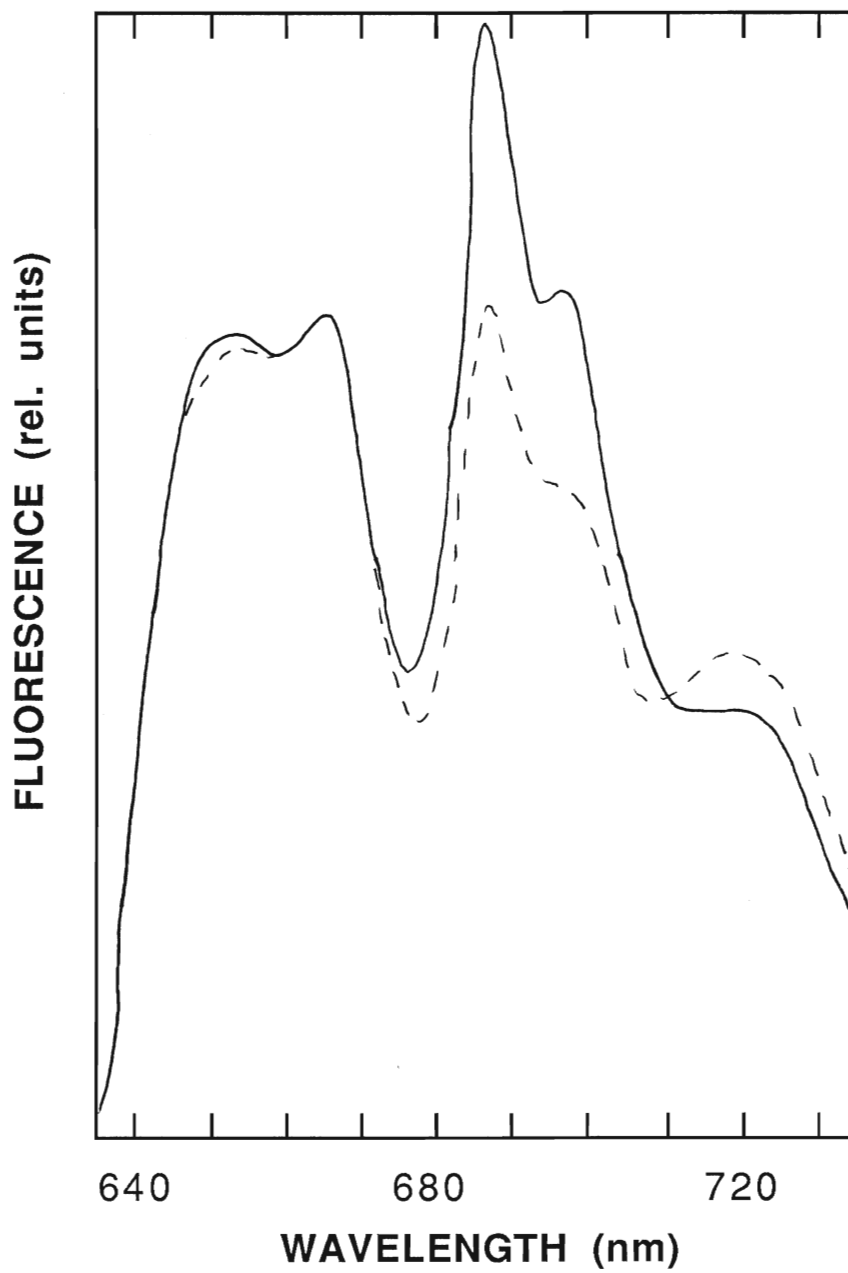
Figure 10. 77K fluorescence emission spectra of wild-type cells of Synechococcus sp. PCC 7002. The solid line is for cells preilluminated with blue light (see Materials and Methods) for two minutes (state 1) and the dashed line is for cells preilluminated with orange light (see Materials and Methods) for two minutes (state 2). Excitation wavelength is 435 nm.
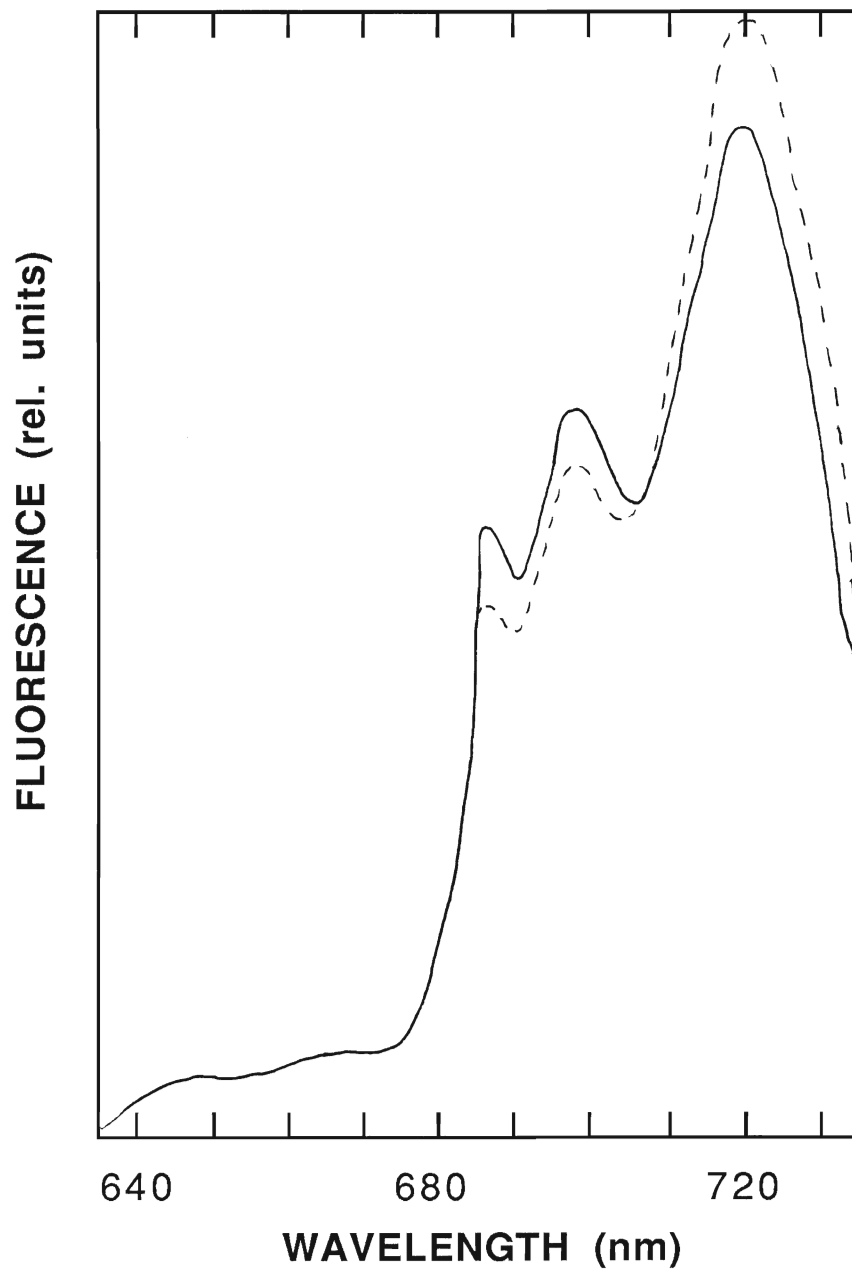
Figure 11. Kinetics of the state 1 to state 2 transition in wild-type cells of <u>Synechococcus sp. PCC 7002</u>. Cells were illuminated in 680 nm light for 1 minute in the presence of 1μM DCMU before the light was turned off. 75μL samples were withdrawn at specific intervals into capillary cuvettes and frozen in liquid nitrogen. A low temperature fluorescence emission spectrum was taken of each sample with 590 nm excitation and the ratio of the F695 peak to the F720 peak measured and plotted versus time. Values represent the average of 4 separate experiments and error bars show the standard errors.
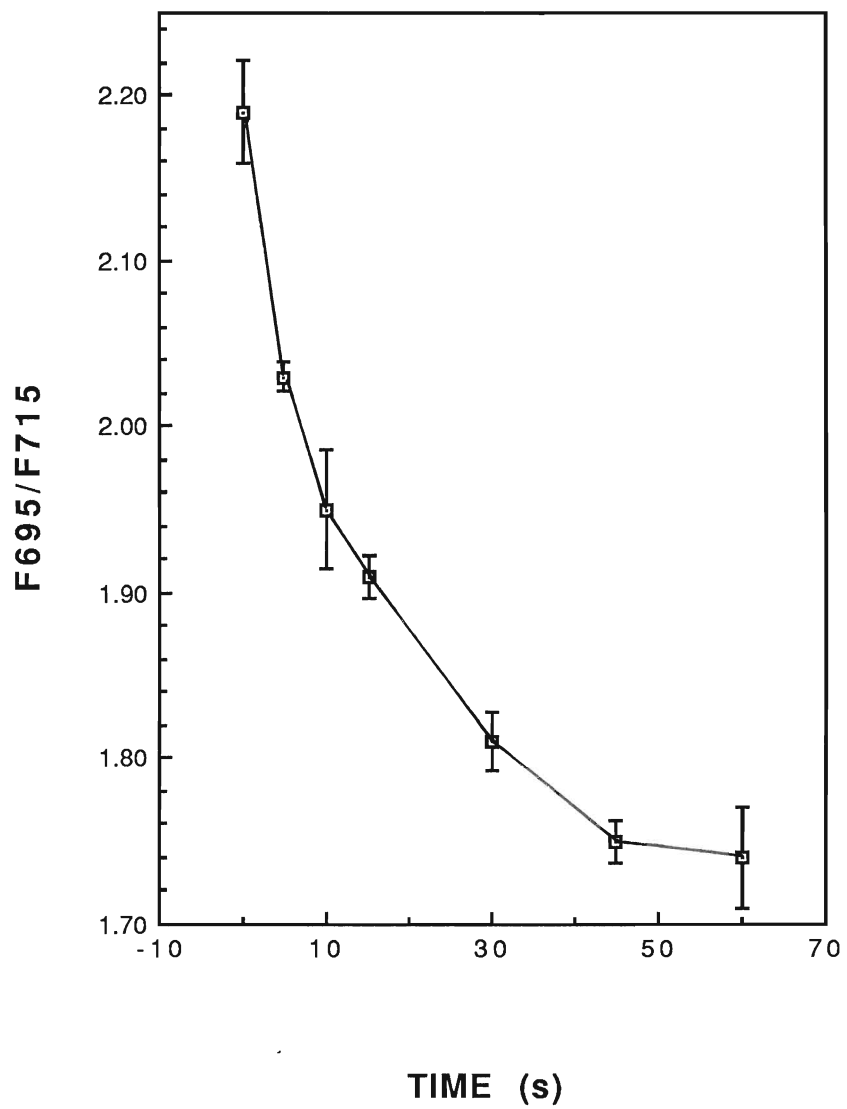
Figure 12. Kinetics of the state 2 to state 1 transition in wild-type cells of <u>Synechococcus sp. PCC 7002</u>. Cells were dark-adapted for 2 minutes in the presence of 1µM DCMU before 680 nm light was turned on. 75µL samples were withdrawn at specific intervals into capillary cuvettes and frozen in liquid nitrogen. A low temperature fluorescence emission spectrum was taken of each sample with 590 nm excitation and the ratio of the F695 peak to the F720 peak measured and plotted versus time. Values represent the average of 5 separate experiments and error bars show the standard errors.

Figure 13. Plot of change in % transmittance at 420 nm (redox state of cyt $\underline{f}$). State 1 (dashed line) represents cells illuminated in presence of DCMU (1 µM) with 590 nm light (wavelength and intensity as indicated) for 20 seconds, followed by 4 seconds in the dark. The measurement begins when the light is turned back on. State 2 (solid line) represents cells dark-adapted in presence of DCMU for 24 seconds, the measurement begins when the light is turned on (wavelength and intensity as indicated). In all measurements the signal was averaged 25 times.

A

light on

590 nm exc.
$200\mu E \cdot m^{-2} \cdot s^{-1}$

$\lceil 0.001\Delta T/T$

500 ms

TIME →

B

light on

590 nm exc.
$150 u E \cdot m^{-2} \cdot s^{-1}$

500 ms

TIME →

$\Delta\%T$ at 420 nm (rel. units)
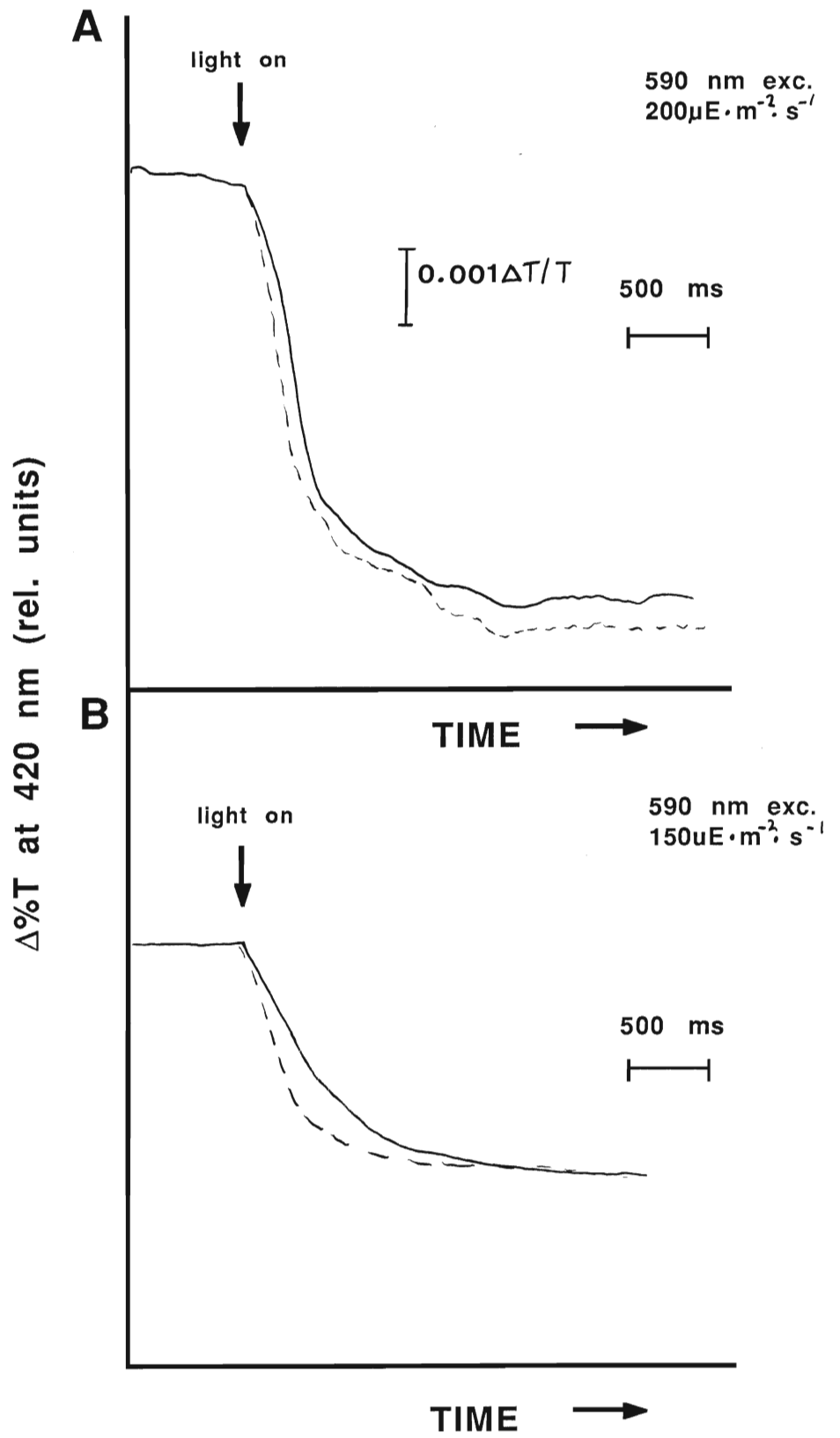
Table 6. The half-times and extents of cyt f (420 nm) and P-700 (700 nm) oxidation kinetics in the wild-type of <u>Synechococcus sp. PCC 7002</u> as a function of the state of the cells is shown for different excitation wavelengths and intensities. One half of the range of the peak-to-peak error is shown in brackets and the method of calculation shown in Appendix A.

ACTINIC LIGHT			HALF-TIME (ms)		EXTENT (mV)	
λ (nm)	INTENSITY (uEm^{-2}s^{-1})	MEASURING λ (nm)	STATE 1	STATE 2	STATE 1	STATE 2
590	200	420	345(38)	519(16)	47.5(3.3)	49.5(1.4)
590	150	420	363(19)	535(28)	28.0(1.0)	24.7(2.3)
590	200	700	175(30)	253(36)	1090(55)	1060(50)
590	64	700	186(16)	379(49)	828(54)	716(47)
590	28	700	241(36)	635(72)	634(41)	488(32)
435	25	700	275(34)	765(72)	547(41)	310(34)

data indicates that cells in state 1 reach a similar cyt $\underline{f}$ redox level as cells in state 2 but do so more rapidly. DCMU is present to inhibit cyt $\underline{f}$ reduction by PS2. 590 excitation should preferentially excite PS2, however the DCMU treatment prevents cyt $\underline{f}$ reduction by PS2, thus the only effect 590 nm light can have is to oxidize cyt $\underline{f}$ either by direct PS1 excitation or by energy spillover from PS2 to PS1.

P-700

The above experiments were repeated while measuring P-700 redox changes rather than cyt $\underline{f}$ changes. Figure 14 shows this experiment at different intensities of 590 nm light as well as with 435 nm light. Dramatic changes are seen in both the half-times and extents of P-700 oxidation, being both faster and greater in state 1 than state 2. An intensity effect is also observed, at higher intensities the difference between states 1 and 2 becomes mimimal (Table 6).

An experiment was done to measure P-700 oxidation with 435 nm light on the wild-type cells which had been dark-treated for varying lengths of time. Figure 15 shows that with increasing time in the dark the half-time for P-700 oxidation increases as well (Table 7). It also appears that at the longer dark treatments a measurable lag is observed between the time when the light is turned on and the time when redox changes first begin.

Absorption

Absorbance spectra for the wild-type and mutant (ΔapcAB cpc-) cells of <u>Synechococcus</u> sp. PCC 7002 are shown in Figure 16.
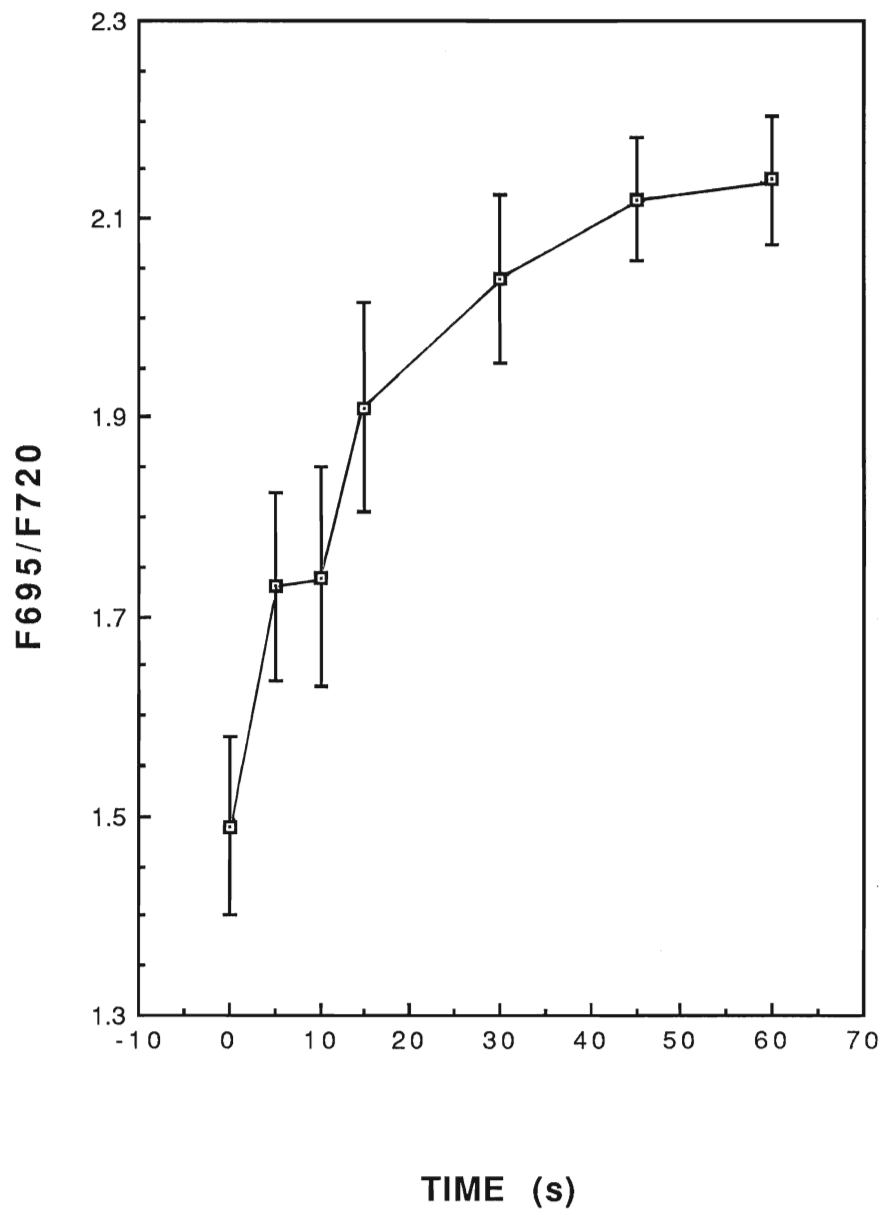
Figure 14. Plot of change in % transmittance at 700 nm (redox state of P-700). State 1 (dashed line) represents cells illuminated in presence of DCMU (1 μM) with 435 nm light for 20 seconds, followed by 4 seconds in the dark. The measurement begins when the light is turned back on (wavelength and intensity as indicated) . State 2 (solid line) represents cells dark-adapted in presence of DCMU for 24 seconds, the measurement begins when light is turned on (wavelength and intensity as indicated). In all measurements the signal was averaged 20 times.

A — 590 exc. 200uE·m^{-2}·s^{-1}

0.002 $\Delta T/T$

500 ms

light on

TIME →

B — 590 exc. 64uE·m^{-2}·s^{-1}

500 ms

light on

TIME →

C — 590 exc. 28uE·m^{-2}·s^{-1}

500 ms

light on

TIME →

D — 435 exc. 25uE·m^{-2}·s^{-1}

500 ms

light on

TIME →

$\Delta\%T$ at 700 nm (rel. units)
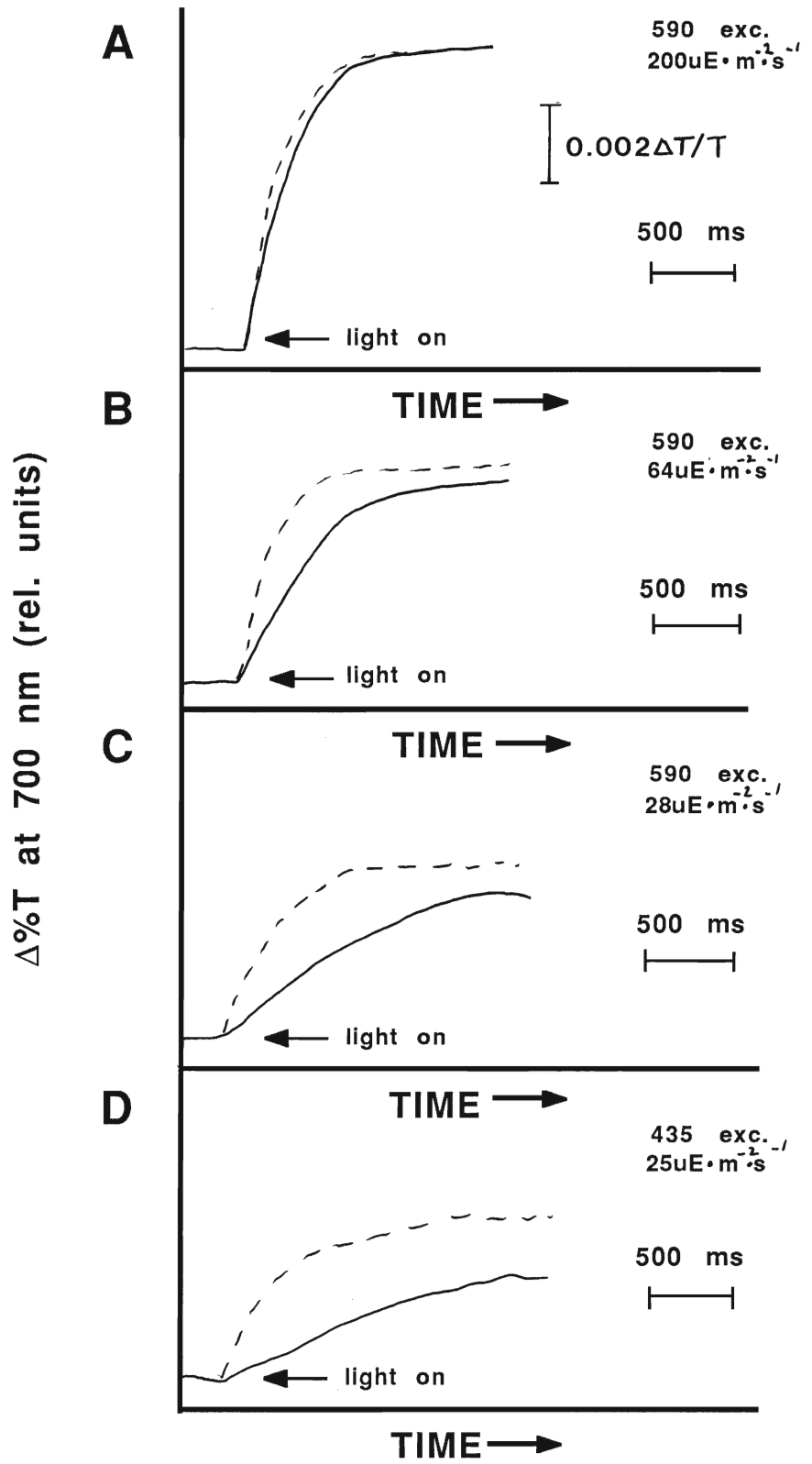
Figure 15. Plot of change in % transmittance at 700 nm (redox state of P-700). Cells were dark-adapted for indicated time in presence of DCMU (1 μM) before 435 nm light was turned on. Signal was averaged 5 times.

$\Delta\%T$ at 700 nm (rel. units)

500 ms

light 20s
dark 30s
dark 60s
dark 120s

light on
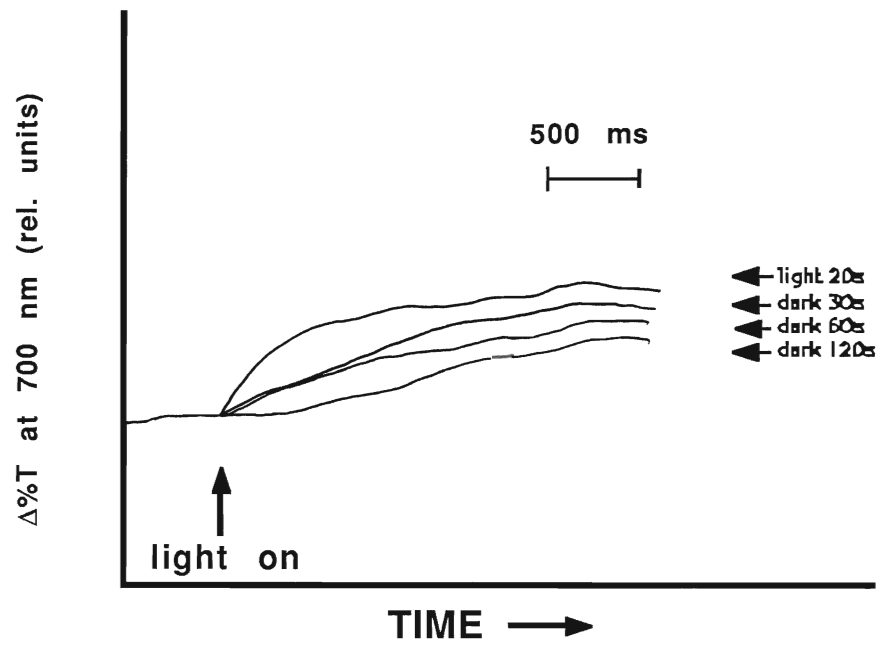
TIME

Table 7. The half-times and extents of P-700 (700 nm) oxidation kinetics in the wild-type of Synechococcus sp. PCC 7002 as a function of a pre-dark adaption period. One half of the range of the peak-to-peak error is shown in brackets and the method of calculation shown in Appendix A.

TREATMENT	HALF-TIME (ms)	EXTENT (mV)
light 1 (20s)	245 (45)	567 (71)
dark (30s)	977 (73)	525 (77)
dark (60s)	956 (84)	414 (40)
dark (120s)	1,316 (56)	352 (27)

Figure 16. Room-temperature absorbance spectra of intact wild-type and phycobilisome-less mutant cells of <u>Synechococcus sp. PCC 7002</u>. The dashed line is for the wild-type and the solid line for the mutant.
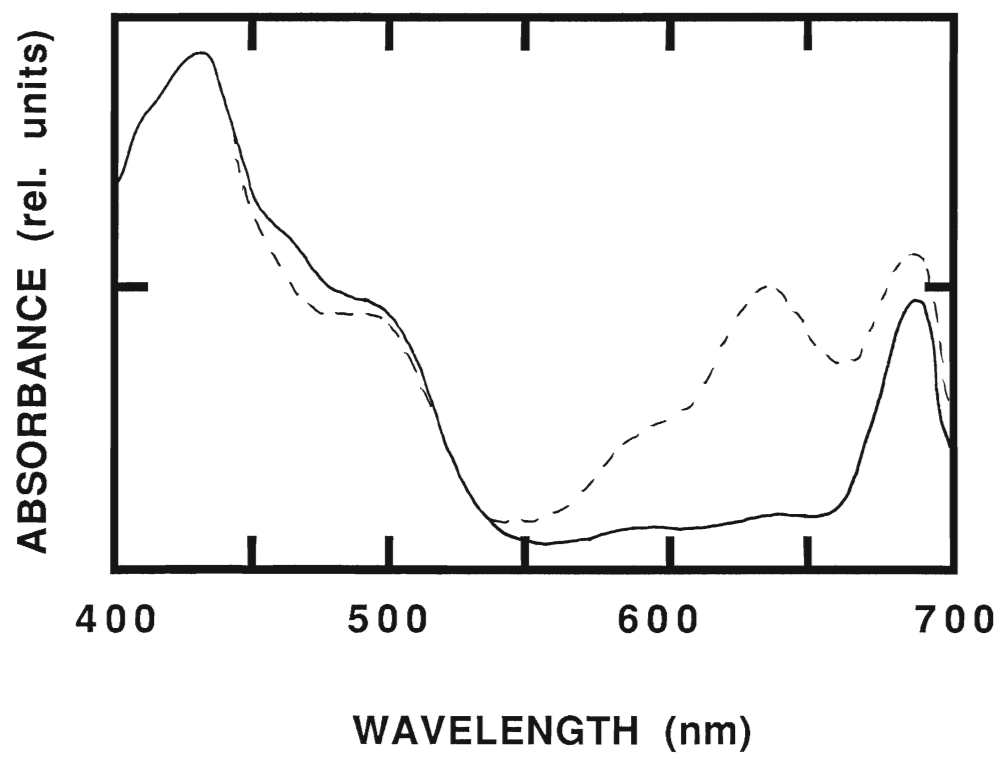
The wild-type cells (dashed line) show a significant contribution by PC and APC in the 580 nm to 650 nm region, in contrast to the mutant (solid line) which shows only Chl $\underline{a}$ Q_x and Q_y transitions in this region. The mutant cells show higher absorption in the 450 nm to 530 nm region than the wild-type which reflects an increase in the carotenoid to Chl $\underline{a}$ ratio.

Fluorescence (PBS-less Mutant)

Figure 17 shows 77K fluorescence emission spectra of 75 µl aliquots of PBS-less mutant cells taken sequentially from a 10 ml sample as described in **Materials and Methods**. Due to the absence of phycobilins in these organisms the actinic wavelength used was 435 nm, exciting both PS1 and PS2 Chl $\underline{a}$. In this figure the solid line represents cells preilluminated in the presence of DCMU and shows a high F695/F720 ratio, the dotted line is for dark-adapted cells and shows a low F695/F720 ratio; and the dashed line shows dark-adapted cells treated with DCMU. These cells also show a high F695/F720 ratio, indicative of state 1. Figure 18 shows spectra from the same cells used in Figure 17 following treatment with 1 mM KCN to inhibit cytochrome oxidase activity. In Figure 17 the addition of DCMU in the dark (dashed line) results in an increase in F695/F720 and the spectrum is very similar to that for cells preilluminated in the presence of DCMU. However, in Figure 18 the KCN treated cells show only a slight increase in F695/F720 after the addition of DCMU in the dark (dashed line) similar to the response of the wild-type cells shown in Figure 4. The preillumination/dark

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Figure 17. 77K fluorescencee emission spectra of PBS-less mutant cells of <u>Synechococcus sp. PCC 7002</u>. The solid line is for DCMU-poisoned cells ($1\mu M$) illuminated for 2 minutes, the dashed line for dark-adapted cells with DCMU, and the dotted line for dark-adapted cells. Excitation wavelength is 435 nm.
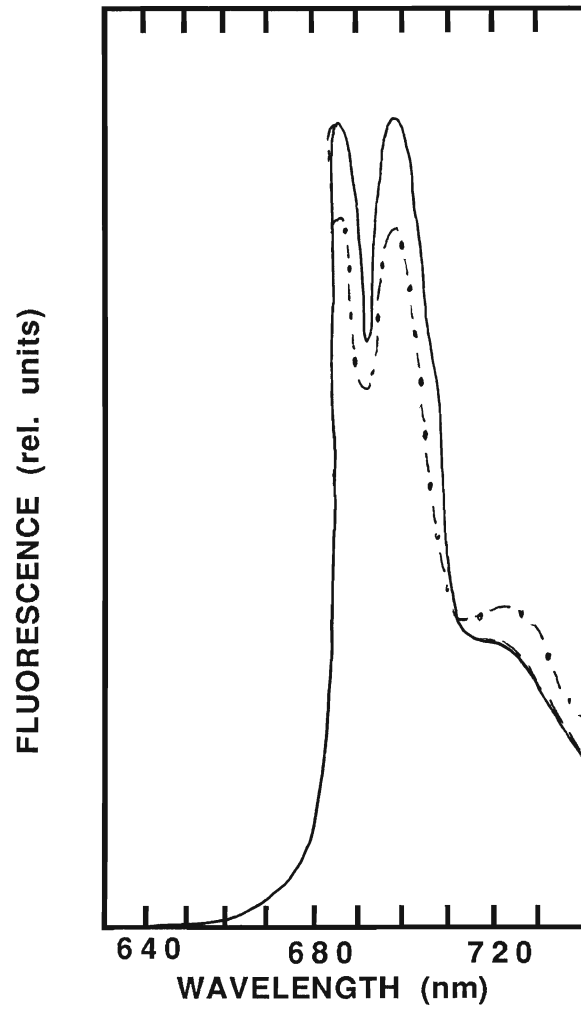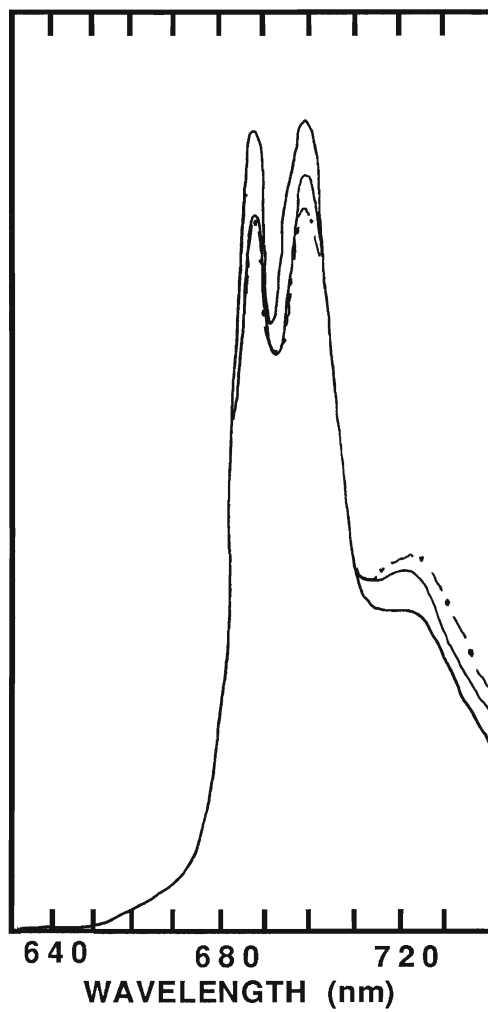
Figure 18. 77K fluorescence emission spectra of PBS-less mutant cells of <u>Synechococcus sp. PCC 7002</u>. The solid line is for DCMU-poisoned cells (1μM) illuminated for 2 minutes, the dashed line for dark-adapted cells with DCMU, and the dotted line for dark-adapted cells. All samples were treated with 1 mM KCN. Excitation wavelength is 435 nm.

adaptation induced changes in F695/F720 in the presence of DCMU and KCN were completely reversible in the mutant as were the preillumination/dark adaptation induced changes in the presence of DCMU in the wild-type. KCN has been shown to inhibit respiratory O_2-uptake in the PBS-less mutant. After a 2 minute incubation with 1 mM KCN the rate of O_2 uptake in the PBS-less mutant was decreased to 20% of the control rate. In contrast, the rate of O_2 evolution was only inhibited to 60% of the control rate by the same treatment (D. Bruce, personal communication).

Excitation spectra at 77K for PS1 and PS2 Chl $\underline{a}$ fluorescence emission of the wild-type and mutant were recorded to compare the pigment complements of PS2 and PS1. The spectra for the wild-type are shown in Figure 19. Wavelengths in the 560 nm to 660 nm region excite PS2 preferentially (dashed curve) due to the presence of PC and APC associated with PS2. Absorption in the Soret and Q_y transitions of Chl $\underline{a}$ excite PS1 preferentially, solid curve, showing that most of the Chl $\underline{a}$ is associated with PS1. In contrast to the wild-type, the excitation spectra for PS2 and PS1 emission, normalized to the 680 nm Chl $\underline{a}$ peak, are very similar in the mutant, seen in Figure 20. However, some preferential contributions to PS2 or PS1 are apparent. The 435 nm and 670 nm peaks contribute more to PS2 emission and the 470 nm and 505 nm peaks more to PS1 emission.

With the excitation spectra of the mutant in mind, four wavelengths were chosen for preillumination, two as light 1 (520 nm and 700 nm) and two as light 2 (435 nm and 670 nm). Table 8 compares the F695/F720 values for these preilluminations to the

Figure 19. Excitation spectra for 77K fluorescence emission from PS2 (695 nm), dashed line, and PS1 (720 nm), solid line of wild-type cells of <u>Synechococcus sp. PCC 7002</u>.

REL. FLUORESCENCE INTENSITY

WAVELENGTH (nm)
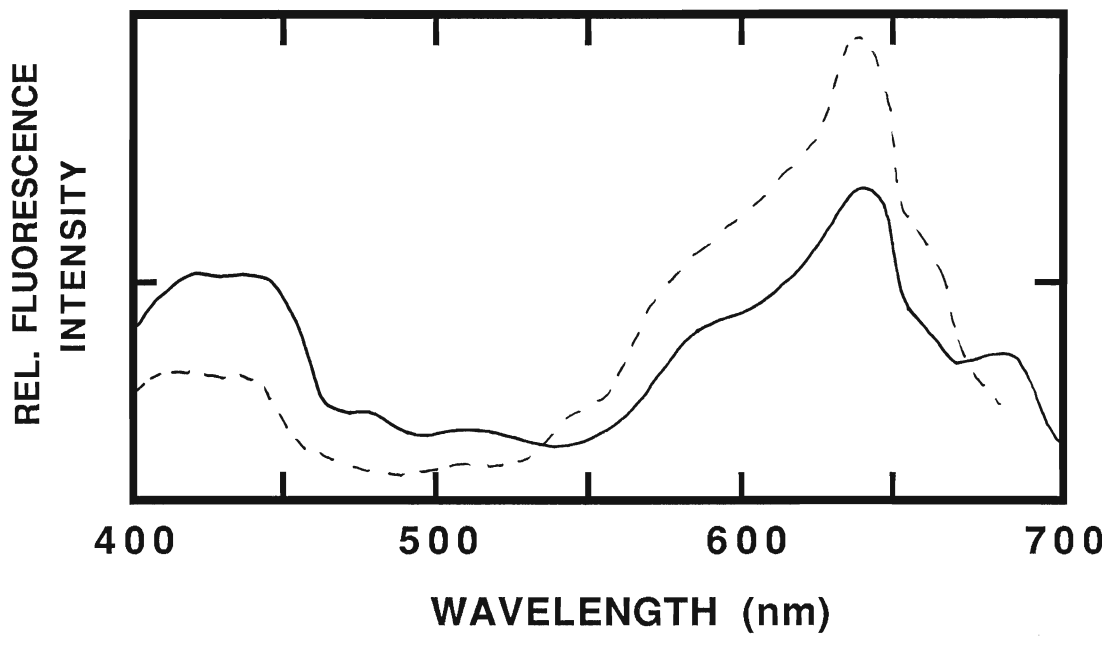
Figure 20. Excitation spectra for 77K fluorescence emission from PS2 (695 nm), dashed line, and PS1 (720 nm), solid line of PBS-less mutant cells of <u>Synechococcus sp. PCC 7002</u>. The spectra were normalized at 680 nm.
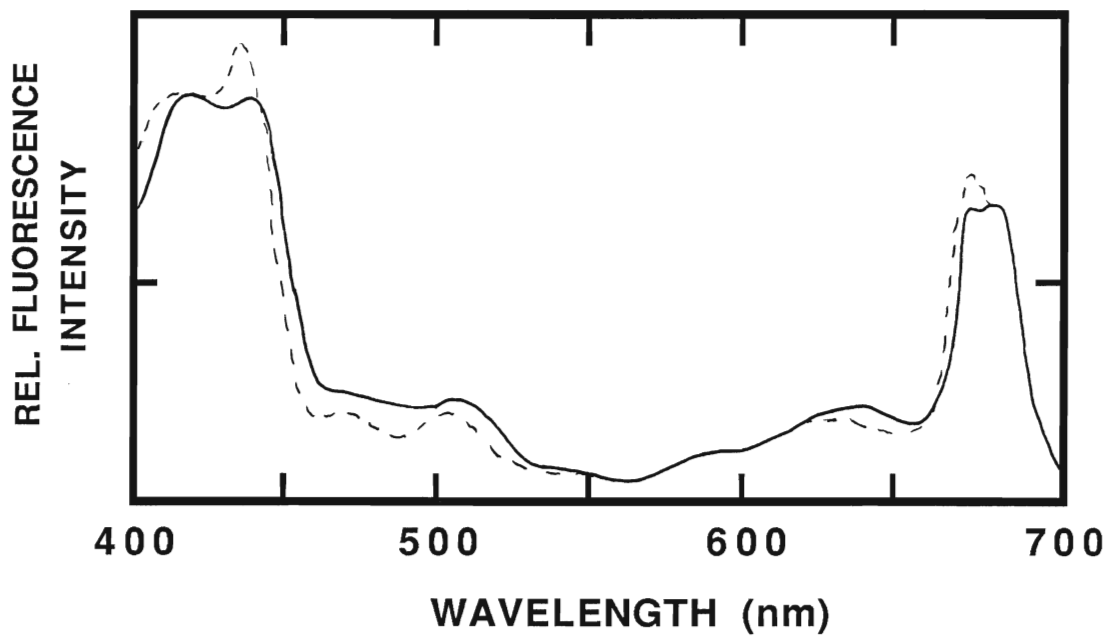
Table 8. The ratio of the F695 (PS2) to F720 (PS1) peaks in the PBS-less mutant of <u>Synechococcus sp. PCC 7002</u> is shown under the described conditions. Excitation wavelength was 435 nm.. The decrease in this ratio relative to cells in state 1 (light + DCMU or light 1) is also shown. The error in these ratios is 2%.

TREATMENT	F695/F720	%DECREASE	ILLUMINATION WAVELENGTH	F695/F720	%DECREASE
DARK	2.18	26	light 2, 435 nm	2.78	4.5
DARK + DCMU	2.80	4.8	light 1, 520 nm	2.85	
ILLUMINATED + DCMU	2.94		light 2, 670 nm	2.73	6.2
DARK +KCN	1.90	35	light 1, 700 nm	2.91	
DARK + DCMU + KCN	2.10	29			

values of dark-adapted cells, cells preilluminated in the presence of DCMU, and dark-adapted KCN-treated cells. Preillumination at 520 nm and 700 nm resulted in 77K emission spectra with a significantly higher F695/F720 than the spectra for cells preilluminated at 435 nm and 670 nm, indicative of a successful light-induced state transition. Comparison of these ratios with those for dark-adapted cells and cells preilluminated in the presence of DCMU indicate that the changes observed in the light-induction experiments were within the limits of the larger changes observed in the redox-induced experiments. Table 8 also shows that the addition of KCN to dark-adapted cells caused a decrease in the F695/F720 ratio.

Protein Isolation and Phosphorylation

As described in the **Materials and Methods** section an improved method of whole cell solubilization was developed. A comparison of two techniques for solubilization after an acetone kill is shown in Figure 21. Cells which were acetone washed twice after the initial acetone kill and subsequently solubilized in an SDS buffer are shown in Lane D. Lane B represents cells which were washed twice with acetone followed by a 30 minute lysozyme treatment and a subsequent acetone wash. In both cases equal amounts of protein were added to the solubilization buffer, however, a greater amount of protein was solubilized in the sample that had been lysozyme-treated. This was observed both when the samples were centrifuged and on the gel. A greater amount of insoluble material was apparent in the sample only acetone-washed and less protein was apparent on the gel. The greatest amount was observed in Lane B using the

Figure 21.   Silver-stained SDS-polyacrylamide gel of whole cell protein lysate of wild-type cells of <u>Synechococcus sp. PCC 7002</u>. Lane B shows cells acetone washed twice and lane D is for cells acetone washed, lysozyme-treated and acetone washed.   See **Materials and Methods** for procedures.   Lane A shows molecular weight standards.

A   B   C   D
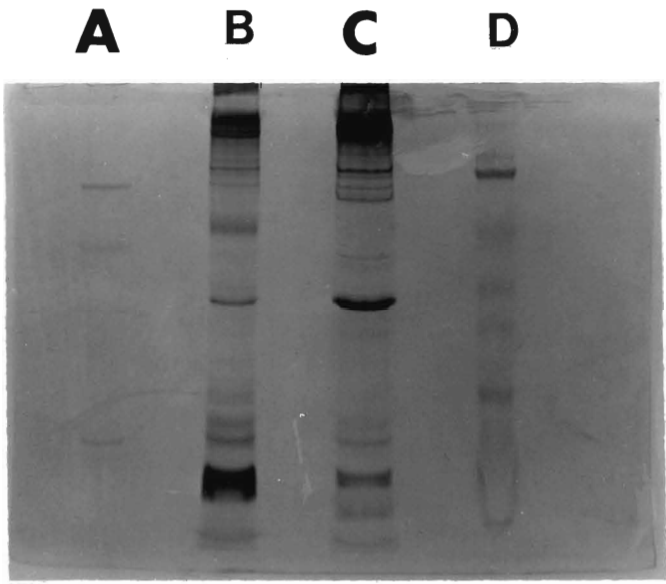
lysozyme while the acetone-washed cells revealed much lower staining levels of protein. Microscopic examination of living cells, acetone washed cells, and the lysozyme treated cells.revealed that the majority of the cells after acetone treatment were intact although the cells clumped very easily. After the lysozyme treatment many of the cells had ruptured, providing more accessible protein to the detergent. A two-dimensional gel of whole cell extracts isolated in this manner is shown in Figure 22.

An experiment was performed to determine the phospho-protein profiles of cells in state 1 and 2. The gel in Figure 23 reveals Coomassie Blue staining patterns of cells in states 1 and 2 (lanes D and C). The protease-treated samples are shown in lanes B and A, revealing that most of the staining bands are protein. The autoradiograph of this gel revealed that several proteins are phosphorylated both in the light and dark in states 1 and 2 (Figure 23). Densitometric scans of the autoradiographs, seen in Figure 24, revealed no obvious differences between the phosphoprotein profiles of cells in states 1 and 2. The phosphorylated proteins appeared to be of the following approximate molecular weights: 21, 22, 24, 27, 30, 39, 42, 50, 51.5, 61.5, 68.5, 71.5, 79, and 82.5kD. The protease-treated samples show little radioactivity, indicating the phosphorylated species are indeed protein. While two-dimensional gels were performed, very little labelling could be seen. It is apparent that with this higher resolving gel, a greater amount of radioactive labelling will be required.

Figure 22. Two dimensional SDS-polyacrylamide gel (silver-stained) of whole cell extracts of the wild-type cells of <u>Synechococcus sp. PCC 7002</u> isolated as described in **Materials and Methods**. Protein was isolated according to its iso-electric point on the horizontal axis and size on the vertical axis.
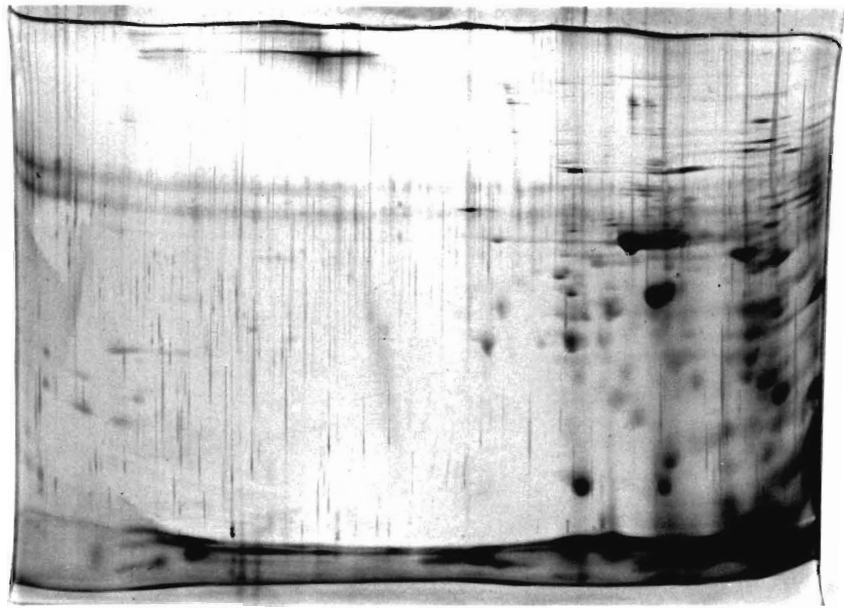
Figure 23.   Coomassie blue-stained SDS-polyacrylamide gel of whole cell lysate of ^{32}P-labelled wild-type cells of <u>Synechococcus sp. PCC 7002</u>.   Lane C shows cells killed in state 2 (15 μg protein), lane D shows cells killed in state 1 (11 μg protein).   Lanes A and B show cells from lanes C and D, respectively which were protease treated (100μg/ml pronase and 100μg/ml proteinase K).   Repeats are shown in lanes E, F, and G with standards in lane H.

A B C D E F G H
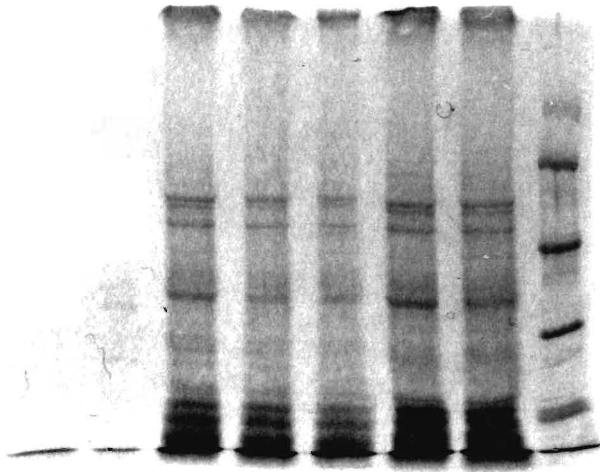
Figure 24. Autoradiogram of gel shown in Figure 23. As indicated before, Lane C shows cells killed in state 2, lane D shows cells killed in state 1. Lanes A and B show cells from lanes C and D, respectively which were protease treated.
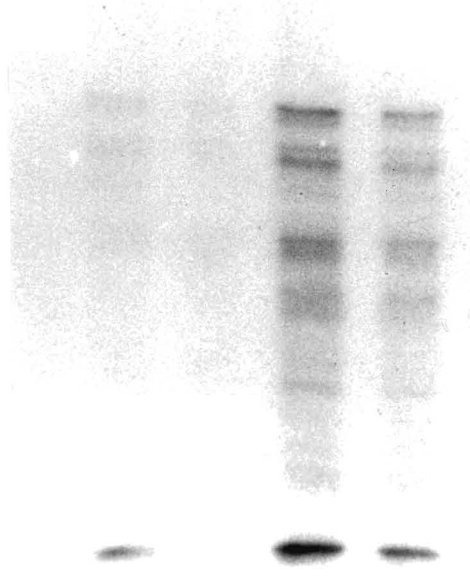
Figure 25. Densitometric scans of lanes B and C from the autoradiogram in Figure 24. Scans were normalized at the dye front.

state 1 - - - -
state 2 ———

Absorbance

Molecular Weight (KDa)

front   21   31   48   65   78
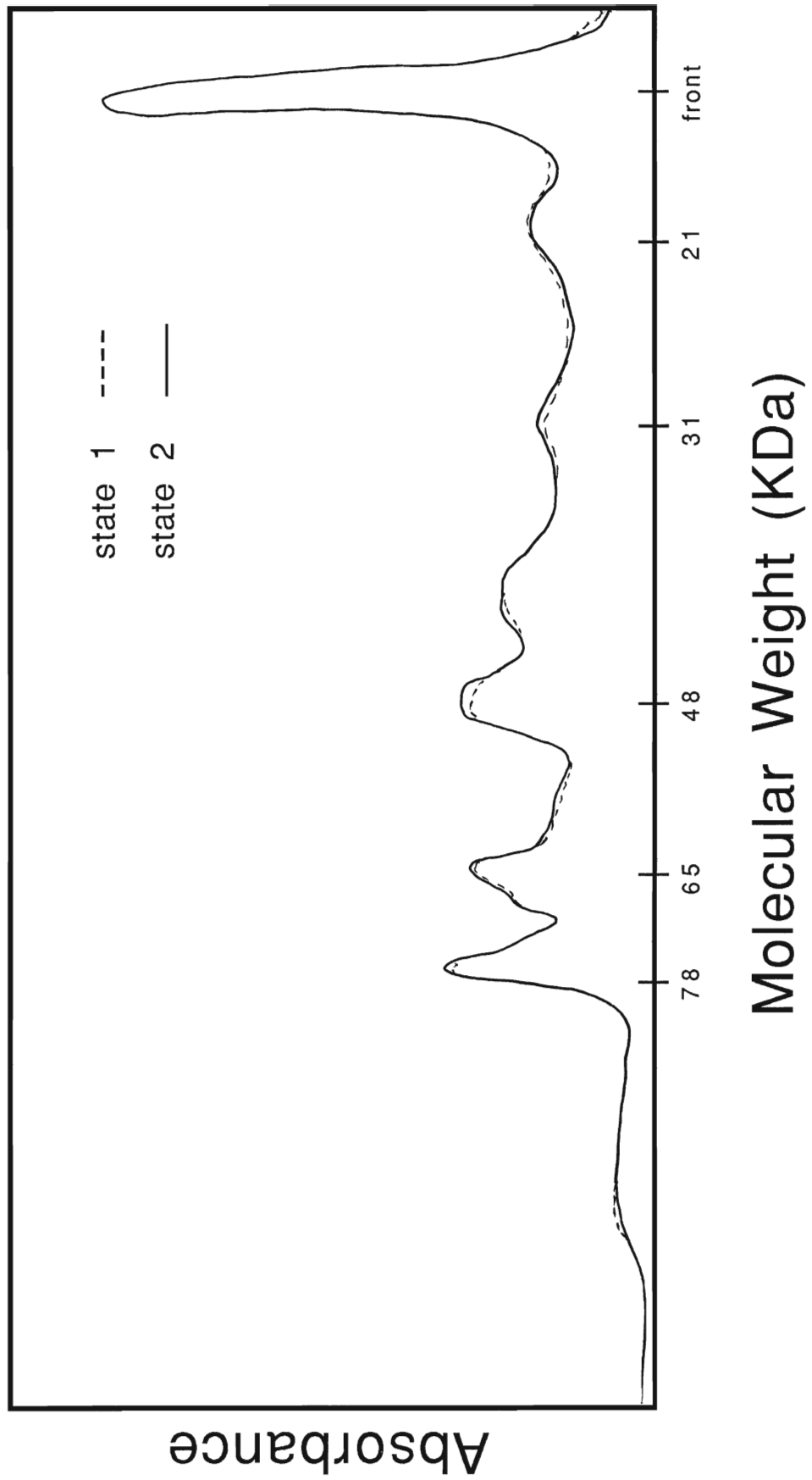
DISCUSSION

State Transitions in Wild-type

In confirmation of earlier studies (Mullineaux and Allen, 1986; Olive *et al.*, 1986) it is shown here that illumination after DCMU poisoning in cyanobacteria induces a transition to state 1 while the dark state is similar to state 2. It has been suggested that the dark state in cyanobacteria is state 2 as a result of respiratory electron transport reducing the PQ pool, a proposed trigger for transition to state 2 (Olive *et al.*, 1986). Structural evidence that state 2 and the dark represent the same state has been provided by electron microscopic data (Olive *et al.*, 1986) and linear dichroism spectroscopy (Bruce and Biggins, 1985; Brimble and Bruce, 1989). Large redox-induced state transitions have been reported in cyanobacteria under conditions designed to reduce or oxidize PQ. An extreme state 2 is reached under dark anaerobic conditions (used to inhibit PQ oxidation by cytochrome oxidase) and an extreme state 1 in cells preilluminated in the presence of DCMU (Olive *et al.*, 1986). Dominy and Williams (1987) have shown a strict correspondence between light-induced and redox-induced state transitions in Synechococcus sp. PCC 6301 which is best explained by a competition between PS1 and cytochrome oxidase for electrons from PQ and/or the cytochrome b_6/f complex.

The changes in 77K fluorescence emission spectra observed here between dark-adapted cells and cells preilluminated in the presence of DCMU indicate that the wild-type is capable of large redox-induced state transitions. The dark-adapted state was found

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to be characterized by a low F695/F720, indicative of state 2. This would suggest, in terms of a model for the state transition involving control by the redox state of PQ and/or the cyt b_6/f complex, that these electron carriers were reduced in the dark. Cyt $\underline{f}$ has been shown to be reduced in the dark in Synechococcus sp. PCC 7002 (Myers, 1986). The dark reduction was proposed to be by respiratory electron transport via an NADH and/or NADPH dehydrogenase and the rate of oxidation of PQ and/or cyt b_6/f via cytochrome oxidase was assumed to be slower than the reduction rate (Myers, 1986). In the same study it was found that DCMU partially inhibited the dark reduction of cyt $\underline{f}$. These results are in agreement with our finding that the addition of DCMU in the dark causes a slight increase in F695/F720 as an inhibition of the dark reduction would allow the cytochrome oxidase activity to oxidize partially PQ and/or cyt b_6/f.

Kinetics

The kinetics of the state transitions in the wild-type are comparable with other studies already done. The half-times of ten seconds for both the state 1 and state 2 transition are somewhat slower than observed in previous studies (Ried and Reinhardt, 1980; Biggins and Bruce, 1985). While it is often difficult to compare studies done under different conditions one probable explanation for the apparently slower kinetics is the fact that dark rather than light 2 was used in the present study to induce state 2. This may be consistent with the study of Catt *et al.* (1984) who showed in green

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algae that the light state 2 transition had a half-time of one minute while the dark transition had a half-time of seven minutes.

Some effort has been made in the past to determine whether changes in energy distribution between the two photosystems occur in response to changes in the light quality using techniques other than fluorescence emission. One way in which to do this has been to measure either cyt $\underline{f}$ or P-700 kinetics. The principle of this is that redistribution of energy between the photosystems should alter the rates of oxidation and reduction of these components under light-limiting conditions. In the presence of DCMU (to negate any electron transfer from PS2 to PS1) cells adapted to state 2 should show faster cyt $\underline{f}$ oxidation times than state 1 adapted cells when activated by PS2 light according to the spillover model. However, the results here show the opposite trend, oxidation times were faster in state 1 than state 2. This was the case whether PS1 or PS2 light was used although the effect was greater using the 590 nm light, exciting the PBS.

One of the problems with using cyt $\underline{f}$ is that it is not directly oxidized by photo-excited PS1. It is therefore possible that changes in electron transfer characteristics between the cyt b_6/f complex and PS1 may result as well as the changes in energy distribution. To avoid this possibility it was decided that a measure of P-700 oxidation kinetics could alleviate this problem. P-700 is the first electron donor in PS1 and thus is directly oxidized by the light. However, as the results clearly show, the same trend was observed and the difference was even greater. Oxidation was fastest in state 1 and the extent was greater. In addition the difference was minimal

when light became saturating. This result is inconsistent with other results which have shown in cyanobacteria and red algae that the rate of PS1 oxidation driven by light absorbed by the PBS is dependent on the state transition and does increase in state 2 (Biggins, 1983; Fork and Satoh, 1983). While it may be argued that the PBS dissociation model is consistent with these results as the PBS decouples from both PS2 and PS1 this is not the case. These results are also observed with light selected to excite Chl a; the PBS dissociation model predicts the same result as the spillover model. The results with Synechococcus 7002 are not tenable with any of the current models. It is possible that the dark adaptation has some affect on electron transport rates which is independent of the state transition and the results shown here support this. Apparently with increasing time in the dark net oxidation of P-700 is partially inhibited. While this may represent a direct inhibition of P-700 it would appear to be more likely that this represents a change in the dark reduction rate. It must be kept in mind when performing these types of measurements that net oxidation of P-700 represents the combined oxidation of P-700 and its re-reduction through the respiratory pathway. The fact that a net oxidation of P-700 is observed indicates that the photo-oxidation rate is faster than the respiratory reduction rate. Increasingly slower rates of P-700 oxidation may indicate an increasing rate of reduction. This represents a problem with this type of measurement that has not been considered in previous studies. The solution to this problem is to use single turnover flashes with a short enough pulse that the re-reduction step does not have time to occur on the time scale of the

measurement. This type of experiment would give a direct number of P-700 centres turned over with each flash as a result of the pre-illumination conditions rather than a net oxidation rate which would require further analysis to make any conclusions.

An interesting study on various red algae (Rehm *et al.*, 1989) showed that at limiting light 1 intensities the photooxidation rate of both cyt f and P-700 was reduced by 40-60% after preillumination with light 1 as compared to light 2. The results could not be attributed to the effect of the state transition as the PS1 light used (699 nm) is absorbed almost completely by PS1 (95%). While a small change should be observed with this wavelength as a result of the state transition the size of this effect is too large. They found the effect to be abolished when DCMU was added prior to the pre-illumination, however this is not surprising as the light transition to state 2 would also be inhibited under these conditions. As mentioned above, however, this study (Rehm *et al.*, 1989) did not consider the possibility of a change in the re-reduction rate which could occur, either by cyclic electron transport or chlororespiration.

It has been assumed in higher plants that the redox state of the PQ pool acts as the trigger for the state transition, apparently controlling the activity of the kinase responsible for phosphorylating the LHC chl a/b complex. A study by Wollman and Lemaire (1988) showed that mutants deficient in the cyt b_6/f complex were unable to regulate energy distribution. This suggests that either some component of the cyt b_6/f complex or some interaction with the complex may trigger the kinase. To date, however, no attempt has been made to correlate the kinetics of the state transition with

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changes in the redox state of components of the photosynthetic electron transport chain. It has been shown that the respiratory electron transport chain intersects the photosynthetic chain as shown in Figure 2. Studies with inhibitors suggest that PQ may also be the trigger in these organisms (Mullineaux and Allen, 1986). However, it is technically difficult to measure changes in the redox state of PQ as the absorption change occurs at 263 nm. Alternative means of measuring the change are indirect (McCauley and Melis, 1986). The results clearly show that the cyt $\underline{f}$ kinetics are considerably faster than the state transition kinetics (see Figures 11, 12 and Table 6), however this does not necessarily mean that cyt $\underline{f}$ is not the trigger. Several steps would be required between the change in the redox state and the change in the fluorescence emission including the actual physical movement of the pigment-protein complexes necessary for the state transition. The kinetics of the state transition should correlate with the rate-limiting step. An indepth study of the cyt $\underline{f}$ kinetics with the state transition would be required, using changes in light intensity and inhibitors to ascertain whether cyt $\underline{f}$ may have a role in the triggering of the state transition. However it has been shown that cells poisoned with DBMIB are unable to perform the state 1 transition yet cyt $\underline{f}$ still reduces in the dark, indicating that cyt $\underline{f}$ reduction can be separated from the transition to state 2 (D. Bruce, personal communication).

State Transitions in PBS-less Mutant

A comparison of dark-adapted cells and cells preilluminated in the presence of DCMU suggests the operation of a redox-induced

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state transition mechanism in the PBS-less mutant cells. However, the addition of DCMU to dark-adapted cells caused an increase in F695/F720 to the same level as that in illuminated, DCMU-poisoned cells. This result is in contrast to that for wild-type cells which only showed a partial transition to state 1 after DCMU treatment in the dark. One possible explanation in keeping with a redox control of the state transition is a lower reduction rate or higher oxidation rate of PQ and/or cyt b_6/f in the mutant as compared to the wild-type. Under these conditions the partial DCMU inhibition of the reduction rate could be enough to make the reduction rate slower than the oxidation rate in the dark. This idea is supported by the experiments with KCN-poisoned PBS-less cells. In the presence of the cytochrome oxidase and/or plastocyanin inhibitor, the addition of DCMU in the dark no longer causes the cells to go to a high F695/F720 state (state 1). In contrast, the KCN-poisoned and DCMU-poisoned PBS-less cells respond in a fashion similar to the DCMU-poisoned wild-type cells. Illumination of the cells, causing oxidation of PQ and/or cyt b_6/f by PS1 activity, induces transition to state 1 and dark adaptation, causing reduction of PQ and/or cyt b_6/f by respiration, induces transition to state 2.

These results indicate that <u>Synechococcus</u> sp. PCC 7002 does not require the presence of the PBS to be competent to undergo redox-induced state transitions. More convincing, however, are the light-induced state transitions observed in the mutant. Even though the excitation spectra show that the pigments contributing to the PS2 and PS1 antenna were very similar it was possible to induce small reversible light-induced changes in F695/F720 by careful selection

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of preillumination wavelength. No claim is made concerning the physiological significance of the state transition in the PBS-less mutant. The pigment complements of PS2 and PS1 are so similar that preferential excitation of either one is extremely difficult to accomplish and would not likely occur in a natural environment. However, even though the autotrophic growth rates of the PBS-less mutant are slow compared to the wild-type, the mutant is photosynthetically competent and the mechanism of the state transition appears intact.

The ability of the PBS-less mutant to undergo reversible, light-induced state transitions is convincing evidence that the PBS is not required in the mechanism of the state transition in <u>Synechococcus</u> sp. PCC 7002. These data do not support models for the state transition in PBS-containing organisms which depend on phosphorylation of the PBS and its subsequent dissociation from PS2 (Allen *et al.*, 1985; Mullineaux and Allen, 1988).

<u>Phosphorylation</u>

Further evidence against the phosphorylation models is provided here. The results indicate that, at the resolution of one-dimensional electrophoresis of whole cell extracts, no obvious differences are apparent in phosphorylated protein levels between cells in states 1 or 2. This suggests that a phosphorylation event may not be a necessary requirement for the state transition. While this result is consistent with that of Biggins *et al.* (1984a) it is in contrast to the study of Allen *et al.* (1985) (described in Literature Review section **Phosphorylation**). However, an important aspect of the

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methodology of the present study different from some previous phosphoprotein analyses is the manner in which the state transition is conserved in the cells. In Allen *et al.* (1985) cells adapted to states 1 or 2 were incubated in the presence of lysozyme for 90 minutes. Such treatment is known to impair photosynthetic function and cause the dissociation of the PBS from the membrane. It is an unlikely assumption that the state transition has remained intact in cells subject to these conditions. No experiments were performed using low temperature fluorescence emission to ascertain whether the state transition was indeed intact after the lysozyme treatment. In the present study, as in that of Biggins *et al.* (1984a), cells which were in state 1 or 2 were quickly killed by precipitation in ice-cold acetone. As a further restraint in this study cells were precipitated in the presence of the phosphatase inhibitor NaF to ensure that no phosphatase activity remained after the acetone wash. While this may have been a problem in the Biggins et al. (1984a) study they could not demonstrate any non-specific phosphatase activity as measured through the addition of an exogenous histone substrate. The present study was also an improvement on the previous one (Biggins et al., 1984a) as an enhancement of intracellular and presumably thylakoid proteins were extracted from acetone-killed cells through a subsequent lysozyme incubation. This results in greater confidence that the phosphoprotein profiles are indicative of whole cell profiles.

There are some general similarities between the whole cell phosphoprotein profile of the cyanobacterium examined in this study and the red alga P. cruentum (Biggins *et al.*, 1984a). At least 14

bands could be resolved in this study while 12 were observed in the red alga. Some similarities in the size of the phosphoproteins could also be drawn between the two studies, with at least six bands of similar molecular weight. These were the 21, 39, 42, 50, 61.5, and 68.5kD bands. Although no attempt has been made to identify these polypeptides the 30.5 kD could conceivably correspond to the 32kD Q_B peptide of PS2. While a light-dependent phosphorylation of this protein has been shown in the red alga P. cruentum (Kirschner and Senger, 1986) it is not likely that this light-dependency could be observed under the conditions in this study. The light treatment and dark treatments were only four minutes and would not likely lead to large differences between the phosphorylation level of this protein. The light-dependent phosphorylation of the Q_B protein in the red alga and LHC-containing organisms is thought to be independent of the state transition but instead is part of the degradation process known to occur. It is somewhat surprising that no band corresponding to the 32 kD protein could be observed in the Biggins *et al.* (1984a) study as in the Kirschner and Senger study (1986). However, phosphorylation of the 32kD protein was not observed in the cyanobacterium Fremyella diplosiphon (Schuster *et al.*, 1984). In this study thylakoids were isolated from labelled cells and examined for phosphoproteins. A polypeptide of 14.5kD as well as a doublet of 21kD were observed. This doublet may correspond to the phosphoproteins of similar molecular weight observed in this present study. While polypeptides of this approximate weight are present in the PBS it is of interest to note that in both F. diplosiphon and P. cruentum no phosphorylation of the PBS components could be seen.

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Conclusions

Several conclusions can be drawn from this study. Firstly, the cyanobacterium Synechococcus sp. 7002 is capable of performing both light-induced and redox-induced state transitions as has been shown in other cyanobacteria (Dominy and Williams, 1987). In the presence of DCMU cells under illumination undergo transition to state 1 while cells in the dark go to state 2. In addition it has been shown that the cytochrome oxidase inhibitor KCN induces an exaggerated state 2 in the dark but does not effect the state 1 transition in the light. Thus an even larger transition occurs when both DCMU and KCN are used.

As a result of experiments with a PBS-less mutant of this cyanobacterium it is clear that the presence of the PBS is not a necessary requirement for cells to perform a state transition. This refutes both the mobile PBS and PBS dissociation models which presume that a phosphorylation event of a PBS component is a necessary requirement for transition to state 2.

Results with measurements of cyt $\underline{f}$ and P-700 oxidation are inconsistent with low temperature fluorescence measurements and suggest a problem with classical measuring techniques. It is suggested that a proper analysis of cyt $\underline{f}$ or P-700 oxidation kinetics can only be achieved through the use of single turnover flashes.

The kinetics of the state transition are somewhat slower than that observed in other PBS-containing organisms. However, the other studies involved measurements of light-induced rather than redox-induced transitions. As well, the redox state of cyt $\underline{f}$ is likely not the trigger for the state transition.

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Lastly, no correlation between a change in protein phosphorylation and the state transition could be observed in this cyanobacterium. The experimental design used in the present study has ensured that no phosphatase activity is present and that an increased yield of intracellular proteins has been extracted over previous whole cell solubilization techniques (Biggins *et al.*, 1984a). Some caution must be used however as there were limits to the phosphorylation analysis. Only proteins in the approximate molecular weight range of 20 kD to 100 kD could be resolved. It is also possible that a minor component in this range may show changes in phosphorylation between states but cannot be clearly seen due to overlapping bands. It is crucial that two-dimensional electrophoresis be carried out before more critical conclusions can be made. This tentative result is consistent with other studies in cyanobacteria and red algae but is in contrast to the study of Allen *et al.* (1985). All of these results are most consistent with the spillover model for the state transition and refute the PBS dissociation and mobile PBS models.

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Appendix A

Calculation of Error in Low Temperature Fluorescence Emission Measurements

A sample of 10 ml of cells were stirred under room light for 5 minutes. After this time 5 samples of 75 uL were removed, frozen and fluorescence emission spectra taken as described in **Materials and Methods**. The ratio of the PS2 peak (695 nm) to the PS1 peak (720 nm) was measured for each sample. This was repeated five times to ensure that each time the same sample was measured the pipette was in a slightly different position with respect to the detector. The ratios were collected and the standard deviation was found to be 1.7% for the 25 measurements. The same experiment was repeated with a different sample of cells on another day. The standard deviation of these measurements was 2.0%. Thus an error of 2% was assumed for all ratio calculations.

Calculation of Error in Cyt f and P-700 Oxidation Kinetics

Below is a hypothetical oxidation curve for P-700.
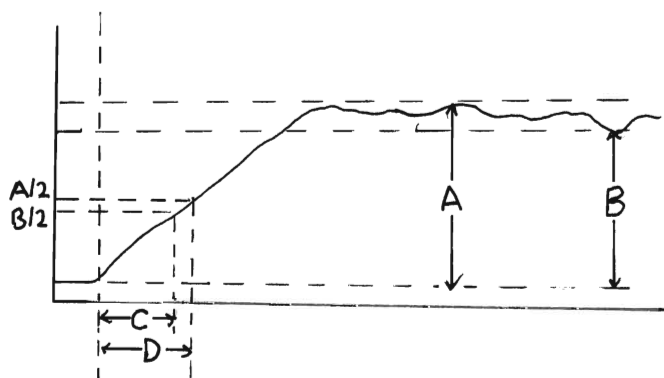
The distance A is the measured maximum extent while distance B is the minimum extent. From these measurements the extent is determined to be:

$(A+B)/2$ +/- $(A-B)/2$. To determine the half-time for the oxidation curve the following is done. The value on the x-axis (time) corresponding to a y-value (extent) of $A/2$ is taken from the curve. This is then repeated for the extent $B/2$. These values are labelled C and D respectively. The half-time measurement is then taken to be: $(C+D)/2$ +/- $(C-D)/2$.

Appendix B

Composition of A+ Medium

Chemical	Final Concentration (g/L)
NaCl	18.0
KCl	0.6
NaNO$_3$	1.0
KH$_2$PO$_4$	0.05
MgSO$_4$.7H$_2$O	5.0
CaCl$_2$	0.272
NaEDTA	0.03
FeCl$_3$.6H$_2$O	0.00389*
P1 metals	1 ml of stock**
TrisHCl pH 8.2	10 mls of 100 g/L stock
Vitamin B12	0.008

*Use 1 ml of a 3.89 g/L stock made up in 0.1N HCl.
**P1 Metals Stock:

H$_3$BO$_3$	34.26
MnCl$_2$.4H$_2$O	4.32
ZnCl$_2$	0.315
MoO$_3$(85%)	0.03
CuSO$_4$.5H$_2$O	0.003
CoCl$_2$.6H$_2$O	0.012