An investigation into the functional role of the D1:1 and D1:2 polypeptides in photosystem II in cyanobacteria. The effect of changing PSI/PSII ratio on photoinhibition in

Synechococcus sp. PCC 7942

By

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Abbreviations:

PPFDs, photosynthetic photon flux density.

q_N, dissipation of excitation energy non-photochemically as heat or energy transfer to an adjacent pigment molecule or system.

$\emptyset psII$, photochemical yield of photosystem II electron transport, (the difference in fluorescence yield at maximum and steady-state fluorescence levels, divided by maximum fluorescence).

F_V'/F_M', the efficiency of excitation energy capture (trapping efficiency of the excitation energy) by open PSII reaction center , F_V' is a variable fluorescence yield ($F_{max.}-F_{min.}$) and F_M' is a maximum fluorescence yield)

Z, redox active tyrosine residue, primary electron donor to photosystem II.

P680, photosystem II reaction center chlorophyll dimer.

$P680^*$, the excited form of P680.

Phe, pheophytin.

Q_A and Q_B, quinone electron acceptors in photosystem II.

$Cytb_6f$, cytochrome b_6f complex.

PC, plastocyanin.

P700, photosystem I reaction center chlorophyll dimer.

$P700^*$, the excited form of P700.

A_0, chlorophyll primary electron acceptor of photosystem I

A_1, vitamin K1 a phylloquinone, electron acceptor of photosystemI.

FeS_x, FeS_b, and FeS_a, membrane-bound iron-sulfur proteins.

F_d, ferredoxin.

CF_1, the head piece of the ATP synthase enzyme

CF_0, the base piece of the ATP synthase enzyme

F_p, flavoprotein ferredoxin-NADP reductase.

NADP, nicotinamide adenine dinucleotide phosphate.

PSII, photosystem II.

PSI, photosystem I.

Abstract

The cyanobacterium *Synechococcus* sp. PCC 7942 (*Anacystis nidulans* R2) adjusts its photosynthetic function by changing one of the polypeptides of photosystem II. This polypeptide, called D1, is found in two forms in *Synechococcus* sp. PCC 7942. Changing the growth light conditions by increasing the light intensity to higher levels results in replacement of the original form of D1 polypeptide, D1:1, with another form, D1:2. We investigated the role of these two polypeptides in two mutant strains, R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present) in cells with either high or low PSI/PSII.

R2S2C3 cells had a lower amplitude for 77 K fluorescence emission at 695 nm than R2K1 cells. Picosecond fluorescence decay kinetics showed that R2S2C3 cells had shorter lifetimes than R2K1 cells.

The lower yields and shorter lifetimes observed in the D1:1 containing cells suggest that the presence of D1:1 results in more photochemical or non-photochemical quenching of excitation energy in PSII. One of the most likely mechanisms for the increased quenching in R2S2C3 cells could be an increased efficiency in the transfer of excitation energy from PSII to PSI.

However, photophysical studies including 77 K fluorescence measurements and picosecond time resolved decay kinetics comparing low and high PSI/PSII cells did not support the hypothesis that D1:1 facilitates the dissipation of excess energy by energy transfer from PSII to PSI.

In addition physiological studies of oxygen evolution measurements after photoinhibition treatments showed that the

two mutant cells had no difference in their susceptibility to photoinhibition with either high PSI/PSII ratio or low PSI/PSII ratio. Again suggesting that, the energy transfer efficiency from PSII to PSI is likely not a factor in the differences between D1:1 and D1:2 containing cells.

A c k n o w l e d g m e n t s

I wish to express my appreciation and thanks to my supervisor prof. D. Bruce. His numerous suggestions and help gave me the chance to successfully accomplish this work in his lab and allowed me to present this work in this form. I would like also to extend my thanks to Dr. Sergej Vasil'ev for his valuable suggestions. I would like to thank also the Department of Biological Sciences for their help and support.

Introduction

Cyanobacteria are prokaryotes which share with plants the ability to utilize light energy to do photosynthesis. Thylakoid membranes in cyanobacteria are similar to those found in higher plants. Many protein complexes in these organisms have been isolated and characterized. Some of these protein complexes are involved in the first part of the photosynthetic activity called light reactions. In these reactions light energy is used to drive electron transfer. The main compounds produced are NADPH and ATP.

Photosystem II (PSII) contains the major components involved in the first part of the light reactions which can be summarized as the light-driven oxidation of water and reduction of a primary quinone electron acceptor.

The PSII reaction center contains two structurally similar but functionally different polypeptides, D1 and D2, besides many other components. Within the thylakoid membrane the D1 and D2 exist as a heterodimer and they house the active components involved in the primary charge separation.

One of the unique features in the cyanobacteria which makes them distinct from other photosynthetic organisms is that they possess small multigene families that code for two distinct forms of the D1 polypeptide. In the genome of the cyanobacterium *Synechococcus* sp. PCC 7942 there are three genes for the D1 polypeptide. One of them (called *psbAI*) codes for the first form of the D1 polypeptide, D1:1, and two other genes called *psbAII* and *psbAIII* code for the other form, D1:2. The two forms of D1 differ in only 25 of the total 360 amino acids, and 12 of these differences occur in the first 16 amino acids and 7 within the putative transmembrane helices II and III (Clarke *et al.*

1993b). The functional significance of the differences between D1:1 and D1:2 are unclear.

Inactivation mutants have been prepared by Susan Golden (Golden et al. 1986), the R2S2C3 mutant contains only D1:1 and the R2K1 mutant contains only D1:2. From that time up until the present a number of studies have been done on these two strains to investigate the functional roles of D1:1 and D1:2 (Bustos 1990; Krupa 1991; Kulkarni 1992; Clarke 1993a,b).

From chlorophyll a fluorescence studies Clarke et al. (1993), suggested that open PSII reaction centers in the R2K1 cells were approximately 25% more efficient in trapping excitation energy than R2S2C3.

Photoinhibition studies also demonstrated that induction of D1:2 polypeptide synthesis during high light stress was essential for limiting the extent of photoinhibition as well as facilitating a rapid recovery of photosynthesis (Krupa et al. 1991; Clarke et al. 1993a; Golden et al. 1996; Campbell et al. 1996).

The main conclusion drawn from the previous observations was that PSII reaction centers containing D1:2 have a higher intrinsic resistance to photoinhibition and are more photochemically efficient than PSII centers with D1:1. These observations were consistent with the transient increase in expression of D1:2 under transition to light intensities higher than the normal growth light intensities.

Recently, Campbell et al. (1998) found that the rapid exchange of the two forms of D1-polypeptide D1:1 and D1:2 in *Synechococcus* is related directly to increased cellular resistance to UV-B inhibition of photosystem II activity and photosynthetic electron transport. Exposing the cells to increased UV-B results in

replacement of D1:1 with the D1:2 form. The D1:2 remains until UV-B is decreased when the D1:2 is replaced by D1:1.

Regarding the observed higher photochemical efficiency and photoinhibition resistance in PSII reaction centers containing D1:2, many mechanisms could explain the possible pathways by which PSII reaction centers are able to dissipate excess excitation energy. One of the possible mechanisms is dissipation of excess excitation energy non-photochemically.

Non-photochemical quenching (q_N) results in dissipation of excess excitation energy by different pathways other than photochemistry such as heat dissipation or transferring the excitation energy to an adjacent pigment system such as energy transfer from PSII to PSI. Non-photochemical quenching is in competition with fluorescence and photochemistry.

One of the possible mechanisms which increases non-photochemical quenching of PSII could be the transfer of excitation energy from PSII to PSI. If that is the case then the differences in non-photochemical quenching between R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present) should be dependent on the cellular PSI/PSII ratio. The objective of this thesis was to test the possibility that differences between the two mutants R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present) would be dependent on the PSI/PSII ratio. Changes in PSI/PSII ratio were successfully accomplished by growing the cyanobacterium *Synechococcus* sp. PCC 7942 under two different light conditions: white light of 55 μmol photons m^{-2} s^{-1} (high PSI/PSII ratio); and red light of 90 μmol photons m^{-2} s^{-1} (low PSI/PSII ratio). Photosynthetic measurements include photophysical measurements (77 K fluorescence measurements, picosecond time resolved fluorescence decay kinetics and pulse amplitude modulated fluorescence

measurements). Also physiological measurements were done of oxygen evolution measurements after photoinhibitory treatments.

Literature   review

Z-scheme (photosynthetic electron transport chain in PSI and PSII):   There is much evidence which has led to the discovery of two structurally separated and functionally related photosystems, one called PSI and the other called PSII.   Many chemical reactions are involved in electron transfer during photosynthesis, these reactions take place within and between those two photosystems.

The   coordinated   electron   transport   by   the   two photosynthetic reaction centers allows for reduction of $NADP^+$ to NADPH.   ATP synthesis depends on the electron transport chain, generating a proton gradient across the thylakoid membrane. ATP is made using the proton motive force, via an ATP synthase enzyme.   The enzyme consists of a head piece (CF_1) which contains the catalytic side of the enzyme and a base piece (CF_0) which is embeded in the thylakoid membrane and forms a proton channel.   ATP is synthesized as protons move down the H^+ electrochemical gradient through the ATP synthetase.

Fig. 1, shows the Z-scheme in which the electron carriers which function in electron flow from H_2O to $NADP^+$ are arranged according to their midpoint redox potentials (Kirk 1994).

Photosystem II (PSII):

The PSII reaction center of cyanobacteria is a multisubunit protein complex of numerous polypeptides with a large number of membrane spanning regions.   PSII is called the water oxidase-plastoquinone reductase by many researchers because it is involved in the first part of the photosynthetic light reactions which result in the oxidation of water and the reduction of the

Figure 1:   Diagram shows the electron flow in Photosystem I and Photosystem II in the Z-scheme for oxygenic photosynthesis (adapted from Kirk 1994).
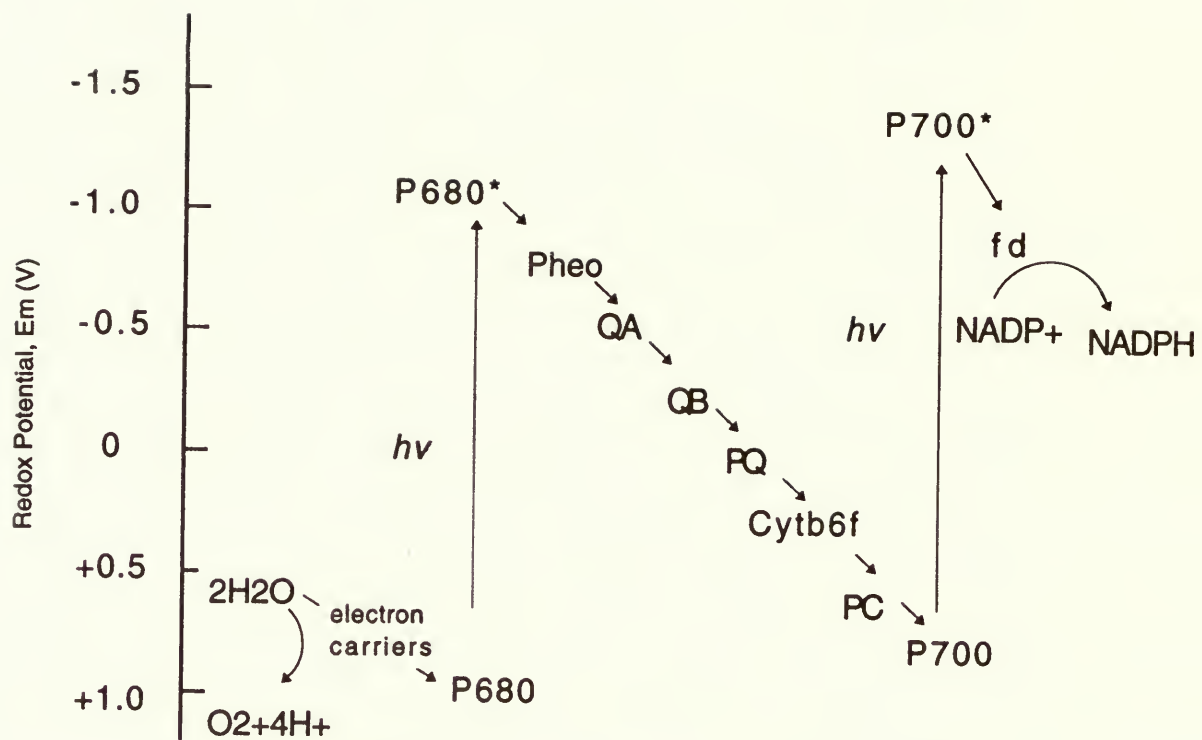
plastoquinone electron acceptor. PSII (the light harvesting complex II) holds

half of the chlorophyll in nature, and therefore harvests half of the light energy that is converted to chemical energy in photosynthesis.

In cyanobacteria there are thylakoid structures like the structures found in chloroplasts. In these organisms, PSII is attached to unique light harvesting antenna called phycobilisomes (PBS) (found also in red algae). Phycobilisomes are macromolecular structures containing between 300 and 800 tetrapyrrole chromophores which absorb light over much of the visible spectrum. Mainly they absorb light in portions of the visible spectrum that are poorly utilized by chlorophyll and, through energy transfer, convey the energy to chlorophyll at the photosynthetic reaction center. Phycobilisomes transfer the excitation energy to PSII reaction center at nearly 100% efficiency (Glazer 1984). In this section four points will be covered, localization of PSII, composition of PSII, function of PSII, and the photoinhibition phenomena which targets mainly PSII.

Localization of PSII: In cyanobacteria as well as in higher plants the thylakoid membrane contains photosystem II and photosystem I. There are, however, some noteworthy differences in cyanobacteria in that they lack the stroma and grana structures found in chloroplasts, but their thylakoid membranes are otherwise similar to the thylakoid membranes found in higher plants.

Composition of PSII: PSII has at least 30 protein subunits (Barber 1998). Results from many studies (Kirk 1994; Svensson 1996) recently reviewed by (Barber 1998) have shown that the

cyanobacterial PSII core is composed of many protein complexes including, a chlorophyll-binding protein of 47 kDa (CP47), a chlorophyll-binding protein of 43 kDa (CP43), two membrane-spanning hydrophobic proteins D1 and D2 (they form a heterodimer which binds all the redox components involved in electron transport from the primary donor P680 to the primary and secondary acceptors Q_A and Q_B), a 33 kDa extrinsic protein or manganese stabilizing protein (MSP), cytochrome b_{559}, (a heterodimer of two subunits of about 8 and 4 kDa), a 9 kDa (or 12 kDa) protein, cytochrome c_{550}, PSII also contains small polypeptides ($\leq$ 10 kDa).

Function of PSII: Photosystem II (PSII) or the water oxidase complex as many researchers prefer to call it has a number of unique features which distinguish it from many other protein complexes associated with the bioenergetic reactions of photosynthesis and respiration found in plants, animal and bacteria (Barber 1998). PSII consists of many polypeptides, some of them are extrinsic proteins while the other are integral membrane proteins. The polypeptides which are required for oxygen evolution in cyanobacteria are 47 kDa (CP47), the 43 kDa (CP43), D1, D2, the 33 kDa extrinsic protein, and two subunits of a cytochrome b_{559}. Cyanobacterial PSII does not contain the two extrinsic proteins of 24 and 18 kDa found in higher plants. Instead of these two polypeptides, cyanobacteria have two different extrinsic proteins a 9 kDa (or 12 kDa) protein and a cytochrome c_{550}. The role of these two polypeptides is to sequester the calcium and chloride ions that are required for catalysis (Wallace 1989; Shen 1992; Shen 1993; Shen 1997). The CP47 and CP43 bind chlorophyll a and form the inner light-

harvesting complex. These polypeptides are also important in stabilizing the reaction center core and modify quinone binding. The CP47, especially a sequence forming a large hydrophobic loop, is involved in stabilizing the oxygen evolving complex (OEC) (Nugent 1996). The 33 kDa extrinsic protein or manganese stabilizing protein (MSP) proposed function is in influencing the properties of the manganese catalytic site, but the exact machinery by which these effects are mediated is not known (Burnap 1991). The proposed function of cytochrome b_6f is in noncyclic electron transport between the reducing side of PSII and the oxidizing side of PSI from plastoquinol (PQH_2) in the membrane to a soluble electron carrier plastocyanin (PC) or cytochrome c_{553} (Kallas 1994). The possible function of cytochrome b_{559} is providing the electrons for the quenching of $P680^+$ in the PSII reaction center (Thompson et al. 1988; Thompson et al. 1989; Satoh 1993).

Another distinguished feature of PSII which makes it unique among all other protein complexes is the fact that the D1 protein turns over rapidly compared to any other protein in the thylakoid membrane. D1 exhibits a high rate of light dependent turnover, it has been found that the D1 turning over is at a rate 50 to 60 times more rapid than that of the large subunit of carboxylase enzyme or the apoprotein of the chlorophyll *a/b* light-harvesting antennae (Mattoo 1981). Light stress causes damage to PSII, in particular to D1. Once the D1 is damaged it is degraded and digested by at least one serine-type proteinase that is tightly associated with the PSII complex (Aro 1993). The PSII complex is partially disassembled and D1 protein synthesized *de*

novo. The new D1 protein is then inserted into the disassembled PSII complex.

Photoinhibition: Excess light induces photoinactivation and degradation in PSII. This phenomenon is called photoinhibition and it was studied intensively by many researchers. It is well known now that photoinhibition starts by the degradation of one of PSII key components, D1 protein. The D1 protein was found to have a high rate of light dependent turnover (Mattoo 1989). Photoinhibitory light was found to block electron transfer from Q_A^-, the first quinone acceptor, to the secondary quinone acceptor Q_B, resulting in an increase in charge recombination events between $P680^+$ and Phe^-. The increased charge recombination enhances the formation of ($^3P680$ Phe) which may interact with oxygen and generate harmful singlet oxygen (Keren et al. 1997). The main target of singlet oxygen is the D1 protein which degrades and starts the PSII photoinactivation process (called acceptor side mediated photoinhibition) (Yruela 1996). Acidification of the thylakoid lumen as a result of high difference in $_pH$ across the thylakoid membrane is considered another way to cause PSII inactivation. Acidification of the thylakoid lumen may lead to a partial release of Ca^{2+} from the oxygen evolving complex, lower the Q_A redox potential and down-regulate PSII activity (called donor side mediated photoinhibition) (Keren 1997). The inactivation of the oxygen evolving complex (Jegerschold 1990) and the oxidative damage due to the formation of PSII reaction center's long-lived highly oxidizing cation radical (Tyr_z^+ and $P680^+$) would trigger the degradation of the D1 protein inducing inactivation of PSII electron transport activity and oxidation of nearby pigments or redox components (Yruela 1996;

Keren 1997).   Specific D1 cleavage is found to yield a 23.5 kDa polypeptide (Greenberg 1987).   Kettunen et al. 1996 reported that the main cleavage generated an 18 kDa N-terminal and a 20 kDa C-terminal degradation fragment of the D1 protein.   The cleavage site was mapped to be located clearly N-terminally of the DE loop.

Recently studies with *Synechocystis* species PCC 6803 mutants with a modified D-E loop of the photosystem II reaction center polypeptide D1 showed less tolerance to photoinhibition compared with the wild type strain (Mulo 1998).

In conclusion the light-induced damage to PSII correlates directly to the susceptibility of the D1 polypeptide to degrade. The oxidative damage to D1 protein results in conformational changes to D1 structure, thereby making it susceptible to proteolysis.   The damage and subsequent degradation of the damaged D1 polypeptide requires *de novo* synthesis and the incorporation of a new D1 copy into the PSII complex (Mulo 1998).

D1-polypeptide: This polypeptide (~32 KDa) with the D2-polypeptide forms a heterodimer of hydrophobic polypeptides and they house the key components that are involved in electron transfer within PSII.   In plants there is only one form of the D1 polypeptide.   By contrast in cyanobacteria two distinct forms of the D1 polypeptide were found (Golden 1986; Jansson 1987). Form I of the D1 polypeptide (D1:1), is coded by a gene called *psbAI*, whereas form II (D1:2) is encoded by two genes called *psbAII* and *psbAIII*.   The two forms of D1 differ in only 25 of the total 360 amino acids, and 12 of these differences occur in the first 16 amino acids and 7 within the putative transmembrane

Figure 2: Amino acid sequences of the D1:1 polypeptide found in R2S2C3 mutant cells. The amino acid sequence for the D1:2 polypeptide found in R2K1 mutant cells is identical to D1:1 with differences in only twenty five amino acids out of 360 amino acids which comprise the polypeptide. The twenty five different amino acids in D1:2 are presented in parenthesis in their actual positions.

All amino acids are presented by their one letter code.

The one letter code of the amino acids in alphabetical order

A=Ala	M=Met
C=Cys	N=Asn
D=Asp	P=Pro
E=Glu	Q=Gln
F=Phe	R=Arg
G=Gly	S=Ser
H=His	T=Thr
I=Ileu	V=Val
K=Lys	W=Trp
L=Leu	Y=Tyr

NH3+

M T S(T) I(A) L R(Q) E(R) Q(R)R(E) R(S) D(A) N(S) V(L)

W D(Q) R(Q) F C E W V T S T D N R I(L) Y V G W F G V L

M I P T L L T A T I C F I V A F I A A P P V D I D G I R

E P V A G S L M Y G N N I I S G A V V P S S N A I G L H

F Y P I W E A A S L D E W L Y N G G P Y Q L V V F H F

L L(I) G I(V) S(F) C Y M G R Q(E) W E L S Y R L G M R P

W I C V A Y S A P L(V) S(A) A A F(T) A V F L I Y P I G

Q G S F S D G M P L G I S G T F N F M F V F Q A E H N I

L M H P F H M L G V A G V F G G S L F S A M H G S L V

T S S L V R E T T E T E S Q N Y G Y K F G Q E E E T Y N I

V A A H G Y F G R L I F Q Y A S F N N S R S L H F F L

G(A) A W P V V G I W F T S M(L) G I S T M A F N L N G

F N F N Q S V L D S Q G K(R) V I N T W A D V L N R A N

L G M E V M H E R N A H N F P L D L A A G E A T P V A

L T A P S(A) I H(N) G

COO-

helices II and III (Clarke et al. 1993b). Fig. 2, shows the linear sequence of amino acids which constitute the two forms of D1-polypeptide. Those amino acids found in D1:2 which are different from D1:1 are presented in parenthesis (Golden et al. 1986).

Fig. 3, shows the folding pattern of D1:1 and D1:2 polypeptides and the positions of the 25 different amino acids between these two forms of polypeptides. Amino acid number 130 has been suggested to be associated with hydrogen bonding to the redox active pheophytin (Svensson 1996), this position is changed from glutamine in D1:1 to glutamic acid in D1:2.

It has been found that the normally predominant D1:1 polypeptide is rapidly replaced with D1:2 during photoinhibition. The exchange between D1 forms is reversible, and when cells are allowed to recover under normal growth light conditions D1:1 became the major D1 species (Clarke 1993b).

The differential expression of *psbA* genes in response to light was done by insertion of *Escherichia coli* lacz gene between each of the *Synechococcus psbA* genes (Schaefer et al. 1989a). The insertion was in the chromosome of wild-type *Synechococcus* sp. at each of the *psbA* genes loci. The *Escherichia coli* lacz gene was inserted to observe in vivo the expression of beta-Galactosidase activities and to indicate if the differential expression of the *psbA-lacz* gene occurred under different light conditions. Under similar light conditions the expression of *psbAI* (produces D1:1) was 500-fold greater than the expression of *psbAII* and 50-fold greater than *psbAIII* (*psbAII* and *psbAIII* produce D1:2) (Schaefer et al. 1989b).

Decreasing the light intensity from 600 µmol photons m^{-2} s^{-1} to 2 µmol photons m^{-2} s^{-1} increases the *psbAI* expression 8-

Figure 3: Predicted folding pattern for the D1:1 and D1:2 polypeptides found in two mutant strains of *Synechococcus* sp. PCC 7942, R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present). Twenty five amino acids are found to be different between the two polypeptides and they are shown with filled circles. D1:1-Gln130, is suggested to function as a specific binding site to the pheophytin. The specific binding of the redox active pheophytin is mostly defined by the hydrogen bond from D1-Gln130 (Svensson 1996). In D1:2 this amino acid is found to be changed to Glu. Residues conserved between the D1:1 and D1:2 polypeptides are shown in hollow circles.
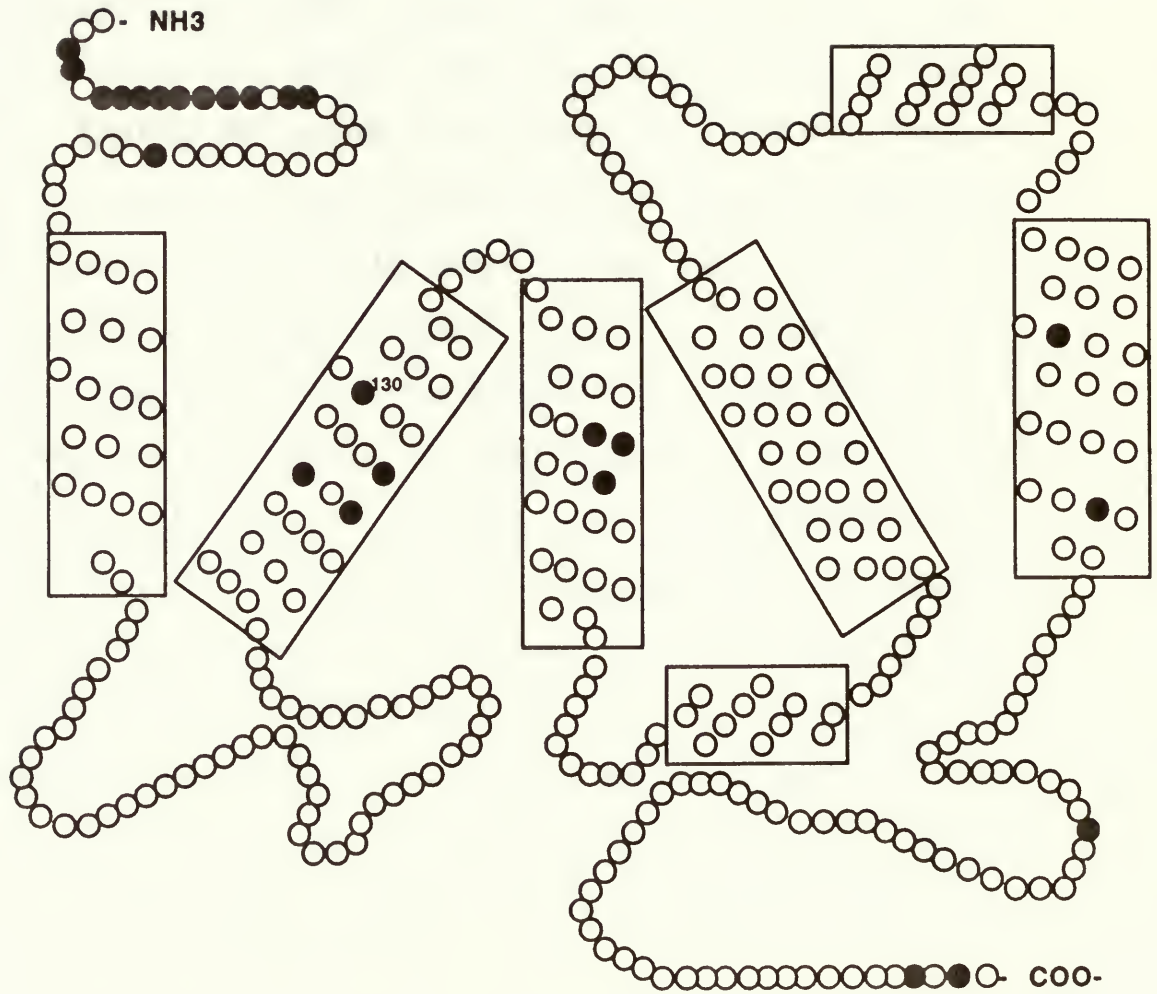
fold whereas the expression of *psbAII* and *psbAIII* decreased 10-fold. The conclusion drawn from the previous results is that the two forms of D1 polypeptide, D1:1 and D1:2 in PSII of the cyanobacterium *Synechococcus* sp PCC 7942 are produced at different rates under different light conditions (Schaefer 1989a,b).

Synechococcus sp PCC 7942 wild type cells showed the same behaviour mentioned above when the experimental protocol changed. Shifting the growth light intensity of 125 μmol photons $m^{-2} s^{-1}$ to higher or lower intensities altered levels of mRNA for the *psbAI, psbAII* and *psbAIII* genes. It has been found that the wild type *psbA* messages respond rapidly to changes of light intensities. *psbAI* message levels increases when cells shifted to a lower light intensity while at higher light intensities *psbAI* message levels decrease dramatically. The opposite happened to *psbAII* and *psbAIII* messages, they decreased when cells shifted to a lower light intensity and increased when cells shifted to a higher light intensities (Bustos 1990).

Knowing that the synthesis of D1:1 and D1:2 is differentially induced over a wide range of light intensities was the starting point for many different studies to find any possible functional differences between D1:1 and D1:2.

Inactivation mutants have been prepared by Susan Golden (Golden et al. 1986), the R2S2C3 mutant contains only D1:1, and the R2K1 mutant contains only D1:2. What are the functional differences between the two forms of D1 polypeptide?.

Results from previous studies:

Interchange between D1:1 and D1:2:

The unicellular cyanobacterium *Synechococcus* sp PCC 7942 contains two distinct forms of D1 polypeptide D1:1 and D1:2. These two forms are encoded by three different genes called *psbA* genes. Changes in light intensity were found to regulate the three genes responsible for the two forms. In low light conditions 80% of the total mRNA messages detected were *psbAI* transcripts. Therefore the only detectable form of D1 polypeptide was D1:1 (Bustos 1990; Kulkarni 1992; Clarke 1993a; Kulkarni 1994; Kulkarni 1995; Golden 1995).

Clarke et al. 1993a, observed that in cells grown under a light intensity of 50 μmol photons m^{-2} s^{-1}, D1:1 represented over 95% of the total D1 protein. D1:2 became the predominant species when cells were shifted to a higher light intensity of 500 μmol photons m^{-2} s^{-1}. Within 1 hr of high light treatment the interchange from D1:1 form to D1:2 form was complete and D1:2 became the predominant species. When cells were allowed to recover under light intensity of 50 μmol photons m^{-2} s^{-1} the interchange from the D1:2 form to the original form D1:1 took about 2 hr and D1:1 became the predominant species again (Clarke 1993a; Clarke 1995).

Degradation of D1:1 and D1:2 proteins:

The D1 protein has a unique feature in that its rate of turnover increases with increasing irradiances (Mattoo 1981; Kyle 1984).

The immuno-blot technique was used to measure the amount of D1:1 and D1:2 polypeptides during different light

treatments (Clarke 1993a; Clarke 1995; Kulkarni 1995). In these experiments the rate of D1:1 and D1:2 degradation was measured. The rate for both D1:1 and D1:2 degradation increases with higher irradiances. It has been found that the degradation rate for both forms was similar even under very high light intensities (e.g. >500 μmol photons m^{-2} s^{-1}). The similarity between D1:1 and D1:2 forms in their differential rate of degradation cannot explain why wild-type cells replace the predominant D1:1 form under normal light conditions with D1:2 during high light stress. Photosynthetic measurements were of crucial importance to test whether any significant differences would exist or not.

Photosynthetic measurements:

By using the two mutant strains, R2S2C3 contains (D1:1 only) and R2K1 contains (D1:2 only), chlorophyll a fluorescence studies showed that the open PSII reaction centers in R2K1 cells were 25% more efficient in capturing excitation energy. This conclusion came from experiments done to measure the efficiency of excitation energy capture (trapping efficiency of the excitation energy) by open PSII reaction center (F_V'/F_M', F_V' is a variable fluorescence ($F_{max.}-F_{min.}$) and F_M' is a maximum fluorescence yield), photochemical yield of photosystem II electron transport which is an estimation for the changes in the quantum yield of non-cyclic electron transport ($\emptyset_{psII}$, defined by the difference in fluorescence yield at maximum and steady-state fluorescnce, divided by maximum fluorescence), and oxygen evolution rates derived from chlorophyll a fluorescence obtained from pulse amplitude modulated (PAM) fluorescence measurements (Clarke *et al.* 1993b).

Photoinhibition measurements:

Photoinhibition studies also demonstrated that the induction of D1:2 polypeptide synthesis during high stress was essential for limiting the extent of photoinhibition as well as facilitating a rapid recovery of photosynthesis in the cyanobacterium *Synechococcus* sp. PCC 7942 (Clarke et al. 1993a). This main functional difference between the two forms of D1 was in agreement with other observations (Krupa et al. 1991; Campbell et al. 1996; Golden et al. 1996).

The conclusion obtained from the previous observations is that there is a clear difference in the photochemical efficiency between the two mutants R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present). The difference between the two mutants is 25 amino acids within the 360 amino acids that comprise the D1 polypeptide. This suggests that some if not all of these differences in the 25 amino acids between D1:1 and D1:2 are significant. Most of the amino acid differences between the two forms D1:1 and D1:2 are located at the amino terminus of the two forms. The other differences are located in helices II and III, 3 differences were found in each helix. In helix V two differences were found. The cytoplasm exposed loop between helices II and III contain one amino acid difference. The other three differences are located on the lumen-exposed carboxyl terminus. The amino acid number 130 is suggested to be associated with hydrogen bonding of the redox active pheophytin (Svensson 1996). This position was found to change from glutamine in D1:1 to glutamic acid in D1:2. Glutamine is an uncharged amino acid but glutamic acid carries a negative charge. The charge stabilization process (electron transfer from Phe$^-$ to Q_A) would definitely be affected by the

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The conclusion obtained from the previous observations is that there is a clear difference in the photochemical efficiency between the two mutants R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present). The difference between the two mutants is 25 amino acids within the 360 amino acids that comprise the D1 polypeptide. This suggests that some if not all of these differences in the 25 amino acids between D1:1 and D1:2 are significant. Most of the amino acid differences between the two forms D1:1 and D1:2 are located at the amino terminus of the two forms. The other differences are located in helices II and III, 3 differences were found in each helix. In helix V two differences were found. The cytoplasm exposed loop between helices II and III contain one amino acid difference. The other three differences are located on the lumen-exposed carboxyl terminus. The amino acid number 130 is suggested to be associated with hydrogen bonding of the redox active pheophytin (Svensson 1996). This position was found to change from glutamine in D1:1 to glutamic acid in D1:2. Glutamine is an uncharged amino acid but glutamic acid carries a negative charge. The charge stabilization process (electron transfer from Phe^- to Q_A) would definitely be affected by the

negatively charged amino acid (Glutamic acid number 130) in the D1:2 polypeptide which is found in R2K1 cells.

Previous studies concluded that the D1:2 polypeptide provides a unique protective mechanism for limiting the severity of photoinhibition as well as facilitating a rapid return to normal photosynthetic activity. The observed higher photochemical efficiency and an increased resistance to photoinhibition in R2K1 cells may be explained by an enhanced ability to dissipate excitation energy compared to R2S2C3 cells. One of the possible mechanisms for the dissipation of excitation energy is energy transfer from PSII to PSI.

In all studies mentioned above R2K1 and R2S2C3 mutant cells were grown in conditions favoring high PSI/PSII ratios. In this study we decided to test the transfer of excitation energy from PSII to PSI as a possible way to explain the differences observed between D1:1 and D1:2 containing cells. We were successfully able to decrease the PSI/PSII ratio and observe the effects on photophysical parameters and physiological parameters.

Data were collected from a series of experiments. These experiments employed 77 K fluorescence spectroscopy, room temperature time resolved fluorescence decay, pulse amplitude modulated (PAM) fluorescence measurements and oxygen evolution measurements.

Materials   and   Methods:

Cells and growth conditions: The two mutants R2S2C3 and R2K1 of the cyanobacterium *Synechococcus* sp. PCC 7942 were grown in BG-11 media (Appendix A) in batches of about 250 ml in 500 ml capacity flasks (Campbell et al. 1996).   In control cells, growth was under a continuous white light supplied by fluorescent lamps with an intensity of about 55 µmol photons m^{-2} s^{-1}.   To decrease the PSI/PSII ratio, cells were grown under red light by covering the flasks with a red cellophane paper.   Also the light source was a tungsten filament bulb, the final intensity was about 90 µmol photons m^{-2} s^{-1}.

Chlorophyll fluorescence measurements: 77 K fluorescence emission spectra were obtained with a spectrofluorimeter based on a 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 486-compatible computer.   Excitation light (10 nm bandwidth) was supplied by a 100W tungsten halogen lamp dispersed by a Jobin Yvon H20 spectrometer (Brimble et al. 1989)

For the determination of 77 K emission spectra, excitation was at 435 nm which is in the chlorophyll absorption region. Samples were prepared to a concentration of 6 µg chlorophyll/ml (the amount of Chl a was assayed by measuring the Chl concentration in 100% methanol) and then incubated in the dark for 10 min to induce state II (state II, low yield of PSII fluorescence).   Samples were then snap frozen in liquid nitrogen and kept in the dark until measurements.   For the representative

fluorescence spectra presented, the absorbance spectra of the two strains were nearly identical.

PSI/PSII ratio: A sum of gaussian distributions was used to fit the emission spectra. Three components were used to model the fluorescence emission spectra with peak emissions at 685, 695, and 715 nm. The areas for the components were used to calculate the PSI/PSII ratios (Murakami 1997).

Room temperature time resolved fluorescence decay kinetics: A single photon timing apparatus which is described in detail in (Bruce et al. 1993), was used to study the room temperature time resolved fluorescence decay kinetics. Laser pulses of about 60 ps duration at a wavelength of 665 nm were generated by a Hamamatsu PLP-01 pulse laser diode. Cell culture suspensions of about 200 mls were used for the experiments when Chl concentration reached 10 μg/ml.

Fluorescence measurements were done on cells pre-adapted in darkness for about 5 min. For each experiment fluorescence decay curves were collected at 675, 680, 685, 690, 695, and 700 nm. Model curve fits were generated by a convolution of the instrument response function with a sum of exponential decay components. The decay curves for all six emission wavelengths were modeled simultaneously using a global fitting routine, to determine the spectral shape of each decay component.

The fitting used an optimized Levenberg/Marquardt algorithm developed by Vasil'ev, S. (1998). Fluorescence was detected at 90° to the excitation. Instrument response functions were measured by setting the emission monochromator to the excitation wavelength (665 nm) and collecting the signal from a dilute milk suspension in water. Fluorescence decay data were

captured in 1024 channels, and collection continued until the peak channel contained 40,000 counts.

Pulse amplitude modulated (PAM) fluorescence measurements: These measurements were done by using a pulse amplitude modulated (PAM), fluorimeter (Walz, Effeltrich, Germany) to measure Chl fluorescence parameters at room temperature. Samples were prepared to a concentration of 6 μg chlorophyll/ml.

Minimal fluorescence (F_O) was determined using low intensity modulated light at 1.6 KHz. Determination of maximal fluorescence (F_M) in the dark adapted state was done by using a 1 s flash of saturating white light (10,000 μmol photons m^{-2} s^{-1}). Steady state fluorescence, was reached by using an actinic light of 52 μmol photons m^{-2} s^{-1}, and the modulated light was switched to 100 KHz for a better signal to noise ratio. F_M' under steady state conditions was determined by a saturating light pulse as described above. Finally, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) was injected into the cuvette (4μM) to stop electron transport, and maximal fluorescence F_M was reached. Data were used to calculate the relative efficiency of open PSII reaction centers (F_V/F_M) where $F_V = F_M - F_O$ (Fig. 17).

To study the susceptibility of cells to photoinhibition, a pulse amplitude modulated (PAM) fluorometer was used to study the sensitivity to photoinhibition. In this study Chl fluorescence parameters at room temperature were measured. The sensitivity to photoinhibition was determined by preilluminating cells for 5 min at light intensities varying from 1000 to 10,000 μmol photons m^{-2} s^{-1}.

Normalized F_V/F_M is plotted versus preillumination intensity for R2S2C3 and R2K1.

Photoinhibitory treatments and oxygen evolution: Cells of two strains of *Synechococcus* sp. PCC 7942, R2S2C3 (D1:1 only) and R2K1 (D1:2 only) were grown under two different light conditions as described previously in growth conditions.

For photoinhibition experiments cell cultures were used when the Chl a concentration reached 2 μg/ml. The cell culture was incubated under low light intensity after a short period of dark adaptation at 30°C. After taking the control samples, the cells were illuminated under PPFDs of 0, 2000, 4000, 6000, 8000, 10000, 12000, 14000, and 16000 μmol photons m^{-2} s^{-1} at 30°C for 5 min for the white light grown cells.

Red light grown cells were illuminated similarly under PPFDs of 0, 4000, 6000, 11000, 18000, 20000, and 25000 μmol photons m^{-2} s^{-1} at 30°C for 5 min. For every treatment a cell culture sample of 4 mls was used.

Photoinhibition treatments which act directly on photoinhibition of PSII were followed by measuring light-saturated oxygen evolution in cells with a Clarke-type electrode chamber connected to a strip chart recorder. The artificial electron acceptors, p-benzoquinone (1 mM final concentration) together with potassium ferricyanide (1 mM final concentration) were added just after photoinhibitory illumination prior to the photosynthetic oxygen evolution measurements.

Results:

77 K fluorescence emission spectroscopy: Three peaks could be distinguished, when the fluorescence emission spectra at 77 K was collected with excitation of chlorophyll at 435 nm. The three peaks appeared at three distinct wavelengths 685, 695, and 715 nm (Figures 4, 5, 6). The emission peaks at 685 nm and 695 nm are associated with PSII antenna chlorophyll and PSII reaction centers respectively whereas the 715 nm peak is associated with PSI (Cleland, 1988)

Comparison of F695/F685 and F695/F715 ratios (obtained from 77 K fluorescence spectra) were done between the two mutant cells R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present) (Table 1). Values show a relative increase in F695/F685 and F695/F715 in R2K1 compared to R2S2C3 (relative increase was expressed as a percentage). In white light grown cells (high PSI/PSII ratio) the F695/F685 ratio was 4.8% $\pm$ 2% higher in R2K1 compared to R2S2C3. In red light grown cells (low PSI/PSII ratio) the same ratio was 18.3% $\pm$ 3% higher in R2K1 compared to R2S2C3 (Table 2).

Comparison of F695/F715 values also show a relative increase in R2K1 compared to R2S2C3. In white light grown cells (high PSI/PSII ratio) the F695/F715 ratio was 13% $\pm$ 2% higher in R2K1 compared to R2S2C3. In red light grown cells (low PSI/PSII ratio) same ratio was 17% $\pm$ 2% higher in R2K1 compared to R2S2C3 (Table 2)

Cells grown under red light had a lower PSI/PSII ratio this can be observed when we look to the three peaks distinguished from the fluorescence emission spectra at 77 K collected with excitation of chlorophyll at 435 nm. By using a decomposition of

Figure 4: Fluorescence emission spectra at 77 K with excitation of chlorophyll at 435 nm. All cells were preadapted to state II by dark incubation immediately prior to freezing. Cells were grown under white light (high PSI/PSII ratio). R2K1 mutant cells (only D1:2 present) have a higher 695 nm peak compared to same peak in R2S2C3 (only D1:1 present).
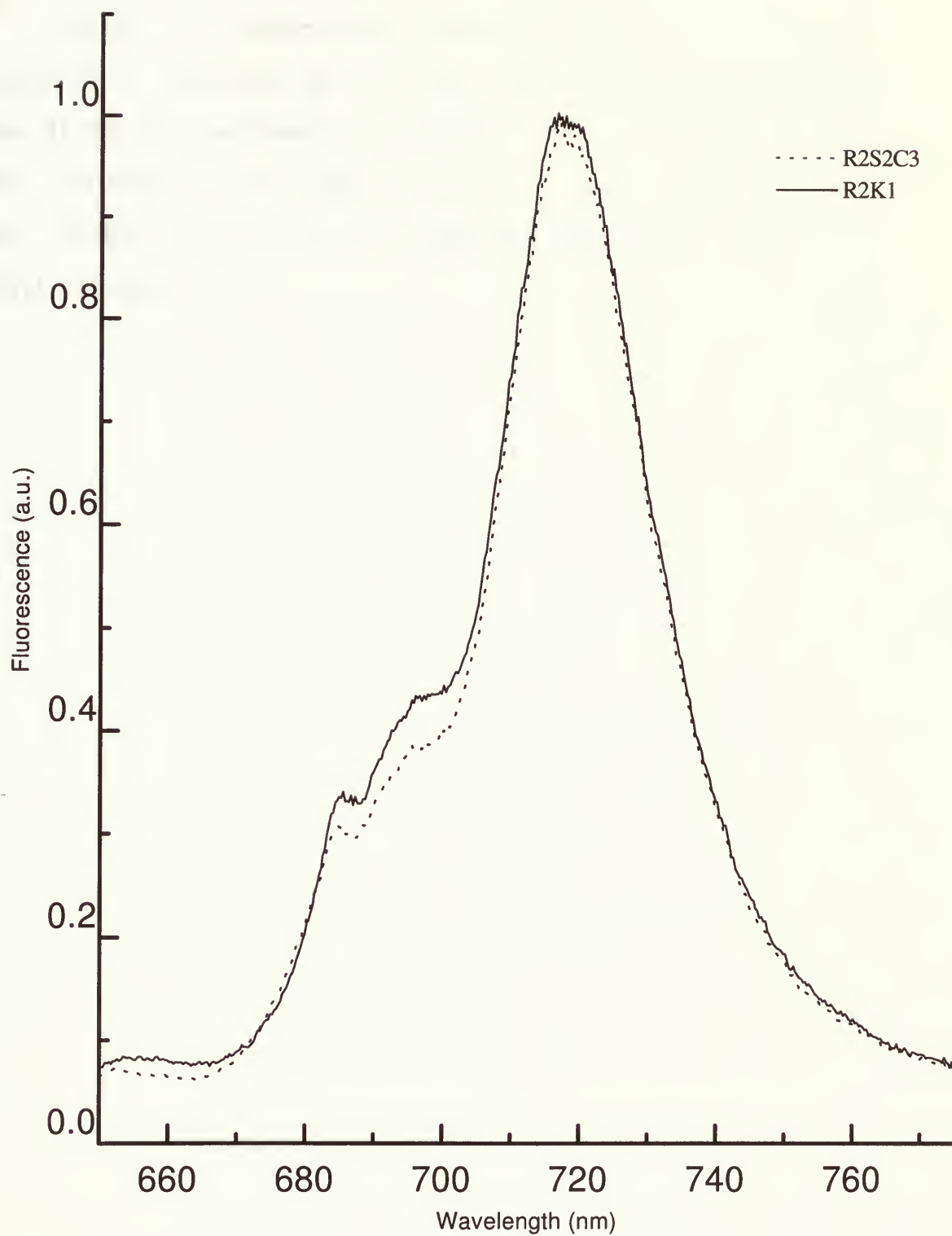
Figure 5: Fluorescence emission spectra at 77 K with excitation of chlorophyll at 435 nm. All cells were preadapted to state II by dark incubation immediately prior to freezing. Cells were transferred to red light for only 1 day to decrease PSI/PSII ratio. R2K1 mutant cells have a higher 695 nm peak compared to R2S2C3 mutant cells.
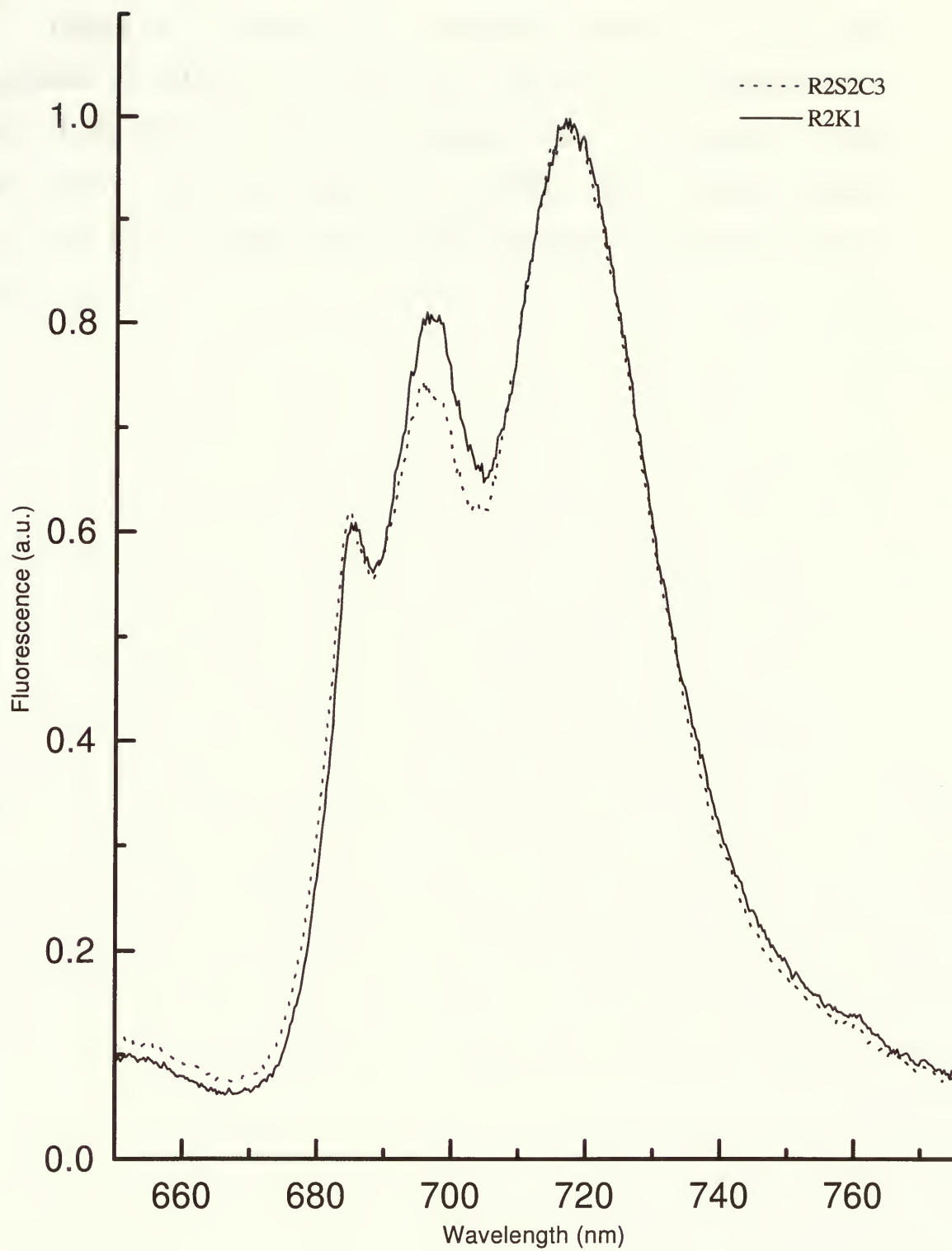
Figure 6: Fluorescence emission spectra at 77 K with excitation of chlorophyll at 435 nm. All cells were preadapted to state II by dark incubation immediately prior to freezing. Cells were grown under red light (low PSI/PSII ratio). R2K1 mutant cells still have a higher 695 nm peak compared to R2S2C3 mutant cells.
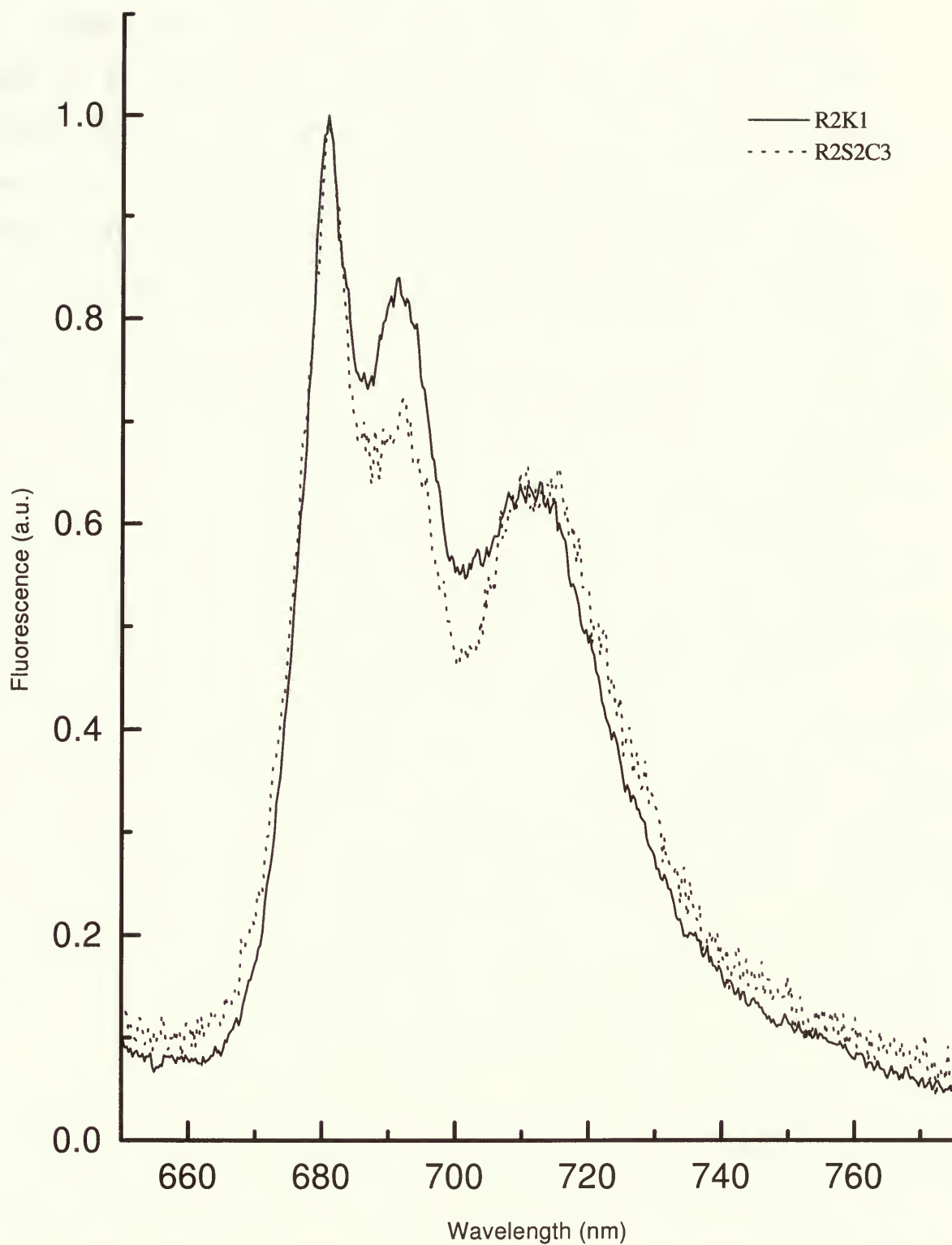
Table (1): (F695/F685) and (F695/F715) ratios (obtained from 77 K fluorescence spectra) between the two mutant cells R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present). Cells were grown under three different light conditions:

-White light at an intensity of 55 μmol photons m^{-2} s^{-1}.

-Transient red light for only 1 day at an intensity of 90 μmol photons m^{-2} s^{-1}.

-Red light at an intensity of 90 μmol photons m^{-2} s^{-1}.

Growth light Condition	Mutant strain	F695/F685	F695/F715
White Light	R2K1	1.29 ± 0.011	0.43 ± 0.002
	R2S2C3	1.23 ± 0.019	0.38 ± 0.005
Red light (1 day after transferring cells to red light)	R2K1	1.33 ± 0.001	0.81 ± 0.001
	R2S2C3	1.20 ± 0.003	0.75 ± 0.002
Red light	R2K1	0.84 ± 0.005	1.31 ± 0.013
	R2S2C3	0.71 ± 0.011	1.12 ± 0.006

Table 2: Comparison of F695/F685 and F695/F715 ratios (obtained from 77 K fluorescence spectra) between the two mutant cells R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present). Values show a relative increase in F695/F685 and F695/F715 in R2K1 compared to R2S2C3 (relative increase was expressed as a percentage).

Growth light Condition	Relative increase in F695/F685 in R2K1 compared to R2S2C3	Relative increase in F695/F715 in R2K1 compared to R2S2C3
White light	4.8% ± 2%	13% ± 2%
Red light (1 day after transferring cells to red light)	10.8% ± 1%	8% ± 1%
Red light	18.3 ± 3%	17% ± 2%

the 77 K fluorescence spectra into Gaussian components and calculating the areas of the three peaks, it was found that the PSI/PSII changed as expected. Cells grown under white light of about 55 μmol photons m^{-2} s^{-1} had a high PSI/PSII ratio, it was 5 in R2S2C3 cells and 5.2 in R2K1 whereas cells grown under red light of about 90 μmol photons m^{-2} s^{-1} showed a dramatic decrease in PSI/PSII ratio. In R2S2C3 the PSI/PSII ratio was 0.8 and in R2K1 the same ratio was 0.9 (Figures 7-12 and Table 3).

Room temperature time resolved fluorescence decay: Fluorescence decay profiles were deconvoluted using a global analysis of the emissions at all six wavelengths. The lifetime of each component is fixed over all detection wavelengths but the amplitude of each component is permitted to vary between wavelengths.

In the global analysis four components have been resolved. Table 4 shows the lifetimes in picoseconds and the amplitudes for the four components resolved. The third component which is related to charge stabilization (electron transfer from Phe$^-$ to Q_A) was found to vary between the two mutants R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present). The three other components showed no significant differences between the two mutants. In high PSI/PSII ratio containing R2S2C3 cells shorter lifetime had been observed for the third component 940ps $\pm$ 190ps, whereas in R2K1 cells the lifetime for the same component was 1200ps $\pm$ 50ps. Low PSI/PSII ratio containing cells showed shorter lifetime for the third component in R2S2C3 cells, 530ps $\pm$ 4ps compared with 670ps $\pm$ 160ps in R2K1 cells.

Amplitudes for the same component (third component) also varied between the two mutants. In high PSI/PSII containing

Figure 7: Gaussian distribution fit for R2S2C3 cells (D1:1 only) grown under white light at an intensity of 55 µmol photons m^{-2} s^{-1}. Three components were used in a Gaussian distribution analysis to model the fluorescence emission spectra, these components (A1, A2, A3) peaked at three distinct wavelengths 685, 695, and 715 nm, respectively. The amplitudes were normalized by setting the largest component to 1.

R2S2C3 (White light)
Model: Gauss

A1= 0.10 ± 0.009
A2= 0.09 ± 0.009
A3= 1 ± 0.008

Figure 8: Gaussian distribution fit for R2K1 cells (D1:2 only) grown under white light at an intensity of 55 μmol photons m^{-2} s^{-1}. Three components were used in a Gaussian distribution analysis to model the fluorescence emission spectra, these components (A1, A2, A3) peaked at three distinct wavelengths 685, 695, and 715 nm, respectively. The amplitudes were normalized by setting the largest component to 1.
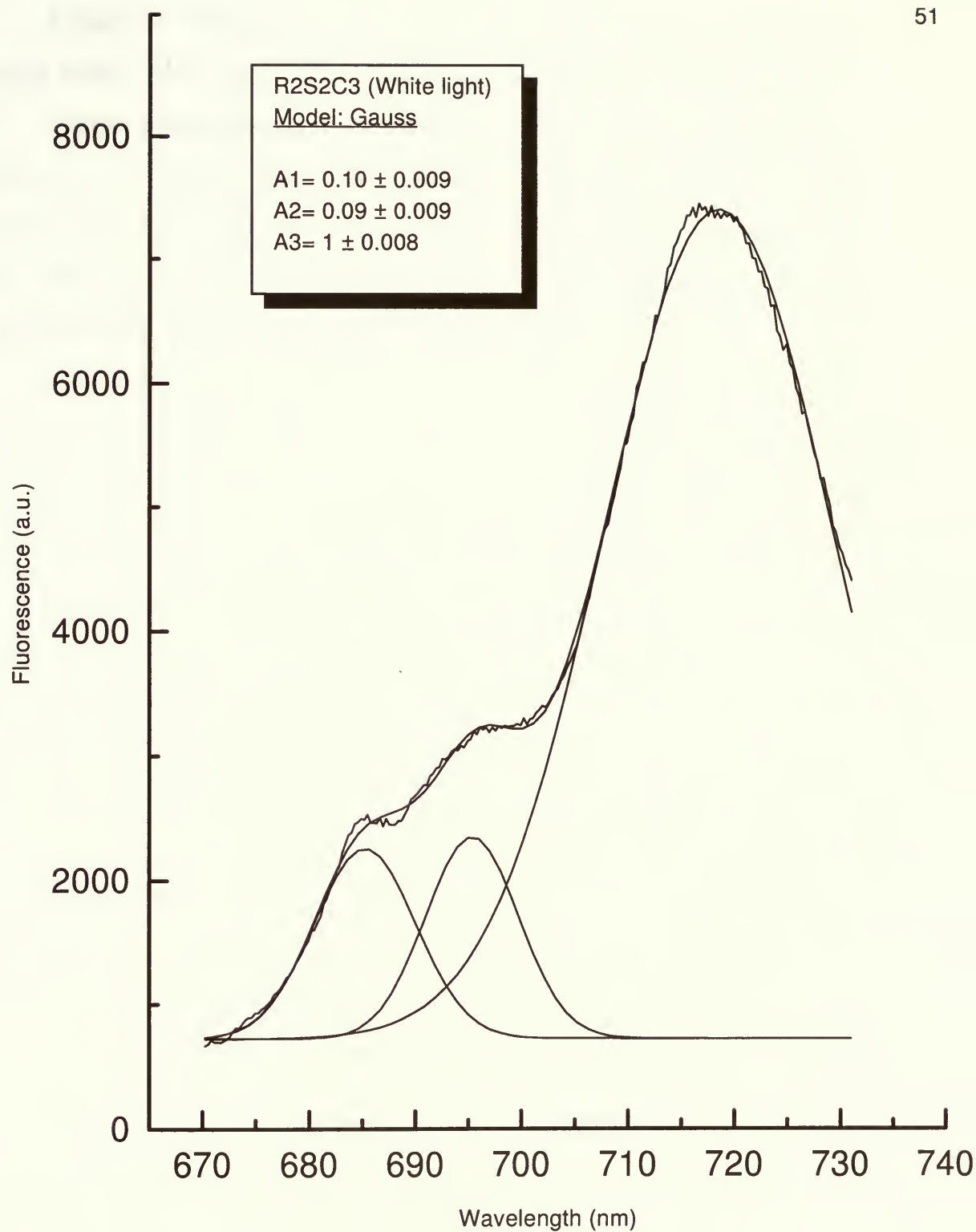
Figure 9: Gaussian distribution fit for R2S2C3 cells grown under red light at an intensity of 90 μmol photons m^{-2} s^{-1} for 1 day. Three components were used in a Gaussian distribution analysis to model the fluorescence emission spectra, these components (A1, A2, A3) peaked at three distinct wavelengths 685, 695, and 715 nm, respectively. The amplitudes were normalized by setting the largest component to 1.
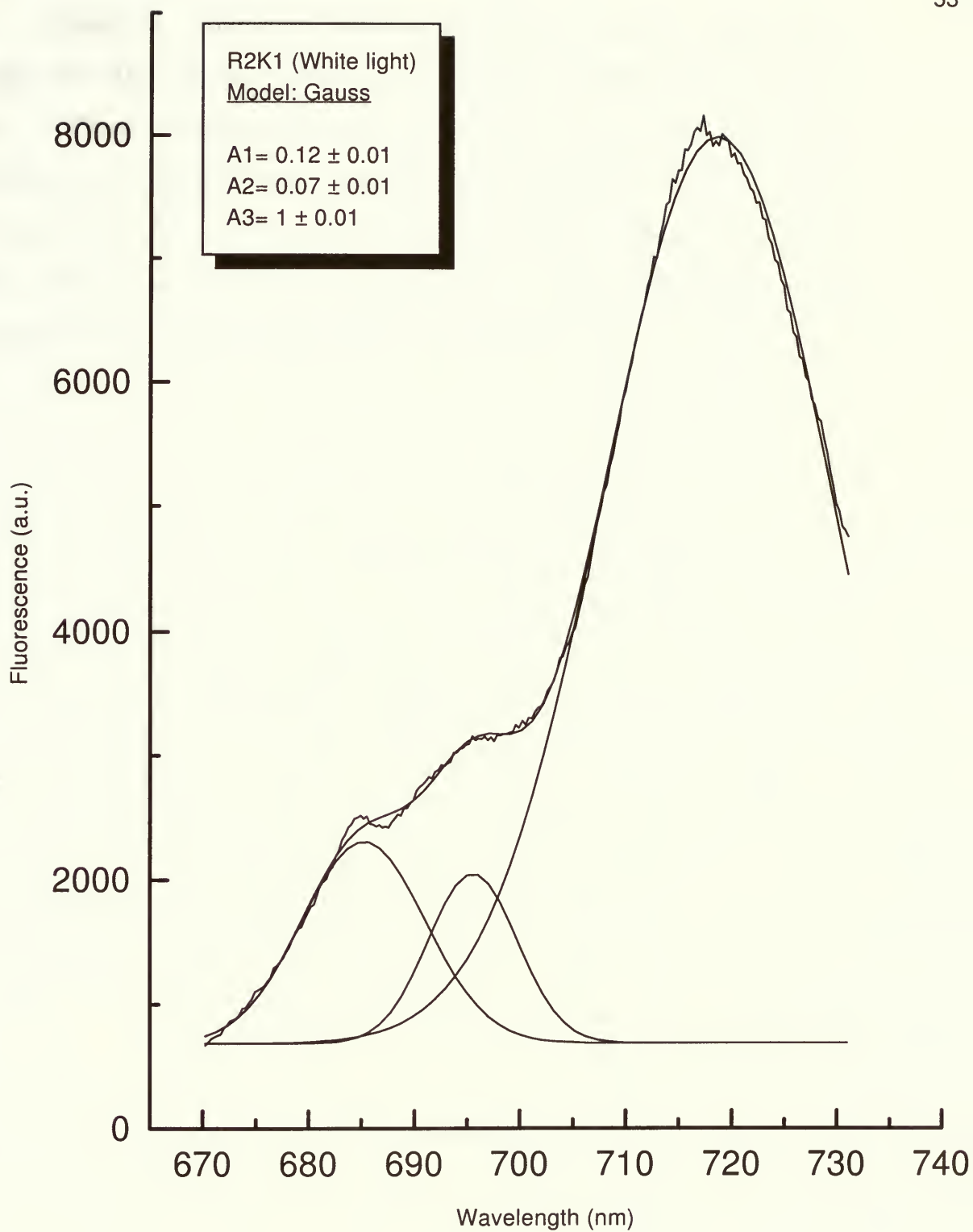
Figure 10: Gaussian distribution fit for R2K1 cells grown under red light at an intensity of 90 μmol photons m^{-2} s^{-1} for 1 day. Three components were used in a Gaussian distribution analysis to model the fluorescence emission spectra, these components (A1, A2, A3) peaked at three distinct wavelengths 685, 695, and 715 nm, respectively. The amplitudes were normalized by setting the largest component to 1.
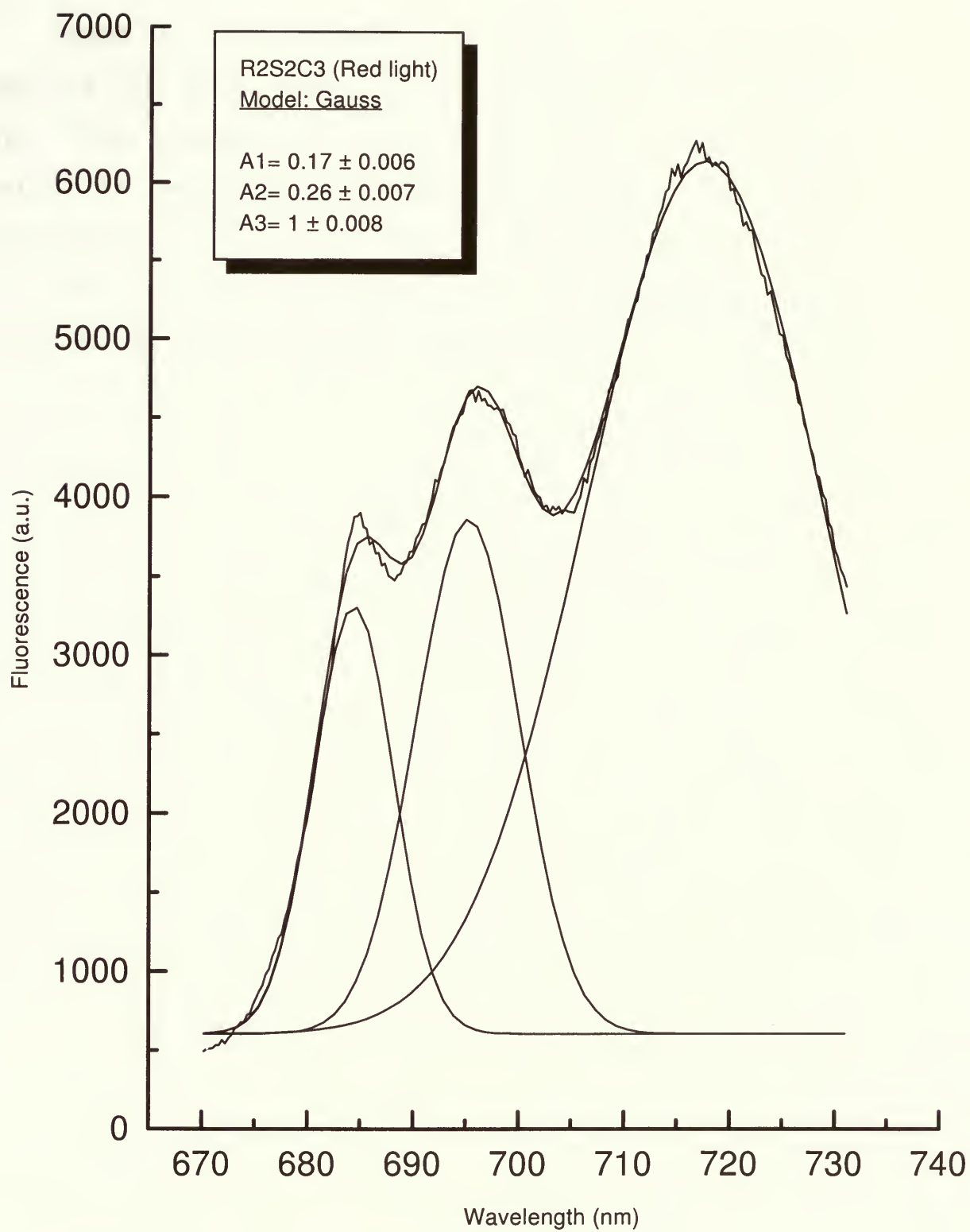
R2K1 (Red light)
Model: Gauss

A1= 0.15 ± 0.005
A2= 0.29 ± 0.007
A3= 1 ± 0.008

Figure 11: Gaussian distribution fit for R2S2C3 cells grown under red light at an intensity of 90 μmol photons m^{-2} s^{-1}. Three components were used in a Gaussian distribution analysis to model the fluorescence emission spectra, these components (A1, A2, A3) peaked at three distinct wavelengths 685, 695, and 715 nm, respectively. The amplitudes were normalized by setting the largest component to 1.
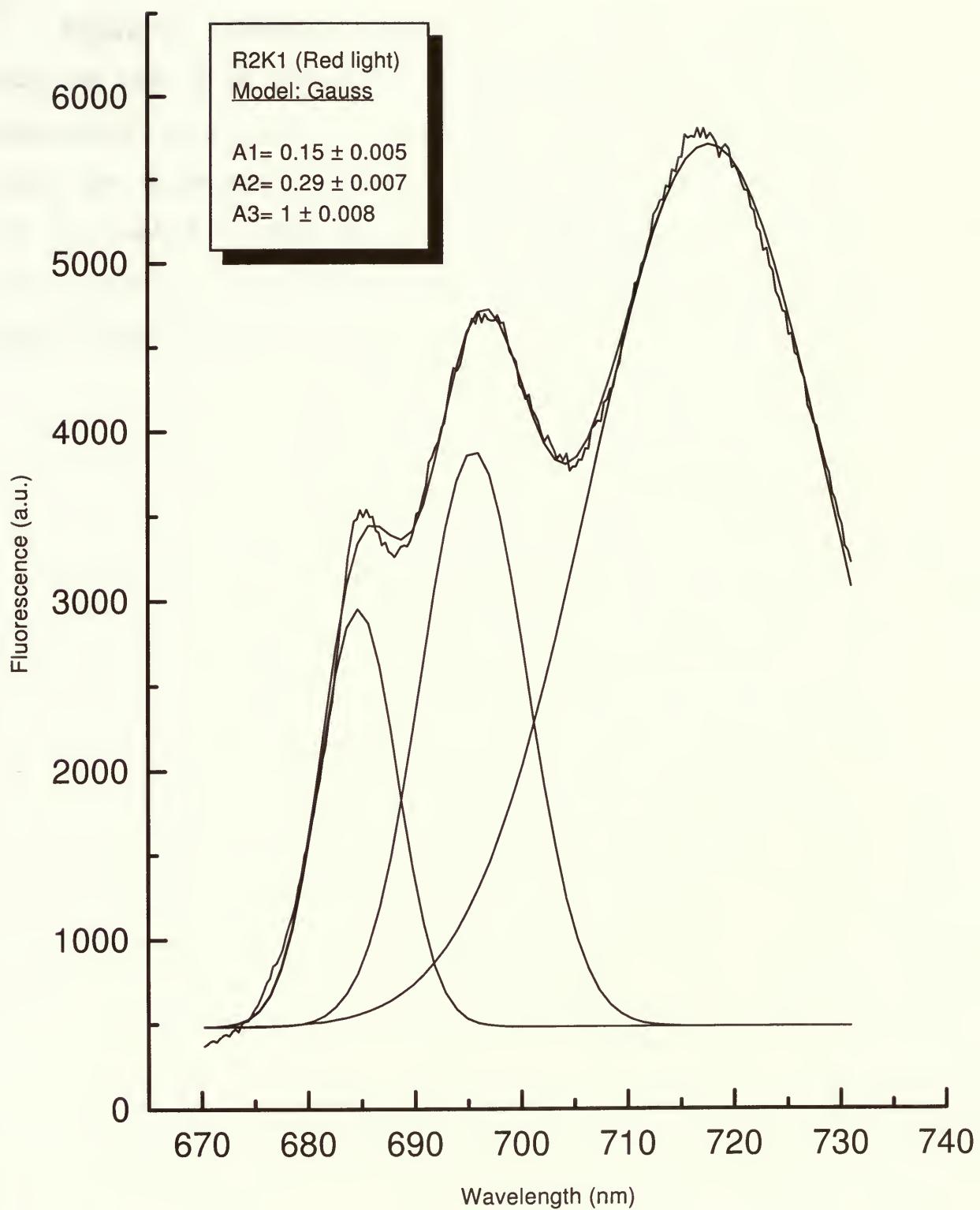
Figure 12: Gaussian distribution fit for R2K1 cells grown under red light at an intensity of 90 μmol photons m^{-2} s^{-1}. Three components were used in a Gaussian distribution analysis to model the fluorescence emission spectra, these components (A1, A2, A3) peaked at three distinct wavelengths 685, 695, and 715 nm, respectively. The amplitudes were normalized by setting the largest component to 1.
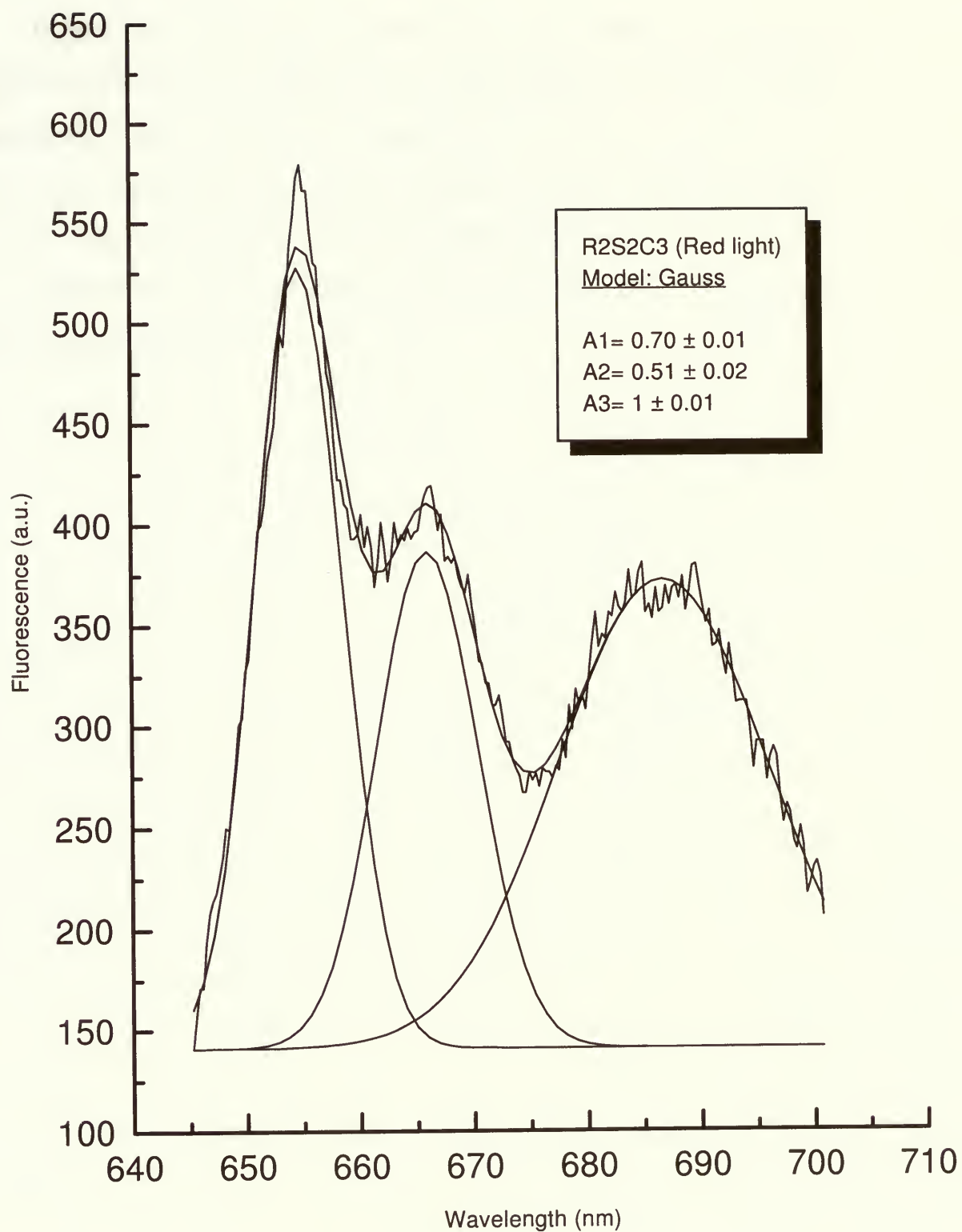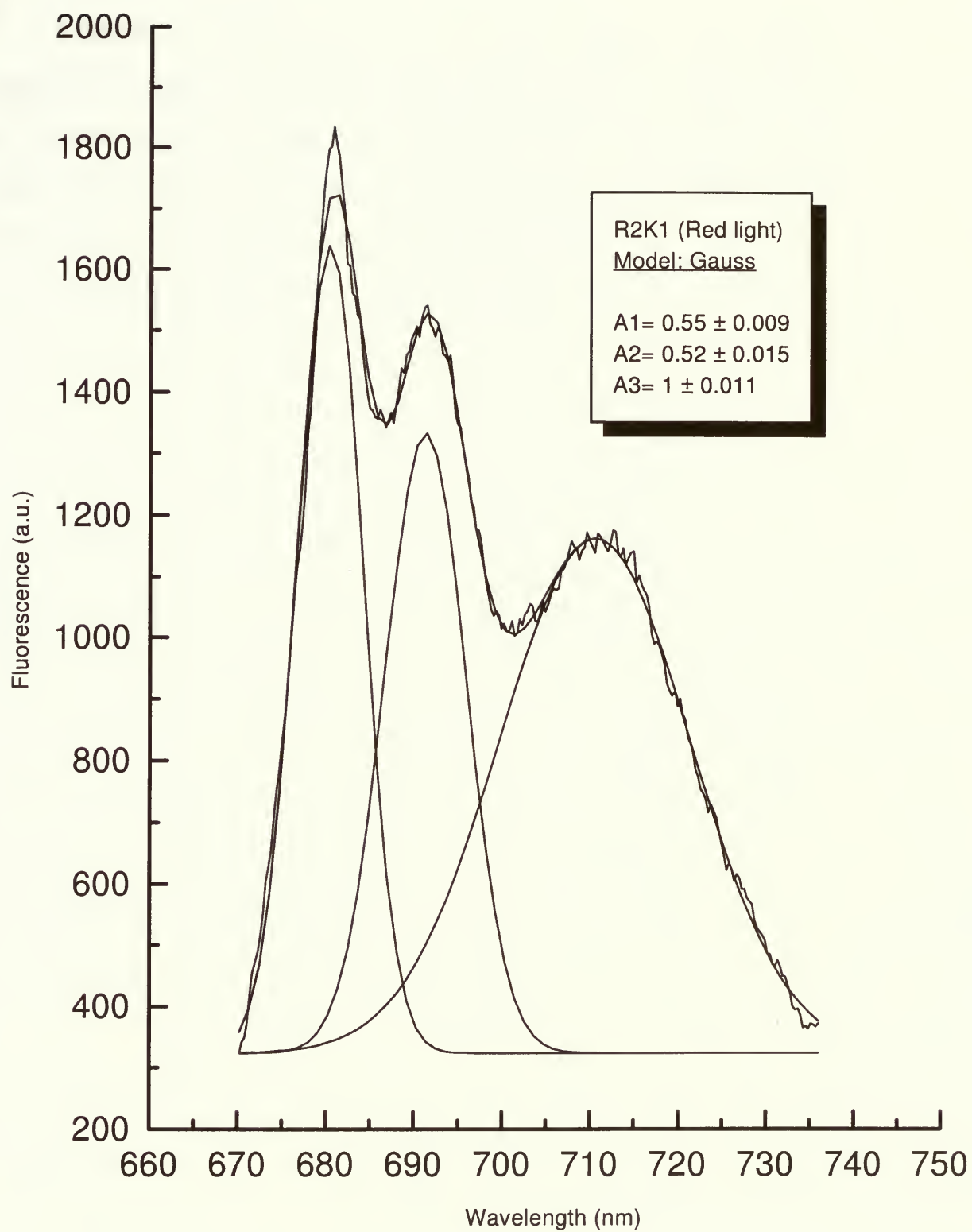
R2K1 (Red light)
Model: Gauss

A1= 0.55 ± 0.009
A2= 0.52 ± 0.015
A3= 1 ± 0.011

Table(3): PSI/PSII ratios in the two mutant cells R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present). Ratios reflect an increase in the amount of PSI/PSII ratio when cells grown under white light at an intensity of 55 μmol photons m^{-2} s^{-1}. In cells grown under red light at an intensity of 90 μmol photons m^{-2} s^{-1} PSI/PSII reached the minimum value.

Light condition / Mutant strain	R2S2C3	R2K1
White light	5.0 ± 0.42	5.2 ± 0.48
Red light (1 day after transferring cells to red light)	2.4 ± 0.76	2.3 ± 0.70
Red light	0.8 ± 0.44	0.9 ± 0.45

cells the relative value of the amplitude for the third component was 0.02 in R2S2C3 and 0.09 in R2K1 cells. Low PSI/PSII ratio containing cells showed also a large difference between the two mutants in R2S2C3 the value of the amplitude for the third component was 0.02 whereas in R2K1 it was 0.06. Amplitudes for the other components were similar in both mutant cells (Figures 13-16 and Table 4).

Pulse amplitude modulated (PAM) fluorescence measurements: To study the susceptibility of cells to photoinhibition, a pulse amplitude modulated (PAM) fluorometer was used to assess PSII activity. In this study Chl a fluorescence parameters at room temperature were measured. These parameters include minimal fluorescence in the dark adapted state, steady state fluorescence and maximal fluorescence.

The sensitivity to photoinhibition was determined by preilluminating cells for 5 min at light intensities varying from 1000 to 10000 μmol photons m^{-2} s^{-1} before determining the fluorescence parameters as described in (Fig. 17). Normalized F_V/F_M is plotted versus preillumination intensity for R2S2C3 and R2K1.

These results showed no difference in susceptibility to photoinhibition between the white light grown cells (high PSI/PSII) and the red light grown cells with low PSI/PSII (Figures 18 and 19). In both, treating cells with about 10000 μmol photons m^{-2} s^{-1} inhibited the relative efficiency of PSII reaction centers (F_V/F_M) down about 70% compared to the control. Again no difference in the susceptibility to photoinhibition was observed between R2S2C3 and R2K1 mutant cells.

<u>Oxygen evolution capacity and photoinhibition measurements:</u> In this study the two mutants R2S2C3 (D1:1 only), and R2K1 (D1:2 only) of the cyanobacterium *Synechococcus* sp. PCC 7942 grown under two different light conditions were used to measure the oxygen evolution after photoinhibition treatments.

Results from the oxygen evolution measurements after photoinhibition treatments are shown in (Fig. 20). These results showed no difference between the two mutant cells in their susceptibility to photoinhibition with either high PSI/PSII ratio or low PSI/PSII ratio. However there was a difference in the susceptibility to photoinhibition observed between cells grown under white light and cells grown under red light.

Figure 13: Decay associated spectra from the global analysis of time-resolved fluorescence decay kinetics from R2S2C3 cells (D1:1 only) grown under white light at an intensity of 55 μmol photons m^{-2} s^{-1}. Emission was from cells with open PSII reaction centers, at the F_O level of fluorescence. Four components have been resolved. Lifetimes for the four components are presented.

Figure 14: Decay associated spectra from the global analysis of time-resolved fluorescence decay kinetics from R2K1 cells (D1:2 only) grown under white light at an intensity of 55 µmol photons $m^{-2} s^{-1}$. Emission was from cells with open PSII reaction centers, at the F_O level of fluorescence. Four components have been resolved. Lifetimes for the four components are presented.

Figure 15: Decay associated spectra from the global analysis of time-resolved fluorescence decay kinetics from R2S2C3 cells (D1:1 only) grown under red light at an intensity of 90 μmol photons $m^{-2} s^{-1}$. Emission was from cells with open PSII reaction centers, at the F_O level of fluorescence. Four components have been resolved. Lifetimes for the four components are presented.
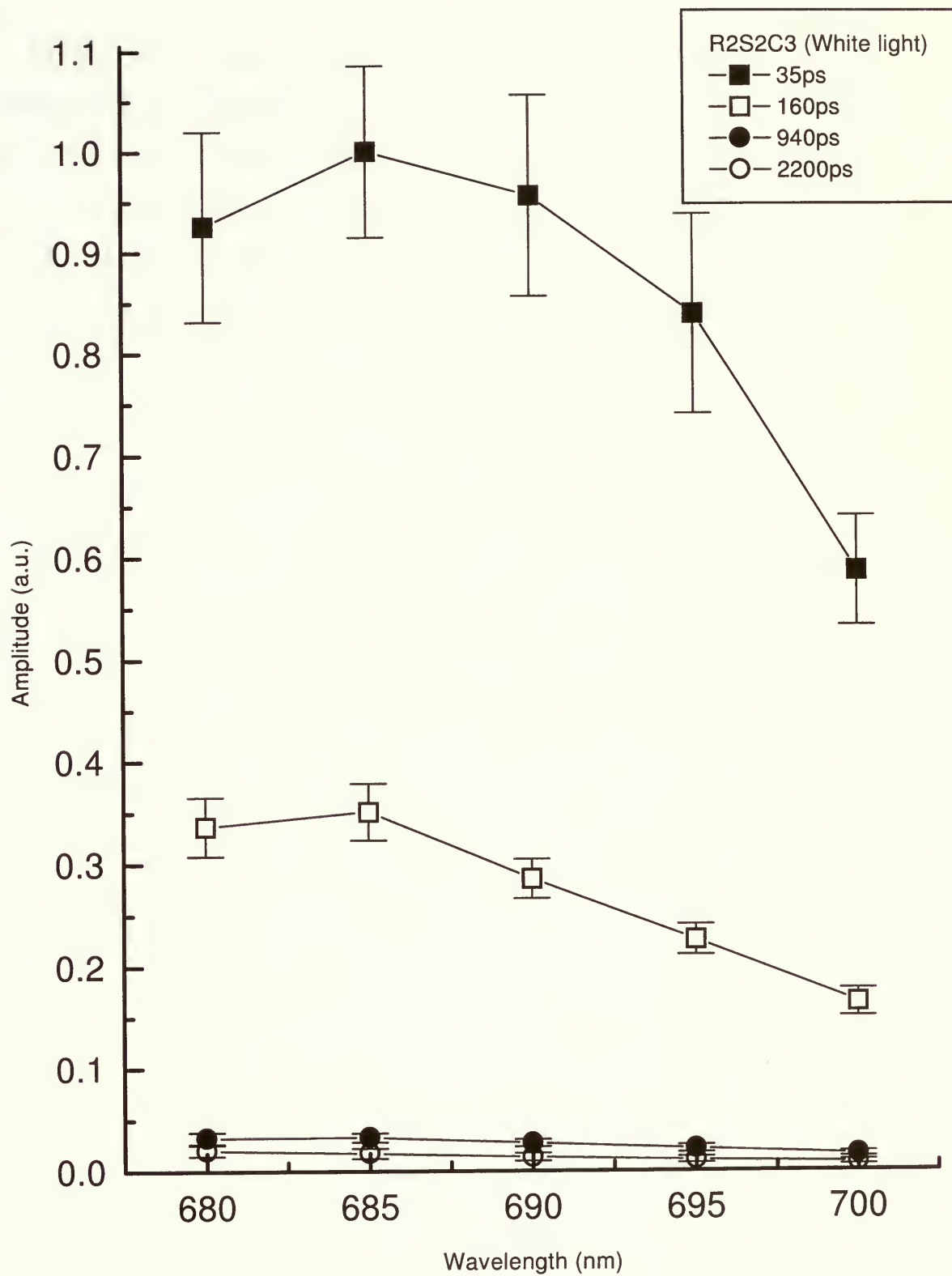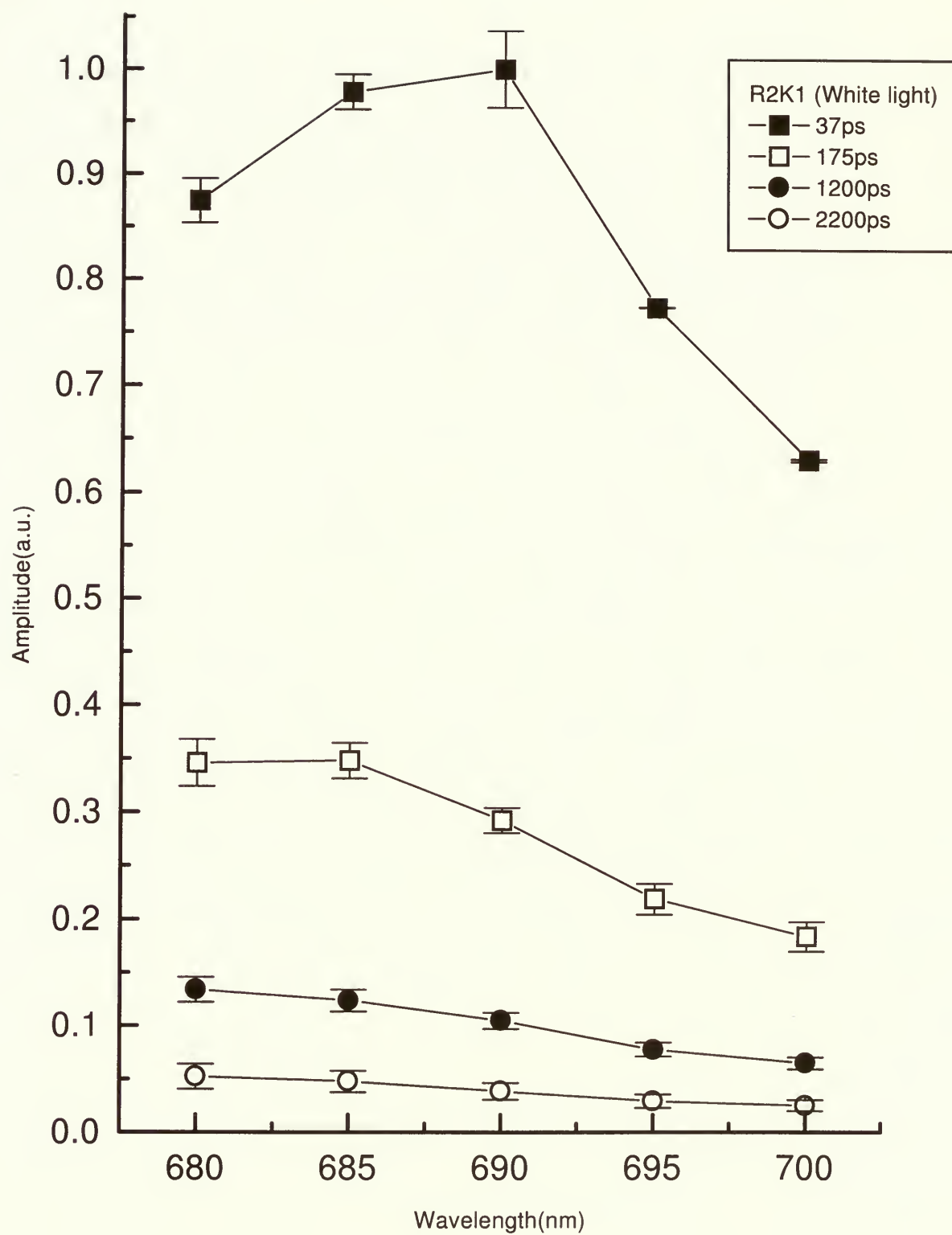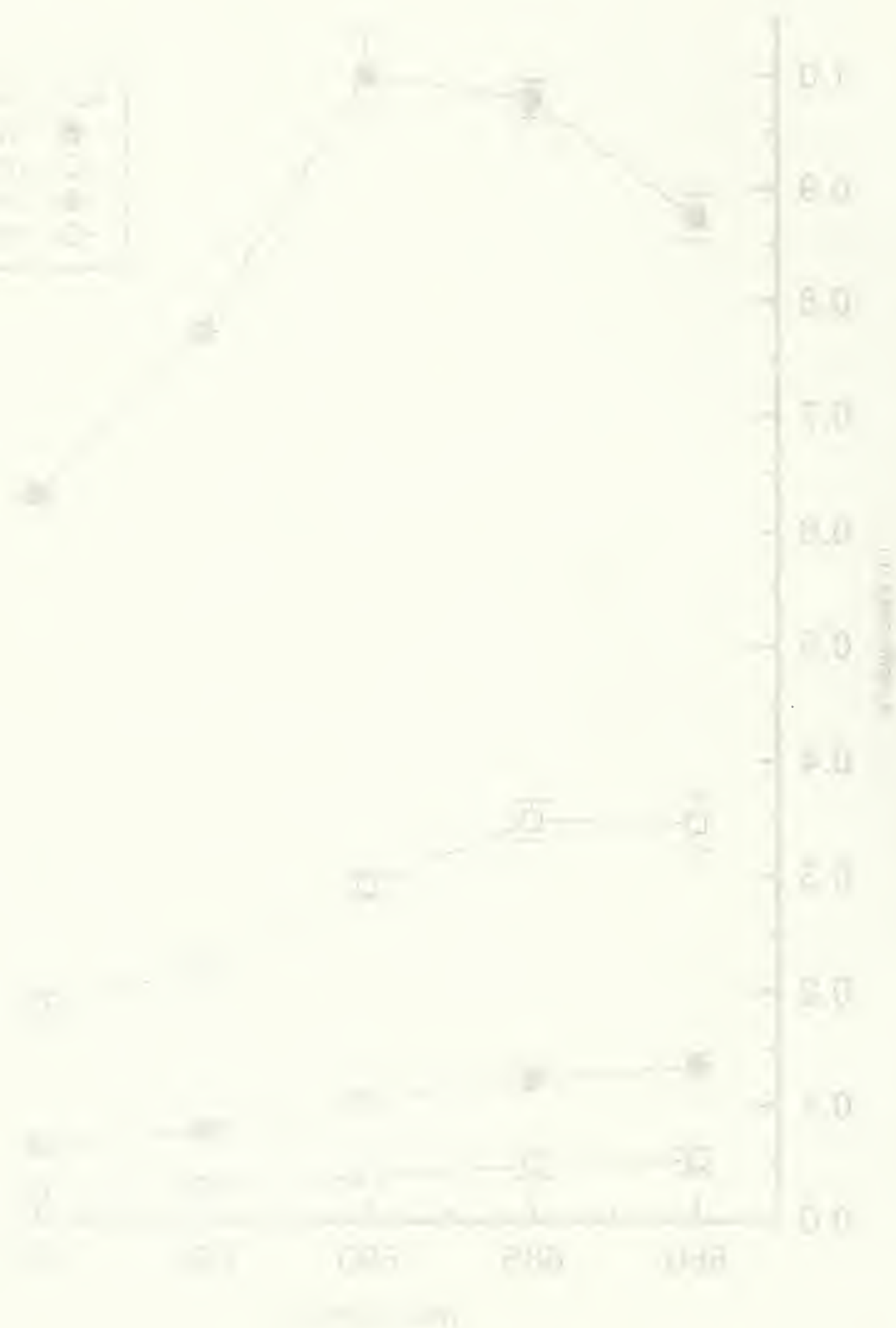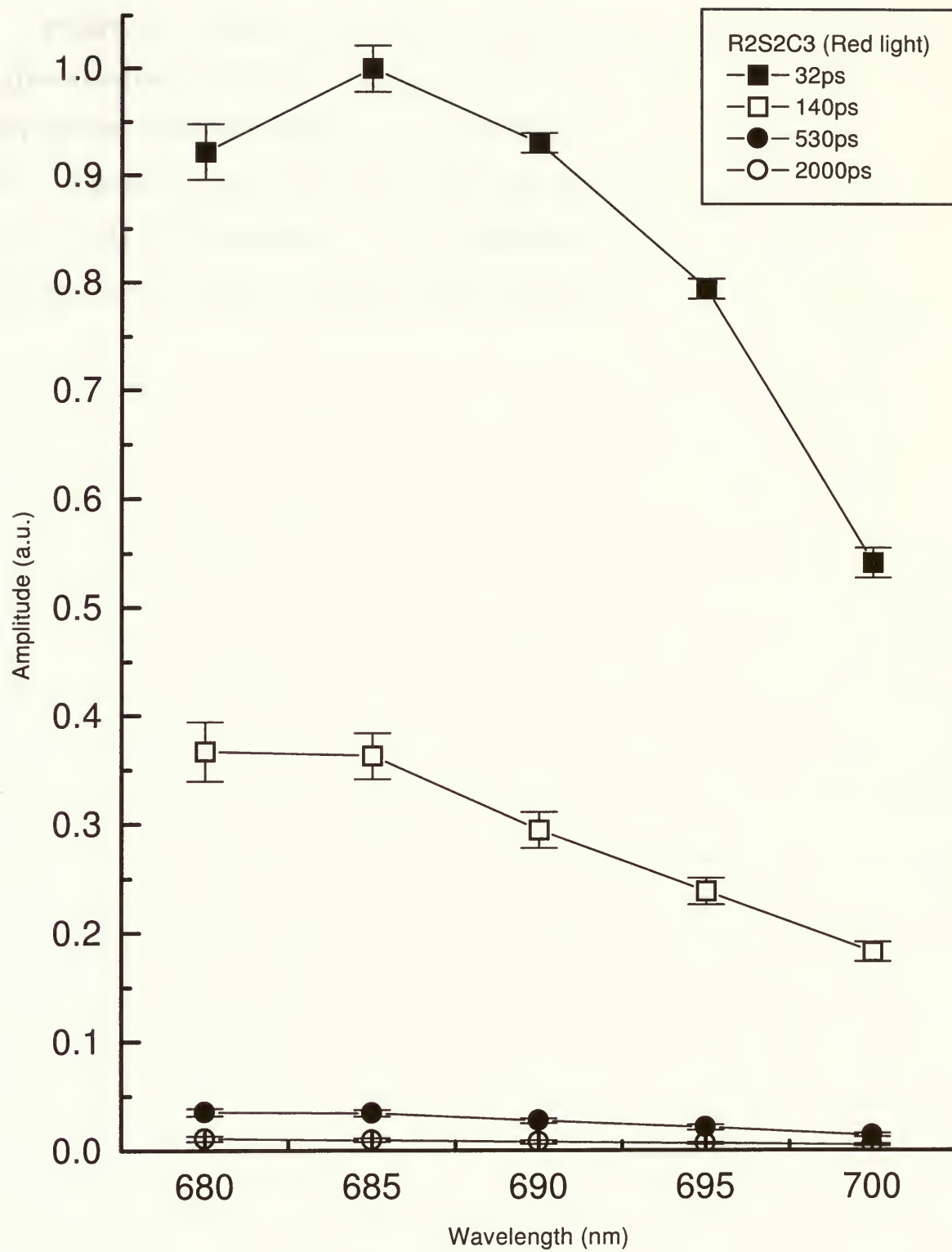
Figure 16: Decay associated spectra from the global analysis of time-resolved fluorescence decay kinetics from R2K1 cells (D1:2 only) grown under red light at an intensity of 90 μmol photons m^{-2} s^{-1}. Emission was from cells with open PSII reaction centers, at the F_O level of fluorescence. Four components have been resolved. Lifetimes for the four components are presented.
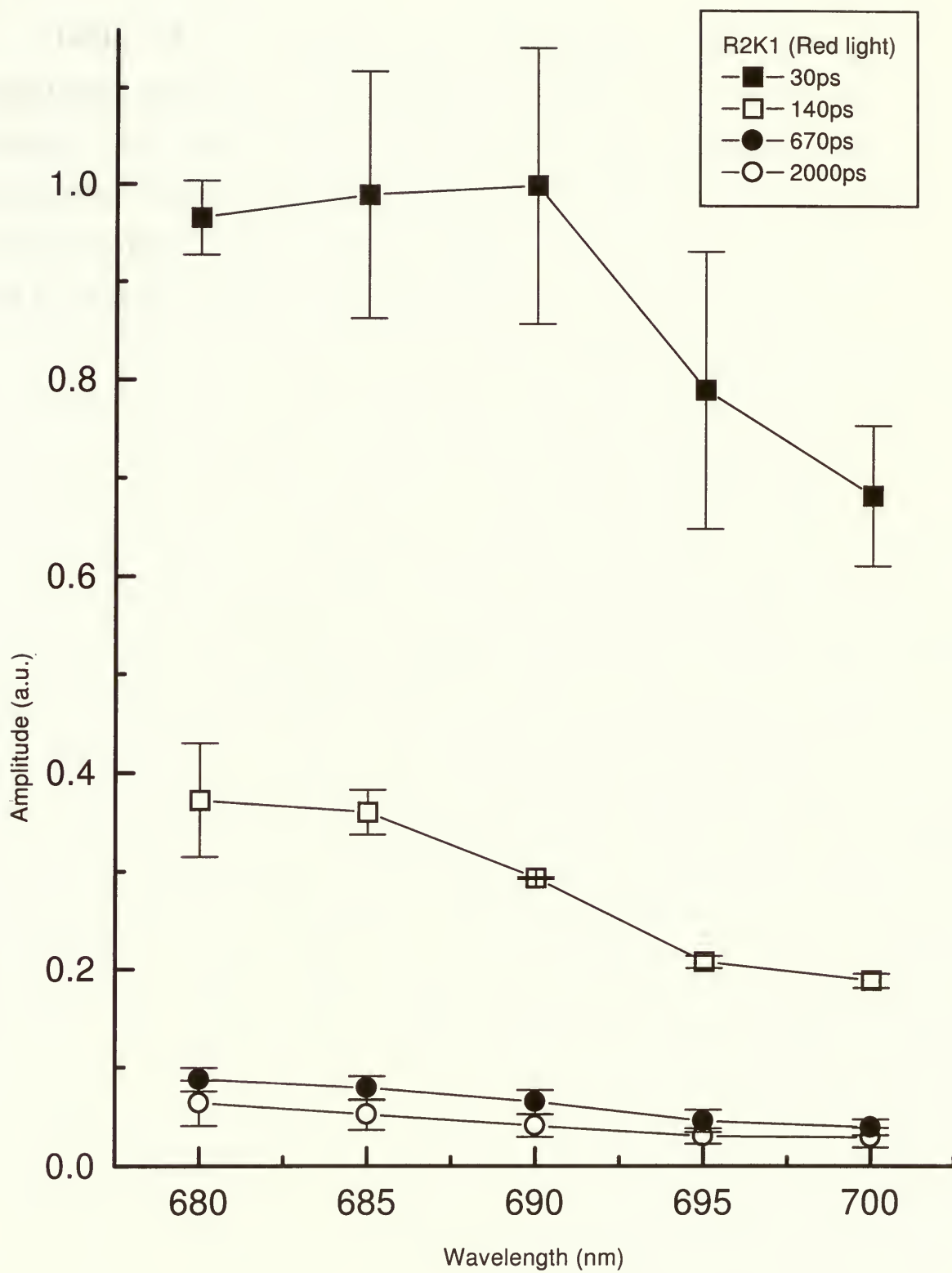
Table (4): Lifetimes (in picoseconds) and relative amplitudes (shown in parenthesis) for the four components resolved by using global analysis for time-resolved room temperature fluorescence emission.

-A-High PSI/PSII ratio containing cells.

-B-Low PSI/PSII ratio containing cells.

A- <u>High PSI/PSII ratio:</u>

i-R2S2C3 cells. χ^2 =1.07-1.12

Lifetime /(Amplitude)	35±2(0.77)	160±7(0.20)	940±190(0.02)	2200±200(0.01)
Maximum wavelength (nm)	685	685	680	680

ii-R2K1 cells. χ^2 =1.12-1.13

Lifetime /(Amplitude)	37±1(0.68)	175±4(0.20)	1200±50(0.09)	2200±120(0.03)
Maximum wavelength (nm)	690	680	680	680

B- <u>Low PSI/PSII ratio:</u>

i-R2S2C3 cells. χ^2 =1.19-1.24

Lifetime /(Amplitude)	32±2(0.67)	140±3(0.30)	530±4(0.02)	2000±100(0.01)
Maximum wavelength (nm)	685	680	680	680

ii-R2K1 cells. χ^2 =1.10-1.20

Lifetime /(Amplitude)	30±3(0.70)	140±10(0.20)	670±160(0.06)	2000±100(0.04)
Maximum wavelength (nm)	690	680	680	680

Figure 17: General diagram showing the PAM Chl a fluorescence parameters. These parameters were used to measure the photoinhibition sensitivity at room temperature;

-F_O was determined using low intensity modulated light at 1.6 KHz.

-Determination of maximal fluorescence in the dark-adapted state, $F_M{}^1$ was measured by using a 1 s flash of saturating light (10000 μmol photons m^{-2} s^{-1}).

-Steady state fluorescence, was reached by using an actinic light of 52 μmol photons m^{-2} s^{-1}, and the modulated light was switched to 100 KHz for a better signal to noise ratio.

-$F_M{}'$ under steady state conditions was determined by a saturating light pulse as described above.

-Finally, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) was injected into the cuvette (4 μM) to stop electron transport and maximal fluorescence F_M was reached.

Data were used to calculate the relative efficiency of open PSII reaction centers (F_V/F_M).

Figure 18: The sensitivity to photoinhibition in R2S2C3 mutant cells (only D1:1 present) and R2K1 mutant cells (only D1:2 present) grown under white light at an intensity of 55 µmol photons m^{-2} s^{-1}. Sensitivity to photoinhibition was determined by preilluminating cells for 5 min at light intensities varying from 1000 to 10,000 µmol photons m^{-2} s^{-1} before determining the fluorescence parameters as described in Fig. 17. Normalized F_V/F_M is plotted versus preillumination intensity (± S.E.)

White light

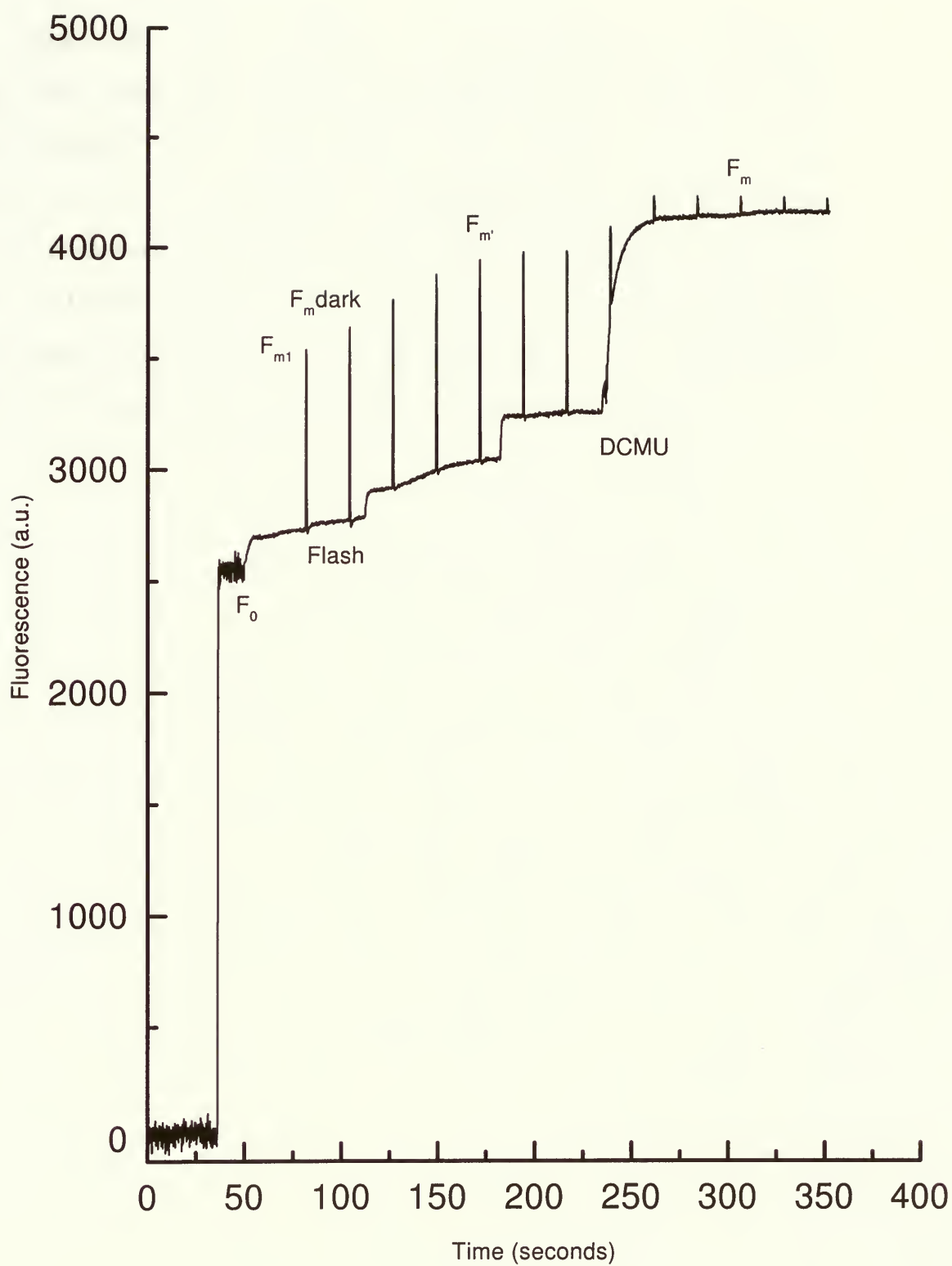F_V/F_M (normalized)

Preillumination intensity μmol photons m⁻² s⁻¹
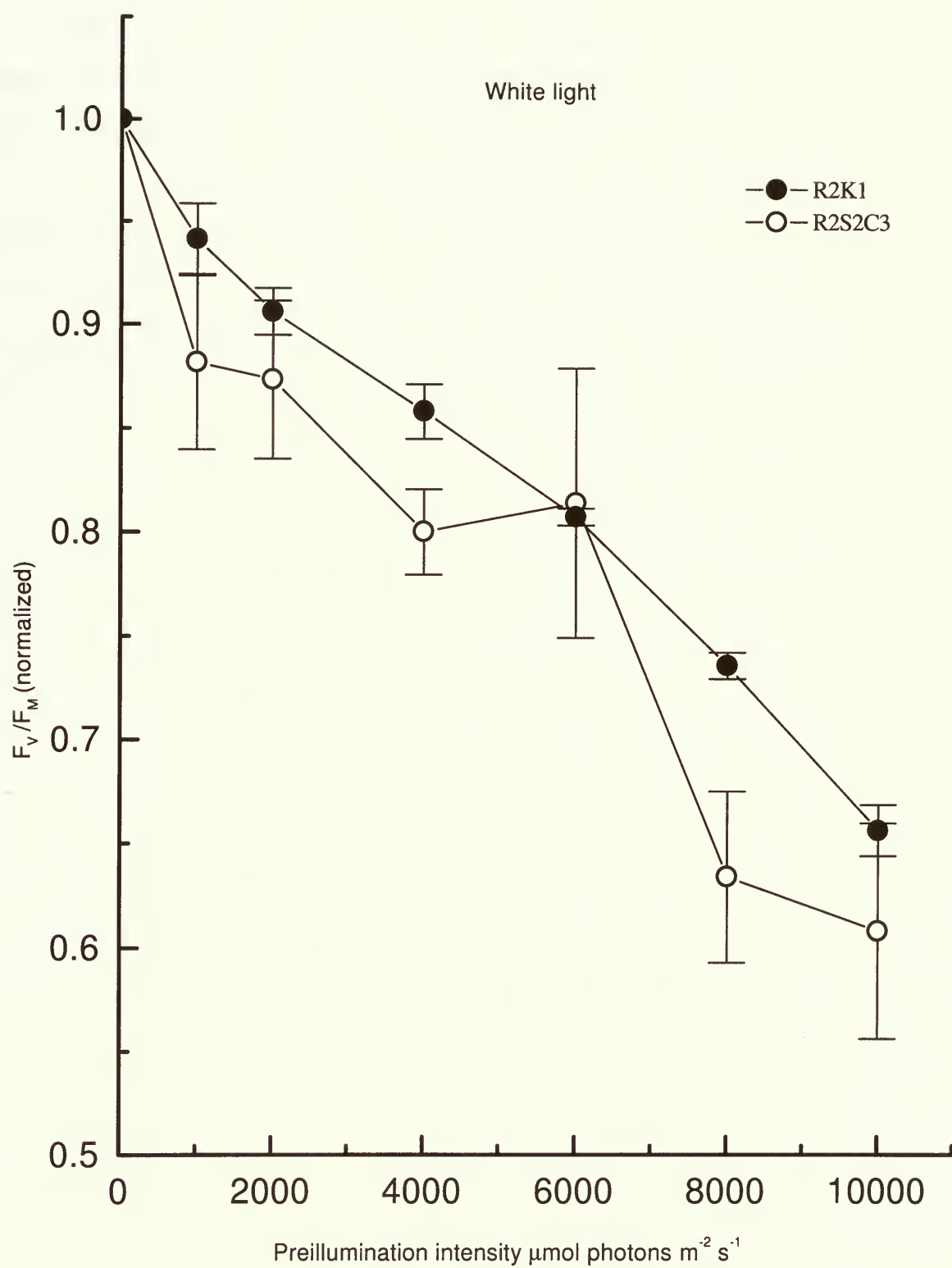
Figure 19: The sensitivity to photoinhibition in R2S2C3 mutant cells (only D1:1 present) and R2K1 mutant cells (only D1:2 present) grown under red light at an intensity of 90 μmol photons $m^{-2} s^{-1}$. Sensitivity to photoinhibition was determined by preilluminating cells for 5 min at light intensities varying from 1000 to 10,000 μmol photons $m^{-2} s^{-1}$ before determining the fluorescence parameters as described in Fig. 17. Normalized F_V/F_M is plotted versus preillumination intensity.
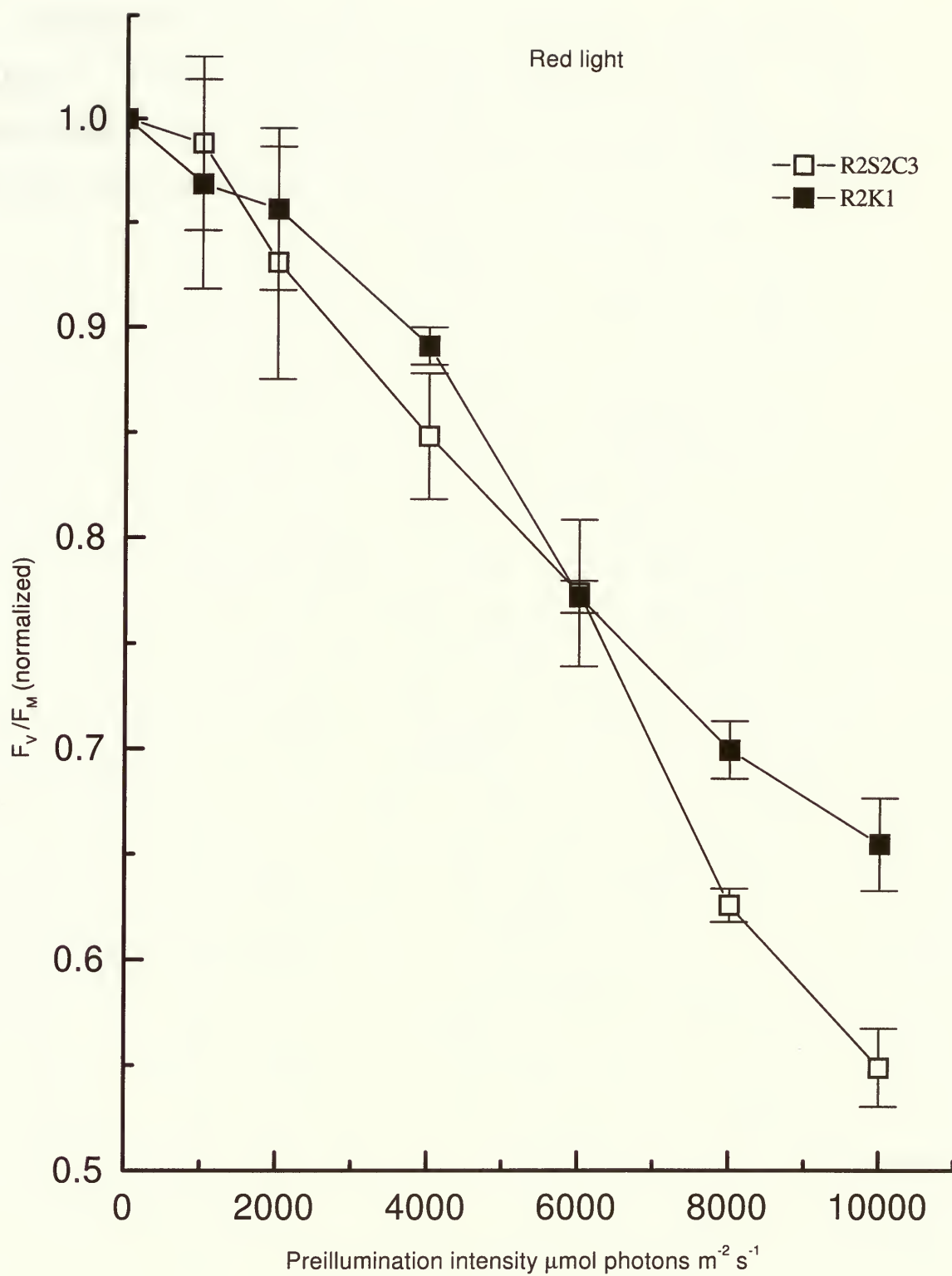
Figure 20: Photosynthetic O_2 evolution after photoinhibition treatment. These results showed a clear distinction in the susceptibility to photoinhibition between cells grown under white light and cells grown under red light.

Figure legend (in plot):
- R2S2C3 (Red light grown cells)
- R2K1 (Red light grown cells)
- R2S2C3 (White light grown cells)
- R2K1 (White light grown cells)

Y-axis: O_2 evolution rate (rel. u.)

X-axis: Preillumination intensity (μmol photons m^{-2} s^{-1})
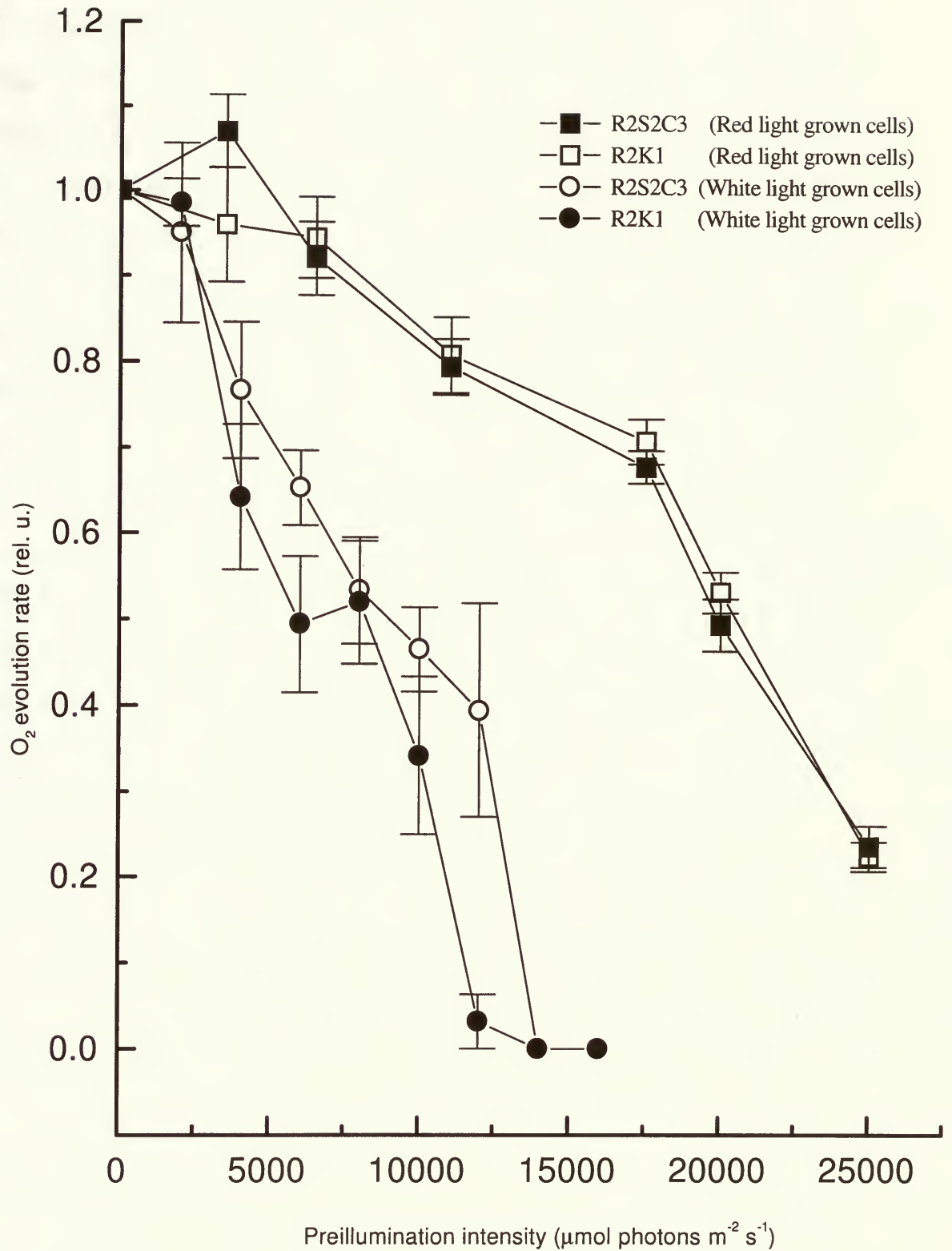
Discussion:

In *Synechococcus* sp PCC 7942 the D1-polypeptide is found in two forms, D1:1 and D1:2. The predominant D1 species is D1:1, the form which is found to be replaced transiently by the other form D1:2 upon shifts to higher light irradiances. Previous analysis of the functional differences between the two forms of D1-polypeptide have shown that R2S2C3 mutant cells (D1:1 form) were functionally more efficient than R2K1 mutant cells (D1:2 form). Another functional clue about the possible role of D1:2 was found in studies which have shown that this form predominates when cells were exposed to UV-B light. My results support the idea that D1:1 containing PSII is functionally more efficient than the D1:2 containing PSII.

Previous studies in cells with high PSI/PSII ratios showed that R2S2C3 cells (only D1:1 present) have a relatively lower amplitude for 77 K fluorescence emission at 695 nm than R2K1 cells (only D1:2 present). This study has confirmed these earlier results. The emission peak at 695 nm is associated with the PSII reaction center (Cleland 1988). It is believed that the 695 nm peak arises mainly from a specific population of low energy chlorophylls associated with CP47 (Campbell et al. 1996). The decreased amplitude of emission at 695 nm in D1:1 containing cells indicates a specific quenching of these low energy chlorophylls not present in D1:2 containing cells.

In a previous study global lifetime analysis of time resolved room temperature fluorescence emission resolved four components in fluorescence decay from intact cells of R2S2C3 and R2K1 (Campbell et al. 1996). The first component had a very short lifetime and was suggested to belong to PSI. The three other

components assigned to PSII. The second component to energy transfer and trapping processes by PSII reaction center including primary charge separation (i.e. formation of P680$^+$ Phe$^-$). The third component was related to the charge stabilization process (electron transfer from Phe$^-$ to Q_A). The fourth component had the longest lifetime and was suggested is arise from uncoupled chlorophyll or closed reaction centers. In that study the major difference between R2S2C3 and R2K1 cells was that R2S2C3 cells had a shorter lifetime for the third decay component. This was confirmed in the present study, R2S2C3 had a faster lifetime for the third component, it was 940ps $\pm$ 190ps compared with 1200ps $\pm$ 50ps in R2K1 cells.

The lower yields and faster lifetimes observed in R2S2C3 cells suggest that PSII containing D1:1 have increased photochemical or non-photochemical quenching of excitation energy in PSII compared to D1:2. <u>What could increase the quenching of fluorescence in R2S2C3 cells?.</u> Three possible mechanisms may explain the increase in generation of photochemical or non-photochemical quenching of excitation energy in PSII. These three mechanisms are increased photochemical yield, increased quenching of PSII antennae, or increased energy transfer from PSII to PSI (Fig. 21). The objective of this thesis was to test the third possibility by changing the PSI/PSII ratio and observing the effects on photophysical parameters and physiological parameters

To test this hypothesis 77 K fluorescence emission measurements and picosecond time resolved decay kinetics were used on cells with a low PSI/PSII ratio. The 77 K fluorescence measurements showed that R2S2C3 cells had a lower yield of 695

Figure 21: Major pathways for dissipation of excitation energy from photosystem II reaction center (adapted from Krause 1988).
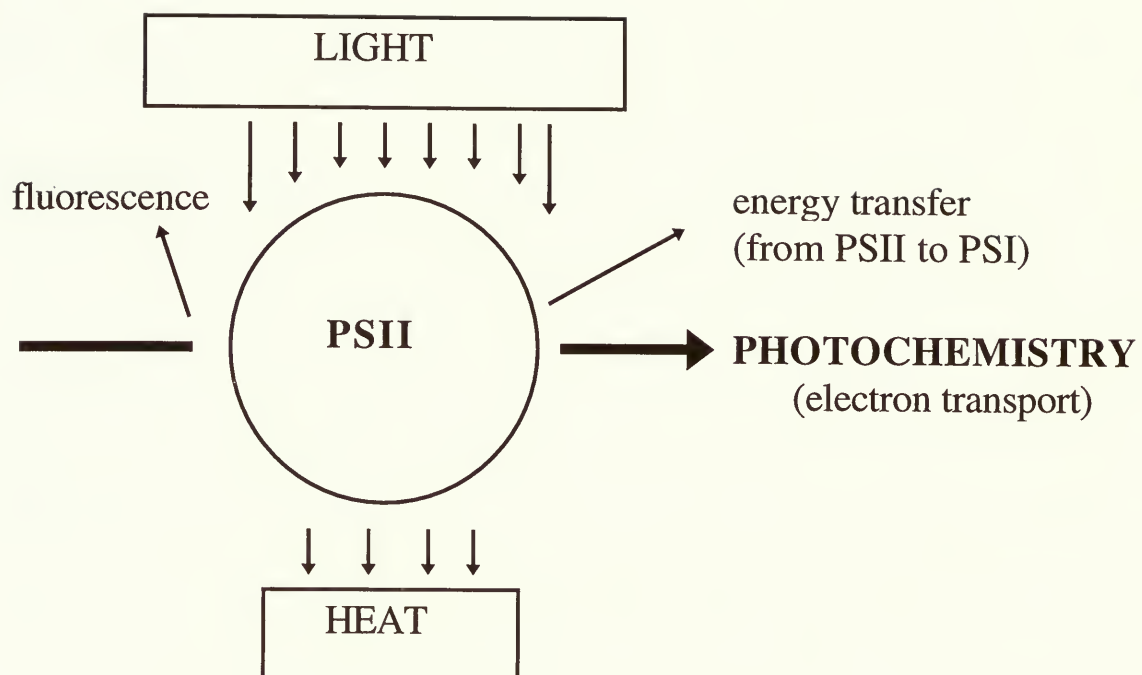
nm emission compared with R2K1 cells. A 18.3% $\pm$ 3% lower amplitude for the 695 nm peak (calculated as F695/F685) was observed in R2S2C3 cells. Comparison of F695/F715 values also show a relative decrease in R2S2C3 compared to R2K1, the ratio was 17% $\pm$ 2% lower in R2S2C3 compared to R2K1. The differences between the two mutants still exist even though the PSI/PSII ratio has changed. Decreasing the PSI/PSII ratio caused the differences between the two mutants for the 695 nm peak to became even larger.

Picosecond time resolved fluorescence decay kinetics showed that R2S2C3 cells had a faster lifetime for the third decay component than R2K1 cells. R2S2C3 cells showed a lifetime for the third component as 530ps $\pm$ 4ps whereas in R2K1 cells it was 670ps $\pm$ 160ps.

Decreasing the PSI/PSII ratio did not diminish the observed differences between the two mutants. In fact decreasing the PSI/PSII ratio made the difference in 77 K spectra between the two mutants larger. Therefore, data collected from 77 K fluorescence measurements and picosecond time resolved fluorescence decay kinetics did not support the possible explanation that D1:1 facilitates the dissipation of excess energy by energy transfer from PSII to PSI.

<u>Photoinhibition:</u>

Another approach to clarify the functional role of the two forms of the D1-polypeptide was to study whether changing the PSI/PSII ratio would affect the difference in susceptibility to photoinhibition between the two mutants.

The hypothesis was that sensitivity to photoinhibition in *Synechococcus* sp PCC 7942 will be dependent on cellular PSI/PSII

ratio. The null hypothesis was that there will be no difference between R2S2C3 (only D1:1 present) and R2K1 (only D1:2 present) in their susceptibility to photoinhibition when their cellular PSI/PSII ratio decreased.

Photoinhibition studies were divided into two different approaches, a photophysical approach using pulse amplitude modulated (PAM) fluorescence measurements and a physiological approach using oxygen evolution measurements.

Again if energy transfer from PSII to PSI is responsible for increased energy quenching in R2S2C3 then cells with a low PSI/PSII ratio should show smaller differences in the relative susceptibility of the two mutants to photoinhibition. Surprisingly, no differences in susceptibility to photoinhibition between R2S2C3 and R2K1 were found in high PSI/PSII or in low PSI/PSII cells.

In addition, physiological studies of oxygen evolution measurements after photoinhibition treatments showed that the two mutant cells had no differences in their susceptibility to photoinhibition with either high PSI/PSII containing cells or low PSI/PSII containing cells. Again this suggests that energy transfer from PSII to PSI is likely not a factor in the differences observed between D1:1 and D1:2 containing cells.

Recently, Campbell et al (1998) found that the rapid exchange of the two forms of D1-polypeptide D1:1 and D1:2 in *Synechococcus* related directly to increased cellular resistance to UV-B inhibition of photosystem II activity and photosynthetic electron transport. Exposing the cells to increased UV-B results in predomination of the D1:2 form and when UV-B is decreased the D1-polypeptide switchs to the more functional form D1:1 which is

predominant form under most environmental growth light conditions.

It had been reported that higher plants possess only one form of D1-polypeptide and this form is similar to D1:2 (Fig. 2) and it is known that higher land plants suffer from increased exposure to UV-B as compared to cyanobacteria in an aquatic environment.

The oxygen evolution measurements showed that the two mutant cells had no difference in their susceptibility to photoinhibition with either high PSI/PSII ratio or low PSI/PSII ratio. However there was a large difference in susceptibility to photoinhibition between the red light and white light grown cells.

Regarding the observed difference in susceptibility to photoinhibition due to PSI/PSII ratio itself, oxygen evolution requires three active components functioning simultaneously. These components are an active PSII complex, an electron acceptor and an oxygen evolving complex (OEC). PAM measurements showed that PSII was active. Potassium ferricyanide and p-penzoquinone were added as an electron acceptors during the oxygen evolution measurements. It is clear that the (OEC) was not functioning properly in the high PSI/PSII containing cells.

We have to point out that one factor was not controlled in the growth conditions used to change cellular PSI/PSII ratio, the red light had a higher intensity compared to white light and the cells growth rate was also higher in the red light grown cells. Light quality (white or red) and light quantity (lower and higher irradiances) were both changed. Changing just one factor and keeping the other factors constant like light quality and growth

rate has to be highly considered in future studies. This may have been a factor in the oxygen evolution results which showed red light (higher intensity) grown cells were less susceptible to damage of the oxygen evolving complex.

Summary:

Both photophysical and physiological studies suggest that the energy transfer efficiency from PSII to PSI is likely not a factor in the observed increase of photochemical and/or non-photochemical quenching of excitation energy in PSII containing D1:1.

Photophysical studies including 77 K fluorescence and picosecond time resolved decay kinetics are consistent with previous results which suggest that the D1:1 containing cells (R2S2C3) are photosynthetically more efficient than the D1:2 containing cells (R2K1). However these results do not depend on PSI/PSII ratio of cells. Photoinhibition studies, including pulse amplitude modulated fluorescence measurements (photophysical) and oxygen evolution measurements (physiological) showed no differences between the mutant cells. As a conclusion the energy transfer efficiency from PSII to PSI is likely not a factor in the differences between D1:1 and D1:2 containing cells. It is possible that other mechanisms are involved in the higher intrinsic efficiency observed in the D1:1 containing cells.

Because the only difference between the two mutant cells lies in the form of D1 polypeptide, the twenty five amino acid differences between D1:1 and D1:2 must be involved in the observed functional differences between the two mutant cells. Site-directed mutagenesis studies are important to investigate the effect(s) of any changes in these twenty five amino acids on the photosynthetic efficiency.

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Appendix A: Liquid BG-11 components

H_2O..1000ml

100x BG-FPC.......................................10ml

Fe ammonium citrate stock...........1ml

Na_2Co_3 stock.......................................1ml

K_2HPO_4 stock......................................1ml

Autoclave

(a precipitate will form after autoclaving).