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Site directed mutagenesis of the heme axial ligands of cytochrome b559 affects the stability of the photosystem II complex

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Cytochrome (cyt) b559, an integral membrane protein, is an essential component of the photosystem II (PSII) complex in the thylakoid membranes of oxygenic photosynthetic organisms. Cyt b559 has two subunits, α and β, each with one predicted membrane spanning α-helical domain. The heme cofactor of this cytochrome is coordinated between two histidine residues. Each of the two subunit polypeptides of cyt b559 has one His residue. To investigate the influence of these His residues on the structure of cyt b559 and the PSII complex, we used a site directed mutagenesis approach to replace each His residue with a Leu residue. Introduction of these missense mutations in the transformable unicellular cyanobacterium, *Synechocystis* 6803, resulted in complete loss of PSII activity. Northern blot analysis showed that these mutations did not affect the stability of the polycistronic mRNA that encompasses both the *psbE* and the *psbF* genes, encoding the α and the β subunits, respectively. Moreover, both of the single His mutants showed the presence of the α subunit which was 1.5 kd smaller than the same polypeptide in wild type cells. A secondary effect of such a structural change was that D1 and D2, two proteins that form the catalytic core (reaction center) of PSII, were also destabilized. Our results demonstrate that proper axial coordination of the heme cofactor in cyt b559 is important for the structural integrity of the reaction center of PSII.

Key words: absorption spectroscopy/cytochrome b559/photosystem II/site directed mutagenesis/*Synechocystis* 6803

Introduction

Photosystem II (PSII) is a multisubunit pigment-protein complex in the thylakoid membranes of green plants and cyanobacteria. PSII contains a cytochrome, cytochrome b559 (cyt b559), as an integral membrane protein. The physiological role of this metalloprotein in photosynthesis is currently unclear. However, genetic, biochemical and biophysical experiments have indicated that this protein is an integral component of an active PSII complex (Pakrasi *et al.*, 1988, 1989; Cramer *et al.*, 1990). Cyt b559 is present in the most purified PSII reaction center complex (Nanba and Satoh, 1987) in which light induced charge separation between the reaction center primary donor chlorphyll(s), P680 and the immediate acceptor, pheophytin, can occur. In the presence of artifical quinones, illumination of this purified complex results in rapid reduction of cyt b559 which in turn slowly reduces oxidized P680 (Chapman *et al.*, 1988; Satoh *et al.*, 1990). Furthermore, at cryogenic temperature, cyt b559 can act as an electron donor to P680 (Vermeglio and Mathis, 1974). These observations indicate that the heme in cyt b559 is physically located close to P680.

In cyanobacteria and chloroplasts of green plants, two neighboring genes, *psbE* and *psbF* encode two subunits, α and β of cyt b559, respectively (Herrmann *et al.*, 1984; Pakrasi *et al.*, 1988). From the sequence and organization of these two genes, it was postulated that the α and β subunits form a heterodimeric structure where the heme cofactor crosslinks the two subunits (Herrmann *et al.*, 1984). Spectroscopic analysis of isolated cyt b559 protein from spinach and maize indicates that, like other *b* type cytochromes, the axial ligands of cyt b559 are two histidine residues (Babcock *et al.*, 1985). The derived amino acid sequence of the α and β subunits of cyt b559 show the presence of only two His residues, one in each subunit. Thus, it was also hypothesized that these two His residues coordinate the heme iron in cyt b559.

We are using a genetic approach to assess the roles of various amino acid residues and protein domains of cyt b559 in the biogenesis and stability of PSII as well as in various electron and proton transfer reactions associated with it. Toward this goal we initially created a deletion strain of the unicellular, transformable cyanobacterium, *Synechocystis* sp. PCC 6803 (hereafter called *Synechocystis* 6803). In addition to the *psbE* and *psbF* genes, two very closely linked genes, *psbL* and *psbJ*, were also deleted from the chromosome of this organism (Pakrasi *et al.*, 1988). The resultant mutant lost all PSII activity, but its PSI activity was not affected. Detailed analysis of this mutant indicated that in the absence of these four genes and their products, the two reaction center proteins of PSII, D1 and D2, were not assembled in the thylakoid membrane. However, two other chlorophyll-antenna proteins, CP47 and CP43, were stably inserted in the thylakoids (Pakrasi *et al.*, 1987). In order to determine unequivocally whether the absence of one or more of these four proteins is involved in this destabilization process, we created targeted specific deletions of the *psbE* and the *psbF* genes (Pakrasi *et al.*, 1990; H.B.Pakrasi and R.Wu, unpublished observation). In both cases, the properties of the resultant deletion mutants were very similar to those of the original deletion mutant. Thus, both the α and the β subunit polypeptides need to be present for stable formation of the PSII reaction center.

In *Synechocystis* 6803, complete deletion of the genes encoding any one of D1, D2, CP47 and cyt b559 proteins of PSII results in destabilization of the complex (Vermaas *et al.*, 1988; Pakrasi *et al.*, 1989; Yu and Vermaas, 1990). In order to gain more detailed insight into the role of cyt b559, we are using a site specific mutagenesis approach to alter various individual amino acid residues. In the present

study we have mutagenized the heme ligand His residues in each subunit, individually as well as jointly, to Leu, a residue that cannot coordinate the heme iron. In the absence of proper coordination of the heme, the reaction center of PSII was destabilized. However, when only one of the two His residues was changed, the α subunit of cyt b559 was present in the membrane, although its size was decreased.

Results

Choice of histidine 22 as the target site for site specific mutagenesis of the cyt b559 protein

An earlier analysis of the nucleotide derived amino acid sequence of the α and the β subunits of the cyt b559 protein of *Synechocystis* 6803 indicated that each subunit of cyt b559 has one putative membrane-spanning α-helical domain (Pakrasi *et al.*, 1988). This structural organization appears to be a common feature of cyt b559 protein in all organisms examined thus far (Cramer *et al.*, 1990). Determination of the N-terminal amino acid sequence of the two subunit polypeptides in a highly resolved PSII core preparation from *Synechocystis* 6803 indicated that the N-terminal Met residues are post-translationally removed from both polypeptides (H.B.Pakrasi, H.Koike, M.Ikeuchi and Y.Inoue, unpublished observation). The currently proposed structure of cyt b559 is shown in Figure 1. Salient features of this model are: (i) the 80 amino acid long α subunit has a predicted membrane-spanning α-helical domain between residues 18 and 43; (ii) the 43 amino acid long β subunit has a predicted membrane-spanning α-helical domain between residues 18 and 42; (iii) the N-terminal ends of both of the subunits are shown as exposed to the stromal side of the thylakoid membrane and, as a consequence, their C-terminal ends are exposed to the lumen side of the thylakoid membrane. This model was drawn in analogy to the orientation of these subunits in spinach thylakoid membranes (Tae *et al.*, 1988; Cramer *et al.*, 1990).

As shown in Figure 1, His22 in each subunit acts as an axial ligand for the iron in the heme cofactor of this cytochrome. According to this model, the two subunits of this protein are held together inside the membrane through the heme−histidine bonds (Babcock *et al.*, 1985).

The strategy for the introduction of specific changes in the cyt b559 protein is summarized in Figure 2. We used an efficient site directed mutagenesis procedure (see Materials and methods, also Eggenberger *et al.*, 1990) to introduce single site mutations in the *psbE* and/or *psbF* genes in the plasmid pSL132 that contains a 2.2 kb *Hin*dIII−*Eco*RI fragment of *Synechocystis* chromosome encompassing the entire *psbEFLJ* region (Pakrasi *et al.*, 1988). After any plasmid DNA with a desired mutation was identified, we introduced a 1.2 kb spectinomycin (Sp^r)/streptomycin (Sm^r) resistance gene cartridge (Ω fragment) at an *Nhe*I site, immediately downstream of the *psbEFLJ* gene cluster. We have previously shown that insertion of an antibiotic resistance gene at this site does not affect PSII activity of the cells nor presumably the function of all of the proteins encoded at the *psbEFLJ* gene cluster (Pakrasi *et al.*, 1988).

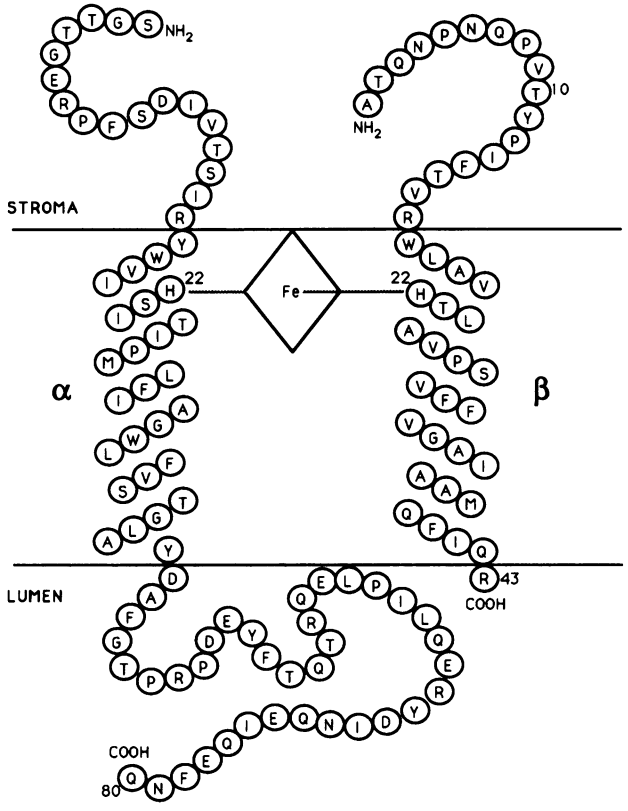

Fig. 1. Structural model of the heterodimeric cytochrome b559 protein in the thylakoid membrane of *Synechocystis* 6803. In this model, the N-terminal ends of both the α and the β subunits are exposed to the stroma, in analogy to the orientation of these two polypeptides in spinach thylakoids (Cramer *et al.*, 1990). The iron in the heme is coordinated between two His residues, His22 in the α subunit and His22 in the β subunit.

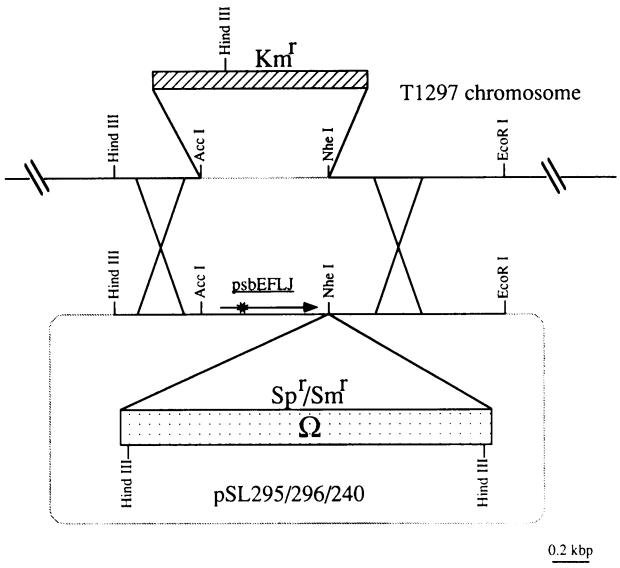

Fig. 2. Scheme for site specific mutagenesis of the *psbEFLJ* region of the genome of *Synechocystis* 6803. The upper line depicts the structure of the genome in this region in the recipient deletion strain, T1297, in which the DNA fragment between the *Acc*I and *Nhe*I sites, containing the *psbEFLJ* region, has been replaced with a kanamycin resistance (Km^r) gene cartridge (Pakrasi *et al.*, 1988). For the introduction of site specific mutations in the *psbE* and *psbF* genes, *in vitro* mutagenized plasmids (pSL295, pSL296 and pSL240) are engineered where a 2.0 kb Sp^r/Sm^r Ω cassette is inserted at the *Nhe*I site. Upon transformation of T1297 cells with such a plasmid, spectinomycin resistant colonies are obtained when double reciprocal recombination events (denoted by two large Xs) lead to the replacement of the Km^r cassette with the mutated *psbEFLJ* gene cluster along with the Sp^r/Sm^r cassette. The * indicates the site of the engineered mutation(s). Further details are given in the text.

Three such recombinant plasmids were used in this study: pSL295 contained an A to T transversion at the CAC codon for His22 in the α subunit resulting in a CTC codon for Leu; pSL296 had a similar change that should change His22 to Leu in the β subunit, whereas pSL240 had both of these mutations. These donor plasmids were used to introduce the mutations into the T1297 strain that has the entire *psbEFLJ* region deleted from its chromosome. The derived Sm^r mutants are designated as T295 (αH22L), T296 (βH22L) and T240 (αH22L plus βH22L). Southern hybridization analysis of the chromosomal DNA of these three mutants indicated that the desired mutations were introduced into the cyanobacterial chromosome via the expected double recombination process (data not shown).

Polymerase chain reaction (PCR) methods were used to amplify asymmetrically the *psbEFLJ* region in the chromosome of the three mutants as well as wild type cells (see Materials and methods). Single-stranded DNA products of such PCR reactions were used to determine the nucleotide sequences of the entire amplified region. Figure 3A shows the presence of the desired A to T change in the *psbF* gene of the mutants T296 and T240. No such change was observed in DNA from wild type or T295 cells. Figure 3B shows the presence of the A to T transversion mutations in the *psbE* gene of the mutants T295 and T240, but not in the T296 mutant nor in wild type cells. No other mutation was present in the *psbEFLJ* region of any of the mutants. Thus the T295, T296 and T240 mutants are expected to have αH22L, βH22L and αH22L plus βH22L respectively, mutations in their cyt b559 protein.

Direct sequencing of PCR amplified single-stranded DNA in this study is a newly applied method in determining the sequence of DNA regions around the sites of directed mutations in *Synechocystis* 6803. This cyanobacterium can have up to 12 copies of its chromosomal DNA in an individual cell (Labarre *et al.*, 1989). Thus, after the introduction of any mutagenic donor DNA and its incorporation into the chromosome, repeated restreaking is necessary to segregate the mutated chromosome from the non-mutated ones (Williams, 1988). Since the direct sequencing of PCR amplified chromosomal DNA determines the presence of any mutation in all copies of the amplified DNA, it is easy to follow the segregation of the mutated DNA from the non-mutated ones.

Single site mutations in T295, T296 and T240 mutations did not destabilize the mRNA product of the psbEFLJ gene cluster

In bacterial systems, a small but significant number of single site mutations in different protein coding regions are known to destabilize the corresponding messages (Kennell, 1986). To eliminate such possibilities and to ensure that the phenotypes of the engineered mutants are due to changes in the primary sequence of their cyt b559 protein only, we examined the presence of the mRNA corresponding to the *psbEFLJ* region (Figure 4). As shown in lane 1, a 0.8 kb mRNA species in wild type cells hybridizes to a DNA probe that encompasses the entire *psbEFLJ* gene cluster. In separate experiments we have determined that this RNA species is recognized by small restriction fragments as well as synthetic oligonucleotide probes corresponding to each of the *psbE*, *psbF*, *psbL* and *psbJ* open reading frames (H.Pakrasi, unpublished observation). Hence, transcription of the *psbEFLJ* region results in a single polycistronic mRNA species.

In a control experiment, RNA from the T1297 deletion strain was fractionated on the same gel and examined by Northern hybridization (lane 5). As expected, the deletion strain does not show the presence of any detectable level of the *psbEFLJ* message. In comparison, all three of the site specific mutants, T295 (lane 2), T296 (lane 3) and T240 (lane 4) had detectable amounts of stable *psbEFLJ* mRNA. Actually, on a total RNA basis, the levels of this message in the three mutant cells were significantly higher than in the wild type cells. The molecular mechanisms underlying these effects are currently unknown. However, other research groups have reported similar increases in the levels of various PSII transcripts when one or more genes are inactivated (Yu and Vermaas, 1990). In any case, it is quite clear that the αH22L and βH22L mutations did not decrease the stability of the *psbEFLJ* message.

His22 – Leu mutations inactivate photosystem II

In the absence of PSII function, *Synechocystis* cells can survive when their growth media are supplemented with 5 mM glucose (Williams, 1988). As shown in Table I, all three of the engineered mutants, T295, T296 and T240 were unable to grow in liquid BG11 medium in the absence of glucose. However, in the presence of glucose, the mutants

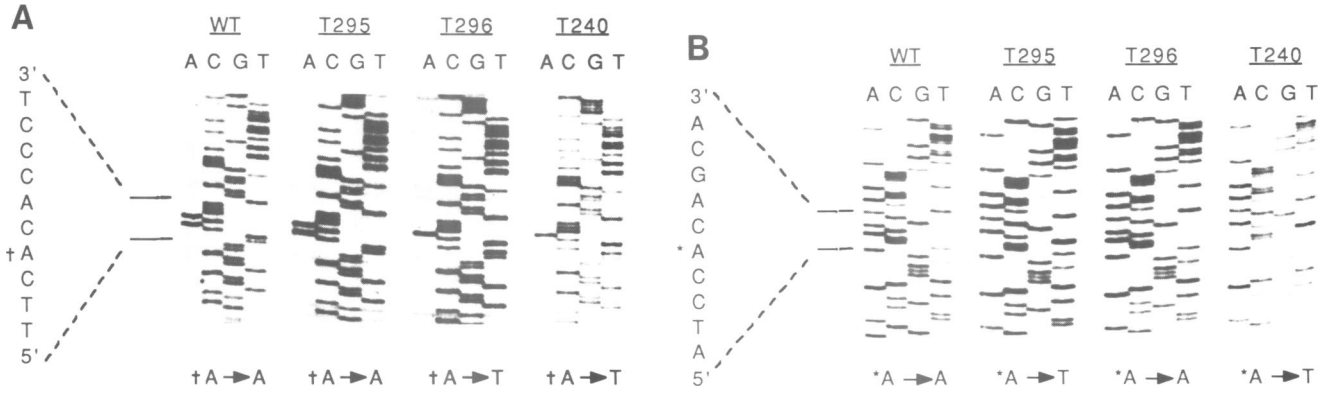

Fig. 3. Nucleotide sequence of single-stranded DNA molecules that are the amplified products of asymmetric polymerase chain reactions on chromosomal DNA from wild type (WT), T295, T296 and T240 cells. The region around the CAC codon for His22 of the β subunit is shown in panel **A** whereas the region around the CAC codon for His22 of the α subunit is shown in panel **B**. The nucleotide sequence of the wild type genes are shown on the left of each panel. * and † indicate the two sites of engineered mutations. Further details are given in the text.

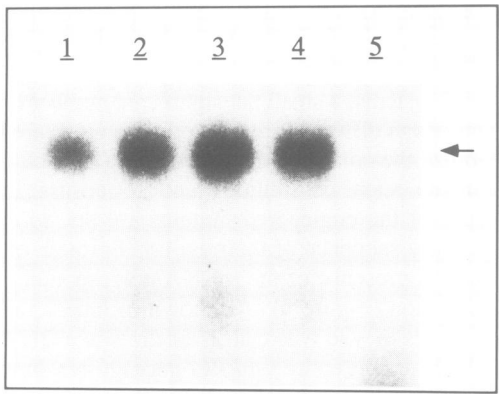

Fig. 4. Northern analysis of RNA from *Synechocystis* 6803, wild type and mutant cells. RNA was isolated from mid log phase cultures of wild type (lane 1), T295 (lane 2), T296 (lane 3), T240 (lane 4) and T1297 (lane 5) cells; and fractionated on a formaldehyde–agarose gel. RNA blotted on nitrocellulose filter was probed with a 0.8 kb *AccI–NheI* ^{32}P-labeled DNA fragment from pKW1261 plasmid (see Figure 2). The arrow indicates a 0.8 kb mRNA species recognized by this *psbEFLJ* probe.

Table I. Growth properties and PSII mediated activities of the cyt b559 H22L mutants

	Wild type	T295	T296	T240
Doubling time (h)				
(a) BG 11	10	NG[a]	NG[a]	NG[a]
(b) BG 11 + glucose	7	20	19	22
PSII mediated O_2 evolution rate[b] (μmol/mg chl/h)	395	0	0	0

[a]NG, no growth.
[b]Light-induced O_2-evolution rates from whole cells in the presence of 0.5 mM 2,6-dichloro-*p*-benzoquinone and 1 mM potassium ferricyanide. The reaction medium was BG 11 + 10 mM $NaHCO_3$.

were able to grow, although at a 3-fold slower rate than that of the wild type cells. These results indicate that T295 and T296 as well as T240 may have impaired PSII activity. To verify this, we measured light induced O_2 evolution rates from whole cells of wild type, T295, T296 and T240 strains. As shown in Table I wild type cells exhibited normal PSII activity, whereas all of the three mutants examined had no detectable PSII activity. Thus the single H22L mutation in each of T295 and T296 mutants and double H22L mutations in T240 resulted in inactivation of PSII.

Steady state levels of photosystem II proteins in the mutants

As discussed above, complete inactivation of individual PSII genes of *Synechocystis* 6803 results in pleiotropic loss of various other PSII proteins (Pakrasi *et al.*, 1988; Yu and Vermaas, 1990). In order to assess the effects of the single nucleotide changes in the *psbE* and *psbF* genes in T295, T296 and T240 mutants, we used monospecific polyclonal antibodies to examine the presence of various PSII proteins in the thylakoid membranes of these mutants (Figure 5). As shown in Figure 5A, D1 was present in wild type cells (lane 1) whereas only faint traces of it were detected in T295 (lane 2), T296 (lane 3) and T240 (lane 4). As described earlier, the deletion mutant T1297 shows the presence of a very low level of D1 (lane 5). A similar picture emerged from the analysis of the D2 protein in these mutants (Figure 5b). We conclude that only trace amounts of the two reaction center proteins, D1 and D2, were present in the membranes of these mutants.

Our earlier experiments have indicated that the CP47, CP43 and the manganese-stabilizing '33 kd' (msp) proteins are present in the thylakoid membrane fraction of the *psbEFLJ* deletion strain, T1297 (Pakrasi *et al.*, 1987, 1989). As shown in Figure 5C and D, the presence of the CP47 apoproteins could be detected in wild type (lane 1), T295 (lane 2), T296 (lane 3), T240 (lane 4) and T1297 (lane 5) thylakoids. Interestingly, the steady state levels of both CP47 and CP43 apoproteins were significantly lower in the three transversion mutants than in either the wild type cells or the T1297 deletion strain. Since each lane in the protein gel contained equivalent chlorophyll-containing material, these results indicate that the stabilities of these two proteins are diminished in these mutants. In six different experiments, the level of the CP43 apoprotein in the three mutants was always found to be $<60\%$ (by densitometric analysis) of that in the wild type cells whereas the amount of the CP47 apoprotein in the mutants was $<80\%$ of that in the wild type cells. As shown in Figure 5E, the extrinsic msp protein was also present in all of the mutants. Thus, the three new mutants in this study have identical composition of PSII polypeptides other than cyt b559, although the relative amounts of various proteins were varied in different mutants.

The most interesting observation was with respect to the stability of the cyt b559 protein itself. We used a polyclonal antibody preparation directed against the α subunit of cyt b559 from *Synechococcus vulcanus*, a thermophilic cyanobacterium. Amino acid sequence analysis demonstrates a high degree of homology between the α subunits of cyt b559 from this organism and *Synechocystis* 6803 (Ikeuchi *et al.*, 1989). As shown in Figure 5F, this antibody preparation recognized the 9.5 kd α subunit of cyt b559 in thylakoids of wild type *Synechocystis* cells (lane 1). As expected, the deletion strain T1297, did not show the presence of any hybridizing band (lane 5). Interestingly, both T295 and T296 thylakoids showed the presence of a faster migrating band ($\sim$8 kd) that was recognized by these antibodies. The double mutant, T240, did not show the presence of such a band. Thus, in the absence of any one of the two axial His ligands of cyt b559, its α subunit migrates at an anomalous position.

Flash induced reduction of cytochrome b559

The light induced reduction of cyt b559 was assayed by illuminating thylakoid membranes in the presence of tetraphenylboron and ferricyanide. In spinach thylakoids, Velthuys (1981) demonstrated that tetraphenylboron, in the presence of ferricyanide, can act as a two-electron donor following a short actinic flash. The first electron reduces a PSII donor oxidized by the flash; the second electron reduces cyt b559. This same reaction has been shown to occur in wild type *Synechocystis* 6803 cells, but not in a deletion mutant that lacks the cytochrome (Pakrasi *et al.*, 1989). The spectrum of the flash induced absorbance increase observed in *Synechocystis* 6803 thylakoid membranes had a peak at 559 nm, the same as the α-band of the protein in a purified PSII complex (H.B.Pakrasi, H.Koike, M.Ikeuchi and Y.Inoue, unpublished observations), indicating reduction of the cytochrome (Figure 6A). Under identical experimental conditions the mutants T240, T295 and T296 did not exhibit a peak associated with cyt b559

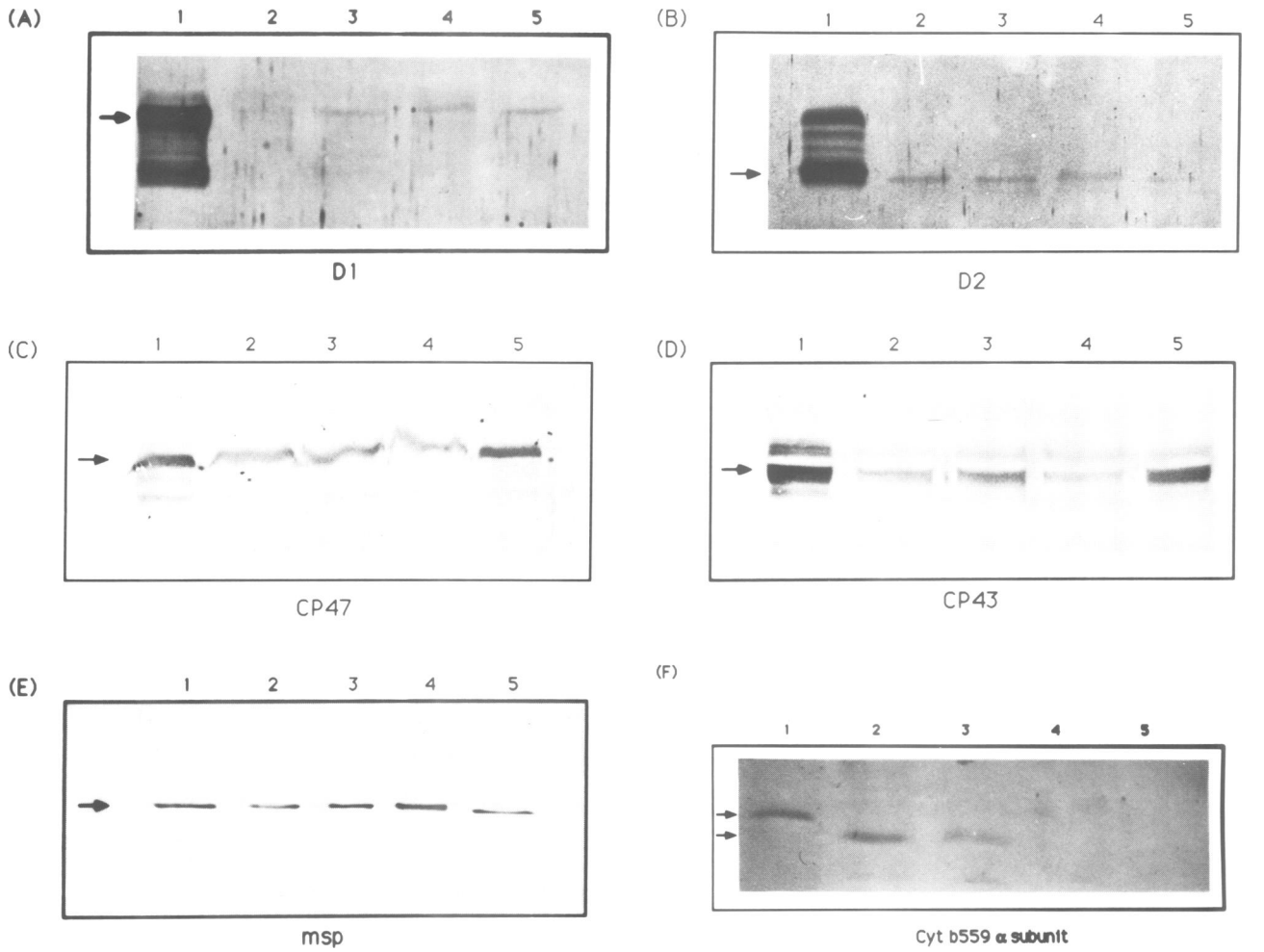

Fig. 5. Western blot analysis of thylakoid proteins. Thylakoids from wild type (lane 1), T295 (lane 2), T296 (lane 3), T240 (lane 4) and T1297 (lane 5) cells were fractionated on gradient acrylamide gels and electrophoretically transferred on nitrocellulose filters. Such filters were subsequently probed with monospecific antibodies against the D1 protein (panel **A**), D2 protein (**B**), CP47 apoprotein (**C**), CP43 apoprotein (**D**), extrinsic manganese stabilizing protein, msp (**E**) and the α subunit of cyt b559 (**F**). 10 μg chlorophyll-containing material was loaded in each lane.

reduction. The only discernable structure observable in the spectra of the mutants was a small peak at 563 nm, due to the reduction of cyt b_6. The difference spectrum between wild type and the mutant T295 cells shows the α band of cyt b559 (Figure 6B). In this assay technique, the oxidation of the *c* type cytochromes by ferricyanide, and the loss of the soluble cytochromes during membrane isolation, makes their contribution to the spectrum negligible. Based on the spectrum shown in Figure 6B, the amount of cyt b559 undergoing light induced reduction is ~1 cyt b559 heme/840 chlorophyll. This is a functional assay that demonstrates the existence of at least one cyt b559 heme per 840 chlorophyll molecules in photosynthetic membranes of wild type *Synechocystis* cells. In membranes of wild type cells, the chemical difference spectrum (dithionite minus ferricyanide) shows a higher concentration of the cytochrome, indicating that the light induced assay does not reveal turnover of the entire complement of cyt b559 present in the membrane (data not shown). Due to the contribution of overlapping spectra from other cytochromes, the chemical difference spectrum was not accurate enough to test the existence of cyt b559 in membranes isolated from the mutants. As shown in Figure 6A, in the mutants T240, T295 and T296 there is no detectable functional cyt b559 undergoing light induced reduction.

Discussion

The primary goal of our work was to determine whether the heme axial ligands have any role in the structural stability of the cyt b559 protein. The unequivocal answer is that they do. To answer such a question, we have used a site directed mutagenesis approach to replace specifically the individual heme ligand His residues in the α and the β subunits of cyt b559 with Leu, a hydrophobic amino acid that is unable to coordinate the heme iron. The overall effect of such mutations was dramatic in that they destabilized the structure of the reaction center of the PSII complex.

The functional role of cyt b559 in electron and/or proton transfer reactions mediated by the PSII complex is poorly understood. Recently Buser *et al.* (1990) reported that cyt b559 is involved in an alternative electron transfer pathway leading to P680, the primary donor chlorophyll molecule in PSII, even though, under physiological conditions, the competing electron transfer reaction from Y_Z, the normal electron donor, to P680 is three orders of magnitude faster than the cyt b559 mediated reaction. Although the function of cyt b559 remains to be elucidated, it is clear that this protein plays a critical role in the stabilization of a functional PSII complex. Mutants in which the entire *psbEFLJ* gene cluster (Pakrasi *et al.*, 1988), the individual *psbE*

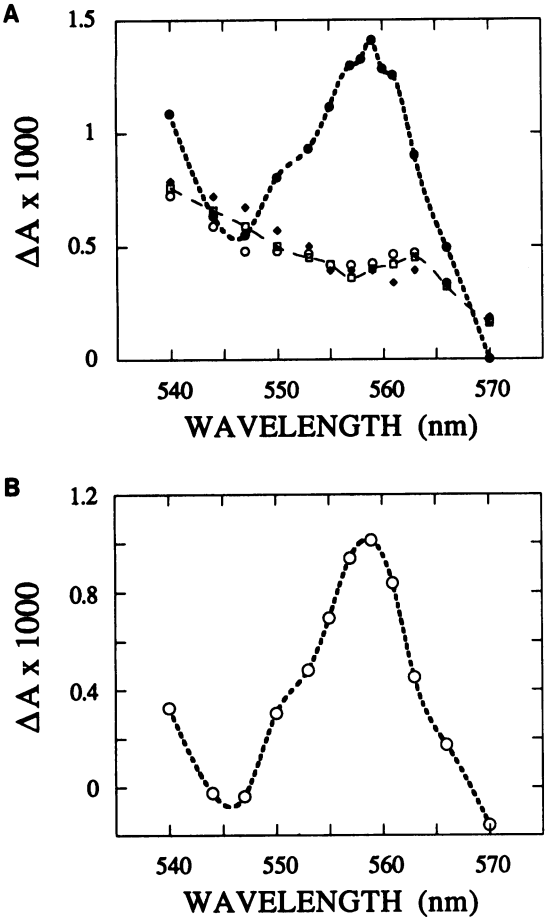

Fig. 6. A. Spectra of flash induced absorption changes measured using thylakoid membranes from *Synechocystis* 6803 wild type (solid circles), T295 (open squares), T296 (solid diamonds), and T240 (open circles) cells. Thylakoid membranes were suspended at a chlorophyll concentration of 56 μM in a reaction medium consisting of 4 mM HEPES−KOH pH 7.5, 10 mM NaCl, 200 μM $K_3Fe(CN)_6$, and 40 μM Na-tetraphenylboron. Flash induced absorbance changes were measured between 10 and 50 ms after the flash. Samples were dark adapted for 2.5−3 min. Further details are given in the text. **B.** Wild type minus T295 difference spectrum of the flash induced absorbance change measured using thylakoid membranes of *Synechocystis* 6803. The difference spectrum is taken from the data shown in A.

(unpublished observation) or *psbF* (Pakrasi *et al.*, 1990) gene is deleted from the chromosome of *Synechocystis* 6803 show a complete absence of all PSII reaction center activity. Moreover, in addition to the cyt b559 subunit(s), they have lost the two reaction center proteins, D1 and D2. Thus, it seems that in the absence of any one or both of the subunits of cyt b559, stable assembly of the D1−D2 reaction center complex of PSII is not possible.

Transcription of psbEFLJ gene cluster is markedly different between chloroplast and cyanobacteria

In order to ensure that the primary effects of single site mutations resulting in the H22L mutation in the three mutants, T259, T296 and T240 are not at a transcriptional level, we examined the relative amounts of the *psbEFLJ* message in wild type and mutant cells (see Figure 4). In wild type cells, a single species of mRNA (0.8 kb) was the only stable transcript detected for this region of the chromosome. In contrast, in plastids of vascular plants, multiple heterogeneous transcripts arise from this gene cluster region (Westhoff *et al.*, 1985; Haley and Bogorad, 1990). In maize chloroplasts, transcripts of six different sizes, 4.0 kb, 2.8 kb, 1.7 kb, 1.4 kb, 1.1 kb and 0.8 kb, are detected that hybridize to the *psbEFLJ* gene probes (Haley and Bogorad, 1990). Among these, the smallest transcript (0.8 kb) may correspond to the single 0.8 kb transcript in wild type *Synechocystis* 6803 cells. A similar phenomenon has been observed with the *psbB* gene of *Synechocystis* 6803 where a single 2.0 kb stable transcript was observed (Yu and Vermaas, 1990). The corresponding region of the plastid chromosomes gives rise to a large number of overlapping transcripts (Westhoff and Herrmann, 1988).

The single site mutations in T295, T296 and T240 mutants did not result in destabilization of the 0.8 kb transcript. On the contrary, the relative levels of these transcripts were increased ~2.3-fold (determined by densitometric scanning) in each of the three mutants (see Figure 4). In *Escherichia coli* and *Bacillus subtilis*, two widely studied bacterial systems, only nonsense mutations in the protein coding regions of various genes are known to affect the stability of the corresponding message (Kennell, 1986). Thus, it is not surprising that the introduction of missense mutations in the three mutants of interest in this study did not destabilize the 0.8 kb message.

αH22L and βH22L replacements in cyt b559 destabilizes the PSII complex

The profiles of various polypeptide components of PSII in these three mutants were similar to those found in the deletion mutants T1297 or T256 (Pakrasi *et al.*, 1990) where respectively the entire *psbEFLJ* or *psbF* gene was deleted. In all three mutants, T295, T296 and T240, the steady state levels of the reaction center proteins D1 and D2 were severely diminished. Interestingly, the transcripts of *psbA* (encoding D1) and *psbD* (encoding D2) were present in near normal amounts in these three mutants (data not shown), although only trace amounts of these two proteins were detected in the thylakoid membranes (see Figure 5). Thus, it seems likely that the D1 and D2 proteins are synthesized and inserted in the thylakoid membranes and then turned over rapidly in the absence of a functional cyt b559 protein. Vermaas and coworkers (Vermaas *et al.*, 1987, 1988) have reported similar findings when the presumptive P680 chlorophyll binding His197 residue in the D2 protein of *Synechocystis* 6803 was changed to Tyr. As a result of this mutation, both D1 and D2 proteins as well as the CP43 apoprotein are absent under steady state growth conditions. In the present study, the absence of cofactor binding in the cyt b559 protein decreases the steady state levels of the D1 and D2 proteins.

In contrast, the apoproteins of CP47 and CP43 are present at significant although reduced levels in the three mutants (Figure 5C and D). We have earlier shown that these two proteins are present at wild type levels in the deletion strains T1297 (Δ*psbEFLJ*) and T256 (Δ*psbF*). It seems that the synthesis, insertion and stability of these two proteins are not severely affected by any mutation in the cyt b559 protein studied thus far. Similar to T1297 and T256, the fluorescence emission peak at 695 nm (F695 at 77K) is absent in these three mutants (data not shown). The origin of F695 is known to be on the CP47 protein (Nakatani *et al.*, 1984). Perhaps an interaction between the CP47 protein and other proteins of PSII is responsible for the proper organization of

chlorophyll molecule(s) on CP47 so that it may emit F695. However, we do not know whether the apoproteins of CP47 and CP43 bind their normal complements of chlorophylls in these mutants. The relative amounts of the CP47 and CP43 apoproteins in the thylakoids of T295, T296 and T240 were significantly lower than those in the wild type cells. However, the *psbEFLJ* deletion strain, T1297, had amounts of CP47 and CP43 apoproteins that are very similar to those in wild type cells. Thus, it seems that in the absence of any cyt b559 protein (as in T1297), the levels of CP47 and CP43 remain normal. On the other hand, if any cyt b559 protein of unusual structure (as in T295, T296 and T240) are even transiently present in the thylakoids, the steady state levels of CP47 and CP43 are significantly decreased. The molecular mechanism for this effect is currently unknown.

'msp', an extrinsic protein associated with PSII is present in near normal amounts in these thylakoids. Recent deletion experiments by Burnap and Sherman (1991) have shown that active PSII complexes are synthesized in *Synechocystis* 6803 cells that do not have the msp protein. Thus, it is conceivable that the synthesis and stability of the msp protein is relatively independent of the presence of other integral protein components of the PSII core.

An interesting aspect of our results is the presence of an aberrantly migrating α subunit of cyt b559 in the membrane fractions of the single point mutants T295 (αH22L) and T296 (βH22L). As shown in Figure 5, the presence of the α subunit could not be detected in the double mutant, T240, nor could it be detected in the deletion strain, T1297. A simple mechanistic explanation for this observation could be that cyt b559 assumes a different conformation depending on whether the heme prosthetic group is coordinated in the normal way. However, the apoproteins are synthesized and inserted in the membrane. In each of the two mutants, T295 and T296, one of the two axial ligands for the heme iron is missing, but the other histidine still acts as the fifth ligand. Some endogenous protease may recognize the α subunit in an abnormal conformation and cleave off a membrane exposed fragment. In the double mutant T240, the heme cannot be coordinated to either of the two subunits. In this case, both of the subunits become extremely unstable and cannot be detected at any appreciable level. In this scenario, the binding of the cofactor heme is crucially important for the proper conformation and stability of the cyt b559 protein. A consequence of this picture is that cyt b559 is a centrally important protein in PSII, without which neither D1 nor D2 protein can be stably assembled. In contrast, the absence of the D1 protein allows stable integration of cyt b559 in the membranes of *Synechocystis* 6803 (Nilsson *et al.*, 1990).

Recently, a number of studies have documented the effect of site specific changes of the heme binding His residues in various membrane-bound cytochromes (Friden and Hederstedt, 1990; Fang *et al.*, 1989). In a directed mutant of *B.subtilis* in which a His residue is changed to Tyr in the cyt b558 protein, the apoprotein is inserted in the membrane but does not bind heme. This change also results in a decrease in the amount of the cyt b558 protein (Friden and Hederstedt, 1990). In a detailed study on cyt *d* from *E.coli*, Fang *et al.* (1989) mutagenized 10 different His residues to Tyr, and found that two of them were essential for the coordination of the hemes in cyt b558, cyt b595 and cyt *d*. In these two His to Tyr mutants, the protein components of the enzyme complex (subunits I and II) are stably inserted in the membrane. Interestingly, subunit I becomes more susceptible to proteolysis in one of the mutants. Sligar *et al.* (1987) have mutagenized cyt b_5 of hepatic endoplasmic reticulum to change one of the heme ligand His residues to Met. This mutant protein was capable of incorporating heme, although the heme environment was substantially modified. Thus, there are several examples in which heme ligand His residues have been modified in *b* type cytochromes where the protein folding and stability have not been changed significantly.

Why is the cytochrome b559 protein so much more labile than other *b* type cytochromes when the cofactor binding residue is altered? The answer may lie in the unusual structure of cyt b559. It is the only known membrane-bound multisubunit cytochrome in which the different subunits are bound together through the ligands between the heme iron and His nitrogen (see Figure 1). Thus, if this cytochrome has a heterodimeric structure, the loss of even one of the two axial ligands is expected to destabilize the structure of the protein severely. In contrast, other membrane-bound cytochromes such as cyt b558 and cyt b595 have multiple membrane spanning α-helical stretches in each polypeptide subunit. In these cytochromes, the heme coordination is not the sole binding force between two different membrane-spanning α helices and the proteins are thus structurally stable even in the absence of heme coordination.

Materials and methods

Materials

All chemicals used were of reagent grade. Enzymes for recombinant DNA work were either from New England Biolab or Promega. [α^{35}S]dATP (>1000 Ci/mmol) for nucleotide sequencing and [α-^{32}P]dCTP (2000 Ci/mmol) for radioactive labeling of DNA fragments were obtained from Amersham. Deoxy- and dideoxynucleoside triphosphates were from Pharmacia. DNA sequencing was performed using modified T7 DNA polymerase (Sequenase, US Biochemicals).

Bacteria and plasmids

Synechocystis 6803 wild type cells were grown as described earlier (Pakrasi *et al.*, 1988). PSII deficient mutants, T1297, T295, T296 and T240 were maintained on solid BG11 media supplemented with glucose (5 mM), atrazine (5 μM) and either kanamycin (T1297) or spectinomycin (T295, T296 and T240) (Elhai *et al.*, 1990). *Escherichia coli*, DH5αF' (Bethesda Research Labs) strain was used for propagation of various plasmids. For site specific mutagenesis, RZ1032 (Kunkel *et al.*, 1987) and BMH 71-18 *mutL* (Kramer *et al.*, 1984) strains of *E.coli* were used (see below).

pUC118 was used as the basic cloning plasmid (Vieira and Messing, 1987). pSL132 is pUC118 containing a 2.2 kb genomic DNA fragment of *Synechocystis* 6803 that contains the *psbEFLJ* region (Pakrasi *et al.*, 1988, 1990).

Isolation of nucleic acids, Southern and Northern hybridization

High molecular weight chromosomal DNA from *Synechocystis* 6803 cells was isolated as described earlier (Pakrasi *et al.*, 1988). ^{32}P-labeled DNA probes were synthesized by using random oligonucleotides (Feinberg and Vogelstein, 1983). DNA fragments fractionated on 0.8% agarose gels were transferred on Gene Screen Plus nylon membrane (New England Nuclear) and hybridization was performed under high stringency condition, as suggested by the manufacturer. For the isolation of RNA from wild type and various mutant strains, 500 ml cultures at mid log phase of growth were harvested and the procedure of Reddy *et al.* (1990) was followed. 10 μg of cellular RNA was fractionated on agarose–formaldehyde gels, transferred on 0.45 μm nitrocellulose filter and hybridized to ^{32}P-labeled DNA probes under stringent conditions (Yarger *et al.*, 1986).

Site specific mutagenesis

For oligonucleotide directed mutagenesis of the *psbE* and *psbF* genes, we first introduced the pSL132 plasmid into *dut*$^-$, *ung*$^-$ *E.coli* strain RZ1032 (Kunkel *et al.*, 1987). To engineer a His to Leu change in the α subunit

of cyt b559, the following synthetic oligonucleotide was used: 5′-TGATCCTCAGCATCACCA-3′ (oligo 1). A second oligonucleotide, 5′-CGGTTCTCACCCTGGAGG-3′ (oligo 2) was used to create a similar change in the β subunit of the protein (the underlined nucleotide indicates the base substitution to be introduced). After annealing of each primer to single-stranded pSL132 DNA (from RZ1032 host), strand extension by Klenow polymerase and ligation, the DNA was introduced into mismatch repair deficient BMH 71-18 *mutL* strain (see Eggenberger *et al.*, 1990, for more detail). The desired mutated plasmid was identified by direct sequencing of double-stranded plasmid DNA. Among these newly created plasmids, pSL295 contained the mutation in the *psb*E gene whereas pSL296 contained the desired mutation in the *psbF* gene. Single-stranded forms of pSL296 and oligo 1 were subsequently used to create pSL240 plasmid that contained both of the mutations. Furthermore, pSL295, pSL296 and pSL240 plasmids also contained a Sp^r/Sm^r resistance gene cartridge (Ω fragment; Prentki and Krisch, 1984) at an *Nhe*I site downstream of the *psbEFLJ* region (Pakrasi *et al.*, 1990).

These donor plasmids were used to introduce the desired mutations into T1297 strain which lacks the entire *psbEFLJ* gene cluster. Transformation of cyanobacterial cells with plasmid DNA was performed as described elsewhere (Pakrasi *et al.*, 1988). Desired transformants were selected in the presence of spectinomycin (7 μg/ml) in the growth medium.

Direct sequence determination of PCR amplified DNA from mutants

A rapid procedure was developed to isolate chromosomal DNA from a small patch of *Synechocystis* cells on solid BG11 medium. Cells were resuspended in 700 μl of TE (10 mM Tris−Cl, 1 mM Na-EDTA, pH 8.0), 1% SDS. After incubation at 65°C for 5 min, proteinase K (Boehringer) was added to 0.2 mg/ml. The mixture was incubated at 45°C for 60 min. RNase A was then added to a concentration of 50 μg/ml and the mixture was further incubated at 37°C for 15 min. After two extractions with phenol−chloroform and one extraction with chloroform, DNA in the aqueous solution was precipitated by the addition of sodium acetate and ethanol. Typical yield of isolated DNA was 10−20 μg.

Polymerase chain reaction (PCR) was carried out on 50 ng chromosomal DNA, using an oligonucleotide that binds to a sequence 5′ to the *psbE* gene (5′-TAGGCGGCTCACAAAATAGT-3′; oligo 3) and another one that hybridizes to the complementary strand of DNA on the 3′ edge of the *psbJ* gene (5′-TTACATGGAAGAACCTAA-3′; oligo 4). For asymmetric amplification of the non-coding strand, 0.25 pmol of oligo 3 and 25 pmol of oligo 4 were used in a PCR reaction mixture (Gyllensten and Erlich, 1990). The reaction conditions were identical to those described elsewhere (Pakrasi *et al.*, 1990). Products of each PCR reaction were analyzed on 0.8% agarose gels, single-stranded DNA was eluted from the gel and directly used in sequencing reactions for the determination of the nucleotide sequence at and around the site(s) of mutation.

Protein analysis and immunoblotting

Membranes from wild type and various mutant cells were isolated by using a procedure described elsewhere (Pakrasi *et al.*, 1990). Chlorophyll *a* concentration was measured in methanol (MacKinney, 1941).

For the analysis of large PSII proteins (i.e. D1, D2, CP47, CP43 and msp), membrane proteins were fractionated on a lithium dodecyl sulfate−polyacrylamide gel system described earlier (Pakrasi *et al.*, 1990). Western immunoblotting was performed according to the procedure described elsewhere (Pakrasi *et al.*, 1989). To analyze the presence of low molecular weight polypeptides (e.g. the α subunits of cyt b559), a highly resolving urea−SDS gel (16−22% acrylamide gradient) was used according to Ikeuchi and Inoue (1988). The fractionated proteins were transferred onto 0.2 μm nitrocellulose filters on a semi-dry gel blotter (Fisher), the transferred proteins were visualized by staining with amido black and then immunostained. Rabbit polyclonal, monospecific antibodies raised against D1, D2, CP47, CP43 and msp proteins of spinach PSII complex were kind gifts from Drs Ikeuchi and Inoue. Polyclonal antibodies against the 8 kd α subunit of cyt b559 of *Synechococcus vulcanus* were raised in rabbits (H.B.Pakrasi, H.Koike, M.Ikeuchi and Y.Inoue, unpublished observations).

Spectral analysis of cytochrome b559

Light induced absorbance changes were measured using a single-beam spectrophotometer described elsewhere (Whitmarsh and Ort, 1984). Actinic flashes were provided by two xenon flash lamps (FX200, E.G &G Electro-Optics, Salem, MA) oriented perpendicular to the measuring beam. The response time of the instrument was 1 ms. The pathlength of the cuvette was 10 mm and the half-bandwidth of the measuring beam was 3 nm. Thylakoids in the reaction medium were incubated in the dark for 5 min before illumination. To improve signal to noise ratio 8−16 traces were averaged. Samples were maintained at 25−27°C and were dark adapted for 2.5−3 min between flashes. The concentration of cyt b559 was calculated using a reduced minus oxidized extinction coefficient of 17.5 $mM^{-1} \cdot cm^{-1}$ (Cramer *et al.*, 1986), using the absorbance measured at 559 minus that measured at 570 nm.

Other methods

PSII mediated O_2 evolution activities of whole cells were determined using a Clark type O_2 electrode, as described elsewhere (Pakrasi *et al.*, 1988). Growth of cyanobacterial cultures was estimated by measuring light scattering at 730 nm on an SLM DW 2000 spectrophotometer. Relative amounts of RNA and proteins in different lanes in either a Northern or a Western blot were determined on a computing densitometer (Molecular Dynamics, Model 300A).

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