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Transcription of a “silent” cyanobacterial *psbA* gene is induced by microaerobic conditions

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ABSTRACT

Cyanobacteria, contrary to higher plants, have a small *psbA* gene family encoding the reaction centre D1 protein subunit of photosystem II, the first macromolecular pigment-protein complex of the photosynthetic electron transport chain. Modulation of expression of multiple *psbA* genes in the family allows cyanobacteria to adapt to changing environmental conditions. To date, two different strategies for regulation of the *psbA* genes have emerged. One, characterized in *Synechocystis* PCC6803 and *Gloeobacter violaceus* PCC7421 involves the increased expression of one type of D1 protein to cope with the increased rate of damage. The other strategy, in *Synechococcus* PCC7942 and *Anabaena* PCC7120, is to replace the existing D1 with a new D1 form for the duration of the stress. However, most of the *psbA* gene families characterized to date contain also a divergent, apparently silent *psbA* gene of unknown function. This gene, present in *Synechocystis*, *Anabaena* and *Thermosynechococcus elongatus* BP-1 was not induced by any stress condition applied so far. Our data shows a reversible induction of the divergent *psbA* gene during the onset of argon-induced microaerobic conditions in *Synechocystis*, *Anabaena* and *Thermosynechococcus elongatus*. The unitary functional response of three unrelated cyanobacterial species, namely the induction of the expression of the divergent *psbA* gene as a reaction to the same environmental cue, indicates that these genes and the protein they encode are part of a specific cellular response to microaerobic conditions. There are no specific primary structure similarities between the different microaerobic inducible D1 forms, designated as D1′. Only three amino acid residues are consistently conserved in D1′. These modifications are: G80 to A, F158 to L and T286 to L. *In silico* mutation of the published D1 structure from *Thermosynechococcus* did not reveal major modifications. The point by point effects of the mutations on the local environment of the PSII structure are also discussed.

1. Introduction

D1 protein is part of the multi-subunit protein complex of photosystem II (PSII) at the core of the photosynthetic apparatus and is one of the main sites of damage by a wide variety of environmental factors [1,2]. Damaged D1 proteins are replaced with newly synthesized ones encoded by the *psbA* gene. While higher plants have one *psbA* gene, cyanobacteria generally display a small gene family (http://www.kazusa.or.jp/cyano/, http://genome.jgi-psf.org/mic_home.html). The *psbA* genes in cyanobacteria encode several forms of the D1 protein [3–7]. Transcriptional regulation studies have confirmed two distinct regulation strategies to cope with stress conditions. One is to enhance the production of the same type of D1 protein, compensating the higher rate of damage under stress [8,9]. This behavior is best characterized in *Synechocystis* where only one type of D1 is encoded by both *psbAII* (*slr1311*) and *psbAIII*(*sll1867*). The transcript of *psbAII* accounts for about 90% of the total *psbA* transcript pool under basic growth conditions. When a stress condition is detected, the cells respond by increasing the total *psbA* transcript pool by *psbAIII* induction, as has been shown by a highly selective S1 nuclease assay [8]. Subsequent microarray and Northern blotting studies showed a general induction of both *psbAII* and *psbAIII* genes [10,11].

Another strategy is the use of two distinct types of D1 protein, D1:1 and D1:2, where the former, present under normal growth conditions, is replaced by the latter under stress conditions [12–14]. This strategy was described in *Synechococcus* 7942, *Anabaena* 7120 and *Thermosynechococcus elongatus*, where the overall regulation of D1 forms involves environmentally dependent transcription, translation and D1 turnover changes [6,15].

A divergent *psbA* sequence, *psbA1*(*slr1181*), encoding a different D1 protein was found in *Synechocystis* sp. PCC6803 [16]. While the

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Abreviations: Synechocystis, Synechocystis sp. PCC6803; Anabaena, Anabaena/Nostoc sp. PCC7120; Thermosynechococcus elongatus, Thermosynechococcus elongatus BP-1

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expression of the *psbAI* gene could theoretically be relevant to stress response, this gene has been considered silent and its function has been a mystery [5]. Artificial induction of the *psbA1* gene through site-directed mutagenesis of the upstream of the gene [17] produces a functional, albeit aberrant D1 protein called D1′ [18,19]. Apart from *Synechocystis*, divergent, low-expressed and non-responsive *psbA* genes have recently been found in *Anabaena* sp. PCC7120 (*psbA0-alr3742*) [6], *Thermosynechococcus elongatus* BP-1 [12,13] (*psbA2-tlr1844*) and possibly *Gloeobacter violaceus* PCC7421 [9]. In fact, out of the five cyanobacteria species where the expression of the *psbA* gene family has been characterized, *Synechococcus* sp. PCC7942 is the only one that does not contain such a gene [20]. We present here the effect of short-time microaerobic exposure on the expression of the *psbA* gene families from *Synechocystis*, *Anabaena* and *Thermosynechococcus*. Under microaerobic conditions, the divergent *psbA* gene that was previously believed to be silent was induced to significant levels, even more than the previously described stress-inducible *psbA* genes. We conclude that the three *psbA* genes present in the different cyanobacterial species encode a novel functional class of D1 proteins (D1′) that are more common than previously believed.

2. Materials and methods

2.1. Strains, growth and treatment conditions

All cyanobacterial strains were cultivated in BG-11 liquid medium [21] in polycarbonate cell culturing bottles bubbled with air.

Synechocystis 6803 and *Anabaena* 7120 were obtained from Pasteur Culture Collection and grown at 30 °C and 50 μmol photons $m^{-2}s^{-1}$ light.

Thermosynechococcus elongatus BP-1 was a kind donation of Dr. Teruo Ogawa and was grown at 45 °C and 50 μmol photons $m^{-2}s^{-1}$ light.

All strains were grown until 6.5 μg chl ml^{-1} as assessed by 100% methanol extraction [22]. Air was then replaced with Ar by bubbling for 90 min. Within 10 min of starting the Ar treatment, the amounts of O_2 and N_2 in cyanobacterial cultures was found to be reduced to below 5% of their original values, as measured by gas chromatography (Fig. 1). Subsequent bubbling with air for 60 min returned the culture to aerobic conditions. 10 ml aliquots for RT-Q-RT-PCR were taken after 0, 30, 60 and 90 min of Ar exposure, as well as after the 60 min of bubbling with air.

2.2. Flash-induced fluorescence measurements

Flash-induced chlorophyll fluorescence and its subsequent decay were measured using a double-modulation fluorimeter from PSI Instruments (Brno, Czech Republic) [23]. The F_m parameter, proportional to the number of PSII centers capable of forming Q_A^-, was recorded in the presence and absence of the PSII inhibitor 3-(3′,4′- dichlorphenyl)-1,1-dimethylurea (DCMU), as described earlier in Sicora et al. 2006 [6]. The decay of the flash-induced fluorescence in the presence of DCMU reflects the Q_A^- recombination with the donor side of PSII. We monitored the decay with a logarithmic sequence of measuring flashes over a 100 s time range [26]. The measured curves were deconvoluted using an equation with one exponential and one hyperbolic components:

$$F(t) - F0 = A1 * \exp(-(x-x0)/T1) + A2/((1+(x-x0)/T2))\hat{}(1/(b-1));$$

where $F(t)$ is the variable fluorescence yield; $F0$ is the fluorescence level before the flash; A1 and A2 are the amplitudes; T1 T2 are the time constants from which the half-lives can be calculated as $t1/2 = \ln 2\ T$ for the exponential component and T for the hyperbolic component. b=2, x=0.00119. The curves were fitted using Origin 8.0 OriginLab Corporation, Northampton, MA, USA.

Table 1
Oligonucleotide sequences used as primers in the Real-Time PCR experiments

Strain	Name	Orientation	Primer sequence 5′–3′
Synechocystis 6803	*psbA1*	Forward	AGCTTAAACCCAAAATCTTACTTCGT
	psbA2	Forward	CCTTTAGACTAAGTTTAGTCAGTTCCA
	psbA3	Forward	GAGCTTGAGGCCAAATCCTTTGAACA
	psbA common	Reverse	CCATCAGAGAAGGAGCCTTGACCA
Anabaena 7120	psbA0	Forward	CCTTGTCGGTTGATATTTCACCATC
	psbA1	Forward	GCGTCTGAATTAATCTGCACAAATCC
	psbA2	Forward	TTGTTACAGGCAAAAAACAACTGCC
	psbA3	Forward	CCGCCTGATTCATAAATAATTAATCGC
	psbA4	Forward	TGTAACAGGTGGAAACGCCTACAA
	psbA common	Reverse	GGAACCGTGCATTGCAGAGAA
Thermosynechococcus elongatus BP-1	psbA1	Forward	GCTTAAGCCCTTGCAGTTGATGAC
	psbA2	Forward	TACTGCTGCTTTGGTCCCGTTGTTA
	psbA3	Forward	CTGAAGTCTTCCAGGCACTCAACT
	psbA common	Reverse	GCCAGAAACAGGCTCACGGAT
Common rnpB primers (reference gene)	*rnpB-F*	Forward	CTCCCGAAAGACCAGACTTG
	rnpB-R	Reverse	CCGGGTTCTGTTCTCTGTGT

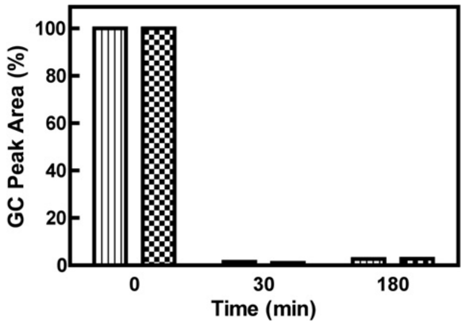


Fig. 1. Induction of microaerobic conditions by argon flushing. The figure shows the change in the concentration of oxygen (vertical lines) and nitrogen (checkered pattern) in the culture flask induced by the replacement of air with pure argon in the bubbling system for up to 180 min.

2.3. Real-time quantitative RT-PCR

RNA-isolation, DNA-ase treatment and first strand cDNA synthesis were performed as previously described in Sicora et al. 2006 [6]. Gene-specific primer pairs were designed (see Table 1) for the amplification of products of similar but different sizes that can subsequently be discriminated by melting curve analysis. The primers were then tested on a 10C gradient real-time RT-PCR. For each individual pair, the temperature range of maximum amplification efficiency was determined and the annealing temperature used in the real-time RT-PCR was set within those limits. Using serial dilutions of template, the dynamic range of the reaction was identified to ensure that the changes in the gene expression are well placed within this range. NTC (no-template control) and –RT control (reverse transcriptase replaced with water) were used to check for foreign template as well as genomic template contaminations, respectively. The *rnpB* gene (encoding RNase P subunit B) was consistently used as a reference gene in all real-time RT-PCR experiments, as its transcript level was unaffected by microaerobic treatments (data not shown). The efficiency of each real-time PCR was estimated using the LineReg program [24] and an average was then used in the subsequent calculations. The change in the transcripts for each gene during the treatment as well as their recovery was then quantified relative to the transcripts for the respective genes in the control samples:

$$\text{Target transcript relative to Time Zero control} = eff^{(\text{CT target at control} - \text{CT target at specific time point})}$$

where CT is the cycle value when the amplicon fluorescence emission reached the detection threshold, and *eff* is the amplification efficiency. Values for target transcripts were plotted as multiples of the control level at time zero (Fig. 3).

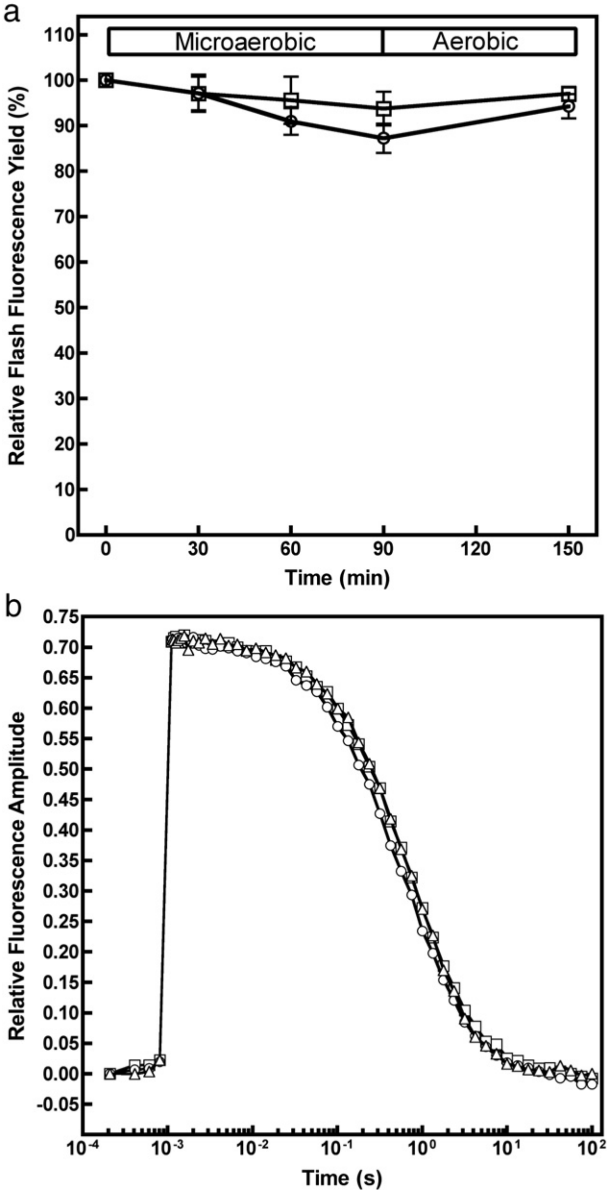


Fig. 2. Panel (a) shows the effect of microaerobic conditions on the amplitude of flash fluorescence in the presence and absence of DCMU. The cells were exposed to microaerobic conditions for 90 min followed by return to air for 60 min. 1 ml samples for flash-fluorescence measurements were taken at T_0 control, and 30, 60, 90 and 150 min during the treatment and fluorescence of each sample was measured in the absence (open square) and presence (open circle) of DCMU. Effects of microaerobic conditions on the relaxation of flash-induced fluorescence in the presence of DCMU in *Synechocystis* PCC6803 are shown in panel (b). Cells were treated for 90 min and then allowed to recover in aerobic conditions for a further 60 min. The control (open squares), 90 min treated (open circles) and 60 min recovery (open up triangles) curves were plotted normalized to the control maximum. The 30 min and 60 min treated curves were omitted for clarity.

To calculate the relative transcript levels we used the CT of the *rnpB* housekeeping gene as a reference point, using the formula:

$$\text{Relative Transcript Level} = eff^{(\text{CT reference gene} - \text{CT target gene})}$$

where *eff* represents the efficiency of the reaction, as mentioned above.

2.4. Modeling and alignments

Alignment of the *psbA* sequences and the phylogenetic tree were built with CLC Free Workbench by CLC bio A/S (www.clcbio.com).

The molecular modeling of the D1 and D1′ protein subunits was performed using Discover Studio 2.0 (Accelrys Software Inc.). The atomic co-ordinates of the native D1 subunit were taken from the 3.0 Å resolution crystal structure of PSII by Loll et al. [25] and Ferreira et al. [26]. The structure of the D1′ subunit was obtained by mutating the relevant residues *in silico* (G80A, F158L, T286A). In order to allow the newly substituted residues in the D1′ subunit to relax, and to allow comparison with D1, both subunits were then atom-typed with the CHARMm forcefield, and allowed to energy minimize (RMS gradient <0.0001) before being compared. The $CaMn_4$ cluster, non-heme iron, P680 and pheophytin co-factors were also included in the energy minimization, but their positions were constrained. The generalized Born with simple switching (GBSW) model was used to implicitly include the presence of solvent ($\varepsilon=80$).

3. Results

3.1. Effect of microaerobic conditions on PSII function

During the experiments, the air in the cell cultures was actively replaced with a continuous flow of argon for 90 min, followed by a 60 min recovery phase of bubbling with air. The replacement with Ar caused rapid and drastic reductions in nitrogen and oxygen concentrations to levels below 10% of their initial values (Fig. 1). The microaerobic state was steady in time (Fig. 1) and was found to be sufficient to induce a specific cellular response. In order to assess the effect of these conditions on the general function of PSII, we estimated the number of centers capable of Q_A^- reoxidation using flash-induced chlorophyll fluorescence measurements. The amplitude of the fluorescence signal (F_m) in the measurements of the flash-induced fluorescence is proportional to the number of PSII centers capable of Q_A reduction regardless of D1 isoform present. The PSII inhibitor DCMU (3-(3′,4′- dichlorophenyl)-1,1-dimethylurea), blocks the forward electron transport from Q_A to Q_B, forcing Q_A^- to recombine with the donor side components of PSII. The effect of 90 min of microaerobic conditions on the number of PSII centers capable of Q_A reduction was minimal (Fig. 2a), and full recovery to normal values was achieved during the subsequent 60 min of returning to aerobic conditions. This suggests that the effect of the microaerobic conditions was either minimal or well buffered by the repair mechanisms of the cell, leading to only a limited net influence on PSII function (Fig. 2a). The microaerobic treatment did not induce consistent modifications of the shape of the decay curve in the absence of DCMU (data not shown), which reflects the process of forward electron transfer. In the presence of DCMU (Fig. 2b), Q_A^- back recombination was slightly accelerated by 90 min of microaerobic treatment, reflecting a modification of the S_2/Q_A^- recombination characteristics (Fig. 2b and Table 2). This effect was reversed fully after 60 min of returning to aerobic conditions (Fig. 2b and Table 2). In addition, this acceleration of recombination was not detected after 30 min of Ar treatment (data not shown).

3.2. Microaerobic effect on the expression of psbA gene family

The expression of the *psbA* gene family in *Synechocystis* has been the topic of many previous studies under various oxidoreductive stress conditions, and the typical stress response has always been the increase in the amount of D1 protein by induction of *psbA2* and *psbA3* genes

Table 2
Characteristics of flash induced chlorophyll fluorescence yield decay

Parameter	A1	T1(ms)	A2	T2(ms)
Control	$19.4*10^{-3}$	18.8	$705*10^{-3}$	661.4
90 min. Argon	$34.3*10^{-3}$	31.5	$690*10^{-3}$	572.8
60 min. Recovery	$14*10^{-3}$	25.4	$721*10^{-3}$	672.2

[5,10,15]. The *psbA1* gene has always been apparently silent, though more recently it has been found to be expressed at trace levels [6]. However, in none of the previous studies were the cells exposed to microaerobic or anaerobic conditions. Thus it was intriguing to record a significant induction of the *psbA1* gene when *Synechocystis* cells were exposed to Ar-induced microaerobic conditions (Fig. 3a). 90 min of cellular exposure to microaerobic conditions increased the expression of psbA1 by 175 fold on average (Fig. 3a), and this induction was reflected in a significant accumulation of *psbA1* transcripts as part of the total *psbA* transcript pool (Fig. 3b). The induction of the other two genes *psbA2* and *psbA3*, was slightly increased, by 2 and 4 fold, respectively (Fig. 3a), a behavior that is in line with a mild stress response in *Synechocystis* [8]. These effects were rapidly reversed when the cells were returned to aerobic conditions (Fig 3).

In order to determine whether the microaerobic induction of the *psbA1* gene was a particular response of *Synechocystis* or rather a more general cyanobacterial reaction to environmental changes, we extended our studies to *Anabaena* and *Thermosynechococcus elongatus* In both of these two cyanobacterial species, divergent and uninducible genes that are similar to *psbA1* of *Synechocystis* have previously been reported [6,12,13]. Treating these species in the same way as for *Synechocystis* (90 min of microaerobic exposure followed by 60 min of recovery in air) they both showed strong induction of their respective divergent *psbA* genes (i.e. *psbA0* in *Anabaena* and *psbA2* in *Thermosynechococcus*: Fig. 4). A mild, but typical stress response of the expression of the other *psbA* genes was recorded here as well. In *Anabaena* and *Thermosynechococcus*, the constitutively expressed *psbA1* genes encoding the D1:1 isoform were down-regulated, whereas the stress responsive *psbA2*, *3* and *4* (*Anabaena*) and *psbA3* (*Thermosynechococcus*) encoding the D1:2 isoform were slightly induced (Fig. 4). The stronger induction of the divergent *psbA* genes due to microaerobic conditions was therefore even more surprising (Fig. 4a and c). As in the case of *Synechocystis*, the response was fast and directly correlated with the onset of microaerobic conditions, and it quickly reversed when the cells were returned to aerobic conditions (Fig. 4). Furthermore, in direct correlation with the induction of the genes, the transcripts also accumulated to significantly increased levels during the 90 min of Ar treatment, and returned to almost control levels within 60 min of the restoration of aerobic conditions (Fig. 4b and d). Given the fact that, in contrast to *Synechocystis*, *Anabaena* and *Thermosynechococcus* are cyanobacteria that use a different strategy of *psbA* regulation in response to other environmental stresses, the common type of regulation in response to microaerobic exposure for all three species is even more intriguing.

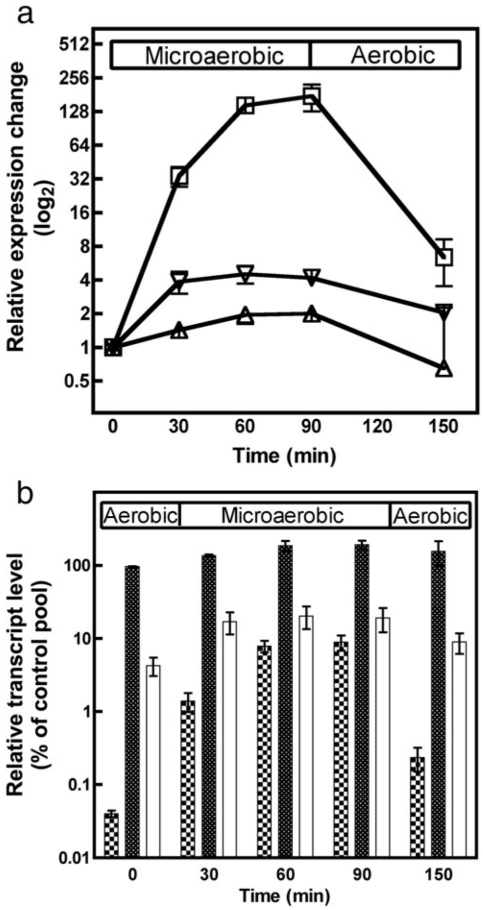


Fig. 3. Effect of microaerobic conditions on the expression of cyanobacterial *psbA* gene family in *Synechocystis sp*. PCC 6803. Panel (a) shows the changes in expression of each *psbA* gene relative to its time 0 control level, during the Ar-treatment and subsequent recovery. *psbA1* (open squares) was induced significantly more then the other two *psbA* genes, *psbA2* (open up triangle) and *psbA3* (open down triangle). The relative abundances of the constitutively expressed *psbA2* (solid black columns), stress inducible *psbA3* (solid, white columns) and microaerobic inducible *psbA1* genes (checkered columns) are presented in panel (b).

3.3. Comparison of the D1 isoforms from Synechocystis, Anabaena and Thermosynechococcus elongatus

Based on frequently observed relationship between protein structure and function, we decided to compare the *psbA*-encoded protein sequences from the cyanobacterial strains studied here in an effort to identify common structural features that could be linked to the same cellular function of the protein (Fig. 5).

The eight different D1 polypeptides encoded by the *psbA* gene families in the three cyanobacterial species studied here belong to four distinct D1 isoform types. The three divergent *psbA* genes, one for each species, encode a D1 form that has until now been called D1′ in *Synechocystis* only. Here, we expand the use of the nomenclature D1′ to encompass the products of all of the divergent, microaerobically induced *psbA* genes in each of these strains. With respect to the other remaining isoforms, *Synechocystis* possesses another D1 form, encoded by *psbA2* and *psbA3*, and is simply called D1. *Anabaena* and *Thermosynechococcus elongatus* have, apart from D1′, two different D1 isoforms: firstly, a constitutively expressed form known as D1:1 that is encoded in both species by *psbA1*, and secondly, a stress-inducible form known as D1:2 that is encoded by *psbA2*, *psbA3*, *psbA4* in *Anabaena* and by *psbA3* in *Thermosynechococcus elongatus*. We have aligned the eight different D1 isoforms in an effort to detect similarities in the primary structure as an indication of common functional roles (Fig. 5).

To see whether the genes encoding D1′ proteins in the different species originate from the same source, the *psbA* genes encoding the corresponding D1/D1′ proteins across the three species of cyanobacteria were compared with each other. It was found that the D1/D1′ encoding genes from the same family were in fact more closely related to each other than to the corresponding isoform of another cyanobacterial species. This indicates an independent evolution of these D1 isoforms providing the cells with a selective advantage under specific environmental conditions and may not be the result of random horizontal gene transfer. This also suggests that their common function does not stem from conserved primary structure domains. In fact, there are only three amino acid residues that are both conserved in all traditional D1 forms and mutated but conserved across all the D1′ proteins (Fig. 5). These modifications are G80A, F158L, and T286A (Fig. 6).

3.4. Modeling of D1′ conserved differences

To better appreciate the structural significance of the three conserved mutations, each of the G/A80, F/L158 and T/A286 residues

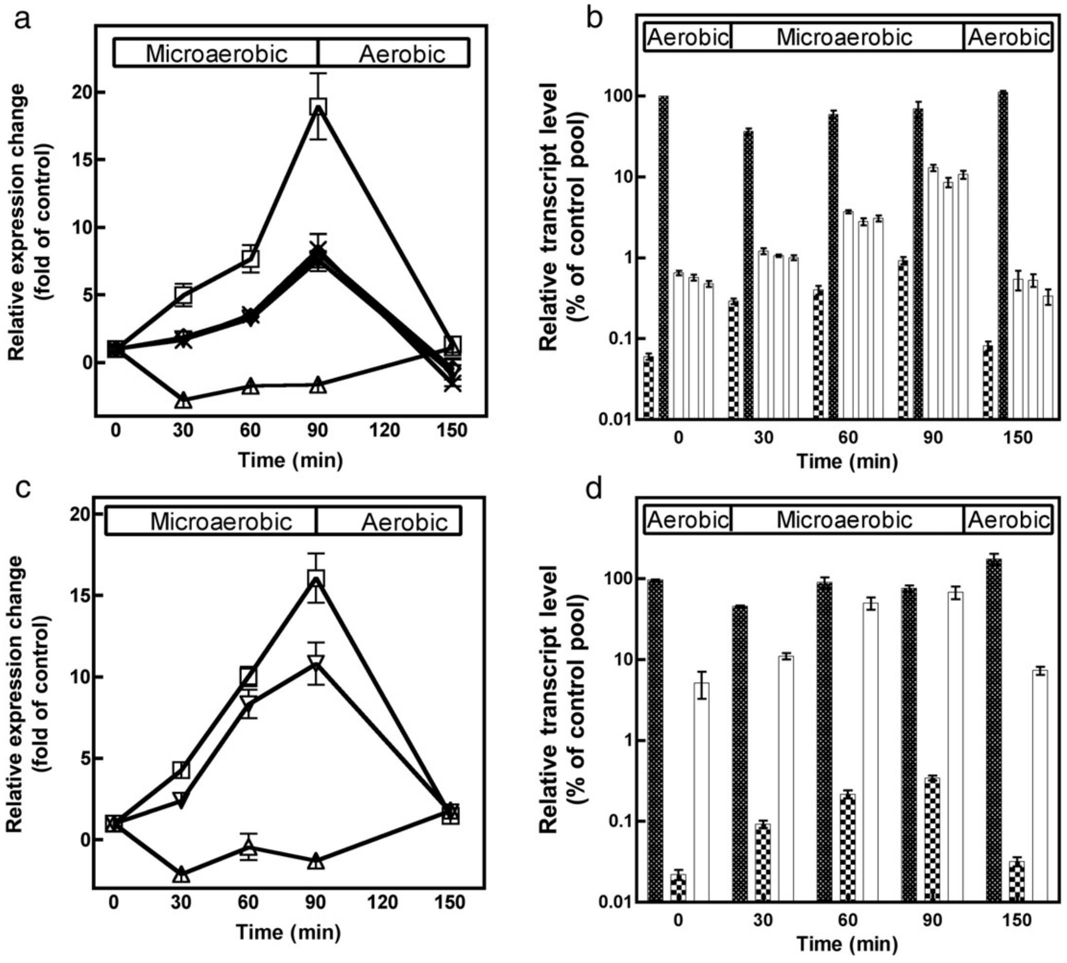


Fig. 4. Effect of microaerobic conditions on the expression of cyanobacterial *psbA* gene families in *Anabaena sp*. PCC 7120 (panels (a) and (b)) and *Thermosynechococcus elongatus* BP-1 (panels (c) and (d)). Panels (a) and (c) show the changes in expression of each *psbA* gene relative to its time 0 control level, during the Ar-treatment and subsequent recovery (open squares represent the divergent *psbA*) in *Anabaena* and *Thermosynechococcus*, respectively. A mild stress response materialized by induction of D1:2 encoding genes: *psbA2* (open down triangle, panel (a)), *psbA3* (open diamond, panel (a)), *psbA4* (star, panel (a)) in *Anabaena*, *psbA3* (open down triangle, panel (b)) in *Thermosynechococcus elongatus* and a down-regulation of D1:1 encoding *psbA1* (open up triangle, panels (a) and (b)) in both species. The relative abundances of the constitutively expressed (solid black columns), stress inducible (solid, white columns) and microaerobic inducible *psbA* genes (checkered columns) are presented in panel (b) (*Anabaena*) and d (*Thermosynechococcus*).

on the D1 and D1′ subunits were visualized by molecular modeling, using the crystal structure of PSII from *Thermosynechococcus elongatus* as the model for the D1 subunit [25,26]. The overall high degree of D1 conservation across different organisms suggests that this is a reasonable approximation for our comparative purposes, despite the presence of some divergence in the amino acid sequence in different species. The conserved D1′ subunit point mutations in question were then constructed by substitution *in silico* of the relevant amino acid residues. To avoid possible steric strain and clashes due to the substitutions, energy minimization was performed on the mutated D1′ subunit to allow the structure to relax as necessary. The native D1 structure was also allowed to energy minimize to permit direct comparison of the two structures. Fig. 7 compares each of the three mutations in turn.

Mutation of G80 to A80 (Fig 7(a) and (b)) led to the introduction of an extra methyl group at this position, where there was previously only a hydrogen atom. This gave rise to an increase in the size of the amino acid side chain. The methyl group from A80 was found to point inwards to the center of the A-B loop of the D1′ protein. Closer examination of this loop region revealed that the extra methyl group from A80 in D1′ extends into an unoccupied space in D1 (not shown). As such, no significant changes in the position of the neighboring residues were necessary to accommodate the bulkier alanine residue. The other residues that were found to surround this unoccupied space in D1 are F52, G69, S70, L71, N75, N76, I77 and A81. All of these residues were found to be conserved across the D1 and D1′ subunits, with the minor exception of A81, where it is T81 in the D1′ subunit obtained from *Anabaena*.

Replacement of F158 with L158 led to very similar orientations of the side chains (Fig. 7c and d). As expected, the lysine side chain protruded less into the region between backbones of the B and C helices of the subunit. When the D1 and D1′ subunits were superimposed, it was found that the positions of the methyl groups of L158 approximately matched those on the proximal half of the phenyl ring of F158 (inset in Fig. 7c). However, since the aromaticity of the phenyl ring is lost upon replacement by lysine, the planarity of the side chain is also lost (inset in Fig. 7d).

In contrast to the other two conserved point mutations in D1′ that led to an increase in the size of the amino acid side chain, the T286A mutation on the E helix gave rise to a decrease in the size of the side chain. The methyl and hydroxyl groups of threonine are replaced by protons in alanine (Fig. 7e and f). Furthermore, the loss of the hydroxyl group in D1′ at this position (insets in Fig. 7e and f) led to a decrease in hydrophilicity, whereas hydrophobicity of the side chains is essentially unchanged for the G80A and F158L mutations. From both the energy minimized and crystal structure of the D1 subunit, this hydroxyl group would be hydrogen bonded to the carbonyl oxygen of G282 (inset in Fig. 7e).

4. Discussion

Most of cyanobacterial species possess a small *psbA* gene family whose members are differentially expressed in response to changing environmental conditions [3–6]. Besides the *psbA* genes that have been shown so far to respond to environmental changes, *Synechocystis* also has an apparently silent *psbA1* gene [16]. Its intactness, despite random mutagenesis over the long evolutionary time period, points to a strong selective pressure for preservation of the *psbA1* gene, most probably due to a unique importance of its transcripts and protein products under hitherto unspecified environmental conditions. The recent mounting evidence that *psbA* genes with apparently silent behavior also exist in other cyanobacterial species [9,12,13] has lent support to the argument that the *psbA1* gene of *Synechocystis* is not an accidental exception, but possibly an essential part of a more general acclimation mechanism to a specific environmental cue.

Intriguingly, we have been able to show here that those members of the *psbA* gene families from different cyanobacterial species that have until now been believed to be silent or uninducible can all be induced by exposure of the cells to microaerobic conditions (Figs. 3 and 4). This places the “silent” *psbA* genes in a distinct functional group with an independent regulation mechanism responding to specific cellular needs. Microaerobic conditions also induce the bidirectional hydrogenase activity in *Synechocystis*, which has been regarded as a safety valve for electrons under anaerobic conditions [27]. It can be speculated that the microaerobic conditions expose the PSII centers to a specific stress that is partially compensated by the exchange of the D1 protein in PSII centers to the D1′ form. In keeping with the current literature, we have decided to extend the use of the term D1′, originally designating only the product of the *psbA1* gene in *Synechocystis*, to the whole group of microaerobically inducible *psbA* gene products, which in *Anabaena* is encoded by *psbA0* and in *Thermosynechococcus elongatus* by *psbA2*.

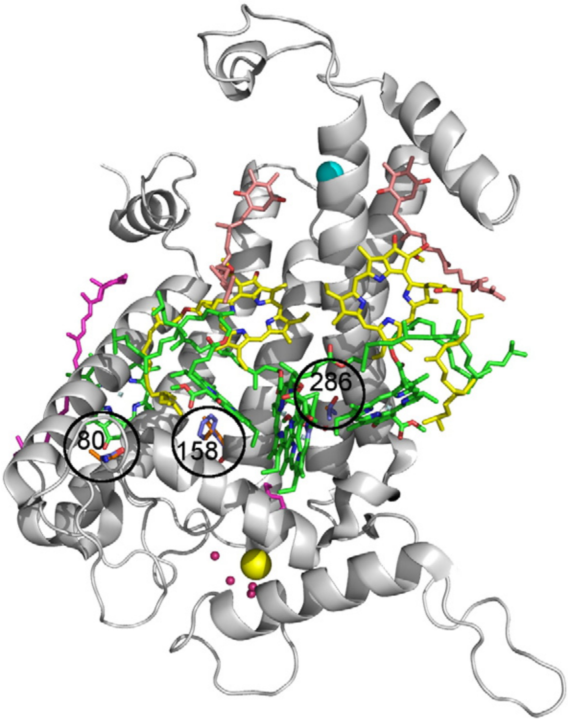
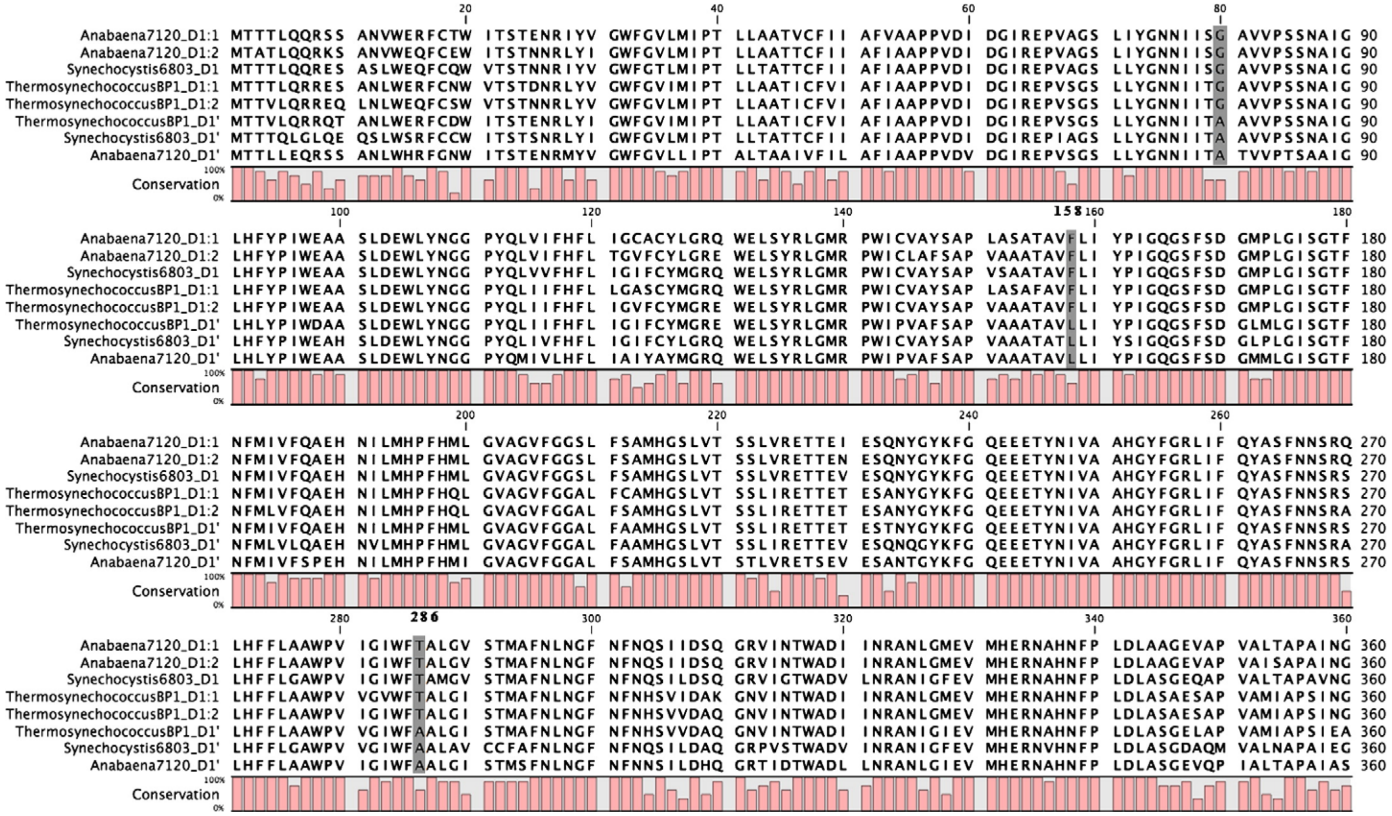


Fig. 6. An superimposed overview of the *Synechocystis PCC6803* D1 and D1′ proteins (shown as grey ribbon) in complex with cofactors, P_{D1}/P_{D2} (green), Chl_{D1}/Chl_{D2} (green), $Pheo_{D1}/Pheo_{D2}$ (yellow), Q_A/Q_B (light red), $Chlz_{D1}$, Car_{D1} (pink), Car_{D2}(pink), the Mn_4Ca cluster (Mn in red and Ca in yellow sphere) and Fe^{2+}(cyan sphere). Carbon atoms in residues at positions 80, 158 and 286 are shown as sticks in blue in D1 (G80; F158; T286) and orange in D1′ (A80; L158; A286)



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Anabaena7120_D1:1            MTTTLQQRSS ANVWERFCTW ITSTENRIYV GWFGVLMIPT LLAATVCFII AFVAAPPVDI DGIREPVAGS LIYGNNIISG AVVPSSNAIG 90
Anabaena7120_D1:2            MTATLQQRKS ANVWEQFCEW ITSTNNRLYI GWFGVLMIPT LLAATTCFII AFIAAPPVDI DGIREPVAGS LIYGNNIISG AVVPSSNAIG 90
Synechocystis6803_D1         MTTTLQQRES ASLWEQFCQW VTSTNNRIYV GWFGTLMIPT LLTATTCFII AFIAAPPVDI DGIREPVAGS LLYGNNIISG AVVPSSNAIG 90
ThermosynechococcusBP1_D1:1  MTTTLQRRES ANLWERFCNW VTSTDNRLYV GWFGVIMIPT LLAATICFVI AFIAAPPVDI DGIREPVSGS LLYGNNIITG AVVPSSNAIG 90
ThermosynechococcusBP1_D1:2  MTTVLQRREQ LNLWEQFCSW VTSTNNRLYV GWFGVLMIPT LLAATICFVI AFIAAPPVDI DGIREPVSGS LLYGNNIITG AVVPSSNAIG 90
ThermosynechococcusBP1_D1'   MTTVLQRRQT ANLWERFCDW ITSTENRLYI GWFGVIMIPT LLAATICFVI AFIAAPPVDI DGIREPVSGS LLYGNNIITA AVVPSSNAIG 90
Synechocystis6803_D1'        MTTTQLGLQE QSLWSRFCCW ITSTSNRLYI GWFGVLMIPT LLTATTCFII AFIAAPPVDI DGIREPIAGS LLYGNNIITA AVVPSSNAIG 90
Anabaena7120_D1'             MTTLLEQRSS ANLWHRFGNW ITSTENRMYV GWFGVLLIPT ALTAAIVFIL AFIAAPPVDV DGIREPVSGS LLYGNNIITA TVVPTSAAIG 90

Anabaena7120_D1:1            LHFYPIWEAA SLDEWLYNGG PYQLVIFHFL IGCACYLGRQ WELSYRLGMR PWICVAYSAP LASATAVFLI YPIGQGSFSD GMPLGISGTF 180
Anabaena7120_D1:2            LHFYPIWEAA SLDEWLYNGG PYQLVIFHFL TGVFCYLGRE WELSYRLGMR PWICLAFSAP VAAATAVFLI YPIGQGSFSD GMPLGISGTF 180
Synechocystis6803_D1         LHFYPIWEAA SLDEWLYNGG PYQLVVFHFL IGIFCYMGRQ WELSYRLGMR PWICVAYSAP VSAATAVFLI YPIGQGSFSD GMPLGISGTF 180
ThermosynechococcusBP1_D1:1  LHFYPIWEAA SLDEWLYNGG PYQLIIFHFL LGASCYMGRQ WELSYRLGMR PWICVAYSAP LASAFAVFLI YPIGQGSFSD GMPLGISGTF 180
ThermosynechococcusBP1_D1:2  LHFYPIWEAA SLDEWLYNGG PYQLIIFHFL IGVFCYMGRE WELSYRLGMR PWICVAYSAP VAAATAVFLI YPIGQGSFSD GMPLGISGTF 180
ThermosynechococcusBP1_D1'   LHLYPIWDAA SLDEWLYNGG PYQLIIFHFL IGIFCYMGRE WELSYRLGMR PWIPVAFSAP VAAATAVLLI YPIGQGSFSD GLMLGISGTF 180
Synechocystis6803_D1'        LHFYPIWEAH SLDEWLYNGG PYQLIVFHFL IGIFCYLGRQ WELSYRLGMR PWICVAYSAP VAAATATLLI YSIGQGSFSD GLPLGISGTF 180
Anabaena7120_D1'             LHLYPIWEAA SLDEWLYNGG PYQMIVLHFL IAIYAYMGRQ WELSYRLGMR PWIPVAFSAP VAAATAVLLI YPIGQGSFSD GMMLGISGTF 180

Anabaena7120_D1:1            NFMIVFQAEH NILMHPFHML GVAGVFGGSL FSAMHGSLVT SSLVRETTEI ESQNYGYKFG QEEETYNIVA AHGYFGRLIF QYASFNNSRQ 270
Anabaena7120_D1:2            NFMIVFQAEH NILMHPFHML GVAGVFGGSL FSAMHGSLVT SSLVRETTEN ESQNYGYKFG QEEETYNIVA AHGYFGRLIF QYASFNNSRQ 270
Synechocystis6803_D1         NFMIVFQAEH NILMHPFHML GVAGVFGGSL FSAMHGSLVT SSLVRETTEV ESQNYGYKFG QEEETYNIVA AHGYFGRLIF QYASFNNSRS 270
ThermosynechococcusBP1_D1:1  NFMIVFQAEH NILMHPFHQL GVAGVFGGAL FCAMHGSLVT SSLIRETTET ESANYGYKFG QEEETYNIVA AHGYFGRLIF QYASFNNSRS 270
ThermosynechococcusBP1_D1:2  NFMLVFQAEH NILMHPFHQL GVAGVFGGAL FSAMHGSLVT SSLIRETTET ESANYGYKFG QEEETYNIVA AHGYFGRLIF QYASFNNSRA 270
ThermosynechococcusBP1_D1'   NFMIVFQAEH NILMHPFHML GVAGVFGGAL FAAMHGSLVT SSLIRETTET ESTNYGYKFG QEEETYNIVA AHGYFGRLIF QYASFNNSRS 270
Synechocystis6803_D1'        NFMLVLQAEH NVLMHPFHML GVAGVFGGAL FAAMHGSLVT SSLIRETTEV ESQNQGYKFG QEEETYNIVA AHGYFGRLIF QYASFNNSRA 270
Anabaena7120_D1'             NFMIVFSPEH NILMHPFHMI GVAGVFGGAL FSAMHGSLVT STLVRETSEV ESANTGYKFG QEEETYNIVA AHGYFGRLIF QYASFNNSRS 270

Anabaena7120_D1:1            LHFFLAAWPV IGIWFTALGV STMAFNLNGF NFNQSIIDSQ GRVINTWADI INRANLGMEV MHERNAHNFP LDLAAGEVAP VALTAPAING 360
Anabaena7120_D1:2            LHFFLAAWPV IGIWFTALGV STMAFNLNGF NFNQSIIDSQ GRVINTWADI INRANLGMEV MHERNAHNFP LDLAAGEVAP VAISAPAING 360
Synechocystis6803_D1         LHFFLGAWPV IGIWFTAMGV STMAFNLNGF NFNQSILDSQ GRVIGTWADV LNRANIGFEV MHERNAHNFP LDLASGEQAP VALTAPAVNG 360
ThermosynechococcusBP1_D1:1  LHFFLAAWPV VGVWFTALGI STMAFNLNGF NFNHSVIDAK GNVINTWADI INRANLGMEV MHERNAHNFP LDLASAESAP VAMIAPSING 360
ThermosynechococcusBP1_D1:2  LHFFLAAWPV IGIWFTALGI STMAFNLNGF NFNHSVVDAQ GNVINTWADI INRANLGMEV MHERNAHNFP LDLASAESAP VAMIAPSING 360
ThermosynechococcusBP1_D1'   LHFFLAAWPV VGIWFAALGI STMAFNLNGF NFNHSVVDAQ GNVINTWADI INRANIGIEV MHERNAHNFP LDLASGELAP VAMIAPSIEA 360
Synechocystis6803_D1'        LHFFLGAWPV VGIWFAALAV CCFAFNLNGF NFNQSILDAQ GRPVSTWADV INRANIGFEV MHERNVHNFP LDLASGDAQM VALNAPAIEG 360
Anabaena7120_D1'             LHFFLAAWPV IGIWFAALGI STMSFNLNGF NFNNSILDHQ GRTIDTWADL LNRANLGIEV MHERNAHNFP LDLASGEVQP IALTAPAIAS 360
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Fig. 5. Sequence comparison of the D1 isoforms encoded by the *psbA* gene families in *Synechocystis*, *Anabaena* and *Thermosynehococcus*. The positions of conserved amino acids in D1′ and simultaneously consistently different in all other D1 forms are highlighted and their positions are marked.

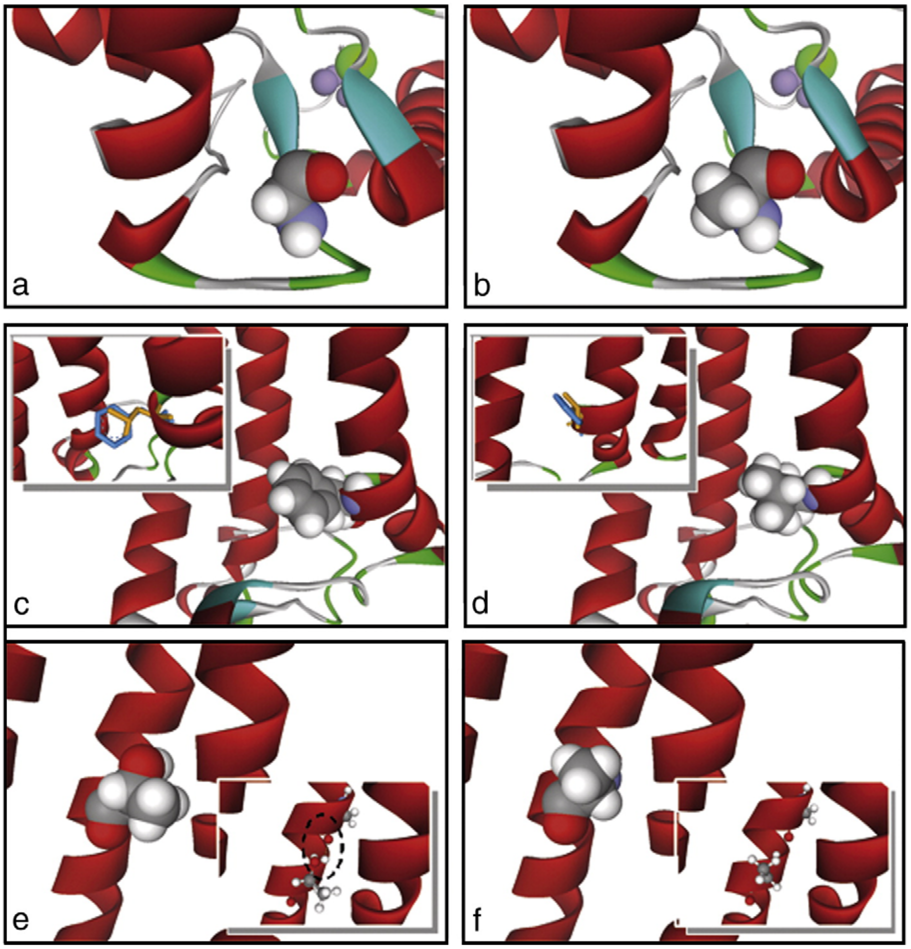

Fig. 7. Comparisons of the point mutations G/A80, F/L158 and T/A286 observed in the D1 and D1′ subunits. (a) and (b): Space-filling (CPK) representation of the residues G80 and A80, respectively, which are located in the A-B loop of the protein subunits. Hydrogen atoms have been omitted for clarity. The $CaMn_4$ cluster (green: Ca; purple: Mn) can be seen in the background. The extra methyl group of A80 was found to point inwards into a space in the centre of the loop. (c) and (d): Space-filling (CPK) representation of the residues F158 and L158, respectively, which are located on the C-helix of the protein subunits. Hydrogen atoms have been omitted for clarity. The inset in (c) shows the overlap of the F158 and L158 residues in D1 and D1′, respectively, demonstrating the close correspondence of the methyl groups in L158 with half of the F158 phenyl ring. The loss of the planar structure of the residue upon the F→L mutation is shown in the inset in (d). (e) and (f): Space-filling (CPK) representation of the residues T286 and A286, respectively, which are located on the E-helix of the protein subunits. Hydrogen atoms have been omitted for clarity. A magnified view of these residues, including hydrogen atoms and colored according to the elements, are show in the insets. In addition, the hydrophilic hydroxyl group that is lost upon the T→A mutation, with its hydrogen bonding partner G282, is highlighted in the insets in (e)

Due in part to the relatively minor differences between the D1 and D1′ isoforms, but mostly because of the low percentage of D1′ proteins present, relative to the total pool of D1 proteins, our mass spectrometry approaches have been unable to identify the presence of the D1′ polypeptide in PSII centers, despite the induction of the D1′ encoding genes in *Synechocystis*. However, we were able to detect a small but distinct modification of PSII flash-induced fluorescence characteristics after 60 and 90 min of microaerobic conditions (Fig. 2b). The results reflect changes in the kinetics of S_2/Q_A^- recombination, and this technique is a sensitive way to detect modifications of PSII function. Furthermore, this method was also previously demonstrated to be useful in documenting the exchange of D1 isoforms [6]. The acceleration of the flash-induced fluorescence decay (Fig. 2b) indicates that a small fraction of the PSII centers has a slightly modified donor side [23]. This modification can be specifically attributed to the donor side, since the forward electron flow in the absence of DCMU is not changed (data not shown). This effect is unlikely to be an artifact caused by the microaerobic environment, as it was not yet present after 30 min of Ar treatment, when the cellular environment was already microaerobic (Fig. 1).

The fast induction/repression regulation of the *psbA* gene family in response to the application and removal of the stress condition is typical for cyanobacteria [4–8]. In all published studies, such regulation has always directly reflected the translation of the encoded D1 isoforms and the regulation of PSII function, due to the short lifetime of the functional D1 protein. Therefore, in combination with the fact that artificially-induced translation of the *psbA1* gene in *Synechocystis* has been shown to produce the D1′ protein [17], our data indicate that microaerobic conditions could be able to induce the hitherto uninducible protein D1′ in the wild-type of all three cyanobacterial species studied here.

An alignment of the D1 forms studied here (Fig. 5), shows that there are three residue positions where there are fully conserved mutations in D1′ that also correspond to fully conserved residues in D1. An interesting question is whether these conserved mutations in the D1′ subunit lead to any significant structural differences from the D1 subunit that could be of functional importance. An initial investigation was performed by *in silico* mutation of the relevant amino acid residues, and a number of comments can be made about the observations outlined above.

Given the core importance of the D1 protein in PSII, and the generally high degree of conservation of the amino acid sequences for the D1 and D1′ subunits, it is unlikely that the overall protein folding structure of PSII would be drastically altered. The importance of the three conserved point mutations, therefore, is likely to lie in the steric conformations and chemical natures of the mutated side chains. For the G80A and F158L mutations, the chemical nature of the side chains is not substantially altered by the mutations. In essence, they merely consist of the replacement of one apolar side chain for another. No bonding interaction between residues is obviously disrupted. The main difference instead lies in the steric bulk of the replacement side chain. Nevertheless, these point mutations may have an effect on the local flexibility of the polypeptide chain under dynamic conditions. The increase in flexibility of the single T286A mutation is highly unlikely to cause severe disruptions to the overall structure of the E-helix. The filling of the previously unoccupied space in the center of the loop region due to the G80A mutation could lead to a reduction in the flexibility here, due to increased steric hindrance of the extra methyl group in the alanine side chain.

Furthermore, it is interesting to note that the F158 and T286 residues are located very close to the chlorophyll molecules $\mathrm{Chl_{D1}}$ and $\mathrm{P_{D1}}$ in $\mathrm{P_{680}}$, respectively. For F158, the closest phenolic C atom of the side chain is located 3.5 Å from the 3^2 carbon attached to the A ring of the $\mathrm{Chl_{D1}}$, and 8.5 Å from the $\mathrm{Mg^{2+}}$ ion of $\mathrm{Chl_{D1}}$. For T286, the central C of the threonine side chain is located 3.3 Å from the carbonyl O connected to the 13^2 carbon extending from the E-ring of $\mathrm{P_{D1}}$, and 8.0 Å from the $\mathrm{Mg^{2+}}$ ion of the same chl. Both the F158L and T286A mutations located on the donor side of PSII could slightly modify the molecular environment around key cofactors, resulting in modifications of the recombination characteristics as seen in Fig. 2b. Also, the extra space could simply lead to more room for movement for the side chains, but it is interesting to consider the possibility that the bulkier side chains in the original D1 sequence might be involved in hindering access of oxygen to $\mathrm{P_{680}}$. As has been suggested previously, PSII may have some mechanism for preventing such oxygen access in order to avoid the formation of harmful singlet oxygen species [28–30].

It would be interesting to examine in more detail the structural changes due to the conserved mutations in the future. There may be subtler, dynamic effects, such as cooperativity between specific residues, the blocking or opening up of channels or cavities within the protein, and/or modified access to co-factors by solvent water/oxygen. The investigation of such effects would require careful and extensive molecule dynamics simulations, and is beyond the scope of the present study.

Although rare, the conditions requiring the presence of D1′ have a sufficient evolutionary importance that the gene is selectively maintained in the genome. While under standard laboratory growth conditions the cells do not experience periods of microaerobic growth, in the natural environment these do occur both as an effect of imbalanced cellular metabolism or specific environmental conditions. These conditions can range in time from minutes, in the case of temporary reduced oxygen evolution rate caused by a sudden decrease in light intensity to weeks or longer when a frozen lake gets anoxic during the winter months. Although the specific functional role of D1′ isoform in PSII centres still remains elusive, the presence of an anaerobically induced *psbA* gene in three unrelated cyanobacteria: a mesophilic unicellular (*Synechocystis*), a filamentous nitrogen fixer (*Anabaena*) and a thermophilic one (*Thermosynechococcus*) leads us to conclude that a new form of D1 is present in PSII centres and important for acclimation of cyanobacteria to microaerobic conditions.

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