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Molecular-genetic approaches to study photosynthetic and respiratory electron transport in thylakoids from cyanobacteria

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Abstract

Molecular-genetic techniques provide a powerful approach to study photosynthetic and respiratory electron transport pathways in thylakoids of the transformable cyanobacterium *Synechocystis* sp. PCC 6803. Either or both of the photosystems can be genetically deleted from this organism, and resulting mutants can be propagated in the presence of glucose. This provides the opportunity for targeted mutagenesis of particular components of one of the photosystems, and analysis of resulting mutants has yielded useful information regarding residues or domains of protein subunits. In addition, by selection for secondary mutations neutralizing the functional impairment caused by an introduced mutation, the sequence flexibility and structural vicinity of domains can be determined. As an example, a preliminary characterization of a photoautotrophic pseudorevertant of the site-directed D2 mutant E69Q, which is an obligate photoheterotroph, will be presented here. Mutant analysis also has proven to be useful in the determination of the functional link between photosynthetic and respiratory electron transfer in cyanobacterial thylakoids. In Photosystem-I-less mutants, light-induced electron transport occurs involving water oxidation (oxygen production) in Photosystem II, and oxygen reduction by a terminal oxidase, thus without net oxygen production or consumption. The net result of this electron transport 'cycle' should be the generation of a proton gradient over the thylakoid membrane, which can be used for ATP production.

Key words: Photosynthesis; Respiration; Cyanobacterium; Photosystem II; Photosystem I

1. Introduction

The cyanobacterium *Synechocystis* sp. PCC 6803 has been established as a suitable system for targeted molecular-genetic modification of the photosynthetic apparatus. Analysis of resulting mutants has led to important insights regarding the structure and function of the photosystems (for example, see [1–4] for reviews). Because of the facts that *Synechocystis* 6803 (1) is spontaneously transformable with linear or circular DNA, (2) integrates foreign DNA into its genome by homologous recombination, and (3) can survive in the presence of glucose even if photosynthetic electron transport has been inactivated, this cyanobacterium has been widely utilized for studies on Photosystem II (PS II) [1,3–6]. However, *Synechocystis* 6803 now has been made suitable also for studies involving the Photosystem I (PS I) core complex [7,8], and respiratory electron transport in thylakoids [9].

Synechocystis strains have now been obtained in which part of the *psa*AB operon (coding for the PS I core proteins) has been genetically deleted. Initially, deletion of *psa*AB was observed to lead to an extremely light-sensitive phenotype, and the PS-I-less mutant was concluded [7] to be viable only under light-activated heterotrophic growth (LAHG) conditions (in darkness, with several minutes of light per day [10]). However, a very long adaptation time (on the order of weeks) is needed for the conversion from light to LAHG conditions and vice versa [8,11], and this most likely is the reason why PS-I-less cells originally were deemed to be propagatable only under LAHG conditions, where growth on plates is poor. PS-I-less cells now have been shown to grow quite well at light intensities that are 10% of those used for wild type (5

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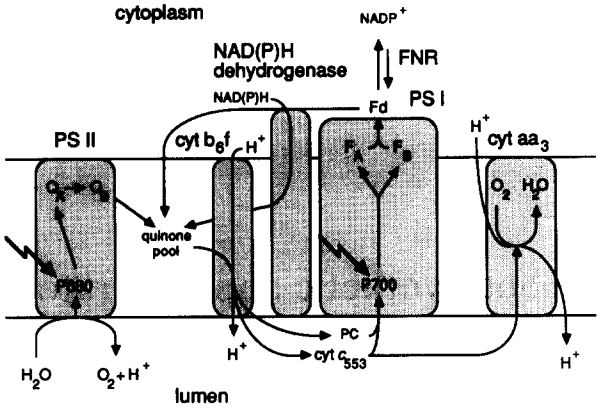

Fig. 1. Scheme of the photosynthetic and respiratory electron transport chains in cyanobacterial thylakoid membranes. Photosynthetic electron flow includes Photosystems II and I (PS II and PS I, respectively), and electrons are transported between these photosystems via the quinone pool, the cytochrome b_6f complex and a soluble carrier (cytochrome *c*-553 or plastocyanin (PC)). PS I electrons are utilized for NADP reduction (via ferredoxin (Fd)-$NADP^+$ oxidoreductase (FNR)), and for cyclic electron flow around PS I, possibly involving NAD(P)H dehydrogenase and probably reintroducing electrons into the quinone pool. Reduction of the quinone pool by NAD(P)H also may occur directly via a NAD(P)H dehydrogenase. The pathway(s) of respiratory electron flow in cyanobacterial thylakoids are uncertain, but a cytochrome aa_3-containing cytochrome *c* oxidase has been shown to occur in thylakoids of these organisms. An alternate respiratory pathway (not indicated) involving direct quinol oxidation via a quinol oxidase cannot be excluded. See text and references for further details.

vs. 50 $\mu E m^{-2} s^{-1}$), but not at normal light intensity [8]. This light sensitivity of PS-I-less strains can be alleviated by genetic reduction of the PS II antenna size: upon deletion of the *apc*E gene (coding for a protein anchoring phycobilisomes to thylakoids and establishing the functional connection between phycobilisomes and PS II) PS-I-less cells can be propagated at normal light intensity [8]; this implies that PS II-generated electrons are the main reason for the light-sensitive phenotype of the PS-I-less strain.

In thylakoid membranes of cyanobacteria, both photosynthetic and respiratory electron transport occurs. The two electron transport chains intersect at the level of the cytochrome b_6f complex and perhaps a soluble cytochrome (cyt *c*-553) [12,13]. An integrated model of the electron transport pathways in cyanobacterial thylakoids is shown in Fig. 1. This situation is different from that in the evolutionarily related chloroplasts of plants, as in these systems the primary function of thylakoids is photosynthetic electron transport, and a major contribution of respiratory electron flow has been lost. However, in the green alga *Chlamydomonas reinhardtii* thylakoids have measurable respiratory activity [14,15], particularly when cells have been grown under nitrogen-limited conditions [16]. Also in plants, functional evidence exists for respiratory electron transport in chloroplast thylakoids ('chlororespiration') [17,18], and genes coding for proteins resembling NADH dehydrogenase components are present in the chloroplast genome [19,20].

If PS I has been removed, light-generated reducing equivalents on the acceptor side of PS II are consumed rapidly [9]. This oxidation of the PS II acceptor side is best visualized by fluorescence measurements: As Q_A, the primary stable electron acceptor in PS II, quenches chlorophyll fluorescence in its oxidized but not in its reduced form, the kinetics of chlorophyll fluorescence decay after a period of strong illumination (reducing all of the plastoquinone pool) are indicative of the rate with which electrons are removed from PS II by respiratory electron transport or other means. In the absence of PS I, reducing equivalents are removed from Q_A^- only slightly slower than in the presence of PS I, indicating the presence of a highly effective electron sink for PS-II-generated electrons even when PS I is absent [9]. The respiratory electron transport chain was implicated as a sink of PS-II-generated electrons, as in the presence of KCN, which is a potent inhibitor of the terminal oxidase, rapid oxidation of Q_A^- after illumination has been inhibited [9].

Thus, targeted genetic modification of both photosystems can be achieved in *Synechocystis* 6803, and the interaction between photosynthetic and respiratory electron flow can be studied in this system. In this paper, some examples and applications will be summarized.

2. Materials and methods

Strains and growth conditions. Synechocystis 6803 strains generated by my group and that can be used for genetic modification of selected PS II components (D2, CP43, CP47, and PS II-K) have been described [20–22]. Creation of a somewhat light-sensitive strain carrying a deletion in the operon coding for the PS I core complex, and of a strain with normal light tolerance and carrying a deletion of part of both the PS I core operon and of the *apc*E gene has been reported in [8]. It is important to note that the PS-I-less strains are provided with 15 mM glucose, 3-fold more than usual for PS II mutants. Particularly mutants with deletions of both PS I and PS II components (thus having respiration as a sole source for energy) depend on a higher glucose concentration (up to 50 mM is easily tolerated [11]), and it is important to restreak such mutants every 2 weeks.

Mutant characterization. Methods for characterization of PS II properties have been described previously [8,20–22]. For measurements involving intact cells, cells were grown to an optical density of about 0.7 at 730 nm, corresponding to the late-logarithmic growth phase.

3. Results and discussion

Approaches towards modification of the photosynthetic apparatus. A series of different molecular-genetic approaches can be utilized to obtain desired modifications of the photosynthetic apparatus. In the first place, protein subunits can be removed from one of the photosystems by gene inactivation or deletion. Removal of one of the large subunits generally leads to structural loss and/or functional inactivation of the entire photosystem, but small or peripheral subunits may be removed without large perturbations in the stability or function of remaining subunits of the complex [24–27]. A functional loss of a photosystem and a structural loss of its major components may be desired for the design of a suitable experimental organism. For example, deletion of PS II yields an organism in which all chlorophylls in the thylakoid essentially are part of PS I, thus providing an appropriate organism to study events associated with PS I [28,29], and genetic deletion of PS I leads to an organism in which chlorophyll is associated only with PS II [8,9]. In such systems, thylakoids generally are appropriate for detailed functional assays, and the need for purification of one photosystem away from the other reaction center complex has been eliminated.

Even though deletion mutagenesis involving entire genes may be informative, it often is too crude. Site-directed mutagenesis has been employed frequently in PS II (for example, see Refs. [1,3,4] for reviews), but to employ this approach efficiently, one needs to have a reasonably well-defined idea of which residues are important and need to be targeted. A derivative of site-directed mutagenesis is oligonucleotide-directed deletion of small sequences within a coding region (for example, 1–10 codons coding for hydrophilic domains). In this way, the structural or functional importance of a region can be determined. If small deletions in a certain region do not affect photoautotrophic properties significantly and do not alter important functional parameters, this suggests that such a region is not critical to function of the complex. For example, a number of deletions can be introduced into a large hydrophilic loop of CP47 without losing photoautotrophic capacity [30], while similar deletions in CP43 have much more significant effects [31].

An informative follow-up to targeted mutagenesis leading to an obligate photoheterotrophic phenotype is the selection for spontaneous (pseudo)revertants. If multiple residues have been altered, reversion to the original gene sequence is unlikely, and in a number of cases the organism has found other, and sometimes surprising, ways to restore photoautotrophic growth. For example, after introduction of a frame-shift mutation in the gene coding for the D2 protein, which led to a loss of photoautotrophic function, a number of pseudorevertants were isolated, in which the frameshift was reversed by introducing another frameshift several codons away, or by duplication of a large gene region, resulting in the insertion of a 31-residue long stretch of a novel protein sequence into the protein [32]. The resulting pseudorevertants have a significantly altered sequence in a highly conserved loop region of the D2 protein, but are functionally viable with photoautotrophic growth rates close to those of wild type under laboratory conditions, even though acceptor side properties (including inhibitor binding affinity) have been affected [33].

An example of a pseudorevertant in which the site of the secondary mutation is not in the same gene as the primary mutation is a photoautotrophic pseudorevertant of the D2 mutant E69Q. In the E69Q mutant, Glu-69 of the D2 protein, located in the lumen-exposed loop between the first two membrane-spanning regions of D2, has been mutated to Gln, leading to a loss of photoautotrophic growth [34]. Spontaneous photoautotrophic mutants have been isolated from this strain, and selected mutants were found to retain the E69Q mutation. The growth curve of one of these pseudorevertants under photoautotrophic and photoheterotrophic conditions has been shown in Fig. 2. The doubling time of the pseudorevertant was found to be about 24 h under photoautotrophic conditions, almost 2-fold slower than wild type. Under photoheterotrophic conditions, the wild type and pseudorevertant had very similar doubling times. Upon amplification of the *psb*DI gene from the pseudorevertant by PCR (polymerase chain reaction), and transformation of the original E69Q mutant with this DNA, no transformation to photoautotrophic phenotype was observed (Apel, W. and Vermaas, W., data not shown). This indicates that the secondary mutation is not located in the *psb*DI gene (note that in site-directed D2 mutants the other D2 gene, *psb*DII, has been deleted out [21]). The site of the secondary mutation has not yet been established, but preliminary results indicate that it is not located in one of the major integral PS II proteins or in the peripheral PS II-O (manganese-stabilizing) protein (W. Apel and W. Vermaas, unpublished). The secondary mutation is unlikely to be located in a tRNA, as in that case the growth rate under photoheterotrophic conditions would be expected to be decreased compared to wild type similar to the photoautotrophic growth rates. This obviously is not observed. It will be important to localize the site of the secondary mutation; however, preliminary results indicate the DNA region carrying the secondary mutation is difficult to clone in *Escherichia coli*, which is a common problem for cyanobacterial genes coding for membrane proteins.

Respiration in cyanobacterial thylakoids. Development of light-tolerant PS-I-less strains of *Synechocystis*

6803 has raised the interesting question what the sink for PS-II-generated electrons is. It is clear that the reason for light sensitivity of the strain in which only *psa*AB has been inactivated is related to PS-II-generated electrons, in that reduction of the PS II antenna size leads to a significant reduction in light sensitivity of the PS-I-less strain [8]. As described in [9], in the absence of PS I, PS-II-generated electrons are utilized efficiently by a KCN-sensitive oxidase. If this oxidase were to be the terminal oxidase of the respiratory electron chain, O_2 would be expected to be the terminal electron acceptor. If O_2 is the terminal electron acceptor, then one would expect that light-induced electron transport in PS-I-less strains in the absence of an artificial electron acceptor would not lead to net oxygen production, but rather to a partial or complete

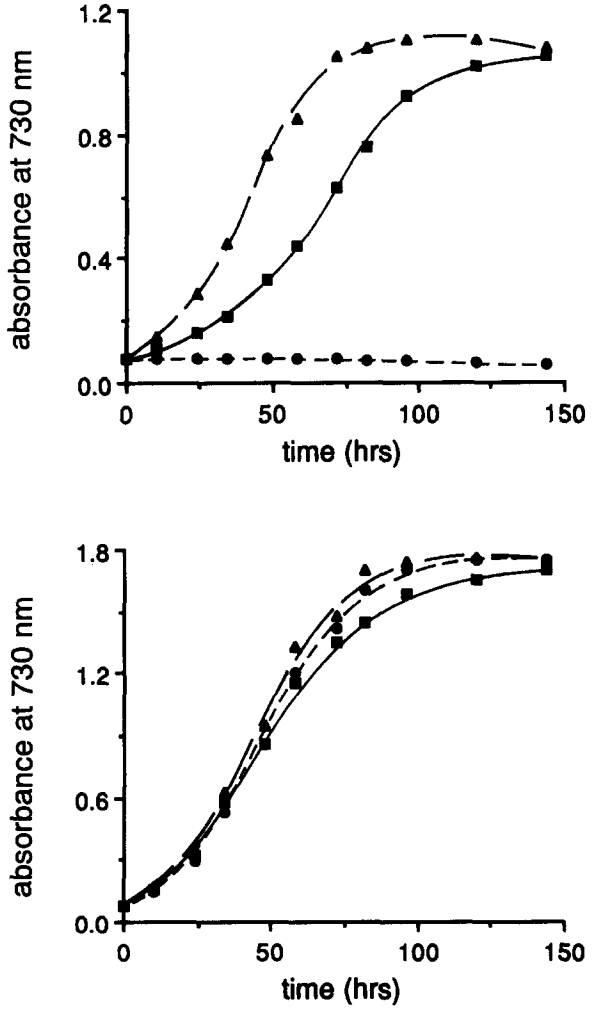

Fig. 2. Growth curve of a photoautotrophic pseudorevertant of the obligate photoheterotrophic D2 mutant E69Q in comparison with wild type and the E69Q mutant. Cells were grown in BG11 medium (photoautotrophic growth; top) or in BG11 medium in the presence of 5 mM glucose (photomixotrophic growth; bottom). Strains used are the E69Q mutant (○, - -), the photoautotrophic pseudorevertant (□, ———), and the control strain that is identical to E69Q except that it does not carry a mutation in the D2 protein (▲,––).

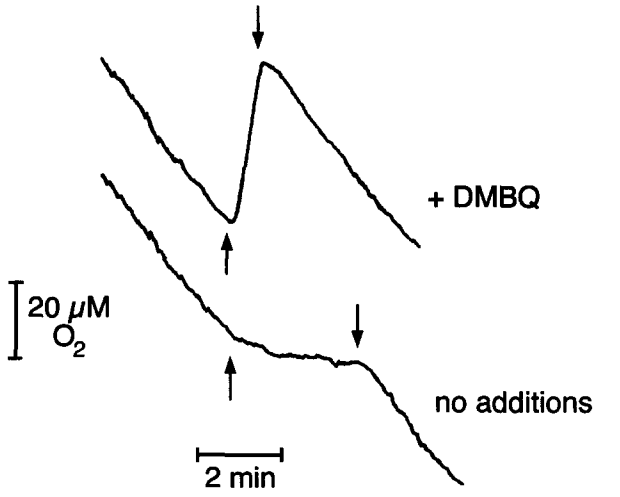

Fig. 3. Oxygen uptake and evolution in intact cells of the PS-I-less mutant. Cells of the PS-I-less mutant were suspended in 25 mM Hepes/NaOH (pH 7.5) to a chlorophyll concentration of 2 μg/ml. After equilibration, oxygen uptake and light-induced oxygen evolution were monitored. Upper trace: 0.5 mM 2,6-dimethyl-*p*-benzoquinone has been added; lower trace: no additions have been made. Arrow up: actinic illumination (1000 $\mu E m^{-2} s^{-1}$) on; arrow down: the illumination was turned off.

suppression of respiratory oxygen uptake. To test this scheme, oxygen evolution was measured in intact cells of the PS-I-less strain of *Synechocystis* 6803 in the presence and absence of an artificial electron acceptor. The results are presented in Fig. 3. In the absence of an artificial electron acceptor, no net oxygen evolution was observed upon illumination, but the oxygen uptake occurring in darkness (respiration) was suppressed almost completely in the light. As expected, when 2,6-dimethyl-*p*-benzoquinone (DMBQ) was added, an artificial electron acceptor that can accept reducing equivalents from the quinone pool, net oxygen evolution occurred at a significant rate (1860 μmol O_2(mg chlorophyll)$^{-1}$ h^{-1}); the respiratory oxygen uptake rate in darkness was 385 μmol O_2(mg chlorophyll)$^{-1}$ h^{-1}, regardless the presence of an artificial quinone. Note that PS-I-less strains are enriched in PS II on a chlorophyll basis by a factor of 6–7 [8]; hence, electron transport rates expressed on a chlorophyll basis are rather high in these strains.

The observation that, in the absence of exogenous electron acceptors in the PS-I-less strain studied, oxygen uptake is found in darkness while no net oxygen uptake or evolution is seen in the light could have the following explanations: (1) No oxygen evolution occurs in the light in this strain in the absence of exogenous acceptors, and respiration is inhibited in the light, (2) by chance the residual rate of oxygen evolution in the absence of exogenous acceptors in the light is about the same as the rate of oxygen uptake by respiration, and no coupling of respiratory and photosynthetic electron transport occurs, or (3) coupling between photo-

synthetic and respiratory pathways is tight in PS-I-less strains of *Synechocystis* 6803, and all PS-II-generated electrons are fed into the repiratory chain and as much oxygen is produced by PS II (one O_2 per four e^-) as is taken up by the terminal oxidase. There is no indication that in the PS-I-less strain respiration is inhibited in the light: in the presence of diuron, an inhibitor of PS II electron transport, oxygen uptake in the light proceeds at a rate essentially indistinguishable from that in darkness (data not shown). This eliminates the first explanation. The second explanation, i.e., no contact between the photosynthetic and respiratory electron transport chains, and a residual rate of oxygen evolution that is about the same as that of respiration, can be excluded because of a rapid oxidation of the PS II acceptor side in a KCN-sensitive fashion if PS I is absent [9]. This shows that photosynthetic and respiratory electron transport chains are closely linked in *Synechocystis* 6803. The third explanation, invoking tight coupling between photosynthetic and respiratory electron transport in thylakoids of the organism of study, by far is the most likely option. The results indeed suggest that the terminal electron acceptor for PS-II-generated electrons is oxygen, and that in the PS-I-less cyanobacterium light-induced electron transport exists from water via PS II and the quinone pool to a KCN-sensitive oxidase with oxygen as terminal electron acceptor. As water splitting leads to oxygen production (4 electrons generated per O_2 produced) in the same electron/O_2 stoichiometry as the terminal electron acceptor uses oxygen, in the PS-I-less strain a light-activated electron transport 'cycle' exists with oxygen as initial product and terminal substrate. The proton gradient over the thylakoid membrane that presumably will result from this electron transfer may be used for ATP synthesis. Thus, in this system a rather complex form of 'cyclic electron flow' appears to occur, with light energy being used for ATP synthesis only, without concomitant net production or use of other compounds.

A promising aspect of the use of molecular genetics to alter and tailor experimental organisms is that the options are almost unlimited, and that the practical limitations mostly are (1) the number of different antibiotic-resistance cartridges that can be inserted to replace genes or to allow for mutant selection, (2) the availability of particular genes from *Synechocystis* 6803, and (3) the limited understanding of the metabolic complexity of a cyanobacterium to determine the genes that are best targeted for particular experiments. In any case, the ability to genetically modify electron transport processes provides the opportunity to study particular processes in far more detail than was possible previously, and to do such measurements in more or less in vivo conditions. The option to delete out complexes that can introduce contaminating signals or artifacts alleviates the need for extensive biochemical purification procedures. For example, the development of PS-I-less mutants essentially has eliminated the need for preparation of PS II particles. This has important ramifications particularly for biophysical characterization of electron transport processes.

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