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Minireview

Gene-targeted and site-directed mutagenesis of photosynthesis genes in cyanobacteria

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Abstract

This historical minireview traces the development and application of methods for gene-targeted and site-directed mutagenesis of photosynthesis genes in cyanobacteria (mainly *Synechocystis* sp. PCC 6803). This approach allowed important data to be obtained on the structure and function of Photosystem I and Photosystem II complexes. I describe some of the major contributions of molecular genetics and subsequent mutant analysis in the 1980s and early 1990s that led to substantial advances in our knowledge of basic principles regarding the organization of the photosynthetic apparatus. This molecular-genetic research on cyanobacteria has initiated a fresh wave of photosynthesis research and created a solid foundation for rapid progress at the threshold of the twenty-first century.

Methodology of gene-targeted mutagenesis

A new era in the history of molecular biology of oxygenic photosynthesis began in the 1980s when the prerequisites for employing cyanobacteria in molecular genetics and genetic engineering had been fulfilled. Initial steps of cyanobacterial genetics, including gene transfer and gene cloning tools, have been thoroughly reviewed (Tandeau de Marsac and Houmard 1987; Shestakov and Reaston 1987; Haselkorn 1991; Thiel 1994). Chromosomal recombination via transformation was discovered in the obligate phototrophic cyanobacterium *Anacystis nidulans* (*Synechococcus* sp.) (Shestakov and Khyen 1970). Efficient transformation systems were developed for *Synechococcus* sp. PCC 7002 (Stevens and Porter 1980) and *Synechocystis* sp. PCC 6803 (hereafter called *S*. 6803) (Grigorieva and Shestakov 1978, 1982). Both strains are photoheterotrophic and can grow without photosynthesis (PS) in the presence of an organic-carbon source. Thus, they are extremely suitable for selection of mutants and recognition of recombinants that are PS-defective. Such mutants can be obtained by two methods: (a) random mutagenesis with selection for a PS-minus phenotype or (b) targeted mutagenesis in which a cloned gene (or DNA fragment) is disrupted by insertion/deletion or is specifically modified. Site-directed mutagenesis allows the investigation of the function of the gene product and of residues or domains in the protein.

At the request of the editor Govindjee, I have included here a photograph of myself, with Clint Fuller, taken in 1991 (Figure 1).

The molecular genetic advantages of *S*. 6803 as a model photosynthetic 'Drosophila' were exactly the reason why this strain was among the first organisms (and the first photosynthetic organism) whose entire genome was completely sequenced (Kaneko et al. 1996); in turn, this made it possible to amplify and specifically mutagenize any genes of this cyanobacterium. The idea of recruiting *S*. 6803 for molecular analysis of PS-systems developed in a conversation between John Williams and myself during the 4th International Symposium on Photosynthetic Prokaryotes (Bombanne, France 1982). Using the experience obtained in developing a method of integrating foreign DNA into the chromosome of *Synechococcus* sp.

Figure 1. Sergey Shestakov (the author) at the International Symposium on Photosynthetic Prokaryotes at the University of Massachusetts, Amherst, in 1991. He is shown receiving a copy of Hugo Scheer's edited treatise 'Chlorophylls' from Clint Fuller, Chair of the symposium, in recognition of Shestakov's research on the molecular genetics of the cyanobacteria.

PCC 7942, John Williams created a system for gene-targeted mutagenesis of PS-genes in *S.* 6803. This kind of approach was not available at that time for green algae and higher plants. The methodology was based on application of an integration platform carrying a cyanobacterial gene interrupted by an antibiotic resistance cartridge serving as a selective marker. This construction was cloned in a nonreplicating vector plasmid that was transferred to the cyanobacterial host with selection for integration of the platform into the chromosome. The procedure permitted inactivation of the gene by insertion or deletion and the ability to replace a wild type gene with a copy altered by site-specific mutagenesis. Gene replacement occurred as a result of homologous recombination due to double crossover (or gene conversion). This method was presented by Williams at the 5th International Symposium on Photosynthetic Prokaryotes (Grindelwald, Switzerland, 1985) and described in detail in his seminal paper that outlines the methodology, which in essence is still being followed (Williams 1988). Virtually all genes for both photosystems were cloned and then examined by gene-targeted mutagenesis.

Mutational analysis of Photosystem II

Pioneering work on Photosystem II (PS II) genes of *S.* 6803 was done in the mid-1980s by Williams and the talented young researchers in C.J. Arntzen's laboratory. Wim Vermaas and colleagues created mutants with inactivation of *psbB,* encoding the CP47 protein (Vermaas et al. 1986, 1987a), and the two *psbD* genes both coding for the protein D2 of the PS II core complex (Vermaas et al. 1987b). Himadri Pakrasi et al. (1987, 1988) deleted *psbE* and *psbF*, both encoding cytochrome b_{559} subunits. All these mutants were unable to grow photo-autotrophically and were PS II deficient. At the same time, Lee McIntosh's group was the first to construct a mutant of *S.* 6803 in which all three *psbA* genes encoding the D1 protein had been deactivated (Jansson et al. 1987). No components of the PS II core complex were detected in the triple deletion *psbA* mutant or the double-deletion *psbD* mutant (Vermaas et al. 1988a), suggesting that the D1 and D2 proteins are required for stabilization of other proteins in the thylakoid. Real progress in understanding the structure and function of the PS II complex and the role of individual amino acid residues was achieved by subsequent region- and site-directed mutagenesis of the *psb* genes.

A great deal of attention has been paid to investigating the D1 and D2 proteins that form the heterodimer of the catalytic core of PS II. In analogy with the purple bacterial system, the function of certain predicted residues was examined on the basis of data accumulated from sequence and hydropathy analysis. Studies of *S.* 6803 site-specific mutants provided the first direct indication that redox-active tyrosine residues in D1 and D2 serve as electron donors to the reaction center chlorophyll (Debus et al. 1988a, b; Vermaas et al. 1988b; Metz et al. 1989). It was shown that Tyr 161 of protein D1 acts as intermediate Y_Z donor in electron transfer from water to P680 while a symmetrical Tyr 160 of D2 is the Y_D component of the heterodimer. Much important information obtained by site-directed mutagenesis of PS II genes was gathered (reviewed by Pakrasi and Vermaas 1992; Vermaas 1993; Pakrasi 1995). Two histidine residues, His 198 in D1 and His 197 in D2, are needed for coordination of chlorophyll likely to be part of P680. The crucial role of Trp 253 of D2 in the binding of Q_A (the first quinone type electron acceptor) and the involvement of His 215 and Ser 264 of D1 protein in binding of Q_B (the secondary electron acceptor) was demonstrated. Mutation of His 268 in D2 resulted in complete inhibition of electron transfer between Q_A and Q_B and showed a prominent role of the nonheme iron in determining the properties of the Q_A and Q_B niches in the PS II complex. Site-directed mutagenesis confirmed that some changes in the Q_B binding pocket of the D1 protein lead to the development of resistance

to certain herbicides blocking photosynthesis. Studies of mutants carrying deletions or site-specific changes (substitutions of Ala 251) in the hydrophilic D–E loop of the D1 polypeptide have demonstrated the importance of this region in the conformation of the acceptor side of PS II (Mulo et al. 1997; Mäenpää et al. 1998).

Systematic site-directed changes of conserved residues in the D1 protein (Nixon and Diner 1992; Boerner et al. 1992; Chu et al. 1995a, b) allowed identification of the functions of some of them: Asp 170, His 332, Glu 333 and others are involved in ligation of manganese and/or assembly of the Mn-cluster; Asp 59 and Asp 61 may bind calcium; and His 190 influences the redox properties of the secondary electron donor. Mutation in Ala 344 position of D1 prevents the cleavage of C-terminal extension of this protein. Such processing is essential for PS II activity because only mature D1 can ligate the Mn-cluster (Nixon et al. 1992).

Analysis of mutants carrying a truncation or small deletions in the C-terminal lumenally exposed tail of the D2 protein revealed its critical role for proper folding of D2 through the membrane and for functioning of the water-splitting complex (Eggers and Vermaas 1993). The Glu 69 residue in D2 is also important for water-splitting activity: replacement of this residue by Gln led to destabilization of PS II activity (Vermaas et al. 1990b). It was shown that Arg 233 and Arg 251 of the D2 polypeptide are involved in the binding of bicarbonate, which causes a reversible stimulation of electron flow in PS II (Cao et al. 1991). Efficient and reliable systems for site-specific and region-targeted mutagenesis of the D2 protein were developed (Vermaas et al. 1990a; Vermaas 1998). A genetically engineered *S.* 6803 recipient strain lacking PS I activity and both the *psbDI* and *psbDII* genes was constructed. This facilitated a positive selection for PS II mutants and also biochemical and biophysical analysis of such mutants. A number of residues in D2 whose functions could not be predicted were revealed by using targeted random chemical mutagenesis (Ermakova-Gerdes et al. 1996). Characterization of more than 100 mutations generated by targeted mutagenesis in D2 protein provided valuable insights into the organization of the PS II complex (Vermaas 1998).

Evidence was obtained that particular pairs of His residues located in transmembrane regions of the CP47 protein are involved in chlorophyll binding. Replacement of these His residues led to destabilization of the PS II complex (Shen et al. 1993a). It was shown that a large lumenally exposed hydrophilic loop of CP47 plays an important role in the function of PS II. A randomly generated *psbB* mutant carrying a mutation of Trp 167 to Arg in this loop was unable to grow photoautotrophically (Ermakova et al. 1993). The replacement of Trp 167 to serine dramatically increased the light sensitivity of PS II (Wu et al. 1996). Deletion between Gly 351 to Thr 365 and some small deletions within this loop yielded mutants impaired in PS II function (Eaton-Rye and Vermaas 1991; Haag et al. 1993). T.M. Bricker's laboratory modified specifically all of the conserved charged residues located in this loop and identified a domain which is crucial for stable assembly of the water-splitting system (Putnam-Evans et al. 1996). The gene *psbC* encoding the CP43 protein (another component of the antenna) was also probed by targeted mutagenesis (Vermaas et al. 1988a; Rögner et al. 1991). Small deletions in the long hydrophilic loop of CP43 led to a failure of overall PS II function (Kuhn and Vermaas 1993).

Inactivation of *S.* 6803 genes encoding cytochrome b_{559} resulted in destabilization of the reaction center but did not affect the stability of core antenna proteins (Pakrasi et al. 1990). Each subunit of cyt b_{559} has one His 22 residue, which acts as a heme ligand. Replacement of His by Leu (which cannot coordinate a heme iron) in either of two subunits led to a complete loss of PS II activity (Pakrasi et al. 1991). Region-specific modification of the lumenally exposed C-terminal tail of the a-subunit revealed that the region between Asp 52 and Ile 71 influences PS II function. Mutational analysis enabled us to design a model of topographical orientation of both subunits and suggested a possible involvement of cyt b_{559} in regulation of cyclic electron flow around the PS II complex to protect it from photoinhibition (Pakrasi 1995).

Several research groups have independently shown by targeted mutagenesis that the manganese-stabilizing protein (MSP) encoded by *psbO* is not absolutely essential for the water-splitting function, although MSP-less mutants are more sensitive to photo-inhibition. Among *psb* genes encoding small proteins, only *psbL* is necessary for PS II function (Anbudurai and Pakrasi 1993). The *psbJ* and *psbH* genes presumably are involved in regulation of electron flow within the PS II complex.

Mutational analysis of Photosystem I

The first publications on gene-targeted mutagenesis of *psaD* and *psaE* coding for Photosystem I (PS I) components appeared in 1989. Chitnis et al. (1989a, b) deciphered the functions of the accessory subunits of PS I through mutational analysis of *S*. 6803 genes. Disruption of the *psaE* (Chitnis et al. 1989b), *psaF* (Chitnis et al. 1991), *psaL* (V.P. Chitnis et al. 1993), and *psaJ* (Xu et al. 1994) genes did not lead to loss of photoautotrophic growth and PS I activity. Studies of gene-directed mutants suggested that PsaD is involved in the docking of ferredoxin to PS I reaction centers and assembly of other peripheral subunits. The PsaL protein was proposed to function in the formation of PS I trimers.

L. McIntosh's group carried out directed deactivation of *S*. 6803 *psaA* (Smart et al. 1991) and *psaB* (Smart and McIntosh 1993) genes encoding two core proteins of the PS I reaction center. The mutants were unable to grow photoautotrophically and were characterized by a low chlorophyll content. A similar mutant (Shen et al. 1993b) was found to grow at low but not at high light intensity, with the light sensitivity presumably caused by toxicity of an overproduced plastoquinone pool. Site-specific changes of Cys 556 and Cys 565 residues in PsaB showed that these cysteines act as ligands for the F_x cluster (4Fe–4S) and participate in assembly of the PS I complex (Smart et al. 1993; Warren et al. 1993). Studies of *Synechococcus* sp. PCC 7002 mutant showed that PsaE protein is required for cyclic electron flow around PS I (Yu et al. 1993).

Pakrasi's team has successfully employed the heterotrophic *Anabaena variabilis* strain for gene-targeted mutagenesis. They deactivated the *psaC* gene encoding the iron-sulfur protein (Mannan et al. 1991), and the *psaA* and *psaB* genes (Nyhus et al. 1993). The PsaC protein is necessary for a stable association of the PsaD, PsaE and PsaL proteins in the PS I complex (Mannan et al. 1994, 1996).

Concluding remarks

In a minireview, it is impossible to cover all significant research endeavors and results obtained through gene-directed mutagenesis in many other leading laboratories (E.M. Aro, L. Bogorad, D.A. Bryant, B.A. Diner, J.K. Golbeck, S. Golden, Govindjee, R. Haselkorn, M. Ikeuchi, I. Ohad, L. Sherman, and numerous others) elucidating key principles of the structural and functional organization of the photosynthetic apparatus. In any case, the major impetus to research progress came from integration of new molecular genetic approaches (including so-called 'reverse genetics') with modern biophysical and biochemical methods. This integration initiated a fresh wave of photosynthesis research and created a solid foundation for rapid progress in this field at the threshold of the twenty-first century.

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