DOE/ER/14031-5

FINAL REPORT, DE-FG02-89ER14031
"The chlorophyll-binding protein CP47 in photosystem II"

Willem F.J. Vermaas
Department of Botany
Arizona State University
Tempe AZ 85287

Unexpended funds: none

A. Publications resulting from the project:

Bittersmann, E. and Vermaas, W.F.J. (1991) Fluorescence lifetime studies of cyanobacterial photosystem II mutants. *Biochim. Biophys. Acta* **1098**, 105-116.

Eaton-Rye, J.J. and Vermaas, W.F.J. (1991) Oligonucleotide-directed mutagenesis of *psbB*, the gene encoding CP47, employing a deletion mutant strain of the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Mol. Biol.* **17**, 1165-1177.

Pakrasi, H.B. and Vermaas, W.F.J. (1992) Protein engineering of photosystem II. *In:* The Photosystems: Structure, Function, and Molecular Biology, Current Topics in Photosynthesis, Volume 11 (J. Barber, ed.), pp. 231-256, Elsevier, Amsterdam.

Eaton-Rye, J.J. and Vermaas, W.F.J. (1992) Characterization of a histidine to glutamine substitution at residue 469 in CP47 of photosystem II. *In:* Research in Photosynthesis (N. Murata, ed.), Vol. I, pp. 239-242, Kluwer, Dordrecht.

Haag, E., Eaton-Rye, J.J., Renger, G., and Vermaas, W.F.J. (1993) Functionally important domains of the large hydrophilic loop of CP47 as probed by oligonucleotide-directed mutagenesis in *Synechocystis* sp. PCC 6803. *Biochemistry* **32**, 4444-4454.

Shen, G., Eaton-Rye, J.J., and Vermaas, W.F.J. (1993) Mutation of histidine residues in CP47 leads to destabilization of the photosystem II complex and to impairment of light energy transfer. *Biochemistry* **32**, 5109-5115.

*Shen, G., and Vermaas, W.F.J. (1994a) Chlorophyll in a *Synechocystis* sp. PCC 6803 mutant without photosystem I and photosystem II core complexes: Evidence for peripheral antenna chlorophylls in cyanobacteria. *J. Biol. Chem.* **269**, 13904-13910.

*Shen, G., and Vermaas, W.F.J. (1994b) Mutation of chlorophyll ligands in the chlorophyll-binding CP47 protein as studied in a *Synechocystis* sp. PCC 6803 photosystem I-less background. *Biochemistry* **33**, 7379-7388.

*Gleiter, H.M., Haag, E., Shen, J.-R., Eaton-Rye, J.J., Inoue, Y., Vermaas, W.F.J., and Renger, G. (1994) Functional characterization of mutant strains of the cyanobacterium *Synechocystis* sp. PCC 6803 lacking short domains within the large, lumen-exposed loop of the chlorophyll-protein CP47 in photosystem II. *Biochemistry* **33**, 12063-12071.

*Gleiter, H.M., Haag, E., Shen, J.-R., Eaton-Rye, J.J., Seeliger, A.G., Inoue, Y., Vermaas, W.F.J., and Renger, G. (1995) Involvement of the CP47 protein in stabilization and photoactivation of a functional water-oxidizing complex in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochemistry* **34**, 6847-6856.

*: a copy has been attached. Copies of earlier publications have been submitted with previous progress reports.

B. Introduction

Over the past decades, the process of photosynthesis has been studied in detail, and the pathways of light-induced electron transfer have been relatively well-established. In plants and cyanobacteria, two pigment-protein complexes in the thylakoid membrane, photosystem II and photosystem I (PS II and PS I, respectively), act in tandem to catalyze light-induced electron transport, resulting in water oxidation (oxygen evolution), $NADP^+$ reduction, and formation of

a proton gradient across the thylakoid membrane. PS II consists of at least a dozen membrane proteins (not counting peripheral proteins), some of which bind pigments. The PS II complex catalyzes light-induced electron transfer resulting in oxidation of water and reduction of plastoquinone. Reducing equivalents of plastoquinol can be used by PS I via the cytochrome b_6f complex. In both PS II and PS I, 50-100 chlorophylls (the "core antenna") are in close vicinity to the reaction center, and serve to absorb light and to transfer light energy to chlorophyll in a specific environment (the primary donor of the reaction center in each of the photosystems); this reaction center chlorophyll, upon excitation, can reduce a nearby electron acceptor, and thus initiate a series of redox reactions. In this way, light energy is converted into chemical energy, which eventually is used for production of ATP and NADPH.

The core antenna of PS II is composed mainly of chlorophyll associated with two chlorophyll-binding proteins, CP47 and CP43. These proteins (about 47,000 and 43,000 M_r, respectively) are thought to contain six transmembrane domains, with the N- and C-terminal ends exposed to the cytoplasmic/stromal side of the thylakoid. Many of the putative transmembrane domains in the two chlorophyll-binding antenna proteins contain pairs of His residues that are very much conserved throughout evolution. The His residues of each pair are separated by 2 or 12-13 other residues, corresponding to one or four turns of an α-helix. These His residues generally are considered to be very strong candidates to be involved in chlorophyll binding, as His is known to be ligand to the central Mg of bacteriochlorophyll in bacterial reaction centers. Also, both in plant and bacterial antenna systems His residues can serve as ligands to (bacterio)chlorophyll. Another striking property of both the CP47 and CP43 proteins is a particularly long hydrophilic region between transmembrane domains V and VI; this region is about-180 residues long for CP47, and about 130 residues in the case of CP43.

In spite of detailed knowledge about the pathways of electron transfer through the photosynthetic electron transport chain, little is known about the factors governing the processes of energy transfer in the core antenna complex. A primary aim of the DOE-sponsored project in my laboratory during the past several years was to investigate chlorophyll-binding domains in the core antenna, and to elucidate whether subtle changes in the environment and in possible ligands of the chlorophyll have effects on energy transfer characteristics. To do so, we have introduced mutations into *psbB*, the gene coding for CP47, at codons for conserved His residues in hydrophobic regions of the protein. The results of this work will be summarized in section C.

Another aim of the project was to determine the function of the large lumenally exposed loop in CP47. As will be discussed in section D, this loop appears to be involved in binding peripheral component(s) of the PS II complex, but also contains domains that are critical for PS II function, assembly and/or stability.

For our research, the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis* 6803) has been used. This strain is a facultative photoautotroph (can grow in the absence of PS II activity if glucose has been added) and is spontaneously transformable. A major advantage of this strain is that plasmid DNA that is introduced generally is integrated into the cyanobacterial genome by homologous recombination. In this way, genes can be interrupted, deleted, or replaced by a gene copy carrying mutations or deletions. Thus, using targeted genetic modification it can be designed to optimally suit the experimental aims that have been set. For example, analysis of PS II pigments *in vivo* or in thylakoids is greatly facilitated by using a background strain that lacks PS I (with which most of the chlorophyll is associated in cyanobacteria) or that is depleted in phycobilisomes, the peripheral antenna in cyanobacteria. Deletion of PS I can be achieved by deletion of the *psaAB* operon, coding for the core proteins of PS I, and depletion of phycobiliproteins results from deletion of the *apcE* gene, which codes for the L_{CM} protein

serving as an anchor for the phycobilisome into the thylakoid membrane. Upon deletion of PS I, a mutant that can be maintained at low light intensity (5 $\mu E.m^{-2}.s^{-1}$) results. Subsequent deletion of *apcE* removes the light sensitivity of the PS I-less mutant, suggesting that excess PS II activity in the absence of PS I is a main factor in light sensitivity of the strains.

Synechocystis 6803 strains lacking PS I and/or functional phycobilisomes have been instrumental towards measuring convincingly the effects of single mutations in His residues. As will be pointed out in section C, with this approach we have been able to detect the formation of one or several extra pheophytins in PS II upon mutation of His residues that appear to serve as chlorophyll ligands. Another finding that was dependent on the ability to generate PS I-less *Synechocystis* strains was the observation that in the absence of both PS II and PS I still chlorophyll is associated with cyanobacterial thylakoids. This is described in Section E and in Shen and Vermaas (1994a).

C. Site-directed mutations in His residues

As indicated, CP47 contains a number of conserved His residues in hydrophobic regions of the protein. These residues have been indicated in a topological model (Figure 1 of Shen et al. (1993)). With DOE support, we have designed a system for site-directed mutagenesis of a significant part of *psbB* (Eaton-Rye and Vermaas, 1991), enabling us to introduce site-directed mutations in the C-terminal 80% of CP47 (which covers all but the first transmembrane span). We have designed *Synechocystis* deletion strains lacking intact *psbB* both in a wild-type background (Eaton-Rye and Vermaas, 1991) and in a background lacking PS I and *apcE* (Shen and Vermaas, 1994b). These strains have been used to introduce mutations in His100, His114, His142, His157, His201, His202, His216, His455, His466, and His469 (Eaton-Rye and Vermaas, 1992; Shen et al., 1993; Shen and Vermaas, 1994b). To introduce site-directed mutations in His9, His23, and His26 of CP47, recently another *psbB* deletion construct was made, in which the first part of the gene was deleted (see Shen and Vermaas (1994b) regarding details on the plasmid construct). This construct was introduced into PS I-less and PS I-containing *Synechocystis* 6803 strains. The resulting cyanobacterial strains carrying a deletion in the first part of *psbB* were used for transformation with *psbB* constructs carrying mutations in one of the His codons near the 5' end of the gene (corresponding to one of the 60 N-terminal residues of CP47).

Mutations that have been introduced in His residues of CP47 have been described in Eaton-Rye and Vermaas (1992), Shen et al. (1993), and Shen and Vermaas (1994b). The reader is referred to these publications for detailed information regarding the outcome of our studies. A summary of the most noteworthy findings and concepts is provided below.

The following general trends were apparent in our studies on mutagenesis of His residues in hydrophobic regions of CP47: (1) mutation of such His residues to Asn or Gln had less effect on the function and apparent stability of PS II in thylakoids than mutation to Tyr; (2) mutation of His to Tyr led to a decrease in the light-harvesting efficiency of the PS II antenna; and (3) mutation of His to Tyr (but not to Asn or Gln) led to the formation of a pigment that spectroscopically resembled pheophytin *a*. As discussed in Shen et al. (1993) and Shen and Vermaas (1994b), we interpret these general trends to indicate that the conserved His residues in hydrophobic regions of CP47 that we have mutated are chlorophyll ligands. Mutation of these residues to other potential chlorophyll ligands (for example, Gln or Asn) had no effect on the pigment composition of the antenna, even though in some cases the PS II complex was somewhat destabilized when Gln or Asn replaced His. However, mutation of His to a residue (such as Tyr) without an N-containing side group appeared to lead to an antenna containing a pheophytin *a*, which either may have been incorporated at the time of biosynthesis of the

protein, or may have been formed upon dissociation of the central Mg from the chlorophyll (due to the absence of a fifth ligand) after biosynthesis and assembly of the pigment-protein.

The presence of this pheophytin was best visualized by low-temperature (77 K) fluorescence excitation spectra (measuring 695 nm (CP47) emission) of thylakoids from His-to-Tyr mutants in PS I-less, *apcE*$^-$ backgrounds. With these samples, a 531 nm peak in the fluorescence excitation spectrum is visible, together with increased amplitude around 415 nm. This is not seen in mutants in which His was changed to Asn (Shen and Vermaas, 1994b). Thus, our main conclusion based on site-directed mutagenesis of His residues in CP47 is that the His residues studied are chlorophyll ligands, and without a proper ligand pheophytin *a* replaces the native chlorophyll.

A main question that we addressed is what the consequences are of having a pheophytin *a* in the antenna system. Three phenomena have been observed in His-to-Tyr mutants: (a) a lower antenna efficiency, (b) a shorter lifetime of PS II-related chlorophyll fluorescence, and (c) a decrease in the amount of PS II on a chlorophyll basis in the thylakoid membrane. The lower antenna efficiency is apparent from the amount of light needed for half-saturation of PS II electron transfer. For all His-to-Tyr mutants studied, 10-50% more light was needed to half-saturate oxygen evolution with dimethyl-*p*-benzoquinone (DMBQ) as acceptor in the mutants as compared to in the wild type (Shen et al., 1993; Shen and Vermaas, 1994b). In His-to-Asn mutants, no significant loss of antenna function as compared to wild type was observed (Shen et al., 1993).

The shorter lifetime of PS II fluorescence in His-to-Tyr mutants is best seen in a PS I-less/*apcE*$^-$ background, as in such systems the relative contributions of PS II-related fluorescence decay components are much more significant than in a PS I-containing background. All three phases with a lifetime of less than 1 ns (corresponding to both open and closed PS II reaction centers) had a decreased lifetime in the His-to-Tyr mutants studied (Shen and Vermaas, 1994b). This indicates to us that the apparent presence of pheophytin *a* in the chlorophyll antenna leads to increased excitation quenching. The reason(s) for this increased excitation quenching at this moment remain in the realm of speculation. However, one possible explanation is that after transfer of excitation energy from chlorophyll to pheophytin, excitation transfer back to chlorophyll may not be as efficient, and excited pheophytin may have a reasonable probability to relax to the ground state before the excitation is transferred back to chlorophyll. This would provide an explanation not only for the decreased fluorescence lifetime, but also for the decreased antenna efficiency in His-to-Tyr mutants.

An important question is why the apparent presence of pheophytin *a* in CP47 affects light-harvesting efficiency, while presence of pheophytin *a* in the reaction center from PS II and purple bacteria naturally has evolved and has been sustained throughout evolution. As discussed in Shen and Vermaas (1994b), the excitation spectrum of the pheophytin *a* in CP47 mutants is shifted considerably to the blue as compared to that of the PS II reaction center complex. Thus, it is likely that energy transfer from excited pheophytin to chlorophyll is much more efficient in the reaction center than in the mutant antenna complex.

The observation that the PS II / chlorophyll ratio in most CP47 His mutants (and particularly in His-to-Tyr mutants) is lower than in wild type appears to indicate that the stability of PS II in the mutants is decreased, as the degradation rate of PS II components is a major mechanism by which the amount of PS II in thylakoids is regulated. Even though His-to-Tyr mutants generally showed a more pronounced decrease in the PS II / chlorophyll ratio than corresponding His-to-Gln or His-to-Asn mutants, suggesting that removal of the chlorophyll ligand decreased the stability of the PS II complex, it would be a major oversimplification to state that the apparent decrease in stability would be due only to a change

in the pigment at the corresponding binding site. Also in many His-to-Gln or His-to-Asn mutants, in which there is no evidence for the formation of additional pheophytin *a* (Shen and Vermaas, 1994b), an apparent destabilization of PS II occurs. Therefore, it is reasonable to assume that the mutation itself causes a certain destabilization of the complex. On top of this, a change in the pigment associated with the residue can result in further destabilization. The apparent destabilization of the PS II complex is in line with what is generally seen upon introduction of small mutations in PS II.

A somewhat unexpected observation was that mutations in His114 had unique consequences with respect to fluorescence emission characteristics at low temperature. Mutations in only His114 were found to lead to a shift in the position of the 77 K fluorescence emission peak at 695 nm that is associated with CP47. Upon mutation of His114 to Asn or Gln, the spectrum was shifted by 2-3 nm to the blue. Upon mutation to Tyr, no PS II accumulated, and therefore no PS II-related emission could be detected. The 695 nm fluorescence emission at low temperature is thought to originate from one or very few "low-energy" pigments with the Q_Y transition dipole tilted more than 35° with respect to the membrane plane. This pigment(s) cannot efficiently transfer excitation energy back to other chlorophylls unless sufficient vibrational energy is available. The shift in the 695 nm emission maximum to lower wavelength seen upon mutation of His114 to Gln or Asn suggests that the pigment associated with His114 is a main contributor to long-wavelength fluorescence emission in PS II. The chlorophyll associated with His114 may have obtained its unusual spectral characteristics by a particular interaction with the protein environment, and small perturbations in this environment already may have significant effects on the energy level of the first excited state.

The presence of low-energy pigments in the core antenna seems to be a general phenomenon, in that in all reaction center complexes that have been investigated long-wavelength fluorescence emission at low temperature has been observed. However, this is the first instance in which one of the low-energy pigments has been tentatively assigned to a particular position in the core antenna. This tentative assignment, together with the hypothesis that most photosynthetic reaction centers share a common ancestry, provides a viable opportunity to start a targeted search for low-energy pigments in other reaction center complexes. Nonetheless, this tentative assignment does not answer the important question of what the functional essence may be of one or very few low-energy chlorophylls in the antenna of photosynthetic reaction center complexes.

D. The long hydrophilic loop of CP47

Between membrane-spanning regions V and VI of CP47, a 180-190 residues long hydrophilic loop exists that appears to be on the lumenal side of the thylakoid. A domain in this loop has been implicated to be involved in interaction with a 33 kDa peripheral protein, which is encoded by *psbO* and involved in stabilizing the Mn cluster in the water-splitting system of PS II. However, such a long loop in a *bona fide* membrane protein is quite unusual, and we set out to investigate the function of different domains in this loop. Our approach towards identifying functionally important regions of this long hydrophilic loop between helices V and VI of CP47 initially was to delete small conserved domains (3-8 residues), to determine the effect of each deletion on the structure and function of PS II, and then to focus on the domains that appear to be of particular importance. As described by Haag et al. (1993), twelve deletions in the long hydrophilic loop have been made, in addition to two deletions that were discussed in Eaton-Rye and Vermaas (1991). Of the twelve new deletions, five led to an obligate (photo)heterotrophic phenotype, indicating that the deletions led to an impairment of PS II. Several other deletion mutants were somewhat impaired in PS II activity, but were capable of photoautotrophic growth. These mutants have been analyzed in terms of

photoautotrophic growth rates, PS II / chlorophyll ratios in cells (Haag et al., 1993), interaction with peripheral PS II proteins, kinetics of photoactivation, and thermodynamics of charge recombination in PS II (Gleiter et al., 1994, 1995). Characterization of the photoautotrophic deletion mutants clearly established an involvement of a specific domain of the loop in interaction with the manganese-stabilizing protein. This is in agreement with observations by other groups. See Haag et al. (1993) and Gleiter et al. (1994, 1995) for details on the deletions introduced in *psbB*, and on the characterization of the resulting mutants.

E. Chlorophyll-binding proteins in cyanobacteria

Generally, light-harvesting chlorophyll-binding proteins (LHCP) of the Cab family that are prevalent antenna systems in plants are thought to be absent in cyanobacteria. Therefore, it often is tacitly assumed that in cyanobacteria all chlorophyll is associated with the PS II and PS I core antenna. For this reason, it was of interest to investigate what the effect would be of genetic deletion of both the PS I core complex and the PS II core antenna in *Synechocystis*. Therefore, a mutant was made in which the *psaAB* genes for the PS I core were deleted, in addition to deletion or inactivation of *psbB* and/or *psbC* (coding for CP43). In this series of mutants, also *apcE* was deleted. In the absence of both CP47 and CP43, also the PS II reaction center proteins D1 and D2 were not detectable in the thylakoid membrane. Thus, both PS II and PS I were deleted in the resulting strains. Nonetheless, a significant amount of chlorophyll (about 15% of that present when PS II was left intact) was found to remain in the PS I-less, *psbB*$^-$, *psbC*$^-$, *apcE*$^-$ mutant (Shen and Vermaas, 1994a). This chlorophyll had fluorescence characteristics resembling those of LHC II in higher plants, with a 678 nm emission maximum at 77 K. The properties of this chlorophyll remaining in the absence of PS II and PS I in *Synechocystis* did not resemble those of chlorophyll bound to a CP43-like protein that has been found in cyanobacteria and that is expressed under iron-stress conditions. However, some similarities in terms of fluorescence emission were observed with the isolated 22 kDa protein encoded by *psbS*. The role and association of the remaining chlorophyll in the PS I-less, *psbB*$^-$, *psbC*$^-$, *apcE*$^-$ mutant remains unclear. However, as discussed in some detail in Shen and Vermaas (1994a), this chlorophyll protein is expected to be functionally connected to PS II when this photosystem is present.