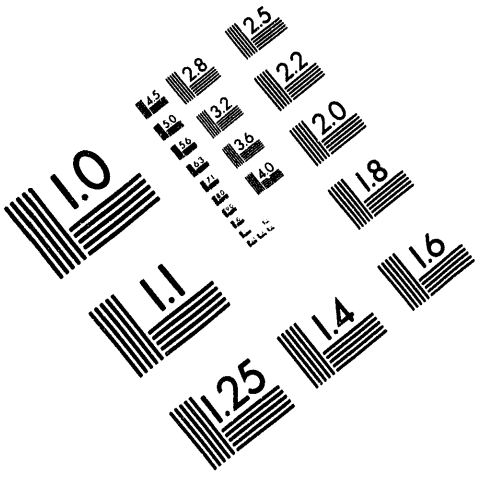

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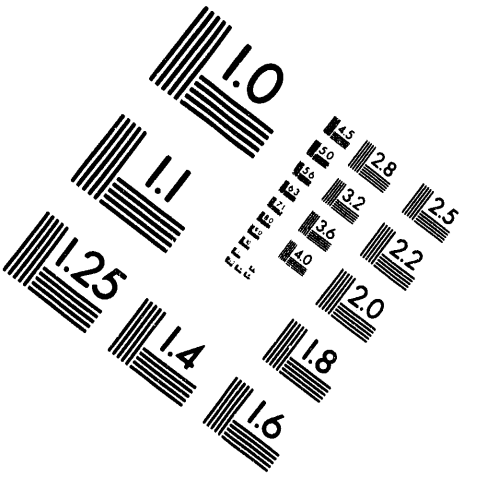

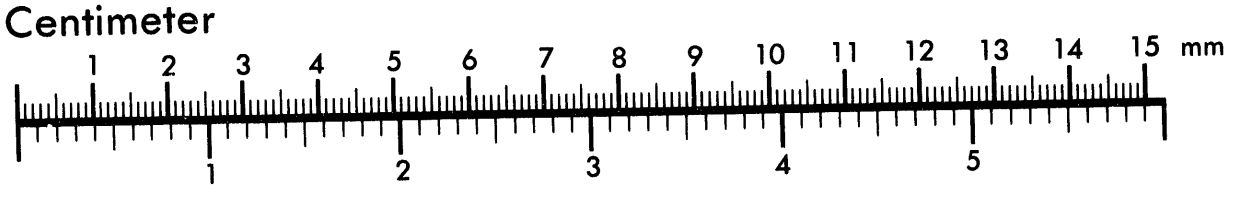

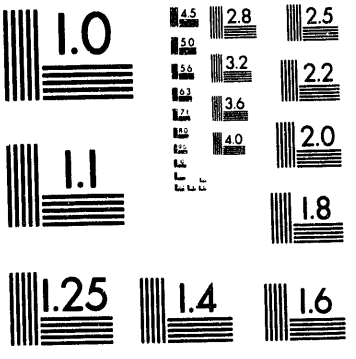

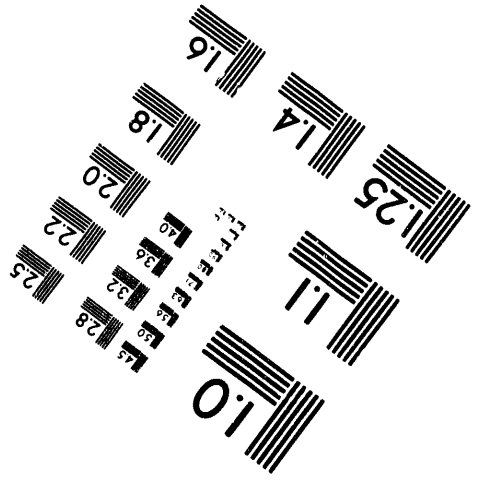

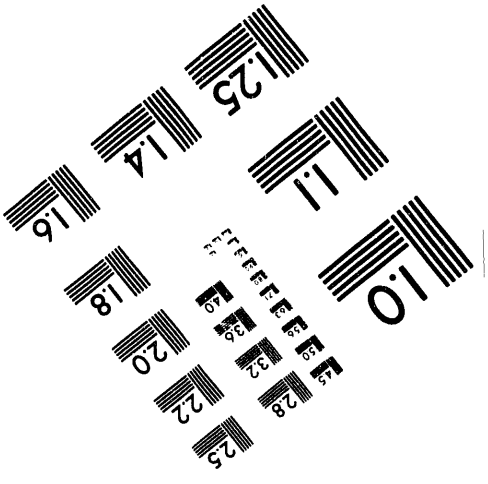

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PROGRESS REPORT AND APPLICATION FOR CONTINUATION AWARD

for The Chlorophyll-binding Protein CP47 in Photosystem II
(DE-FG02-89ER14031)
Willem F.J. Vermaas

In 1992, funds for a two-year period (June 1992 through May 1994) were granted for the above-mentioned project. The project was reviewed in July 1993 as part of the Peer Review Assessment of the DOE/BES Biosciences Program, and an extensive progress report was submitted at that time. The progress report that follows here duplicates some of the information that was provided for the review in July 1993, and highlights progress that has been made since that time.

Introduction:
In many laboratories around the globe, the primary steps in the photosynthesis process are studied in terms of electron transfer reactions and cofactor properties in photosynthetic reaction center proteins. However, little attention is paid to factors affecting the efficiency of light harvesting and energy transfer in antenna proteins of photosynthetic systems, in spite of the fact that efficient light harvesting is of critical importance for photosynthesis under light-limiting conditions. The aim of the DOE-sponsored project in my laboratory is to determine the role of specific conserved residues and domains of chlorophyll-binding antenna proteins, and we designed a system in which targeted mutations could be introduced in an important light-harvesting antenna protein, CP47 of photosystem II, and in which mutation-induced effects could be studied *in vivo*.

The CP47 protein is encoded by the *psb*B gene, binds 10-20 chlorophylls, and has an apparent molecular weight of about 47 kDa. Specific mutations can be introduced into this protein using the transformable, (photo)heterotrophic cyanobacterium *Synechocystis* sp. PCC 6803. This cyanobacterium takes up foreign DNA spontaneously, and can incorporate it into its genome by homologous recombination. As this cyanobacterium can survive in the absence of PS II activity if glucose is present, it is possible to take out the wild-type *psb*B gene and subsequently replace it by a construct containing the desired mutation(s) in *psb*B.

Sequence analysis of the CP47 protein predicts six transmembrane regions, interspersed by relatively hydrophilic loops. Five of the putative transmembrane regions contain conserved His pairs that are spaced 13-14 residues apart. His residues have been found to serve as bacteriochlorophyll ligands, and therefore His residues are logical candidates to serve as chlorophyll ligands in chlorophyll-binding proteins. An important part of the DOE-sponsored project on CP47 is to analyze effects of mutations in conserved His residues in hydrophobic regions of the protein.

In addition to serving as a chlorophyll-binding antenna protein, CP47 also appears to be involved in providing a suitable environment for an efficient and stable oxygen-evolving complex. CP47 contains a long hydrophilic loop (about 190 residues) between transmembrane regions V and VI, and part of this loop appears to interact closely with the peripheral protein MSP (the manganese-stabilizing protein), which is important for efficient and stable oxygen evolution. Also other areas of this and other lumen-exposed loops of CP47 may interact with components of the water-splitting system, and we are investigating this issue in more detail.

Progress report and research directions:

Publications acknowledging DOE support that were published/submitted between June 1992 and January 1994:

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., and Vermaas, W. (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.

*Haag, E., Gleiter, H.M., 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*, submitted.

*Shen, G., and Vermaas, W.F.J. (1994) 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.*, submitted.

(*: Three copies of these papers have been enclosed.)

Energy transfer: Energy transfer processes in chlorophyll antenna systems have been studied mostly by biophysically oriented methods. Detailed information regarding requirements of efficient energy transfer has thus been obtained. We have complemented this approach by generation of targeted mutations in chlorophyll-binding proteins, and analysis of the resulting mutants. As indicated in Shen et al. (1993), a mutation of one of several conserved His residues in hydrophobic regions of CP47 to Tyr leads to a decrease of the PS II/chlorophyll ratio in the thylakoid membrane, and to a decrease in the efficiency by which PS II can utilize absorbed light energy. Mutation of the same His residues to Asn leads to less significant effects. We interpret these observations as evidence that the targeted His residues indeed serve as chlorophyll ligands (see Shen et al. (1993) for a discussion regarding this point).

Additional evidence for His involvement in chlorophyll binding comes from analysis of a mutant, in which His114 of CP47 was changed to Gln. At low temperature, one or more of the CP47 chlorophylls fluoresce at higher wavelength (695 nm) as compared to other chlorophyll associated with PS II (685 nm). Most other photosynthetic reaction centers, both in plants and bacteria, also contain an antenna pigment with particularly high fluorescence emission. Mutation of His114 to Gln does not significantly affect PS II function, but shifts the 695 nm fluorescence emission peak 2-3 nm to the blue, while other CP47 mutations do not affect the position of the 695 nm peak (manuscript in preparation). We interpret this as evidence that the 695 nm fluorescence emission peak is related to chlorophyll associated with His114 of CP47. To our knowledge, this is the first time the location of a low-energy chlorophyll in a photosynthetic reaction center complex has been determined.

For a more detailed characterization of His mutants, an *in vivo* system in which most pigments are associated with PS II would be advantageous. In *Synechocystis* 6803, PS I and phycobilisomes bind many more pigments than the PS II core complex. To obtain a "cleaner" system for analysis of the chlorophyll antenna associated with PS II, we have genetically deleted out the PS I reaction center complex and the *apc*E gene. The latter codes for the L_{CM} anchor protein, which is important for assembly of the phycobilisome as well as for attachment of phycobilisome components to the thylakoid. We have found conditions under which full genetic deletion of the PS I reaction center in *Synechocystis* sp. PCC 6803 can be obtained in dim continuous light (5 $\mu E.m^{-2}.s^{-1}$) (G. Shen, S. Boussiba and W. Vermaas, *Plant Cell* 5 (1993) 1853-1863). Upon subsequent deletion of the *apc*E gene, phycobilisome components no longer are functionally attached to PS II and degrade quickly. The resulting PS I-less, *apc*E-less mutant strain grows at normal light intensity with essentially wild-type rates. In thylakoid preparations from such a strain, remaining phycobilin-binding proteins are easily washed off, resulting in thylakoids devoid of phycobilins and lacking PS I.

This strain is very suitable for introduction of site-directed mutations in PS II components. We have introduced a large number of His-to-Tyr mutations in CP47 in a PS I-less, *apc*E$^-$ background. The PS II characteristics of the resulting mutants are very similar to those obtained in a PS I-containing background, thus illustrating that no inadvertant artifacts (for example, due to changes in redox poising or in electron transport rates through PS II) have been introduced. The PS I-less system is very useful for detailed mutant analysis, and has provided us with direct experimental evidence that upon mutation of selected His residues of CP47 to Tyr pheophytin has been formed, presumably caused by the loss of the 5th ligand to the central Mg^{2+} in chlorophyll: by measuring fluorescence excitation spectra of mutants at 77K, monitoring 695 nm (CP47) emission, bands were found at positions (412 nm and 531 nm) where pheophytin absorbs. These bands were absent in the PS I-less background strain and in strains in which His residues have been replaced by residues that can serve as chlorophyll ligands (such as Asn). Also strains with a highly unstable PS II complex did not show such 412 and 531 nm bands in the fluorescence excitation spectrum, showing that these bands are not due to unspecific chlorophyll degradation. This provides the first direct evidence that upon mutation of His residues to Tyr in CP47 pheophytinization occurs, and could not have been observed in systems still containing PS I: in such systems too much chlorophyll is present to see the conversion of one or very few PS II-related chlorophylls to pheophytin.

In addition, fluorescence lifetime measurements have been carried out on thylakoids from one of the His-to-Tyr mutants in CP47 (H202Y; Shen et al., 1993) in the PS I-less, *apc*E-less background strain, and on thylakoids from the background strain itself. In both the PS I-less, *apc*E$^-$ background strain and the mutant, open PS II reaction centers have a predominant fluorescence lifetime of approximately 40 ps (the time it takes for energy migration through the antenna to the reaction center pigment), while closing of the reaction centers leads to the induction of 0.6 and 1.6 ns components. However, in His-to-Tyr CP47 mutants, the fluorescence lifetimes of components associated with open and closed reaction centers consistently are 10-15% shorter than in systems with wild-type CP47. We interpret these results to indicate that a pheophytin present in CP47 acts as an excitation sink, perhaps caused by less efficient energy transfer from pheophytin back to chlorophyll. A manuscript detailing these findings is in a final stage of preparation.

An interesting side line. Upon genetic deletion of the PS I reaction center complex, we observed the chlorophyll/PS II ratio to be approximately 120. This is more than the 40-70 chlorophylls assumed to be associated with the isolated PS II core complex. This suggests that in cyanobacterial thylakoid membranes chlorophyll also is associated with proteins

other than those of the PS II and PS I core complexes. Indeed, in a mutant in which the PS I core as well as CP47 and CP43, the two core antenna proteins associated with PS II, have been genetically deleted, some chlorophyll remains present in the thylakoid membrane even though none of the major PS I or PS II core proteins can be observed in thylakoids of such a mutant. The absorption and emission spectrum of the remaining chlorophyll suggests it to be associated with protein, and on the basis of 77 K fluorescence emission spectra of various mutants we have concluded that the remaining chlorophyll under physiological conditions may serve as a PS II antenna complex (Shen and Vermaas, 1994). If time allows, we are interested in identification of the protein component(s) with which this remaining chlorophyll is associated.

Interaction of CP47 with components of the water-splitting apparatus: Mostly on the basis of biochemical evidence, an interaction of the long hydrophilic loop of CP47 with the peripheral manganese-stabilizing protein (MSP), optimizing water-splitting activity, has been suggested. The interaction between CP47 and the MSP appears to be centered between residues 360 and 420 of CP47. We have generated two mutants in which domains between residues 373 and 392 have been deleted; analysis of these mutants suggests that this region of CP47 contributes to tight binding of MSP to the PS II complex, but that some functional association between MSP and PS II can be observed even in the absence of this CP47 domain (Haag et al., 1994). Deletion of other domains in this loop did not very much affect the functional interaction between MSP and the PS II complex.

Perhaps even more interesting is the question whether CP47 plays a more direct role in water splitting events (not mediated via attachment of the MSP). The fact that a number of deletions led to a disappearance of the PS II reaction center complex (Haag et al., 1993) indicates that these regions are functionally or structurally important for PS II. To determine why these domains are so important, at the site of two deletions we are reintroducing one or more copies of oligonucleotides of the same length (but with different sequence) of that what was deleted out. We have selected some photoautotrophic and photoheterotrophic transformants, and are in the process of analyzing the DNA sequence of the various mutants in this region. From comparison of sequences that restore photoautotrophic growth vs. those that do not, important residues and domains can be identified. In this way, we will be able to distinguish between a structural requirement for a certain number of residues, and a functional requirement for specific residues in a particular location.

A complementary approach we have taken is selection for spontaneously occurring photoautotrophic pseudorevertants of obligate photoheterotrophic deletion mutants. Spontaneous duplication of nearby DNA regions in *Synechocystis* 6803 occurs at reasonable frequency, and a duplication of the right size may restore PS II function in some obligate photoheterotrophic CP47 mutants carrying a deletion in the large hydrophilic loop. We have isolated various pseudorevertants of two different deletion mutants, and have determined by complementation experiments that the site of the secondary mutation in all cases is located in *psb*B. From the sequence of *psb*B in the pseudorevertants we expect to obtain information on structural adjacencies in the protein, and on sequences that can functionally substitute for the deleted protein domain.

Other experimental systems: About two years ago, when we had not yet established that a PS I-less *Synechocystis* strain could be maintained in continuous light, we set out to explore other organisms that would be suitable to study PS II in a PS I-less background. The most appropriate alternative was the green alga *Chlamydomonas reinhardtii*, for which a suitable chloroplast transformation system has been developed. A number of PS I-less mutants of *Chlamydomonas* are available, and some double-mutants have been generated, in which both PS I and most of the light-harvesting chlorophyll-protein complexes are

lacking. We obtained one mutant from Dr. L. Mets (LM15), and three mutants from Dr. Ladygin in Pushchino (Russia). Particularly the three Russian mutants were quite stressed; they generally died when the culture reached a certain density. To prepare a background strain in which targeted mutagenesis of CP47 can be done routinely, it is important to first delete out *psb*B from one of these mutants depleted in PS I and LHC. To this purpose, wild type and the four mutants were transformed with two plasmids, one deleting out *psb*B, and one generating a mutation in the 16S RNA gene, leading to spectinomycin resistance. Wild type routinely yielded spectinomycin-resistant transformants in transformation experiments. However, 15 transformation experiments (done under a variety of different conditions) with the four PS I-less, LHC-depleted *Chlamydomonas* strains yielded only a total of two spectinomycin-resistant transformants, both of which retained their *psb*B.

Even though this does not imply that *Chlamydomonas* mutants lacking both PS I and LHC cannot be transformed, it is clear that progress with such mutants is predestined to be considerably slower than that with a well-behaving PS I-less *apc*E$^-$ strain of *Synechocystis*. For this reason, we will complete a fluorescence lifetime study we are doing on the PS I-less *Chlamydomonas* mutants, but we are not continuing our efforts to utilize PS I-less *Chlamydomonas* strains as a transformation system for *psb*B.

In conclusion, the PS I-less *Synechocystis* system we are now utilizing for studies of His mutations in the CP47 protein is a very useful strain for detailed studies of pigment-protein interactions and of the effects of limited pheophytinization in the antenna on light-harvesting efficiency. This is providing new information on parameters influencing energy transfer efficiency in photosynthetic systems. Moreover, studies regarding the hydrophilic regions of the CP47 protein are contributing to an understanding of the role of this protein in optimizing water-splitting activity. During the coming year, we look forward to a further investigation of the long hydrophilic loop of CP47, as well as to a full assessment of the structural and functional effects of the loss of the central Mg^{2+} from specific chlorophyll molecules in the PS II antenna.

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