Site-Directed Mutations of Two Histidine Residues in the D2 Protein Inactivate and Destabilize Photosystem II in the Cyanobacterium *Synechocystis* 6803

Wim F. J. Vermaas*, John G. K. Williams, and Charles J. Arntzen

E. I. du Pont de Nemours and Company, Central Research and Development Department, Building 402, Wilmington, DE 19898, USA

Z. Naturforsch. **42c**, 762–768 (1987); received December 30, 1986

Site-Directed Mutagenesis, Herbicide Binding, Photosystem II Proteins, Photosynthesis, Cyanobacteria

Site-directed mutations were created in the cyanobacterium *Synechocystis* 6803 to alter specific histidine residues of the photosystem II (PS II) D2 protein. In one mutant (tyr-197), the his-197 residue was replaced by tyrosine, in another mutant (asn-214), his-214 was changed into asparagine. The tyr-197 mutant did not show any low-temperature fluorescence attributable to PS II, but contained a PS II chlorophyll-protein, CP-47, in significant quantities. Another PS II chlorophyll-protein, CP-43, was absent, as was PS II-related herbicide binding. The asn-214 mutant showed a blue-shifted low-temperature fluorescence maximum around 682 nm, but did not have a significant amount of membrane-incorporated CP-43 or CP-47. Herbicide binding was also absent in this mutant. These data indicate a very important role of the his-197 and his-214 residues in the D2 protein, and are interpreted to support the hypothesis that the D2 protein and the M subunit from the photosynthetic reaction center of purple bacteria have analogous functions. According to this hypothesis, his-197 is involved in binding of P680, and his-214 forms ligands with Q_A and Fe^{2+}. In absence of a functional D2 protein, the PS II core complex appears to be destabilized as evidenced by loss of chlorophyll-proteins in the mutants.

Introduction

Photosystem II (PS II) catalyzes the light-induced reduction of plastoquinone by H_2O, the first step of the photosynthetic process in thylakoid membranes. The PS II complex consists of seven or more proteins, several of which are integrated into the membrane (see [1–3] for reviews). The precise function(s) of the individual polypeptides in PS II electron transport is generally unknown. Even less is known about the function of protein domains within individual polypeptides, with the exception of select regions of the D1 protein known to be involved in binding PS II herbicides such as atrazine and diuron [2, 4].

Recent advances in divergent research areas have opened new opportunities to link structural aspects of the PS II complex to the functional roles of individual polypeptides. These include the following:

1) The crystallization and subsequent X-ray diffraction analysis of the reaction center complex from the purple photosynthetic bacterium *Rhodopseudomonas viridis* [5] has provided a detailed insight into the three-dimensional structure of the bacterial reaction center, which is functionally analogous to PS II.

2) It has been recognized that the 32–34 kDa PS II proteins D1 (the "herbicide-binding protein") and D2 share amino acid homology with the L and M subunits from purple bacteria [5–7]. In particular, amino acid residues in the L and M subunits of bacteria that are required for binding electron transport carriers in the reaction center can be identified in corresponding positions in the D1 and D2 proteins [4, 5, 7]. This has led to the suggestion that D1 and D2 together form the binding environment for the PS II reaction center and acceptor side, as the L and M subunits do in bacterial reaction centers.

3) Very recently, Satoh and coworkers reported on the development of detergent fractionation techniques which allow the isolation of a very small but functionally active PS II particle which contains only D1, D2 and Cyt b_{559} [8]. This is the first direct evidence to indicate that D1 and D2 are the proteins primarily involved in charge separation in PS II.

4) Molecular-genetic techniques have been developed for the specific modification of PS II proteins by either gene deletion or inactivation [9–12] or by gene replacement with a copy carrying a site-directed

* Present address: Department of Botany and Microbiology, Arizona State University, Tempe, AZ 85287 USA.

Reprint requests to Dr. W. F. J. Vermaas.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0600–0762 $ 01.30/0

mutation [13]. Characterization of the properties of the mutants obtained by gene inactivation or deletion has led to the conclusion that all polypeptides of the PS II core complex investigated thus far (D2, Cyt b_{559}, and the chlorophyll-binding proteins CP-47 and CP-43) must be coordinately synthesized *in vivo* to obtain a photosynthetically active PS II complex ([11, 14] and W. F. J. Vermaas, J. G. K. Williams, H. B. Pakrasi, and C. J. Arntzen, unpublished results).

While the deletion or insertional inactivation of PS II genes was useful to show that these genes are essential to PS II function, little information was obtained on the function of the individual PS II proteins encoded by these genes since in each case the PS II complex could not assemble normally. We conclude that modifications made in the PS II complex by the specific deletion of one protein changes the conformation of the entire PS II complex. For this reason, experiments were started to introduce mutations of a more subtle nature, namely single-amino-acid changes in regions of the PS II complex suspected to have defined functional roles [13]. To this end, we mutated two histidine residues of the D2 protein (his-197 and his-214), which can be predicted to be involved in binding of P680, and of Q_A and Fe^{2+}, respectively, on the basis of the analogy of M with D2 [4, 5, 7]. In one mutant, his-197 was changed to a tyrosine residue, and in the other one his-214 was replaced by asparagine. In both cases, the mutations caused an inactivation of the PS II complex [13]. In this manuscript we describe the properties of the mutants with respect to the presence of chlorophyll-binding PS II proteins, herbicide binding, and low-temperature fluorescence emission. It was found that these single-amino-acid mutations in the D2 protein have a pronounced destabilizing effect on the PS II complex. The data presented here confirm the hypothesis that D2 plays a central role in the structure and function of the PS II reaction center.

Materials and Methods

Synechocystis 6803 was cultivated as previously described [9]. The procedure to create the site-directed mutants has been outlined in [13]. The method of DNA sequencing was as in [9], and low-temperature fluorescence emission experiments and immunoblotting were performed as described in [14]. The antibodies raised against CP-47 and CP-43 of *Chlamydomonas reinhardii* [15] were a kind gift of Dr. N.-H. Chua.

Results

Synechocystis 6803 contains two *psb*D genes, *psb*D I and *psb*D II, which encode the D2 polypeptide. The mutants described in this study have only the *psb*D I gene; the *psb*D II gene was deleted prior to the construction of the site-directed mutants [13]. As described [13], mutation of histidine-197 to tyrosine, or of histidine-214 to asparagine, caused a loss of PS II function. To verify that this defect was due only to the site-directed mutation, and not to unintended lesions, we first localized the mutation to a 0.5 kb fragment of the *psb*D I gene by transforming each mutant with this fragment from the wild-type gene; in each case, recovery of wild-type transformants confirmed that the mutation is located on this fragment of the *psb*D I gene, which encodes the histidine residues at positions 197 and 214. To map precisely the mutation at position 214, the nucleotide sequence of the 0.5 kb fragment was compared between the DNA from wild type and the mutant asn-214. Indeed, the only difference was found at the codon which determines the amino acid at position 214: histidine in the wild-type sequence and asparagine in the mutant (Fig. 1).

The large advantage of the two mutants obtained by site-directed mutagenesis over those obtained by random mutagenesis is that the mutation sites of the tyr-197 and asn-214 mutants are precisely defined.

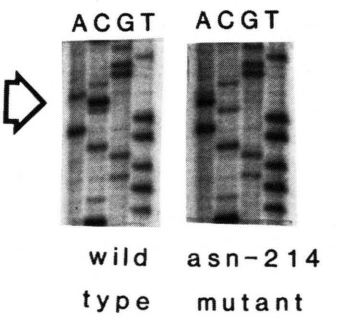

Fig. 1. Sequence of the region of the *psb*D-I gene, cloned from wild type of *Synechocystis* 6803 (left) and from the asn-214 mutant (right) of *Synechocystis* 6803, showing the histidine (codon CAC) to asparagine (codon AAC) mutation at arrow. Sequencing gels are read from bottom to top.

Functional and structural characterization of these mutants will help to define the role of his-197 and his-214 in the D2 protein. To investigate the reason for the absence of PS II activity in the mutants, three approaches were taken: (a) comparison of the low-temperature fluorescence spectra of the mutants with that of the wild type; (b) measurements of binding of PS II herbicides to wild type and mutant cells, and (c) probing for the presence of the PS II proteins CP-43 and CP-47 using antibodies.

Low-temperature fluorescence spectra

Fig. 2 shows 77 K fluorescence spectra from wild-type and mutant thylakoids. Wild-type thylakoids show the normal emission maxima near 685 and 695 nm (both indicative of PS II) and at 722 nm (indicative of PS I). The fluorescence emission at wavelengths below 650 nm originate from phycobiliproteins. Because of several factors, including the relative abundance of PS I in cyanobacterial thylakoid membranes, the 722 nm maximum is much higher than the 685 and 695 nm emission.

In contrast to the wild type, the tyr-197 mutant did not show any significant emission maxima between 670 and 700 nm, which suggests that either the PS II fluorescence is entirely quenched, or that no chlorophyll is associated with the PS II complex.

The asn-214 mutant shows a blue-shifted fluorescence emission maximum near 681 nm, and no appreciable maxima at 685 or 695 nm. This indicates that chlorophyll-protein interactions in PS II are disturbed; however, these data alone do not allow conclusions on the nature of the mutation-induced decrease of the maximum of the "PS II-related" emission wavelengths.

Herbicide binding

To monitor the intactness of the PS II herbicide-binding site in the mutants, binding of [^{14}C]atrazine and [^{14}C]diuron was measured in wild-type and mutant cells. Neither the tyr-197 nor asn-214 mutants showed any specific atrazine or diuron binding (specific binding being defined as that fraction of binding that is competitive with other PS II-directed herbicides). However, wild-type cells showed normal high-affinity herbicide binding (data not shown). We interpret these data to indicate that both of the site-directed mutants have lost herbicide affinity, indicating that in the mutants the herbicide-binding D1 protein is either no longer in its wild-type conformation, or is physically absent from the thylakoids.

Probing for CP-43 and CP-47

Since the protein composition of cyanobacterial thylakoid membranes is very complex, it is not yet technically feasible to obtain an unequivocal answer about the presence of PS II proteins by analysis of a stained protein gel. For this reason, antibodies raised against the PS II chlorophyll-binding proteins CP-43

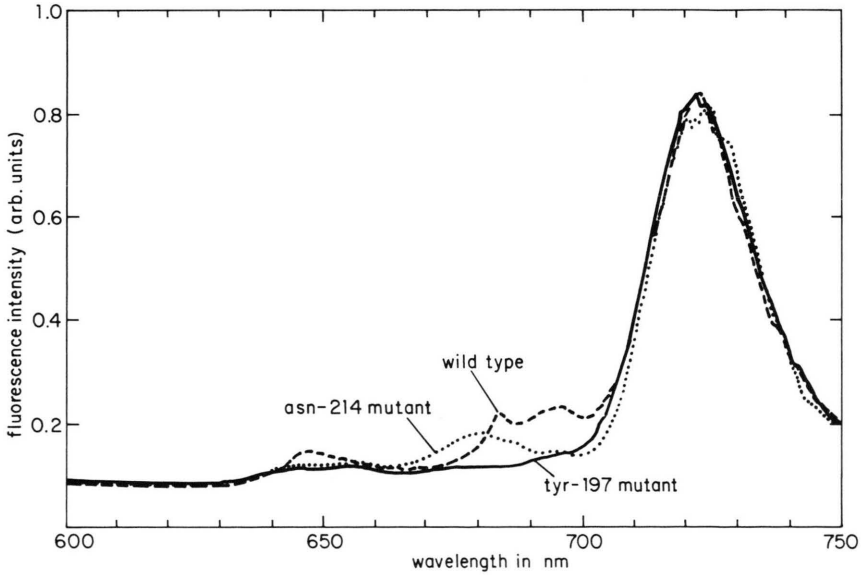

Fig. 2. 77 K fluorescence spectra of thylakoids from wild type (– – –), and the tyr-197 (———) and asn-214 (·····) mutants. The samples contained 50% glycerol. Excitation wavelength, 435 nm; band width 8 nm. Emission band width, 2 nm.

and CP-47 from *Chlamydomonas reinhardii* [15] were utilized to probe for the presence of these proteins in thylakoid membranes from the mutants. Unfortunately, no antibodies against D1 or D2 were available that, in our hands, showed sufficient specificity in recognizing these proteins from *Synechocystis* 6803.

Fig. 3 shows that CP-43 is greatly depleted in both mutants. Overexposure of the protein blot for the asn-214 mutant is shown to indicate that we could detect a trace amount of CP-43 in this mutant. Fig. 4 shows that CP-47 is almost totally absent from thylakoids of the asn-214 mutant. However, CP-47 is present in thylakoids from the tyr-197 mutant.

We conclude that the two site-directed mutants differ from each other and from wild type in their ability to accumulate chlorophyll-proteins of PS II. We do not wish to hypothesize about the nature of these differences in phenotype since it is still premature to evaluate how altered integration or folding of a mutated protein might influence the incorporation and stability of its neighbors in a membrane-protein complex. Clearly, however, this research approach offers a new tool to begin exploring these parameters.

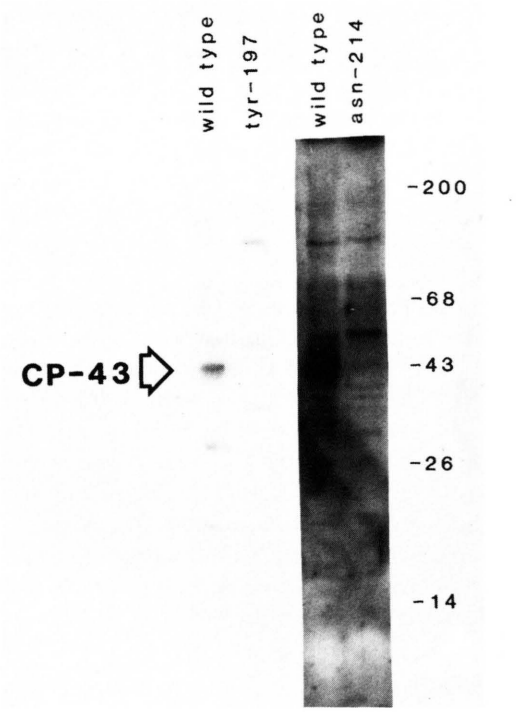

Fig. 3. Immunoblots of thylakoid proteins from wild type and the tyr-197 and asn-214 mutants probed with antibodies raised against CP-43 from *Chlamydomonas reinhardii*. The blot with wild type and asn-214 mutant thylakoids was overexposed to visualize a trace of CP-43 in the asn-214 mutant. As is obvious from these data, the antibody is not very specific for *Synechocystis* CP-43 under the experimental conditions used. The numbers to the right indicate the size (in kDa) of molecular weight markers.

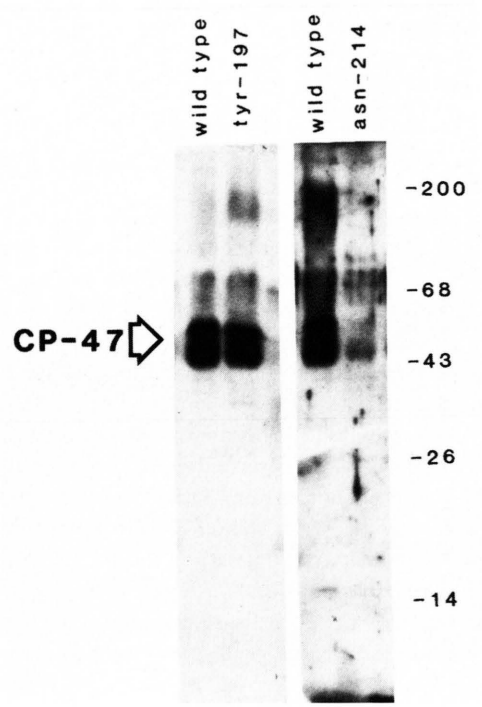

Fig. 4. Immunoblots of thylakoid proteins from wild type and the tyr-197 and asn-214 mutants probed with antibodies raised against CP-47 *Chlamydomonas reinhardii*. The numbers to the right indicate the size (in kDa) of molecular weight markers.

Discussion

The results presented herein show that single amino acid changes at the 197 or 214 positions of the D2 protein have pronounced effects on the structure and function of the PS II complex. However, it is as yet difficult to precisely explain these consequences other than to interpret the phenotypes of the individual mutants. This discussion follows.

The tyr-197 mutant

Replacement of his-197 by tyr leads to a loss of CP-43, but not of CP-47, and to a loss of herbicide

binding and 77K fluorescence emission maxima attributable to PS II. At a first glance, the presence of CP-47 and the absence of PS II fluorescence seems contradictory; previous studies have shown this purified pigment protein to fluoresce at 695 nm [16]. From the presence of this chlorophyll-binding PS II protein in the thylakoid membrane, therefore, it would be expected that the fluorescence emission spectrum of the tyr-197 mutant would have some PS II features. The absence of PS II fluorescence emission in this mutant can be explained in at least four ways: (1) the fluorescence yield of PS II in the mutant is very low due to either structural or functional changes in PS II, (2) CP-47 in the mutant does not bind chlorophyll, (3) the abnormal microenvironment within CP-47 does not allow the creation of the unique pigment species responsible for wild-type 695 nm fluorescence, or (4) the CP-47 content of the mutant may be considerably less than that in the wild type since the results obtained with immunoblotting techniques are not necessarily quantitative. As this moment no data are available to allow a choice between these explanations.

If there is analogy between the M-subunit from purple photosynthetic bacteria and the D2 protein, his-197 of the latter would bind one of the chlorophylls of the "special pair" [4, 5, 7]. (It should be noted that the cyanobacterial D2 amino acid sequence is one amino acid shorter than the D2 protein sequence derived from sequencing the corresponding gene in spinach; his-197 is equivalent to his-198 of the spinach protein [17]). Thus, the primary lesion in the tyr-197 mutant would be expected to be at the level of P680 binding, and there was no *a priori* reason to expect any other PS II abnormality. However, the total loss of PS II traits such as herbicide-binding and typical 77K chl fluorescence suggests that his-197 has a crucial structural role in PS II which cannot be substituted by tyr-197. Although definitive proof is still lacking, the hypothesis that the his-197 residue of the D2 protein binds a P680 chlorophyll is still fully compatible with the data presented, since the integration of the specialized reaction center chlorophyll may be necessary to stabilize the proteins surrounding it.

The asn-214 mutant

This mutant lacked CP-43 and CP-47, possibly due to a rapid turnover of these proteins. In this respect it resembles *Chlamydomonas* mutants in which a faster rate of turnover of PS II proteins was observed in cell lines carrying a (randomly generated) mutation in the genes for either D1 or D2 [18, 19], or in unknown loci of the nuclear or chloroplast genome [20]. Interestingly, the asn-214 phenotype differs from the tyr-197 mutant in this respect; in the latter CP-47 was still present. Other examples of partially intact, but inactive PS II complexes include those from mutants in which the gene for either CP-47 or Cyt b_{559} had been inactivated or deleted [11, 14]. Therefore, it appears that a change in, or the absence of, specific proteins of the PS II complex may lead to the partial loss of other proteins of the complex, but the extent of these depletions is variable. To date we do not have enough examples to formulate rules governing stable PS II assembly. In fact, the examples presented herein show how unpredictable it seems whether a mutation will cause loss of certain PS II complex components from the thylakoid membrane or not: the change in his-197 of D2 leads to a loss of CP-43 but not of CP-47, but the change in his-214 of D2 causes the disappearance of both CP-43 and CP-47. Further results obtained by different site-directed changes at these and other residues, together with use of other techniques will be needed to gain a deeper insight into the structural requirements of the complex which determines the synthesis and assembly of a stable PS II complex.

Thylakoids from the asn-214 mutant had a fluorescence emission peak which was blue-shifted from that of the wild-type PS II emission. This appears somewhat puzzling in that neither CP-43 or CP-47 accumulate in the thylakoid membrane in this mutant. It is possible to explain these observations by at least two mechanisms: (1) the red-shifted fluorescence originates from another "PS II antenna" chlorophyll protein, or (2) the fluorescence originates from the traces of aberrant forms of CP-43 and CP-47 present in the membrane.

In favor of the first mechanism, Pakrasi *et al.* [21] have obtained evidence that there is a chlorophyll-binding protein (not identical to CP-43 or CP-47) associated with PS II in cyanobacteria, especially under stressed conditions. Absence of CP-43 and CP-47 could result in fluorescence of this "antenna" chlorophyll protein since energy transfer to the PS II trap and the antenna from CP-43 and CP-47 is not feasible under these conditions. However, if this were the case, then the data obtained with the

tyr-197 mutant (as described above) have to be interpreted by a low fluorescence yield of CP-47 in the tyr-197 mutant. Otherwise in the tyr-197 mutant the blue-shifted fluorescence maximum would also have been expected to occur.

The second possible mechanism to explain the shifted fluorescence (as originating from the little CP-43 and CP-47 present in the membrane) is plausible only if it is assumed that the fluorescence yield of the residual (aberrant) CP-43 and CP-47 is significantly higher than normal.

Whatever the reason for the 682 nm fluorescence maximum, it is obvious that replacement of his-214 by asn in the D2 protein seriously impairs the function and organization of the PS II complex. This is not surprising in view of the hypothesis that D2 and the bacterial M-subunit are analogous. This hypothesis predicts his-214 to be involved in binding Q_A, and to be acting as one of the ligands for the Fe^{2+} at the acceptor side of PS II. Accordingly, the apparent instability of the PS II complex could possibly be not only caused by the structural change of the amino acid replacement, but also by the inability to properly accommodate Q_A and/or Fe^{2+}, which in turn could be required for stable PS II assembly.

Comparison of PS II mutants created by directed mutagenesis

Other *Synechocystis* 6803 mutants which have a defined genetic change have previously been analyzed. These include one in which the *psb*B gene (encoding CP-47) has been inactivated [9, 13], and one in which the *psb*E/F genes (encoding Cyt b_{559}) have been deleted [11]. Although the genetic modifications for those mutants inactivated the expression of an entire gene, the phenotypic changes due to the site-directed mutations described herein appear to be equally damaging to the PS II complex. For example, the *psb*B gene-inactivation mutant still showed a normal 685 nm fluorescence emission peak and the presence of CP-43 in the thylakoid membrane. This supports the hypothesis that the D2 residues targeted for site-directed mutagenesis (his-197 and his-214) bind vital PS II cofactors (P680 and Q_A, respectively), and that the CP-47 and Cyt b_{559} proteins are more peripherally located in the PS II core, thereby being less essential for assembly of other proteins in the complex.

This manuscript has shown that generation of site-directed mutants can help to test to validity of hypotheses, and to obtain more information on the possible function of individual proteins in the PS II complex. Ultimately, these data, in conjunction with a detailed static model obtained from X-ray diffraction studies of crystals from the same or a related protein complex, will enable a further understanding of the processes taking place in PS II.

Acknowledgements

We thank Dr. N.-H. Chua for providing us with antibodies against CP-43 and CP-47, Dr. Himadri Pakrasi for discussions, and JoAnne Zeroka for technical assistance.

[1] K. Satoh, Photochem. Photobiol. **42,** 845–853 (1985).
[2] C. J. Arntzen and H. B. Pakrasi, in: Encyclopedia of Plant Physiology, New Series, Photosynthesis III (L. A. Staehelin and C. J. Arntzen, eds.), pp. 457–467, Springer, Berlin 1986.
[3] B. Andersson, in: Encyclopedia of Plant Physiology, New Series, Photosynthesis III (L. A. Staehelin and C. J. Arntzen, eds.), pp. 447–456, Springer, Berlin 1986.
[4] A. Trebst, Z. Naturforsch. **41c,** 240–245 (1986).
[5] J. Deisenhofer, O. Epp, K. Miki, R. Huber, and H. Michel, Nature **318,** 618–624 (1985).
[6] J.-D. Rochaix, M. Dron, M. Rahire, and P. Malnoe, Plant Mol. Biol. **3,** 363–370 (1984).
[7] H. Michel and J. Deisenhofer, in: Encyclopedia of Plant Physiology, New Series, Photosynthesis III (L. A. Staehelin and C. J. Arntzen, eds.), pp. 371–381, Springer, Berlin 1986.
[8] O. Nanba and K. Satoh, Proc. Natl. Acad. Sci. USA **84,** 109–112 (1987).
[9] W. F. J. Vermaas, J. G. K. Williams, and C. J. Arntzen, Plant Mol. Biol. (1987), in press.
[10] J. G. K. Williams, Meth. Enzymol., in preparation.
[11] H. B. Pakrasi, J. G. K. Williams, and C. J. Arntzen, in: Progress in Photosynthesis Research, **Vol. IV** (J. Biggins, ed.), pp. 813–816, Martinus Nijhoff, Dordrecht 1987.
[12] S. S. Golden, J. Brusslan, and R. Haselkorn, Meth. Enzymol. (1987), in press.
[13] W. F. J. Vermaas, J. G. K. Williams, D. A. Chisholm, and C. J. Arntzen, in: Progress in Photosynthesis Research, **Vol. IV,** (J. Biggins, ed.), pp. 805–808, Martinus Nijhoff, Dordrecht 1987.
[14] W. F. J. Vermaas, J. G. K. Williams, A. W. Rutherford, P. Mathis, and C. J. Arntzen, Proc. Natl. Acad. Sci. USA **83,** 9474–9477 (1986).
[15] N.-H. Chua and F. Blomber, J. Biol. Chem. **254,** 215–223 (1979).
[16] H. Y. Nakatani, B. Ke, E. Dolan, and C. J. Arntzen, Biochim. Biophys. Acta **765,** 347–352 (1984).
[17] J. G. K. Williams and D. A. Chisholm, in: Progress in Photosynthesis Research, **Vol. IV** (J. Biggins, ed.), pp. 809–812, Martinus Nijhoff, Dordrecht 1987.
[18] P. Bennoun, M. Spierer-Herz, J. Erickson, J. Girard-Bascou, Y. Pierre, M. Delosme, and J.-D. Rochaix, Plant Mol. Biol. **6,** 151–160 (1986).
[19] J. M. Erickson, M. Rahire, P. Malnoe, J. Girard-Bascou, Y. Pierre, P. Bennoun, and J.-D. Rochaix, EMBO J. **5,** 1745–1754 (1986).
[20] K. H. Jensen, D. L. Herrin, F. G. Plumley, and G. W. Schmidt, J. Cell Biol. **103,** 1315–1325 (1986).
[21] H. B. Pakrasi, H. C. Riethman, and L. A. Sherman, Proc. Natl. Acad. Sci. (USA) **82,** 6903–6907 (1985).