Biochimica et Biophysica Acta 933 (1988) 444–449
Elsevier

BBA 42769

Changes in phosphorylation of thylakoid membrane proteins in light-harvesting complex mutants from *Chlamydomonas reinhardtii*

C. de Vitry and F.-A. Wollman

Service de Photosynthèse, Institut de Biologie Physico-Chimique, Paris (France)

(Received 4 January 1988)

Key words: Thylakoid membrane; Light harvesting complex mutant; Chlorophyll *b* deficiency; State 1–State 2 transition; Photosystem II protein phosphorylation; (*C. reinhardtii*)

Phosphorylation of thylakoid membrane proteins was assessed in vivo in the BF3 and BF4 mutants from *Chlamydomonas reinhardtii* which are deficient in light-harvesting complex (LHC). Thylakoid membranes from the BF3 mutant, which was lacking in chlorophyll *b*, nevertheless contained significant amounts of LHC apoproteins. The phosphorylation pattern of thylakoid membrane proteins in the mutant was similar to that in the wild type. Incubation of BF3 cells in reducing conditions induced an increase in LHC phosphorylation and a fluorescence quenching at room temperature, thereby indicating that kinase-controlled state transitions still occurred in this mutant. In contrast to the BF3 mutant, the BF4 mutant still contained chlorophyll *b* but lacked most of the LHC apoproteins. It contained no LHC phosphoproteins and did not undergo state transitions as shown by the absence of fluorescence quenching in reducing conditions in vivo. Surprisingly, phosphorylation of the PS II subunits D2 and 6 (CP IV apoprotein) was totally abolished in this mutant. However, the two PS II subunits of low molecular weight, L5 and L6, were still slightly phosphorylated.

Introduction

Although the number and nature of the phosphoproteins detected in the thylakoid membranes from higher plants [1,2] and green algae [3–5] are somewhat different, they nevertheless belong to the same two families of phosphoproteins: one consists of polypeptides involved in the organization of the peripheral antenna, LHC II, but significant phosphorylation also occurs on some PS II subunits [6–8]. The existence of several kinases was suggested by the difference in phosphorylation behaviour between LHC II and PS II subunits [9–12] and by the purification of at least two kinases from spinach thylakoid membranes [13,14].

Whether interactions between these different phosphoproteins are required for the occurrence of state transitions is of particular interest. The availability of various photosynthetic mutants of the unicellular green alga *C. reinhardtii* provides a unique tool to investigate this possibility. This allowed us to show previously that the presence of the PS II reaction centre was not required for either LHC phosphorylation or for the occurrence of state transitions [4,5,15–17]; in contrast, the

Abbreviations; LHC, light-harvesting complex; CP, chlorophyll-protein complex; PS, Photosystem; SDS, sodium dodecyl sulfate; α, antibody; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-di-methylurea; F_{max}, maximal yield of fluorescence.

Correspondence: C. de Vitry, Service de Photosynthèse, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France.

presence of cytochrome b_6f was required for the phosphorylation of a subset of LHC apoproteins and for the occurrence of state transitions in vivo, but it was not required for PS II phosphorylation [18,19]. This involvement of the b_6f complex in the control of kinase activity has been confirmed in *Lemna perpusilla* [20]. Here we report on the phosphorylation of thylakoid membrane polypeptides in mutants deficient in LHC and conclude that PS II phosphorylation depends on the presence of some LHC apoproteins.

Material and Methods

Wild-type and LHC mutants, BF3 and BF4 strains, of *C. reinhardtii* were grown in Tris-acetate-phosphate medium. These mutants were isolated by P. Bennoun and partial characterization of the BF4 mutant has been previously reported by Olive et al. [21].

Purified thylakoid membranes were isolated according to Chua and Bennoun [22]. These were prepared for electrophoresis by membrane solubilization in the presence of 1% SDS at a chlorophyll concentration of 1 mg/ml for the wild-type, 0.5 mg/ml for the BF3 mutant and 0.25 mg/ml for the BF4 mutant. Solubilization and gel electrophoresis were performed at 4°C to separate the chlorophyll-protein complexes and at room temperature to separate the polypeptides.

SDS polyacrylamide gel electrophoresis were run according to Laemmli [23] as modified by Delepelaire and Chua [24] using 7.5–15% polyacrylamide gradients or using 12–18% polyacrylamide gradients containing 8 M urea according to Piccioni et al. [25]. Polypeptides were stained by Coomassie brillant blue R-250 or detected by autoradiography of the dried gels using Agfa-Gevaert industrial P films.

Electrophoretic transfers were carried out according to Towbin et al. [26]. Protein saturation of the nitrocellulose sheets was performed using 5% low-fat milk in phosphate-buffered saline. Binding of the α-LHC II, prepared according to Vallon et al. [27], was detected using radioiodinated protein A as described by Burnette [28].

Chl *a*/Chl *b* molar ratios were calculated according to the procedure of Ogawa and Shibata [29].

Fluorescence induction experiments using intact cells at room temperature were performed as reported in Ref. 30. The algae were placed either in oxidizing conditions, analogous to state I, by a white light preillumination (1000 lx) for 20 min in the presence of 10^{-5} M DCMU, or in reducing conditions, analogous to state II, by a 20 min dark incubation in anaerobic conditions (2 mg/ml glucose oxidase/20 mM glucose).

In vivo [^{32}P]orthophosphate labelling of the thylakoid membrane polypeptides was carried out in the above-described states according to Wollman and Delepelaire [4].

Results

Shown in Fig. 1 are the chlorophyll-protein complexes from the wild-type, BF3 and BF4 mutants, separated by an SDS-polyacrylamide gel electrophoresis run at 4°C. As first described by Delepelaire and Chua [24], these electrophoresis conditions allow the observation of several chlorophyll-protein complexes in the wild-type thylakoid membranes. These corresponded to the PS I core antenna (CP I), the PS II core antennae (CP III and CP IV) and the peripheral antennae CP II (a complex containing LHC I and LHC II subunits [31,32]) and CP V (containing an LHC II subunit associated with a extrinsic 17 kDa polypeptide of unknown function [32]). As seen in Fig. 1, the two mutants were lacking in CP II and CP V, which should be indicative either of a lower stability upon electrophoresis of these chlorophyll-protein complexes in the mutants or in the absence of the LHC apoproteins.

In Fig. 2 are shown the thylakoid membrane polypeptides in each strain as viewed by Coomassie blue staining of a 12–18% acrylamide gel containing 8 M urea. Most of the bands previously attributed to subunits of either LHC I [31] or LHC II [32] were absent in the BF4 mutant, whereas the BF3 mutant showed only a reduced content in LHC II subunits.

The presence of LHC II in the thylakoid membranes from the mutants was further assessed in immunoblotting experiments using iodinated protein A coupled to an antiserum against LHC II from *C. reinhardtii* (Fig. 3). This antiserum mainly recognized the main LHC II subunit from *C.*

reinhardtii, numbered 11 (Fig. 3a) against which it has been prepared [27]. As observed after prolonged exposure of the autoradiograms (Fig. 3b), it cross-reacted weakly with three other subunits of the same complex, numbered 13, 16, 17 according to Ref. 22, which were previously shown to share common antigenic determinants with polypeptide 11 [33]. These experiments confirmed that the LHC II subunits were lacking in the BF4 thylakoid membranes, only trace amounts of subunits 11 being detected in overexposed autoradiograms. In contrast, LHC II subunits, although in a reduced amount, were immunodetected in the BF3 thylakoid membranes.

Comparison of the Chl *a*/Chl *b* ratios in the wild-type and mutant strains (Table I) showed that the BF3 mutant was devoid of Chl *b*, whereas the BF4 mutant still contained significant amounts

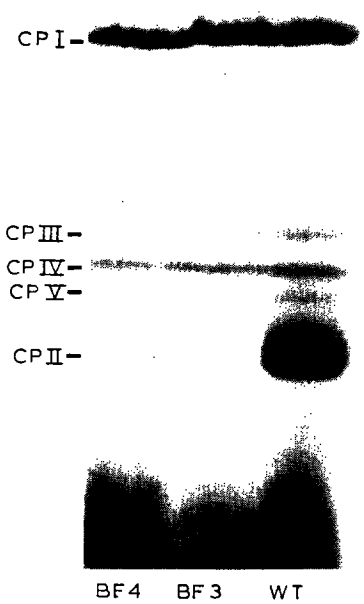

Fig. 1. Chlorophyll-protein complexes of thylakoid membranes from the wild-type (WT) and from LHC mutant strains lacking in chlorophyll *b* (BF3) or lacking most of the LHC apoproteins (BF4) after SDS polyacrylamide (7.5–15% acrylamide gradient) gel electrophoresis at 4° C. The gel is not stained: only the chlorophyll-protein complexes (CP) and the free pigments at the front of the gel are visible. Note that the LHC mutants BF3 and BF4 are lacking in the peripheral antenna complexes CP II and CP V.

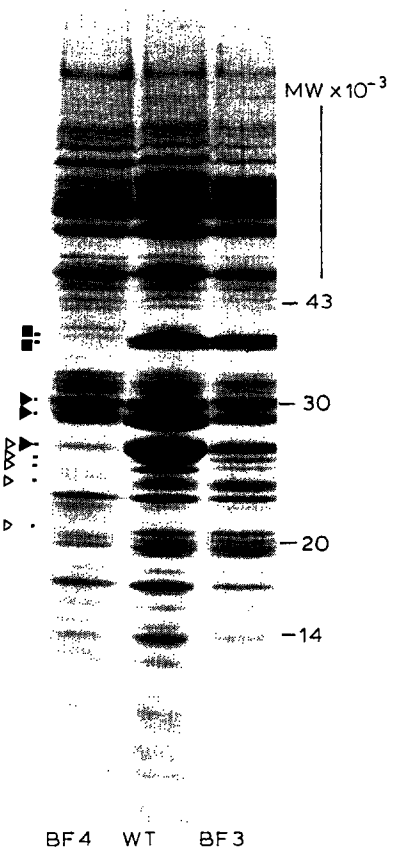

Fig. 2. Polypeptide patterns of thylakoids membranes from the wild-type (WT) and from the LHC mutant strains lacking in Chl *b* (BF3) or lacking most of the LHC apoproteins (BF4). Analysis was by SDS-urea polyacrylamide (8 M urea, 12–18% polyacrylamide gradient) gel electrophoresis at room temperature. The gel was stained with Coomassie blue. LHC I polypeptides (▶), LHC II polypeptides (▷) and two other chlorophyll-binding polypeptides of the peripheral PS II antenna (■) are indicated. They are in reduced amount in the BF3 mutant but most of them are lacking in the BF4 mutant.

of Chl *b*. Thus, we were led to conclude from the above experiments that the BF3 mutant is a *b*-less mutant similar to that previously described by Michel et al. [34], whereas the BF4 mutant is an LHC mutant showing no accumulation of most of the subunits of the peripheral antenna in the thylakoid membrane. The significant content in Chl *b* of the thylakoid from this mutant cannot be accounted for by the trace amounts of subunit 11 which were immunodetected. Therefore the thylakoid membrane polypeptides in positions of LHC II subunits 11 and 13 which were stained

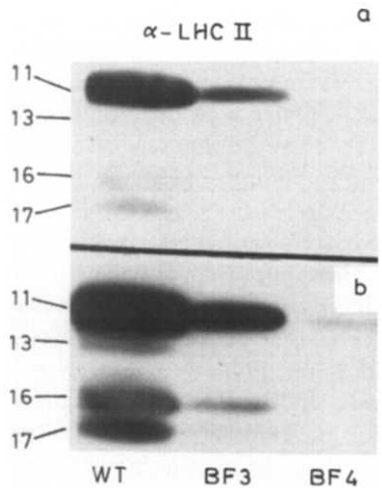

Fig. 3. Immunoblots on thylakoid membranes from wild-type (WT) and LHC mutant strains lacking in Chl *b* (BF3) or lacking most of the LHC apoproteins (BF4) after SDS-polyacrylamide gel electrophoresis; using α-LHCII protein coupled to radioiodinated protein A, with a short (a) or a long (b) exposure. The BF3 mutant is deficient in the four LHC II polypeptides. The BF4 mutant shows only a slight labelling of polypeptide 11.

but did not react with α-LHC II in the BF4 mutant (Fig. 2) could correspond to other Chl *a*/*b*-binding proteins specifically synthesized in this mutant.

We then investigated the patterns of thylakoid membrane polypeptide phosphorylation in vivo in these mutants (Fig. 4). These were compared in oxidizing and reducing conditions which mimic state transitions [4]. We could detect no phosphorylation of antenna proteins in the BF4 mutant, even under reducing conditions. This could be compared with the set of antenna phosphopolypeptides, numbered 9, 10, 11, 13 and 17, previously identified in the wild-type [4], which could also be detected in the BF3 mutant. Although less labelled in this mutant, these antenna polypeptides were more heavily phosphorylated when BF3 cells were placed in reducing than in oxidizing conditions, as was the case with wild-type cells.

The characteristics of antenna polypeptide phosphorylation in the two mutant strains were consistent with their fluorescence behaviour at room temperature (Table I). Whereas the BF3

TABLE I

CHLOROPHYLL *a*/*b* MOLAR RATIO AND FLUORESCENCE QUENCHING BETWEEN STATE I AND STATE II.

In contrast to the wild-type (WT) and to the Chl *b*-lacking mutant (BF3), the mutant lacking most of the LHC apoproteins (BF4) does not show a fluorescence quenching characteristic of state transitions. R, reducing conditions analogous to state II; O, oxidizing conditions analogous to state I.

Strain	WT	BF4	BF3
Chl *a*/Chl *b*	2.3	5.2	> 50
F_{max} (R)/F_{max} (O)	80%	105%	70%

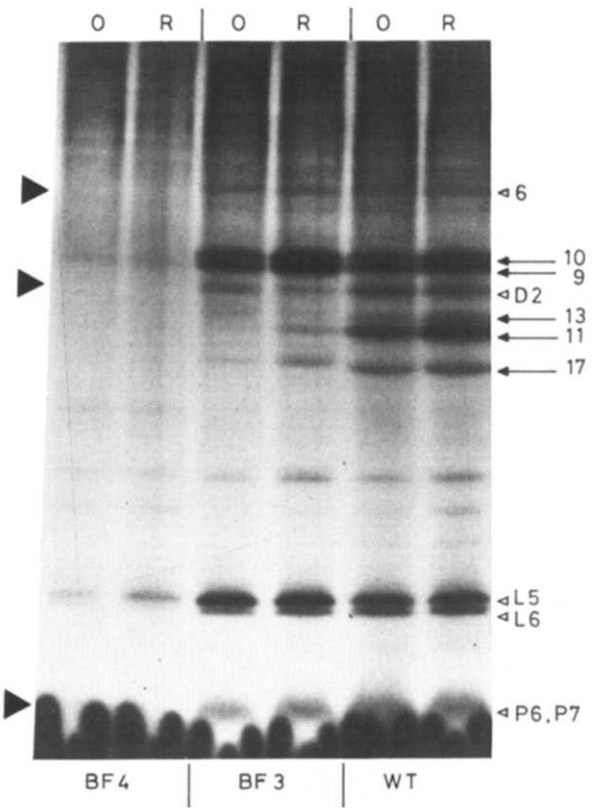

Fig. 4. Autoradiogram of thylakoid membrane polypeptides from ^{32}P-labelled cells of wild-type and of LHC mutant strains lacking in Chlb (BF3) or lacking most of the LHC apoproteins (BF4). O: oxidizing conditions, analogous to state I. R: reducing conditions, analogous to state II. Note (▶) that the phosphorylated bands corresponding to PSII subunits (◁), except L5 and L6, and those corresponding to LHC subunits (←) are absent in the mutant BF4. In contrast, the BF3 mutant shows the same patterns of phosphorylation of the thylakoid membrane polypeptides as the wild-type.

mutant showed a quenching at F_{max} in reducing conditions, as is the case with wild-type cells, the BF4 mutant displayed about the same maximal fluorescence wild-type under reducing and oxidizing conditions. We could even observe a slight increase at F_{max} in the anaerobic state, indicative of the reduction of the plastoquinones. We have reported a similar increase with b_6f mutants from *C. reinhardtii* which showed no state transitions in vivo [18,19].

Fig. 4 also shows that the same set of phosphorylated PS II subunits (indicated by open arrowheads on Fig. 4) was observed in the thylakoid membranes of the wild-type and BF3 mutant. In contrast, PS II phosphorylation was deeply altered in the BF4 mutant: polypeptides 6, D2, P6 and P7 were no longer phosphorylated, whereas the two other PS II subunits of low molecular weight, L5 and L6, were only slightly phosphorylated. The polypeptides P6 and P7 had not been detected in previous studies on ^{32}P-labelled thylakoid membranes [4,5] since they migrate near the pigment/lipid front. However they have been recently identified as genuine PS II subunits since they were found in isolated PS II reaction centres from *C. reinhardtii* cells grown in the presence of [^{32}P] orthophosphate [7].

Discussion

Since state transitions in green algae and higher plants occur through a reversible phosphorylation of some LHC subunits, we have investigated this regulation process in two LHC mutants from *C. reinhardtii*, the BF3 and BF4 strains. Although they both lacked CP II upon electrophoresis at 4° C in the presence of SDS, they differed widely in thylakoid membrane composition. The BF3 mutant is a Chl *b*-less mutant, since it displays a Chl *a*/Chl *b* ratio higher than 50. However, its thylakoid membranes contain significant amounts of LHC apoproteins. In this respect the BF3 mutant is similar to several pigment mutants, showing Chl *a*/Chl *b* ratios larger than 20, which have been previously characterized in barley [35,36], sweet clover [37] and *C. reinhardtii* [34]. It has been reported that the LHC apoproteins which remain in all these Chl *b*-less mutants were phosphorylated [37,39]. This is also the case in the BF3 mutant. However, state transitions could not be detected either in vitro in the chlorina f2 mutant from barley [38] or in vivo in a sweet-clover mutant [37]. This is at variance with our observation that state transitions corresponding to changes in the level of phosphorylation of peripheral antenna proteins still occurred in vivo in the BF3 mutant. Therefore the corresponding state transitions must involve some phosphoproteins of the periferal antenna now binding Chl *a* only. The discrepancy between the behaviour of the BF3 mutant and of the other Chl *b*-less mutants could arise merely from differences in the experimental procedures used to mimic state transitions in each case. However, we note that there is a larger number of peripheral antenna subunits which undergo reversible phosphorylation in *C. reinhardtii* than in higher plants. In particular, the phosphoproteins 9 and 10 (respective apparent molecular weight of 35 kDa and 33 kDa), which bind mainly Chl *a* (Bassi, R., personal communication) may function as a mobile antenna responsible for the occurrence of state transitions in the absence of Chl *b* in *C. reinhardtii*.

We showed that the BF4 mutant was deficient in most of the LHC apoproteins. It showed almost no thylakoid protein kinase activity and was unable to undergo state transitions.

It is particularly interesting that this LHC-deficient mutant BF4 showed almost no PS II kinase actvity. This is consistent with, the almost total absence of phosphorylated subunits in the PS II reaction centres isolated from various *C. reinhardtii* strains, containing the BF4 mutation, when grown in the presence of [^{32}P]orthophosphate [7]. There is no reciprocity, since we have previously shown that in the PS II-deficient mutants F34 and FUD7, the LHC kinase activity was similar to that in the wild-type [4,5,19]. Although the PS II subunits 6 and D2 were still membrane-inserted in such mutants [6,38], they were not phophorylated [5,19]. Therefore, there is no PS II phosphorylation either when PS II reaction centres are not properly assembled or when LHC subunits are lacking in the thylakoid membranes. We suggest that PS II phosphorylation can occur only in a PS II-LHC complex. Such a possibility is supported by the recent finding of a protein kinase activity in granal membranes enriched in PS II-LHC com-

plexes [14]. This 64 kDa kinase cofractionates with LHC upon dissociation of the PS II-LHC complexes [40]. We have suggested recently that the LHC moiety of the complex could control PS II phosphorylation either by eliciting a structural interaction between the kinase and the PS II subunits or by the transfer of phosphate groups from LHC to PS II subunits [41].

The control of PS II phosphorylation by LHC does not prevent some phosphorylation occurring on polypeptides L5 and L6. Whether this is due to differential access of PS II subunits to the LHC kinase or to the existence of an additional PS II kinase in the thylakoid membranes remains to be determined.

Acknowledgements

We thank R. Bassi, B. Diner and P. Delepelaire for fruitful discussions, and Ms Couratier for the photographs. This work was supported by the C.N.R.S. (V.A. 1187).

References

1 Bennet, J. (1977) Nature 269, 344–346.
2 Bennet, J. (1979) Eur. J. Biochem. 99, 133–137.
3 Owens, G.C. and Ohad, I. (1982) J. Cell. Biol. 93, 712–718.
4 Wollman, F.A. and Delepelaire, P. (1984) J. Cell. Biol. 98, 1–7.
5 Delepelaire P. and Wollman F.A. (1985) Biochem. Biophys. Acta 809, 277–283.
6 Delepelaire, P. (1984) EMBO J. 3, 701–706.
7 De Vitry, C., Diner, B.A. and Lemoine, Y. (1986) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. 2, pp. 105–108, Martinus Nijhoff, Dordrecht.
8 Millner, P.A., Marder, J.B., Gounaris, K. and Barber, J. (1986) Biochim. Biophys. Acta 852, 30–37.
9 Steinback, K.E., Bose, S. and Kyle, D.J. (1982) Arch. Biochem. Biophys. 216, 356–361.
10 Millner, P.A., Widger, W.R., Abbott, M.S., Cramer, W.A. and Dilley, R.A. (1982) J. Boil. Chem. 257 (4), 1736–1742.
11 Farchaus, J., Dilley, R.A. and Cramer, W.A. (1985) Biochim. Biophys. Acta 809, 17–26.
12 Bennet, J., Shaw, E.K. and Bakr, S. (1987) FEBS Lett. 210 (1), 22–26.
13 Lin, Z.F., Lucero, H.A. and Racker, E. (1982) J. Biol. Chem. 257, 12153–12156.
14 Coughlan, S.J. and Hind G. (1986) J. Biol. Chem. 261 (30), 14062–14068.
15 Bennet, J., Steinback, K.E. and Arntzen, C.J. (1980) Proc. Natl. Acad. Sci. USA 77, 5253–5257.
16 Allan, J.F., Bennet, J., Steinback, K.E. and Arntzen, C.J. (1981) Nature (Lond.), 291, 25–29.
17 Horton, P., Allen, J.F., Black, M.T. and Bennet, J. (1981) FEBS Lett. 125, 193–196.
18 Lemaire, C., Girard-Bascou, J. and Wollman, F.A. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. 4, pp. 655–658, Martinus Nijhoff, Dordrecht.
19 Wollman, F.A. and Lemaire, C. (1988) Biochim. Biophys. Acta 933, 85–94.
20 Gal, J.N., Shahak, Y., Shuster, G. and Ohad, I. (1987) FEBS Lett. 221, 205–210.
21 Olive, J., Wollman, F.A., Bennoun, P. and Recouvreur, M. (1981) Arch. Biochem. Biophys. 208 (2), 456–467.
22 Chua, N.H. and Bennoun, P. (1975) Proc. Natl. Acad. Sci. USA 72, 2175–2179.
23 Laemli, U.K. (1970) Nature (Lond.) 227, 680–685.
24 Delepelaire, P. and Chua, N.H. (1979) Proc. Natl. Acad. Sci. USA 76, 111–115.
25 Piccioni, R.G. and Bennoun, P., Chua, N.H. (1981) Eur. J. Biochem. 117, 93–102.
26 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
27 Vallon, O., Wollman, F.A. and Olive, J. (1986) Photobiochem. Photobiophys. 12, 203–220.
28 Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
29 Ogawa, T. and Shibata, K. (1965) Photochem. Photobiol. 4, 193–200.
30 Wollman, F.A. and Diner, B.A. (1980) Arch. Biochem. Biophys. 201, 646–659.
31 Wollman, F.A. and Bennoun, P. (1982) Biochim. Biophys. Acta 680, 352–360.
32 Delepelaire, P. and Chua, N.H. (1981) J. Biol. Chem. 256 (17), 9300–9307.
33 Chua, N.H. and Blomberg, F. (1979) J. Biol. Chem. 254, 215–223.
34 Michel, H., Tellenbach, M. and Boschetti, A. (1983) Biochim. Biophys. Acta 725, 417–424.
35 Thornber, J.P. and Highkin, H.R. (1979) Eur. J. Biochem. 41, 109–116.
36 Bellemare, G., Barlett, S.G. and Chua, N.H. (1982) J. Biol. Chem. 257 (13), 7762–7767.
37 Markwell, J.P., Webber, A.N., Danko, S.J. and Baker, N.R. (1985) Biochim. Biophys. Acta 725, 417–424.
38 Haworth, P., Kyle, D. and Arntzen, C.J. (1982) Arch. Biochem. Biophys. 218 (1), 199–206.
39 Bhalla, P. and Bennet, J. (1987) Arch. Biochem. Biophys. 252 (1), 97–104.
40 Coughlan, S.J. and Hind, G. (1987) Biochem. 26, 6515–6521.
41 Wollman, F.A. and Lemaire, C. (1988) in Photocatalytic Production of Energy-Rich Compounds. (Hall, D.O. and Grassi, G., eds.), Elsevier Applied Science Publishers, London, In Press.