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Isolation and characterization of an oxygen-evolving Photosystem II reaction center core preparation and a 28 kDa Chl-*a*-binding protein

Demetrios F. Ghanotakis, Demetris M. Demetriou and Charles F. Yocum

Division of Biological Sciences, The University of Michigan, Ann Arbor, MI (U.S.A.)

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An oxygen-evolving Photosystem II reaction center complex was characterized by using both biophysical and biochemical techniques. A low-temperature EPR study of this preparation has revealed that cytochrome *b*-559 has been converted to its low-potential form(s); although in the presence of Ca^{2+} and Cl^- the PS II reaction center complex shows high rates of oxygen-evolution activity, cytochrome *b*-559 is not converted to its high-potential form. The same study also demonstrated that Ca^{2+} and Cl^-, not the 17 and 23 kDa proteins, are the cofactors required for the generation of the multiline signal which is associated with the S_2 state. Further solubilization of the PS II reaction center complex, followed by gel filtration chromotography, resulted in the isolation of a purified oxygen-evolving PS II reaction center core and a 28 kDa Chl-*a*-binding protein. The purified oxygen-evolving preparation contains polypeptides with molecular masses of 47, 43, 32, 30 and 9 kDa as well as the extrinsic 33 kDa polpeptide. These proteins, along with manganese, chloride and calcium, appear to form the simplest structure thus far reported to retain the enzymatic activity necessary for oxidation of water to molecular oxygen.

Introduction

The oxidizing side of Photosystem II operates at unusually high redox potentials and this implies the presence of a specialized environment which can promote the stabilization of the reactive chemical intermediates formed during the photochemical splitting of water [1–3]. The isolation of PS II membranes has advanced our knowledge of the structural organization of PS II and has also provided information about the roles of Mn, Cl^- and Ca^{2+} as cofactors of oxygen evolution (for a review see Ref. 1). In oxygen-evolving preparations of PS II, the inorganic cofactors are closely associated with the hydrophilic domain of PS II, which consists of three proteins with molecular masses of 17, 23 and 33 kDa [1,4–6]; another protein with an estimated molecular mass of 10 kDa has been also implicated as a component of the hydrophilic structural arrangement of the oxidizing side of PS II [7,8]. A substantial body of experimental evidence now supports the view that the 17 and 23 kDa polypeptides are required both for high-affinity binding of Ca^{2+} and Cl^- [4,5] and as essential components of a structure which allows only small reductants (for example H_2O and NH_2OH) to penetrate and react with the Mn complex [9]. Various studies have provided data to indicate that the 33 kDa species is somehow associated with manganese binding to PS II membranes

Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; Mes, 4-morpholineethanesulfonic acid; EPR, electron paramagnetic resonance; LHCP, light harvesting protein complex; PS, Photosystem; RC, reaction center.

Correspondence: C.F. Yocum, Division of Biological Sciences, The University of Michigan, Ann Arbor, MI 48109, U.S.A.

[10,11]; since under certain conditions this polypeptide can be removed without concurrent release of manganese, the 33 kDa protein is obviously not the only manganese-binding protein [12–15]. Highly refined preparations of a PS II 'core' complex incapable of oxygen-evolution activity have made it possible to define the polypeptide composition of the PS II hydrophobic domain and to identify the components of PS II which are associated with this dmain [16,17]. Recently, a series of O_2-evolving PS II reaction center complexes depleted of the LHCP have been isolated from spinach and cyanobacteria [18–20]. These isolation procedures consist of solubilization of PS II membranes by a non-ionic detergent (such as digitonin or *n*-octyl β-D-glucopyranoside) followed by purification using sucrose gradients and/or column chromatography. More recently, we reported a new method for isolation of an oxygen-evolving PS II reaction center complex: a method which does not require ultracentrifugation, sucrose gradients or chromatographic separations and which produces an oxygen-evolving complex in high yields which is depleted of LHCP [21].

In this communication we report new results on the isolation and characterization of a purified oxygen-evolving PS II RC core and a 28 kDa Chl-*a*-binding protein. We show here that one can, by biochemical methods, define more closely the polypeptide requirement for oxygen evolution activity.

Materials and Methods

Subchloroplast PS II membranes having high rates of oxygen evolution were prepared as described in Ref. 22. The PS II oxygen-evolving reaction center complex was prepared by the method described below. PS II membranes (2.5 mg Chl/ml), resuspended in a solution containing 0.4 M sucrose, 50 mM Mes (pH 6.0) and 10 mM NaCl (solution A), were mixed with an equal volume of a solution containing 1.0 M sucrose, 50 mM Mes (pH 6.0), 0.8 M NaCl, 10 mM $CaCl_2$ and 70 mM octyl glucopyranoside (solution B). After a 10 min incubation in the dark at 4°C, one part of the solubilized membranes was mixed with two parts of a solution containing 1.0 M sucrose, 50 mM Mes (pH 6.0), 0.4 M NaCl, 5 mM $CaCl_2$ (solution C); mixing was followed by 5 min incubation and a 90 min centrifugation step (40 000 × *g*) to pellet to LHCP (see Ref. 21). The supernatant was desalted and the sucrose was removed by two 30 min dialysis steps against a solution containing 50 mM Mes (pH 6.0), 10 mM NaCl and 5 mM $CaCl_2$ (solution D) (a Spectrapor No. 6 dialysis tubing with M_r 50 000 cutoff was used), further diluted 50% with solution D and subsequently centrifuged for 60 min at 40 000 × *g*. The pellet which resulted from the last centrifugation step was resuspended in a solution containing 0.4 M sucrose, 50 mM Mes (pH 6.0), 10 mM NaCl and 5 mM $CaCl_2$ (solution E), whereas the colorless supernatant was discarded. We should note that the increased amounts of sucrose in solutions B and C result in recovery of the PS II reaction center complex in high yields (about 20% of the Chl in the starting PS II membranes was recovered in the PS II RC complex preparation).

To isolate the purified PS II oxygen evolving core the PS II reaction center complex, prepared as described above, was solubilized with 0.5% of the detergent dodecyl β-D-maltoside and was subsequently subjected to gel-filtration chromatography on a Superose-12 Pharmacia column (attached to a Pharmacia FPLC system) which had been previously equilibrated with a solution containing 50 mM Mes (pH 6.0), 20 mM NaCl and 0.05% dodecyl maltoside (solution F). The various fractions were eluted from the column with solution F. Throughout the experiment precautions were taken to maintain the temperature below 10°C in order to avoid destruction of oxygen evolution activity.

Gel electrophoresis was carried out as described in Ref. 23 with the modifications described in the figure captions. O_2 evolution activity was measured with a Clark-type oxygen electrode. EPR measurements were carried out by using a Bruker ER200D spectrometer. An Oxford ESR-9 liquid helium cryostat was used to maintain sample temperature; frequencies were measured with a Hewlett-Packard frequency counter, and magnetic fields were determined with a Bruker gaussmeter accessory. Optical spectroscopy was carried out on a Perkin-Elmer Lambda 5 UV/Vis spectrophotometer.

Results

The PS II reaction center complex isolated by exposure of PS II membranes to the non-ionic detergent octyl glucoside in the presence of high ionic strength is depleted of the water-soluble 17 and 23 kDa polypeptides and thus requires the presence of non-physiological concentrations of Ca^{2+} and Cl^- for maximal oxygen evolution activity [24]. A Mn and a Z^+ (the primary donor to $P680^+$) quantitation in this new oxygen-evolving PS II reaction center complex has demonstrated a 3.7-fold enrichment in trap concentration (on a Chl basis) [21]. Since the PS II RC complex shows high oxygen-evolution capacity in the presence of $CaCl_2$, we studied the state of cytochrome *b*-559 and the S_2 state by use of low-temperature EPR spectroscopy. As shown in Fig. 1a, in the PS II RC complex cytochrome *b*-559 has been converted to its low-potential form(s) as revealed by its *g* values ($g_z = 2.94$; $g_y = 2.26$). Such a conversion causes cytochrome *b*-559 to be oxidized in the dark; illumination of the system at 77 K to oxidize any high-potential cytochrome *b*-559 had very little effect on the EPR signal of cytochrome *b*-559, which suggests that conversion to the low-potential form(s) is complete (data not shown). Even though addition of $CaCl_2$ restores high rates of oxygen evolution activity, it does not restore cytochrome *b*-559 to its high-potential forms. When the PS II RC complex was illuminated at 200 K we observed the generation of the multiline signal which is attributed to the S_2 state of the oxygen-evolving enzyme [25]. Since the RC complex is lacking the 17 and 23 kDa proteins it is apparent that these two species are not necessary for the generation of the multiline signal when high concentrations of Ca^{2+} and Cl^- are present. Once the system was depleted of either Ca^{2+} (by treatment with EGTA) or Cl^- (by dialysis against a Cl^- free

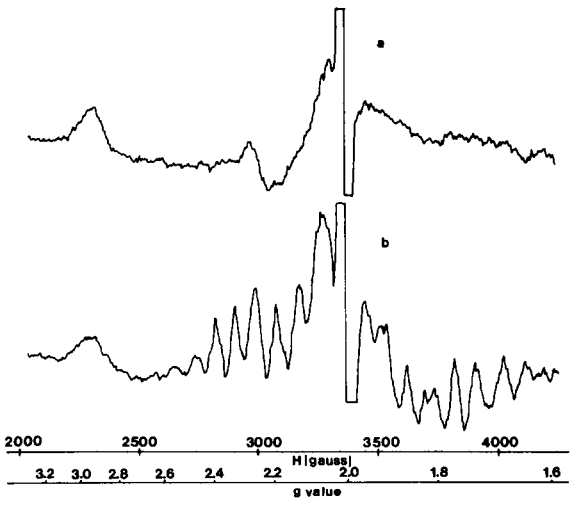

Fig. 1. EPR spectra of the oxygen evolving PS II RC complex (1.8 mg Chl/ml) in the presence of 10 mM $CaCl_2$: (a) dark adapted (4 h in ice); (b) illuminated for 3 min at 200 K ($T = 8$ K; microwave power = 100 mW; modulation amplitude = 15 Gpp).

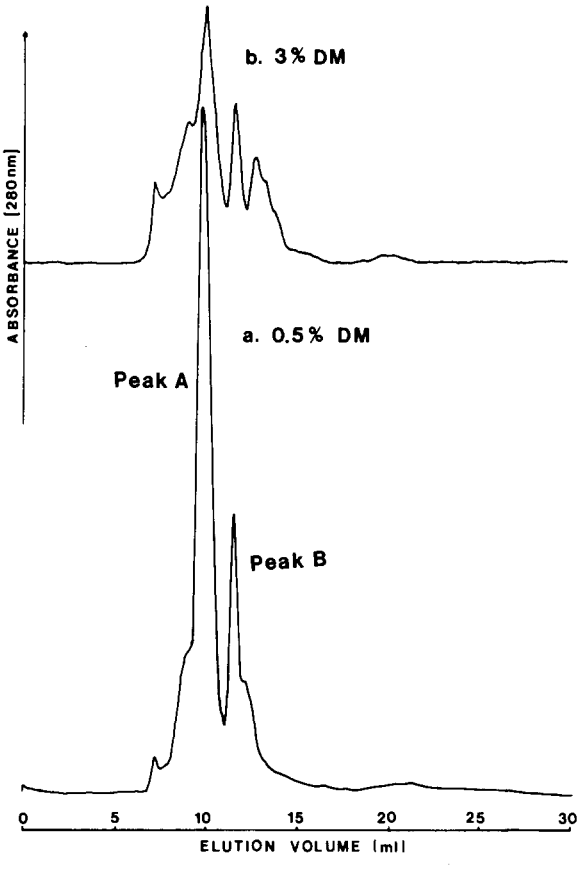

Fig. 2. Elution profile of the PS II RC complex monitored by asorption at 280 nm. The complex was solubilized with the indicated concentration of the detergent dodecyl maltoside and subsequently subjected to gel-filtration chromatography. The running buffer contained 50 mM Mes (pH 6.0), 20 mM NaCl and 0.05% dodecyl maltoside and was maintained at 4°C.

medium) we were unable to generate the multiline signal (data not shown). A similar behavior was observed by De Paula et al. [26] in salt-washed PS II membranes which had been depleted of the 17 and 23 kDa species.

The principal proteins of the PS II RC complex, resolved on a 6.5 M urea gel, are: the 47 kDa and the 43 kDa Chl-binding species, the hydrophilic 33 kDa kDa and the 43 kDa Chl-binding species, the hydrophilic 33 kDa protein, the hydrophobic 32 and 30 kDa, and four other proteins with molecular masses of 28, 20, 10 and 9 kDa. In the presence of 6.5 M urea in the running gel we resolve two components at the 43 kDa region; it is possible that one of the two species is either a phosphorylated form or a proteolytic product. The 10 kDa protein is the species which is removed by Tris treatment [8] and has been implicated as a constituent required for activity on the oxidizing side of Photosystem II, whereas the 9 kDa species is identified as the protein associated with cytochrome *b*-559 [27]. To investigate the minimal structure required for O_2-evolution activity, we further purified the PS II RC complex by using gel-filtration chromatography (Pharmacia FPLC system) (see Materials and Methods for details). When the PS II RC complex was solubilized with relatively high concentrations of dodecyl maltoside and subsequently chromatographed on a gel-filtration column, various protein complexes were resolved (Fig. 2), but none of these species retained oxygen evolution activity (data not shown). Solubilization of the system with a low concentration of dodecylmaltoside (0.5%), however, resulted in isolation of two main fractions (Figs. 2 and 3). One of these, designated as peak A, was a complex with a reduced number of polypeptides (Fig. 3) which retained high rates of oxygen evolution activity when ferricyanide was used as an artificial electron acceptor (Table I). As shown in Table I, for the new preparation (designated as the PS II reaction center core to distinguish it from the less highly resolved PS II reaction center complex), ferricyanide is a more effective acceptor when compared to DCBQ; in addition, sensitivity of electron transfer capacity to DCMU has almost disappared in the new preparation. At this point it is not clear whether changes in the activity of the reducing side of PS II result from the release of certain proteins, or are due to modifications to the reducing side arising from exposure to dodecyl maltoside.

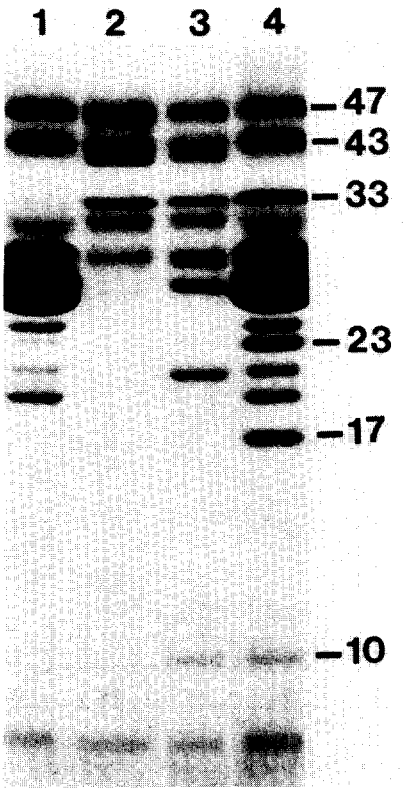

Fig. 3. Gel electrophoresis patterns of (1) Tris-treated PS II membranes; (2) peak A (PS II RC core); (3) PS II RC complex; (4) intact PS II membranes (13.5% acrylamide gel in the presence of 6.5 M urea).

An examination of the polypeptide content of the PS II RC core shows that the main components of this new complex are species with molecular masses of 47, 43, 33, 32, 30 and 9 kDa (and probably the 6 kDa species) (Fig. 3). It is very interesting to note that the 10 kDa polypeptide which was previously implicated in manganese binding is not present in this new oxygen-evolving core preparation. Other proteins which have been removed from the PS II RC complex are the 22 and 28 kDa species.

The second fraction isolated along with the PS II RC core contains only a 28 kDa protein which binds mainly Chl *a* (designated as peak B in Fig. 2) (data not shown). Since this protein has a molecular mass which is characteristic of LHCP

TABLE I

OXYGEN-EVOLUTION ACTIVITY OF VARIOUS PS II PREPARATIONS

Additions	Activity (μmol O_2/mg Chl per h)		
	PS II membranes [a]	PS II RC complex [b]	PS II RC core [c]
DCBQ (500 μM)	680	1050	640
DCBQ (500 μM) + DCMU (5 μM)	60	340	520
Ferricyanide (2 mM)	160	620	1100
Ferricyanide (2 mM) + DCMU (5 μM)	20	180	980

[a] The PS II membranes were assayed for oxygen-evolution activity in a medium containing 0.4 M sucrose, 50 mM Mes (pH 6.4) and 10 mM NaCl.

[b] The PS II RC complex was assayed in a medium containing 0.4 M sucrose, 50 mM Mes (pH 6.4), 10 mM NaCl and 10 mM $CaCl_2$.

[c] The PS II RC core was assayed in a medium containing 0.4 M sucrose, 50 mM Mes (pH 6.4) 10 mM NaCl and 10 mM $CaCl_2$, in the presence of 0.01% dodecyl maltoside, which was present because of the isolation procedure followed (see Materials and Methods for details).

components (28 kDa), we carried out a comparison of this Chl-binding protein to the LHCP. As shown in Fig. 4, while LHCP contains both Chl *a* and Chl *b*, the 28 kDa protein contains mainly Chl *a*. In adition to Chl *a*, the 28 kDa protein also contains carotenoids, as indicated by the 468

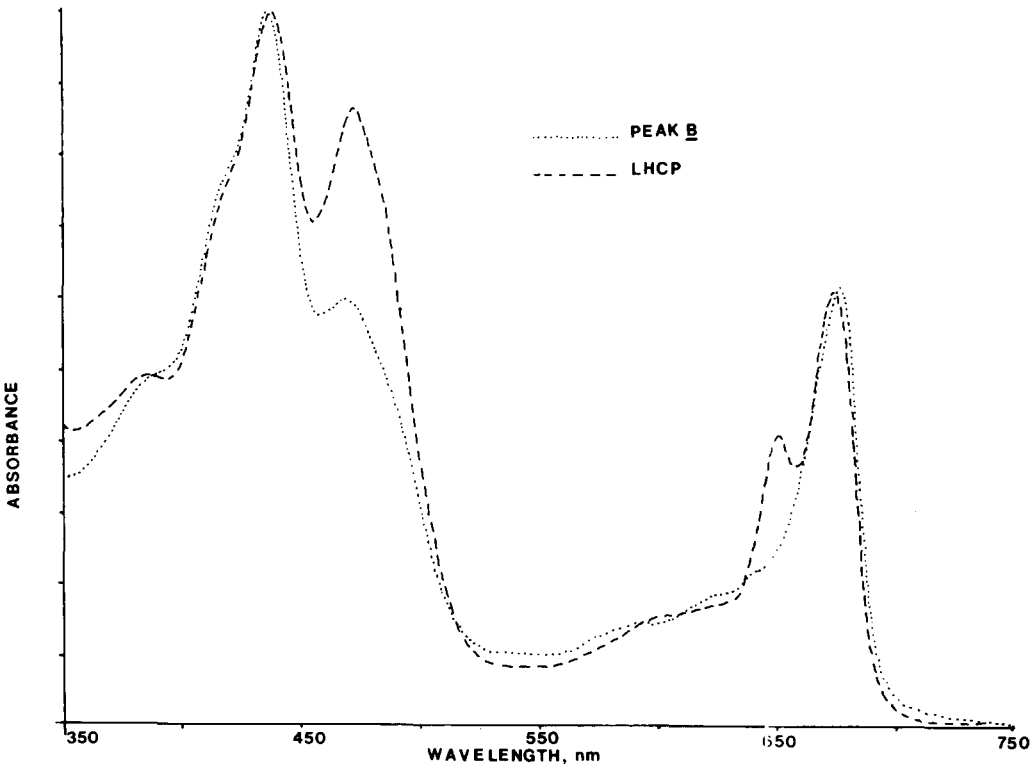

Fig. 4. Room-temperature absorption spectra of the 28 kDa protein and the LHCP (LHCP was purified from the crude LHCP (first pellet during the isolation of the PS II RC complex) by use of the FPLC system (data not shown).

nm absorption peak (Fig. 4). At this point we suggest that the 28 kDa protein is either a separate Chl-*a*-binding protein closely associated with the LHCP and the PS II RC complex, or a component of the LHCP released during the isolation of the PS II RC complex. The second possibility seems unlikely, since we have been unable to isolate such a component from LHCP solubilized with various detergents (data not shown).

Discussion

Photosystem II reaction center preparations which have been depleted of the LHCP as well as of other polypeptides are very useful systems for both the biochemical and the biophysical study of the oxygen evolving system. In this communication we have carried out a low-temperature EPR study of an oxygen-evolving PS II reaction center complex in order to examine the properties of cytochrome *b*-559 and the S_2 state of the oxygen-evolving complex.

An accumulation of circumstantial evidence has linked the high-potential form of cytochrome *b*-559 to oxygen-evolution activity [28]. A series of experiments carried out by Matsuda and Butler [29] has clearly demonstrated that the integrity of the photosynthetic membrane is required for high-potential cytochrome *b*-559. Thus, it was not surprising that in the PS II RC complex, which was prepared by extensive solubilization of the photosynthetic membrane, cytochrome *b*-559 had been converted to its low-potential form(s). Addition of high concentrations of Ca^{2+} and Cl^- reconstituted high rates of oxygen evolution activity but, under our experimental conditions, did not convert cytochrome *b*-559 to its high-potential form(s) (Fig. 1). This observation suggests that either the high-potential form of the cytochrome is not required for oxygen evolution activity or that conversion from the low-potential form(s) to the high-potential species requires the interaction of cytochrome *b*-559 with S-states higher than S_2 (Fig. 1 shows the generation of the multiline signal (S_2) while cytochrome *b*-559 remains low potential.) A similar behavior for cytochrome *b*-559 was observed in PS II membranes depleted of the water-soluble 17 and 23 kDa proteins [30,31]. A series of experiments are now in progress in order to study the effect of these two polypeptides on the structure of cytochrome *b*-559.

There have been conflicting reports in the literature regarding the effect of the 17 and 23 kDa polypeptides on the S-states. Toyoshima et al. [32], Jansson et al. [33], Franzen et al. [34], Blough and Sauer [35] and Imaoka et al. [36] have reported that the S_2 multiline signal was suppressed in their 17, 23 kDa-depleted PS II membranes, whereas the Paula et al. [26] observed a strong multiline signal in salt-washed PS II preparations. Our results in this communication suggest that Ca^{2+} and Cl^- affect the environment of the Mn complex, not the 17 and 23 kDa proteins. In the absence of the 17 and 23 kDa polypeptides, Ca^{2+} can be depleted from the photosynthetic membrane either by illumination [37] or by treatment with EGTA [4]; it is therefore possible that the disagreement among various groups as to the requirements for generation of the S_2 multiline signal is due to a requirement for Ca^{2+} to produce the EPR multiline signal.

Solubilization of the PS II RC complex followed by gel-filtration chromatography has made it possible to isolate an even more highly resolved oxygen-evolving PS II RC core as well as a Chl-*a*-binding protein. This new core material has a reduced number of polypeptides and to an extent establishes that the hydrophobic 'core' along with the hydrophilic 33 kDa protein and associated inorganic cofactors constitutes a minimal structure capable of oxygen-evolution activity. Since the PS II RC core has been depleted of all proteins with molecular masses from 10 to 28 kDa, previous structural models of the oxidizing side of PS II should be reviewed in light of this purified preparation. One species which is separated from the PS II RC complex during isolation of the oxygen-evolving core is a Chl-*a*-binding protein. Since this 28 kDa species is consistently observed as a component of the less purified PS II RC complex, we would propose that it is a discrete antenna-binding protein. It is possible that this Chl-*a*-binding protein is the CP29 protein isolated by Camm and Green [38]. The fact that the PS II RC core, which contains only Chl *a*, interacts with this Chl-*a*-binding 28 kDa protein indicates that Chl *a* is more closely associated with the reaction center of PS II (P-680).

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References

1 Ghanotakis, D.F. and Yocum, C.F. (1985) Photosynthesis Res. 7, 97–114
2 Renger, G. and Govindjee (1985) Photosynthesis Res. 6, 33–55
3 Van Gorkom, H.J. (1985) Photosynthesis Res. 6, 97–112
4 Ghanotakis, D.F., Topper, J.N., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 170, 169–173
5 Andersson, B., Critchley, C., Ryrie, I.J., Jansson, C., Larsson, C. and Anderson, J.M. (1984) FEBS Lett. 168, 113–117
6 Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539
7 Ljungberg, U., Akerlund, H.E. and Andersson, B. (1984) FEBS Lett. 175, 255–258
8 Ljungberg, U., Akerlund, H.E., Larsson, C. and Andersson, B. (1984) Biochim. Biophys. Acta 767, 145–152
9 Ghanotakis, D.F., Topper, J.N. and Yocum, C.F., (1984) Biochim. Biophys. Acta 767, 524–531
10 Cammarata, K., Tamura, N., Sayre, R. and Cheniae, G. (1984) in Advances in Photosynthesis Research (Sybesma, C. ed.) Vol. 1, pp. 311–320, Nijhoff, Junk, Dordrecht
11 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, K. (1981) FEBS Lett. 133, 265–268
12 Ono, T.A. and Inoue, Y. (1983) FEBS Lett. 164, 255–260
13 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1985) Biochim. Biophys. Acta 809, 173–180
14 Franzen, L.G. and Andreasson, L.E. (1984) Biochim. Biophys. Acta 765, 166–170
15 Miyao, M. and Murata, N. (1984) Biochim. Biophys. Acta 765, 253–257
16 Satoh, K., Nakatani, H.Y., Steinback, K.E., Watson, J. and Arntzen, C.J. (1983) Biochim. Biophys. Acta 724, 142–150
17 Diner, B.A. and Wollman, F.A. (1980) Eur. J. Biochem. 110, 521–526
18 Ikeuchi, M., Yuasa, M. and Inoue, Y. (1985) FEBS Lett. 185, 316–322
19 Satoh, K., Ohno, T. and Katoh, S. (1985) FEBS Lett. 180, 320–330
20 Tang, X.S. and Satoh, K. (1985) FEBS Lett. 179, 60–64
21 Ghanotakis, D.F. and Yocum, C.F. (1986) FEBS Lett. 197, 244–248
22 Ghanotaks, D.F., Babcock, G.T. and Yocum, C.F. (1984) Biochim. Biophys. Acta 765, 388–398
23 Chua, N.H. (1980) Methods Enzymol. 69, 434–446
24 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 167, 127–130
25 Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274–278
26 De Paula, J.C., Li, M.P., Miller, A.F., Wu, B.W. and Brudvig, G.W. (1986) Biochemistry, in the press
27 Babcock, G.T., Widger, W.R., Cramer, W.A., Oertling, W.A. and Metz, J. (1985) Biochemistry 24, 3638–3645
28 Butler, W.L. (1978) FEBS Lett. 95, 19–25
29 Matsuda, H. and Butler, W.L. (1983) Biochim. Biophys. Acta 725, 320–324
30 Ghanotakis, D.F., Yocum, C.F. and Babcock, G.T. (1986) Photosynthesis Res. 9, 125–134
31 Briantais, J.M., Vernotte, C., Miyao, M., Murata, N. and Picaud, M., (1985) Biochim. Biophys. Acta 808, 348–351
32 Toyoshima, Y., Akabori, K., Imaoka, A., Nakayama, H., Ohkouchi, N. and Kawamori, A. (1984) FEBS Lett. 176, 346–350
33 Jansson, C., Hansson, O., Akerlund, H.E. and Andreasson, L.E. (1984) Biochem. Biophys. Res. Commun. 124, 269–276
34 Franzen, L.G., Hansson, O. and Andreasson, L.E. (1985) Biochim. Biophys. Acta 808, 171–179
35 Blough, N.V. and Sauer, K. (1984) Biochim. Biophys. Acta 767, 377–381
36 Imaoka, A., Akabori, K., Yanagi, M., Izumi, K., Toyoshima, Y., Kawamori, A., Nakayama, H. and Sato, J. (1986) Biochim. Biophys. Acta 848, 201–211
37 Dekker, J.P., Ghanotakis, D.F., Plijter, J.J., Van Gorkom, H.J. and Babcock, G.T. (1984) Biochim. Biophys. Acta 767, 515–523
38 Camm, E.L. and Green, B.R. (1980) Plant Phyisol. 66, 428–432