Biochimica et Biophysica Acta 894 (1987) 453–459
Elsevier

BBA 42667

The location of the chloride binding sites in the oxygen-evolving complex of spinach Photosystem II

William J. Coleman [a,*], Govindjee [a] and H.S. Gutowsky [b]

[a] *Departments of Physiology and Biophysics and Plant Biology, and* [b] *Department of Chemistry, Univeristy of Illinois, Urbana, IL (U.S.A.)*

(Received 4 June 1987)
(Revised manuscript received 27 August 1987)

Key words: Photosystem II; Chloride binding; NMR, ^{35}Cl-; Oxygen evolving complex; Polypeptide; (Spinach)

^{35}Cl-NMR studies are presented here for spinach Photosystem II membranes inhibited by hydroxylamine (to remove Mn), Tris (to remove Mn and 18, 24 and 33 kDa polypeptides), and salt-washing (to remove 18 and 24 kDa; and 33 kDa polypeptides). Removal of Mn affects the ^{35}Cl-NMR binding curve only slightly, indicating that not all of the bound Mn is directly required for Cl-binding. Removal of both Mn and extrinsic polypeptides eliminates almost all of the Cl^--specific binding observable by NMR. Removal of the extrinsic 18 and 24 kDa polypeptides drastically changes the ^{35}Cl-NMR binding pattern; this effect is partially restored by the addition of 2 mM $CaSO_4$, and, to a lesser extent, by the partial rebinding of the polypeptides. Existence of Cl^- binding to the intrinsic polypeptides (e.g., D_1/D_2), with a peak at 0.5 mM Cl^-, is shown in samples lacking 18, 24 and 33 kDa polypeptides. Thus, both intrinsic (i.e., on the D_1/D_2 membrane protein) and extrinsic (i.e., on the 33 kDa protein) binding sites for Cl^- are suggested to exist.

Introduction

A primary objective of research into the mechanism of Cl^- activation of O_2 evolution is to locate the Cl^- binding sites. The pioneering work of Izawa et al. [1] and Kelley and Izawa [2] (see also Ref. 3) with Cl^--depleted thylakoids narrowed the search for the funcitonal site to the electron donor side of Photosystem II (PS II). More recently, with the development of procedures to isolate PS II-enriched membrane sheets, it has become possible to dissect the structure of the oxygen-evolving complex by selectively removing extrinsic polypeptides that are electrostatically bound to the inner thylakoid membrane in the vicinity of the OEC (for reviews, see Refs. [4–6]). This selective approach has provided a considerable amount of information on the effect of polypeptide removal on the kinetics of Cl^- activation.

Other studies have shown that complete removal of the three extrinsic polypeptides (masses of 18, 24 and 33 kDa) from PS II membranes lowers the maximum attainable activity to about 10–20% of the control rate and raises the Cl^- requirement several-fold, to about 100–200 mM

* Present address: Department of Applied Biological Sciences, Massachusetts Institute of Technology, Building 56-212, 77 Massachusetts Avenue, Cambridge, MA 02139, U.S.A.

Abbreviations: PS II, Photosystem II; OEC, oxygen-evolving complex; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

Correspondence: Govindjee, Department of Plant Biology, 289 Morrill Hall, 505 South Goodwin Avenue, Urbana, IL 61801, U.S.A.

[7–10] from the 10 mM required for the native complex [9]. Supplementation of the assay medium with 5–10 mM $CaCl_2$ raises the activity to about 40% of the control rate [7,9,10]. When only the 33 kDa extrinsic polypeptide is present, the system requires 20–30 mM NaCl to reach 26–40% of the original activity [9,11,12], but this level can be increased to 80% by substituting 10 mM $CaCl_2$ [12]. When the 18 and 24 kDa polypeptides are removed by salt washing, dialyzed against EGTA to remove Ca^{2+}, and added back to the depleted membranes, the observed Ca^{2+} requirement for maximum activity is about 500–700 μM [13]. The above experiments demonstrate that although progressive depletion of extrinsic polypeptides consistently lowers the apparent Cl^- and Ca^{2+} affinity of the OEC, complete removal of polypeptides does not eliminate the stimulatory effect of these ions. Harsher treatment, such as incubation in 0.8 M Tris, not only removes the extrinsic polypeptides, but also disrupts the catalytic Mn site, thereby eliminating O_2 evolution [14,15].

These results suggest that Cl^- and Ca^{2+} function over an extended region of the OEC, including sites that are close to the Mn active site. Following the trail of Cl^- and Ca^{2+} by monitoring only O_2 evolution does not, however, provide enough specific information about the interaction of these ions with each of the polypeptides. The ^{35}Cl-NMR results described in the accompanying paper [16] indicate that Cl^- probably binds to several types of site comprised of highly structured protein domains. The experiments in this paper were designed to locate these domains with respect to the OEC polypeptides and the Mn-active site. To do this, we examined the Cl^- binding properties of PS II membranes exposed to salt washing (to remove some or all of the extrinsic polypeptides), Tris washing (to remove the extrinsic polypeptides and the catalytic Mn), and hydroxylamine treatment (to remove Mn). Murata et al. [15] have summarized the effects of these treatments on O_2 evolution and polypeptide/Mn depletion. By directly monitoring the effects of these treatments on Cl^- binding, we have been able to construct a more detailed map of the Cl^- binding sites within the OEC. Our preliminary observations on salt-washing have been presented, in an abstract form, in the proceedings of a conference [17].

Materials and Methods

PS II membranes were prepared by the method of Berthold et al. [18], as described in Ref. 16. Following the first centrifugation after Triton treatment, the pellet was rinsed with Cl^- free buffer containing 400 mM sucrose and 20 mM Mes (pH 6.0). The upper pellet was resuspended in a small volume of Cl^- free buffer and then diluted into a larger volume of the same buffer containing a final concentration of 1.0 M NaCl, 1.0 M $CaCl_2$, or 1.5 mM NH_2OH (sulfate salt). For Tris treatment, the buffer employed was 0.8 M Tris-phosphate at pH 8.0. The final chlorophyll concentration was approx. 200 $\mu g \cdot ml^{-1}$. The suspension was placed on ice and stirred in darkness for 25 min. For reconstitution experiments, the suspension that had been incubated in 1.0 M NaCl was diluted with Cl^- free buffer to a final NaCl concentration of 50 mM [19]. This suspension was then allowed to stir on ice for an additional 25 min.

After treatment, the samples were transferred to centrifuge tubes and centrifuged at $60000 \times g$ for 40 min. The pellet was resuspended in a large volume of Cl^- free buffer and centrifuged for 20 min at $40000 \times g$. This washing cycle was repeated two more times to remove loosely associated Cl^-. The preparation was then given a final pH jump treatment to remove tightly bound Cl^- (see Materials and Methods [16]).

Thylakoids, thoroughly depleted of Cl^- as described in Ref. 16, were given a 25 min treatment in 0.8 M Tris-phosphate (pH 8.0) and then washed twice in Cl^- free buffer (50 mM Hepes, pH 7.2). They were then resuspended in the same buffer.

Details for Hill reaction activity measurements and ^{35}Cl-NMR assays are given in Ref. 16 and in the figure legends. NMR spectra were obtained at 24508 kHz with a 250 MHz spectrometer.

Results and Discussion

Fig. 1 shows the effect of treatment with 1.5 mM NH_2OH on the Cl^- binding properties of PS II membranes. It is clear from comparison with untreated PS II membranes (Ref. 16; also see below – Fig. 3A) that NH_2OH pre-treatment does not alter the overall shape of the ^{35}Cl-NMR bind-

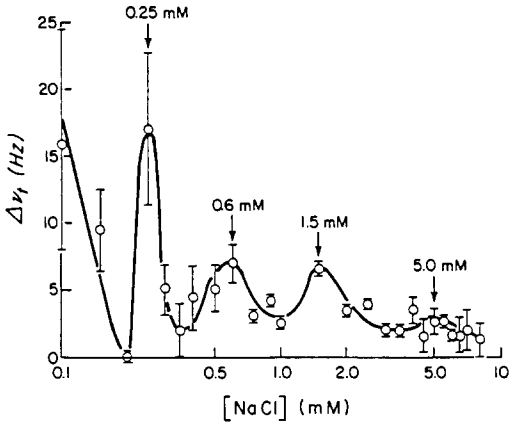

Fig. 1. ^{35}Cl-NMR binding curve for PS II membranes treated with 1.5 mM NH_2OH. The preparations showed no detectable O_2-evolution after NH_2OH treatment. Error bars show the sample standard error for 2–3 measurements on four different preparations. See Ref. 16 for NMR details.

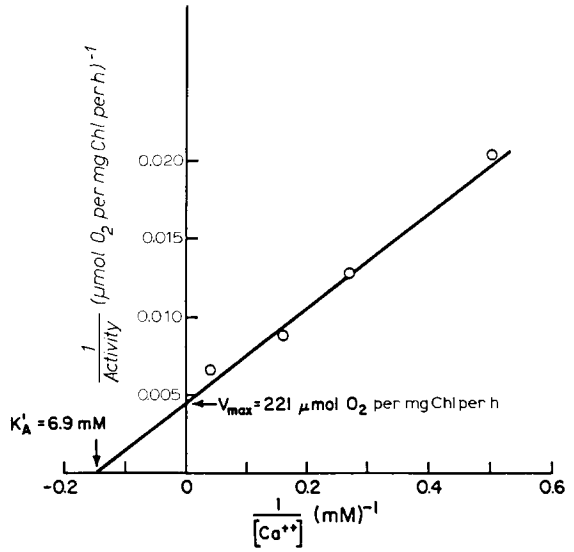

Fig. 2. Double-reciprocal plot for Ca^{2+} activation of PS II membranes washed with 1.0 M NaCl. Oxygen evolution was measured at pH 6.0 in the presence of 50 mM Cl^-.

ing curve. Murata et al. [15] have shown that while treatment with 1.5 mM NH_2OH removes more than half of the PS II Mn, it depletes the membranes of only 15% of the 18 and 24 kDa polypeptides and does not affect the 33 kDa polypeptide. The only observable effect on Cl^- binding of this selective depletion of Mn is a small decrease in the [Cl^-] for each of the ^{35}Cl-NMR linewidth maxima: from 0.3 to 0.25 mM (17%), from 0.75 to 0.6 mM (20%), from 2.0 to 1.5 mM (25%), and from 7.0 to approx. 5.0 mM (29%). The latter peak falls within the noise level.

The reason for the shift in the Cl^- concentration dependence of the binding curve is not known. It is possible that Mn depletion relieves constraints on the motion of the polypeptide chains, and consequently makes them more accessible to the Cl^-, or that partial depletion of the 18 and 24 kDa polypeptides alters the binding of Cl^-. In any case, the observation that removal of Mn does not substantially affect the Cl^- binding properties of the OEC (as observed by ^{35}Cl-NMR) indicates that the Mn itself is probably not a binding site for Cl^- in dark-adapted PS II membranes.

Ca^{2+} reactivation of oxygen evolution activity in PS II membranes, in which the 18 and 24 kDa polyppetides have been removed, by washing with 1.0 M NaCl [19], is shown in Fig. 2. The apparent activator dissociation constant for Ca^{2+} is raised to about 6.9 mM in the presence of 50 mM Cl^-. The double reciporcal plot gives the maximum attainable activity under these conditions to be 221 μmol O_2 per mg Chl per h, or about 60% of the control rate. The level of reactivation is consistent with reports from other laboratories (see Ref. 12), and indicates an apparent OEC requirement for Ca^{2+} that is beyond typical physiological concentrations.

The effect of the NaCl washing on the ^{35}Cl-NMR binding curve for PS II membranes is striking. Comparison of the binding curve for untreated membranes (Fig. 3A) with that for the washed membranes (Fig. 3B) shows that this treatment causes two major changes: (1) it substantially reduces the line-broadening at very low [Cl^-] (approx. 0.1 mM) and at high [Cl^-] (1.0–10 mM), indicating that the enzyme affinity for Cl^- is also reduced; and (2) it apparently shifts the first high point and the two linewidth maxima to higher [Cl^-], apprently replacing the linewidth maxima at 0.3 mM and 0.75 mM Cl^- with linewidth minima, while simultaneously broadening the linewidths at 0.2 mM and 0.4–0.5 mM Cl^-. The above shift may be consistent with an approx. 2-fold shift in [Cl^-] dependence on O_2 evolution, when 33 kDa polypeptide is retained, as noted earlier [9,11,12]. However, in view of the fact that NMR data are on dark-adapted membranes, and

O_2 evolution data are in light, such correspondence (or absence of it) requires further study. The correspondence between the linewidth maxima in Fig. 3A and the linewidth minima in Fig. 3B begins to disappear at $[Cl^-] > 1.0$ mM.

The persistence of sharp, concentration-dependent changes in $\Delta\nu_1$ in NaCl-washed membranes suggests that Cl^--dependent conformational changes can still occur in these samples. However, the changes from increased affinity (linewidth maxima) to decreased affinity (linewidth minima) at 0.3 mM and 0.75 mM Cl^-, along with the overall loss of O_2-evolution (to approx. 7% of the control activity in 50 mM Cl^-), suggest that removal of the 18 and 24 kDa polypeptides generates enzyme conformations less favorable to Cl binding.

Since it is known that the addition of high concentrations of Ca^{2+} may partially replace the function of the 18 and 24 kDa polypeptides in stimulating O_2-evolution in these preparations, we added a fixed concentration of Ca^{2+} (in the form of 2.0 mM $CaSO_4$) to the ^{35}Cl-NMR assay buffer and measured the ^{35}Cl-NMR binding curve again. Fig. 3C shows that the addition of 2.0 mm Ca^{2+} largely restores three of the four ^{35}Cl-NMR linewidth maxima that were observed in the control (Fig. 3A). This restoration in the NMR curve is consistent with our expectation that Ca^{2+}-restored memrbane should have lower $[Cl^-]$ requirement for O_2 evolution than the salt-washed membrane. We, however, emphasize that comparison of NMR data in dark with the O_2 evolution data in light may not be meaningful; detailed data on $[Cl^-]$ dependence of O_2 evolution $\pm Ca^{2+}$ are not available; and the only available data [20] on this point appear inconsistent with our expectation. Restoration of the linewidth maximum at 7.0 mM was not observed, however, and the linewidth maximum at 0.3 mM Cl^- appears to be shifted to a slightly higher concentration (0.35 mM Cl^-). Although sulfate, added here as calcium sulfate, may be a competitive inhibitor of Cl^- binding (see, e.g., Itoh and Uwana [21]), fairly high concentrations are required to compete effectively with Cl^-. We believe that the sulfate concentration, used here, does not noticeably alter Cl^- binding to PS II (also see Sandusky and Yocum [22]), and, the ineffectiveness of Ca^{2+} in completely replacing the

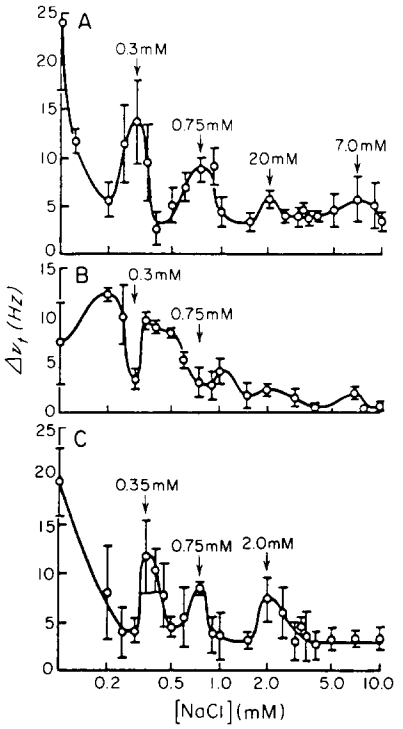

Fig. 3. Effect of polypeptide depletion and reconstitution on the ^{35}Cl-NMR binding curve for PS II membranes. Error bars show the sample standard error for 2–5 measurements on 3–6 different preparations. (A) Untreated membranes (mean activity = 383 μmol O_2 per mg Chl per h in 50 mM Cl^-). (B) Membranes washed in 1.0 M NaCl (mean activity = 28 μmol O_2 per mg Chl per h in 50 mM Cl^-). (C) Membranes washed in 1.0 M NaCl and assayed in the presence or 2.0 mM $CaSO_4$ (mean activity = 102 μmol O_2 per mg Chl per h in 50 mM NaCl + 2.0 mM $CaSO_4$).

18 and 24 kDa polypeptides is a more likely explanation of the failure to observe a linewidth maximum of 7.0 mM Cl^-.

Our ^{35}Cl-NMR results with Ca^{2+} parallel the findings of a number of other studies on O_2 evolution, in which Ca^{2+} was found to partially replace the function of the 18 and 24 kDa polypeptides. The reappearance of the ^{35}Cl-NMR linewidth maxima after Ca^{2+} addition indicates to us that this cation reorganizes the complex into a quasinative structure that is then able to bind Cl^- and evolve oxygen. The Cl^- binding curve in Fig. 3C for the Ca^{2+} supplemented membranes is largely similar to that in Fig. 3A for the untreated PS II membranes. This experiment also indicates that the 18 and 24 kDa polypeptides are not directly involved in Cl^- binding to the sequestered sites.

The effects of NaCl-washing on the ^{35}Cl-NMR binding curve was partially reversible (data not shown): dilution of the PS II suspension from 1.0 M to 50 mM NaCl during preparation appeared to enable the functional complex to reassemble, although the restoration was incomplete. The linewidth maxima at 0.3 mM and 2.0 mM Cl^- appeared to be restored more completely (with $\Delta\nu_t$ mesured at 90% and 86% of the control value, respectively) than were the maxima at 0.75 mM and 7.0 mM Cl^- (with $\Delta\nu_t$ measured at 49% and 47% of the control value, respectively). The line broadening at 0.1 mM Cl^- was, however, substntially reduced. The observation of partial reconstitution of the ^{35}Cl-NMR linewidth maxima is consistent with the partial reconstitution of O_2 evolution in these preparations (approx. 30% of the control rate in 50 mM Cl^- with no added Ca^{2+}). These ^{35}Cl-NMR results, although preliminary, support the findings of Miyao and Murata [23], who reported that dilution of NaCl-treated PS II membranes to 34 mM NaCl partially restored the control rate of O_2 evolution and enabled the 18 and 24 kDa polypeptides to rebind to the membrane.

The ability of Ca^{2+} (at high concentrations) to substitute for the missing 18 and 24 kDa polypeptides (as reflected in the large restoration of both Cl^- binding and enzyme activity) suggests that it may function by coordinating to negatively charged residues on the remaining proteins. It is possible that these negative charges are normally associated with positive charged residues on the 18 and/or kDa polypeptide(s) in the native complex. Ca^{2+} might then replace these two polypeptides, but with reduced effectiveness. It is also possible that these two polypeptides raise the Ca^{2+} affinity of the complex directly, by supplying ligands to a specific Ca^{2+} binding site, or indirectly, by increasing the affinity of other proteins for Ca^{2+}. As far as Cl^- binding is concerned, it is apparent from these data that the sequestered Cl^- binding sites revealed by the ^{35}Cl-NMR linewidth maxima are not part of the 18 and 24 kDa polypeptides, although these two polypeptides play an important role in stabilizing their structure.

To determine whether the 33 kDa extrinsic polypeptide contains the binding sites for Cl^-, we treated PS II membranes with 1.0 M $CaCl_2$. Ono and Inoue [24] have shown that this treatment removes all of the extrinsic polypeptides. The ^{35}Cl-NMR binding curve for this preparation (Fig. 4) exhibits a single, broad linewidth maximum centered at about 0.5 mM Cl^-. Very similar results (not shown) were obtained in two other sets of experiments. The exact position of this maximum in the native membrane may be quite different, since [Cl^-] dependence for O_2 evolution is drastically altered upon the removal of the three extrinsic polypeptides [7–10]. No other maxima were observed in the Cl^- concentration range between 0.1 and 10 mM, and supplementation of the medium with 2.0 mM $CaSO_4$ had no additional effect in this concentration range (data not shown). We conclude from this result that a significant amount of high affinity Cl^- binding is still possible after depletion of the extrinsic polypeptides. If this binding involves portions of the intrinsic D1/D2 proteins (see Fig. 1 in Ref. 6 for a current picture of PS II structure), then it may be associated with the residual O_2-evolving activity at the Mn-active site. In any case, it is clear from a comparison of this ^{35}Cl-NMR binding curve, shown in Fig. 4, with that of the intact system (Fig. 3A) or the preparation washed with NaCl and supplemented with Ca^{2+} (Fig. 3C), that the 33 kDa polypeptide must be present in order to observe the multiple linewidth maxima characteristic of the native complex.

When PS II membranes are treated with 0.8 M

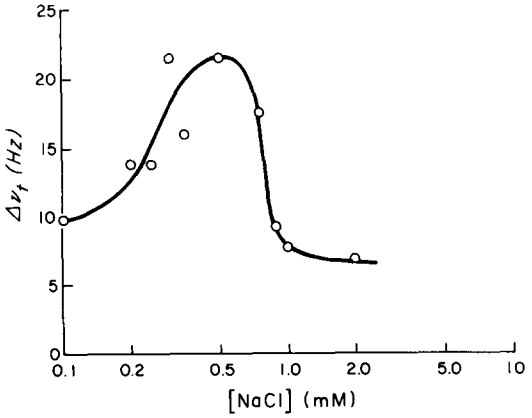

Fig. 4. ^{35}Cl-NMR binding curve for PS II membranes washed with 1.0 M $CaCl_2$. Activity was approx. 46 μmol O_2 per mg Chl per h in the presence of 25 mM $CaCl_2$.

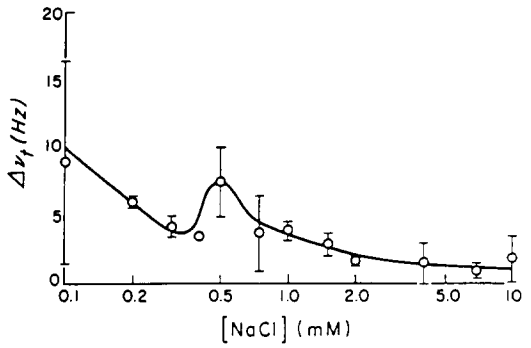

Fig. 5. ^{35}Cl-NMR binding curve for PS II membranes washed with 0.8 M Tris. The mean rate of O_2 evolution after Tris treatment was 16 μmol O_2 per mg Chl per h (approx. 96% inhibition). Error bars show the variation in $\Delta\nu_1$ for two measurements on two different preparations.

Tris, which inactivates O_2 evolution and releases the catalytic Mn [13,14], most of the specific Cl^- binding is eliminated (Fig. 5). This result is a further indication that the Cl^- binding observed in the $CaCl_2$-washed PS II membranes is associated with proteins involved in Mn binding. It is noteworthy that the ^{35}Cl linewidths are much smaller for the Tris-washed membranes than for those washed in $CaCl_2$ (FIg. 4) indicating that the latter are more intact.

Concluding remarks

We can briefly summarize the significance of the results presented in this paper by concluding: (1) under dark-adapted conditions, most of the Cl^- does not bind directly to the Mn in the OEC; (2) the Cl^--binding mechanism in the intact complex involves the cooperation of both intrinsic sites (possibly on D1/D2) and extrinsic sites (on the 33 kDa polypeptide); (3) Cl^- binding to the extrinsic sites is enhanced by the indirect cooperation of the 18 and/or 24 kDa polypeptides; the function of these two polypeptides can be partially replaced by a high concentration of Ca^{2+} (2.0 mM); and (4) although an approximate relationship between O_2 evolution and ^{35}Cl linewidth, reflecting Cl^- binding, is observed (see also Ref. 16), a quantitative relationship may be very complex; for example, O_2 evolution requires binding but binding may not be a sufficient condition for O_2 evolution. Furthermore, we need to obtain both NMR and O_2 evolution data under identical conditions.

A hypothesis to explain the mechanism of Cl^- activation of the OEC is presented in Ref. 25.

Acknowledgements

This work was supported by National Science Foundation grants PCM 79-11148 to HSG and 83-06061 to G. Also, the work has benefited from the use of facilities made available through the UIUC-NSF Regional NMR Instrumentation Facility (grant CHE-79-16100). This work was also supported by grants from the University of Illinois Research Board and the Biomedical Research Committee to G. We thank Kristy Hendrich of the University of Illinois Molecular Spectroscopy Laboratory for her technical assistance.

References

1 Izawa, S., Heath, R.L. and Hind, G. (1969) Biochim. Biophys. Acta 180, 388–398
2 Kelley, P.M. and Izawa, S. (1978) Biochim. Biophys. Acta 502, 198–210
3 Critchley, C., Baianu, I.C., Govindjee and Gutowsky, H.S. (1982) Biochim. Biophys. Acta 682, 436–445
4 Ghanotakis, D.F. and Yocum, C.F. (1985) Photosynthesis Res. 7, 97–114
5 Govindjee, Kamabra, T. and Coleman, W. (1985) Photochem. Photobiol. 42, 187–210
6 Murata, N. and Miyao, M. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. I, pp. 453–462, Martinus Nijhoff, Dordrecht
7 Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–286
8 Miyao, M. and Murata, N. (1984) FEBS Lett. 170, 350–354
9 Miyao, M. and Murata, N. (1985) FEBS Lett. 180, 303–308
10 Kuwabara, T., Miyao, M., Murata, T. and Murata, N. (1985) Biochim. Biophys. Acta 806, 283–289
11 Nakatani, H.Y. (1984) Biochem. Biophys. Res. Commun. 120, 299–304
12 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1985) FEBS Lett. 192, 1–3
13 Ghanotakis, D.F., Topper, J.N., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 170, 169–173
14 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268
15 Murata, N., Miyao, M. and Kuwabara, T. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 213–222, Academic Press Japan, Tokyo
16 Coleman, W.J., Govindjee and Gutwosky, H.S. (1987) Biochim. Biophys. Acta 894, 443–452
17 Coleman, W.J., Govindjee and Gutowsky, H.S. (1987) in

Progress in Photosynthesis Research (Biggins, J., ed.), Vol. I, pp. 619–632, Martinus Nijhoff, Dordrecht
18 Berthold, D.A., Babcock, G.T., and Yocum, C.F. (1981) FEBS Lett. 134, 231–234
19 Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93
20 Ikeuchi, M. and Inoue, Y. (1986) Arch. Biochem. Biophys. 247, 97–107
21 Sandusky, P.O. and Yocum, C.F. (1986) Biochim. BIophys. Acta 849, 85–93
23 Miyao, M. and Murata, N. (1986) Photosynthesis Res. 10, 489–496
24 Ono, T. and Inoue, Y. (1983) FEBS Lett. 164, 255–260
25 Coleman, W. and Govindjee (1987) Photosynth. Res. 13, 199–223