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A possible role for hydrogen peroxide as a naturally occurring electron donor in photosynthetic oxygen evolution

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Oxygen absorption in the light exhibited by spinach chloroplasts is traditionally explained by assuming that oxygen functions as Hill acceptor (Mehler, A.H. (1951) Arch. Biochem. Biophys. 34, 339–351). In this paper it is suggested that the light-driven absorption of oxygen results in the production of superoxide and that this process is catalyzed by a 58 kDa catalase. Superoxide then dismutates to hydrogen peroxide and oxygen. It is further suggested that the production of hydrogen peroxide takes place in the lumin of the thylakoid and that the hydrogen peroxide is used as electron donor for photosynthesis. There will only be little loss of oxidizing equivalents, since the oxygen-evolving complex does not have to reach higher oxidation steps. This will be an advantage for light-limited plants. The use of hydrogen peroxide as electron donor will have the consequence that the production of oxygen is higher on the second flash in a series of flashes than when water is used as electron donor. It is also suggested that hydrogen peroxide can deactivate the higher oxidation steps in the oxygen-evolving complex.

Introduction

In this classic work Mehler [1–3] showed that the O_2 absorption by spinach chloroplasts exposed to light gave H_2O_2. He assumed that this reaction takes place at the reducing side of PS I, and that O_2 should function as an electron acceptor. This paper gives an alternative explanation of O_2 absorption in the light. It is suggested that superoxide is produced from O_2 in a process catalyzed by a 58 kDa catalase type protein. Superoxide dismutates to H_2O_2 and O_2. H_2O_2 is then used as electron donor instead of H_2O. The evolution of one molecule of O_2 from two molecules of H_2O requires the accumulation of four oxidizing equivalents in the oxygen-evolving complex [4]. The S-states, S_2 and S_3 in the S-state clock of Kok et al. [4], are unstable; the two S-states can be deactivated by back-reaction [5]. This loss of oxidizing equivalents is a problem for photosynthetic organisms suffering from light limitation. Using H_2O_2 as electron donor, the oxygen-evolving complex only has to accumulate two oxidizing equivalents to evolve one molecule of O_2. This system will be quite stable, since H_2O_2 is metastable. The system with the 58 kDa catalase working in parallel with the oxygen-evolving complex, will to some extent be equivalent to having interconnected photosynthetic units. Therefore, plants which use H_2O_2 as an electron donor will have raised levels of O_2 evolution on the second flash in a series of flashes, relative to plants which do not use H_2O_2 as an electron donor.

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ESR, electron spin resonance; Mops, 3-(*N*-morpholino)propane sulfonic acid; PS, Photosystem.

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Deactivation of S_2 and S_3 in the S-state system [4] shows remarkable differences in *Chlorella* cells and in spinach chloroplasts [5]. This may be attributed the presence of H_2O_2 production in spinach [1–3], but not in *Chlorella* [6].

A series of results is presented which shows that the electron chain can be completely inhibited with little or no effect on the O_2 absorption in the light. This is strong evidence that the O_2 absorption does not take place at the reducing side of the electron-transport chain. The paper presents a simple procedure to purify the 58 kDa catalase-type protein from spinach thylakoids. The 58 kDa catalase exhibits O_2 absorption in the light. The same purification procedure also yields a 14 kDa protein associated with manganese, a yellow pigment and a brown pigment.

Materials and Methods

The purification of the 58 kDa and the 14 kDa proteins is outlined in the flow sheet.

Homogenization of spinach leaves
↓
Filtration
↓
Centrifugation 16000 × *g*, 20 min
↓
Pellet, resuspension and centrifugation
↓
Pellet solubilized in SDS
↓ dialyzed 3 × 12 h against 10 mM ammoniumsulphate
Ammoniumsulphate precipitation 0.5 M
↓ centrifugation 16000 × *g*, 10 min
Supernatant, dialyzed 2 × 12 h against distilled water
↓
Ion exchange
↓
Concentration
↓
Gel filtration

Spinach leaves (from the local market) were cut into pieces and homogenized for 20–30 s in distilled water using a Polytron Kinematica, Switzerland. The homogenate was filtered through two layers of cheese cloth with a layer of cotton wool between them and the thylakoid membranes were collected by centrifugation at 16000 × *g* for 20 min. The pellet was resuspended with the polytron and washed twice with distilled water. Finally, the washed membranes were resuspended in distilled water (3 mg Chl/ml) and solubilized with SDS (4 mg SDS per mg Chl). SDS was added from a 10% stock. The solubilized thylakoids were dialyzed three times for 12 h against distilled water. The dialyzed solution was then made 0.5 M with respect to ammonium sulfate and left for 1 h. Then most of the membrane lipids and the Chl-proteins were precipitated by centrifugation at 16000 × *g* for 10 min. The supernatant was dialyzed twice for 12 h against distilled water.

The proteins were concentrated and purified by ion-exchange chromatography. The chromatography was performed on a short column packed with 20 ml Trisacryl(M) (LKB), and equilibrated with 20 mM Mops buffer (pH 7.5). The 14 kDa protein was eluted with the equilibrating buffer made 0.2 mM with respect to NaCl. The protein was eluted in a few ml. The protein is coffee-brown because of the quinone. The 14 kDa protein can be further purified using gel-filtration chromatography. This was done on a Sephadex G 50 superfine column (90 cm long and with a diameter of 2.5 cm) (Pharmacia) using distilled water as eluent. All procedures were done at 0–4° C. The yellow 58 kDa protein was eluted with the buffer made 1 M with respect to NaCl. When the Trisacryl(M) ion-exchange material is used for the first time much of the catalase is not liberated from the ion-exchange material. This part of the catalase was liberated from the ion-exchange material by adding the Mops buffer made 1% with respect to SDS. Further purification was performed on an Agarose (Bio-rad) 0.5 M fine column (90 cm long and with a diameter of 2.5 cm). The column was equilibrated with 20 mM Tris-HCl (pH 7.6), 50 mM NaCl. This procedure was performed at room temperature. Protein measurements were done according to Lowry [7].

Manganese measurements were performed with a Perkin-Elmer 303 atomic absorption spectrometer using an acetylene flame as reducing agent. Samples were dialyzed and lyophilized before analysis. Iron measurements were done with the bathophenanthrolin method [8]. SDS polyacrylamide gel electrophoresis was done according to Ref. 9. The stacking gel was 6% acrylamide and the separation gel 15%; Coomassie blue G 250 was used for staining. Molecular weight standards were

the low molecular weight kit from Pharmacia. O_2 measurements were done with a Clark-type electrode constructed in this laboratory. Chlorophyll measurements were done according to Ref. 10 on a Carl Zeiss, PMQII instrument.

Results

Iron content. The 58 kDa protein contains one iron atom per molecule. It has an absorption maximum at 425 nm (Fig. 1) when freshly isolated. Exposed to air the absorption maximum shifts to 405 nm.

O_2 absorption in the light. The 58 kDa protein catalyzes O_2 absorption in the light; see Fig. 2. The rate of O_2 absorption depends on the redox state of the buffer system. If the protein is isolated in the presence of dithiothreitol as in Ref. 11 it can be reactivated by adding an oxidant. Bovine liver catalase also catalyzes O_2 absorption in the light.

Superoxide production. It was assumed that the O_2 absorption in the light catalyzed by catalase resulted in the production of superoxide. The formation of superoxide was qualitatively demonstrated as light-driven oxidation of NH_2OH to NO_2^- [12] (see Fig. 3). Samples of catalase were immobilized in Petri dishes using glutaraldehyde [13]. 20 ml reaction mixture was placed on top of the immobilized proteins. The Petri dishes were exposed to a light intensity of 100 W/cm^2 and 2 ml samples were assayed at given time intervals.

Effect of inhibitors. A set of experiments was made in order to determine what effect poisoning the electron-transport chain would have on O_2

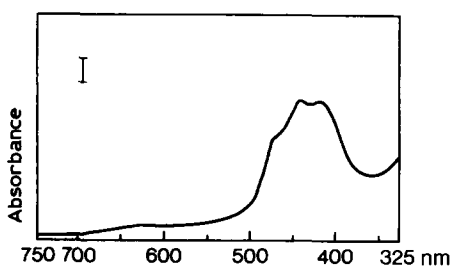

Fig. 1. The absorption spectrum of the 58 kDa catalase in the visible range of the spectrum. The protein is suspended in 20 mM Mops buffer (pH 7.5). The spectrum was made on a Carl Zeiss DMR 21, beamsplit instrument. The bar denotes 0.1 absorbance units.

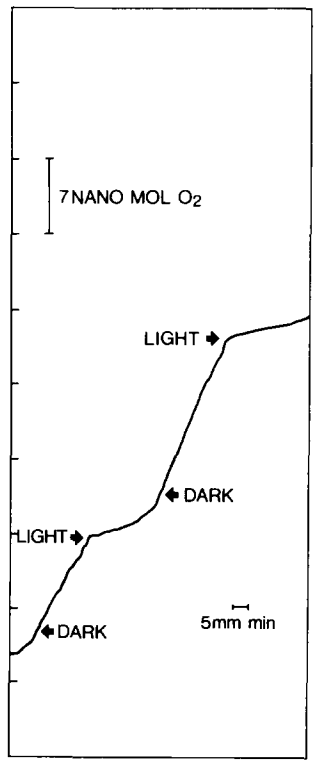

Fig. 2. Light-driven O_2 absorption catalyzed by the 58 kDa catalase at a concentration of 5 mg protein per ml; 20 mM Mops buffer (pH 7.5). The rate of O_2 absorption at saturating light intensity is 40 nmol O_2 absorbed per mg protein per h.

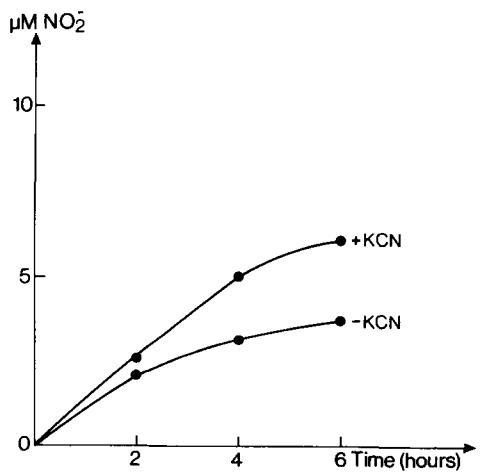

Fig. 3. Light-driven formation of nitrite from hydroxylamine. The reaction mixture was 0.1 M potassium phosphate buffer (pH 7.5) made 1 mM with respect to hydroxylamine. In one experiment potassium cyanide was added to a concentration of 1 mM.

absorption in the light. Intact and broken chloroplasts were made according to Nakatani and Barber [14]. Broken chloroplasts at a concentration of 10 μg Chl per ml in the assay buffer [14] were incubated with 10 mM NH_2OH for 1 h.

This treatment is known to destroy electron transport completely by displacing manganese from the oxygen-evolving complex [15,16]. After incubation, excess NH_2OH was removed by two washes with the assay buffer at $2500 \times g$ for 2 min. The pellet was resuspended in the assay buffer and the volume was adjusted to give a final concentration of 10 μg Chl per ml. No O_2 evolution was found after the NH_2OH incubation, using ferricyanide as Hill acceptor [17]. O_2 absorption in the light was typically about 10–20 μmol O_2 per mg Chl per h after the NH_2OH incubation. Similar results were obtained by incubating with 0.8 M Tris (pH 8) or by exposing chloroplasts to 55 °C for 5 min. The results are summarized in Table I.

The 14 kDa protein. The purification procedure used here ensures that the mananese is specifically associated with the 14 kDa protein, since neither ammonium sulfate precipitation nor extensive dialysis disrupts the binding. When the 14 kDa and 58 kDa proteins are eluted from the ion-exchange column, the manganese is associated only with the 14 kDa protein. There is typically 0.1–0.3 manganese atom per protein molecule. This is unsatisfactory and a less harsh purification procedure will be developed. The yellow pigment which is also associated with the 14 kDa protein is dissociated from it when manganese is. When the protein is heated for 5 min at 50 °C, exposed to low pH, exposed to high ionic-strength or other treatments known to abolish O_2 evolution, both the manganese and the yellow pigment are removed. The brown pigment associated with the 14 kDa protein has the ESR line spectrum (Fig. 4a) usually attributed to Z [18,19]. Fig. 4b shows the ESR spectrum of the oxidized form of the brown

TABLE I

EFFECT OF DIFFERENT TREATMENTS OF BROKEN CHLOROPLASTS ON OXYGEN ABSORPTION IN THE LIGHT

Incubation	Addition	O_2 absorption (μmol O_2 per mg Chl per h)
10 mM NH_2OH		9.6
10 mM NH_2OH	1 mM FeCN	20.3
0.8 M Tris		8.5
0.8 M Tris	1 mM FeCN	9.6
0.8 M Tris	1 mM FeCN	22.3
Heat treatment		4.3
Heat treatment	1 mM FeCN	14.8

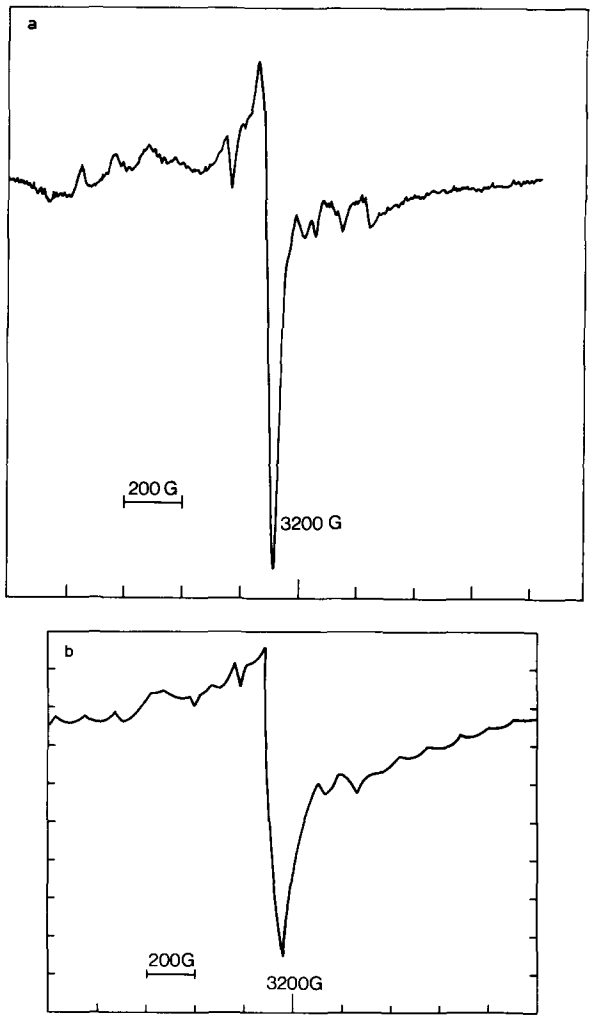

Fig. 4. (a) The ESR spectrum of the quinone on the 14 kDa protein. The ESR spectrum was made on a Jeol ME-IX spectrometer from Japan Electronic Company. Temperature was −160 °C; the magnetic field was 3200 G ± 1000 G; gain, 6300×; response 3 s; modulation 20 G; power 20 W. (b) The ESR spectrum of the oxidized form of the quinone obtained with excess ammonium peroxodisulfate being used as oxidant. Instrument settings were as described under (a).

pigment. The brown pigment is liberated from the 14 kDa protein by adding excess base. Exposed to the oxygen of the air, the brown pigment polymerises and the low-molecular form of this compound is never seen. This indicates that the brown pigment is a quinone bound to the 14 kDa protein in the dihydroxyl form as suggested in Ref. 20. The polymerized brown pigment is separated from the protein by precipitating the latter with acid.

Discussion

The presence of the 58 kDa catalase in thylakoids indicates that the light-driven production of H_2O_2 is an advantage to those organisms which possess catalase. The use of H_2O_2 as electron donor for the oxygen-evolving complex, instead of H_2O, will give a quite stable system. Two photons are used for the production of one molecule of H_2O_2 [21]:

$$2h\nu + \tfrac{1}{2}O_2 + H_2O \rightarrow H_2O_2$$

and two photons are used for inducing charge separation in the oxygen-evolving complex, which then can oxidize H_2O_2 in an S_2–S_0 or S_3–S_1 cycle:

$$2h\nu + H_2O_2 + H_2O \rightarrow 4e^- + 4H^+ + \tfrac{3}{2}O_2$$

The sum of the two reactions is the same as the oxidation of two H_2O molecules.

Several predictions can be made from the model presented in this paper. The photosynthetic organism is assumed to possess the 58 kDa catalase unless the opposite is stated.

The site of O_2 absorption is in the lumen of the thylakoids

In the classic work of Mehler [1–3] it was assumed that O_2 functions as a Hill acceptor at the reducing side of PS I. However, some of Mehler's experiments indicate that the O_2 absorption in the light takes place in the lumen of the thylakoids. In one experiment ascorbic acid and quinone are mixed before being added to thylakoids [2]. In this experiment the O_2 absorption proceeds at the same rate as in experiments with thylakoids with no additions. In another experiment, quinone is added to the thylakoid suspension, and, after some time, ascorbic acid is also added, both in the dark. In this case there is an O_2 absorption on subsequent illumination, which is 2–3-times faster than in the preceding experiment [2]. The only important difference between the two experiments is that in the latter case, hydroquinone is probably present in the lumen of the thylakoids. Two assumptions can explain this: (a) quinone penetrates the thylakoid membrane while hydroquinone does not; and (b) ascorbic acid is able to reduce quinone to hydroquinone through the membrane via a redox carrier. This could well be plastoquinone. In the buffer outside the thylakoids, the concentration of hydroquinone will be almost the same in both experiments, as the volume of the thylakoids is negligible. This indicates that H_2O_2 is produced in the lumen of the thylakoids and not at the reducing side of PS I. Other groups also find that O_2 absorption takes place at the PS II [19,22].

Effect of light limitation

Exposed to light limitation, but saturating amounts of electron acceptor (CO_2 or artificially), the photosynthetic material should show relatively higher levels of O_2 absorption, since the O_2 production from H_2O is slow. Heber et al. [23] showed with spinach chloroplasts that the amount of O_2 absorption drops relative to the amount of O_2 evolution when increasing the light intensity.

Adaption to light limitation

The 58 kDa catalase should not be expected to occur in all photosynthetic organisms, since it is only an advantage to those species that experience light limitation. Patterson and Meyers [6] demonstrate that cells of *Anacystis nidulans* grown under light limitation produce more H_2O_2 at raised light intensity than cells grown under high light intensity. It is unlikely according to the hypothesis of Mehler [1–3] that O_2 absorption would show much variation among different species, as the electron acceptors at the reducing side of PS I are common to all photosynthetic organisms. At least one would expect that values of O_2 absorption would reflect evolutionary status, but this is not the case. Contrary to spinach, soybean cells show very little O_2 absorption [24] and several algae do not produce H_2O_2 in the light, e.g., *Chlorella*, while other algae do [6].

Heterogenous photosystems

Joliot and Joliot [16] show that the O_2 evolution on the second flash (Y_2) relative to the O_2 evolution in the steady state (Y_{ss}) in a series of flashes depends on the biological material. Double photoreactions will have the consequence that the O_2 evolution on the second flash relative to the O_2 evolution in the steady state (Y_2/Y_{ss}) will be high. This phenomenon will be observed in organisms having interconnected photosystems or with heterogenous light-absorbing systems functioning in parallel as suggested in this paper. In Ref. 16 it is shown that white light is more efficient in producing O_2 evolution on the second flash than laser light (694 nm) is. This would be expected according to the model presented here and it almost certainly rules out a system which is entirely based on chlorophyll as the absorbing pigment.

Double photoreactions are observed in spinach [16], but not in pea chloroplasts [24] or in *Chlorella* [16]. This is to be expected, since spinach chloroplasts exhibit O_2 absorption in the light [1–3], while soybean cells do not [20] and *Chlorella* does not evolve H_2O_2 in the light [6].

Modified oxygen-evolving complexes

The amount of O_2 evolution on the second flash (Y_2/Y_{ss}) in a series of flashes with spinach chloroplasts will be particularly high when the oxygen-evolving complex has been damaged. In these cases only H_2O_2, but not H_2O, can be used as electron donor because of some chemical modification of the oxygen-evolving complex. Pan and Izawa [26] demonstrated by using mild treatment with NH_2OH, that non-water-splitting chloroplasts can oxidize H_2O to O_2 through PS II at substantial rates. In a work of Carpentier and Nakatani [27] O_2 evolution was abolished by adding different kinds of herbicides to chloroplasts. Carpentier and Nakatani suggest that the action of the herbicides is to displace active chloride and calcium. The electron transport was restored by using H_2O_2 as electron donor. Spinach chloroplasts exposed to saturating concentrations of DCMU show an abnormal high value of O_2 evolution on the second flash in a series of flashes [16]. This is simply explained by assuming that H_2O_2 is the electron donor. It indicates an action of DCMU on the oxidizing side of PS II as suggested in Refs. 27 and 28. Increasing the dark-adaption time at 0 °C from 30 min to 7 h increases the O_2 evolution on the second flash in a series of flashes 30% [16]. This indicates a relative increase in the use of H_2O_2 as electron donor in aged spinach chloroplasts. Diner [28] reports a similar result after 'aging' chloroplast on the polarized O_2-electrode.

Deactivation of S-states

Deactivation or disappearance of S-state S_3 is observed by measuring O_2 evolution of one measuring flash given after various dark intervals following two oversaturating flashes [5]. Joliot and Kok [5] describe deactivation of S_3 in *Chlorella* cells and spinach chloroplasts. There are several pronounced differences: deactivation is faster in whole *Chlorella* cells than in spinach chloroplasts. Chloroplasts show considerable variation not readily correlated with the conditions of preparation. In spinach chloroplasts the decay of S_3 is biphasic, this is sometimes more pronounced than at other times. Deactivation is the loss of oxidizing equivalents. As pointed out in Ref. 5 this happens in two ways. S_3 may react with an electron donor which replaces H_2O or by back-reaction (hole-electron annihilation). The electron donor which replaces H_2O could well be H_2O_2. This could explain why biphasic decay is seen in spinach and not in *Chlorella*.

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References

1 Mehler, A.H. (1951) Arch. Biochem. Biophys. 33, 65–77.
2 Mehler, A.H. (1951) Arch. Biochem. Biophys. 34, 339–351.
3 Mehler, A.H. (1952) Arch. Biochem. Biophys. 38, 365–370.
4 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
5 Joliot, P. and Kok, B. (1975) in Bioenergetics of Photosynthesis (Govinjee, ed.), pp. 400–404, Academic Press, New York.
6 Patterson, C.O.P. and Myers, J. (1973) Plant Physiol. 51, 104–109.

7 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
8 Strickland, J.D.H. and Parsons, T.R. (1968) A Practical Handbook of Seawater Analysis, Bull. 167, pp. 97–103, Fish. Res. Bd. Can. Ottawa.
9 Laemmli, U.K. (1970) Nature 227, 680–685.
10 Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
11 Nakatani, H.Y. and Barber, J. (1981) Photobiochem. Photobiophys. 2, 69–78.
12 Elstner, E.F., Stoffer, C. and Heupel, A. (1975) Z. Naturforsch. 30 c, 53–56.
13 De la Rosa, M.A., Krishna Rao, K. and Hall, D.O. (1976) Photobiochem. and Photobiophys. 11, 173–187.
14 Nakatani, H.Y. and Barber, J. (1977) Biochim. Biophys. Acta 461, 510–512.
15 Cheniae, G.M. and Martin, I.F. (1971) Plant Physiol. 47, 568–575.
16 Joliot, P. and Joliot, A. (1981) Biochim. Biophys. Acta 638, 132–140.
17 Hill, R. (1939) Proc. Roy. Soc. (London), Series B, No. 847, 127, 192.
18 Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 315–328.
19 Von Kameke, E. and Wegman, K. (1978) in Photosynthetic Oxygen Evolution (Metzner, H., ed.), pp. 371–380, Academic Press, London.
20 O'Malley, P.J., Babcock, G.T. and Prince, R.C. (1984) Biochim. Biophys. Acta 766, 283–288.
21 Losada, M. and Guerrero, M.G. (1979) in Photosynthesis in Relation to Model Systems (Barber, J., ed.), pp. 393, Elsevier/North-Holland, Amsterdam.
22 Sayre, R.T. and Homann, P.H. (1979) Arch. Biochem. Biophys. 196, 523–533.
23 Heber, U., Egneus, H., Hanck, U., Jensen, M. and Koster, S. (1978) Planta 143, 41–49.
24 Behrens, P.W., Marsho, T.V. and Radmer, R.J. (1982) Plant Physiol. 70, 179–185.
25 Jursinic, P. (1981) Biochim. Biophys. Acta 635, 38–52.
26 Pan, R.L. and Izawa, S. (1979) Biochim. Biophys. Acta 547, 311–319.
27 Carpentier, R. and Nakatani, H.Y. (1985) Biochim. Biophys. Acta 808, 288–292.
28 Diner, B. (1974) Biochim. Biophys. Acta 368, 371–385.