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Properties of the oxidizing site of Photosystem I

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The bimolecular electron transfer from plastocyanin to P-700$^+$ after flash excitation was investigated in inside-out vesicles from spinach chloroplast lamellae with randomly distributed photosystems and in vesicles from stroma membranes. Incubation of the vesicles with increasing amounts of Triton X-100 at room temperature revealed two opposing effects on the electron transfer rate. The maximum value of the rate constant which was $1.4 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ in the absence of detergent, increased to the extremely high value of $5.3 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at 0.05% (w/v) Triton X-100. However, at 1% (w/v) Triton X-100 the rate constant dropped to the range of 10^5 $M^{-1} \cdot s^{-1}$. The effect of monovalent and divalent ions on the rate constant was used to characterize the electrostatic interaction between plastocyanin and the oxidizing site of Photosystem I under the following three conditions. (i) In intact membranes, the rate constant increased with increasing salt concentration as would be expected from the negative charges on both plastocyanin and Photosystem I. (ii) In the presence of 0.05% Triton X-100, the rate constant decreased with increasing salt concentrations, indicating two oppositely charged reactants. Extrapolation of the rate constant to infinite salt concentration gave a value of $2.5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ in the absence as well as in the presence of 0.05% Triton X-100. It is concluded that Triton X-100 solubilizes at this concentration a negatively charged component leaving a positively charged oxidizing site of Photosystem I, but does not change the local structure which is essential for efficient reaction with plastocyanin. (iii) In the presence of 1% Triton X-100, high cation concentrations (more than 0.5 M) were found to stimulate the severely inhibited rate constant to values of 10^6 $M^{-1} \cdot s^{-1}$. The effect of different cations indicates an about 10-times more negative surface charge density than in inside-out vesicles. The data are consistently described by a subunit which provides a positive charge at the highly negative oxidizing site on the Photosystem I reaction center protein, thereby facilitating the fast electron transfer from plastocyanin, and is removed by high Triton X-100 concentrations. The identity of the subunit and its occurrence in maize is discussed.

Introduction

The reaction center complex of PS I in higher plants is assembled from the reaction center protein of two almost identical subunits with 60–70 kDa and at least six additional polypeptides with molecular weights between 25 and 5 kDa which give PS I the full competence for the light-induced electron transfer (for recent reviews, see Refs. 1

Abbreviations: Chl, chlorophyll; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; octylglucoside, *n*-octyl-ß-D-glucopyranoside; PG, phosphatidylglycerol; PS I, Photosystem I; PS II, Photosystem II; P-700, reaction center chlorophyll *a* of Photosystem I; SQDG, sulphoquinovosyldiacylglycerol; Triton X-100, polyoxyethylene(9,10) *p-t*-octylphenol.

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and 2). P-700, the primary electron donor of PS I, is localized in the reaction center protein near the lumenal surface of the thylakoid membrane. Therefore, P-700 is accessible to exogenous plastocyanin in inside-out vesicles or after detergent fractionation of the thylakoid membranes but not in intact thylakoids or in vesicles with right-side out orientation of the membrane [3]. The method of membrane disruption strongly affects the electron transfer from plastocyanin to $P\text{-}700^+$. Three different ranges for the second-order rate constant of this reaction have been reported $(0.8\text{–}1.5) \cdot 10^8$ $M^{-1} \cdot s^{-1}$, $(1\text{–}2) \cdot 10^7$ $M^{-1} \cdot s^{-1}$, and $(2\text{–}3) \cdot 10^5$ $M^{-1} \cdot s^{-1}$ in digitonin particles of PS I [4–6], in sonicated chloroplasts and Triton X-100 particles of PS I [4,7,8], and in highly purified PS I reaction centers [9–11], respectively. These differences are not yet understood. It is also not known if any of these preparations conserve the in vivo environment of the reaction site of P-700, which is important for studies of functional details of the molecules. Changes of the local lipid or protein composition cannot be excluded after detergent fractionation [12,13]. In inside-out vesicles the reaction site of P-700 is exposed to the external medium in the absence of detergent [3,14]. We have used inside-out vesicles isolated from thylakoid membranes with randomized distribution of the complexes [15], since those from normal grana thylakoids contain only minor amounts of PS I [16].

Important for a characterization of the reaction site of plastocyanin at the PS I complex is the effect of charges on the electron transfer. At pH values above five both species are negatively charged as inferred from the isoelectric points of plastocyanin from spinach, of inside-out vesicles and of PS I particles, which are 3.0 [17] to 3.9 [18], 4.1 [19] and 4.9 [20] to 5.0 [21], respectively. This aspect has been studied by the effect of salt on reactions of PS I [6–8, 22–24] and analyzed in terms of the Debye-Marcus theory [25,26] or of the Gouy-Chapman theory (Ref. 23, cf. Refs. 27–29) of a diffuse layer of ions. In addition, the effect of local charges on the electron transfer has been considered for plastocyanin [7,20] and for the analogous cytochrome *c* [30,31]. The reported effects of cations on the electron-transfer range from a large stimulation by 3 and 30 mM of divalent and monovalent cations, respectively, [6–8,32] to a negligible effect of 200 mM monovalent cations [11]. This indicates different situations at the oxidizing site of PS I in thylakoid membranes [33,34] and PS I particles [4,6–11,22] as reflected in the rate constant. We have studied the effect of different salts to compare and characterize different states of the oxidizing site of PS I.

Another problem in the understanding of the reaction between plastocyanin and P-700 is the function of a subunit of PS I, termed subunit III, by Bengis and Nelson [35]. This subunit with an apparent molecular weight of 18–20 kDa is removed by 1% Triton X-100 during the isolation of the PS I complex. The removal was accompanied by an inhibition of the reduction of $P\text{-}700^+$ by plastocyanin [35]. This slow electron transfer is stimulated by a high concentration of 100 mM magnesium ions similar to that in highly purified PS I reaction centers [9,10] and isolated PS I from *Chlamydomonas* [36]. It has been proposed that subunit III provides the proper conformation for the binding of plastocyanin [5] and for the rapid electron transfer to $P\text{-}700^+$ [33]. However, direct evidence for a function of subunit III is missing (cf. Ref. 18). Recently, Nechushtai et al. [37] concluded from the cross-reactivity of antibodies that all subunits of PS I found in C3-plants, except subunit III, are present in PS I isolated from chloroplasts of the C4-plant maize. If subunit III is indeed necessary for an efficient electron transfer from plastocyanin to $P\text{-}700^+$ in C3-plants [35,5], then either plastocyanin or the PS I reaction center protein of maize should be considerably different from that of C3-plants. We have tested this possibility.

Materials and Methods

Spinach leaves were obtained either from the local botanical garden or from the local market. Inside-out vesicles were isolated from spinach thylakoids after destacking and lateral randomization of the integral complexes essentially as described by Andersson et al. [15]. The inside-out vesicles were collected by centrifugation for 30 min at $40\,000 \times g$ from the bottom phase at 3°C after three phase partitions in a system made from

5.8% (w/v) polyethylene glycol 4000 and 5.8% (w/v) dextran T-500. The concentrations were optimized for a maximal difference of the Chl *a*-to-Chl *b* ratio between the top and the bottom phase after press treatment of stacked thylakoids [14]. The pellet was washed twice with 100 mM sorbitol to remove salts. Stroma lamellae vesicles were isolated from spinach thylakoids with stacked membranes as described for the Y-100 fraction [14]. After two passages of the thylakoids, previously kept for 30 min on ice in 150 mM NaCl and 50 mM sodium phosphate (pH 7.4), at 9.81 MPa through a Yeda press the stroma lamellae were collected from the supernatant of a centrifugation at $40\,000 \times g$ for 30 min by a centrifugation at $100\,000 \times g$ for 90 min. All chemicals were of analytical grade. Polyethylene glycol 4000 was purchased from Union Carbide, Dextran T-500 from Pharmacia, Triton X-100 and octylglucoside from Sigma.

Incubation with Triton X-100 or octylglucoside was at room temperature (22–24° C) for 2.5 min in the sample cuvette at 10 μg Chl/ml immediately before the measurements. The concentration of Triton X-100 is given as g per 100 ml solution, i.e., % (w/v) in all experiments. No measurement was carried out later than 10 min after the addition of Triton X-100. The measuring solution contained vesicles or chloroplasts at chlorophyll concentration of 10 μg/ml, 1 mM sodium ascorbate, 0.1 mM DAD, 0.2 mM methyl viologen, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes) buffer (pH 7.0), 0.1 M sorbitol and 2 μM plastocyanin. Deviating conditions are given in the figure legends. The 1 × 1 cm cuvette contained a 3 ml sample. The absorbance changes of P-700 were measured at 703 nm after excitation with a flash of 2 μs duration (FWHM) as described previously [33], except that the signal was digitized in a transient recorder (Datalab DL 912) and directly transferred to a mini-computer (Hewlett Packard A-700) and averaged. Triggering was controlled by the computer. The electrical bandwidth ranged from d.c. to 0.1 MHz or to 0.3 MHz if the time resolution was 5 μs per address.

Maize was soaked for one day, kept for 7 days on vermiculite in the dark, grown with a cycle of 14 h light and 8 h dark and harvested after 12–14 days. Isolation of chloroplasts from mesophyll cells followed a given procedure [38]. Plastocyanin was isolated from thylakoids of spinach, pea, maize, and poplar after two freeze-thaw cycles from the supernatant of the pelleted chloroplasts by ion-exchange chromatography on DEAE cellulose (DE52, Whatman) using a gradient of 80–300 mM NaCl, followed by gel filtration with Sephadex G 75. The purity as determined from the ratio of the absorbance at 278 nm to that at 597 nm was between 1.2 and 1.4. The concentration of plastocyanin was determined after oxidation with ferricyanide using an extinction coefficient of 4.9 mM at 597 nm [39]. Isoelectric focusing was performed with an Ultrophor apparatus (LKB) at 10° C in a polyacrylamide gel with a pH gradient formed from two different mixtures of immobilines with pK 3.6, 4.6 and 6.2 (LKB). The pH gradient was examined with the low p*I* Calibration Kit (pH 2.5–6.5) by neglecting a possible shift at 10° C in the isoelectric points of the marker proteins given for 24° C (Pharmacia).

Results

Fig. 1A shows the reduction kinetics of P-700$^+$ following flash excitation of an inside-out vesicle preparation isolated from thylakoid membranes with randomized distribution of the integral complexes. The half-time $t_{1/2}$ of the fast component of the biphasic time course is 3.9 ms in the presence of 2 μM plastocyanin and 1 mM $MgSO_4$. In several experiments (not shown) we verified our preliminary result [40] that the reciprocal value of the half-time of this kinetic component is proportional to the plastocyanin concentration up to 80 μM. Thus, the second-order rate constant k_2 of the electron transfer from plastocyanin to P-700$^+$ in the absence of detergent can be estimated ($k_2 = \ln 2/([\text{plastocyanin}] \cdot t_{1/2})$) as $8.9 \cdot 10^7$ $M^{-1} \cdot s^{-1}$. The relative amplitude of the fast component represents 50% (in other preparations up to 65%) of the total P-700 amplitude and indicates the fraction of total P-700$^+$ accessible to added plastocyanin [3]. The half-time of the slow component of about 30 ms did not depend on the concentration of added plastocyanin.

Fig. 1B shows the kinetics of the P-700 absorbance changes after addition of 0.05% Triton X-100 to the sample used for the measurement in

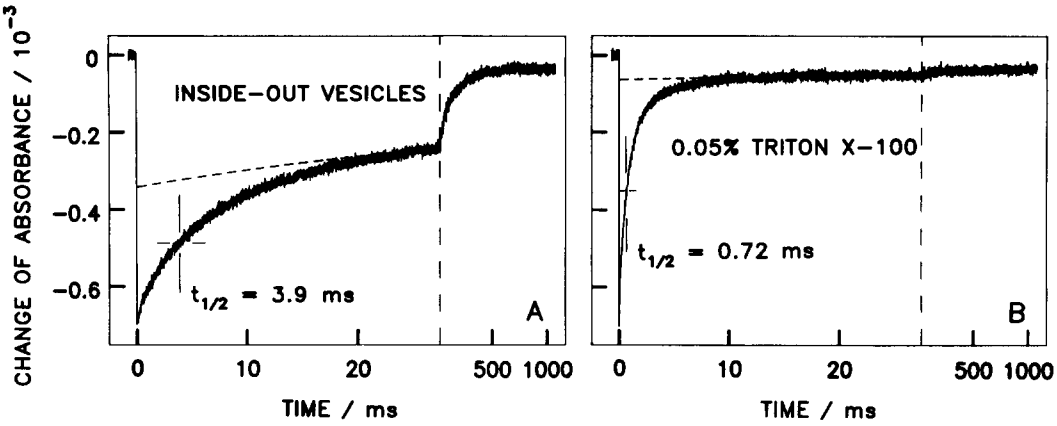

Fig. 1. Reduction of P-700$^+$ by plastocyanin after a short flash in an inside-out vesicles fraction isolated from thylakoids with uniform distribution of the integral membrane complexes. To minimize additional ions a buffer was omitted from the reaction mixture with 1 mM $MgSO_4$. A pH of 6.8 ± 0.05 was determined in the samples. 20 signals were averaged at a repetition rate of 0.3 Hz. At the time indicated by the vertical dashed line the time per address was switched from 10 μs to 1 ms. The extrapolation of the slow component was derived from an exponential fit of the time-course. (A) Inside-out vesicle fraction, plastocyanin 2 μM, gramicidin D 1.5 μM. The half-time of the fast component is 3.9 ms. (B) Sample used for A after incubation with 0.05% Triton X-100 for 2.5 min in the sample cuvette. The half-time of the fast component of 0.72 ms is indicated.

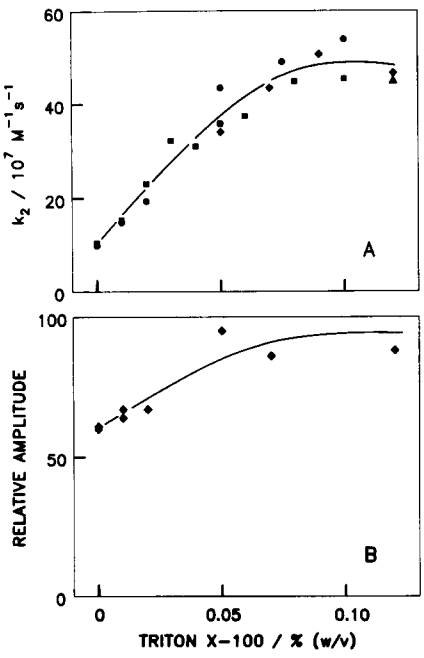

Fig. 2. Effect of the Triton X-100 concentration on the reduction of P-700$^+$ by 2 μM plastocyanin in an inside-out vesicle fraction. (A) Rate constant. (B) Relative amplitude. The reaction mixture contained in addition 2 mM $MgSO_4$. The different symbols indicate different samples of inside-out vesicles.

Fig. 1A. The electron transfer from plastocyanin to P-700$^+$ is considerably faster than that in the inside-out vesicles. About 90% of the total signal in Fig. 1B follows a rapid pseudo first-order time-course. The half-time of 0.72 ms indicates the very high second-order rate constant of $4.8 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ of the electron transfer from plastocyanin to P-700$^+$. This effect of Triton X-100 is different from that expected from the low rate constant of $2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ in the presence of Triton X-100 reported previously [4] and was therefore investigated in more detail.

Fig. 2A shows the second-order rate constant of the electron transfer from plastocyanin to P-700$^+$ as a function of the concentration of Triton X-100 in the presence of 2 mM $MgSO_4$. The rate constant increases from a value of 10^8 $M^{-1} \cdot s^{-1}$ in the inside-out vesicles almost proportional to the concentration up to 0.08% Triton X-100. At concentrations of Triton X-100 higher than 0.5% (not shown) the rate constant decreases from its maximal value of $5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$. The amplitude of the fast component of the P-700$^+$ reduction as a function of the Triton X-100 concentration is shown in Fig. 2B. The relative amplitude increases from about 60% of the total signal amplitude in the inside-out vesicle preparation in parallel to the

rate constant to about 94% at 0.05% Triton X-100 and decreases again at Triton X-100 concentrations higher than 0.12%. The increase in the amplitude indicates that inaccessible P-700 probably in contaminating right-side out vesicles becomes accessible to exogenous plastocyanin. Incubation with 0.05% Triton X-100 made P-700 also accessible to exogenous plastocyanin in stromal lamellae isolated after Yeda-press treatment of thylakoids (Y-100 fraction) as well as in intact thylakoids (data not shown). We have also compared the effect of Triton X-100 with that of octylglucoside and found almost the same dependencies as those shown in Fig. 2, except that the concentrations (% w/v) of octylglucoside had to be 20-times higher than those of Triton X-100. The major reason for the difference in the effect of the two detergents is likely to be the critical micelle concentration, which is 0.015% (0.24 mM) and 0.73% (25 mM) of Triton X-100 and octylglucoside, respectively [41].

Fig. 3 shows the reduction kinetics of P-700$^+$ 2 min after addition of 1% Triton X-100 at room temperature. The initial portion of the signal shows at a high time resolution that the fast decay seen in the presence of 0.05% Triton X-100 in Fig. 1B is converted to an extremely slow decay with a half-time $t'_{1/2}$ of 275 ms recorded at the 200-times slower dwell time of the second portion. The reduction is primarily due to plastocyanin used at a concentration of 4.8 μM in the presence of 10 mM $MgSO_4$. DAD is used as mediator for the slow electron transfer from ascorbate to plastocyanin [42]. Its contribution to the reduction of P-700$^+$ with a halftime $t_{1/2}^{DAD}$ of 1.2 s is subtracted to determine the halftime $t_{1/2}^{plastocyanin}$ of plastocyanin by $1/t_{1/2}^{plastocyanin} = 1/t'_{1/2} - 1/t_{1/2}^{DAD}$.

The changes in the rate of the plastocyanin-P-700 reaction due to Triton X-100 could originate from changes of the structure as well as of the charges at PS I. The effect of the surface charge density on the reaction rate can be characterized by the addition of different salts (cf., e.g., Refs. 23 and 28). We have used salts of monovalent and divalent cations, including $MgSO_4$, $MgCl_2$, $CaCl_2$, NaCl and KCl. Fig. 4 shows the rate constant of P-700$^+$ reduction by plastocyanin in inside-out vesicles as a function of these cations. The rate constant increases from $1.8 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ to a maximal value of $1.4 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at increasing concentrations from 0.2 to about 6 mM and from 2 to 65 mM of divalent and monovalent cations, respectively. At higher concentrations the rate constant decreases again. For comparison we have measured the electron transfer from plastocyanin to P-700 after sonication of stroma lamellae vesicles (Y-100) at different $MgSO_4$ concentra-

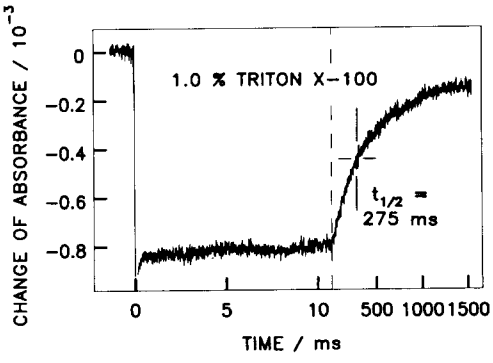

Fig. 3. Reduction of P-700$^+$ by plastocyanin after a short flash in an inside-out vesicles fraction 2.5 min after increasing the Triton X-100 concentration to 1% in a sample used previously for a measurement similar to that in Fig. 1B, except that the concentration of plastocyanin was 4.8 μM and $MgSO_4$ 10 mM. 35 signals were averaged at a repetition rate of 0.16 Hz. At the time indicated by the vertical dashed line the time per address was switched from 5 μs to 1 ms.

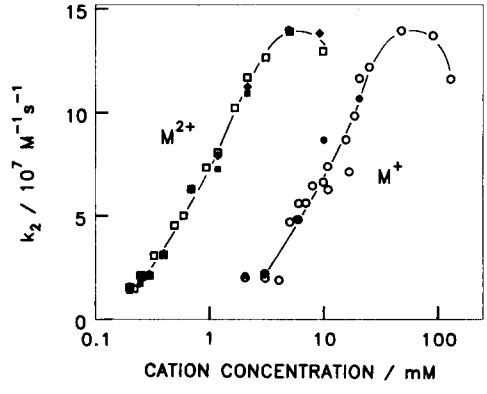

Fig. 4. Rate constant of the reduction of P-700$^+$ by plastocyanin in inside-out vesicles from thylakoids with uniform distribution of the membrane complexes as a function of the concentration of monovalent and divalent cations. The reaction mixture contained 2 μM plastocyanin; ○, NaCl; ●, KCl; □, $MgSO_4$; ■, $MgCl_2$; ◆, $CaCl_2$. The given concentration of monovalent cations includes 1 mM sodium ascorbate, that of divalent cations 0.2 mM methyl viologen both present in all samples.

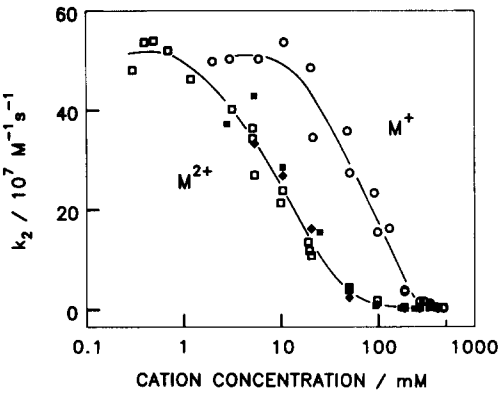

Fig. 5. Rate constant of the reduction of P-700$^+$ by plastocyanin in stroma lamellae vesicles incubated with 0.05% Triton X-100 as a function of the concentration of monovalent and divalent cations. Measuring conditions as in Fig. 1B, symbols as in Fig. 4.

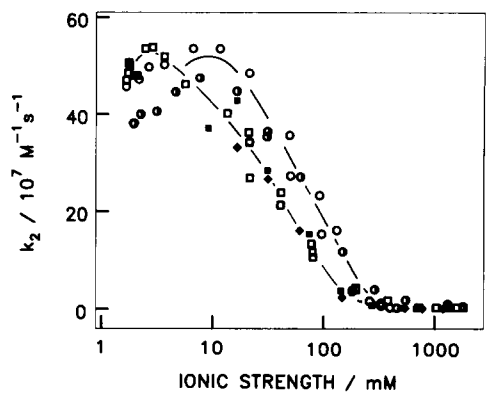

Fig. 6. Rate constant of the reduction of P-700$^+$ by plastocyanin in the presence of 0.05% Triton X-100 as a function of the ionic strength in the measuring solution. The values are taken from Fig. 5, except for those measured in the presence of Na_2SO_4 (Φ), other symbols as in Figs. 4 and 5.

tions. The reduction rate of the accessible fraction of total P-700 is the same as that observed in inside-out vesicles within the error of the experiments.

Fig. 5 shows the effect of the cation concentration on the electron transfer from plastocyanin to P-700$^+$ in stroma lamellae vesicles in the presence of 0.05% Triton X-100. The rate constant shows a remarkably high value of $5.3 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at the low concentrations of 0.5 mM and 10 mM of divalent and monovalent cations, respectively. Increasing cation concentrations decrease the rate constant in contrast to the effect in Fig. 4. At 0.4 and 0.5 M divalent and monovalent cations, respectively, its value was as low as $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$.

The reaction rate of molecules carrying point charges is related to the ionic strength as described by the Bronsted-Debye-Hückel theory. To test whether this relation holds for the data in Fig. 5 and to compare the effect of anions and cations on the reaction rate we have plotted these data as a function of the ionic strength in Fig. 6 and included measurements in the presence of Na_2SO_4 as an additional salt with a divalent anion. The values of the rate constant measured in the presence of Na_2SO_4 and NaCl follow a curve above those measured in the presence of $MgSO_4$, $MgCl_2$ and $CaCl_2$. This is not consistent with an interaction between simple point charges, and indicates a more pronounced effect of divalent cations than that of divalent anions.

After incubation of stroma lamellae vesicles with 1% Triton X-100 the rate constant of the reduction of P-700$^+$ by plastocyanin shows the effect of the cation concentration of Fig. 7. Monovalent cations up to a concentration of 180 mM do not stimulate a detectable reduction by plastocyanin. The data are corrected for the reduction by DAD. The rate constant of the P-700$^+$ reduction by the uncharged DAD with an average value $5.8 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ ($\pm 0.5 \cdot 10^3$ $M^{-1} \cdot s^{-1}$, 16 measurements) did not depend on the salt concentration (experiments not shown). Only high concentrations of cations stimulate the electron transfer from plastocyanin to P-700$^+$. At the extreme concentration of 3.4 M NaCl the value of the rate constant was not greater than 10^6 $M^{-1} \cdot s^{-1}$ and the value of $1.85 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ at 530 mM $MgSO_4$ is still two orders of magnitude smaller than the maximal values in Figs. 4 and 5. In all of our experiments we saw no evidence for effects of specific cations or anions.

The Debye-Marcus theory predicts that the logarithm of the rate constant for the reaction of a charged species at a charged membrane surface is proportional to the square root of the reciprocal concentration of a symmetrical monovalent salt above a certain level of about 10 mM (cf. Ref. 23).

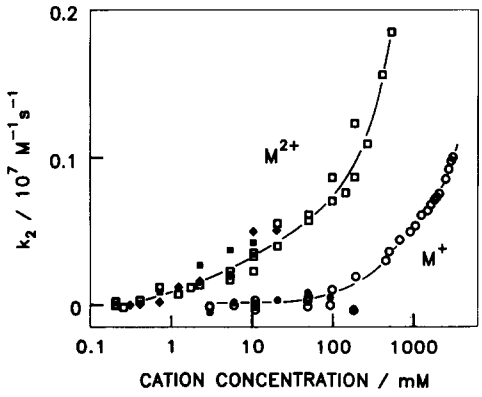

Fig. 7. Rate constant of the reduction of P-700$^+$ by plastocyanin in stroma lamellae vesicles incubated with 1% Triton X-100 as a function of the concentration of monovalent and divalent cations. The reduction of P-700$^+$ by DAD was subtracted as given in the text. Measuring conditions as in Fig. 3, symbols as in Fig. 4.

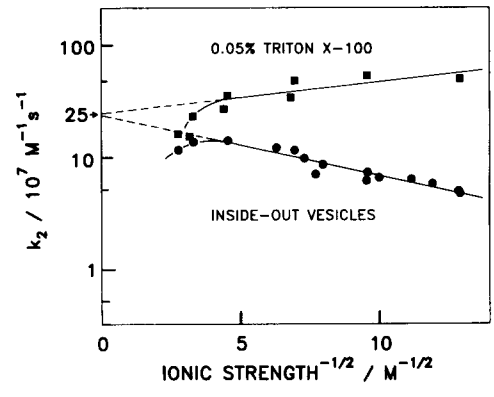

Fig. 8. Plot of the apparent rate constant of the electron transfer from plastocyanin to P-700$^+$ on a logarithmic scale as a function of the reciprocal square root of the concentration of monovalent salt. The values measured in the presence of NaCl are taken from Fig. 4 (circles) and from Fig. 5 (squares).

Fig. 8 shows that this relation holds indeed for the data of Figs. 4 and 5 measured in the presence of NaCl, except for concentrations greater than 60 mM where the values of the rate constant in Fig. 4 start to decrease after reaching a maximum. The slope found in the presence of 0.05% Triton X-100 indicates a density of positive surface charges at PS I with 45% the density of the negative surface charges in inside-out vesicles. Extrapolation to infinite concentrations (zero on the abscissa) where the effect of charges on the reaction rate becomes negligible indicates the same rate constant of $2.5 \cdot 10^8\ M^{-1} \cdot s^{-1}$ for both experiments.

To test for a possible absence of subunit III of PS I in C4-plants [37] we have compared the reduction of P-700$^+$ in thylakoids from spinach with that in mesophyll chloroplasts from maize

TABLE I

ELECTRON TRANSFER FROM PLASTOCYANIN TO P-700$^+$ IN THE PRESENCE OF TRITON X-100 IN THYLAKOIDS OF SPINACH AND MAIZE

The rate constants have been estimated assuming the same extinction coefficient at 597 nm for plastocyanin from maize as for plastocyanin from spinach. The half-time of P-700$^+$ reduction in the absence of plastocyanin was 0.16 and 0.6 s in spinach and maize thylakoids, respectively, in the presence of 0.05% Triton X-100. Measurements were performed at 2.5 and 5 μM spinach plastocyanin and at 1.5 and 3 μM maize plastocyanin, respectively. 4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer was 20 mM (pH 7.5), KCl 10 mM. Thylakoids instead of inside-out vesicles have been used. 0.05% Triton X-100 is sufficient to make total P-700 accessible to plastocyanin (cf. Fig. 2B and text).

Source of		Triton X-100 (% (w/v))	Fast component of P-700$^+$-reduction	
thylakoids	plastocyanin		rate constant ($M^{-1} \cdot s^{-1}$)	relative amplitude (%)
spinach	spinach	0.05	$1.6 \cdot 10^8$	80
spinach	maize	0.05	$2.0 \cdot 10^8$	80
spinach	spinach	1.05	$2.1 \cdot 10^8$	15
maize	spinach	0.05	$3.2 \cdot 10^8$	63
maize	maize	0.05	$3.4 \cdot 10^8$	59
maize	spinach	0.44	$2.4 \cdot 10^8$	13
maize	spinach	0.83	$2.0 \cdot 10^8$	8

TABLE II

ISOELECTRIC POINTS OF PLASTOCYANIN

Plastocyanin was isolated from all plants as described in Materials and Methods for spinach. Isoelectric focusing was on a polyacrylamide gel with a gradient from pH 3.6 to 4.4.

Source	Isoelectric point
Spinach	3.97
Pea	3.94
Maize	3.92
Poplar	3.93
Poplar (major band)	3.905

using plastocyanin either from spinach or from maize. The results are summarized in Table I. The rate constant shows no significant difference between the two species, except that the rate constants of plastocyanin from maize are slightly higher than those of plastocyanin from spinach. But the fraction of total P-700 which is rapidly reduced by plastocyanin is smaller in thylakoids from maize as compared to thylakoids from spinach at 0.05% Triton X-100 and it decreases more rapidly after addition of the detergent (not shown) or at increasing Triton X-100 concentrations. Binding of the subunit needed for rapid electron transfer from plastocyanin to PS I appears to be more sensitive to Triton X-100 in maize than it is in spinach.

Differences in the structure and charges between plastocyanins from maize and spinach might be expected to be reflected in different isoelectric points. Our measurements of the isoelectric points at a resolution of about 0.005 pH units are shown in Table II and include for comparison the values of plastocyanin from pea and from poplar (*Populus nigra* var. Italica). The value of plastocyanin from maize is only 0.05 pH units lower than the value of plastocyanin from spinach, and similar to those of the other C3-plants. With poplar plastocyanin we found two bands which differ by 0.025 pH values. This suggests the presence of two different plastocyanins in *Populus nigra* var. Italica. It remains to be shown which one is the crystallized one [43]. A polymorphism of plastocyanin has previously been reported for Compositae [44].

Discussion

Reduction of P-700 by plastocyanin in the absence of detergent

One aim of our investigations was to find conditions at which molecular details of the electron transfer from plastocyanin to P-700 can be studied as they occur in situ. In inside-out vesicles from membranes with randomized distribution of the complexes [15] the environment of P-700 should not be disturbed by possible delipidation which cannot be excluded if detergent is used for membrane fractionation. The flash-induced kinetics of P-700 enabled us to study the fraction of total P-700 which is directly reduced by the exogenous plastocyanin. Any heterogeneity in the reduction of $P\text{-}700^+$ (Fig. 1A) caused by different accessibilities of P-700 due to contaminating right-side out vesicles or structural changes at the reaction site can be quantitatively estimated from the different kinetic components. The flash-induced electron transfer is not limited by any other reaction which may occur during fast turnover in strong light (cf. Ref. 18).

The rate of electron transfer from plastocyanin to $P\text{-}700^+$ in the inside-out vesicles increased with increasing cation concentration (Fig. 4). This implies shielding of the negative surface charges by cations and a decrease in the repelling electrostatic interaction as described by the Gouy-Chapman theory (cf. Refs. 27 and 28). Using the concentrations of monovalent and divalent cations corresponding to the same rate constant Eqn. 4 yields a value of $-0.023\ C \cdot m^{-2}$ for the surface charge density. The maximal value of the second-order rate constant of $k_2 = 1.4 \cdot 10^8\ M^{-1} \cdot s^{-1}$ in inside-out vesicles is found at 6 and 65 mM of divalent and monovalent cations, respectively. An effect of the anions Cl^- or SO_4^{2-} or a specificity of Ca^{2+} or Mg^{2+} is not observed in Fig. 4, consistent with other investigations of PS I [6,7]. The salt concentrations at which the rate constant starts to decrease are similar to those in PS I particles isolated with digitonin [6,7,23]. This effect is not fully understood and has been discussed in terms of an additional short range interaction between species of asymmetric charge distribution and dipole-dipole interaction [20,25,26].

The value of the surface charge density in our

inside-out vesicles is higher than the values of -0.015 [24] and -0.0108 C · m^{-2} [7] reported for the reduction of P-700$^+$ in sonicated chloroplasts by ferricyanide and in PS I particles by plastocyanin, respectively, and it is lower than the value of -0.037 C · m^{-2} estimated for the inner surface of appressed membranes in the vicinity of PS II [45]. Therefore, the value estimated from our data could be due to an averaged surface charge density resulting from the random distribution of the two photosystems in the inside-out vesicles. However, we investigated also the reduction of P-700$^+$ by plastocyanin as a function of salt concentrations in sonicated stroma membranes isolated as Y-100 vesicles after Yeda press fractionation of stacked thylakoid membranes [14]. First experiments (not shown) did not indicate differences between the reaction rate of P-700 in these vesicles almost devoid of PS II [16] and in the inside-out vesicles in Fig. 3. This suggests that it is the local surface charge density at PS I which controls the reaction rate of plastocyanin.

Effect of low Triton X-100 concentration on the reduction of P-700 by plastocyanin

The electron transfer from plastocyanin to P-700$^+$ shows a maximal rate constant of $5.3 \cdot 10^8$ M$^{-1} \cdot$ s^{-1} in the presence of 0.05% Triton X-100 which is 3-times higher than the values reported previously [5,6,46]. The value is close to $8.2 \cdot 10^8$ M$^{-1} \cdot$ s^{-1} for the reaction between cytochrome c_2 and the reaction center of *Rhodobacter sphaeroides* [47], which is the fastest known reaction rate between proteins. Previous investigations have reported a lowering of the electron-transfer rate by Triton X-100. Different from the isolation procedures of PS I [48] or PS II [49] at 0–4 °C, 2–3 mg Chl/ml and a ratio of Triton X-100/Chl of about 20–30 for extended periods, which is sufficient to solubilize the PS I complex from the membrane [50], our incubation was for a few min at room temperature at an about three times higher molar ratio of Triton X-100/Chl. Under our conditions, Triton X-100 as well as octylglucoside (data not shown) increase the rate constant of the reduction of P-700$^+$ and its accessibility below their critical micelle concentration (Fig. 2).

The decrease in the fast reaction rate which is seen with increasing salt concentration (Fig. 5) is opposite to the effect in inside-out vesicles. This is evidence that Triton X-100 induces a change from electrostatic repulsion to attraction between the reactants (see also Fig. 2A). Since plastocyanin with its nine negative charges [51] cannot be made positive by the neutral detergent, a change in the charge from negative to positive at the oxidizing site of PS I must occur. This could be due either to removal of a loosely bound subunit of PS I or to dispersal of negatively charged lipid molecules. Possible candidates for negative lipid molecules are PG which has been suggested to function in the regulation of electron transfer into PS I [52] and SQDG which is preferentially located on the inner leaflet of the thylakoid membrane (cf. Ref. 53). SQDG has been shown to be specifically associated with other complexes as the ATP synthase [54] and the light-harvesting complex [55].

We have analyzed the changes at PS I in Fig. 6 and found that the electron-transfer rate is more inhibited by divalent cations than by divalent anions. This is evidence that shielding of negative surface charges by cations is a dominating effect in controlling the reaction rate. The absolute value of the negative surface charge density at plastocyanin may be higher than that of the positive one at PS I. Using the molecular dimensions of poplar plastocyanin [43] an averaged density of -0.174 charges per nm^2 on the surface or -0.028 C · m^{-2} can be estimated for spinach plastocyanin.

A more quantitative analysis is possible by the Debye-Marcus theory which is based on the idea that a rapid tumbling protein presents a spherically averaged field of its net charge Z_p at a large distance from a charged membrane. For monovalent symmetrical salts of the concentration $c_{1,1}$ in the bulk solution the simplified relation of Gouy-Chapman between the surface potential and the surface charge density σ may be used as described by Itoh [23] for the rate constant k_2 of the P-700$^+$ reduction if the salt concentration is not too low:

$$\ln k_2 = \ln k_2^0 - \text{const.}\ Z_p \sigma c_{1,1}^{-1/2} + \ln f'$$

where f' is the ratio of the activity coefficient of plastocyanin in the bulk solution to that at the membrane surface. Fig. 8 shows for the data in Fig. 4 and 5 that the logarithm of the rate constant is indeed proportional to the square root of

the reciprocal concentration of NaCl above 5 mM, except for concentrations greater than 60 mM. At any given cation concentration all parameters can be assumed to be the same in both experiments, except for the surface charge density at PS I. Thus, the slopes of the two lines give the relative values of the surface charge densities and indicate that in the presence of 0.05% Triton X-100 the density of positive surface charges at PS I is 45% of the density of the negative surface charges in inside-out vesicles. Another interesting result is that the two lines extrapolate to the same rate constant of $2.5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at a negligible effect of charges. The extrapolation suggests also that apart from the charges the protein conformation at the oxidizing site of PS I was not changed by the low Triton X-100 concentration.

The maximal rate constant between uncharged particles is estimated by the Smoluchowski approach to a diffusion-controlled reaction, $k_2 = 4\,RT/3\eta$, where η is the viscosity coefficient. Relative to the theoretical value of $32 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ for one component at 25 °C, the rate constant of $2.5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ for the oxidation of plastocyanin indicates that about 8% of its encounters with PS I result in an electron transfer. This is a remarkably high value because a reactive amino acid residue represents a fraction smaller than 8% of the total protein surface. We conclude that a hydrophobic interaction and/or local charges are very effective in orienting the plastocyanin molecule (cf., e.g., Ref. 56).

Properties of the oxidizing site of PS I at high Triton X-100 concentrations

Incubation of thylakoids with Triton X-100 at a high concentration of 1% inhibits the electro transfer from plastocyanin to $P\text{-}700^+$ in the presence of 2 mM $MgSO_4$ by more than 3 orders of magnitude. A low value of the second-order rate constant in the range of $3 \cdot 10^5$–10^6 $M^{-1} \cdot s^{-1}$ and a stimulation by high salt concentration appears to be characteristic for the isolated PS I reaction center protein or highly purified PS I complexes [11,32,35,57]. The stimulation of the slow rate by increasing cation concentration (Fig. 7) is in contrast to the effect on the rate at 0.05% Triton X-100 (Fig. 4). Following the same line of arguments as above we conclude that the net charge on the oxidizing site of PS I has been changed from positive to negative. These changes indicate that a subunit of PS I with positive charges is removed. An extremely negative surface charge density at the oxidizing site of PS I of -0.38 $C \cdot m^{-2}$ can be estimated from the data in Fig. 7 and Eqn. 4 in the appendix. A strong repulsion of plastocyanin is inferred which is likely to cause the slow electron transfer to purified PS I reaction center protein. Clustering of negative charges is not obvious from the amino acid sequence derived for the two homologous PS I reaction center protein genes [58] and the predicted 10–12 transmembrane α-helices [59]. It may be local clustering of negative charges due to protein folding of one or more exposed segments which constitute the electron-transfer site near P-700.

Function of a subunit with positive charges at the oxidizing site of PS I

Our data indicate that a subunit with positive charges is necessary for a rapid electron transfer from plastocyanin to the oxidizing site of the PS I reaction center protein. This is consistent with the function of subunit III proposed by Bengis and Nelson [35], but it is in contradiction to the conclusion that plastocyanin donates electrons directly to the largest peptide of the PS I reaction center complex with a minor role of the small PS I subunits [10,11]. The low value of the rate constant reported in the latter studies [10,11] suggests that the subunit involved in the electron transfer from plastocyanin might be lost during the isolation of the PS I complex.

By comparing the reaction of plastocyanin with PS I of spinach with that of maize we find no evidence for an essential difference between the C3- and the C4-plant neither from the reaction rates nor from the isoelectric point of the plastocyanins which differ by not more than 0.05 pH units. The suggestion that subunit III is absent in chloroplasts of maize [37] was based on the cross-reactivity of antibodies raised against PS I subunits of C3-plants. Our results suggest for example that either another small subunit with binding properties similar to those of subunit III can be the subunit for efficient electron transfer from plastocyanin to P-700 or that the antigenic but not the functional site of subunit III might be differ-

ent in C4-plants from that in C3-plants.

Our data are consistently described by a subunit of PS I which provides a positive charge at the oxidizing site of plastocyanin. The effect of the positive charge is prerequisite for the fast electron transfer to P-700 at the reaction center protein with an extremely negative reaction site. In intact thylakoid membranes the positive subunit is associated with a negatively charged component which does not change the structure at the electron-transfer site

Appendix

The relationship between the electrical potential Ψ_s at the surface of a biological membrane and the surface-charge density σ resulting from negative lipids and amino-acid residues of integral and peripheral proteins is given by the equation derived by Gouy and Chapman (cf. Refs. 27 and 28)

$$\sigma = \pm\left\{2\varepsilon_r\varepsilon_0 RT\sum_i c_i(e^{-z_i F\Psi_s/RT}-1)\right\}^{1/2} \tag{A-1}$$

where ε_r is the permittivity of the solution relative to that of the vacuum, ε_0, c_i is the concentration of the ith ion in the bulk solution carrying the charge z_i. Our solution is composed from a mixture of symmetrical monovalent and divalent electrolytes with concentrations $c_{1,1}$ and $c_{2,2}$, respectively. This simplifies Eqn. 1 to

$$\sigma = \pm(4\varepsilon_r\varepsilon_0 RT)^{1/2}\left\{2c_{2,2}\left(\cosh^2\frac{F\Psi_s}{RT}-1\right)+c_{1,1}\left(\cosh\frac{F\Psi_s}{RT}-1\right)\right\}^{1/2} \tag{A-2}$$

(Eqn. 12 of Ref. 29 is erroneous). If we assume that the surface potential controls the reaction rate, for example, by controlling the actual concentration of the charged plastocyanin at the reaction site, the surface potential should be the same in the experiments (labeled by single and double primes ′ and ″), where the same rate constant k_2 is found but different mixtures of electrolytes $c'_{1,1}$ plus $c'_{2,2}$ and $c''_{1,1}$ plus $c''_{2,2}$ have been used. Thus, the surface potential is derived from Eqn. 2 using the rules for hyperbolic functions

$$\Psi_s = \frac{RT}{F}\operatorname{arccosh}\left\{\frac{c''_{1,1}-c'_{1,1}}{2(c'_{2,2}-c''_{2,2})}-1\right\} \tag{A-3}$$

Combining Eqns. 2 and 3, one obtains

$$\sigma = \pm(8\varepsilon_r\varepsilon_0 RT)^{1/2}\left(c'_{2,2}\left[\left\{\frac{c''_{1,1}-c'_{1,1}}{2(c'_{2,2}-c''_{2,2})}-1\right\}^2-1\right]+c'_{1,1}\left\{\frac{c''_{1,1}-c'_{1,1}}{4(c'_{2,2}-c''_{2,2})}-1\right\}\right)^{1/2} \tag{A-4}$$

Numerical substitution for 25 °C gives for $(8\varepsilon_r\varepsilon_0 RT)^{1/2} = 0.1174$ when σ is measured in $C\cdot m^{-2}$ and the concentrations in $mol\cdot dm^{-3}$. Eqn. 4 is easily solved on a calculator and has been used to analyze our data in Figs. 4, 5 and 6. Measurements in the presence of either monovalent or divalent electrolytes alone [8,11,45] were previously used to estimate the surface-charge density. Under these conditions Eqn. 4 reduces to (cf. Ref. 60)

$$\sigma = \pm(8\varepsilon_r\varepsilon_0 RT)^{1/2}\left(\frac{c_{1,1}^2}{4c_{2,2}}-c_{1,1}\right)^{1/2} \tag{A-5}$$

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References

1 Malkin, R. (1982) Annu. Rev. Plant Physiol. 33, 455–479.
2 Wollman, F.-A. (1986) in Encyclopedia of Plant Physiology, New Ser., Vol. 19, Photosynthetic Membranes and Light Harvesting Systems (Staehelin, L.A. and Arntzen, C.J., eds.), pp. 487–495, Springer-Verlag, Berlin.
3 Haehnel, W., Berzborn, R. and Andersson, B. (1981) Biochim. Biophys. Acta 637, 389–399.
4 Wood, P.M. and Bendall, D.S. (1975) Biochim. Biophys. Acta 387, 115–128.
5 Haehnel, W., Hesse, V. and Pröpper, A. (1980) FEBS Lett. 111, 79–82.
6 Olsen, L.F. and Cox, R.P. (1982) Biochim. Biophys. Acta 679, 436–443.
7 Tamura, N., Itoh, S., Yamamoto, Y. and Nishimura, M. (1981) Plant Cell Physiol. 22, 603–612.
8 Tamura, N., Yamamoto, Y. and Nishimura, M. (1980) Biochim. Biophys. Acta 592, 536–545.
9 Davis, D.J., Krogmann, D.W. and San Pietro, A. (1980) Plant Physiol. 65, 697–702.
10 Bhardwaj, R., Burkey, K.O., Tripathy, B.C. and Gross, E.L. (1982) Plant Physiol. 70, 424–429.
11 Takabe, T., Ishikawa, H., Niwa, S. and Itoh, S. (1983) J. Biochem. 94, 1901–1911.
12 Murphy, D.J. and Prinsley, R.T. (1985) Biochem. J. 229, 31–37.
13 Lyon, M.K. and Miller, K.R. (1984) J. Ultrastruct. Res. 88, 229–243.
14 Andersson, B. and Åkerlund, H.-E. (1978) Biochim. Biophys. Acta 503, 462–472.
15 Andersson, B., Sundby, C. and Albertsson, P.-A. (1980) Biochim. Biophys. Acta 599, 391–402.
16 Andersson, B. and Haehnel, W. (1982) FEBS Lett. 146, 13–17.
17 Siegelman, M.H., Rasched, I.R., Kunert, K.-J., Kroneck, P. and Böger, P. (1976) Eur. J. Biochem. 64, 131–140.
18 Haehnel, W. (1986) in Encyclopedia of Plant Physiology, New Ser., Vol. 19, Photosynthetic Membranes and Light Harvesting Systems (Staehelin, L.A. and Arntzen, C.J., eds.), pp. 547–559, Springer-Verlag, Berlin
19 Åkerlund, H.-E., Andersson, B., Persson, A. and Albertsson, P.-A. (1979) Biochim. Biophys. Acta 552, 238–246.
20 Tamura, N., Itoh, S. and Nishimura, M. (1983) Plant Cell Physiol. 24, 215–223.
21 Burkey, K.O. and Gross, E.L. (1981) Biochemistry 20, 2961–2967.
22 Lockau, W. (1979) Eur. J. Biochem. 94, 365–373.
23 Itoh, S. (1979) Biochim. Biophys. Acta 548, 579–595.
24 Itoh, S. (1979) Biochim. Biophys. Acta 548, 596–607.
25 Takabe, T., Takenaka, K., Kawamura, H. and Beppu, Y. (1986) J. Biochem. 99, 833–840.
26 Takenaka, K. and Takabe, T. (1984) J. Biochem. 96, 1813–1821.
27 McLaughlin, S. (1977) in Current Topics in Membranes and Transport, Vol. 9, (Bronner, F. and Kleinzeller, A., eds.), pp. 71–144, Academic Press, New York.
28 Barber, J. (1982) Annu. Rev. Plant. Physiol. 33, 261–295.
29 Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174–181.
30 Koppenol, W.H. and Margoliash, E. (1982) J. Biol. Chem. 257, 4426–4437.
31 Augustin, M.A., Chapman, S.K., Davies, D.M., Sykes, A.G., Speck, S.H. and Margoliash, E. (1983) J. Biol. Chem. 258, 6405–6409.
32 Lien, S. and San Pietro, A.S. (1979) Arch. Biochem. Biophys. 194, 128–137.
33 Haehnel, W., Pröpper, A. and Krause, H. (1980) Biochim. Biophys. Acta 593, 384–399.
34 Matssura, K. and Itoh, S. (1985) Plant Cell Physiol. 26, 1057–1066.
35 Bengis, C. and Nelson, N. (1977) J. Biol. Chem. 252, 4564–4569.
36 Nechushtai, R. and Nelson, N. (1981) J. Bioenerg. Biomembr. 13, 295–306.
37 Nechushtai, R., Schuster, G., Nelson, N. and Ohad, I. (1986) Eur. J. Biochem. 159, 157–161.
38 Jenkins, C.L.D. and Russ, V.J. (1984) Plant Sci. Lett. 35, 19–24.
39 Katoh, S., Shiratori, I. and Takamiya, A. (1962) J. Biochem. 51, 32–40.
40 Haehnel, W., Ratajczak, R. and Mitchell, R. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. II, pp. 521–524, Martinus Nijhoff, Dordrecht.
41 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79.
42 Wood, P.M. (1974) Biochim. Biophys. Acta 357, 370–379.
43 Colman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M. and Venkatappa, M.P. (1978) Nature 272, 319–324.
44 Boulter, D., Gleaves, J.T., Haslett, B.G., Peacock, D. and Jensen, U. (1978) Phytochem. 17, 1585–1589.
45 Mansfield, R.W., Nakatani, H.Y., Barber, J., Mauro, S. and Lannoye, R. (1982) FEBS Lett. 137, 133–136.
46 Bottin, H. and Mathis, P. (1985) Biochemistry 24, 6453–6460.
47 Prince, R.C., Cogdell, R.J. and Crofts, A.R. (1974) Biochim. Biophys. Acta 347, 1–13.
48 Bengis, C. and Nelson, N. (1975) J. Biol. Chem. 250, 2783–2788.
49 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
50 Morrissey, P.J., McCauley, S.W. and Melis, A. (1986) Eur. J. Biochem. 160, 389–393.

51 Guss, J.M. and Freeman, H.C. (1983) J. Mol. Biol. 169, 521–563.
52 Rawyler, A. and Siegenthaler, P.-A. (1981) Biochim. Biophys. Acta 638, 30–39.
53 Siegenthaler, P.A. and Rawyler, A. (1986) in Encyclopedia of Plant Physiology, New Ser., Vol. 19, Photosynthetic Membranes and Light Harvesting Systems (Staehelin, L.A. and Arntzen, C.J., eds.), pp. 693–705, Springer-Verlag, Berlin.
54 Pick, U., Gounaris, K., Admon, A. and Barber, J. (1984) Biochim. Biophys. Acta 765, 12–20.
55 Barber, J. and Gounaris, K. (1986) Photosynth. Res. 9, 239–249.
56 Berg, O.G. (1985) Biophys. J. 47, 1–14.
57 Burkey, K.O. and Gross, E.L. (1981) Biochemistry 20, 5495–5499.
58 Fish, L.E., Kück, U. and Bogorad, L. (1985) J. Biol. Chem. 260, 1413–1421.
59 Fish, L.E., Kück, U. and Bogorad, L. (1985) in Molecular Biology of the Photosynthetic Apparatus (Steinback, K.E. et al., eds.), pp. 111–120, Cold Spring Harbor, New York.
60 Chow, W.S. and Barber, J. (1980) Biochim. Biophys. Acta 589, 346–352.