Biochimica et Biophysica Acta, 593 (1980) 384—399

BBA 47962

EVIDENCE FOR COMPLEXED PLASTOCYANIN AS THE IMMEDIATE ELECTRON DONOR OF *P*-700

WOLFGANG HAEHNEL, ADELHEID PRÖPPER and HELMUT KRAUSE

Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität Bochum, P.O. Box 102148 D-4630 Bochum 1 (F.R.G.)

(Received June 4th, 1980)

Key words: Plastocyanin; P-700; Cytochrome f; Photosystem I; Electron transfer

Summary

The reduction of *P*-700 by its electron donors shows two fast phases with half-times of 20 and 200 μs in isolated spinach chloroplasts. We have studied this electron transfer and the oxidation kinetics of cytochrome *f*.

Incubation of chloroplasts with KCN or $HgCl_2$ decreased the amplitude of the 20 μs phase. This provides evidence for a function of plastocyanin as the immediate electron donor of *P*-700.

At low concentrations of salt and sugar the fast phases of $P\text{-}700^+$ reduction were largely inhibited. Increasing concentrations of $MgCl_2$, KCl and sorbitol (up to 5, 150 and 200 mM, respectively) were found to increase the relative amplitudes of the fast phases to about one-third of the total *P*-700 signal. Addition of both 3 mM $MgCl_2$ and 200 mM sorbitol increased the relative amplitude of the 20 μs phase to 70%. The interaction between *P*-700 and plastocyanin is concluded to be favoured by a low internal volume of the thylakoids and compensation of surface charges of the membrane.

The half-time of 20 μs was not changed when the amplitude of this phase was altered either by salt and sorbitol, or by inhibition of plastocyanin. This is evidence for the existence of a complex between plastocyanin and *P*-700 with a lifetime long compared to the measuring time. The 200 μs phase exhibited changes in its half-time that indicated the participation of a more mobile pool of plastocyanin.

Cytochrome *f* was oxidized with a biphasic time course with half-times of 70—130 μs and 440—860 μs at different salt and sorbitol concentrations. The half-time of the faster phase and a short lag of 30—50 μs in the beginning of the kinetics indicate an oxidation of cytochrome *f* via the 20 μs electron trans-

---

Abbreviation: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

fer to *P*-700. An inhibition of this oxidation by $MgCl_2$ suggests that the electron transfer from cytochrome *f* to complexed plastocyanin is not controlled by negative charges in contrast to that from plastocyanin to *P*-700.

Introduction

The photosynthetic electron transport sequence between plastoquinone and *P*-700, the reaction center chlorophyll of Photosystem I [1], involves plastocyanin and cytochrome *f* [2]. Although this part of the electron transport chain was the subject of many investigations, only a few electron transfer steps have been characterized in situ, and little is known about the interaction between these electron carriers in the membrane. Some of these reactions can be investigated by monitoring the fast reduction of *P*-700$^+$ by its immediate electron donors [3].

After an ultra short flash ($\leq$1 μs), *P*-700$^+$ is reduced in three phases. The half-times of the phases are 10—20 μs, 200 μs and about 10 ms in spinach chloroplasts [3—7]. The two rapid phases are also observed in blue-green algae [8] and in *Chlorella pyrenoidosa* [9,10]. The slow phase is due to a reduction of *P*-700$^+$ via the rate-limiting step of linear electron transport by electrons from Photosystems II [11]. The phase with 200 μs half-time coincides with the oxidation kinetics of plastocyanin, and should therefore originate from a reduction of *P*-700$^+$ by plastocyanin [12,13]. The electron transfer with the 20 μs half-time has not yet been attributed to a known electron donor of *P*-700.

Bouges-Bocquet and Delosme proposed that a previously unknown electron donor functions between plastocyanin and *P*-700 and may be associated with subunit III of Photosystem I reaction center [10]. This subunit III has been demonstrated by Bengis and Nelson to be necessary for the electron transfer from plastocyanin to *P*-700 [14]. Recently we confirmed the latter result, but at the same time we were able to exclude the presence of an electron carrier in this subunit [15]. This again raises the question about the nature of the immediate electron donor of *P*-700.

Another problem concerns the function of this immediate electron donor in the reaction sequence of the electron transport chain. The relative contributions of 40—45, 30—35 and about 25% of the 20 μs, 200 μs and the slow phase of *P*-700$^+$ reduction [4,6], respectively, are not easily explained by either a linear or a parallel arrangement of the two electron donors of *P*-700 [3,9]. An alternative could be the connection of some *P*-700 molecules to the 20 μs and of others to the 200 μs donor [9].

The time course of the electron transfer reactions depends not only on the reaction sequence but also on the cooperation between the photosystems [16]. Instead of an analysis by two exponential phases [3,6] the kinetics of the fast *P*-700$^+$ reduction have been interpreted to follow a second-order time course [17,9]. A second-order time course is evidence for a fast cooperation between reaction centers of Photosystem I during the electron transfer. On the other hand, the independent functioning of Photosystem I reaction centers was indicated by the biphasic reduction of *P*-700$^+$ after a long flash at partial inhibition of plastocyanin with KCN [18]. It was concluded that a part of plasto-

cyanin was tightly associated with *P*-700, and another part formed a pool common to several reaction centers of Photosystem I. Evidence for such a pool inside the thylakoids with a variable concentration of plastocyanin was derived from the increase of electron transport rates at increasing osmolarity of the solution [19,20].

To approach the complex situation between *P*-700 and its immediate electron donors we have measured the fast reduction kinetics of $P\text{-}700^+$, as well as the oxidation kinetics of cytochrome *f*. We provide evidence for the function of complexed plastocyanin as the yet unidentified electron donor of *P*-700. The influence of salt and sorbitol indicates the formation of this complex at a high concentration of plastocyanin inside the thylakoids, when the negative charges of plastocyanin and the membrane are compensated by cations. The interaction between cytochrome *f* and plastocyanin shows more hydrophobic character.

Materials and Methods

Class II chloroplasts [21] and osmotically-shocked chloroplasts [22] were isolated from spinach leaves and freshly used. Incubation of chloroplasts with KCN and $HgCl_2$ was carried out as described in Refs. 23 and 24, respectively. Treatment of chloroplasts with hydroxylamine was essentially as described [25], at a concentration of 10 mM NH_2OH plus 1 mM disodium ethylenediamine-tetraacetate (adjusted to pH 7.5 immediately before use) for 10 min at room temperature. The reaction mixture usually contained chloroplasts at a chlorophyll concentration of 15 μM, 20 mM *N*-tris(hydroxymethyl)methylglycine (Tricine)-NaOH buffer, pH 7.6, 0.1 mM diaminodurene, 1 mM sodium ascorbate, and 0.5 mM 9,10-anthraquinone-2-sulfonate as electron acceptor, unless different concentrations are indicated in the legends. The temperature of the reaction mixture was 21—23°C.

Absorbance changes were measured with a single beam flash photometer [26] of high time resolution. The monitoring light, obtained from a 150 W tungsten halide lamp and either a narrow-band interference filter (Fa. Anders) or a grating monochromator (Bausch & Lomb), had a spectral half-width of 2.2—3 nm.

The measurements of *P*-700 at 703 nm were carried out at an intensity of the monitoring light of 0.8 $W \cdot m^{-2}$. A distance of 50 cm between the 1 × 1 cm cuvette and the silicon photocell (EG & G, type SGD 444), a narrow-band interference filter (703 nm, $\Delta\lambda = 2.2$ nm) in front of the photocell and subtraction without monitoring light [26] were used to minimize disturbance of the *P*-700 signals by flash-induced fluorescence. The signals induced by repetitive blue (Schott-filter BG 23 (6 mm) and KG2 (2 mm)) xenon flashes (Novatron 725, Xenon corporation) were digitized with a transient recorder (Biomation, type 805) and averaged in a signal processor TN 1500 from Tracor. The electrical bandwidth of the set-up was limited by the dwell time of 1 μs per address. During the measurement of cytochrome *f* the intensity of the monitoring light was less than 0.25 $W \cdot m^{-2}$. A polished aluminium light pipe was used to guide most of the scattered light to the photomultiplier EMI 9658B. The exciting flashes were passed through filters Calflex C from Balzers and RG 610

(3 mm) from Schott. The difference of absorbance changes was obtained by subfraction in the signal processor, after storage of the two signals in different memory allocations.

To minimize the effect of time jitter in the xenon flash the recording procedure was triggered by a photodiode which monitored the flash. The transient recorder was used in the pre-trigger recording mode which permitted recording of the signal before the flash. In experiments using the dual time-base of the transient recorder, the trigger of the flash was delayed by a pulse generator (Systron-Donner).

Flashes with a significant tail in the range of the 20 μs half-time of *P*-700 reduction induce double hits on *P*-700 [9]. Therefore, we monitored the time distribution of the actinic light during our flash with the silicon photocell and the transient recorder described above, at a dwell time of 200 ns per address and an electronic bandwidth of 0.1 Hz—1 MHz. The duration of the flash was 2.0 μs, full width at half maximum intensity. The integrated light energy was 50, 90 and 99.9% of the total energy after 2.0, 6.2 and 16 μs, respectively.

Results

Fig. 1 shows the absorbance changes of *P*-700 at 703 nm induced by a short flash. The reduction kinetics of *P*-700, following its oxidation in the flash can be decomposed into two fast decaying phases with respective half-times of about 20 and 140 μs (Fig. 2) and an additional slow phase, with relative weights of 67, 30 and 3%, respectively.

From one batch of chloroplasts to another, the half-times varied between 17 and 23 μs (average 20 μs with a standard deviation of ± 3 μs for 40 measure-

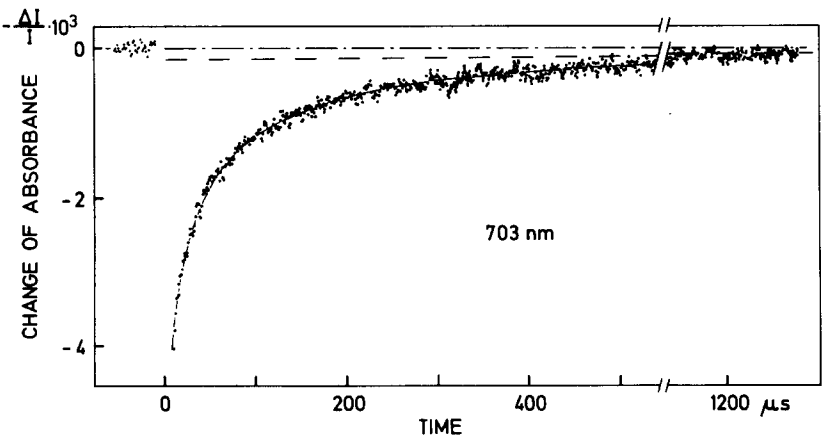

Fig. 1. Absorbance change of *P*-700 at 703 nm in chloroplasts induced by a short flash as a function of time. The standard reaction mixture contained hypotonically broken chloroplasts [22] and in addition 20 mM KCl, 3 mM $MgCl_2$, 200 mM sorbitol, 1.5 μM gramicidin D and 0.2 mM reduced 2,6-dichlorophenolindophenol as electron donor, instead of diaminodurene. 2000 signals were averaged at 1 μs per address with a repetition rate of 4 Hz. The sample was changed after 1000 signals. The dashed line indicates the time course of the slow phase extrapolated from the signal monitored over more than 1800 μs. The origin of the time scale is at 6 μs after ignition of the flash. Some flash-induced fluorescence disturbed the electronic set-up for about 18 μs.

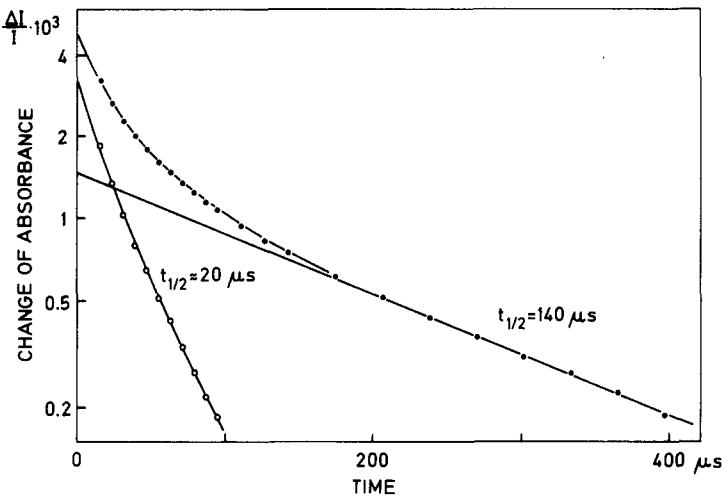

Fig. 2. Semi-logarithmic plot of the fast phases of the *P*-700$^+$ reduction. The closed points are taken from the amplitudes between the thin line through the signal and the dashed line in Fig. 1.

ments) and between 140 and 230 μs, respectively. We will refer to these phases as the 20 μs phase and the 200 μs phase. The 200 μs phase always exhibited exponential decay, which allowed an unambiguous separation of the 20 μs phase by a semi-logarithmic plot (Fig. 2). The separated 20-μs component showed sometimes a slight deviation from an exponential decay. To keep the immediate electron donors of *P*-700 reduced at a relatively high intensity of the oxidizing monitoring light [3,4] the reaction mixture usually contained either reduced 2,6-dichlorophenolindophenol or diaminodurene. We altered the concentration of these electron donors and found no effect on the fast reduction kinetics of *P*-700$^+$, in agreement with previous results [5].

In looking for conditions which affect the interaction between *P*-700 and its immediate electron donors we accounted for the following effects by variation of the concentration of salt and sorbitol. (1) Ionic strength, which affects, in particular, the reaction rate between ions in solution (cf. Ref. 27). (2) Compensation of negative surface charges of a membrane by cations (for a review see Ref. 28). (3) Decrease of the osmotic space inside the thylakoid vesicles by increasing osmolarity of the solution [29,30].

In the presence of buffer alone the 20 μs as well as the 200 μs phase showed relative weights of only 10 and 16% of the total signal, respectively (Table I). To this solution $MgCl_2$, KCl and sorbitol were added at concentrations increasing from 0.5 to 5 mM, 7.5 to 150 mM and 15 to 300 mM, respectively. For the sake of clarity only the results at concentrations sufficient for maximal amplitudes of the fast phases are shown in Table I. Each of the three reagents increased the 20 μs phase to one-third of the total amplitude. The same holds for the 200 μs phase, except that addition of 150 mM KCl stimulated an even larger amplitude. The increases of the fast phases either by a high concentration of 200 mM sorbitol or by only 5 mM $MgCl_2$ doubtless originate from dif-

TABLE I

EFFECT OF IONS AND OSMOLARITY ON THE REDUCTION OF *P*-700$^+$ BY ITS IMMEDIATE ELECTRON DONORS

The standard reaction mixture contained chloroplasts at a chlorophyll concentration of 27.5 μM. Further additions are indicated. Measurement and evaluation of the signals as in Figs. 1 and 2. The total amplitude of the absorbance change at 703 nm of $-\Delta I/I = -9.5 \cdot 10^{-3}$ was not changed by the additions, within an error of 10 per cent.

Additions	'20 μs' phase		'200 μs' phase		'Slow' phase
	Half-time (μs)	Relative amplitude (per cent)	Half-time (μs)	Relative amplitude (per cent)	Relative amplitude (per cent)
—	20	10	210	16	74
5 mM $MgCl_2$	22	32	210	28	40
150 mM KCl	27	34	200	42	24
200 mM sorbitol	18	35	210	32	33
2 mM $MgCl_2$, 20 mM KCl, 200 mM sorbitol	18	53	180	35	12
3 mM $MgCl_2$, 20 mM KCl, 200 mM sorbitol	20	67	140	30	3

ferent effects on the interaction between *P*-700 and its immediate electron donors.

Therefore, we investigated the *P*-700$^+$ reduction at increasing $MgCl_2$ concentrations in the presence of 200 mM sorbitol. As shown in Table I this caused a remarkable increase of the amplitude of the 20 μs phase. In the presence of 3 mM $MgCl_2$ and 200 mM sorbitol (cf. Fig. 1) the 20 μs phase showed the maximum amplitude, between 62 and 73% of the total, in different batches of chloroplasts. The larger percentages were observed with freshly prepared chloroplasts. The half-time of this phase was not affected by any of the additions. The 200 μs phase showed minor effects on its amplitude but a noticeable decrease of the half-time from 210 to 140 μs.

To study the effect of inactivation of plastocyanin on the fast reduction kinetics of *P*-700$^+$ we incubated chloroplasts with KCN, as well as with $HgCl_2$. Fig. 3A shows that the amplitude of the 20 μs phase decreases at increasing time of KCN incubation. The time course of this decrease is very similar to that of the inhibition of linear electron transport [31]. The fact that the total amplitude is constant indicates that *P*-700 is intact even after prolonged KCN incubation.

Equivalent results were observed after incubation with increasing amounts of $HgCl_2$, as shown in Fig. 3B. The amplitude of the 20 μs phase was found to decrease at increasing molar ratios of $HgCl_2$ to chlorophyll from 0.1 to 0.3. This range corresponds to that of inhibition of electron transport [24]. The slight decrease of the total amplitude indicates secondary effects of $HgCl_2$ on Photosystem I at higher $HgCl_2$ concentrations.

Fig. 4 shows that neither KCN incubation nor $HgCl_2$ treatment of the chloroplasts changed the half-time of the 20 μs phase. The averages of the values shown in Fig. 4A and B are 21.5 and 19 μs, respectively.

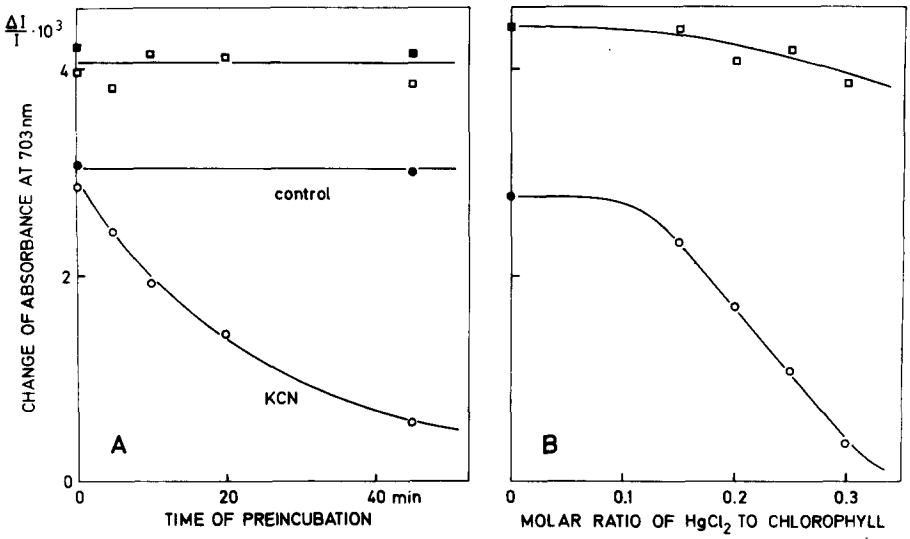

Fig. 3. Effect of KCN or $HgCl_2$ treatment of chloroplasts on the amplitude of the fast $P\text{-}700^+$ reduction. Squares, total amplitudes of the signal at 703 nm, circles, amplitudes of the 20 μs phase of $P\text{-}700^+$ reduction. A, effect of the time of preincubation with KCN (open symbols), control incubation with KOH as in Ref. 23 (closed symbols). B, effect of the $HgCl_2$ concentration during incubation. Measuring conditions and estimation of the amplitudes as in Figs. 1 and 2. The chloroplasts used for A and B were isolated from different batches of spinach leaves.

The 200 μs phase showed a more complex effect of the inhibition. Both treatments caused only a slight decrease of the amplitude of this phase but a pronounced increase of its half-time. For instance, a 20 min KCN incubation increased the half-time from 140 to 320 μs. At the maximal inhibition used for Figs. 3 and 4 this phase was too slow to be separated from the slow phase.

The results above indicate that plastocyanin may be the unknown electron donor of *P*-700. However, in addition to the inhibition of plastocyanin, KCN or $HgCl_2$ might be affecting subunit III or the accessibility of the donor site of Photosystem I. To test this possibility we incubated digitonin particles [32] for 100 min with KCN, following the same procedure as used for chloroplasts

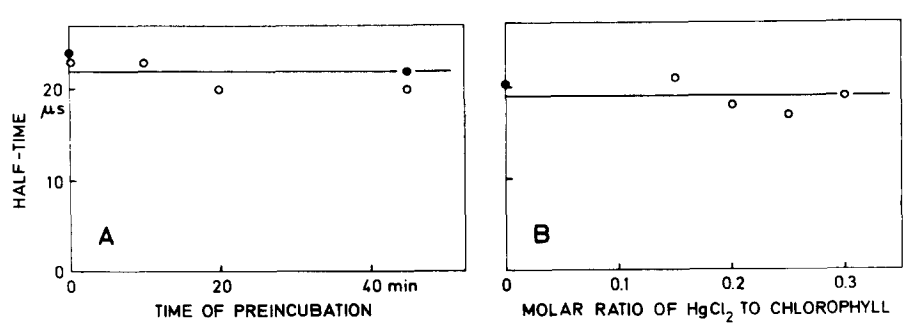

Fig. 4. Effect of KCN or $HgCl_2$ treatment of chloroplasts on the half-time of the 20 μs phase of $P\text{-}700^+$ reduction. Estimation of the half-times as in Fig. 2. All other details as in Fig. 3.

[23]. The electron transfer from added plastocyanin to $P\text{-}700^+$ was measured as described in Ref. 15. We could not detect any effect of the incubation (experiments not shown).

Both the 20 μs and the 200 μs phase were sensitive to disruption of chloroplasts by passage through a Yeda press at a low pressure of 10 MPa or by digitonin treatment. Incubation of chloroplasts with only 0.1% digitonin for 5 min in the cuvette inhibited the fast reduction phases of *P*-700 completely.

Oxidation kinetics of cytochrome f in chloroplasts

Superposition of the absorbance changes of cytochrome *f* in the α-band region and *C*-550 [33,34] caused us to reinvestigate the oxidation kinetics of cytochrome *f*. We used DCMU and either hydroxylamine treatment of the chloroplasts [25] or addition of 10 mM hydroxylamine to inhibit reversible reactions of Photosystem II [35]. Fig. 5A shows the complete time course of the absorbance changes of cytochrome *f* induced by a short flash in the presence of the electron donor diaminodurene. The first half-time of the cytochrome *f* oxidation is 360 μs, as reported recently [15]. A more careful analysis shown in Fig. 5B indicates a bi-exponential decay. An extrapolation of the faster phase with a half-time of about 100—150 μs yields greater amplitudes than actually measured in Fig. 5A. This may indicate a lag in the beginning of the cytochrome *f* oxidation. Variation of the diaminodurene concentration did not change the time course of cytochrome *f* oxidation, but did change its slow re-reduction. The latter followed pseudo-first-order kinetics and gave a second-order rate constant ($k = \ln 2[\text{diaminodurene}]/t_{1/2}$) of $7 \cdot 10^5\ M^{-1} \cdot s^{-1}$ for the electron transfer from diaminodurene to oxidized cytochrome *f*.

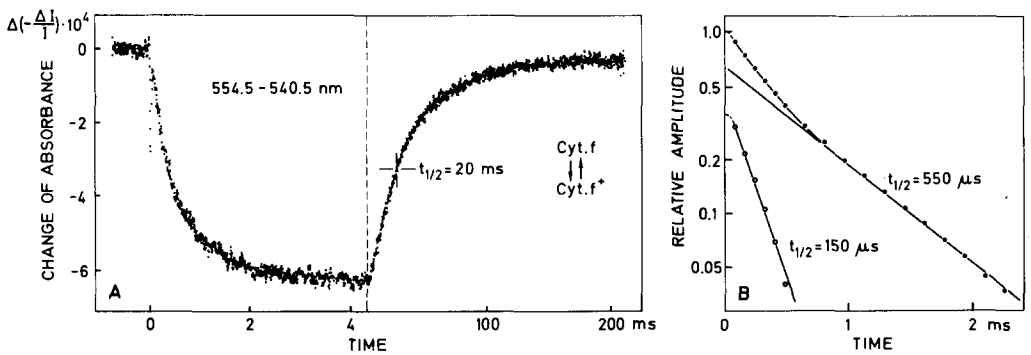

Fig. 5. Time course of the absorbance change of cytochrome *f* after a short flash monitored as the difference of the absorbance changes at 554.5 minus 540.5 nm in hydroxylamine incubated [25] chloroplasts. The standard reaction mixture contained these chloroplasts at a concentration of 30 μM, 20 mM KCl, 2 mM $MgCl_2$, 20 μM DCMU, 50 μM diaminodurene, 1.5 μM gramicidin D and 30 μM anthraquinone-2-sulfonate. 500 signals were averaged with a repetition rate of 2 Hz at each wavelength. A, at the time marked by the dashed line the dwell time of the transient recorder was switched from 5 to 200 μs per address. The re-reduction of cytochrome *f* showed an exponential decay with a half-time of 20 ms as indicated. Downward deflection indicates oxidation of cytochrome *f*. B, semi-logarithmic plot of the relative amplitudes ($\Delta I_{max} - \Delta I_t)/\Delta I_{max}$ of the initial time course shown in A. ΔI_t, amplitude at the time *t* after the flash, ΔI_{max}, maximal amplitude observed at about 4 ms. The oxidation of cytochrome *f* shows a bi-exponential decay with respective half-times of 150 and 550 μs. Abbreviations: Cyt. *f*, reduced form, and Cyt. f^+, oxidized form of cytochrome *f*.

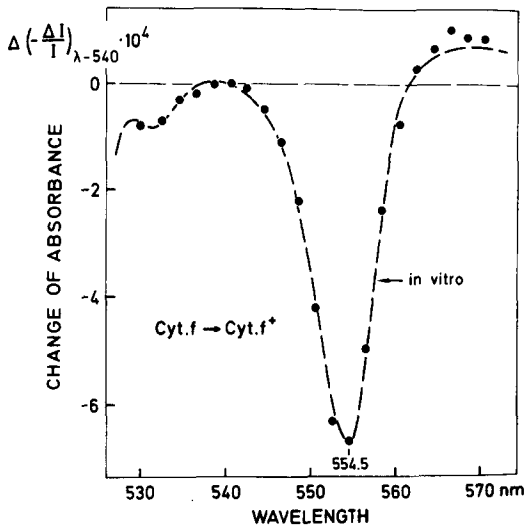

Fig. 6. Difference between absorbance changes as a function of wavelength and the absorbance change at 540.5 nm 4 ms after a short flash. The dots represent the amplitudes of the signals. The dashed line traces the oxidized minus reduced difference spectrum of cytochrome *f* from parsley in vitro (Forti, G., unpublished data). Experimental conditions and abbreviations as in Fig. 5.

Fig. 6 shows the difference spectrum of the absorbance changes in Fig. 5, 4 ms after the flash. It shows excellent agreement with the in vitro difference spectrum of oxidized-minus-reduced cytochrome *f* (measurement by G. Forti) and with that of Ref. 36. Above 560 nm a faster rise compared to that in Fig. 5 indicates a small contribution of other absorbance changes. To estimate

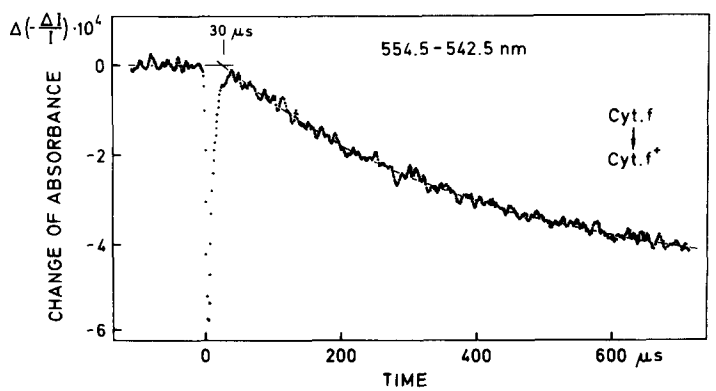

Fig. 7. Initial time course of the cytochrome *f* oxidation after a short flash in chloroplasts, monitored as the difference of absorbance changes at 554.5 minus 542.5 nm. The reaction mixture was as for Fig. 5 except that 0.1 mM diaminodurene was present and inhibition of the donor side of Photosystem II by hydroxylamine treatment of the chloroplasts was replaced by addition of 10 mM hydroxylamine (pH 7.5) to the measuring solution. 10 000 signals were averaged at a repetition rate of 8 Hz. The content of the sample cuvette was changed after every 2000 signals. The intensity of the flash was not saturating to avoid large amplitudes of the carotenoid triplet [37]. The rise-time ($t_{10\text{-}90}$) of the set-up was 3.8 μs and the dwell time 1 μs per address. Abbreviations as in Fig. 5.

more accurately the extent of the lag in the cytochrome *f* oxidation kinetics the initial time course was measured at a high time resolution and is shown in Fig. 7. At the beginning there is superposition of the absorbance change of the carotenoid triplet [37], which decays with a half-time of about 3 μs. This superposition could not be avoided although the flash intensity was kept below saturation. Nevertheless, the lag can be estimated from the limiting slope of the initial time course, as shown in Fig. 7. The lag was not greater than 30—50 μs. The same value was estimated from the initial time course of the difference of absorbance changes at 554.5 minus 563.5 nm. This duration of the lag indicates that it is the 20 μs electron transfer to $P\text{-}700^+$ which precedes the oxidation of cytochrome *f*. To correct for the absorbance changes of the triplet we measured the absorbance change at the 520 nm peak of its difference spectrum. Assuming that the total amplitude of the negative spike in Fig. 7 originates from the triplet we added the stored signal at 520 nm after multiplication with a suitable factor of about 0.075 to the signal difference in Fig. 7. This creates a lag in the resulting time course with a duration as estimated above. However, the procedure seems to be rather arbitrary, because the accuracy of the factor had to be much higher than that of the available triplet spectrum [37].

Table II shows the effect of osmolarity and ions on the oxidation of cytochrome *f* at the concentrations used for the measurements of $P\text{-}700^+$ reduction in Table I. Without additions only a small amount of cytochrome *f* was oxidized, showing a predominant slow phase with a half-time of 860 μs. Addition of 5 mM $MgCl_2$ caused an increase of the slow phase and a slight decrease of the half-time. By contrast, 200 mM sorbitol increased the amplitude of the fast

TABLE II

EFFECT OF IONS AND OSMOLARITY ON THE OXIDATION KINETICS OF CYTOCHROME F

The oxidation of cytochrome *f* was measured as the difference of the absorbance changes at 554.5 minus 540 nm induced by a short flash. Amplitudes and half-times of the kinetics were analysed as in Fig. 5. Each value is the average obtained from measurements with chloroplasts prepared from two different batches of spinach leaves. The standard reaction mixture contained chloroplasts at a chlorophyll concentration of 17.5 μM and, in addition, 0.1 mM diaminodurene, 1 μM gramicidin D, 10 μM DCMU and 10 mM hydroxylamine hydrochloride adjusted to pH 7.5 with NaOH immediately before use. Further additions are indicated. By contrast with previous practise, the measuring light with an intensity of 2.5 $W \cdot m^{-2}$ was switched on by an electronically controlled shutter 20 ms before the transient was recorded and switched off immediately after the monitoring interval of 10 ms. 50 signals were averaged with a repetition rate of 0.5 Hz.

Additions	'Fast' phase		'Slow' phase		Total amplitude $\frac{\Delta I}{I} \cdot 10^4$
	Half-time (μs)	Amplitude $\frac{\Delta I}{I} \cdot 10^4$	Half-time (μs)	Amplitude $\frac{\Delta I}{I} \cdot 10^4$	
—	130	0.22	860	1.25	1.47
5 mM $MgCl_2$	110	0.44	670	1.87	2.31
150 mM KCl	90	0.66	530	2.00	2.66
200 mM sorbitol	73	1.77	490	1.18	2.95
3 mM $MgCl_2$, 20 mM KCl, 200 mM sorbitol	70	1.50	440	1.50	3.00

phase and accelerated the decay from 130 to 73 μs. 3 mM $MgCl_2$ added in addition to sorbitol seems to favor the slow phases at the expense of the fast one. It is obvious, that the half-times of the two phases of cytochrome *f* oxidation and their amplitudes do not coincide with those of the *P*-700$^+$ reduction in Table I. Hydroxylamine plus DCMU as used in the cytochrome *f* measurements did not change the reduction kinetics of *P*-700$^+$, in agreement with previous results [5].

Discussion

We have investigated the polyphasic reduction kinetics of *P*-700$^+$ after a short flash. The electron donor responsible for the fastest phase with a half-time of about 20 μs is presently unknown. Inhibition of plastocyanin and measurement of the oxidation kinetics of cytochrome *f* are expected to provide information on this immediate electron donor of *P*-700.

Evidence for plastocyanin as immediate electron donor of P-700

The reduction of *P*-700$^+$ with a half-time of 20 μs is inhibited by treatment of chloroplasts with either KCN or $HgCl_2$ (Fig. 3). This is evidence for a function of plastocyanin as the unknown electron donor of *P*-700. Both inhibitors have been shown to inactivate plastocyanin in situ [38,39] and to react specifically with plastocyanin [31,39,24], except for minor effects of $HgCl_2$, in the concentration range used (Fig. 3B and Ref. 40). The result is consistent with our recent conclusion that the electron transfer from externally added plastocyanin to *P*-700 with an extremely high rate constant of $1.5 \cdot 10^8\ M^{-1} \cdot s^{-1}$ does not involve an additional electron carrier [15].

Bouges-Bocquet and Delosme [10] have proposed a linear reaction sequence with an electron carrier PD (primary donor to *P*-700) functioning between plastocyanin and *P*-700:

$$\text{plastocyanin} \xrightarrow{200\mu s} \text{PD} \xrightarrow{20\mu s} P\text{-}700\ .$$

However, in such a sequence an inhibition of plastocyanin would not block the 20 μs phase of the *P*-700$^+$ reduction. An additional inhibition site in Photosystem I is excluded by our finding that KCN incubation of digitonin particles has no effect on the electron transfer from soluble plastocyanin to *P*-700.

Effect of osmolarity and surface charges on the reduction of P-700

Previous measurements of the fast *P*-700$^+$ reduction were carried out in the absence of divalent cations [5,6] or in the presence of 1 mM $MgCl_2$ at a low osmolarity [3,4]. We studied the effect of salt and osmolarity on the interaction between *P*-700 and its immediate electron donors. Sorbitol and other membrane impermeable sugars decrease the osmotic space inside the thylakoid vesicles [30]. Addition of 200 mM sorbitol increased the amplitude of both the 20 and the 200 μs phase of *P*-700$^+$ reduction by a factor of about three. This can best be explained if plastocyanin is assumed to be free inside the thylakoids and an increase of the plastocyanin concentration causes more *P*-700 to interact with plastocyanin. This is consistent with the finding of Bendall and Wood [19], and Lockau [20] of a 2—3-fold stimulation of the electron transfer rate

through Photosystem I upon addition of sucrose and glucose, respectively, at a concentration of 200 mM.

Addition of 5 mM $MgCl_2$ does not significantly increase the osmolarity or ionic strength of the solution. Nevertheless, both fast phases of $P\text{-}700^+$ reduction were increased. Mg^{2+} has been shown to control grana stacking of the thylakoids [41], as well as the distribution of quanta between the two Photosystems [42—44] most probably by its effect on the diffuse cation layer compensating the negative surface charges of the thylakoid membrane and the protein complexes [45]. This effect should also be of major importance for the reaction between *P*-700 and plastocyanin because of the low isoelectric points of the internal surface of the thylakoid membrane and plastocyanin of 4.0—4.3 [46—48] and below 4 [49], respectively. Therefore, at physiological pH values the positive ions should compensate the repulsive interaction between *P*-700 embedded in the negatively charged membrane [50,51] and the negatively charged plastocyanin. Our result is evidence for this interaction in situ. For the reaction between soluble plastocyanin and $P\text{-}700^+$ in digitonin particles a similar interaction was indicated by a larger rate constant of the electron transfer in the presence of low Mg^{2+} concentrations compared to that at higher KCl concentrations [15].

Addition of 150 mM KCl not only increases the ionic strength of the solution significantly, but it is also effective in compensating surface charges [28,52] and in diminishing the internal volume of the thylakoids [30]. We conclude that the latter two effects rather than the ionic strength [20] provide an explanation for the influence of KCl. Addition of both 3 mM $MgCl_2$ and 200 mM sorbitol increases the amplitude of the 20 μs phase found in the presence of each alone 2-fold to 70% of the total amplitude of *P*-700. Therefore, compensation of surface charges and a small internal volume of the thylakoids are concluded to be necessary for the optimal interaction between plastocyanin and *P*-700.

Evidence for a complex between P-700 and its immediate electron donor

A second-order reaction between plastocyanin and *P*-700 implies the probability for every molecule of one type to react with those of the other type. In chloroplasts this could be realized either by freely mobile plastocyanin [19] or if there is a possibility of a rapid electron exchange between closely associated complexes of plastocyanin and *P*-700 [9]. In either case a concentration change of one component would lead to a reciprocal change of the half-time for the electron transfer. However, neither the variation of the relative amplitude of the 20 μs phase between 10 and 70 per cent by salt and sorbitol (Table I) nor its decrease from 70 to 20 per cent by inhibition with KCN or $HgCl_2$ (Fig. 3) had any effect on the half-time of 20 μs. Thus, we can exclude a cooperation involving electron exchange between Photosystems I within the measuring time. The result is evidence for a complex between *P*-700 and its immediate electron donor.

Other evidence for a tight association of plastocyanin with *P*-700 was found in our previous study of changes in the path of electron transfer from water to Photosystems I after partial inhibition with KCN [18]. The kinetics of the electron paramagnetic resonance Signal I showed an increasing portion of $P\text{-}700^+$

remaining unreduced upon increasing inhibition of plastocyanin, which is not expected if the remaining plastocyanin is able to reach every *P*-700.

The equilibrium constant for the binding of plastocyanin to *P*-700 cannot be high because a number of factors cause the dissociation of this complex, e.g. low ion concentrations and low osmolarity of the solution, addition of low digitonin concentrations or a disruptive treatment of the chloroplasts at a very low pressure. In addition to compensating the surface charges, a high internal [53,54] concentration of plastocyanin is believed to be prerequisite for the formation of this complex.

Delosme et al. [9] have suggested an electron exchange between the reaction centers of Photosystem I which would have to occur rapidly, i.e. faster than the 20 μs electron transfer to $P\text{-}700^+$. Their conclusion was based on the non-exponential time course of the fast $P\text{-}700^+$ reduction after a strong short flash of saturating intensity compared to an exponential decay with a 15—20 μs half-time after a weak short flash. However, the time course after the strong flash (Fig. 7 in Ref. 9) seems to be better approximated by a bi-exponential decay, as shown in Fig. 2, rather than by a second-order decay. This would be an indication for a partial oxidation of the plastocyanin molecule complexed with *P*-700 during the strong flashes. This interpretation is consistent with the estimation that the short strong flash, induced about 10% of double hits on *P*-700 [9]. For our flash we calculate 15—19 per cent of double hits at an intensity sufficient to oxidize 97—99 per cent of total *P*-700, if we assume a half-time of 17 μs for the reduction. Therefore, probably more than 90% of total *P*-700 is associated with complexed plastocyanin at our optimal conditions.

The half-time of the 200 μs phase of $P\text{-}700^+$ reduction increased at increasing inhibition of plastocyanin by either KCN or $HgCl_2$. This is expected for a second-order reaction and may indicate a pool of plastocyanin common to several reaction centers of Photosystem I. If this plastocyanin is freely mobile within the thylakoids, its concentration and therefore the electron transfer rate should be increased at increasing osmolarity. But addition of 200 mM sorbitol has only a negligible effect on the half-time (Table I). Therefore, the pool of plastocyanin contributing to the 200 μs phase must consist of molecules adsorbed to the membrane surface. On the other hand, the decreasing amplitudes of the two fast reduction phases observed at decreasing salt concentration suggest that plastocyanin is released into a state that is inactive in mediating fast electron transfer to $P\text{-}700^+$.

Plastocyanin has been found to be oxidized with the half-time of the 200 μs phase of *P*-700 [12,13]. A faster oxidation with a half-time of 20 μs has not been observed, which has to be expected according to our present result. However, in these previous studies we have used low concentrations of salt [12] far from optimal for large amplitudes of the 20 μs phase. Small amplitudes of the fast oxidation of plastocyanin could have been missed because of superposition with other absorbance changes. An approach to the oxidation kinetics of plastocyanin in *Chlorella* has to account for additional superposition with absorbance changes [10] not seen in broken chloroplasts. Big uncertainties are introduced by the necessary procedure because it involves the subtraction of the kinetics of three other absorbance changes [10] and because of the small

extinction coefficient of plastocyanin [49].

Plastocyanin can be considered to function at a site analogous to that of cytochrome c_2 in bacterial electron transport. Very recently cytochrome c_2 has been found to react in a bound state as well as in a freely mobile form with the reaction center in *Rhodopseudomonas sphaeroides* [55]. It is very likely that this is the situation between plastocyanin and the Photosystem I reaction center. In both cases the complex is reversibly formed and its lifetime is long, compared to the measuring time.

On the oxidation of cytochrome f

In our previous measurements of cytochrome *f* [12] the oxidation kinetics were superimposed with the absorbance changes of *C*-550. However, the rapid reoxidation of the primary acceptor of Photosystem II with a half-time of about 0.6 ms [11] limits this superposition to the initial 3—4 ms after the flash. The amplitude and the subsequent time course of the reduction are not affected. A similar superposition could interfere with the detection of the initial time course of the cytochrome *f* reduction after a long flash, which is not yet understood in terms of a normal linear electron transport [56,57,40].

The presence of a lag in the oxidation kinetics of cytochrome *f* after a short flash (Fig. 7) is evidence that cytochrome *f* is not an immediate electron donor of *P*-700. This has been previously found in *Chlorella* [10,13]. The duration of the lag of 30—50 μs indicates that the oxidation of cytochrome *f* is preceded by the 20 μs electron transfer from plastocyanin to *P*-700$^+$ but cannot be preceded by a sequence of the 200 and 20 μs electron transfer steps [13]. The time course of the oxidation shows two phases (Fig. 5 and Table II). The half-time of 70—130 μs for the faster phase indicates an oxidation of cytochrome *f* after the 20 μs electron transfer to *P*-700$^+$. This is in agreement with the conclusion based on the duration of the lag. The slower phase of cytochrome *f* oxidation with half-times of 440—860 μs is tentatively ascribed to an electron transfer to plastocyanin which has been oxidized by the 160—210 μs electron transfer to *P*-700$^+$. The two phases of the cytochrome *f* kinetics are probably due to the oxidation via plastocyanin in the two reaction states discussed above.

The effect of salt and sorbitol on the oxidation of cytochrome *f* should depend on both the electron transfer from cytochrome *f* to oxidized plastocyanin and from plastocyanin to *P*-700$^+$. Comparison of Tables I and II indicates little similarity between the effects on cytochrome *f* oxidation and those on *P*-700$^+$ reduction. Although the relation seems to be complex, some qualitative properties can be derived from our data. Addition of salt has only little stimulating or, in the presence of sorbitol even an inhibitory effect on the amplitude of the fast phase of cytochrome *f* oxidation, although there is an increase of the amplitude of the 20 μs phase of *P*-700$^+$ reduction. This may indicate either a hydrophobic interaction between cytochrome *f* and plastocyanin complexed with *P*-700 or the interaction of opposite charges at the reaction site. On the other hand, the amplitude of the slower phase of cytochrome *f* oxidation is increased by addition of salt, which indicates an influence of ionic interaction. This is consistent with an oxidation via the 200 μs electron transfer. The approach of mobile plastocyanin to cytochrome *f* in the mem-

brane should be facilitated by compensation of the surface charges.

Colman et al. [58] have determined the structure of plastocyanin and proposed two different electron paths to the copper center, one starting at a large hydrophobic patch close to the copper atom and the other close to a cluster of four negative carboxylate groups. The stimulation of the electron transfer from plastocyanin to *P*-700 by $MgCl_2$ suggests that the latter is the reaction site with *P*-700. Subunit III of Photosystem I reaction center which carries the recognition site for plastocyanin [15] may specifically interact with the four negative groups. This assignment of one electron path of plastocyanin implies that the patch close to the positive copper [59] is the electron transfer site from plastocyanin to cytochrome *f*. This is consistent with the inhibition of the fast cytochrome *f* oxidation by $MgCl_2$.

Conclusions

We conclude that complexed plastocyanin functions as the immediate electron donor of *P*-700. The half-time of the electron transfer is about 20 μs. The complex is probably formed at a high concentration of plastocyanin inside the thylakoid and at compensation of negative surface charges. Another reaction state of plastocyanin, in which it reduces *P*-700 with a half-time of about 200 μs, shows properties of a more mobile pool which could mediate electron exchange between Photosystems I.

The oxidation kinetics of cytochrome *f* in isolated chloroplasts indicate, in agreement with previous results, that cytochrome *f* is not an immediate electron donor of *P*-700. They also indicate that a larger portion of cytochrome *f* is oxidized via the fast electron transfer from complexed plastocyanin to *P*-700. This oxidation of cytochrome *f* by plastocyanin seems not to be controlled by repulsion of negative charges in contrast to that of plastocyanin by *P*-700.

Acknowledgements

We thank Dr. G. Forti for the measurement of the difference spectrum of cytochrome *f*, Dr. K. Sauer for helpful suggestions during the preparation of the manuscript and Dr. A. Trebst for stimulating discussions. This work has been supported by Deutsche Forschungsgemeinschaft.

References

1 Kok, B. (1957) Acta Bot. Neerl. 6, 316—336
2 Trebst, A. (1974) Annu. Rev. Plant Physiol. 25, 423—458
3 Haehnel, W., Döring, G. and Witt, H.T. (1971) Z. Naturforsch. 26b, 1171—1174
4 Haehnel, W. and Witt, H.T. (1972) in 2nd Int. Congr. Photosynthesis, Stresa, 1971 (Forti, G., Avron, M. and Melandri, A., eds.), Vol. 1, pp. 469—476, Junk, The Hague
5 Mathis, P., Haveman, J. and Yates, M. (1976) Brookhaven Symp. Biol. 28, 267—277
6 Van Best, J.A. and Mathis, P. (1978) Biochim. Biophys. Acta 503, 178—188
7 Warden, J.T. and Bolton, J.R. (1974) Photochem. Photobiol. 20, 263—269
8 Hiyama, T. and Ke, B. (1971) Biochim. Biophys. Acta 226, 320—327
9 Delosme, R., Zickler, A. and Joliot, P. (1978) Biochim. Biophys. Acta 504, 165—174
10 Bouges-Bocquet, B. and Delosme, R. (1978) FEBS Lett. 94, 100—104
11 Witt, H.T. (1971) Q. Rev. Biophys. 4, 365—477
12 Haehnel, W. (1977) Biochim. Biophys. Acta 459, 418—441
13 Bouges-Bocquet, B. (1977) Biochim. Biophys. Acta 462, 362—370
14 Bengis, C. and Nelson, N. (1977) J. Biol. Chem. 252, 4564—4569

15 Haehnel, W., Hesse, V. and Pröpper, A. (1980) FEBS Lett. 111, 79—82
16 Siggel, U., Renger, G., Stiehl, H.H. and Rumberg, B. (1972) Biochim. Biophys. Acta 256, 328—335
17 Bouges-Bocquet, B. (1975) Biochim. Biophys. Acta 396, 382—391
18 Haehnel, W. (1977) in Bioenergetics of Membranes (Packer, L., Papageorgiou, G.C. and Trebst, A., eds.), pp. 317—328, Elsevier, Amsterdam
19 Bendall, D.S. and Wood, P.M. (1977) in Proc. 4th Int. Congr. Photosynthesis (Hall, D.O., Coombs, J. and Goodwin, T.W., eds.), pp. 771—775, The Biochemical Society, London
20 Lockau, W. (1979) Eur. J. Biochem. 94, 365—373
21 Winget, G.D., Izawa, S. and Good, N.E. (1965) Biochem. Biophys. Res. Commun. 21, 438—443
22 Nelson, N., Drechsler, Z. and Neumann, J. (1970) J. Biol. Chem. 245, 143—151
23 Izawa, S., Kraayenhof, R., Ruuge, E.K. and DeVault, D. (1973) Biochim. Biophys. Acta 314, 328—339
24 Kimimura, M. and Katoh, S. (1972) Biochim. Biophys. Acta 283, 279—292
25 Ort, D.R. and Izawa, S. (1973) Plant Physiol. 52, 595—600
26 Döring, G., Stiehl, H.H. and Witt, H.T. (1967) Z. Naturforsch. 22b, 639—644
27 Prince, R.C., Cogdell, R.J. and Crofts, A.R. (1974) Biochim. Biophys. Acta 347, 1—13
28 McLaughlin, S. (1977) Curr. Top. Memb. Transp. 9, 71—144
29 Dilley, R.A. and Rothstein, A. (1967) Biochim. Biophys. Acta 135, 427—443
30 Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54—63
31 Ouitrakul, R. and Izawa, S. (1973) Biochim. Biophys. Acta 305, 105—118
32 Bengis, C. and Nelson, N. (1975) J. Biol. Chem. 250, 2783—2788
33 Knaff, D.B. and Arnon, D.I. (1969) Proc. Natl. Acad. Sci. U.S.A. 63, 963—969
34 Van Gorkom, H.J. (1974) Biochim. Biophys. Acta 347, 439—442
35 Joliot, P., Delome, R. and Joliot, A. (1977) Biochim. Biophys. Acta 459, 47—57
36 Singh, J. and Wasserman, A.R. (1971) J. Biol. Chem. 246, 3532—3541
37 Wolff, Ch. and Witt, H.T. (1969) Z. Naturforsch. 24, 1031—1037
38 Selman, B.R., Johnson, G.L., Giaquinta, R.T. and Dilley, R.A. (1975) J. Bioenerg. 6, 221—231
39 Berg, S.P. and Krogmann, D.W. (1975) J. Biol. Chem. 250, 8957—8962
40 Olsen, L.F. and Cox, R.P. (1979) Eur. J. Biochem. 102, 139—145
41 Izawa, S. and Good, N.E. (1966) Plant Physiol. 41, 544—552
42 Homann, P.H. (1969) Plant Physiol. 44, 932—936
43 Murata, N. (1969) Biochim. Biophys. Acta 189, 171—181
44 Butler, W.L. (1976) Brookhaven Symp. Biol. 28, 338—346
45 Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174—181
46 Mercer, F.V., Hodge, A.J., Hope, A.B. and McLean, J.D. (1955) Aust. J. Biol. Sci. 8, 1—18
47 Berg, S., Dodge, S., Krogmann, D.W. and Dilley, R.A. (1974) Plant Physiol. 53, 619—627
48 Åkerlund, H.-E., Andersson, B., Persson, A. and Albertsson, P.-Å. (1979) Biochim. Biophys. Acta 552, 238—246
49 Katoh, S., Shiratori, I. and Takamiya, A. (1962) J. Biochem. 51, 32—40
50 Itoh, S. (1979) Biochim. Biophys. Acta 548, 579—595
51 Itoh, S. (1979) Biochim. Biophys. Acta 548, 596—607
52 Barber, J. and Chow, W.S. (1979) FEBS Lett. 105, 5—10
53 Hauska, G.A., McCarty, R.E., Berzborn, R.J. and Racker, E. (1971) J. Biol. Chem. 246, 3524—3531
54 Haehnel, W., Berzborn, R.J. and Andersson, B. (1981) Biochim. Biophys. Acta, submitted
55 Overfield, R.E., Wraight, C.A. and DeVault, D. (1979) FEBS Lett. 105, 137—142
56 Haehnel, W. (1973) Biochim. Biophys. Acta 305, 618—631
57 Cramer, W.A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, 133—172
58 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
59 Cookson, D.J., Hayes, M.T. and Wright, P.E. (1980) Nature 283, 682—683