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ON THE FUNCTIONAL ORGANIZATION OF ELECTRON TRANSPORT FROM PLASTOQUINONE TO PHOTOSYSTEM I

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Signal I, the EPR signal of P-700, induced by long flashes as well as the rate of linear electron transport are investigated at partial inhibition of electron transport in chloroplasts. Inhibition of plastoquinol oxidation by dibromothymoquinone and bathophenanthroline, inhibition of plastocyanin by KCN and $HgCl_2$, and inhibition by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide are used to study a possible electron exchange between electron-transport chains after plastoquinone. (1) At partial inhibition of plastocyanin the reduction kinetics of P-700$^+$ show a fast component comparable to that in control chloroplasts and a new slow component. The slow component indicates P-700$^+$ which is not accessible to residual active plastocyanin under these conditions. We conclude that P-700 is reduced via complexed plastocyanin. (2) The rate of linear electron transport at continuous illumination decreases immediately when increasing amounts of plastocyanin are inhibited by KCN incubation. This is not consistent with an oxidation of cytochrome *f* by a mobile pool of plastocyanin with respect to the reaction rates of plastocyanin being more than an order of magnitude faster than the rate-limiting step of linear electron transport. It is evidence for a complex between the cytochrome b_6-*f* complex and plastocyanin. The number of these complexes with active plastocyanin is concluded to control the rate-limiting plastoquinol oxidation. (3) Partial inhibition of the electron transfer between plastoquinone and cytochrome *f* by dibromothymoquinone and bathophenanthroline causes decelerated monophasic reduction of total P-700$^+$. The P-700 kinetics indicate an electron transfer from the cytochrome b_6-*f* complex to more than ten Photosystem I reaction center complexes. This cooperation is concluded to occur by lateral diffusion of both complexes in the membrane. (4) The proposed functional organization of electron transport from plastoquinone to P-700 in situ is supported by further kinetic details and is discussed in terms of the spatial distribution of the electron carriers in the thylakoid membrane.

Introduction

In green plants most of the photosynthetic electron carriers are assembled in three intrinsic complexes, the PS II reaction center complex associated with the oxygen-evolving system, the PS I reaction center complex and the cytochrome b_6-*f* complex (for a review, see Ref. 1). The functional interaction of the electron carriers has been studied by many lines of investigation. A periodicity of four of the oxygen evolution in consecutive flashes [2] and of two in the reduction of the secondary electron acceptor of PS II [3,4] are evidence for isolated electron-transport chains from water to the secondary electron acceptor of PS II. The following electron carriers, however, plas-

Abbreviations: BPh, 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline); DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.

toquinone, the cytochrome b_6-f complex and plastocyanin mediating the electron transport to P-700, the primary electron donor of PS I, do not function as an isolated series of interacting molecules (for a recent review, see Ref. 5). Electron exchange between at least ten chains has been demonstrated [6,7]. This lateral interaction is likely to be at the level of a large common pool of plastoquinone [6,8]. A predominant location of PS II in appressed grana thylakoid membranes and of PS I in stroma thylakoid membranes has been concluded from studies on membrane fragments [1,9,10]. This suggests a long-range lateral electron transfer between the two reaction center complexes as discussed recently by Anderson [11].

Besides plastoquinone the hydrophilic plastocyanin could mediate a long-range electron exchange between electron-transport chains. Plastocyanin is easily released from the inside of the thylakoids [12,13], which suggests that it is either freely moving or loosely bound at the inner surface of the membrane. Furthermore, the proportions of cytochrome f and plastocyanin relative to P-700 are variable [14,15]. Therefore, plastocyanin has been proposed to function as an electron-shuttling agent between the cytochrome b_6-f complex and the PS I reaction center complex [16,17]. In support of this model, a decreasing rate of linear electron transport at decreasing osmolarity has been ascribed to a decreasing concentration of plastocyanin at an increasing internal volume of the thylakoids [17]. However, measurements of the fast reduction of $P\text{-}700^+$ by plastocyanin after a short flash gave evidence for the formation of a complex between the two electron carriers which depends on osmolarity as well as on the presence of $MgCl_2$ [18].

It is possible to discriminate isolated reaction sequences from cooperating ones by kinetic measurements at partial inhibition. Components of isolated sequences will show biphasic kinetics with a fast portion due to intact units and a slow portion due to inhibited ones, while components of cooperating sequences will show monophasic kinetics with an increased half-time. This approach has been applied to fluorescence kinetics to study energy transfer between photosynthetic units [19,20] as well as to reduction kinetics of $P\text{-}700^+$ to study cooperation between linear electron-transport chains [6]. In the latter studies monophasic reduction of $P\text{-}700^+$ was found at partial inhibition of PS II with DCMU. This has been suggested to indicate a cooperation via plastoquinone [6]. The results, however, would be consistent with electron exchange between the chains at any site after the inhibition site of DCMU and before or at P-700. Other inhibitors acting at sites closer to P-700 should help to localize the cooperation more precisely.

Inhibitors known to act between plastoquinone and P-700 (for a review, see Ref. 21) are DBMIB [22] and BPh [23,24], both inhibiting the oxidation of plastoquinol at the high-potential iron-sulfur center of the cytochrome b_6-f complex [25], and KCN [26,27] and $HgCl_2$ [28] which both inactivate plastocyanin in situ [29,30]. Incubation of chloroplasts with EDAC [31] has been proposed to inhibit between plastoquinone and cytochrome f, but may inactivate PS I and plastocyanin as well [32,33].

In extending our preliminary measurements [34] we have studied the effect of partial inhibition of electron transport by the above-mentioned inhibitors on the reduction kinetics of P-700 and on the rate of linear electron transport. Our results indicate a complex of plastocyanin with the cytochrome b_6-f as well as with the PS I reaction center complex. Evidence is given for a cooperation between several of these complexes via plastocyanin.

Materials and Methods

Hypotonically broken chloroplasts were isolated from spinach leaves as described by Nelson et al. [35]. Incubation of chloroplasts with KCN was carried out as described by Izawa et al. [26]. Incubation of control chloroplasts was done by substituting KOH for KCN. After dilution to terminate the treatment [27] the chloroplast suspension was centrifuged at $1500 \times g$ for 3 min. The resulting pellet was resuspended in a small aliquot of the supernatant to give a chlorophyll concentration of 3 mg/ml. $HgCl_2$ treatment followed the procedure described by Kimimura and Katoh [28]. During preincubation of chloroplasts for 30 min with various concentrations of $HgCl_2$ at 0°C the concentration of chlorophyll was adjusted to 1 mg/ml. Finally, the chloroplasts were

pelleted by centrifugation and resuspended in measuring solution to give a chlorophyll concentration of approx. 2 mg/ml in the EPR sample cell. Incubation of chloroplasts with EDAC (from Sigma) was carried out as described by McCarty [31]. The chloroplast suspension with a chlorophyll concentration of 0.25 mg/ml was illuminated in a water bath with light passed through a red filter RG 610/3 mm (Schott) at an intensity of 1 kW/m^2. BPh (from Sigma) was dissolved in methanol. The final concentration of methanol in the reaction mixture was always less than 1%. The concentration of chlorophyll was determined by the method of Arnon [36].

The reaction mixture usually contained 3 mM $MgCl_2$, 20 mM KCl and 20 mM Tricine adjusted to pH 7.6 with NaOH and either 5 mM NH_4Cl, 0.5 mM 9,10-anthraquinone-2-sulfonate and chloroplasts at a chlorophyll concentration of 1–2.5 mg/ml for EPR measurements or 1.5 μM gramicidin D, 0.1 mM 9,10-anthraquinone-2-sulfonate, 0.3 mM NaN_3 and a chlorophyll concentration of 30 μg/ml for measurements of linear electron transport rates. 9,10-Anthraquinone-2-sulfonate reacts rapidly with oxygen to form H_2O_2 [37], similar to methyl viologen. PS I-dependent electron-transport rates were monitored in the same reaction mixture as linear electron transport, except that in addition 0.1 mM DAD, 5 mM sodium ascorbate and 15 μM DCMU were present and the chlorophyll concentration was 15 μg/ml.

Electron-transport rates were estimated from light-induced oxygen uptake monitored routinely for 1.5 min at 20.0°C with a Clark-type electrode (Rank Brothers Ltd.) and a 7047A recorder (Hewlett Packard). Actinic light of saturating intensity of 800 W/m^2, obtained from a 600 W tungsten iodine lamp (Silvana), was filtered by water of 7 cm thickness, a heat-reflecting filter and an orange color filter OG570/3 mm (Schott).

EPR measurements were carried out with a Varian E-9 spectrometer at X band with 100 kHz magnetic field modulation. Other instrument settings were: modulation amplitude, 3.2 G, microwave power, 20 mW, time constant, 3 ms, and receiver gain, $2 \cdot 10^4$. Signal averaging was performed using a TN-1500 digital signal analyser (Tracor). Appropriate timing circuits (100 A, Systron Donner) synchronized the start of the averager sweep and the illumination period. 140 signals were averaged with a repetition rate of 0.4 Hz. To correct for different chlorophyll concentration in individual samples of a series, signal amplitudes were normalized by multiplication with a factor chosen in the signal analyser. Excitation with long flashes was controlled by a Compur-electronic m-1 shutter (Prontor) which enabled illumination with a rise and a decay time shorter than 2 ms. The actinic light from a 250 W tungsten iodine lamp (Osram) was filtered through 7 cm of water, a heat-reflecting filter Calflex C (Balzers) and an OG 570/3 mm filter (Schott). The light was guided through a light pipe to the grid in front of the cavity. The light incident upon the flat sample cell with an optical path length of 0.3 mm had a saturating intensity of about 1.1 kW/m^2. The temperature of the sample was 20–22°C.

Our preliminary experiments [34] were carried out at the peak of the low-field derivative maximum of Signal I. We found in long flashes at this field position a superposition of Signal I with the EPR signal of monodehydroascorbate (cf. Refs. 38 and 39 and Fig. 1) and of anthrasemiquinone-2-sulfonate. In addition, one of the two major lines in the EPR spectrum of DBMIB semiquinone shown in Fig. 1 can also interfere at this field position with Signal I. Therefore, Signal I was monitored at a magnetic field strength 0.5 G higher than that at the peak of the high-field derivative maximum to avoid all of these interferences. In contrast to the absorbance changes of P-700, the kinetics of Signal I are not disturbed by fluorescence or monitoring light.

A previously published EPR spectrum ascribed to $DBMIB^{\cdot -}$ [40] is a superposition of the spectrum in Fig. 1 and a spectrum with five lines ($\Delta H_{pp} = 2.30$ G) of an unknown product. This product is observed if DBMIB dissolved in ethanol is kept for some hours at room temperature. The spectrum of DBMIB-semiquinone monitored in *n*-butanol after addition of a grain of $NaBH_4$ at 1 mW microwave power and 10 mG modulation amplitude (not shown) exhibits no additional hyperfine structure compared to the spectrum in buffer at pH 7.4 (Fig. 1) except that the hyperfine splitting of the four lines is 2.06 G instead of 2.20 G as in Fig. 1.

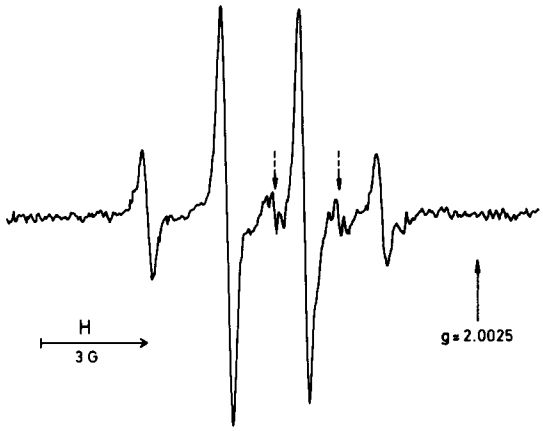
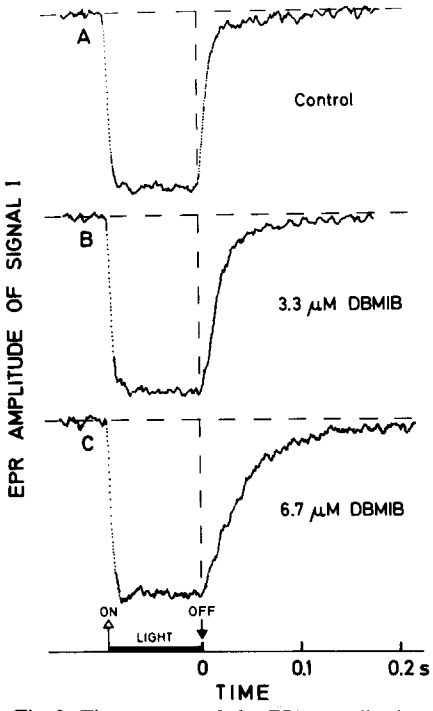


Fig. 1. EPR spectrum of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzosemiquinone. The sample contained 0.2 mM DBMIB, 0.25 mM sodium ascorbate, 10 mM Tricine-NaOH, pH 7.4, and 2% ethanol. All solutions were degassed with argon. Scan with 2.5 G/min at 1 s time constant, modulation amplitude 160 mG, microwave power 5 mW, frequency 9.52 GHz, room temperature. The two lines of the monodehydroascorbate anion radical [38] are marked with dashed arrows. The approximate position of Signal I (g 2.0025) [41] is indicated.

Results

Reduction kinetics of P-700 at partial inhibition of electron transfer

The reduction kinetics of P-700 after a long flash of saturating intensity are measured at partial inhibition of electron transport to distinguish between independent action and cooperation of electron carriers. Fig. 2, trace A, shows the time course in control chloroplasts. After the onset of light P-700 becomes oxidized, i.e., Signal I appears [41], which is indicated by the downward deflection. During the long flash the rate-limiting oxidation of plastoquinol causes an accumulation of electrons in the plastoquinone pool [8] while P-700 stays oxidized. After cessation of light $P\text{-}700^+$ is reduced via cytochrome *f* and plastocyanin by the electrons from plastoquinol. The first half-time is about 7.3 ms as indicated in Fig. 2, trace A. An effect of changing internal proton concentration on this electron-transfer step [42] as a consequence of a reduced electron-transport rate at partial inhibition is avoided by the uncoupler NH_4Cl. Addition of 3.3, 6.7 and 15 μM DBMIB to the reaction mixture increased the half-time of $P\text{-}700^+$ reduction to 14, 31 and 84 ms, respectively. Higher concentrations of DBMIB caused greater half-



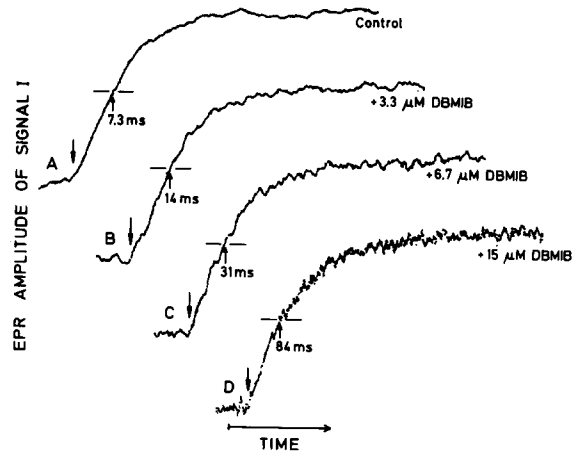


Fig. 2. Time course of the EPR amplitude of Signal I induced by long flashes at various concentrations of DBMIB. Addition to reaction mixture: (A) none; (B) 3.3 μm DBMIB; (C) 6.7 μM DBMIB; (D) 15 μM DBMIB. The chlorophyll concentration is 2 mg/ml. The content of the cuvette was changed after 70 sweeps. Upward open arrow indicates light on, downward full arrow indicates light off. The origin of the time scale is at the end of the illumination period of 96 ms. (Left) Complete time courses. (Right) Kinetics of $P\text{-}700^+$ reduction at time scales chosen in the signal processor to fit the transients with the control. The arrow-labelled time represents an interval of 20, 40, 100 and 265 ms for trace A, B, C and D, respectively. The respective first half-times of 7.3, 14, 31 and 84 ms of $P\text{-}700^+$ reduction are indicated.

times. The amplitude of Signal I was not affected. After appropriate expansion of the time scales of the signals in Fig. 2 (left), the traces in Fig. 2 (right) demonstrate that DBMIB does not change the shape of the P-700 reduction kinetics.

DBMIB binds to the iron-sulfur center of the cytochrome b_6-f complex [25], which is found in chloroplasts at a molar ratio of about 1:600 chlorophylls [15,43]. Presuming this value for our chloroplasts we estimate that at least half of the added DBMIB is specifically bound in the measurement shown in Fig. 2, trace B (about 50% inhibition at a molar ratio of DBMIB to chlorophyll of 1:600).

BPh inhibits linear electron transport similarly to DBMIB [24,44]. Its effect on the time course of Signal I induced by a long flash is shown in Fig. 3. A concentration of 15 and 30 μM BPh decreased the first half-time of the reduction of P-700$^+$ from 7.3 to 12 and 22 ms, respectively. At higher concentrations greater half-times and still monophasic reduction of P-700$^+$ is observed. Thus, the effect on the reduction kinetics of P-700$^+$ is the same as that of DBMIB except for higher concentrations of BPh. The effect of increasing concentration of BPh on the relative rate of linear electron transport at continuous illumination (data not shown) is almost the same as that on the initial rate of P-700$^+$ reduction after a long flash in Fig. 3. A parallel decrease in relative reduction rates of P-700$^+$ and cytochrome f has been found at subzero temperatures [45]. PS I-mediated electron transport was not affected by BPh in agreement with previous findings [23,44]. Both DBMIB and BPh slow down P-700$^+$ reduction but do not affect the distribution of electrons to PS I reaction centers (cf. Fig. 2B). This indicates an interaction between electron-transport chains at a site after the rate-limiting plastoquinol oxidation, possibly via plastocyanin.

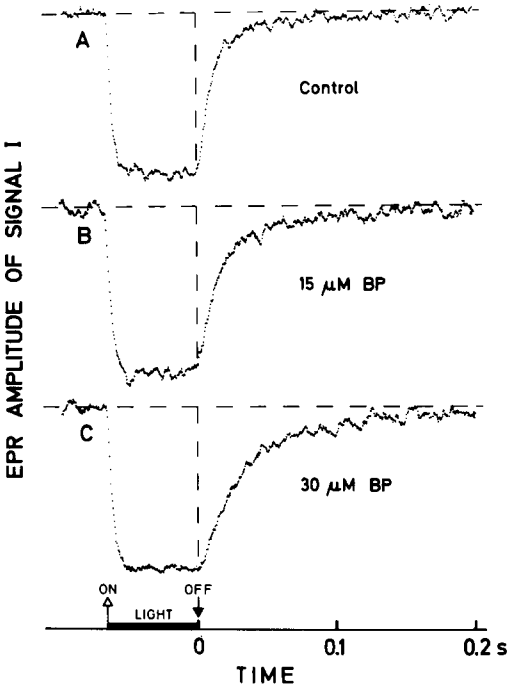


Fig. 3. Time course of the EPR amplitude of Signal I induced by long flashes at various concentrations of BPh (BP). To avoid precipitation, the lipophilic inhibitor was added to chloroplasts suspended at a chlorophyll concentration of 30 μg/ml in 20 mM Tricine-NaOH buffer, pH 7.6, 20 mM KCl and 3 mM $MgCl_2$. Further additions are (A) none, (B) 15 μM BPh, (C) 30 μM BPh. The chloroplasts were centrifuged down and the pellet was resuspended in a small aliquot of the supernatant. Inhibition of linear electron transport (cf. Refs. 24 and 44) was not affected by this centrifugation as checked by oxygen uptake (see Materials and Methods). 280 signals were averaged. The content of the EPR sample cell was changed after 140 signals. Chlorophyll concentration was approx. 1.8 mg/ml. Arrows and origin of the time scale as in Fig. 2. Flash duration is 67 ms.

An approach to investigate a cooperation in electron transport via plastocyanin is partial inhibition of this electron carrier by KCN or $HgCl_2$. Fig. 4 shows the effect of KCN incubation of chloroplasts [26,27] on the EPR signal of P-700 induced by a long flash. An increasing incubation time causes a biphasic reduction of P-700$^+$ with an increasing portion of a new slow phase with 0.3–0.5 s half-time. The half-time of the fast phase increases only slightly from 11 ms to 13, 17 and 33 ms after KCN incubation for 5, 10 and 30 min, respectively (traces B, C and D in Fig. 4). Incubation with KCN for only 15 s did not change the P-700 kinetics as compared to the kinetics in control chloroplasts (Fig. 4, trace A). The presence of uncoupler has no effect on the relative amplitude of the two phases except for a shorter half-time of the fast phase (experiments not shown) (cf. half-times in Fig. 5). Addition of 0.2 mM dichlorophenolindophenol and 5 mM sodium ascorbate to the reaction mixture decreased the half-time of the slow phase in KCN-incubated chloroplasts to 22 ms due to direct reduction of P-700$^+$ by reduced dichlorophenolindophenol in agreement with previous results [26].

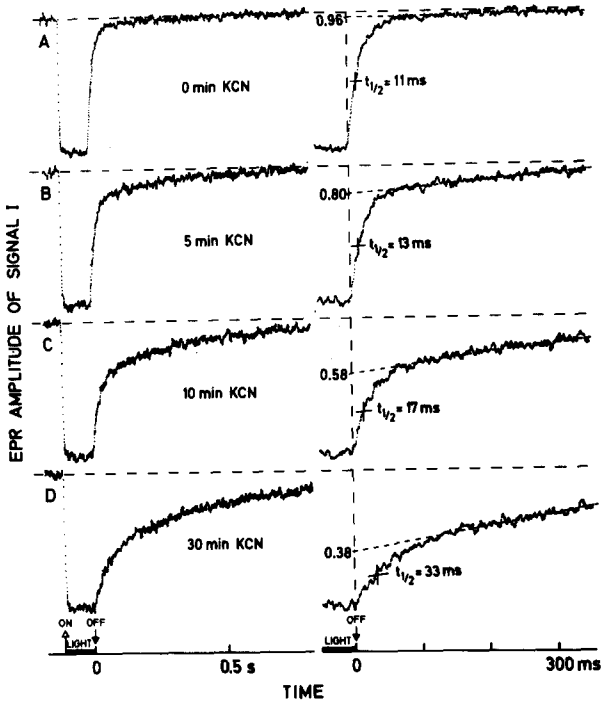


Fig. 4. Time course of the EPR amplitude of Signal I induced by long flashes after preincubation of chloroplasts with KCN. The time of preincubation was 0 min (approx. 15 s needed for addition of KCN and subsequent dilution), 5, 10 and 30 min for trace A, B, C and D, respectively. Left-hand traces: complete time course. Right-hand traces: Section of the left-hand traces at expanded time scale. The slow phase of P-700$^+$ reduction is extrapolated by the dashed line. The resulting relative amplitude of the fast phase is 0.96, 0.80, 0.58 and 0.38 for traces A, B, C and D, respectively. Chlorophyll concentration was between 1.9 and 2.1 mg/ml. NH_4Cl was omitted from the reaction mixture. Arrows and origin of time scales as in Fig. 2. The flash duration was 66 ms.

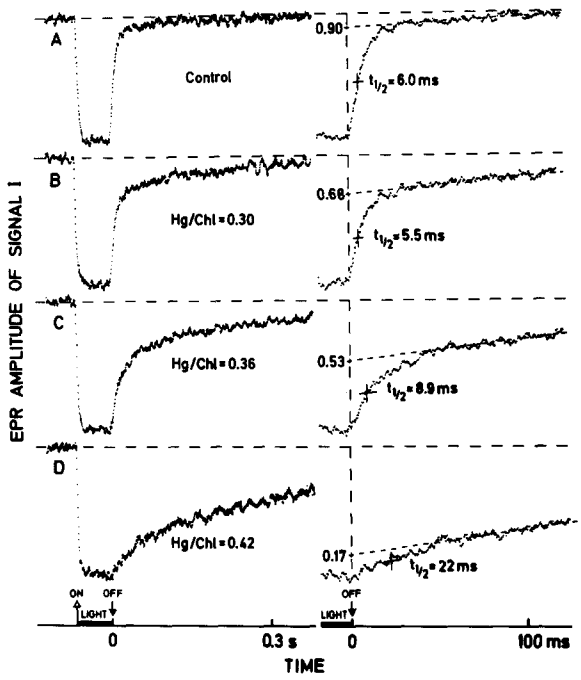


Fig. 5. Time course of the EPR amplitude of Signal I induced by long flashes after incubation of chloroplasts with $HgCl_2$. The molar ratio of $HgCl_2$ to chlorophyll during incubation was 0, 0.30, 0.36 and 0.42 for trace A, B, C and D, respectively. The relative amplitude of the fast phase is 0.90, 0.68, 0.53 and 0.17 for trace A, B, C and D, respectively. The chlorophyll concentration was approx. 2 mg/ml, NH_4Cl 5 mM. Other details as in Fig. 4. Chl, chlorophyll.

The flash-induced kinetics of P-700 after incubation of chloroplasts with increasing $HgCl_2$ concentrations are shown in Fig. 5. Partial inhibition is possible in the narrow concentration range from 0.3 to 0.42 mM $HgCl_2$ at 1 mM chlorophyll in agreement with previous measurements of electron-transport rates [28,46]. The half-time of the fast component of the biphasic P-700$^+$ reduction increased at decreasing amplitude from 6.0 ms in control chloroplasts to 5.5, 8.9 and 22 ms at a molar ratio of $HgCl_2$ to chlorophyll of 0.30, 0.36 and 0.42, respectively (traces B, C and D in Fig. 5). At a molar ratio of 0.46, total P-700$^+$ is reduced slowly after the flash with a half-time of 0.2–0.3 s (not shown). The appearence of the slow phase of P-700$^+$ reduction after partial inhibition of plastocyanin with KCN or $HgCl_2$ is evidence for P-700 molecules not reducible by the residual active plastocyanin.

Incubation of chloroplasts with EDAC has been supposed to inhibit electron transport between the inhibition sites of DBMIB and KCN [31]. Fig. 6 shows that the water-soluble carbodiimide causes a biphasic reduction of P-700$^+$ after a long flash. Incubation of chloroplasts with 0.05, 0.2 and 2.0 mM EDAC increased the half-time of the fast phase from 4.5 ms to 5.7, 7.5 and 12 ms, respectively (traces in Fig 6). The amplitude of the fast phase did not decrease below 40% of the total Signal I amplitude even at high EDAC concentrations as demonstrated by traces C and D measured

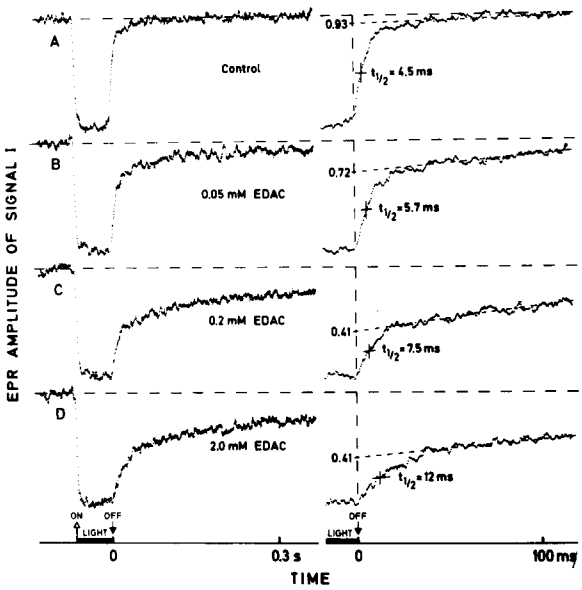


Fig. 6. Time course of the EPR amplitude of Signal I induced by long flashes after incubation of chloroplasts with EDAC. The concentration of EDAC during incubation in the light [31] was 0, 0.05, 0.2 and 2.0 mM for trace A, B, C and D, respectively. The relative amplitude of the fast phase is 0.93, 0.72, 0.41 and 0.41 for trace A, B, C and D, respectively. 280 signals are averaged. The content of the cuvette was changed after 140 signals. Chlorophyll concentration was between 2.1 and 2.5 mg/ml, NH_4Cl 5 mM. Other details as in Fig. 4.

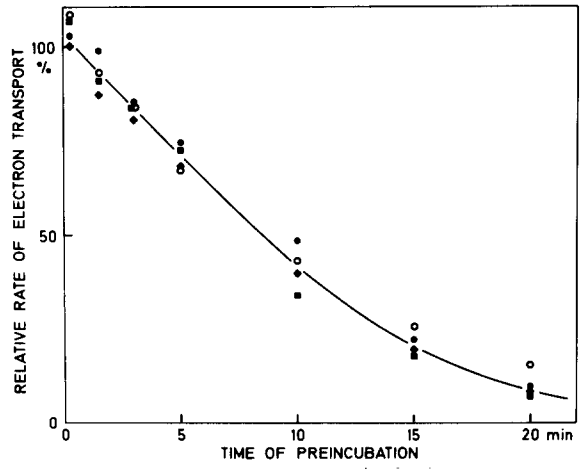


Fig. 7. Effect of preincubation with KCN on electron-transport rates. Full symbols: linear electron transport in chloroplasts prepared from different batches of spinach leaves. The control rate was 810, 1090 and 1130 μequiv./mg chlorophyll per h for the measurements represented by ■, ● and ◆, respectively. Open circles: PS I-driven electron transport from DAD in the chloroplasts used for the measurements represented by full circles. The control rate was 2470 μequiv./mg chlorophyll per h.

after incubation with 0.2 and 2 mM EDAC, respectively. The initial reduction rate of $P\text{-}700^+$ at 2 mM EDAC is about 15% of that in control chloroplasts (Fig. 6A). This is in agreement with the rates of linear electron transport reported by McCarty [31].

Effect of partial inhibition of plastocyanin on the rate of linear electron transport

The effect of partial inhibition of plastocyanin on the rate of linear electron transport is expected to provide additional information on a cooperation between electron-transport chains after the rate-limiting step by plastocyanin. If plastocyanin functions as a pool of molecules the fast bimolecular reactions of plastocyanin [47,48] would not become rate limiting in linear electron transport before most of plastocyanin is inhibited. A sigmoidal dependence of the electron-transport rate as a function of the concentration of active plastocyanin would be expected. Therefore, we have examined the effect of KCN with special regard to the first minutes of incubation. The results in Fig. 7 show an immediate decrease in the rate of linear electron transport at increasing incubation times. Fig. 7 shows also the effect of KCN incubation on the electron-transport rate from DAD to PS I in the presence of DCMU. The active portion of the electron-transport chain in this system includes plastocyanin [21,26] but not the slow electron transfer from plastoquinol to cytochrome *f*. Nevertheless, the time course of inhibition is the same as that of linear electron transport.

Discussion

A functional connection of more than ten PS II [6–8] with a smaller number of PS I [49] has been shown by previous measurements as well as a large variability in the molar ratio of PS II to PS I [50]. These results are consistent with a long-range electron transfer from PS II in the grana partitions to PS I in nonappressed regions of the thylakoids as

suggested recently by the distribution of the protein complexes [10]. Plastoquinone has been proposed to mediate the lateral distribution of electrons [6], and its rate of diffusion in the lipid matrix of the thylakoids may match with the rate-limiting oxidation of plastoquinol (cf. Ref. 11). The experimental evidence, however, for a localization of the site of the electron distribution is rather indirect [6,7]. Plastocyanin is a small molecule [51] which could connect the cytochrome b_6-f with PS I reaction center complexes fast enough by lateral diffusion [52,53] even at large distances to be consistent with the fast reactions found in chloroplasts.

Models for cooperation between electron-transport chains

Our approach to localize sites of cooperation more precisely takes advantage of the slow rate of electron transfer from plastoquinol to cytochrome f which limits the rate of linear electron transport between the two photoreactions. This is obvious from the reduction of the plastoquinone pool [8] and the simultaneous oxidation of cytochrome f, plastocyanin and P-700 during illumination with light of high intensity (cf., e.g., Ref. 54). This state is symbolized in Fig. 8. A plastoquinone pool common to several PS I [6] is assumed but is not prerequisite for the following discussion. The schemes in Fig. 8B–E show relative to an inhibition site, different possibilities of cooperation between the electron-transport chains after the rate-limiting step.

The time course expected for P-700 after a long flash is plotted below the respective scheme at an arbitrarily chosen inhibition of 50%. An electron exchange between electron carriers after the inhibition site, being fast compared to the rate-limiting step, will cause a reduction of total P-700$^+$ with a 2-fold greater half-time than that of the control (Fig. 8B). If the electron-transport chains act independently of each other after the rate-limiting step, P-700 of active chains will be reduced with a half-time as in control chloroplasts while P-700 of inhibited chains is not reduced (Fig. 8C). P-700 of inhibited chains will also not be reduced if a fast electron exchange is possible before the inhibition site (Fig. 8D). At variance with independent chains the oxidation of plastoquinol will not be changed and therefore the half-time of P-700$^+$ reduction in active chains will decrease by a factor of about two.

A fast electron exchange before as well as after the inhibition site is realized by inhibition of a

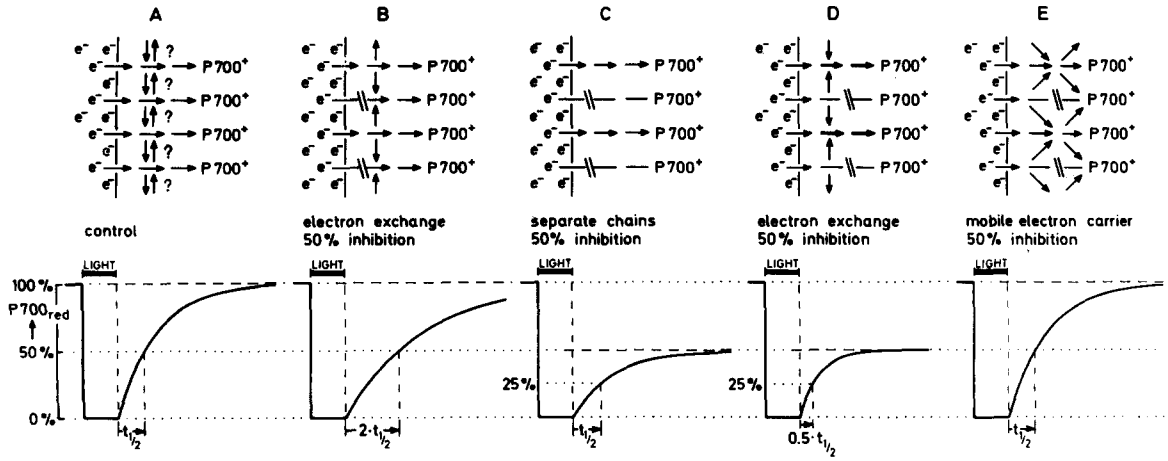


Fig. 8. (Top) Simplified models of linear electron transport through PS I electron carriers with different sites of electron exchange relative to the site of inhibitor action. (Bottom) Time courses of P-700$^+$ reduction after a long flash expected at 50% inhibition in the electron-transport model above the kinetics. e^- symbolize electrons accumulated in the plastoquinone pool during the illumination period [8]. The vertical line represents the rate-limiting step in linear electron transport between PS II and PS I. (A) No inhibition; (B) fast electron exchange after the inhibition site; (C) independent electron-transport chains; (D) fast electron exchange at a site between the rate-limiting step and the inhibition site; (E) inhibition of a mobile electron carrier functioning as a pool. For details see text.

mobile electron carrier (Fig. 8E). This has been proposed for plastocyanin [1,14,17], and needs special consideration of the reaction rates of plastocyanin. The half-time of cytochrome *f* oxidation of 100–500 μs [18,47] and that of the electron transfer from plastocyanin to P-700$^+$ of 20–200 μs [18,48] are more than an order of magnitude faster than the oxidation of plastoquinol with a half-time of about 10–20 ms [8]. In addition, both the in vitro rate constant of plastocyanin reduction by cytochrome *f* [55] and that of plastocyanin oxidation by P-700$^+$ [56,57] indicate extremely fast reactions. Thus, at 50% inhibition of mobile plastocyanin its reactions should still be faster than the rate-limiting reaction. The expected reduction kinetics of P-700$^+$ are very similar to those of control chloroplasts. At progressive inhibition of active plastocyanin by more than 90% a new limitation of the linear electron-transport rate is expected and P-700$^+$ kinetics similar to those in Fig. 8B.

On a complex between plastocyanin and P-700

At partial inhibition of plastocyanin by KCN or $HgCl_2$ the time course of P-700$^+$ reduction after a long flash shows biphasic kinetics with a new slow phase (Figs. 4 and 5). This indicates a fraction of P-700 which becomes not as accessible to electrons from PS II as the other fraction of P-700. The scheme and kinetics in Fig. 8C are a first approximation to the effect on the time course of P-700. This result excludes (i) a fast exchange of electrons between P-700 molecules and (ii) a direct reduction of P-700$^+$ by a pool of mobile plastocyanin. Both of these possibilities would have caused a monophasic reduction of total P-700 similar to that in Fig. 8B or E. P-700 is concluded to be reduced via complexed plastocyanin.

This conclusion is in agreement with the properties of the large 20 μs component of P-700$^+$ reduction after a short flash [18]. The effect of KCN on the smaller component with a half-time of 140–200 μs indicates some P-700 being reduced via a more mobile pool of plastocyanin. In the absence of $MgCl_2$ the latter becomes a major component of the P-700 reduction kinetics [18]. This is in agreement with the analysis by Olsen et al. [58] which indicates a bimolecular reduction of P-700$^+$ at subzero temperatures. If their data are extrapolated to 20°C (Fig. 3 of Ref. 58) a value of about 120 μs is found for their shorter half-time in close agreement with our values. The kinetics of P-700$^+$ after long repetitive flashes, however, do not indicate a significant contribution of this type of mobile plastocyanin to linear electron transport.

The effect of incubation with EDAC on the reduction of P-700$^+$ is very similar to that of KCN and $HgCl_2$. It is likely that the water-soluble carbodiimide accumulates inside the thylakoids during incubation in the light and inactivates plastocyanin by reaction with its carboxyl groups at the low internal pH. Incubation of isolated plastocyanin with 30 mM carbodiimide at pH 5.5 inhibits its reaction with P-700$^+$ but does not affect the spectrum of oxidized plastocyanin (unpublished results). This is in agreement with recent results found with ethylenediamine and EDAC [32].

On a complex between plastocyanin and cytochrome f

The electron-transport rate at continuous illumination provides an independent test for a function of plastocyanin as a mobile electron carrier. As discussed for the scheme in Fig. 8E partial inhibition of mobile plastocyanin is not expected to limit the rate of linear electron transport before the concentration of active plastocyanin is decreased by a factor of about ten. The electron-transport rate, however, decreases immediately without any sigmoidicity at increasing time of KCN incubation (Fig. 7). This result excludes (i) an oxidation of cytochrome *f* by a pool of mobile plastocyanin and (ii) a fast electron exchange between cytochrome b_6-*f* complexes (cf. schemes in Fig. 8E and D, respectively). It indicates that the amount of active plastocyanin controls the rate-limiting step in linear electron transport. A direct electron transfer from plastoquinol to plastocyanin is very slow [59]. But a possible mechanism consistent also with other findings is that a plastocyanin molecule tightly associated to the cytochrome b_6-*f* complex is prerequisite for the function of this complex. Inhibition of the associated plastocyanin would block the electron transfer through the whole complex. This is supported by a decrease in the amplitude of cytochrome *f* absorbance changes at increasing time of KCN in-

cubation (Haehnel, W. and Krause, H., unpublished results). It is concluded that the number of active cytochrome b_6-f-plastocyanin complexes determines the rate limitation in linear electron transport. Ions and osmolarity may affect the reversible association of plastocyanin to cytochrome f which could account for the effect of ions [61] and sucrose [17] on linear electron transport. Ke et al. [62] have found plastocyanin associated with the cytochrome b_6-f complex which is consistent with our conclusion.

An inhibition of plastocyanin proportional to the time of KCN incubation is presumed for the conclusions above. This is supported by the exponential time course of KCN inhibition of plastocyanin in vitro [27] by removal of the copper atom [30] as well as by the PS I-driven electron-transport rate with DAD as electron donor in Fig. 7. The latter electron-transport rate can be assumed to be proportional to the concentration of active plastocyanin [21,26]. KCN incubation of the purified cytochrome b_6-f complex [63] shows no effect on its plastoquinol-plastocyanin-oxidoreductase activity (experiment carried out in collaboration with E. Hurt). It was also tested if KCN-inhibited plastocyanin binds tightly to P-700 in contrast to active plastocyanin. This was not supported by the effect of 9 μM KCN-treated plastocyanin [27] added to a reaction mixture with 3 μM active plastocyanin and PS I particles (cf. Ref. 57). The half-time of P-700$^+$ reduction increased slightly from 1.9 to 2.4 ms, but the flash-induced amplitude was not diminished (experiments not shown).

An important result with respect to possible models of electron transport is the increasing half-time of the fast P-700$^+$ reduction at progressive inhibition with KCN, $HgCl_2$ and EDAC in Figs. 4, 5 and 6, respectively. The finding is not consistent with any of the schemes in Fig. 8 nor with a possible association of the cytochrome b_6-f complex, plastocyanin and PS I in a supramolecular aggregate. The latter seems also unlikely with regard to the variable molar ratio of cytochrome f to P-700 [15]. The increase in the half-time indicates an additional reaction site of plastocyanin which controls the rate-limiting electron transfer at the cytochrome b_6-f complex and is in agreement with the data found for linear electron transport.

Evidence for cooperation of electron-transport chains after plastoquinone

Partial inhibition of electron transport by DBMIB and BPh causes reduction kinetics of P-700$^+$ which indicate rapid electron exchange at a site between cytochrome f and P-700 as discussed for scheme B of Fig. 8. Total P-700$^+$ is found to be reduced even after inhibition of more than 90% of the Rieske iron-sulfur centers (cf. trace D in Fig. 2B). This is evidence that one cytochrome b_6-f complex is capable of distributing electrons to more than ten PS I reaction center complexes. Prerequisite to this conclusion is a strong binding of DBMIB to the inhibition site which is indicated by the shift in the EPR signal [64] and in the redox potential of the Rieske iron-sulfur center [65] as well as by the specific binding of DBMIB. Independent support for a cooperation of the electron-transport complexes is provided by the oxidation kinetics of cytochrome f after a short flash (Haehnel, W. and Krause, H., unpublished results). The half-time of these kinetics increases remarkably at increasing inhibition of plastocyanin by KCN.

This distribution of electrons can be reconciled with the results showing no cooperation between cytochrome b_6-f complexes and between P-700 complexes after inhibition of plastocyanin by a mechanism as proposed in Fig. 9. It is concluded that both the cytochrome b_6-f complex and the PS I reaction center complex are complexed with a plastocyanin molecule. This is consistent with two plastocyanin molecules per P-700 participating in linear electron transport as detected by EPR [66,67] and absorbance changes [54]. The electron transfer between the two complexes could occur via the plastocyanin molecules by lateral diffusion of the complexes as tentatively suggested in Fig. 9. The distance diffused in the plane during a given period of time is equivalent to the root of the mean-square displacement $\sqrt{\overline{x^2}}$ according to Einstein's formula $\overline{x^2} = 4 \cdot D \cdot t$. If a diffusion coefficient $D = 8.3 \cdot 10^{-10}$ cm^2/s as determined for integral proteins in mitochondria [68] is assumed for the cytochrome b_6-f and the PS I complex, each of the complexes

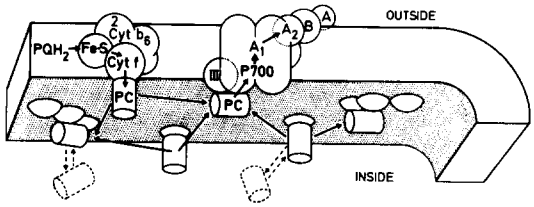


Fig. 9. Tentative model for an organization of electron transport from plastoquinone to PS I in nonappressed thylakoid membranes as an attempt to summarize the results described in the text. The configuration of the cytochrome b_6-f complex and of the PS I reaction center complex is based on the results of Hurt and Hauska [63] and of Bengis and Nelson [74], respectively. Thickness of the membrane is about 6–7 nm. Plastocyanin is symbolized by a cylinder [69]. A_1, A_2, B, A, chain of immediate electron acceptors of PS I (cf. Ref. 75); Cyt, cytochrome; Fe-S, Rieske iron-sulfur center; PC, plastocyanin; PQH_2, plastoquinol; III, subunit III of PS I reaction center complex (Ref. 74, cf. Ref. 57).

could diffuse about 10 nm during the 300 μs half-time of cytochrome f oxidation [54]. This value is larger than the free distance between the protein complexes as estimated from the density and the diameter of the particles observed by electron microscopy [1]. Fig. 9 illustrates the relatively short distances between neighboring complexes in the plane of the membrane. The electron transfer from one complexed plastocyanin to the other may be facilitated by appropriate binding of the two different electron-transfer sites of the cylindrical plastocyanin molecule [69]. This would be a mechanism different from that of cytochrome c [70]. The slow electron-exchange reaction of plastocyanin in solution [71] does not favor the distribution of electrons via free plastocyanin inside the thylakoids.

On the lateral organization of electron transport

Recent investigations [72,73] have shown a rather uniform distribution of the cytochrome b_6-f complex in thylakoid membranes. This suggests that about one-half of the cytochrome b_6-f complex is associated with PS II in appressed membranes and the other half with PS I in nonappressed membranes. The reactions of the cytochrome b_6-f complex in the different environments should differ considerably and depend largely on the site of the long-range electron transfer between the two photosystems. If plastocyanin were to act as the mobile electron carrier, the cytochrome b_6-f complex in appressed membranes would function in linear electron transport and that in nonappressed membranes in some cyclic electron transfer. Our results are not consistent with this model and favor an alternative possibility, that it is the pool of plastoquinone which acts as shuttling electron carrier in the membrane. The cytochrome b_6-f complex in nonappressed membrane regions is concluded to participate efficiently in electron transport to PS I. This is consistent with the finding that less than the chemically determined amount of cytochrome f participates in linear electron transport [49,60]. Questions related to the cytochrome b_6-f complex in appressed regions are the subject of present investigations.

Conclusions

Fig. 9 shows a possible organization of electron transport in the plane of the membrane which is consistent with the reported results as well as with most other known properties of electron-transport components. The rate-limiting electron transfer in linear electron transport is concluded to be controlled by the number of supramolecular complexes of cytochrome b_6-f with plastocyanin. PS I reaction centers are assumed to be associated with another complexed plastocyanin. The cytochrome b_6-f complex can transfer electrons to more than ten PS I reaction center complexes. This distribution of electrons could be possible by lateral diffusion of both complexes localized in nonappressed membranes. The plastoquinone pool is assumed to function as the long-range mobile electron carrier from PS II in appressed to cytochrome b_6-f in nonappressed membrane regions.

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