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ELECTRON TRANSPORT BETWEEN PLASTOQUINONE AND CHLOROPHYLL A_1 IN CHLOROPLASTS

II. REACTION KINETICS AND THE FUNCTION OF PLASTOCYANIN IN SITU

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SUMMARY

The light-induced reaction kinetics of electron carriers between the two light reactions were studied in spinach chloroplasts.

1. The difference spectrum of the absorbance changes of plastocyanin in situ was separated from superimposing absorbance changes by flash titration described in the preceding paper (Haehnel, W. (1973) Biochim. Biophys. Acta 305, 618–631). Relative amounts of 2 : 1 electron equivalents were observed for plastocyanin and chlorophyll a_I (*P*-700).

2. A balance of the electron equivalents released from reduced plastoquinone and simultaneously accepted by oxidized plastocyanin, cytochrome *f* and chlorophyll a_I indicated a quantitative electron transfer. Additional electron carriers between plastoquinone and light reaction I can be excluded with an accuracy of about ± 0.3 electron equivalents per light reaction II.

3. The time course of the absorbance changes of plastocyanin was measured at 584 nm with negligible interference with other absorbance changes. The reduction kinetics show an initial lag followed by a rise with a half time of about 20 ms. The redox states of plastocyanin and chlorophyll a_I during this reduction via the rate-limiting step between the light reactions and during oxidation by weak far-red light suggest a true equilibrium constant of about 20.

4. The simultaneous oxidation and reduction kinetics of plastoquinone, cytochrome *f*, plastocyanin and chlorophyll a_I induced by two successive groups of saturating flashes after far-red illumination were measured. The oxidation kinetics of plastocyanin and the simultaneous reduction kinetics of chlorophyll a_I after the single flashes indicate a quantitative electron transfer with a half time of 200 μs.

5. The fast reduction of chlorophyll a_I by plastocyanin showed no effect of the

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

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inhibitors 3-(3',4'-dichlorophenyl)-1,1-dimethylurea and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone or of reduced phenazine methosulfate. But it was completely inhibited after KCN incubation.

6. The oxidation kinetics of cytochrome *f* were reinvestigated with high time resolution from the difference of absorbance changes at 554 minus 540 nm to minimize the disturbing interference with other absorbance changes. Absorbance changes measured at 554 nm alone do not reflect kinetics of cytochrome *f*. The half time of the oxidation was faster than 40 μs.

7. The observed reaction kinetics gave evidence for a function of cytochrome *f* between plastoquinone and chlorophyll a_I in parallel to plastocyanin. In addition, they indicate that the greater portion of linear electron transport passes plastocyanin. The complex interaction between cytochrome *f* and chlorophyll a_I is discussed.

INTRODUCTION

Plastocyanin was discovered and characterized by Katoh et al. in algae and chloroplasts of higher plants [1, 2]. It was found to contain one copper atom [3, 4] and in spinach two [5] or possibly four [6] copper atoms per molecule. The oxidation and reduction of plastocyanin takes place by a single electron transfer [1, 5, 7]. Evidence from many lines of investigation has established the role of plastocyanin in the photosynthetic electron transport between the two light reactions. However, its exact site of action is still not fully understood.

The wealth of results concerning plastocyanin has been obtained from studies of the effect of externally added soluble plastocyanin on photochemical reactions in plastocyanin-depleted chloroplast fragments obtained by sonic [8–13], detergent [14–17], or French press treatment [18, 19]. Many of these investigations report that the oxidation of cytochrome *f* requires the addition of plastocyanin. This is interpreted in favour of a strict order in linear electron transport of cytochrome *f* → plastocyanin → chlorophyll a_I (*P*-700), which is supported by the rate constants of electron transfer between the isolated proteins in vitro [20, 21]. Whether this is valid for the action of plastocyanin in vivo does not seem to be conclusive, because in intact chloroplasts added plastocyanin has no effect [22, 23] and in fragmented chloroplasts, still retaining bound plastocyanin [23, 24], the reaction of externally added plastocyanin is artifactual [12]. This sequence of electron transport has also been favoured by Gorman and Levine [25] from their experiments with mutants of *Chlamodymonas reinhardii* deficient either in plastocyanin or cytochrome *f*. But in contrast to a linear sequence of the electron carriers the same authors could not detect plastocyanin in one mutant strain which was capable in photosynthetic CO_2 fixation. Another line of evidence was provided by experiments with chloroplasts incubated with $HgCl_2$ [26] and KCN [27, 28]. Both inhibitors inactivate endogenous plastocyanin [29].

However, measurements of the absorbance changes of cytochrome *f* and chlorophyll a_I in intact chloroplasts gave no evidence for this sequence [30–36]. The simple in-series model of the three electron carriers has been excluded not only from the different equilibrium constants derived from the redox states of cytochrome *f* and chlorophyll a_I in the dark and during light-limited excitation by Malkin [30] and by

Marsho and Kok [31], but also from their reduction kinetics, as reported in the preceding paper [34].

The only spectrophotometric measurements of a light-induced absorbance change in the 600 nm region ascribed to plastocyanin are reported by de Kouchkovsky and Fork [37]. This absorbance change was found to be inhibited irreversibly by salicylaldoxime in contrast to the absorbance change of cytochrome *f* [38]. However, salicylaldoxime was shown later to be an inhibitor of Photosystem II and does not react with plastocyanin at any significant rate [39, 40]. Absorbance changes of plastocyanin could not be detected, as yet, by Amesz et al. in different algae [41].

Malkin and Bearden [42] and Visser et al. [43] have succeeded in demonstrating photooxidation and photoreduction of plastocyanin in situ in isolated spinach chloroplasts and different algae, respectively, by ESR spectroscopy at 25 K. Their results confirm the role of plastocyanin as electron carrier between the two light reactions. Kinetic studies of the oxidation and reduction of plastocyanin which would be expected to give more detailed evidence of its reaction site are difficult to achieve by this technique.

Investigations showing the interaction of chlorophyll a_I with its primary electron donors should give an approach to the reactions of plastocyanin in situ. Marsho and Kok accounted for such an electron donor which was supposed to be in true equilibrium with chlorophyll a_I in contrast to cytochrome *f* [31]. Furthermore one of the two fast reduction kinetics of chlorophyll a_I with half times of 20 μs and 200 μs, which we found in spinach chloroplasts [44, 45], should correspond to the oxidation kinetics of plastocyanin.

In the preceding communication [34], a method of flash titration of the electron carriers of Photosystem I has been described. Using this technique, the electron equivalents released by the pool of plastoquinone were found to exceed those accepted by oxidized cytochrome *f* and chlorophyll a_I. The electron equivalent difference enabled to predict the reduction kinetics of plastocyanin if any other electron carrier between plastoquinone and chlorophyll a_I was excluded.

Based on the results in this communication, the separation of the plastocyanin difference spectrum is reported with special regard to superimposing absorbance changes. The simultaneous kinetics of plastocyanin and chlorophyll a_I during reduction and oxidation are resolved. The oxidation kinetics of cytochrome *f* after a flash previously measured at one wavelength [46–48] are reinvestigated with high time resolution from the difference of the absorbance changes at 554 minus 540 nm. The concentrations of the electron carriers of System I, engaged in linear electron transport are quantitatively calculated and, based on the reaction kinetics, their sequence is discussed in detail.

MATERIALS AND METHODS

Hypotonically broken and Class II chloroplasts of spinach were isolated as described in ref. 34. The reaction mixture contained these chloroplasts at a concentration of 10 μM chlorophyll, 20 mM *N*-tris(hydroxymethyl)methylglycine adjusted to pH 7.2 with NaOH, 20 mM KCl, 1 mM $MgCl_2$, 10 μM benzylviologen as electron acceptor and 0.5 μM gramicidin D as uncoupler. Further additions are given in the legends of the figures. KCN treatment of the chloroplasts was carried out as described

by Izawa et al. [28]. At a final concentration of 30 mM KCN, the chloroplasts were incubated for 60 min. The temperature of the sample was 20–22 °C. For registration a flash photometer with double beams and repetitive flash excitation was employed similar to that described by Döring et al. [49]. The actinic flashes of saturating intensity had a half time of 25 μs, except in the experiment shown in Fig. 8 during which ultra short flashes (1 μs) were used [50]. The flashes were passed through Schott filters BG 23 (6 mm) and KG 2 (2 mm) for simultaneous measurement of the absorbance changes of plastoquinone at 265 nm ($\Delta\lambda = 2.5$ nm) and of chlorophyll a_I at 705 nm ($\Delta\lambda = 3$ nm) and through Schott filters RG 610 (3 mm) and KG 2 (2 mm) for the measurement of the absorbance changes of cytochrome *f* as the difference at 554 nm minus 540 nm ($\Delta\lambda = 2$ nm) and of plastocyanin at 584 nm ($\Delta\lambda = 5$ nm). The intensity of the monitoring light was less than 100 ergs $\cdot$ cm^{-2}s^{-1}. The influence of light-induced scattering changes on the absorbance changes at 584 nm and those in Fig. 3 was minimized by placing an opal glass on the cuvette and a light pipe above the photomultiplier [51]. During the measurement of the signals with a repetition rate of 0.2 Hz, the far-red light with an intensity of $1.3 \cdot 10^4$ ergs $\cdot$ cm^{-2}s^{-1} (Schott interference filter IL 720, $\Delta\lambda = 15$ nm) was switched off. The content of the 2 $\times$ 2 cm cuvette was changed after every 64 signals. The electrical bandwidth ranged from d.c. to 100 kHz and in the measurement in Fig. 8 from 1 Hz to 300 kHz and was limited by the chosen distance of the addresses of the signal averager Fabri-Tek 1072 with plug-in SD-72/2A and SW-70 or SD-77 and SW-77, respectively. Deviating conditions were noted.

The difference of the absorbance changes at 554 minus 540 nm was obtained in two ways. At time resolutions up to 40 μs/address, the signals at 554 and 540 nm were simultaneously measured with the dual beam spectrophotometer. The difference was obtained by inverted data transfer of the averaged signal at 540 nm into the memory allocation containing the averaged signal at 554 nm. This technique has been used for our previous investigations [34] as well as for the measurement shown in Fig. 5. At higher time resolution, as used for the transients in Fig. 8, the signals were stored one after the other in the same memory allocation of the averager but the signals at 540 nm with inverted mode.

Separation of absorbance changes by flash titration

To separate absorbance changes of components between plastoquinone and chlorophyll a_I, our technique of flash titration [34] is simplified by using only excitations with one flash or a group of twelve flashes spaced at 1.6 ms. Fig. 1 illustrates the situations in the linear electron transport chain at different times of the experiments. All electron carriers between the light reactions of chlorophyll a_{II} and chlorophyll a_I are oxidized by preillumination with System I light (Fig. 1A). After one flash, one electron and, during a group of twelve flashes spaced at 1.6 ms, about five electrons [34] are accumulated in the pool of plastoquinone. This is due to the electron transfer times of 0.6 ms from water to the plastoquinone pool [52, 53] and of 20 ms via the rate-limiting step to the electron carriers of System I [53]. After one flash, only reduction of chlorophyll a_I is expected as a first approximation because of the difference of 80 mV in the midpoint potentials of plastocyanin [5, 7] and chlorophyll a_I [54, 55] and the negligible reduced amount found for cytochrome *f* [34]. After a group of twelve flashes, all electron carriers of System I must be reduced because their

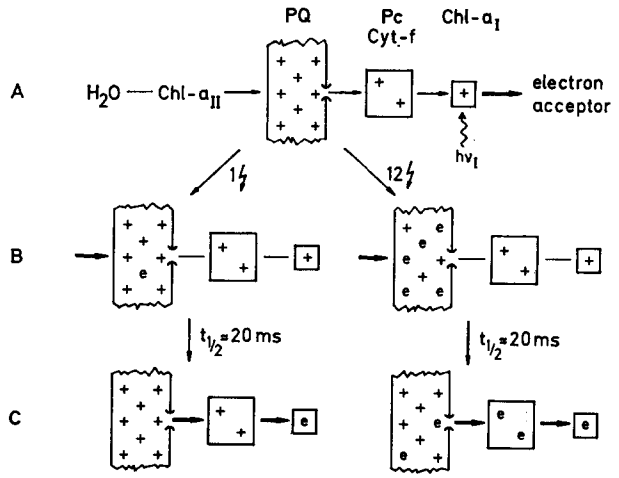

Fig. 1. Simplified scheme of linear electron transport between the two light reactions [53]. Redox states of the electron carriers, A: after preillumination with System I light; B, C (left): after one flash; B, C (right): after a group of twelve flashes. For details see text.

electron capacity is limited to three per light reaction II [53, 31]. Therefore the difference between the amplitudes of the absorbance changes in the visible region after twelve flashes minus those after one flash must originate from the reduction of cytochrome *f* and at least partially from the reduction of plastocyanin if the latter functions as electron carrier between plastoquinone and chlorophyll a_I.

RESULTS

Separation of the light-induced difference spectrum of plastocyanin in situ

Fig. 2 shows the approach to the light-induced difference spectrum of plastocyanin in situ as described in Materials and Methods. Electrochromic absorbance changes [56] were accelerated to non-resolved time courses by addition of gramicidin D [57] to avoid their interference. Fig. 2A shows the difference spectrum of the absorbance changes after one flash and that after a group of twelve flashes.

After one flash, the time course of the absorbance changes resembles at all wavelengths that at 705 nm (cf. refs. 60 and 68). In addition the difference spectrum agrees with the difference spectrum of the reduction of chlorophyll a_I [58, 59]. This indicates a predominant reduction of chlorophyll a_I in agreement with our previous result [60]. The amplitude corresponds to a reduction of 91 % of oxidized chlorophyll a_I (cf. ref. 34).

After twelve flashes, the absorbance changes show complex time courses. This should result from the superposition of the absorbance changes of chlorophyll a_I and plastocyanin with different kinetics and proportions as a function of the wavelength. Therefore, the amplitudes of the absorbance changes after the last flash are separated with regard to kinetics with half times not appreciably greater than 20 ms, in favour of components engaged in linear electron transport. The investigation is restricted to wavelengths greater than 580 nm to avoid the interference with the absorbance changes of cytochrome *f* under these conditions (see Fig. 5B and ref. 34).

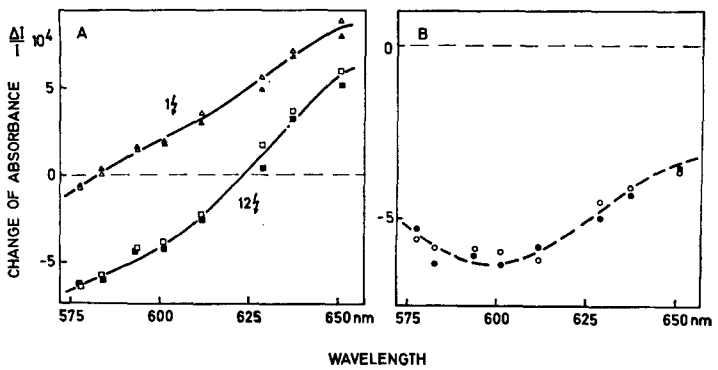

Fig. 2. (A) Absorbance changes as a function of the wavelength after one flash (triangles) and after a group of twelve flashes (squares). Each point was taken from the averaged trace of 128 signals. To separate the amplitudes after twelve flashes more precisely, the starting point of the kinetics was taken from transients resolved with a four times greater time resolution than used for the evaluation of the final level and in Fig. 3. (B) Difference between the absorbance changes shown in Fig. 2A after a group of twelve flashes minus the absorbance changes after one flash multiplied by 1.1 as a function of the wavelength. The open and full symbols represent the results from two different chloroplast preparations. The dashed line traces the reduced minus oxidized difference spectrum of plastocyanin in vitro reported by Katoh [5].

The difference spectrum of cytochrome *f* [61, 62] allows to estimate negligible amplitudes in this region. The complete reduction of chlorophyll a_I after twelve flashes [34] corresponds to an increase of its amplitude after one flash by a factor of 1.1 (equal to 100 % : 91 %). The absorbance changes of the reduction of plastocyanin $(\Delta I/I)_{Pc}$ should cause the difference between the absorbance changes after twelve flashes $(\Delta I/I)_{12\frac{1}{2}}$ minus those after one flash $(\Delta I/I)_{1\frac{1}{2}}$ multiplied by the factor of 1.1:

$$\left(\frac{\Delta I}{I}\right)_{Pc} = \left(\frac{\Delta I}{I}\right)_{12\frac{1}{2}} - 1.1\left(\frac{\Delta I}{I}\right)_{1\frac{1}{2}}$$

The equation is used to calculate from the amplitudes in Fig. 2A the amplitudes in Fig. 2B. They show an excellent agreement with the in vitro difference spectrum of the reduction of plastocyanin from spinach [5].

The amount of reduced plastocyanin is estimated from the amplitude at 597 nm of this difference spectrum by use of the extinction coefficient of oxidized plastocyanin related to a gram-atom copper, $\varepsilon_{597nm} = 4.9 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [2, 5]. The resulting ratio of total chlorophyll to the reduced copper atoms of plastocyanin is 350. The chloroplasts used for these measurements had a ratio of 550 chlorophylls per light reaction II as determined from the oxygen yield per flash (measurement by Dr. G. Renger). Hence 1.6 copper atoms of plastocyanin per light reaction II should be engaged in linear electron transport. This is a first approximation and will be improved below.

Amounts of the electron carriers of System I engaged in linear electron transport

A comparison of the electron equivalents released from the reduced pool of plastoquinone with those accepted by the oxidized electron carriers of System I should not be complicated by an unknown loss of electrons because a quantitative

TABLE I

BALANCE OF ELECTRON EQUIVALENTS IN SYSTEM I

The values were estimated from the absorbance changes of the electron carriers observed after a group of ten or twelve flashes. For details see text. PQ, plastoquinone; PQH_2, plastohydroquinone.

Reaction	Electron equivalents per chlorophyll a_{II}	Related value or assumption
$PQH_2 \rightarrow PQ$	−3.0 [53,34]	$\Delta A_{1/2} \cong$ chlorophyll a_{II} [53]
Cytochrome $f^+ \rightarrow$ Cytochrome f	+0.4 [34]	$\Delta\varepsilon_{554-540\,nm} = 22\ mM^{-1} \cdot cm^{-1}$ [63], oxygen yield per flash
Plastocyanin$^+ \rightarrow$ Plastocyanin	+1.8	$\Delta\varepsilon_{597\,nm} = 4.9\ mM^{-1} \cdot cm^{-1}$ [5], oxygen yield per flash
Chlorophyll $a_I{}^+ \rightarrow$ Chlorophyll a_I	+0.8 (cf. ref. 60)	$\Delta\varepsilon_{705\,nm} = 64\ mM^{-1} \cdot cm^{-1}$ [58], oxygen yield per flash
	+3.0	

electron transfer from water via plastoquinone to chlorophyll a_I has been found after one flash [60]. Table I summarizes our estimations of electron carriers of System I engaged in linear electron transport. The amplitudes of the absorbance changes of plastoquinone related to the amplitude after one saturating flash indicate an amount of three electron equivalents per light reaction II [53]. This estimation avoids the assumption of a questionable difference extinction coefficient. The light-induced changes in the concentrations of the other electron carriers were estimated by use of their known difference extinction coefficients [63, 5, 58]. For a comparison with the result derived from the absorbance changes of plastoquinone, the concentrations were related to the light reactions II. Their number was determined from the oxygen yield per flash in the steady state of System II (measured by Dr. G. Renger).

The value of cytochrome *f* is our previous experimental result [34] and is supported again by the amplitudes of the absorbance changes in Fig. 5B. Deviating from our previous assumption of a ratio of chlorophyll a_I to chlorophyll a_{II} equal to one [34], the amount of chlorophyll a_I was now calculated by use of the difference extinction coefficient of chlorophyll a_I $\Delta\varepsilon_{705\,nm} = 6.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [58] and the maximal absorbance changes at 705 nm, (cf. Fig. 5D). This gave an amount of 0.83 electron equivalents being reduced per chlorophyll a_{II}. An almost equal ratio of chlorophyll a_I to chlorophyll a_{II} of 0.85 has been deduced from comparing the kinetics of plastoquinone and chlorophyll a_I without use of an extinction coefficient. This ratio implies a reduction of the immediate donors of chlorophyll a_I after one saturating flash [60].

Therefore the amount of plastocyanin should be better approximated than above. Small absorbance changes of plastocyanin after one flash would not have remarkably changed the difference spectrum in Fig. 2A from that of chlorophyll a_I but would have reduced the amplitudes of the difference spectrum in Fig. 2B. About 0.16 electron equivalents are expected to reduce plastocyanin after one flash because the reduction of 91 % of chlorophyll a_I corresponds to 0.77 [60] and the reduction of cytochrome *f* to 0.07 electron equivalents per chlorophyll a_{II} [34]. In case of an equilibrium between plastocyanin and chlorophyll a_I according to the midpoint potentials, $E'_0 = 0.37$ V [5, 7] or 0.34 V [64] and $E'_0 = 0.45$ V [54, 55] or 0.46 V [24], respectively, this is the lower limit of reduced plastocyanin after one flash. Thus, plastocyanin should accept at least 1.76 electron equivalents per chlorophyll a_{II} instead of 1.6 as estimated above.

The agreement between the number of electron equivalents released from the reduced pool of plastoquinone and the sum of those being accepted by oxidized cytochrome *f*, plastocyanin and chlorophyll a_I indicate a quantitative electron transfer. Thus, any other electron carrier between plastoquinone and chlorophyll a_I can be excluded from this evaluation, with a maximal error estimated to be ± 0.3 electron equivalents per chlorophyll a_{II}.

Reduction kinetics of plastocyanin

Difficulties in the measurement of kinetics of plastocyanin in situ arising from the superposition of the absorbance changes of plastocyanin and chlorophyll a_I are overcome by measurements at 584 nm. Around this wavelength, the difference spectrum of chlorophyll a_I exhibits an isosbestic point (see Fig. 2A and refs. 58, 59). This wavelength is favoured in addition by the extinction coefficient of plastocyanin

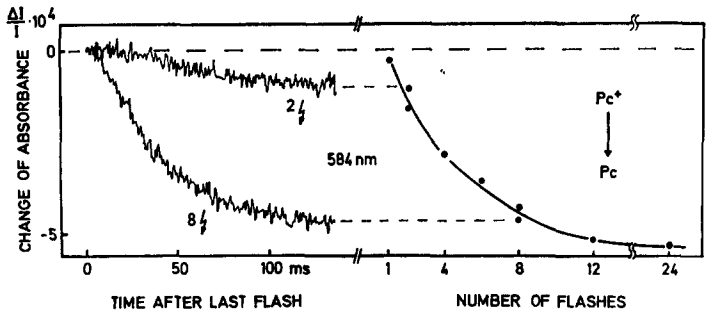

Fig. 3. Absorbance changes of plastocyanin at 584 nm after the last flash of a flash group as a function of the number of the flashes. Left: time course after two and eight flashes. Right: extent of the absorbance changes. Every trace is the average of 512 signals.

being close to that at the maximum of the difference spectrum [5]. Fig. 3 shows the extent of the absorbance changes at 584 nm after the last flash induced by flash groups of an increasing number of flashes. The time course shows in the beginning a lag of about 15 ms after two flashes and of about 5 ms after eight flashes. These lags are followed by reduction kinetics of plastocyanin with half times of 18–20 ms. The amount of reduced plastocyanin increases from a very small value after one flash to its maximal value after a group of twelve flashes.

The equilibrium constant of the electron transfer from plastocyanin to chlorophyll a_I was estimated from their reduction kinetics after eight flashes to be about $K = 20$. This equilibrium being well towards chlorophyll a_I and a fast electron transfer from plastocyanin to chlorophyll a_I is believed to cause the lag in the reduction of plastocyanin. The shortening of the lag corresponds to the decrease of the first half time of the reduction of chlorophyll a_I from about 10 ms after two flashes down to 4 ms after eight flashes [34].

Oxidation of plastocyanin by System I light

Strong white light [30, 31, 65] or a group of many flashes [53] accumulates more electrons in the plastoquinone pool than can be accepted by the oxidized electron carriers of System I during the following electron transfer in the dark. If far-red light of limiting intensity is subsequently submitted the plastoquinone remaining reduced should keep the electron carriers of System I in the reduced state until it is oxidized. Fig. 4 presents the time courses of plastocyanin and chlorophyll a_I under these conditions. The relatively fast reduction of both components via the rate-limiting step of linear electron transport after the flash group is not resolved. The reoxidation of plastocyanin and chlorophyll a_I by far-red light is preceded by a delay as expected. The delay of the reoxidation of plastocyanin was observed after groups with more than ten flashes and increased with the number of flashes at a given intensity of far-red light. This is understood by the increased amount of reduced plastoquinone accumulated during the flash group (data not shown). In addition, the area bounded by the transients of plastocyanin was found to be inversely proportional to the intensity I of the far-red light. This $I \times t$ relationship indicates that the redox state of plastocyanin is determined by the number of electrons transferred by light reaction I to the final acceptor if started from a fixed amount of reduced electron carriers be-

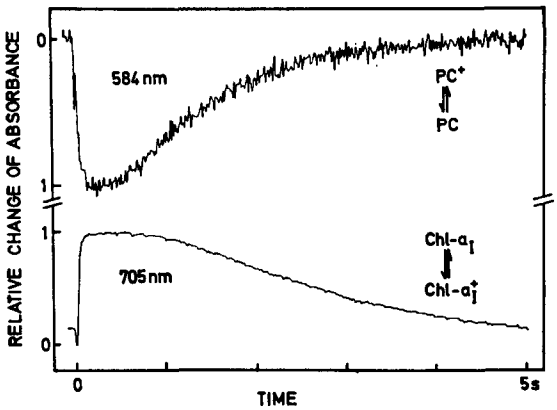

Fig. 4. Absorbance changes as a function of time induced by 16 flashes spaced at 1.6 ms at continuous far-red light with an intensity of 3500 ergs · cm^{-2} · s^{-1}. 32 signals were averaged with a repetition rate of 0.1 Hz. Above: absorbance changes of plastocyanin at 584 nm. Below: absorbance changes of chlorophyll a_I at 705 nm.

tween both light reactions. Similar induction effects were previously observed for cytochrome *f* and chlorophyll a_I [30, 31, 33]. The transients show an oxidation of plastocyanin previous to the oxidation of chlorophyll a_I. The slow rate of electron removal by System I and the considerably faster electron transfer between these components (see below) allow to assume approximated equilibrium conditions between the reaction partners. The equilibrium constant of the electron transfer from plastocyanin to chlorophyll a_I is estimated from the transients in Fig. 4 to be $K \cong 10$. The value is comparable to that estimated from the reduction kinetics.

Kinetics of plastoquinone, cytochrome f, plastocyanin and chlorophyll a_I in subsequent flash groups

Fig. 5 shows the kinetics of absorbance changes of plastoquinone, cytochrome *f*, plastocyanin and chlorophyll a_I induced by a group of twelve flashes followed 200 ms later by a group of six flashes. The preillumination with far-red light caused an oxidation of these electron carriers except about 25 % of chlorophyll a_I [66] which was oxidized in the first flash.

During the first flash group plastoquinone became progressively reduced; cytochrome *f*, plastocyanin and chlorophyll a_I stayed oxidized. This provides further evidence for the location of the rate-limiting step after the pool of plastoquinone and before the electron carriers of System I [67]. The small absorbance change at 584 nm during the flash group is not due to a known component according to its difference spectrum peaking around 584 nm with a small amplitude of $\Delta I/I \cong 2 \cdot 10^{-4}$. However, its relaxation must be very slow because no contribution to the difference spectrum in Fig. 2 is found. The increasing reduction rate of chlorophyll a_I at increasing amounts of reduced plastoquinone [34, 68] is indicated by the increasing spikes caused by chlorophyll a_I which is reduced within 2.5 ms between the flashes.

The kinetics after the flash groups were evaluated from tracings of these signals at a two or four times expanded time scale. A semi-log plot indicated first-order kinetics for the oxidation of plastoquinone and the reduction of cytochrome *f* with

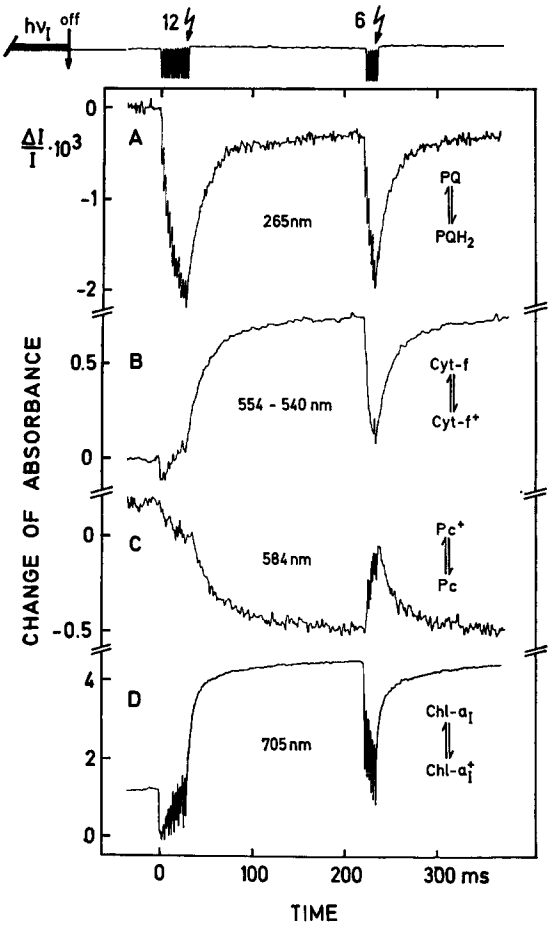

Fig. 5. Absorbance changes as a function of time induced by 12 flashes spaced at 2.5 ms and 200 ms later by 6 flashes spaced at 2.15 ms after far-red preillumination. A, plastoquinone at 265 nm; B, cytochrome *f* at 554 minus 540 nm; C, plastocyanin at 584 nm; D, chlorophyll a_I at 705 nm. Every trace is the average of 128 signals. The upper trace shows the monitored spikes of the flashes. PQH_2, plastohydroquinone.

half times of 10 and 15 ms, respectively. No lag or any sigmoidal time course is observed even from this smooth transient of cytochrome *f*. These kinetics of plastoquinone and cytochrome *f* after the first flash group as well as the time course of chlorophyll a_I reduction, which shows no first-order kinetics and a first half time of 3.5 ms in Fig. 5D, were described in detail in the preceding paper [34]. A comparison of the reduction kinetics of plastocyanin (cf. Fig. 3) and of chlorophyll a_I in Fig. 5C and D indicates that chlorophyll a_I becomes reduced first and plastocyanin more slowly, thus reflecting the estimated equilibrium constant of about 20. The residual amount of plastohydroquinone after the fast reoxidation is significant for a complete reduction of all electron carriers of System I [65, 53] and characterized the situation in the electron transport chain before the second flash group.

During the second group of six flashes, plastoquinone became reduced again.

Reduced cytochrome *f*, plastocyanin and chlorophyll a_I, on the other hand, were oxidized almost completely. This provides evidence for a considerably faster electron transfer from cytochrome *f* and plastocyanin to chlorophyll a_I^+ than via the rate-limiting step. In Fig. 5D, only the amplitude of the slow reduction of chlorophyll a_I can be seen from the spikes after the flashes. Its increasing amplitude indicates a decreasing portion of the fast reduction with increasing number of the flashes. This was paralleled with an increasing amount of oxidized plastocyanin indicated by the amplitudes of the decay after the second flash group, when increasing numbers of flashes were used (not shown).

The kinetics after the second flash group of these four electron carriers resembled those after the first flash group. In particular, the almost equal amount of plastohydroquinone being reoxidized after the flash groups indicated a preceding reoxidation of the electron acceptors during the second flash group in accordance with the amplitudes of these components.

Oxidation kinetics of plastocyanin and simultaneous reduction kinetics of chlorophyll a_I

The kinetics of the small absorbance changes of plastocyanin induced by the first and the second flash of the second flash group in Fig. 5 were resolved at a high time resolution and are shown in Fig. 6 with the coincident kinetics of chlorophyll a_I. The amount of oxidized plastocyanin after the first flash was increased after the second one. Chlorophyll a_I being oxidized in the flashes exhibited repetitive fast reduction. The half time of these reduction kinetics of chlorophyll a_I and of the oxidation kinetics of plastocyanin after the first as well as after the second flash were equal. The average value of several measurements was $t_{\frac{1}{2}} = (200 \pm 30)\ \mu s$. The

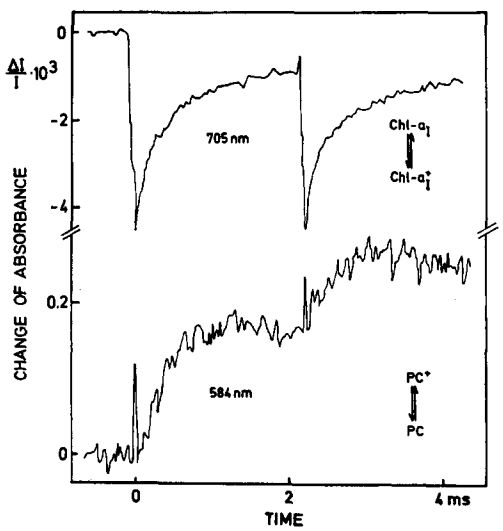

Fig. 6. Absorbance changes as a function of time induced by two flashes spaced at 2.15 ms. Above: absorbance changes of chlorophyll a_I at 705 nm. 128 signals have been averaged. Below: absorbance changes of plastocyanin at 584 nm. 2048 signals have been averaged. The rapid spikes are flash artifacts. The experimental conditions are the same as in Figs. 5C and D, respectively, during the first two flashes of the second flash group, except for a higher time resolution of 20 μs per address.

TABLE II

AMOUNTS OF OXIDIZED PLASTOCYANIN AND SIMULTANEOUSLY REDUCED CHLOROPHYLL a_I

The concentration changes were estimated from the transients in Fig. 6 and the in vitro difference extinction coefficient of plastocyanin at 584 nm of 4.6 $mM^{-1} \cdot cm^{-1}$ [5] and of chlorophyll a_I at 705 nm of 64 $mM^{-1} \cdot cm^{-1}$ [58].

Reaction	First flash		Second flash	
	fast change of absorbance $\Delta I/I \cdot 10^3$	nequiv./l	fast change of absorbance $\Delta I/I \cdot 10^3$	nequiv./l
Plastocyanin oxidation	0.18	8.5	0.12	5.7
Chlorophyll a_I reduction	2.6	8.8	1.7	5.8

time resolution was not sufficient for the reduction kinetics of chlorophyll a_I with a half time of 20 μs [44, 45].

If plastocyanin reduces only chlorophyll a_I, the amount of oxidized plastocyanin should be comparable to the amount of reduced chlorophyll a_I^+. Table II compiles the corresponding absorbance changes ($\Delta I/I$) separated from Fig. 6. To calculate the change of the concentrations in units of equivalents per liter, the extinction coefficient of plastocyanin related to one gram-atom copper at 584 nm, $\Delta\varepsilon_{584\,nm} = 4.6 \cdot 10^3\ M^{-1}\ cm^{-1}$ [5], was used and for chlorophyll a_I $\Delta\varepsilon_{705\,nm} = 6.4 \cdot 10^4\ M^{-1}\ cm^{-1}$ [58]. Both concentration changes after the first and after the second flash differ by less than 7 % from each other. This agreement as well as the equal half times strongly suggest a quantitative electron transfer from plastocyanin to chlorophyll a_I^+ with a half time of 200 μs. The electron capacity of plastocyanin being twice the capacity of chlorophyll a_I should allow only partial oxidation of plastocyanin after one flash. In addition, the oxidation-reduction equilibrium admits only successive oxidation of plastocyanin during a flash group. This is supported by the increase of oxidized plastocyanin not only after the second flash but also after the third and fourth flash (not shown, but cf. amplitudes in Figs. 6 and 5C, second flash group).

Effect of inhibitors on the reduction kinetics of chlorophyll a_I

The oxidation kinetics of plastocyanin are not easily measured. However, the quantitative electron transfer to chlorophyll a_I^+, shown above, enables an estimation of the reduced state of plastocyanin from the 200 μs component of the chlorophyll a_I reduction. Chlorophyll a_I is completely oxidized in the flash. Thus, the reduced state of both electron carriers is indicated by measurements of the kinetics of chlorophyll a_I at a sufficient time resolution.

The transient in Fig. 7A shows a similar time course to that in Fig. 6, upper trace, after the first flash. The first half time of 400 μs is longer than the average value of 200 μs. It is close to the slowest reaction time found for this component and illustrates the variation in different preparations of chloroplasts. Reduced plastocyanin was achieved not by a preceding flash group as in Fig. 6, but by repetitive flash excitation at a low intensity of the far-red monitoring light (cf. ref. 44). If in addition to reduction by electrons from light reaction II reduced phenazine methosulfate is acting

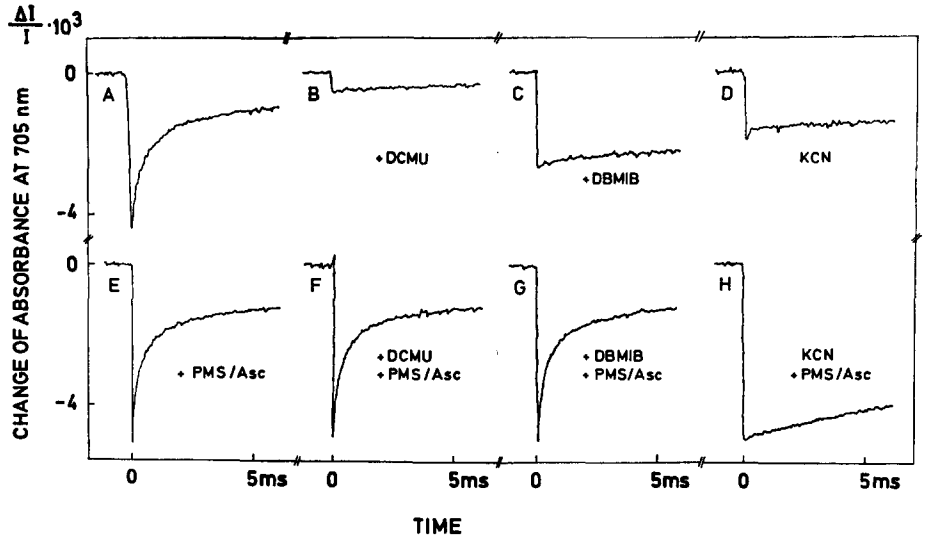

Fig. 7. Absorbance changes of chlorophyll a_I at 705 nm as a function of time. Every trace is the average of 256 signals induced by flashes with a repetition rate of 1 Hz. Additions to standard reaction mixture are, during measurement of trace A, none; B and F, 1.3 μM 3-(3,4-dichlorphenyl)-1,1-dimethylurea (DCMU); C and G, 4 μM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB); E, F, G and H, 6.7 mM sodium ascorbate (Asc) and 3.3 μM *N*-methylphenazonium methosulfate (PMS). For traces D and H, the chloroplasts were pre-incubated with KCN [28].

(Fig. 7E) no influence is observed on the reduction of chlorophyll a_I by plastocyanin, besides a slight increase in the amplitudes.

It should be emphasized that the amplitude and the time course of the transient in Fig. 7A is not changed if gramicidin is omitted from the reaction mixture. The presence or absence of the electric field during this fast reduction was tested by the absorbance change at 520 nm (traces not shown).

The other transients in Fig. 7 show the absorbance changes of chlorophyll a_I in the presence of inhibitors known to block the electron transport between the light reactions at different sites. The amplitudes in Figs. 7B, C and D indicate that the remaining chlorophyll a_I is reduced within the dark time of 1 s between the repetitive flashes. This enables an accurate estimation of smallest reduction rates of chlorophyll a_I. Relative to the reduction of chlorophyll a_I with a half time of 15 ms via the rate-limiting step after a flash [55] the residual reduction was estimated to be 0.3 % in the presence of 1.3 μM DCMU, 1.0 % after KCN incubation and 2.3 % in the presence of 4 μM dibromothymoquinone. The small percentage in the presence of DCMU may be at least partially due to endogenous dark reduction of chlorophyll a_I [30]. The higher values indicated a residual electron transfer from light reaction II. The rate found at 4 μM dibromothymoquinone is consistent with the $NADP^+$ reduction reported by Trebst [69].

The equal time course of the transients in Figs. 7E, F and G demonstrate that the electron transfer from plastocyanin to chlorophyll a_I was neither influenced by DCMU nor by high concentrations of dibromothymoquinone. This is noteworthy because the latter inhibitor acts strongly even at lower concentrations on the site of

the plastohydroquinone reoxidation [70], presumably the site of interaction between plastoquinone and plastocyanin. Direct measurements of plastoquinone will be reported in a subsequent paper. The incubation with KCN completely abolished the fast electron transfer, but not the slower direct reduction of chlorophyll a_I by reduced phenazine methosulfate (Fig. 7H) consistent with the results of Ke [71]. This indicated directly the site of KCN inhibition in linear electron transport to be plastocyanin in agreement with previous suggestions [27, 28]. The estimated equilibrium constant of about 20 of the redox reaction between plastocyanin and chlorophyll a_I is supported by the transient in Fig. 7C. The amplitude indicated a reduced fraction of 65 % of total chlorophyll a_I before the flash. This fraction was in equilibrium with a very small amount of reduced plastocyanin according to the hardly visible amplitude of the fast reduction kinetics. Also consistent with this equilibrium constant is a decrease of the slow component of chlorophyll a_I reduction down to about 30 % of the total signal at considerably higher reduction rates, e.g. as in Fig. 7A or G. Measurements with an insufficient time resolution would detect a decrease of reduced chlorophyll a_I. Thus, misinterpretations of the electron transfer rates via chlorophyll a_I are possible at lower time resolution.

Oxidation kinetics of cytochrome f

The reduction kinetics of chlorophyll a_I show two fast components of 20 μs and 200 μs [45]. Because the 200 μs component can now be attributed to an electron transfer from plastocyanin, the 20 μs component should be due to an electron transfer from cytochrome *f*. This contradicts our previous tentative suggestion [44, 45] based

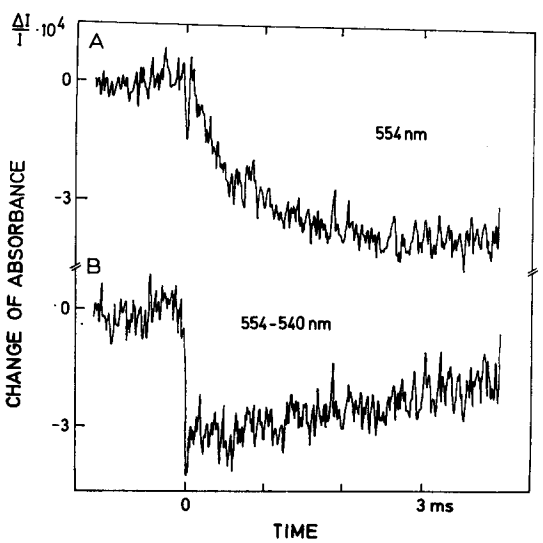

Fig. 8. A, Absorbance changes at 554 nm as a function of time. The trace is the average of 1024 signals. B, Absorbance changes of cytochrome *f* at 554 nm minus 540 nm as a function of time. 1024 signals at 540 nm were subtracted from the trace shown in Fig. 8A. The half bandwidth was 3 nm at both wavelengths. The content of the cuvette was changed after every 512 signals. The repetition rate of the ultra short flashes [50] was 2 Hz. Continuous illumination with System II light (Schott interference filter PAL 650) had an intensity of 2000 ergs · cm^{-2} · s^{-1}.

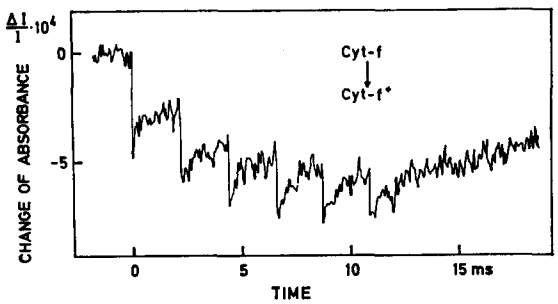

Fig. 9. Absorbance changes of cytochrome *f* at 554 minus 540 nm during excitation with six flashes spaced at 2.15 ms. The experimental conditions are the same as in Fig. 5B during the second flash group except a higher time resolution of 40 μs per address. At both wavelengths, 256 signals have been averaged simultaneously.

on kinetics with a half time of 1 ms [46, 47] and 0.3 ms [48] ascribed to the oxidation of cytochrome *f*. These kinetics were measured with single beam spectrophotometers at one wavelength only. Usually the superposition of absorbance changes of cytochrome *f* and other components is minimized by the difference of the absorbance changes at 554 nm minus 540 nm. Therefore we have reinvestigated the oxidation kinetics of cytochrome *f*.

Reduction of cytochrome *f* was ensured by additional illumination with System II light during the experiment in Fig. 8A. It shows in the upper trace the absorbance change at 554 nm induced by an ultra short flash. The time course and the half time of about 0.4 ms agreed closely with the measurements at this wavelength of Hildreth [47, 48]. Dolan and Hind [72] recently found almost equal kinetics from the difference of the absorbance change at 554.5 nm minus 0.164 times that at 531 nm. This difference was used to remove contributions of the electrochromic absorbance changes similar as previously described by Rüppel et al. [73]. In our measurements this was done by an acceleration of the electrochromic absorbance change with a high concentration of gramicidin D [57] to a non-resolved time course as checked at 520 nm.

Fig. 8B presents the difference of absorbance changes at 554 nm minus 540 nm. The kinetics should be as close as possible to the oxidation kinetics of cytochrome *f*. The half time being not resolved is concluded to be faster than 40 μs according to the time resolution used in this experiment (10 μs/address). The amplitude was only one half of the total amplitude of cytochrome *f* shown in Fig. 5B.

To investigate the conditions for complete oxidation of cytochrome *f* the transient in Fig. 5B during the second flash group was measured and is shown in Fig. 9. Although flashes of 25 μs were used in this experiment instead of 1 μs, the amplitude after the first flash was the same as in Fig. 8B. Complete oxidation was observed after the fourth flash. The total amplitude of this oxidation ($\Delta I/I = -6.5 \cdot 10^{-4}$) was the same as that of the preceding reduction shown in Fig. 5B.

The fast initial rise in Fig. 8B was investigated at a ten times increased time resolution. It was found to be superimposed with a large negative spike which may mask the oxidation kinetics of cytochrome *f*. The spike probably results from the negative difference of absorbance changes at 554 minus 540 nm of the carotenoid triplet [74] and the accelerated electrochromic absorbance change [56] with ampli-

tudes of 36 and 32 % of their maximal amplitude at 520 nm, respectively. The fast kinetics at 520 nm were resolved at this time resolution and fitted to the negative spike at these conditions. However, a digital subtraction of 34 % of the signal at 520 nm seemed to introduce an additional superposition with other absorbance changes.

DISCUSSION

The main problem of the approach to the reaction kinetics of plastocyanin and cytochrome *f* turns out to be the superposition of absorbance changes. Only careful consideration of the difference spectra of all components and their kinetics can result in a sufficient approximation. It should be visualized that the oxidation of plastocyanin and cytochrome *f* occurs simultaneously and cannot be observed without reduction of previously oxidized chlorophyll a_I. In addition, the reduction of these three electron carriers by electrons from light reaction II is again simultaneous.

The resulting interference of absorbance changes as well as the superposition with the electrochromic absorbance change was not contemplated for the first absorbance changes ascribed to plastocyanin [37]. This superposition defies a derivation of the difference spectrum of plastocyanin from the initial off-rate related to the signal amplitude during illumination [76].

With respect to a separation of absorbance changes of cytochrome *f* the difference spectra of plastocyanin [5] and chlorophyll a_I [58] are flat enough to neglect an interference if the difference of the absorbance changes at 554 minus 540 nm is measured. At the larger difference between 565 and 540 nm, a deviation of $\Delta I/I \cong -2 \cdot 10^{-4}$ from the correct difference spectrum of cytochrome *f* reduction has been observed [34]. This is fully consistent with the flat difference spectrum of the simultaneous plastocyanin reduction (Fig. 2B). The amplitude of chlorophyll a_I at 705 nm can be decreased by the largest amplitudes of plastocyanin by not more than 5 % which is near the range of accuracy of these measurements.

Amounts of plastocyanin, cytochrome f and chlorophyll a_I

Excitation of the chloroplasts with flash groups after far-red preillumination and the investigated time range of the kinetics limits our detection to the amounts of electron carriers being reduced via the rate-limiting step by electrons from light reaction II. Additional amounts of electron carriers not as effectively coupled to linear electron transport may become reduced at a still slower rate. This has been shown for chlorophyll a_I [60] and cytochrome *f* [72].

The ratio of plastocyanin to chlorophyll a_I turns out to be very close to 2 : 1 on the basis of electron equivalents. This is in agreement with the relative amounts of plastocyanin and chlorophyll a_I of 1 : 1 estimated from ESR measurements by Malkin and Bearden [42] who implicated two copper atoms per plastocyanin molecule [5], but not with the estimation of Bouges-Bocquet [36] of only one electron carrier between the plastoquinone pool and chlorophyll a_I with the same electron capacity as chlorophyll a_I. The small amount of cytochrome *f* reduced via linear electron transport reported in ref. 34 is supported by the amplitude of the absorbance change in Fig. 5B. The ratio of (cytochrome *f*) : (copper atoms of plastocyanin) : (chlorophyll a_I) in linear electron transport is approximately 0.5 : 2 : 1. If one molecule of plasto-

cyanin consists of four tightly bound subunits [6], this could indicate a structural association of one plastocyanin with two chlorophyll a_I and one cytochrome *f*.

The electron equivalents being accepted by oxidized plastocyanin exceeded those accepted by oxidized cytochrome *f* by a factor of 1.8/0.4 = 4.5 (cf. Table I). An almost equal ratio of 4.7 copper atoms of plastocyanin per molecule cytochrome *f* has been determined by chemical methods in spinach chloroplasts [77]. It should be emphasized that these agreeing ratios are different from a stoichiometric value of one which is frequently implied.

The amount of copper atoms of plastocyanin estimated from the absorbance changes to be 1 : 320 chlorophylls is smaller than the chemically determined total amount of about 1 : 200 [77, 12, 42] or 1 : 300 chlorophylls [2]. A similar discrepancy is found for cytochrome *f* [34]. However, in a previous paper, we presented evidence that only 75 % of total chlorophyll a_I is coupled to chlorophyll a_{II} [60]. With the assumption that the same amount of plastocyanin is associated to every chlorophyll a_I, the ratio of total plastocyanin to chlorophyll can be extrapolated from the absorbance changes to be 1 : 240 in good agreement with the chemically determined values.

Reaction between chlorophyll a_I and plastocyanin in situ

The interaction between plastocyanin and chlorophyll a_I should reflect the difference of their midpoint potentials if equilibrium conditions can be presumed. The equilibrium constant of 20 estimated from their reduction kinetics seems to favour the midpoint potential of 370 mV for plastocyanin [5] before the lower one of 340 mV [64] and is consistent with the indirect estimation of Marsho and Kok [31]. In agreement with this value, the reduction of plastocyanin after the initial lag starts not before more than half of chlorophyll a_I is reduced. A contribution to the lag by the reactions preceding the rate-limiting step can be neglected after a group of more than two flashes [68].

The oxidation of plastocyanin and chlorophyll a_I with far-red light might have been investigated at an intensity not low enough to maintain equilibrium conditions. This would cause an apparently lower equilibrium constant than the actual one and could be an explanation of the value of about 10 estimated from the transients in Fig. 4.

A direct and quantitative electron transfer from plastocyanin to oxidized chlorophyll a_I is shown by their simultaneous fast kinetics and the calculated electron equivalents (Table II). These reduction kinetics of chlorophyll a_I with a first half time of about 200 μs were measured now with a high enough accuracy that in correcting our previous evaluation [44] a semi-log plot indicates a higher order than one. A second order reaction [36] would result in a more complex time course because of the higher concentration of plastocyanin compared to chlorophyll a_I and the oxidation reduction equilibrium between these electron carriers.

The reduced amount of plastocyanin increased with the number of the preceding flashes (Fig. 3). This is in agreement with an increase of the fast reduction kinetics of chlorophyll a_I after a flash from about 20 to 70 % of the total amplitude of chlorophyll a_I if one flash (cf. ref. 60) or a group of twelve flashes (cf. Figs. 5 and 6) is given 200 ms before. The association of the 200-μs component of chlorophyll a_I reduction with a cyclic electron transport around light reaction I is not only excluded by these results but also by the kinetics of chlorophyll a_I after treatment with KCN

(see Fig. 7H). The latter show the oxidation of the total amount of chlorophyll a_I in the flash but only slow reduction.

The fast reduction of chlorophyll a_I with a half time in the range of 200 μs was also detected by Blankenship et al. by ESR measurements of Signal I in spinach chloroplasts [78]. In *Chlorella*, this reaction seems to be slightly faster, as has been concluded from the electrochromic absorbance change at 520 nm by Joliot and Delosme [79] and from the reduction of methylviologen by Bouges-Boquet [36].

The electric field across the membrane seems to have no influence on the electron transfer from plastocyanin to chlorophyll a_I. Thus, at least relative to plastocyanin, there is no change in the midpoint potential of chlorophyll a_I by the field as would be expected, if the electron carriers were located at different levels of the transmembrane field. Consistent with this interpretation, Junge reported no effect of the fast reduction of chlorophyll a_I on the decay of the electrochromic absorbance change at 520 nm [80].

On the reactions of cytochrome f in linear electron transport

The transients of cytochrome *f* shown in Fig. 5B are consistent with previous results (cf. refs. 30, 31, 33). The oxidation of cytochrome *f* by far-red illumination and during a flash group of red light as well as the subsequent reduction with the half time of the rate-limiting step of linear electron transport are in support of a function of cytochrome *f* between the pool of plastoquinone and light reaction I.

However, the apparently changing equilibrium constant between cytochrome *f* and chlorophyll a_I in the light and the dark [30, 31] and the reduction kinetics of cytochrome *f* showing no sigmoidal time course [34] as plastocyanin cannot be reconciled with a simple linear electron transfer mechanism. In support of our results, Cox [35] detected at low temperatures a reduction of cytochrome *f* without any lag in the beginning and with a half time greater by a factor of 3.5 than that of chlorophyll a_I. A lag in the cytochrome *f* reduction could not be detected in case of an equilibrium between cytochrome *f* and chlorophyll a_I with a constant smaller than 5. However, from the kinetics increasing values during the reduction including 10 and 20 could be calculated from the amplitudes (cf. also the reduction kinetics in Fig. 5, B and D). A continuously changing midpoint potential of cytochrome *f* which could cause this effect seems to be improbable. Any influence of the field [35] and the pH gradient can be excluded with respect to the presence of uncoupler in these measurements.

Additional information about the function of cytochrome *f* should be derived from its oxidation kinetics. As pointed out above, kinetics measured at 554 nm reflect the sum of different components and not the oxidation of cytochrome *f* alone [48, 72]. At 554 nm, the kinetics of chlorophyll a_I are inverted compared to those at 705 nm and exceed those of plastocyanin by a factor of 3–4. Thus, at least the greater portion of the time course at 554 nm should result from a contribution of chlorophyll a_I with kinetics similar to that in Fig. 6 (above) and Fig. 7A. This is in agreement with the half times of the absorbance changes at 554 nm of 0.22 ms [72], 0.3 ms [48] and of 0.4 ms in Fig. 8A. The difference of absorbance changes at 554 minus 540 nm indicates that the oxidation of cytochrome *f* is faster than 40 μs. The reduction of chlorophyll a_I with a half time of about 20 μs [45] suggests a corresponding fast electron transfer from cytochrome *f*. This oxidation time of cytochrome *f* would be similar to that of other plants. For red algae, Nishimura reported 15 and 16 μs [81] and Hiama

and Ke for a mutant of *Chlamydomonas* 15 μs with regard to interfering absorbance changes of chlorophyll a_I [82]. The latter authors also detected the very fast reduction of chlorophyll a_I ($t_{\frac{1}{2}} \cong 10$ μs) which has been indirectly supported by the ESR measurements of Warden and Bolton [83].

The amount of cytochrome *f* oxidized after one flash is only one half of the total reduced amount (cf. Figs. 5B and 8B). The small amount is supported by the measurements of Dolan and Hind [72], but it is lower than in red algae [33, 81]. It corresponds to a reduction of only 20 % of the oxidized chlorophyll a_I. This is not easily understood. If thermodynamic equilibrium were presumed, a midpoint potential of cytochrome *f* equal or higher than that of chlorophyll a_I would have to be postulated, as well as a reduction of cytochrome *f* after the flash with 200 μs in parallel to chlorophyll a_I. This was not the case (cf. Fig. 8B). Thus, an interaction between cytochrome *f* and chlorophyll a_I during the reduction of the latter by plastocyanin is not compatible with these kinetics. Cytochrome *f* may interact with separate light reactions I. Another possibility is a fast structural or conformational change of cytochrome *f* induced by the oxidation of chlorophyll a_I which enables an interaction with chlorophyll a_I for only 20 μs. This could be realized by a rotation of cytochrome *f* in the membrane during which the site of oxidation of the protein molecule is exposed to oxidized chlorophyll a_I. An induction of this conformational change by the oxidation of chlorophyll a_I is proposed because the almost equal amounts of oxidized cytochrome *f* after a flash of 1 or 25 μs duration indicate no double hits of chlorophyll a_I as expected for this fast reaction. Although no proof is possible yet, this hypothesis can account for the contradictory effects found in chloroplasts.

Reaction sequence of plastocyanin, cytochrome f and chlorophyll a_I in linear electron transport

The frequently assumed linear reaction sequence plastoquinone → cytochrome *f* → plastocyanin → chlorophyll a_I is not consistent with the reaction kinetics of these electron carriers. The reduction kinetics of cytochrome *f* and chlorophyll a_I exclude this sequence (see above and ref. 34), in particular with respect to the now detected initial lag of the plastocyanin reduction. A function of cytochrome *f* before plastocyanin would cause a similar or greater lag in the reduction of cytochrome *f*. The other more obvious finding against this sequence is the oxidation kinetics of cytochrome *f* being faster than those of plastocyanin.

The latter kinetics could be consistent with a function of cytochrome *f* between plastocyanin and chlorophyll a_I. However, this has to be excluded from the reduction kinetics and because a fast oxidation of cytochrome *f* after a flash, plastocyanin — cytochrome $f \xrightarrow{20\,\mu s}$ chlorophyll a_I^+, should be followed by a reduction of cytochrome f^+ with a half time of 200 μs: plastocyanin $\xrightarrow{200\,\mu s}$ cytochrome f^+ — chlorophyll a_I. This is not observed. In addition, the quantitative transfer from plastocyanin to chlorophyll a_I^+, after one flash (Table II), of more electron equivalents than correspond to the total amount of cytochrome *f* engaged, does not support this sequence.

Thus, the oxidation as well as the reduction kinetics of plastocyanin and cytochrome *f* indicate no interaction between these electron carriers. As a possible arrangement remains only a parallel function of plastocyanin and cytochrome *f* between the

plastoquinone pool and chlorophyll a_I. An open question remains, whether the two electron carriers are oxidized by locally different or the same chlorophyll a_I molecules.

Fig. 10 presents a possible scheme of linear electron transport derived from our measurements of the reaction kinetics in spinach chloroplasts. The number of indicated molecules may illustrate the relative amounts of cytochrome *f*, plastocyanin and chlorophyll a_I. Two [5] or four [6] plastocyanin molecules are possibly associated. Another electron carrier of System I is excluded. The equilibrium constant of about 20 for the redox reaction between plastocyanin and chlorophyll a_I is found in the light and dark. The electron exchange between different Systems I concluded from our statistical calculations [34] is proposed to occur via plastocyanin as indicated (details will be presented elsewhere). The fast equilibration of electrons does not allow to distinguish between this and an electron transfer at the level of chlorophyll a_I [36]. At least 85 % of linear electron transport should run via plastocyanin to chlorophyll a_I as estimated from the initial reduction rates of cytochrome *f* and chlorophyll a_I [34]. If the probability of electron transfer from the pool of plastoquinone were almost equal to plastocyanin and to cytochrome *f* in the membrane, this could be due to the higher concentration of copper atoms of plastocyanin compared to the molecules of cytochrome *f*. The reduction of cytochrome *f* via the plastoquinone pool should occur in parallel to this main electron path. The symbolized switch on the oxidizing side of cytochrome *f* accounts for the difficulties in the relation of the electron carriers and may indicate the tentatively proposed conformational change.

Many previous approaches to the function of plastocyanin and cytochrome *f* were based on the restoration of electron transport in different types of chloroplast fragments by exogenously added plastocyanin. But this reaction seems to be artificial because added plastocyanin should act from the outside of the fragments and did not restore phosphorylation [12]. In vivo plastocyanin is located inside the thylakoids [12, 13]. The oxidation of cytochrome *f* in these fragments was found to be stimulated by exogenous plastocyanin. This was also shown for isolated cytochrome *f* and Photosystem I particles by Nelson and Racker [84] and by Wood and Bendall [21]. The latter authors found from the rate constants of the redox reactions that in solution the fastest pathway of electrons is from cytochrome *f* via plastocyanin to chlorophyll a_I. This is not consistent with the reaction kinetics in the intact system reported above.

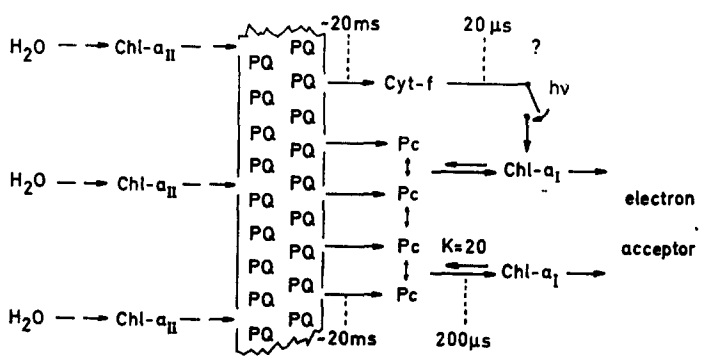

Fig. 10. Scheme of linear electron transport in System I derived from the reaction kinetics in spinach chloroplasts. For a detailed discussion, see text.

However, because of the structural arrangement of the components in the membrane the actual reaction sequence in photosynthesis needs not be the same as in solution.

The investigations of Kimimura and Katoh with $HgCl_2$ [26] as well as those of Izawa et al. with KCN [28] showed an inhibition of the oxidation of cytochrome *f* but not of its reduction. If any other effect of the inhibitors except the inactivation of the redox reaction of plastocyanin in the membrane [29] is excluded, these findings seem to indicate a function of plastocyanin between cytochrome *f* and chlorophyll a_I. However, another possibility could be an indirect effect, e.g. by the mechanism proposed for the oxidation of cytochrome *f*. As shown in Fig. 9, several turn-overs of chlorophyll a_I are necessary to oxidize cytochrome *f*. An inhibition of plastocyanin decreases the reduction rate of chlorophyll a_I and therefore the turnover of reduced to oxidized chlorophyll a_I, which is speculated to initiate the oxidation of cytochrome *f*. Thus, the rate of cytochrome *f* oxidation would be decreased at an inhibition of plastocyanin even in the electron transfer mechanism proposed in Fig. 10. However, more experiments on the reaction kinetics in the intact electron transport system are necessary to solve the problem of the oxidizing mechanism of cytochrome *f*.

After submission of this manuscript, ESR measurements of Signal I by Warden were published [85], showing the fast reduction of chlorophyll a_I with a half time of about 300 μs. These results confirm all kinetics of the absorbance changes of chlorophyll a_I shown in Fig. 7. In addition, they show the same effects of $HgCl_2$ as KCN. However, according to our results presented in this paper the fast kinetics should be assigned to a reduction of chlorophyll a_I by plastocyanin and not by cytochrome *f*.

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