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FLASH PHOTOLYSIS-ELECTRON SPIN RESONANCE STUDIES OF PHOTOSYSTEM I

A FAST REDUCTION COMPONENT OF *P*-700$^+$

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SUMMARY

A 300 μs decay component of ESR Signal I (*P*-700$^+$) in chloroplasts is observed following a 10 μs actinic xenon flash. This transient is inhibited by treatments which block electron transfer from Photosystem II to Photosystem I (e.g. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), KCN and $HgCl_2$). The fast transient reduction of *P*-700$^+$ can be restored in the case of DCMU or DBMIB inhibition by addition of an electron donor couple (2,6-dichlorophenol indophenol (Cl_2Ind)/ascorbate) which supplies electrons to cytochrome *f*. However, this donor couple is inefficient in restoring electron transport in chloroplasts which have been inhibited with the plastocyanin inactivators, KCN and $HgCl_2$. Oxidation-reduction measurements reveal that the fast *P*-700$^+$ reduction component reflects electron transfer from a component with $E_m = 375 \pm 10$ mV (pH = 7.5). These data suggest the assignment of the 300-μs decay kinetics to electron transfer from cytochrome *f* (Fe^{2+}) to *P*-700$^+$, thus confirming the recent observations of Haehnel et al. (Z. Naturforsch. 26b, 1171–1174 (1971)).

INTRODUCTION

The identity of the primary electron donor(s) to *P*-700$^+$, the oxidized, reaction center chlorophyll dimer of Photosystem I, has been the object of much speculation and controversy in recent years. Two candidates for the donor moiety have been proposed: cytochrome *f*, a *c*-type cytochrome [1], and plastocyanin, a copper-containing electron transport protein [2]. However, the precise relationship between cytochrome *f*, plastocyanin and *P*-700 remains to be established. Gorman and Levine [3], utilizing a plastocyanin-less algal mutant, demonstrated that cytochrome *f* could only be photooxidized by Photosystem I in the presence of exogenous plastocyanin.

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Cl_2Ind, 2,6-dichlorophenolindophenol; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

Similar observations have been published by Shneyour and Avron [4]. These data suggest that plastocyanin lies between *P*-700 and cytochrome *f*. However, in contrast, Knaff and Arnon [5, 6] have proposed that cytochrome *f* is an intermediary between plastocyanin and *P*-700, since plastocyanin-depleted chloroplasts retained the capacity to photooxidize cytochrome *f*. Furthermore, recent potentiometric determinations have indicated that plastocyanin in vivo has a significantly lower midpoint potential (+340 mV) than cytochrome *f* (+385 mV) [7]. These contradictory reports on the sites of action of plastocyanin and cytochrome *f* have been complicated by recent proposals that these components may serve as parallel donors to $P\text{-}700^+$ [8, 9].

In recent communications we have utilized flash-photolysis electron spin resonance spectroscopy as a probe for transient light-induced electron transfers involving *P*-700 [10, 11]. Monitoring the $P\text{-}700^+$ ESR signal (Signal I [12]), we have shown that under conditions where the primary donors to $P\text{-}700^+$ were predominantly oxidized, Signal I decayed with a $t_{1/2} \approx 20$ ms after a 100 μs actinic flash [11]. Similar observations have also been presented by Witt [13] and Haehnel et al. [14]. Likewise, these investigators have discovered two additional decay components of $P\text{-}700^+$, having half-lives of 10 and 300 μs. On the basis of the similarity of the 300 μs component to the observed times for cytochrome *f* photooxidation [15], this kinetic component was attributed to electron transfer from cytochrome *f* to $P\text{-}700^+$.

This communication presents a reexamination of $P\text{-}700^+$ reduction with an improved time-response ESR spectrometer. These investigations showed that the 300 μs component described by Haehnel et al. [14] represents the oxidation by $P\text{-}700^+$ of a chloroplast component with $E_m = 375 \pm 10$ mV (pH = 7.5), presumably cytochrome *f*.

MATERIALS AND METHODS

Chloroplasts were isolated from locally grown spinach as previously described [11]. Chlorophyll content for all samples was 3–4 mg/ml as assayed by the method of Arnon [16]. $HgCl_2$ -treated chloroplasts were obtained as described by Radmer and Kok [17]. KCN inhibition was performed by the method of Izawa et al. [18].

Flash photolysis-electron spin resonance studies were performed on a Varian E-9 spectrometer modified as previously described [19]. Instrument-operating parameters are given in the figure captions. Oxidation-reduction measurements were performed according to the method of Dutton [20]. The Ingold platinum microcombination electrode was calibrated using quinhydrone as reference ($E_m = 296$ mV, pH = 7.0) [21].

NADP, spinach ferredoxin and sodium ascorbate were purchased from Sigma Chemical Co. DCMU was obtained from K and K Laboratories and recrystallized from benzene. Cl_2Ind, $HgCl_2$ and KCN were obtained from Fisher Scientific. DBMIB was a generous gift of Dr. David Knaff.

RESULTS

The time-course for reduction of $P\text{-}700^+$ in dark-adapted, broken spinach chloroplasts has recently been demonstrated to be dependent on the duration of the actinic flash [11]. Thus samples subjected to a series of flashes of 100 μs duration

exhibit a $t_{\frac{1}{2}}$ for $P\text{-}700^+$ decay of 15–30 ms. However, photolysis with 10-μs pulses results in a 50 % reduction in the amplitude of the Signal I response. These results suggest that at shorter flash durations a significant portion of Signal I is decaying with a half-life shorter than the limiting time constant for our conventional ESR detection system (> 250 μs) [11]. To test this hypothesis, the transient $P\text{-}700^+$ decay kinetics in chloroplasts was reexamined utilizing an improved time-response spectrometer (minimum system time constant approx. 90 μs). As illustrated in Fig. 1a, P-700 oxidation (Signal I formation) occurred in the time constant of the system; however, $P\text{-}700^+$ reduction exhibited biphasic kinetics. The slower phase of Signal I decay corresponds to the 20 ms component previously observed by optical and ESR techniques [11, 22]. The faster component which was previously undetected by ESR experiments displayed first-order kinetics (within experimental error) with a $t_{\frac{1}{2}}$ of approx. 400 μs. (Fig. 1c) The spectrum of the fast transient, obtained by a systematic variation of the magnetic field, is identical to Signal I observed in steady-state illumination. The ratio of fast to slow kinetic components is 1 : 1 for this experiment, which is in agreement with the reduced amplitude of the slow component previously observed [11]. The time constant for the fast component lies in the range of 300–400 μs and is similar to the half-life of a fast component of $P\text{-}700^+$ decay observed by Haehnel et al. [14], and the risetime for cytochrome f oxidation as reported by Hildreth [15]. The extent of the fast component as well as its half-life are unchanged if benzyl viologen is substituted for NADP/ferredoxin as the Photosystem I electron acceptor.

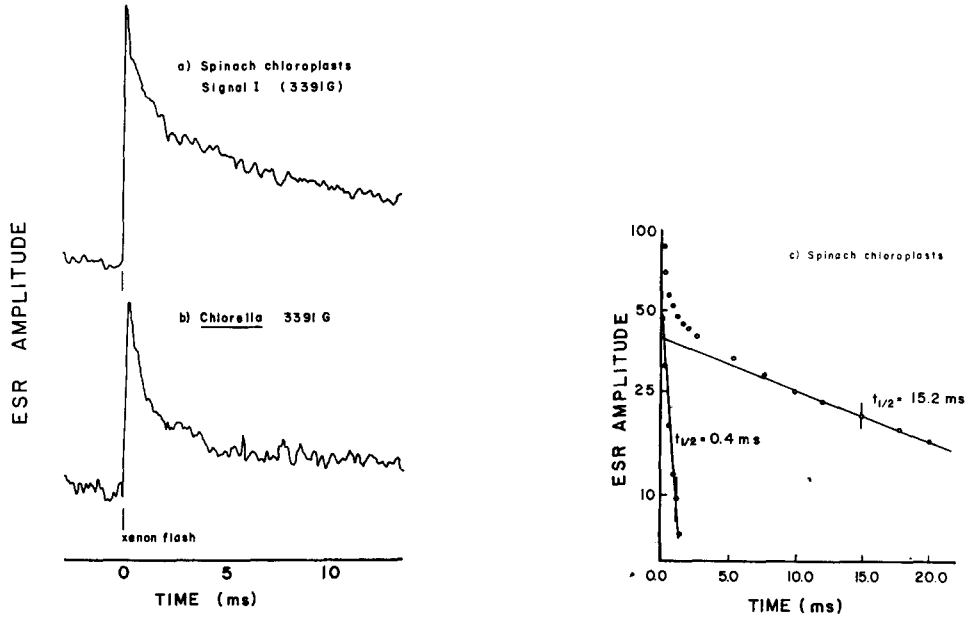

Fig. 1. Flash-induced kinetics for Signal I: a, in spinach chloroplasts; b, in (*C. pyrenoidosa*); c, log plot of kinetics for a. Data shown are the average of 1024 repetitive flashes. Modulation amplitude 5 G; microwave power, 35 mW; field set, 3390 G; flash-excitation frequency, 2 Hz. Kinetic traces are taken from the low-field derivative peak of Signal I.

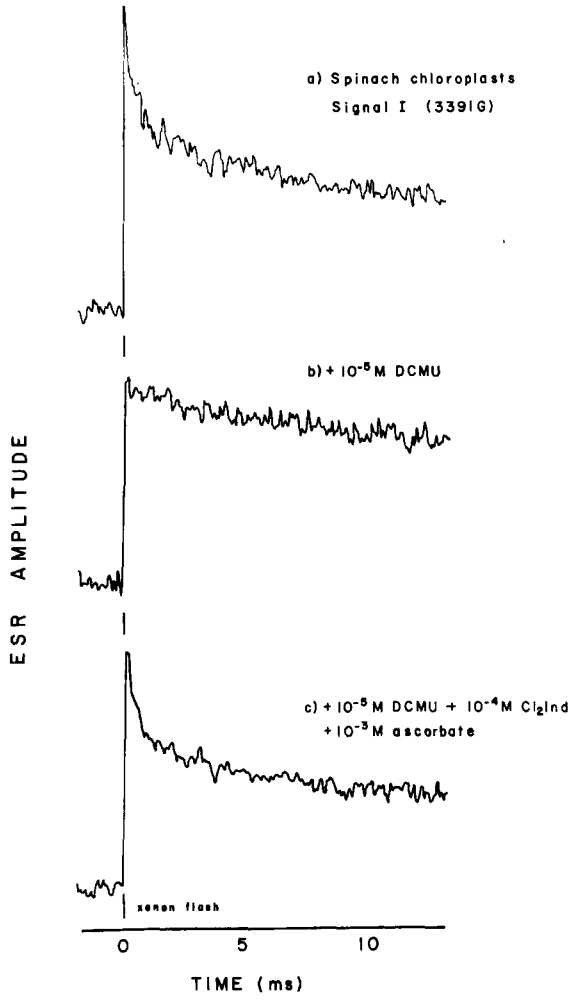

Fig. 2. Kinetic behavior for Signal I in a, control chloroplasts; b, chloroplasts plus 10 μM DCMU, and c, DCMU-treated chloroplasts plus 100 μM Cl_2Ind and 1 mM ascorbate. The traces are averages of 511 scans. Instrumental parameters are given in Fig. 1.

A similar fast reduction of $P\text{-}700^+$ ($t_{\frac{1}{2}} \approx 300$ μs) is also observed in *Chlorella pyrenoidosa* (Fig. 1b). However, in contrast to the biphasic $P\text{-}700^+$ reduction of green plant chloroplasts, *Chlorella* possesses predominantly the fast kinetic component.

If the fast Signal I decay component reflects electron transfer from a component situated between Photosystems I and II, then disruption of the intermediate electron transport chain should eliminate the rapid decay kinetics. Fig. 2b illustrates the effect of 10 μM DCMU on $P\text{-}700^+$ decay kinetics. In the absence of exogenous donors or endogenous cyclic electron transport, total $P\text{-}700^+$ reduction requires several seconds. However, addition of the donor couple Cl_2Ind/ascorbate restores the rapid Signal I kinetics (Fig. 2c), indicating that the DCMU block lies before the site of electron donation by the reduced Cl_2Ind [23].

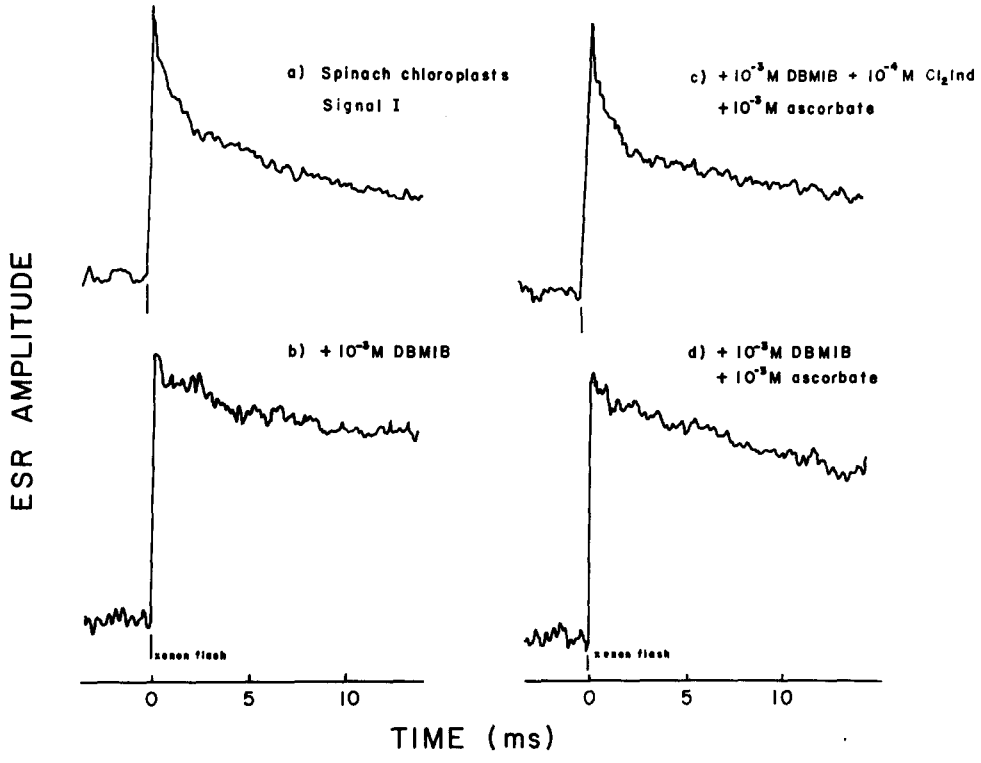

Fig. 3. Signal I kinetic traces in a, control spinach chloroplasts; b, chloroplasts plus 1 mM DBMIB; c, chloroplasts plus 1 mM DBMIB, 100 μM Cl_2Ind and 1 mM ascorbate; d, chloroplasts plus 1 mM DBMIB and 5 mM ascorbate. ESR parameters are as given in Fig. 1.

Trebst and coworkers [24] have introduced an electron-transport inhibitor, DBMIB, which acts at a locus between plastoquinone and cytochrome *f*. Incubation of spinach chloroplasts with 1 mM DBMIB retards Signal I decay in a manner similar to that observed with DCMU (Fig. 3b). Likewise addition of an exogenous donor couple (e.g. Cl_2Ind/ascorbate) to the blocked sample restores the fast reduction component (Fig. 3c). However, if reduced DBMIB (1 mM) is used for inhibition, $P\text{-}700^+$ reduction kinetics is only slightly accelerated over that observed in samples treated with the oxidized inhibitor (Fig. 3d). Hence, reduced DBMIB appears to be a poor donor to Photosystem I [25].

Electron transport in the region of Photosystem I is sensitive to various treatments which inactivate the electron carrier protein, plastocyanin. Specifically, incubation of chloroplasts with polylysine [26], $HgCl_2$ [27] or KCN [28] leads to a marked reduction in the Hill reaction from water to methyl viologen. Recently Selman et al. [29] have demonstrated that a specific inactivation of the chloroplast plastocyanin is associated with the KCN-induced loss in electron transport. $HgCl_2$ or KCN treatment of chloroplasts markedly retards $P\text{-}700^+$ reduction after an actinic flash [18] and specifically inhibits the fast $P\text{-}700^+$ decay component (Figs. 4b and 4c). In contrast with DCMU or DBMIB inhibition, catalytic quantities of the donor couples

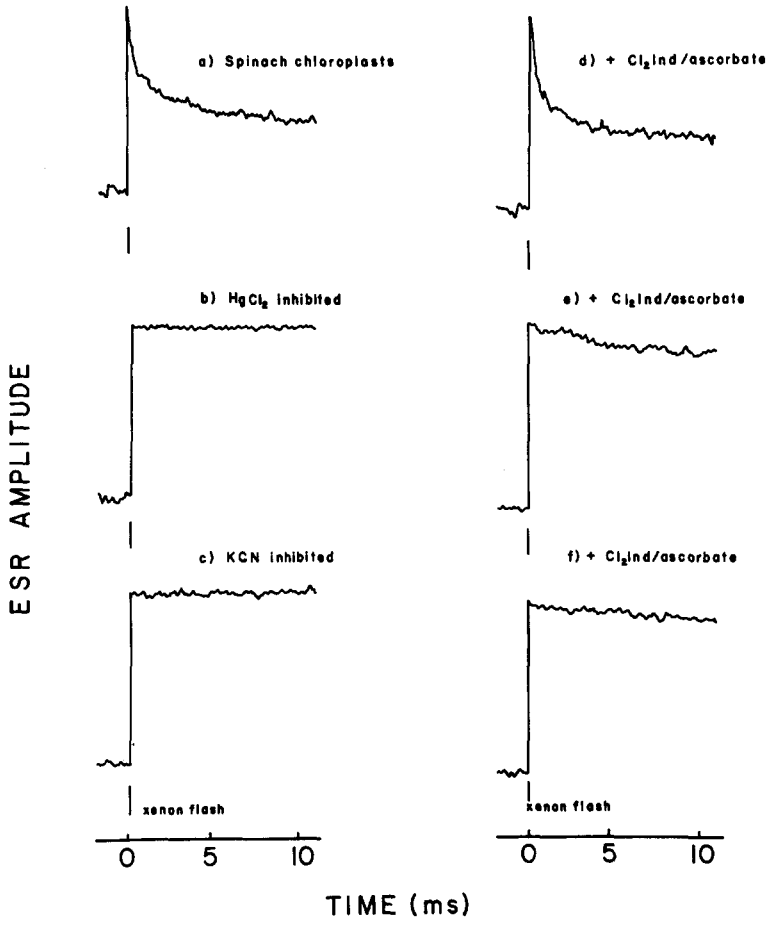

Fig. 4. Transient Signal I kinetics in chloroplasts inhibited by plastocyanin inhibitors: a, control; b, $HgCl_2$-inhibited chloroplasts; c, KCN-blocked chloroplasts; d, control plus 100 μM Cl_2Ind and 1 mM ascorbate; e, $HgCl_2$-inhibited sample plus 100 μM Cl_2Ind and 1 mM ascorbate; and f, KCN-treated chloroplasts plus 100 μM Cl_2Ind and 1 mM ascorbate. Instrumental parameters are given in Fig. 1.

Cl_2Ind/ascorbate or TMPD/ascorbate are ineffective in restoring the fast decay component (Figs. 4e and 4f).

DISCUSSION

In addition to the well-documented 20 ms reduction component of $P\text{-}700^+$, we have presented confirming evidence in this communication for a 300 μs half-time electron transfer to the oxidized reaction center [14, 37, 38]. That this fast decay component represents an electron transfer from a donor located on the Photosystem II side of P-700 rather than a "backreaction" with a reduced acceptor is supported by the observation that both the amplitude and the decay half-life are independent of

the nature of the electron acceptor used for Photosystem I. In contrast, the backreaction between $P\text{-}700^+$ and $P\text{-}430^-$, the primary acceptor for Photosystem I, is highly dependent on the nature of the secondary, exogenous acceptor [30]. Additionally, the inhibition of the 300 μs component in the presence of DBMIB suggests that the electron transfer moiety is located between the plastoquinone pool and P-700 [31–33]. Apparently during illumination in the absence of electron flow from Photosystem II, the primary donor(s) to Photosystem I becomes predominantly oxidized and $P\text{-}700^+$ reduction is subsequently retarded. However, addition of electron donors, such as Cl_2Ind, which can bypass the DCMU or DBMIB inhibition and donate to cytochrome *f* [23] restore the rapid Signal I decay component.

The species responsible for the 300 μs reduction component of $P\text{-}700^+$ has been tentatively assigned to cytochrome *f* by Haehnel et al. [14]. The redox properties of this hemoprotein have been well established with mid-point potentials of 360 [34], 385 [7] and 390 mV [35] reported. By monitoring the percent of the 300 μs decay component of Signal I at well-established redox potentials, we have determined the oxidation-reduction midpoint potential for the electron donor species. A Nernst plot of our data is presented in Fig. 5. The donor species behaves as a one-electron intermediary with a midpoint potential (pH 7.5) of 375±10 mV. This value is remarkably similar to that given above for cytochrome *f* in vivo and, additionally, is significantly higher than the in situ midpoint potential for plastocyanin (340±10 mV), measured by Malkin et al. [7]. On the basis of the midpoint potential associated with the 300 μs component, as well as the similarity of the decay half-life with those reported by Hildreth for cytochrome *f* oxidation, we assign this rapid $P\text{-}700^+$ reduction component to a one-electron transfer from cytochrome *f*.

The assignment of the rapid decay component to a reduction of $P\text{-}700^+$ by cytochrome *f* permits a reexamination of the electron transport sequence on the donor

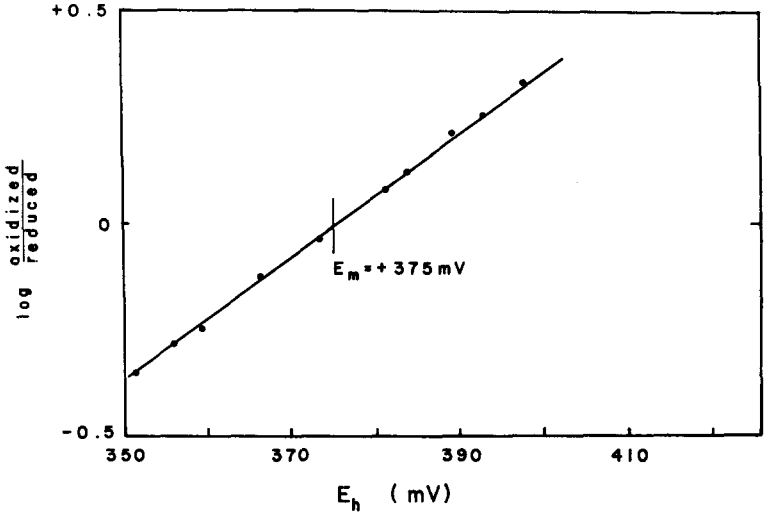

Fig. 5. Oxidation-reduction potential of the rapid decay component of Signal I. ESR parameters are given in Fig. 1.

side of Photosystem I. Three alternative schemes for electron transfer to $P\text{-}700^+$ have been proposed: two sequential models [3, 6] and one parallel formalism [8].

(1) Photosystem II → plastocyanin → cytochrome *f* → *P*-700
(2) Photosystem II → cytochrome *f* → plastocyanin → *P*-700
(3) Photosystem II → cytochrome *f* ⟶ } *P*-700
Photosystem II → plastocyanin ⟶ }

Scheme 1 concurs with the midpoint determinations presented by Malkin et al. [7] and suggests that inhibition of electron flow at plastocyanin would have little effect on $P\text{-}700^+$ reduction kinetics, if an exogenous electron donor to cytochrome *f* were present. However, scheme 2 predicts that inactivation of plastocyanin would eliminate flow to $P\text{-}700^+$ from reduced cytochrome *f*. The results of Fig. 4 clearly indicate that inactivation of membrane-bound plastocyanin blocks electron flow from Photosystem II, an inhibition which is not removed in the presence of catalytic concentrations of reduced Cl_2Ind. These findings are best interpreted as supporting scheme 2, since both schemes 1 and 3 would predict a restoration of the fast decay transient in the presence of the donor to cytochrome *f*. Similar conclusions have been published recently by Izawa and coworkers [18], indicating that cytochrome *f* reduction (by Photosystem II) is not affected by KCN treatment; however, cytochrome *f* oxidation by Photosystem I with the associated $P\text{-}700^+$ reduction is inhibited.

On the other hand, recent flash-titration studies on preilluminated spinach chloroplasts by Haehnel [39] suggest that linear electron flow from Photosystem II occurs predominantly via plastocyanin and that cytochrome *f* lies on a parallel pathway to *P*-700. In this regard simultaneous flash optical and electron-spin resonance [40] quantitation of electron equivalents transferred from Photosystem II will prove invaluable for ascertaining the significance of scheme 3 in electron transport.

The placement of plastocyanin on the oxidizing side of cytochrome *f* is not consistent with the relative in vivo midpoint potentials for these electron carriers [7]. However, electron transfer between these components may occur via a non-equilibrium process (e.g. quantum mechanical tunneling [36]), thereby circumventing the unfavorable potential difference. With these considerations it is noteworthy that Marsho and Kok [8] have demonstrated recently that no true equilibrium exists between cytochrome *f* and *P*-700. Future investigations in this laboratory will attempt to correlate the relative oxidation states of plastocyanin and cytochrome *f* after flash excitation utilizing low temperature ESR spectroscopy. Additionally, planned modification of our ESR spectrometer will therefore provide a time resolution in the 2 μs domain and will allow study of the 10 μs $P\text{-}700^+$ reduction component reported by Haehnel et al. [14].

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