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# STUDIES ON WELL-COUPLED PHOTOSYSTEM-I-ENRICHED SUBCHLOROPLAST VESICLES

## ELECTRON TRANSFER BY *b*- AND *c*-TYPE CYTOCHROMES IN RELATION TO THE ORIGIN OF THE 'SLOW' ELECTRIC POTENTIAL COMPONENT

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**Cytochrome redox changes and electric potential generation are kinetically compared during cyclic electron transfer in Photosystem-I-enriched and Photosystem-II-depleted subchloroplast vesicles (i.e., stroma lamellae membrane vesicles) supplemented with ferredoxin using a suitable electron donating system. In response to a single-turnover flash, the sequence of events is: (1) fast reduction of cytochrome *b*-563 ($t_{0.5} \approx 0.5$ ms) (2) oxidation of cytochrome *c*-554 ($t_{0.5} \approx 2$ ms), (3) slower reduction of cytochrome *b*-563 ($t_{0.5} \approx 4$ ms), (4) generation of the 'slow' electric potential component ($t_{0.5} \approx 15$–20 ms), (5) re-reduction of cytochrome *c*-554 ($t_{0.5} \approx 30$ ms) and (6) reoxidation of cytochrome *b*-563 ($t_{0.5} \approx 90$ ms). Per flash two cytochrome *b*-563 species turn over for one cytochrome *c*-554. These *b*-563 cytochromes are reduced with different kinetics via different pathways. The fast reductive pathway proceeds probably via ferredoxin, is insensitive to DNP-INT, DBMIB and HQNO and is independent on the dark redox state of the electron transfer chain. In contrast, the slow reductive pathway is sensitive to DNP-INT and DBMIB, is strongly delayed at suboptimal redox poising (i.e., low NADPH/$NADP^+$ ratio) and is possibly coupled to the reduction of cytochrome *c*-554. Each reductive pathway seems obligatory for the generation of about 50% of the slow electric potential component. Also cytochrome *b*-$559_{LP}$ (LP, low potential) is involved in Photosystem-I-associated cyclic electron flow, but its flash-induced turnover is only observed at low preestablished electron pressure on the electron-transfer chain. Data suggest that cyclic electron flow around Photosystem I only proceeds if cytochrome *b*-$559_{LP}$ is in the reduced state before the flash, and a tentative model is presented for electron transfer through the cyclic system.**

## Introduction

Cyclic transfer around Photosystem I may be considered as an important energy-conserving pathway in higher plant chloroplasts, supplying part of the ATP needed for carbon assimilation [1,2]. Whereas in the grana thylakoid membranes it occurs concurrently with linear electron transfer [3,4], in the thylakoid stroma lamellae it may be the exclusive electron transfer system using

Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl) methyl]amino}ethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; Chl, chlorophyll; LP, low-potential; HP, high-potential; Qbc, plastohydroquinone-plastocyanin oxidoreductase: cytochrome *b*-*c* complex containing bound plastoquinone and Rieske (FeS) protein; PS, Photosystem.

NADPH (via ferredoxin and ferrodoxin-$NADP^+$ oxidoreductase) or other reductants (like reduced thioredoxin) to poise the redox state of the system for optimal functioning [5]. Also in bundle sheat chloroplasts of C-4 plants [6] and in heterocysts of filamentous cyanobacteria [7,8] only PS-I-associated cyclic electron transfer is active without PS-II involvement, using NADPH, NADH (via a dehydrogenase) or hydrogen (via hydrogenase) as source of reducing equivalents, the latter two donors only being utilized in heterocysts.

The operation of PS-I-associated cyclic electron transfer in spinach chloroplasts in vivo requires ferredoxin as a mediator, while its activity is strongly dependent on a proper redox balance of the electron transfer carriers [5,9]. Further redox mediators are plastoquinone, the cytochrome *b*-563-*c*-554 ('$b_6$-*f*') complex containing the Rieske (Fe-S) protein (denoted further as Qbc complex here), plastocyanin and P-700 [10,11]. A straightforward participation of cytochrome *b*-$559_{LP}$ and ferredoxin-$NADP^+$ oxidoreductase in cyclic electron transfer is less clear [12].

The arrangement of electron transfer carriers has been only partly characterized [11,13–15] and is still under investigation. Schemes of nonserial transfer of electrons through the Qbc redox complex (i.e., Q- and *b*-cycles [11–19]), in analogy with suggestions for that through the Qbc-type redox complexes in mitochondria and photosynthetic bacteria [20], have been proposed to account for the anomalous redox behavior of cytochrome *b*-563 as well as for an extra electrogenic redox step as indicated by the electrochromic carotenoid absorbance changes. According to these schemes, electron transfer through the Qbc complex, which contains two *b*-563 cytochromes, one *c*-554 cytochrome and one Rieske (Fe-S) protein [21], is proposed to proceed as follows. Oxidation of cytochrome *c*-554 by PS I would cause a reduction of cytochrome *b*-563 ('oxidant-induced reduction'), in which plastoquinol is oxidized delivering a high potential electron via the Rieske (Fe-S) protein to cytochrome *c*-554, and a low-potential electron towards cytochrome *b*-563. Cytochrome *b*-563 would be reoxidized by plastoquinone in a cooperative (with ferredoxin, reduced by PS I [14]) or non-cooperative (e.g., more turnovers of Qbc complex per turnover of PS I [22]) way and this would be accompanied by an electrogenic transfer of an electron from the inner to the outer membrane side. Evidence for an 'oxidant-induced reduction' mechanism was presented by Hurt and Hauska [23], using isolated cytochrome '$b_6$-*f*' complex, but their model was modified by others [11,19] to explain kinetic discrepancies in flash-induced redox transients of cytochromes *b*-563 and *c*-554 in whole chloroplasts. The reduction of cytochrome *b*-563 [24], the oxidation of cytochrome *b*-563 [19,25] and also the reduction of cytochrome *c*-554 [11,26] have all been proposed to represent the electrogenic step, that is monitored by the carotenoid absorbance transient ($\Delta A_{518}$) and by the extrinsic field-sensitive probe oxonol VI ($\Delta A_{590}$) [5].

In previous papers we have described the isolation and characterization of well-coupled PS-I-enriched and PS-II-depleted subchloroplast vesicles [27,28]. These thylakoid vesicles, that are derived from stroma lamellae membranes, provide a most useful system to study 'native' PS-I-associated, ferredoxin-mediated, cyclic electron transfer and electric potential generation, the redox system being carefully poised [5]. In this paper we present experiments on the redox changes of *b*- and *c*-type cytochromes in relation to the flash-induced 'slow' carotenoid response when the activity of cyclic electron transfer is manipulated by modulation of the preestablished redox balance of the cyclic system and by addition of electron transfer inhibitors. We present evidence for two separate pathways in the reduction of cytochrome *b*-563 after a flash, in which for one cytochrome *c*-554 two *b*-563 cytochromes turn over. The overall reoxidation of cytochrome *b*-563 is dependent on the presence of some reductant, possibly reduced cytochrome *b*-$559_{LP}$.

## Materials and Methods

PS-I-enriched vesicles were isolated from market spinach as described previously [28]. The standard reaction medium contained 5 mM Tes-KOH (pH 7.8)/2.5 mM $KH_2PO_4$/25 mM NaCl/25 mM KCl/5 mM $MgCl_2$; the chlorophyll concentration was 50 $\mu$g/ml. For optimal cyclic electron transfer [5], ferredoxin and NADPH were added to final concentrations of 5$\mu$M and 0.25 mM, respectively,

while the oxygen concentration was maintained at about 100 $\mu$M by periodical stirring, as controlled by a Clark oxygen electrode. Anaerobic conditions were obtained by adding 6 mM glucose and 8 units/ml glucose-oxidase to the reaction mixture. All experiments were carried out at 20°C (unless stated otherwise) in a thermoelectrically controlled multipurpose cuvette of 2.0 ml [29], the actinic light being provided from the bottom via a fiber-optic light guide by a 250 W tungsten projector lamp (Osram).

Flash-induced absorbance changes were measured with a laboratory-built fast-responding dual-wavelength spectrophotometer [30]. The carotenoid ($\Delta A_{518}$) and cytochrome *c*-554 transients were measured against the reference 547 nm and the transients of the *b*-type cytochromes against the reference 572 nm, unless stated otherwise. Saturated flash activation was provided by a General Electric FG-230 xenon flash tube (2 kV) firing flashes (5 $\mu$s at half-amplitude and tail-depressed) and passed through a filter cutting off below 695 nm (Schott, Mainz, F.R.G.). The signals of 16 or 32 flashes were averaged at 8 or 16 s time intervals. On-line processing and triggering were mediated by a microprocessor-minicomputer system as described before [30].

Cytochrome spectra at 77 K were recorded in an Aminco DW-2a spectrophotometer equipped with a low-temperature accessory (Aminco-J4-9603) under conditions as described before [28]. EPR measurements were principally carried out as described previously [28].

Digitonin (twice recrystallized from ethanol) was obtained from Merck, spinach ferredoxin from Sigma. DBMIB and DNP-INT were generously provided by Prof. A. Trebst (Ruhr-Universität, Bochum, F.R.G.).

## Results

If optimal redox poising of the ferredoxin-mediated cyclic electron transfer system is controlled with NADPH and oxygen, a very prominent slow-rising component is present in the flash-induced carotenoid response in PS-I vesicles (Fig. 1A). In this reconstituted system the carotenoid transient is qualitatively similar to that in intact chloroplasts [11] and whole algae [26] and remains

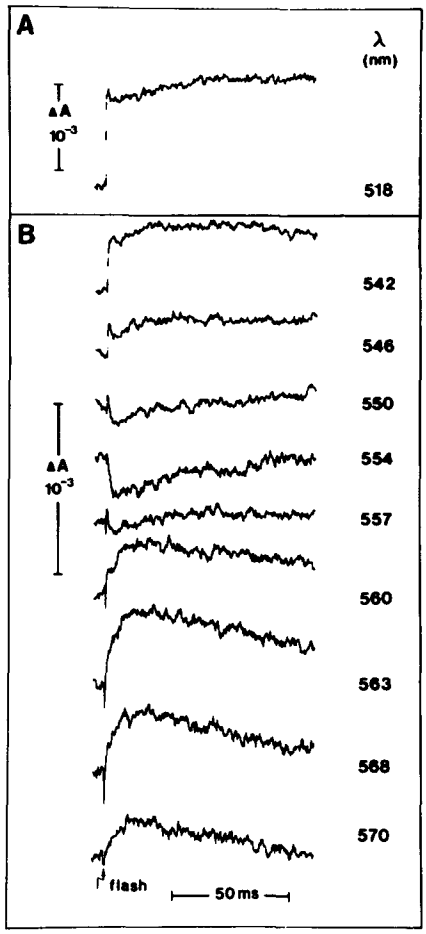


Fig. 1. Flash-induced absorbance changes in PS-I vesicles of carotenoid at 518 nm (A) and in the cytochrome $\alpha$-bands (B) under conditions of optimal redox poising for ferredoxin-mediated cyclic electron transfer.

identical for at least 45 min [5]. To investigate the function of cytochromes in the cyclic system of these PS-I-vesicles on the same time-scale, flash-induced absorbance changes were recorded throughout the cytochrome $\alpha$-band region; a representative series of recordings is shown in Fig. 1B. The extents of absorbance changes relative to the initial dark level, at selected time intervals after the flash, are plotted in the spectra of Fig. 2. Below about 550 nm the absorbance changes are mainly caused by the carotenoid response. At 0.5 ms after the flash a reduction of cytochrome *b*-563 is already evident; at the later time intervals oxidation of cytochrome *c*-554 also appears. The reduc-

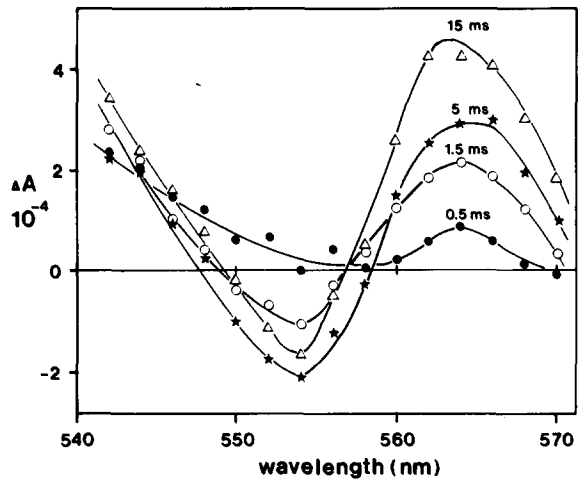


Fig. 2. Time-resolved spectra of flash-induced absorbance changes in the cytochrome $\alpha$-band region. Spectra were constructed from the extents of the transients (cf. Fig. 1B) at selected time-intervals after the flash, as indicated.

tion of cytochrome *b*-563 is biphasic (Fig. 1B); this is also observed for the reoxidation kinetics (computer deconvoluted; not shown). Cytochrome *c*-554 re-reduction kinetics, after the fast flash-induced oxidation, are single-exponential. The biphasic increment in absorbance at 563 nm was also observed in intact chloroplasts under conditions for cyclic electron transfer around PS I, the fast component being interpretated as a result of redox changes of plastocyanin [11]. The spectrum of absorbance changes in this wavelength region (Fig. 2) indicates that in PS-I vesicles the absorbance changes between 0.5 and 1.5 ms are mainly due to a fast reduction of cytochrome *b*-563, but some interference from spectral changes of other origin cannot be excluded. Biphasic kinetics in the reoxidation of cytochrome *b*-563 were also observed in chloroplasts [19]. Following the observed absorbance changes in Fig. 1B (cf. Fig. 2), the sequence of redox changes of cytochromes after the flash is: (1) fast reduction of *b*-563 ($t_{0.5} \approx 0.5$ ms); (2) oxidation of *c*-554 ($t_{0.5} \approx 2$ ms); (3) slower reduction of *b*-563 ($t_{0.5} \approx 4$ ms); (4) re-reduction of *c*-554 ($t_{0.5} \approx 30$ ms) and (5) reoxidation of *b*-563 ($t_{0.5} \approx 90$ ms).

Apparently, maximal cyclic electron transfer in PS-I vesicles occurs relatively slow as compared to that in intact chloroplasts. For instance, Crowther and Hind [11] found $t_{0.5}$ values of 5 and 20 ms for cytochrome *c*-554 re-reduction and *b*-563 reoxidation kinetics, respectively, similar to values observed by Selak and Whitmarsh [19]. The fast reduction of cytochrome *b*-563 preceeds the flash-induced oxidation of cytochrome *c*-554, so reduced ferredoxin or another reductant on the acceptor side of PS I is probably responsible for the fast reduction of cytochrome *b*-563. Relatively little (about 25%) cytochrome *b*-563 and *c*-554 is seen to turnover after a flash. Their contribution ratio is about 2 (measured 14 times in different preparations), indicating that for one cytochrome *c*-554 two *b*-563 cytochromes turn over. In addition, the amplitudes of the fast and slow phases in the reduction of cytochrome *b*-563 are about equal, measured at 1.5 ms and 15 ms after the flash, respectively (Figs. 1B and 2). Therefore, these two *b*-563 cytochromes apparently turn over kinetically different.

The slow-rising component in the carotenoid response has a $t_{0.5}$ of 15–20 ms (Fig. 1A), which is significantly smaller than the $t_{0.5}$ of the reoxidation of *b*-563 (90 ms). So, the reoxidation of cytochrome *b*-563 does not coincide with the slow electric potential generation as proposed for chloroplasts by Selak and Whitmarsh [19].

As is previously shown [5], the electron pressure on (i.e., redox balance of) the cyclic chain in PS-I vesicles can be manipulated by modulating the input and output of electrons at the level of ferredoxin (PS II activity is absent). This can be achieved by varying the concentrations of NADPH and $NADP^+$, determining the redox potential of ferredoxin via ferredoxin-$NADP^+$ oxidoreductase, and by varying the concentration of oxygen, which oxidizes reduced ferredoxin. Fig. 3 shows the effect of different concentrations of NADPH and $NADP^+$ on the flash-induced carotenoid and cytochrome transients under anaerobic conditions. Fig. 4 shows the corresponding spectra of the traces obtained at 130 $\mu$M NADPH in the absence and presence of 1 mM $NADP^+$ (the spectrum of the traces obtained in the presence of 5 $\mu$M NADPH is similar to that in the presence of 130 $\mu$M NADPH plus 1 mM $NADP^+$, result not shown). One can see that at the lower molar ratio of NADPH/$NADP^+$ the flash-induced maximal absorbance increase around 563 nm partly shifts towards 559 nm, indicating a reduction of cyto-

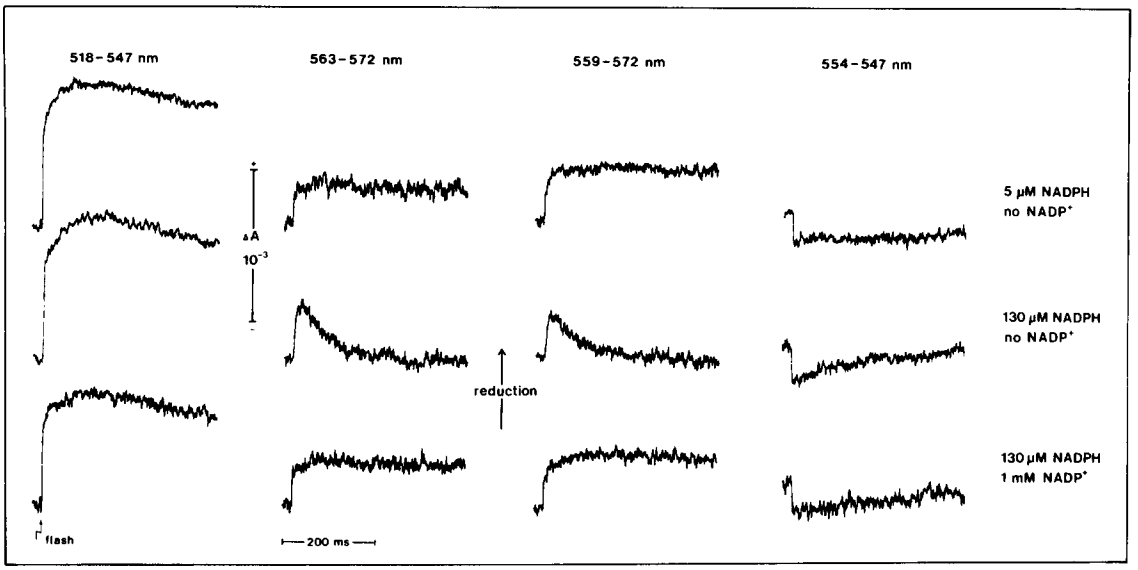


Fig. 3. Flash-induced absorbance changes of carotenoids and *b*- and *c*-type cytochromes at various concentrations of NADPH and $NADP^+$ under anaerobic conditions.

chrome *b*-559 at the cost of the reduction of cytochrome *b*-563. The low-potential form of cytochrome *b*-559 ($b\text{-}559_{LP}$, $E_0' \approx 30$ mV [5]), which is present in PS-I vesicles in relatively high amounts, is candidate for these redox reactions, because cytochrome $b\text{-}559_{HP}$ is absent in PS-I-vesicles [28]. Thus, cytochrome $b\text{-}559_{LP}$ appears to be involved in cyclic electron transfer around PS I, but is only observed at low electron pressure on the cyclic chain. Some cytochrome *b*-563 turnover is still observed, mainly due to the fast cytochrome *b*-563 reduction (Figs. 3 and 4). Remarkably, at the lower ratio of NADPH/$NADP^+$ the overall reoxidation of *b* cytochromes is strongly inhibited (Fig. 3). The rates of the (slow) cytochrome *b* reduction and cytochrome *c*-554 rereduction are delayed as well, whereas the kinetics of the fast cytochrome *b*-563 reduction are not notably changed. The total amount of *b* cytochromes reduced after a flash remains more or less constant at the different NADPH/$NADP^+$ ratios (Figs. 3 and 4). In other words, the reoxidation and slow reduction of cytochrome *b*-563 and the re-reduction of cytochrome *c*-554, but not the fast cytochrome *b*-563 reduction, are strongly dependent on the dark redox state of some other component. Thus, in disharmony with a serial transfer of elec-

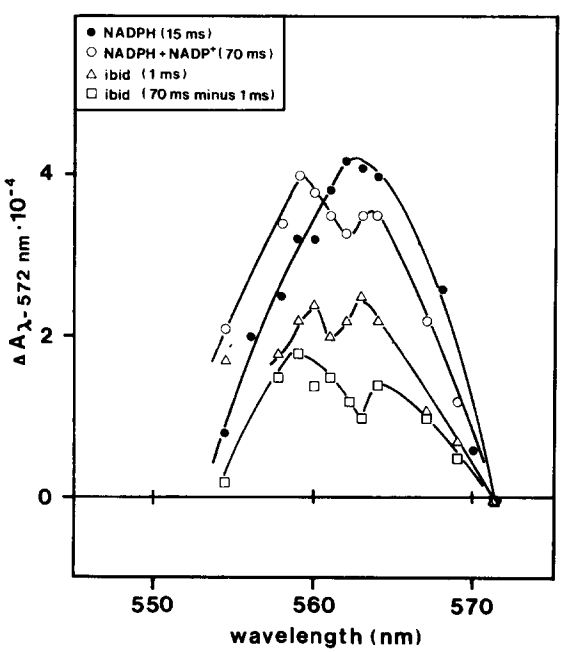


Fig. 4. Spectra of flash-induced absorbance changes in the cytochrome *b* α-band region at various NADPH/$NADP^+$ ratios under anaerobic conditions and at various times after the flash. Traces were obtained as in Fig. 3 and spectra were constructed as in Fig. 2. Concentrations of NADPH and $NADP^+$ were 130 µM and 1 mM, respectively.

trons from cytochrome *b*-563 to cytochrome *c*-554, oxidizing equivalents generated by PS I do not induce the oxidation of cytochrome *b*-563, in accordance with findings in chloroplasts [14].

Fig. 3 also confirms the conclusions from Fig. 1 in that the slow electric potential component does not correlate with the reoxidation of cytochrome *b*-563, because the carotenoid response looks rather similar at variable rates of cytochrome *b*-563 reoxidation.

Fig. 5 shows the effects of the electron transfer inhibitors DNP-INT and HQNO and of the absence of ferredoxin (i.e., no cyclic electron transfer) on the flash-induced carotenoid and cytochrome changes. The plastoquinone-antagonist DNP-INT [31] inhibits the slowly reduced part of cytochrome *b*-563 and its reoxidation. It also inhibits the re-reduction of cytochrome *c*-554, but not the fast reduction of *b*-563. The spectrum of these flash-induced absorbance changes in Fig. 6 indicates that cytochrome *b*-563 is still the major species turning over after addition of 10 μM DNP-INT, maximally 50% of its reduction being inhibited. DBMIB, which is another plastoquinone-antagonist [31], shows the same effects at a concentration of 1 μM (not shown). The inhibitions by DNP-INT and DBMIB point to the involvement of plastoquinone in the slow reduction and reoxidation of cytochrome *b*-563 as well as in the re-reduction of cytochrome *c*-554. Addition of HQNO results in an increase of the total flash-induced reduction level of cytochrome *b*-563 of maximally 20–30% at a concentration of 5 μM, the cytochrome *c*-554 transient not being affected (Figs. 5 and 6). At higher concentrations of HQNO no further increase of the flash-induced reduction level of cytochrome *b*-563 is observed (not shown). In chloroplasts an increase of the flash-induced

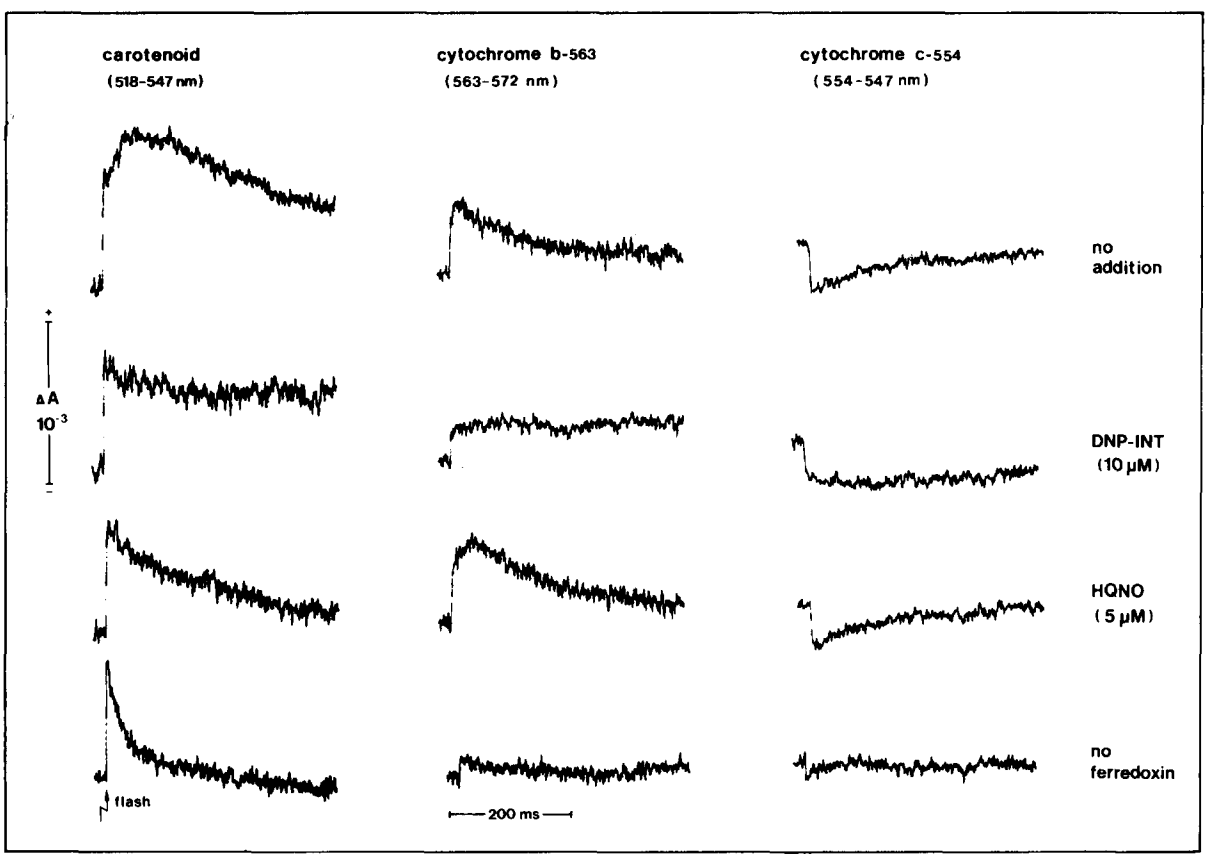


Fig. 5. Flash-induced absorbance transients of carotenoid and cytochromes *b*-563 and *c*-554 in the presence of DNP-INT (10 μM) and HQNO (5 μM) and in the absence of ferredoxin.

reduction level of cytochrome *b*-563 was also observed after addition of HQNO, but to a much larger extent (200–300% [19]). This was interpreted as due to a specific inhibition of cytochrome *b*-563 reoxidation compared to its reduction, confirming the assumption that under non-inhibiting conditions competing reduction and oxidation reactions result in a limited observable turnover of cytochrome *b*-563. However, this explanatoin seems not applicable to PS I vesicles, because the reoxidation of cytochrome *b*-563 is not significantly inhibited (Fig. 5). Fig. 5 furthermore shows that 10 $\mu$M DNP-INT (similar to 1 $\mu$M DBMIB) inhibits the slow electric potential component. The amplitude of the carotenoid response at 80 ms after the flash is lowered to about 50% of its original magnitude (in the absence of ferredoxin this amplitude is almost zero, cf. Fig. 5). Since both the slow reduction phase of cytochrome *b*-563 as well as the re-reduction of cytochrome *c*-554 are fully inhibited under these conditions, each of these processes may be obligatory for the DNP-INT-sensitive part of the slow electric potential generation. However, the re-reduction of cytochrome *c*-554 ($t_{0.5} \approx 30$ ms) is too slow to account for the generation of the slow potential component. The fast and DNP-INT insensitive reduction of cytochrome *b*-563 may be obligatory for the remaining part of this electric potential component. This interpretation is consistent with the finding that in the absence of ferredoxin no cytochrome turnover is observable, while the slow potential component is absent. Both reduction phases of cytochrome *b*-563 are faster than the rise of $\Delta A_{518}$ (slow) (Fig. 5). HQNO at a concentration of 5 $\mu$M inhibits the slow electric potential component to about the same extent as 10 $\mu$M DNP-INT, while both reduction phases of *b*-563 are still present (Fig. 5). This could be interpreted by the fact that the slow reduction phase of cytochrome *b*-563 does not always result in the slow electric potential generation, agreeing with findings in chloroplasts by Van Kooten et al. [32]. However, HQNO can also act as an uncoupler [33] thus decreasing $\Delta A_{518}$ (slow).

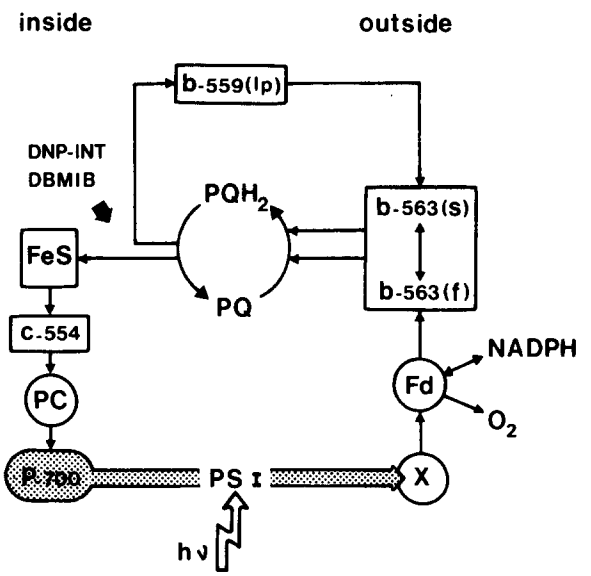


Fig. 7. Tentative scheme for 'native' PS-I and ferredoxin-mediated cyclic electron transfer in PS-I vesicles. PC, plastocyanin; Fe-S, Rieske (Fe-S) protein; PQ, plastoquinone; Fd, ferredoxin; *b*-563 (*f*), fast reducible cytochrome *b*-563; *b*-563 (s), slow reducible cytochrome *b*-563.

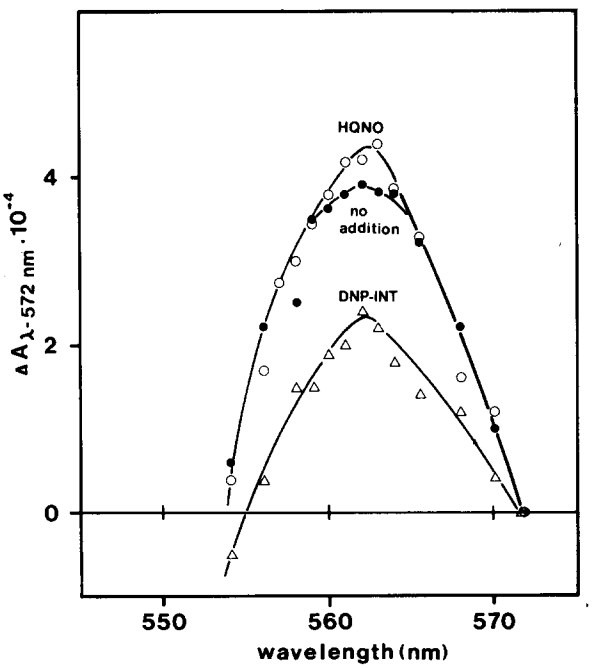


Fig. 6. Spectra of flash-induced absorbance changes in the cytochrome *b* $\alpha$-band region in the absence and presence of DNP-INT (10 $\mu$M) and HQNO (5 $\mu$M). Spectra were constructed as in Fig. 2. Traces were obtained as in Fig. 5.

## Discussion

The experiments presented in this work were aimed at a kinetic comparison of cytochrome redox and electric potential changes in flash-illuminated PS-I subchloroplast vesicles (i.e. stroma lamellae membrane vesicles). Spectral identification of the participating cytochromes, variation of the applied 'electron pressure' and differential effects of electron transfer inhibitors were used to gain information on the electron flow scheme and the location in time of the so-called 'slow' electric potential generation.

The results will be discussed following a tentative model of electron transfer by the Qbc redox complex in PS I and ferredoxin-mediated cyclic electron flow, as shown in Fig. 7.

In PS I vesicles, the Qbc complex is present [28] and its cytochromes *b*-563 and *c*-554 ('*f*') obviously participate in cyclic electron transfer, as in whole chloroplasts [10,11]. In addition, also cytochrome $b\text{-}559_{LP}$ appears to be involved, but is only observed under conditions of suboptimal redox poising of the cyclic system, i.e., at a low $NADPH/NADP^+$ ratio (Figs. 4 and 5). The function of cytochrome $b\text{-}559_{LP}$ is yet poorly understood, in spite of its relatively high content in thylakoid membranes [3,28]. Several authors have proposed a function of $b\text{-}559_{LP}$ in cyclic electron transfer around PS I [28,34], but its turnover, in particular after a flash, was hardly observed without perturbing the membrane and/or electron transfer system [34].

In PS-I vesicles, two kinetically distinguishable cytochrome *b*-563 species turn over after a flash for one cytochrome *c*-554 (Figs. 1 and 2), in accordance with the ratios of the *b*-563 and *c*-554 contents found earlier in PS-I vesicles from a combined spectrophotometric/potentiometric analysis [28], in chloroplasts [3] and in the isolated '$b_6$-*f*' complex [21]. On the other hand, a 1 : 1 turnover ratio of *b*- and *c*-type cytochromes seems evident from other work on flash-illuminated broken and intact chloroplasts [11,13,18,19]. However, in these latter studies the participation of cytochrome *b*-563 may be underestimated, because the flash-induced reduction level of cytochrome *b*-563 is strongly masked by its rapid reoxidation [19]. This suggestion is substantiated by experiments in which addition of the cytochrome *b*-563 oxidation-inhibitor HQNO resulted in a 2–3-fold larger extent of the *b*-563 reduction level after a flash [19]. It is of interest to note that in PS-I vesicles HQNO increases the flash-induced extent of cytochrome *b*-563 reduction with only 20–30% of its original value (Figs. 5 and 6), thus roughly maintaining the 2 : 1 ratio per flash. The *b*-563/*c*-554 turnover ratio of 2 in PS-I vesicles is further substantiated by its constantness at specific inhibition of the re-reduction of *c*-554 and reoxidation of *b*-563 (see e.g. Fig. 3). Alternatively, an underestimated turnover ratio of cytochrome *b*-563 to *c*-554 in chloroplasts may also result from the turnover of cytochrome *c*-554 molecules that are present in the appressed regions of the grana membranes and not associated with PS I [3,28].

The two cytochrome *b*-563 species are reduced via two different pathways, as is visualized in Fig. 7. The fast reductive pathway of cytochrome *b*-563 proceeds probably from reduced ferredoxin, is insensitive to the plastoquinone antagonists DNP-INT and DBMIB and to HQNO (Fig. 5) and is independent on the dark redox state of the electron transfer chain (Fig. 3). This pathway may contribute for about 50% to the slow electric potential generation. In contrast, the slow reductive pathway of cytochrome *b*-563 is sensitive to DNP-INT and DBMIB (Fig. 5) and is strongly delayed at suboptimal redox poising (Fig. 3). This pathway seems obligatory for the generation of the remaining part of the slow potential component and may also involve the reduction of cytochrome *c*-554, because of its analogous behaviour with the slow reduction of cytochrome *b*-563 (Figs. 3 and 5). The slow reduction of cytochrome *b*-563 preceeds the re-reduction of cytochrome *c*-554 (half-times of 4 and 30 ms, respectively, see Figs. 1B and 2), as in whole chloroplasts [11,19], and points to a mechanism in which the $PQH_2/PQ^-$ couple reduces cytochrome *b*-563 and the $PQ^-/PQ$ couple reduces the Rieske (Fe-S) protein and cytochrome *c*-554, as proposed by Selak and Whitmarsh [19]. This mechanism contradicts the so-called 'oxidant-induced reduction' of cytochrome *b*-563 as found in an isolated '$b_6$-*f*' complex [23], in which cytochrome *c*-554 is the oxidizing species that induces the simultaneous reduction of cytochrome *b*-563.

At a preestablished low electron pressure of the cyclic system (i.e., low NADPH/$NADP^+$ ratio), a slow reduction of cytochrome $b$-$559_{LP}$ is obvious, apparently at the cost of the slow reduction of cytochrome $b$-563 (Figs. 3 and 4). This suggests that the slow reduction of cytochrome $b$-$559_{LP}$ preceeds the slow reduction of cytochrome $b$-563. Electron transfer from cytochrome $b$-$559_{LP}$ to $b$-563 is not easily understood when comparing their respective redox midpoint potentials ($E_0'$ are 32 mV and $-132$ mV in PS-I vesicles, respectively [28]). However, their actual redox potentials in the preestablished dark redox state at high NADPH/$NADP^+$ ratio are indeed compatible with electron transfer in this direction, because in this state cytochrome $b$-$559_{LP}$ is almost fully reduced and cytochrome $b$-563 almost fully oxidized (data not shown). In addition, the appearance of flash-induced cytochrome $b$-$559_{LP}$ turnover goes hand in hand with inhibition of reoxidation of all $b$-cytochromes as well as retardation of cytochrome $c$-554 reduction (Fig. 3). This suggests that cyclic electron transfer only proceeds if cytochrome $b$-$559_{LP}$ is in reduced state before the flash. We proposed earlier [35] that the availability of a semiquinone species would be the limiting factor in cyclic electron transfer, because this semiquinone would be the oxidant for reduced cytochrome $b$-563 (in socalled 'reductant-induced oxidation' of cytochrome $b$-563). However, we were not able to detect a stable semiquinone species in the millisecond-to-second time scale by EPR spectroscopy at various electron pressures (results not shown).

## Acknowledgements

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