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Cyt *b*-559 (Fd) Participating in Cyclic Electron Transport in Spinach Chloroplasts: Evidence for Kinetic Connection with the Cyt b_6/f Complex

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A small fraction of low potential Cyt *b*-559, amounting to only 13% of total Cyt *b*-559 in spinach chloroplasts, is analyzed with the help of a highly selective, computer-controlled spectrophotometer, which simultaneously applies 16 pulse modulated narrow band measuring beams with wavelengths in the cytochrome *α*-band (500–600 nm) for recordings of time resolved difference spectra. This Cyt *b*-559 fraction remains oxidized upon dark incubation with ascorbate and is reduced upon illumination. It can be reduced by cyclic PSI in an antimycin A-sensitive reaction or in the course of antimycin A-insensitive linear electron transport via the Cyt b_6/f complex. Reduction by NADPH in the dark requires ferredoxin. Simultaneous recordings of Cyt *b*-563 and Cyt *f* reveal close kinetic connection between this Cyt *b*-559 fraction and the low potential chain of the Cyt b_6/f complex. These results confirm and extend previous observations of Miyake et al. 1995 (*Plant Cell Physiol.* 36: 743) in maize mesophyll thylakoids, which led to the hypothesis that Cyt *b*-559 (Fd) occupies the position of the postulated ferredoxin-plastoquinone reductase (FQR) in cyclic electron transport.

Key words: Cyclic electron transport — Cyt *b*-559 — Cyt b_6/f complex — Q-cycle.

Discovered more than 35 years ago (Lundegårdh 1962, Boardman and Anderson 1967), Cyt *b*-559 has remained an enigma until the present time: Despite numerous extensive studies, the only widely accepted result has been that the high potential form of Cyt *b*-559, which constitutes ca. 70% of total Cyt *b*-559, is an integral part of the PSII reaction center (Pakrasi et al. 1988, 1990, Whitmarsh and Pakrasi 1996). The remaining 30% of low potential Cyt *b*-559 (Cyt *b*-559 LP) has found much less attention in the past, mostly due to technical difficulties to detect its absorbance changes against the background of the much larger absorbance changes of Cyt *f* and Cyt *b*-563. The difficulties to obtain information on Cyt *b*-559 LP were aggravated by uncertainties concerning the existence of a distinct Cyt *b*-560 (Rolfe et al. 1987, Tae et al. 1993). Furthermore, considerable confusion has been caused by the fact that Cyt *b*-559 HP associated with PSII can be converted into a low potential form by various nonphysiological treatments, like heat stress (Erixon et al. 1972), extreme pH (Horton and Cramer 1975, Ortega et al. 1990), or detergent treatment (McNamara and Gounaris 1995). Hence, although Cyt *b*-559 LP clearly occurs as a distinct component in freshly prepared, untreated chloroplasts (Rich and Bendall 1980, Bendall 1982), it remains difficult to determine how—and if—this naturally occurring form can be distinguished from artificially derived low potential forms of Cyt *b*-559.

In studies with thylakoid membranes enriched in PSI, Anderson and Boardman (1973) found evidence for an association of Cyt *b*-559 LP with PSI, and Peters et al. (1983) presented evidence for Cyt *b*-559 LP participating in a cycle around PSI. Bendall (1982) pointed out the ease with which Cyt *b*-559 LP can be reduced by NADPH and ferredoxin. Thylakoid fractionation studies revealed that Cyt *b*-559 LP occurs in stroma thylakoids (Vallon et al. 1987) but not in grana stacks (McNamara and Gounaris 1995). Bendall (1982) already suggested that Cyt *b*-559 LP "might behave as an electron acceptor for ferredoxin in the PSI-dependent cyclic pathway as part of the ferredoxin-plastoquinone reductase of Crowther and Hind (1980)." But, he also noted that the difference spectrum reported by these authors under cyclic conditions is very close to what would be detected for pure Cyt *b*-563. Therefore, it has been clear that convincing evidence for a participation of Cyt *b*-559 LP in cyclic electron transport by absorbance measurements puts extraordinary demands on the sensitivity and selectivity of the applied measuring equipment.

A computer-controlled kinetic spectrophotometer (LED-array spectrophotometer) with outstanding selectivity was introduced by Klughammer et al. (1990). This device is particularly well suited for analysis of the complex absorbance changes in the cytochrome *α*-band region (500–600 nm). With the help of this spectrophotometer, Miyake et al. (1995) succeeded to characterize a Cyt *b*-559 LP in maize mesophyll thylakoids which is involved in cyclic electron flow around PSI. This Cyt *b*-559 (Fd) could be reduced by far-red light in a ferredoxin-dependent reaction, which was inhibited by low concentrations of an-

Abbreviations: DAD, diaminodurene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Fd, ferredoxin; FQR, ferredoxin-quinone-reductase, TPT, triphenyltin-chloride.

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timycin A, known to inhibit ferredoxin dependent cyclic flow (Tagawa et al. 1963, Moss and Bendall 1984, Cleland and Bendall 1992). Hence, Miyake et al. (1995) suggested that their Cyt *b*-559 (Fd) is identical with the long sought-for FQR. However, unequivocal confirmation of this assignment for chloroplasts of C3 plants proved difficult, as the content of Cyt *b*-559 (Fd) in spinach thylakoids was only 10% of that in maize thylakoids (Miyake et al. 1995). Furthermore, Heimann and Schreiber (1996) showed that there are two distinct forms of Cyt *b*-559 LP in spinach chloroplasts, only one of which is oxidized in the dark in the presence of ascorbate.

Here we report on experiments using the LED-array spectrophotometer (Klughammer et al. 1990, 1998) for the characterization of the small Cyt *b*-559 LP fraction in spinach chloroplasts which is not reduced by ascorbate. It will be shown that this fraction of Cyt *b*-559 LP can be reduced via antimycin A sensitive cyclic electron flow as well as by NADPH-ferredoxin in the dark, thus suggesting that it corresponds to the Cyt *b*-559 (Fd) described by Miyake et al. (1995) in maize thylakoids. In addition, evidence in favor of a close association of this Cyt *b*-559 (Fd) with the Cyt b_6/f complex will be presented. It will be shown that Cyt *b*-559 (Fd) can also be reduced by linear electron flow under conditions which exclude cyclic electron flow, presumably via Cyt *b*-563 in the low potential chain.

Materials and Methods

Intact spinach chloroplasts were freshly prepared as previously described (Heimann and Schreiber 1996) except that no additional ascorbate was added during isolation. Thylakoids were obtained directly before each experiment by rupturing intact chloroplasts in ten-fold diluted standard suspension buffer to which 10 mM $MgCl_2$ was added. Isoosmotic conditions were re-established after 30 s by addition of double-strength suspension buffer. In the experiment of Fig. 6C, fresh thylakoids were centrifuged and resuspended in fresh buffer in order to remove residual ferredoxin.

Absorbance changes were recorded with an improved version of a laboratory-built LED-Array-Spectrophotometer (Klughammer et al. 1990, 1998). This computer-controlled kinetic spectrophotometer measures absorbance changes in the 500–600 nm wavelength range quasi-simultaneously at 16 different discrete wavelengths (ca. 2 nm half-band widths) using pulse modulated light emitting diodes (LED) and narrow band interference filters. The time resolved difference spectra were deconvoluted on the basis of model difference spectra for C550, Cyt *f*, Cyt *b*-559 and Cyt *b*-563 and converted to redox changes for the cytochromes. For Cyt *b*-559 (Fd) the same extinction coefficient as for Cyt *b*-559 was assumed. Possible contributions of plastocyanin and P700, whose spectral changes are sufficiently flat in the given wavelength range, were taken into account by including a second order polynomial function in the deconvolution procedure. If not stated otherwise, experiments were carried out at 20°C with 40 μg Chl ml^{-1}. Measuring light intensity was ca. 0.04 μmol m^{-2} s^{-1}, which amounts to about one photon per photosystem every 50 s, or roughly one excitation per photosystem every 150 s, taking into account the actual absorption between 500 and 600 nm. White saturating flashes ($t_{1/2}=7$ μs) were applied with a stroboscope flash lamp (Polytec, Waldbronn, Germany).

Duroquinol was prepared as follows: 20 ml methanol were saturated with duroquinone. Small amounts of borohydride were added until the yellow solution was colorless, with white flakes indicating precipitation of the quinol. Then 20 ml of 2 M HCl were added. When all borohydride had reacted off, the mixture was centrifuged for 5 min at 5,000 × *g*. The pellet was washed once with 40 ml of 2 M HCl and centrifuged again. The resulting pellet was heated slowly until it was completely dry and no acrid smell (HCl) could be detected, and then stored as powder in closed caps. For experiments, small amounts were dissolved in dimethylsulfoxide/1 M HCl.

Results and Discussion

A very low potential form of Cyt b-559 in spinach thylakoids—Fig. 1 shows the redox characteristics of Cyt *b*-559, Cyt *b*-563 and Cyt *f* in freshly prepared spinach thylakoids, as measured with the LED-array spectrophotometer. It may be noted that this device is particularly well suited for the recording of chemically induced absorbance changes over extended periods of time, as the deconvoluted signals are not affected by stirring noise, broad-band unspecific absorbance changes and signal drifts. The specific signals reflecting the chemically induced redox changes of the three cytochromes are deconvoluted by special computer software (see Materials and Methods). Before the start of the experiment, the thylakoids were dark incubated for 10 min in order to assure quantitative oxidation of all low potential forms of Cyt *b*-559. Under these conditions,

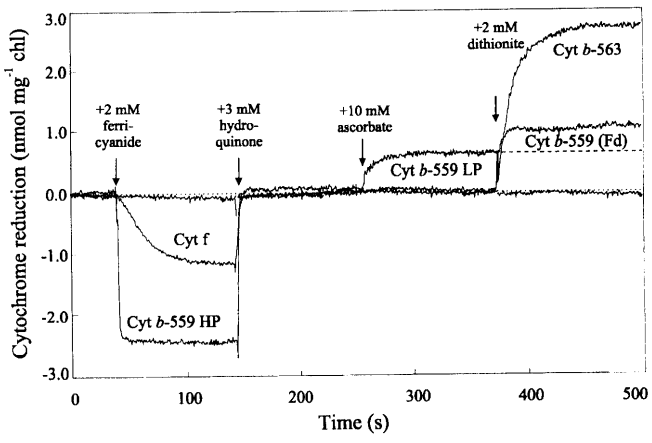

Fig. 1 Chemically induced redox changes of cytochromes in spinach thylakoids freshly prepared by osmotic rupture of intact chloroplasts. Before the experiment, the thylakoids were dark incubated for 10 min in the measuring cuvette in the presence of 25 μM DAD and 1 μM benzylviologen in order to oxidize Cyt *b*-559 LP quantitatively. Where indicated, 2 mM ferricyanide, 3 mM hydroquinone, 10 mM ascorbate and 2 mM dithionite were added. The suspension was continuously stirred. The three different fractions of Cyt *b*-559 are denoted.

in the given thylakoid preparation ca. 30% of total Cyt *b*-559 is oxidized before addition of ferricyanide. The remaining 70%, which is oxidized by ferricyanide, can be re-reduced again by hydroquinone, representing the well known Cyt *b*-559 HP. The existence of two low potential fractions of Cyt *b*-559 (Heimann and Schreiber 1996) is revealed by the successive addition of ascorbate, which reduces 17% of total Cyt *b*-559 (Cyt *b*-559 LP), and dithionite, which reduces the remaining fraction of 13% which we will call Cyt *b*-559 (Fd) following Miyake et al. (1995) (see below). The latter fraction is oxidized in dark-adapted intact chloroplasts, which contain at least 10 mM ascorbate (Foyer et al. 1983), and which is reduced upon illumination (Klughammer et al. 1990).

Fig. 2 shows the light induced redox changes of Cyt *b*-559 in intact spinach chloroplasts upon onset of strong continuous illumination. As explained above, it may be assumed that these changes reflect Cyt *b*-559 (Fd). It is apparent that only a small part of Cyt *b*-559 (Fd) is transiently reduced during the first 20 s of illumination. In view of the small amplitude and the transient character of this light induced change, it is understandable that details of this reaction so far have not been reported. The rapid transient reduction is followed by a much slower stable reduction which is completed in ca. 200 s and only slowly reversible in the dark.

For an interpretation of these transients, it is important to consider that only few components of the photosynthetic electron transport chain have a sufficiently negative midpoint potential to reduce this very low potential form of Cyt *b*-559. These are the acceptor sides of PSII and PSI, as well as the low potential chain of the Cyt b_6/f complex. In the following sections, we will present data arguing for reduction of Cyt *b*-559 (Fd) by both the PSI acceptor side and the low potential chain of the Cyt b_6/f complex.

Reduction of Cyt b-559 (Fd) induced by far-red light

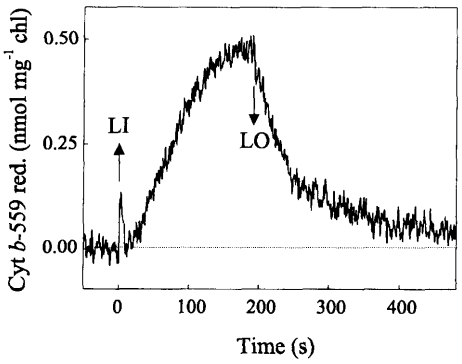

Fig. 2 Light induced redox changes of Cyt *b*-559 in intact spinach chloroplasts after 10 min dark incubation with 5 mM ascorbate. Illumination was by 1,000 μmol m^{-2} s^{-1} red light for 190 s. Continuous stirring during all of the recording.

—In Fig. 3 the redox reactions of all three chloroplast cytochromes are shown upon illumination of intact chloroplasts with strong far-red light combined with weak red light. These conditions are favorable for cyclic electron flow around PSI. Upon onset of illumination, rapid oxidation of Cyt *f* is observed, part of which is slowly reversed during illumination. When antimycin A was added to inhibit cyclic electron transport (Tagawa et al. 1963, Miyake et al. 1995), Cyt *f* was reoxidized again, reaching almost the same level as upon onset of illumination. Uncoupling of linear electron transport by nigericin led to partial reversion of Cyt *f* oxidation due to an increase in the rate of electron transfer from PQH_2 to the cyt b_6/f complex. This demonstrates that the effect of antimycin A was not due to an uncoupling effect, which is observed at higher concentrations (Hosler and Yocum 1985, Hormann 1995). When the light was switched off after 150 s, Cyt *f* oxidation was fully reversible in the dark.

Cyt *b*-563 was partially reduced upon illumination, as can be expected due to its participation in Q-cycle electron transport. Two reduction phases can be distinguished, with a major rapid phase followed by a minor slow phase. Antimycin A distinctly lowered the reduction level, which was only marginally increased by addition of nigericin. Upon

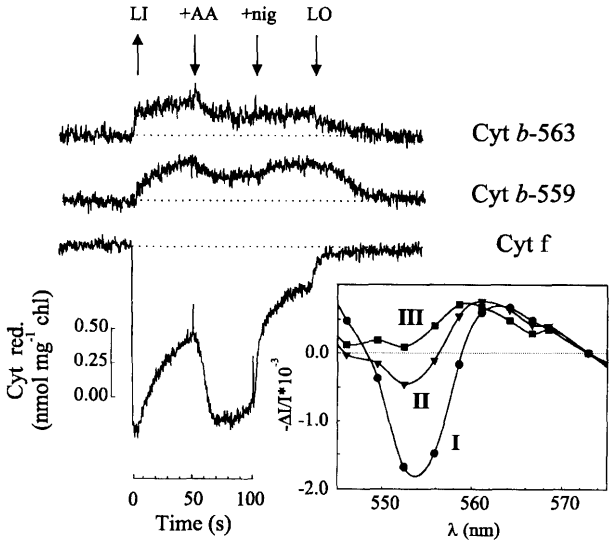

Fig. 3 Redox changes of cytochromes in intact chloroplasts induced by continuous far-red light. Inset: Difference spectra between the initial dark state and various time spans during illumination. Intact chloroplasts dark incubated with 5 mM ascorbate were illuminated for 150 s with 175 μmol m^{-2} s^{-1} far-red light (735 nm) to which 9 μmol m^{-2} s^{-1} red light (655 nm) was added for redox poising of cyclic PSI. Antimycin A (1 μM) was added at 50 s and nigericin (0.5 μM) at 100 s after start of illumination. The on/off arrows denote start/stop of illumination. Abbreviations: AA, antimycin A; nig, nigericin. Inset: Circles, directly before addition of antimycin A; triangles, after addition of nigericin; squares, directly after illumination.

switching off the light, a rapid and a slow reoxidation phase can be distinguished.

Cyt *b*-559 (Fd) displays similar although not identical redox changes as Cyt *b*-563. Upon onset of illumination, the amplitude of the rapid reduction phase was smaller than that of the slow phase. Antimycin A caused partial reoxidation, but as in the case of Cyt *b*-563, approximately 50% of light induced reduction was not affected. In contrast to Cyt *b*-563, upon addition of nigericin the original reduction level was re-established. And when the light was switched off, also differing from Cyt *b*-563, reoxidation set in with sigmoidal kinetics (half time ca. 30 s).

The inset of Fig. 3 shows difference spectra of this experiment comparing the original dark state with the states directly before addition of antimycin A (I), after addition of nigericin (II), and directly after cessation of illumination (III). While it is not possible to identify a contribution of Cyt *b*-559 (Fd) in the difference spectrum I, which is dominated by Cyt *f* oxidation, after uncoupling by nigericin, when the amplitude of Cyt *f* oxidation is decreased, a reduction of Cyt *b*-559 (Fd) may be discerned (difference spectrum II), which becomes even more clear briefly after light-off, as dark relaxation kinetics of Cyt *f* and Cyt *b*-563 are distinctly faster than that of Cyt *b*-559 (Fd).

The data of Fig. 3 allow some tentative conclusions concerning the localization of this particular Cyt *b*-559 fraction which is reduced upon illumination:

(1) Reduction can be driven by PSI light, which shows that cyclic electron flow is involved.

(2) If one may assume that the strong initial oxidation of Cyt *f* reflects donor side limitation of PSI, than the slow re-reduction observed during the first minute of illumination, which is paralleled by Cyt *b*-559 reduction, should reflect the induction of cyclic electron flow which relieves donor side limitation.

(3) While antimycin A clearly does affect Cyt *b*-559 reduction, which may be expected if reduction is driven by cyclic electron flow, only ca. 50% of this reduction is reversed. Therefore, in addition to cyclic flow also reduction associated with linear electron flow should be considered.

(4) The sigmoidal oxidation kinetic of Cyt *b*-559 after light-off could reflect a pool of reductant which becomes only slowly exhausted in the dark and, possibly, could be identical to reduced ferredoxin-NADPH.

(5) While the reduction kinetics of Cyt *b*-559 are similar to those of Cyt *b*-563, also distinct differences are apparent. A minor rapid phase coincides with oxidation of Cyt *f* and with the major phase of Cyt *b*-563 reduction, whereas a major slow phase is paralleled by Cyt *f* re-reduction and the minor phase of Cyt *b*-563 reduction.

These data are in agreement with the working hypothesis that the involved fraction of Cyt *b*-559 is located close to the outer surface of the thylakoid membrane and can accept electrons from ferredoxin, but also from another donor involved in linear electron transport.

Light-induced reduction of Cyt b-559 (Fd) associated with linear electron transport—Cyclic electron transport can be suppressed by addition of methyl viologen which constitutes an efficient electron acceptor of PSI. In this way, the suggested association of Cyt *b*-559 (Fd) with linear electron transport can be further investigated. Fig. 4 shows redox changes of the three chloroplast cytochromes in the presence of methyl viologen induced by single turnover saturating flashes. In a control sample with undisturbed Q-cycle activity (Fig. 4A) the flash-induced changes are fast and very small, such that for an evaluation many recordings had to be averaged. Computer-assisted deconvolution reveals besides the well known responses of Cyt *b*-563 reduction and Cyt *f* oxidation also some Cyt *b*-559 (Fd) reduction, corresponding to ca. 20% of the fraction which is oxidized after dark incubation with ascorbate. These Cyt *b*-559 (Fd) changes, which reflect the reduction of only 3% of total Cyt *b*-559, are too small to be detected by conventional difference spectroscopy. Actually, in view of the similarity between the calculated changes of Cyt *b*-563 and Cyt *b*-559, it has to be considered that the Cyt *b*-559 response could be an artifact of the computer fitting routine. This possibility, however, is rendered unlikely by the data of Fig. 4B. In this case, the experiment

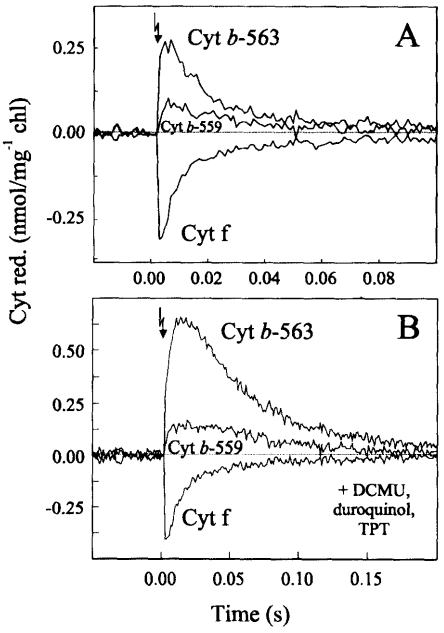

Fig. 4 Redox changes of cytochromes in uncoupled spinach thylakoids induced by a single turnover saturating flash. Freshly prepared thylakoids were dark incubated with 400 μM methyl viologen, 1,100 U ml^{-1} catalase, 0.5 μM nigericin and 0.5 μM nonactin. (A) No further addition; average of 6,000 measurements (2 Hz) on 3 separate samples. (B) In the presence of 100 μM TPT, 10 μM DCMU and 1 mM duroquinol; average of 1,000 measurements (1 Hz) on one sample.

was carried out in the presence of triphenyltin-chloride (TPT), which recently was introduced as a selective inhibitor of the Q_N-site (Klughammer et al. 1998). This inhibitor stimulates flash-induced Cyt *b*-563 reduction by slowing down the rate of dark-reoxidation, thus facilitating registration and computer-assisted analysis of the responses. It is apparent that also flash-induced Cyt *b*-559 (Fd) reduction is stimulated and its reoxidation slowed down by TPT. Most importantly, with the increased signal/noise ratio clear-cut differences in the reoxidation kinetics of Cyt *b*-559 (Fd) and Cyt *b*-563 can be detected. As in the case after continuous far-red illumination (Fig. 3), reoxidation of Cyt *b*-559 (Fd) is distinctly slower than that of Cyt *b*-563. On one hand, these data confirm that the Cyt *b*-559 (Fd) response obtained by computer-assisted deconvolution does reflect a component which is distinct from Cyt *b*-563. On the other hand, the effect of TPT on Cyt *b*-563 and Cyt *b*-559 (Fd) leads to the conclusion that this particular fraction of Cyt *b*-559 is closely associated with the low potential chain of the Cyt b_6/f complex. This is confirmed by the fact that the PSII acceptor side, the only other electron donor with sufficiently negative redox potential, had been blocked by DCMU.

This conclusion is further strengthened by the data of Fig. 5. In this experiment, electron flow via the low potential chain of the Cyt b_6/f complex was slowed down by oxidative pretreatment (Velthuys 1979, Joliot and Joliot 1984). Under the given conditions, the Cyt b_6/f complex is in a particular inactive state ("state-s" in Heimann et al. 1998), with the reoxidation of Cyt *b*-563 being much more slowed down than by any known Q_N-inhibitor. It is apparent that in this particular state the dark-reoxidation of flash-induced Cyt *b*-559 (Fd) reduction is even more slowed down than that of Cyt *b*-563. Hence, despite the small amplitude of reduced Cyt *b*-559 (Fd) accumulating under repetitive flash illumination, its existence can be unequivocally detected even by conventional difference spectra, i.e. without computer-deconvolution (see inset of Fig. 5).

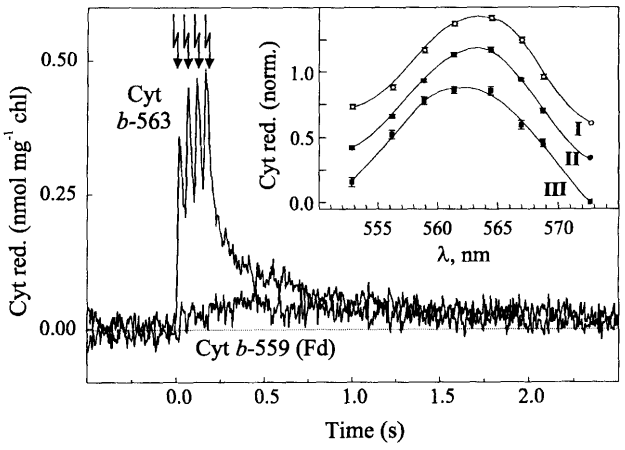

Fig. 5 Redox changes of Cyt *b*-563 and Cyt *b*-559 in spinach thylakoids after oxidative pretreatment induced by four consecutive single turnover saturating flashes. Note: By the oxidative pretreatment the Cyt b_6/f complex had been transformed into a specially inactive state ("state-s"). Inset: Normalized difference spectra between the initial dark state and various time spans in the course of the experiment. Fresh thylakoids containing 0.5 μM nigericin, 0.5 μM nonactin, 2 μM DCMU were incubated with 2 mM ferricyanide for 15 min in the dark. Then, before the actual measurement, 5 mM ascorbate and 1 mM duroquinol were added. Time between flashes, 50 ms. 250 recordings were averaged using 5 separate samples, with 10 s between single recordings. Inset: I, time span from 0.3 to 0.35 s (circles); II, time span from 0.5 to 1.0 s (triangles); III, time span from 1.5 to 2.0 s (squares). The standard error was obtained by averaging over the given time span.

Ferredoxin-dependent Cyt b-559 (Fd) reduction in the dark—While the data of Fig. 2–3 support the notion that the particular fraction of Cyt *b*-559, which is not reduced by ascorbate in spinach chloroplasts, does correspond to the Cyt *b*-559 (Fd) previously identified in maize thylakoids (Miyake et al. 1995), the data of Fig. 4–5 suggest that its reduction does not necessarily require cyclic electron flow. In principle, electron flow via the low potential chain of the Cyt b_6/f complex could account for the observed responses, as this flow is linked to all electrons passing the Cyt b_6/f complex, irrespective of whether the electrons originate from PSII, PSI or an artificial donor (like duroquinol) equilibrating with the plastoquinone pool. Therefore, it was important to test whether Cyt *b*-559 (Fd) can be reduced in the dark by ferredoxin-NADPH in the absence of Q-cycle activity.

As shown in Fig. 6, this is indeed the case. Experiments were carried out with freshly prepared thylakoids in the absence of oxygen, with 1 mM duroquinol added to prereduce the plastoquinone pool. The reaction was started

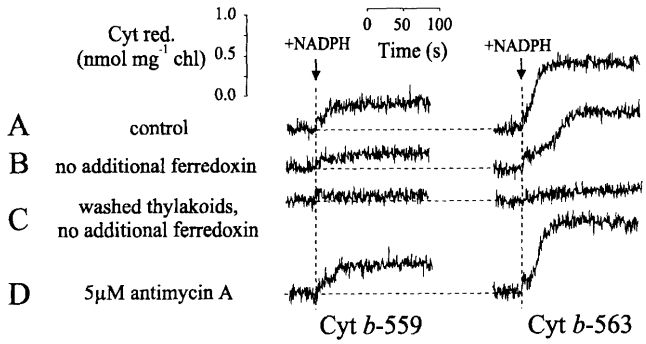

Fig. 6 Reduction of Cyt *b*-559 and Cyt *b*-563 induced by addition of NADPH in dark adapted spinach thylakoids. Fresh thylakoids (80 μg Chl ml^{-1}) containing 5 mM ascorbate, 1 mM duroquinol and 5 μM ferredoxin (where indicated) were made anaerobic with glucose/glucose-oxidase/catalase. After 10 min 3 mM NADPH were added. A, control with ferredoxin added; B, control without ferredoxin added; C, washed thylakoids (see Materials and Methods) without ferredoxin added; D, as (A) but in the presence of 5 μM antimycin A.

by addition of 3 mM NADPH, which reduces ferredoxin (if present) via the ferredoxin-NADP oxido-reductase. Measuring light intensity was extremely low, such that less than one charge separation per reaction center was induced in 150 s. Either 5 μM ferredoxin was added (traces A), or just that amount of ferredoxin was present which remained bound to the thylakoid membranes following osmotic rupture (traces B), or ferredoxin was deliberately removed by a washing step (traces C). It is apparent that the dark-reduction of Cyt *b*-559 (Fd) as well as that of Cyt *b*-563 upon addition of NADPH does require ferredoxin.

We have tried to inhibit the dark-reduction of Cyt *b*-559 (Fd) and Cyt *b*-563 with antimycin A, but the observed inhibitory effect was only weak (see traces D). This finding, however, does not argue against the hypothesis that dark-reduction involves the same pathway as light driven cyclic electron flow, which is known to be antimycin sensitive. It has to be considered that

(1) only very few electrons are needed to reduce the cytochromes,

(2) the reduction is very slow, with $t_{1/2}$ in the order of 10–20 s, and

(3) antimycin A is a *competitive* inhibitor and does not totally block electron transport.

Hence, the same amount of antimycin A which inhibits light driven cytochrome reduction via cyclic flow may not be effective to prevent dark reduction via the same pathway.

Conclusions

In conclusion, the presented data point to the existence of a distinct fraction of Cyt *b*-559 in spinach chloroplasts which is closely associated with Cyt *b*-563 in the Cyt b_6/f complex as well as with the acceptor side of PSI. It can be reduced in an antimycin A-sensitive reaction by PSI-driven cyclic flow, but also in an antimycin A-insensitive reaction by PSII-driven linear electron flow, presumably via the low potential chain of the Cyt b_6/f complex. Its reduction by PSI requires the presence of ferredoxin. Ferredoxin is also required for reduction by NADPH in the dark.

In view of the presented data, in particular of the close kinetic connection between Cyt *b*-559 (Fd) and Cyt *b*-563 observed upon reduction in the light and by NADPH in the dark, we presently favor a localization of Cyt *b*-559 (Fd) close to Cyt *b*-563 HP at the outer surface of the thylakoid membrane, with access to ferredoxin at the acceptor side of PSI (see scheme in Fig. 7). With such a location, linking ferredoxin and Cyt *b*-563 HP, Cyt *b*-559 (Fd) could play a key role in cyclic PSI electron transport.

It is well known that for optimal cyclic electron transport in C3 plants careful redox poising of the PQ-pool is required, which may be achieved by partially suppressing PSII activity e.g. at moderate concentrations of DCMU. This property may be explained by our finding that Cyt *b*-559 (Fd) can be reduced by Cyt *b*-563 (Fig. 4, 5). Such reduction, which will be favored at a high reduction level of the PQ-pool, counteracts electron donation from ferredoxin to plastoquinon via cyt *b*-559 (Fd).

Mano et al. (1995) concluded from post-illumination chlorophyll fluorescence measurements that part of NADPH-driven PQ reduction proceeds via FQR and depends on Q-cycle activity. If Cyt *b*-559 (Fd) does function as FQR, as depicted in the scheme of Fig. 7, one may hypothesize that its reoxidation by the two-electron carrier PQ requires the concerted oxidation of Cyt *b*-563. This hypothesis would provide an explanation for the apparent light activation of the FQR as well as the effect of nigericin reported by Mano et al. (1995). With electron transfer from NADPH to PQ requiring Cyt *b*-563 reduction via the Q-cycle, the potential rate of this electron flow would decline with dark-oxidation of the PQ-pool, which is speeded up by nigericin. Verification of this hypothesis appears important for understanding the regulation of cyclic electron transport.

Our results support the earlier suggestion of Miyake et al. (1995) that Cyt *b*-559 (Fd) is identical to the ferredoxin-plastoquinone reductase (FQR) postulated by Crowther and Hind (1980) and Bendall (1982). As compared to maize mesophyll thylakoids, the content of Cyt *b*-559 (Fd) in spinach thylakoids is very small (Miyake et al. 1995). Its identification has been complicated by the fact that there exists another low potential form of Cyt *b*-559. We cannot rule out that Cyt *b*-559 (Fd) corresponds to the Cyt *b*-560 of Rich and Bendall (1980). Tae et al. (1993) put forward arguments "that the properties of the 560 nm α-band in the stromal membranes are not sufficiently different from that

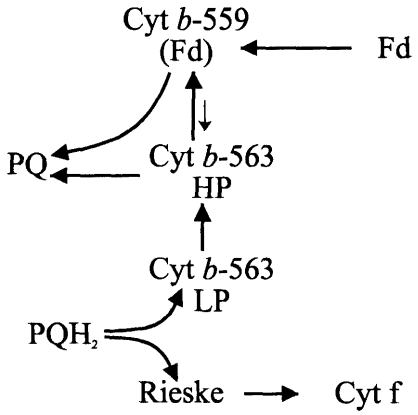

Fig. 7 Scheme depicting the postulated localization of Cyt *b*-559 (Fd) at the outer surface of the thylakoid membrane, connecting ferredoxin (Fd) at the PSI acceptor with the low potential chain of the Cyt b_6/f complex, as a key component of cyclic electron transport.

of PSII Cyt *b*-559 to warrant a conclusion that the stromal Cyt *b*-560 corresponds to a distinct cytochrome". On the basis of our data, we may state that with respect to its redox potential and physiological function the Cyt *b*-559 (Fd) is very different from Cyt *b*-559 HP in PSII reaction centers. However, it also has to be considered that the properties of PSII in stroma membranes and grana margins are largely different from PSII in the grana stacks (for a recent review on PSII heterogeneity see Lavergne and Briantais 1996). If Cyt *b*-559 (Fd) were associated with PSIIβ or non-Q_B PSII centers, this would mean that this type of PSII could play a so far unknown role in cyclic electron flow.

However, so far we do not have any evidence that Cyt *b*-559 (Fd) is identical with Cyt *b*-559 in PSII, i.e. corresponding to the gen products PsbE and PsbF. Lavergne (1983) discovered a so-called carrier G in *Chlorella sorokiana* which subsequently was identified as a high-spin cytochrome c′ (Joliot and Joliot 1988), being kinetically connected to the low potential chain of some but not all Cyt b_6/f complexes. If future work would show that Cyt *b*-559 (Fd) is not PsbE/F, its possible common origin with the carrier G certainly would be worth investigating.

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