A FLASH SPECTROSCOPIC STUDY OF THE KINETICS OF THE ELECTROCHROMIC SHIFT, PROTON RELEASE AND THE REDOX BEHAVIOUR OF CYTOCHROMES *f* AND *b*-563 DURING CYCLIC ELECTRON FLOW

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1. Introduction

Early studies of the redox behaviour of cytochrome *b*-563 in chloroplasts suggested that this cytochrome is involved in cyclic electron flow acting in a linear electron transport chain between ferredoxin and plastoquinone [1]. More recent studies have revealed that the cytochrome also undergoes redox changes under conditions favouring non-cyclic electron flow in such a way that the results may be interpreted as cytochrome *b*-563 participating in a protonmotive Q-cycle [2], (R. K. Chain and R. Malkin, unpublished) along the lines suggested in [3]. A Q-cycle mechanism would predict H^+:e^- ratios of 2 and 3 for cyclic and non-cyclic electron flow, respectively, despite the fact that the evidence for this ratio being 2 under non-cyclic conditions seems almost overwhelming [4–7]. Some reports suggest H^+:e^- ratios of >2 [8–10] but these cannot stand up to a rigorous criticism of the experimental approach used in the measurements [6], (A. B. Hope, L. F. Olsen and J. Barber, unpublished). Therefore at present the only evidence for a Q-cycle in chloroplasts remains with the redox behaviour of cytochrome *b*-563 [2], (R. K. Chain and R. Malkin, unpublished).

In order to investigate further the functional role of cytochrome *b*-563 in chloroplast electron transport we have studied the kinetics of the electrochromic shift, proton release and the redox changes of cytochromes *f* and *b*-563 following a 10 μs flash in DCMU poisoned chloroplasts in which cyclic electron flow has been induced by addition of NADPH plus ferredoxin [11–13]. The redox behaviour of cytochrome *b*-563 under these cyclic conditions is not in accordance with the view that the cytochrome acts as an electron acceptor for ferredoxin. The kinetics of *b*-563 reduction and oxidation could indicate that it was participating in a protonmotive Q-cycle. However we were unable to obtain evidence for a H^+:e^- ratio of 2 under these conditions as would be expected from a scheme involving a Q-cycle. We therefore propose that cytochrome *b*-563 functions as a plastosemiquinone dismutase under our conditions.

2. Materials and methods

Intact chloroplasts from 1–2 week old pea plants were prepared by the method in [14]. The final resuspension medium contained 1 mM KCl and 1 mM $MgCl_2$, adjusted to pH 7.6 with Tris base. The chloroplasts were osmotically shocked in distilled water at 0°C for 30 s followed by an equal addition of a double strength medium to obtain a final solution containing 0.33 M sorbitol, 50 mM Tricine/KOH (pH 8.3), 5 mM $MgCl_2$ and chloroplasts corresponding to 40–60 μM chlorophyll for measurements of the electrochromic shift and cytochrome redox changes. For measurements of the intrathylakoid proton release we used a medium containing 0.33 M sorbitol, 50 mM KCl, 5 mM $MgCl_2$ and 2 mg/ml serum albumin (pH 7.1)

Abbreviations: DBMIB, 2,5 dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide-*p*-trifluoro-methoxy-phenylhydrazone; P700, reaction centre chlorophyll of photosystem I; Tricine, *N*-[2 hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine

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and 40 μM neutral red. Further additions were 20 μM DCMU, 5 μM ferredoxin, 0.5 mM NADPH and finally 2.5–10 mM glucose, 0.1 mg/ml glucose oxidase and 0.2 mg/ml catalase to obtain anaerobic conditions. Valinomycin was present at 2 μM in the reaction mixture during measurements of the proton release and cytochrome redox changes in order to eliminate contributions due to the electrochromic shift.

The experiments were performed in a single beam flash apparatus constructed by Applied Photophysics Ltd. using a cuvette with an optical pathlength of 10 mm. The intensity of the measuring beam was ~0.2 W/m^2. The measuring light was guided to the photomultiplier using optical fibres that were placed right up to the surface of the cuvette in order to minimize loss of light due to scattering effects. Saturating light flashes were provided with a xenon flash tube and had a lifetime of 10 μs (width at half-height). The flash was filtered through a 2 mm Schott RG 665 filter and the photomultiplier was protected by a 4 mm Corning 4-96 filter. The signal from the photomultiplier was fed into a Datalab DL905 transient recorder or, when the signal-to-noise ratio was too low to allow 'single shot' experiments, into a signal averager (Nuclear measurements, model 546 C). The experiments were in general performed with an apparatus time constant of 0.3 ms.

The electrochromic shift was measured at 510 nm. Neutral red was measured at 540 nm and cytochrome *f* and *b*-563 at 554 minus 540 nm and 564 minus 575 nm, respectively, by averaging *n* number of sweeps at the measuring wavelength and subtracting the same number of sweeps at the reference wavelength. For cytochrome *f* *n* = 16 and for cytochrome *b*-563 *n* = 32. The temperature was 20°C ± 1°C in all experiments. Chlorophyll was measured according to [15]. DBMIB was the kind gift of Professor A. Trebst, Bochum. Ferredoxin from *Spirulina maxima* was generously supplied by Dr K. K. Rao, Kings College, London.

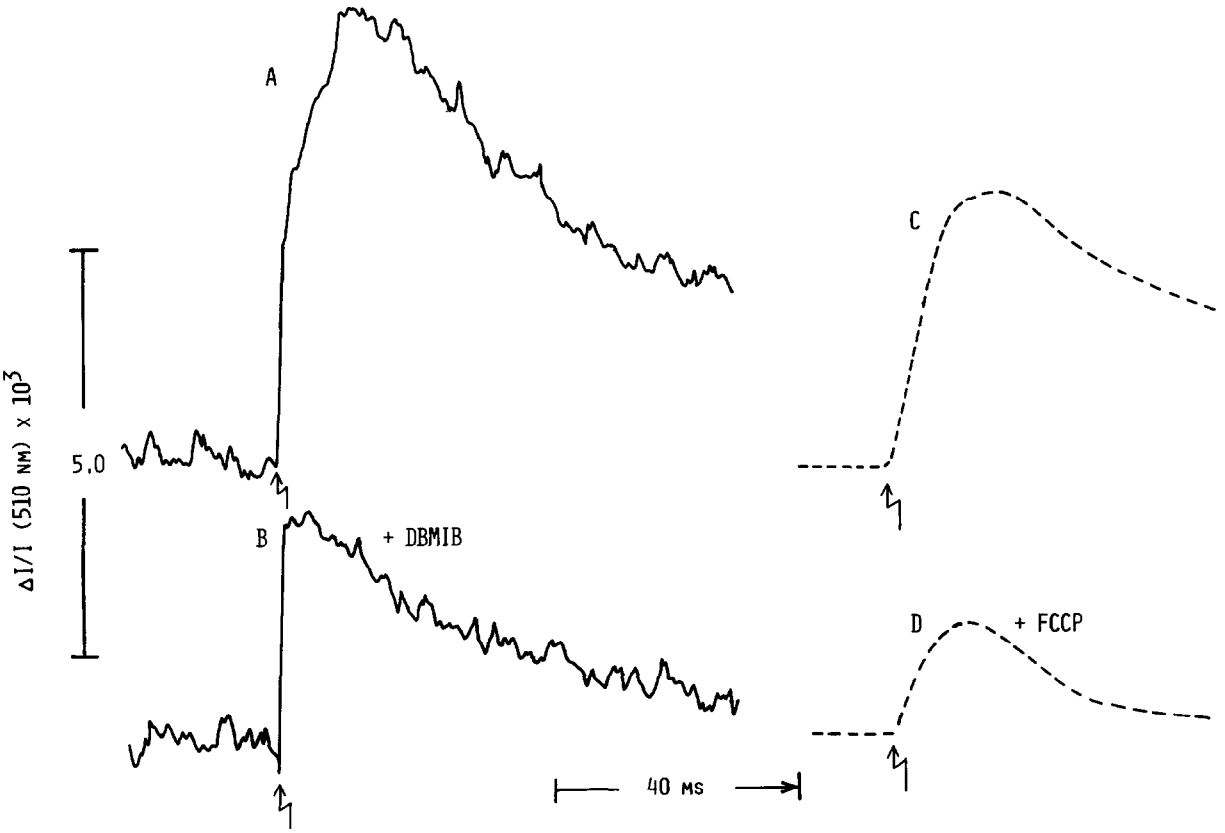

Fig.1. Kinetics of the electrochromic shift following a flash in DCMU-poisoned chloroplasts: (A) 5 μM ferredoxin and 0.5 mM NADPH were added to the reaction mixture; (B) as in A but with further addition of 20 μM DBMIB; (C) A minus B (by hand); (D) the equivalent signal to C obtained in the presence of 1 μM FCCP. Chlorophyll was 40 μM.

3. Results and discussion

The electrochromic shift at 510–520 nm is generally considered to be a 'molecular voltmeter', indicating the formation of an electric field across the thylakoid membrane as a consequence of the photochemical charge separation taking place on illuminating the chloroplasts [16]. Under cyclic conditions (see fig.1) the electrochromic shift induced by a single turnover flash consisted of a fast rise followed by a 20 ms slow rise. A similar slow rise has been observed in intact chloroplasts in which cyclic electron flow was induced by partial inhibition by DCMU [17–19]. The slow component was removed by addition of DBMIB and the ratio of this DBMIB sensitive part to the DBMIB resistant fast component was ~1:1. No signal due to the electrochromic shift was observed in the absence of NADPH. Addition of FCCP to the reaction mixture reduced the size of the slow DBMIB-sensitive component and accelerated the rate of decay. These experiments suggest that the slow part of the electrochromic shift could be due to the release of protons during the oxidation of plastoquinol. A similar conclusion has been reached for the slow rise observed under non-cyclic conditions [9], (A. B. Hope and A. Morland, unpublished). The amplitude of the slow rise, as compared to the fast part, would suggest that the electrogenic contribution from the oxidation of plastoquinol involves the release of $>1\ H^+/e^-$ transferred to P700 [9]. However, the existence of a protonmotive Q-cycle of this type has not been favoured by some workers (A. B. Hope

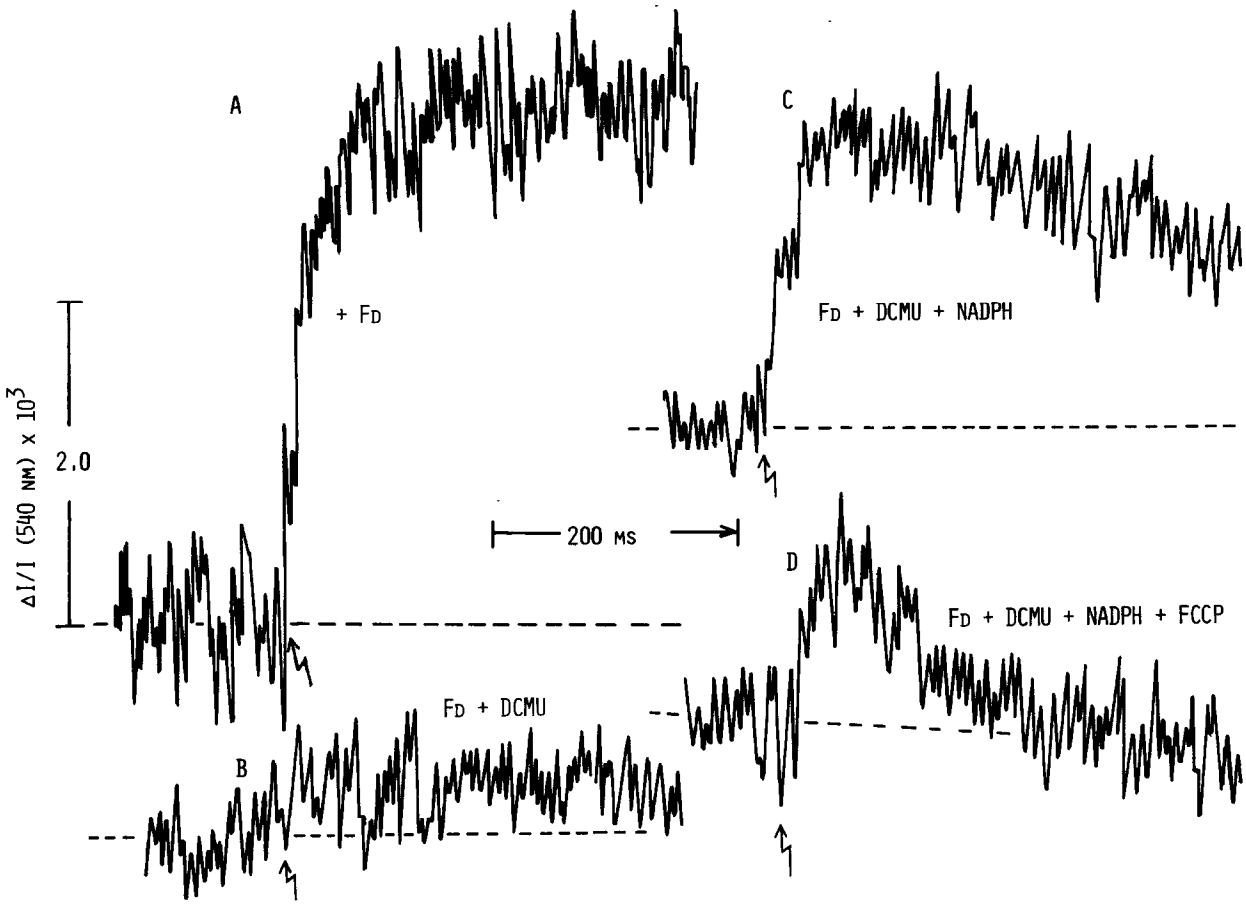

Fig.2. Kinetics of the flash-induced proton release under non-cyclic and cyclic conditions measured at 540 nm using neutral red: (A) with only 5 μM ferredoxin added to the reaction mixture; (B) as in A but plus 20 μM DCMU; (C) as in B but plus 0.5 mM NADPH; (D) as in C but plus 1 μM FCCP. The signals in A–D were obtained in the presence of 2 μM valinomycin. Chlorophyll was 40 μM.

and A. Morland, unpublished) since the H^+:e^- ratio measured using pH indicating dyes only allows for the release of 1 H^+/e^-taken from plastoquinol in chloroplasts in which they also observed a slow rise of the electrochromic shift following a laser flash. To see whether such a Q-cycle was incorporated into cyclic electron flow we have investigated the proton release under cyclic conditions using neutral red and with the external medium buffered by serum albumin. Fig.2 shows the flash-induced ΔA_{540} due to neutral red under different conditions. In the case when ferredoxin was an electron acceptor (non-cyclic) the signal consisted of a fast rise followed by a slow rise with a half-time of 20 ms. The fast component was resistant to DBMIB and is therefore identified as being due to the oxidation of water by photosystem II. The slow rise on the other hand was abolished by addition of DBMIB and is hence identified as the proton release due to oxidation of plastoquinol [5]. The ratio of the $\Delta H^+_{slow}/\Delta H^+_{fast}$ was ~1. The flash-induced ΔA_{540} in the presence of neutral red disappeared following addition of the permeable buffer imidazol suggesting that the entire signal in fig.2A is due to proton release. Addition of DCMU to the reaction mixture also resulted in a decrease in the neutral red response, the signal being almost non-existant after a few flashes (fig.2B). This shows that with ferredoxin only there is no contribution from cyclic electron flow. However, addition of NADPH to the DCMU inhibited chloroplasts resulted in a restoration of the ability to release protons as shown in fig.2C. The proton release under these conditions was completely inhibited by addition of 5 μM DBMIB suggesting that it is due to oxidation of plastoquinol. The extent of proton release under these cyclic conditions was ~50% of the release under non-cyclic conditions (fig.2A) indicating that

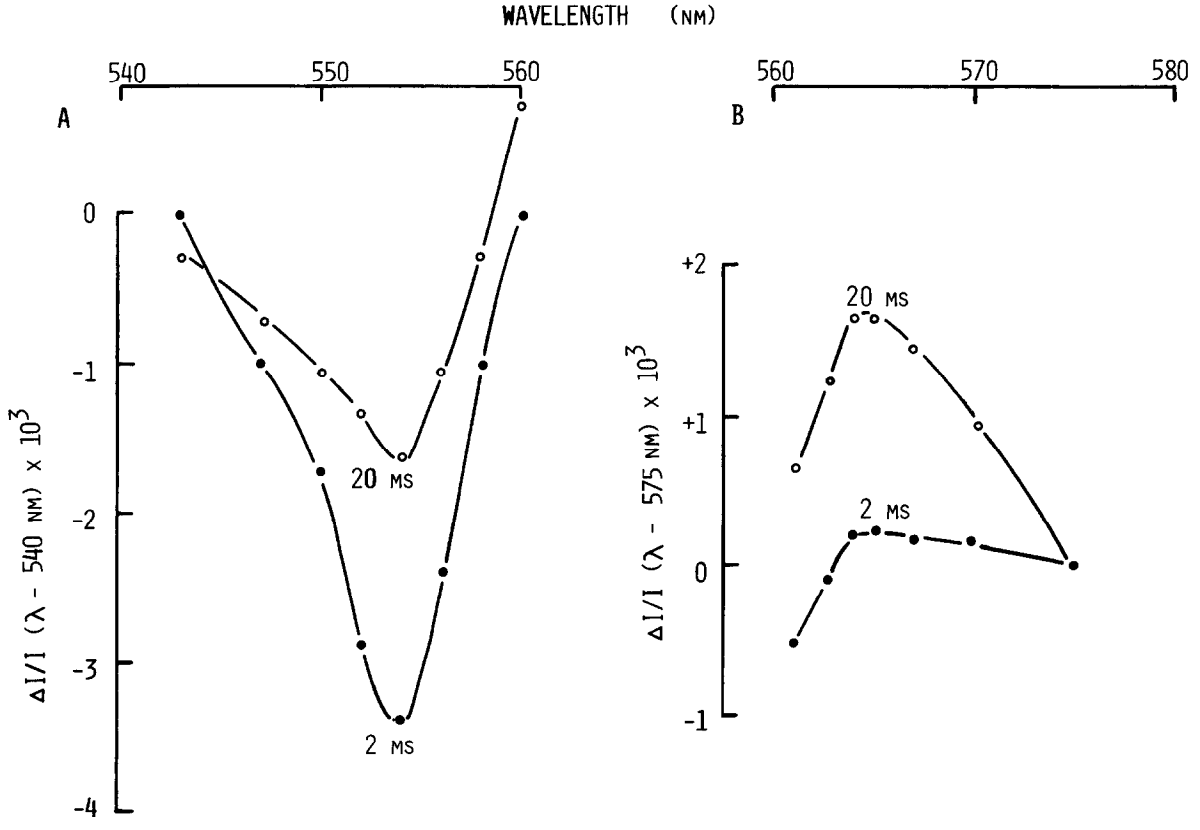

Fig.3. Spectra of the flash-induced absorbance changes in the cytochrome α-band region in DCMU-poisoned chloroplasts supplemented with ferredoxin plus NADPH. The spectra are recorded at 2 ms and 20 ms after the flash with the change at 200 ms subtracted: (A) spectra obtained with 540 nm as the reference wavelength; (B) spectra obtained with 575 nm as the reference wavelength. Valinomycin was present at 2 μM during all measurements. Chlorophyll was 60 μM.

the number of protons released by plastoquinol oxidation under cyclic and non-cyclic conditions are the same. Using the data from fig.2A and assuming that only 1 H^+ is released/e^- taken from water, we conclude that plastoquinol oxidation is associated with the release of only 1 H^+/e^- transferred to P700. The effect of FCCP on the proton release (fig.2D) is very similar to its effect on the slow part of the electrochromic shift (fig.1D). This observation further strengthens the conclusion that the slow rise of the electrochromic shift is associated with the release of protons from plastoquinol oxidation. However, unlike the interpretation made in [9] we conclude with others (A. B. Hope and A. Morland, unpublished) that the slow rise cannot be taken as evidence for release of 2 H^+/e^- transferred to P700.

We have also studied the redox behaviour of cytochromes *b*-563 and *f* following a flash under cyclic conditions. Fig.3 shows the spectra obtained in the cytochrome α-band region at 2 ms and 20 ms after the flash was given. At 2 ms cytochrome *f* was oxidised to an extent of 1 molecule/900 chlorophylls based on $\Delta\epsilon_{554-540} = 2.2 \times 10^4\ M^{-1} . cm^{-1}$ [20]. Such an extent of photooxidation is higher than found under non-cyclic conditions following a single turnover flash [21,22]. We did not observe any contribution from cytochrome *b*-563 2 ms after the flash (fig.3B). However, at 20 ms a portion corresponding

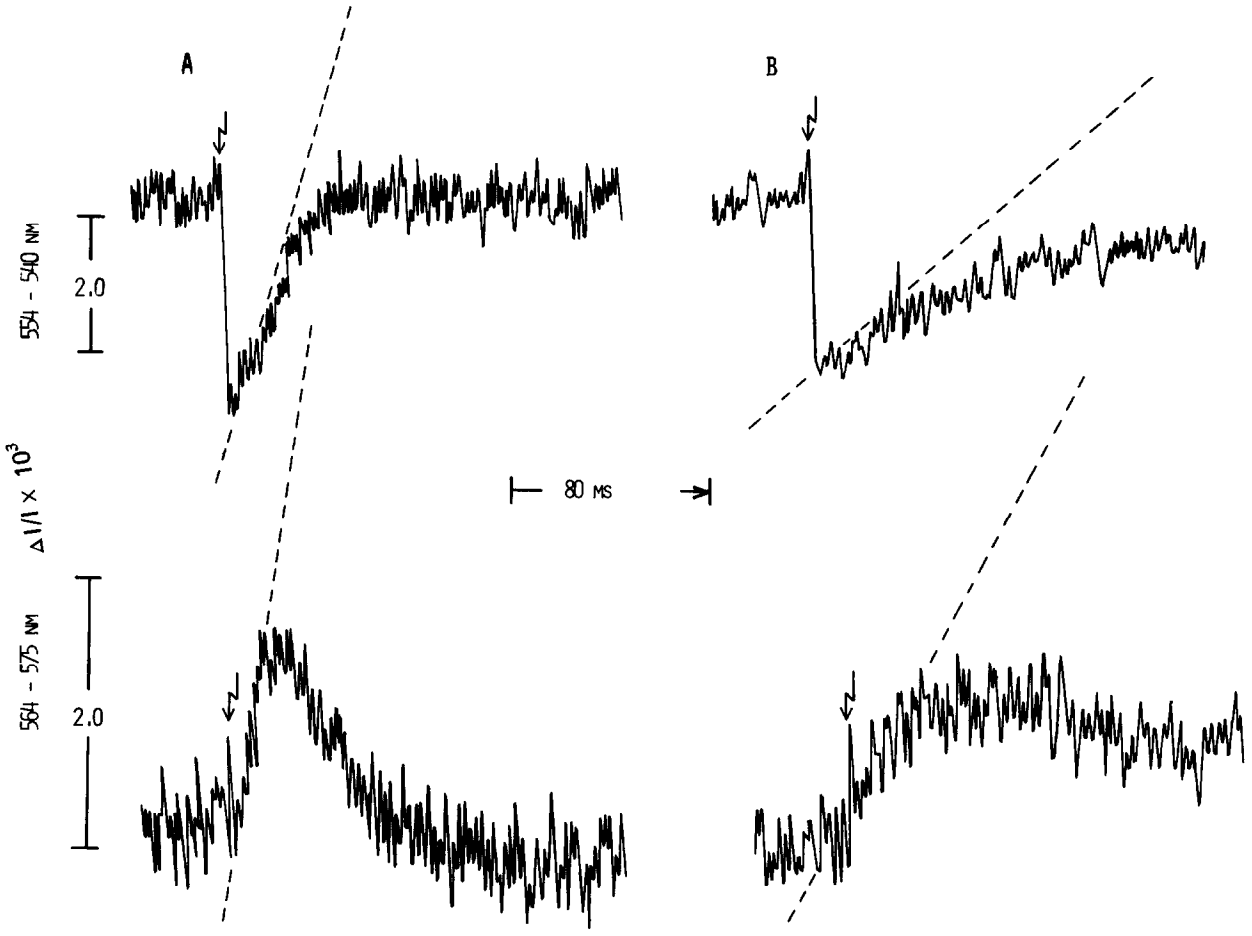

Fig.4. Flash-induced changes due to cytochromes *f* and *b*-563 in DCMU-poisoned chloroplasts supplemented with ferredoxin plus NADPH: (A) conditions as in fig.3; (B) as in A but with further addition of 100 μM bathophenanthroline. The changes due to cytochrome *f* are the averaged of 16 expt. whereas in the case of cytochrome *b*-563, 32 experiments were averaged. Flash frequency 0.25 Hz. Valinomycin was added to 2 μM throughout. The dashed lines are drawn to indicate the initial rates of reduction. Chlorophyll was 60 μM.

to 1 molecule/2000 chlorophylls had become reduced when using $\Delta\epsilon_{564-575} = 2 \times 10^4\ M^{-1} . cm^{-1}$ [23]. The reduction of *b*-563 was accompanied by reduction of cytochrome *f*. The flash-induced changes at 554–540 nm and 564–575 nm are shown in fig.4A. From the slopes indicated in the figure we can estimate that the initial rate of reduction of *b*-563 is equal to the corresponding rate of reduction of *f*. These rates were 0.044 and 0.046 equiv./(s . mol chlorophyll). Addition of bathophenanthroline, which has been found to inhibit electron transfer to cytochrome *f* and P700 under non-cyclic conditions [22,24], resulted in a decrease of the rate of reduction of cytochrome *f* as shown in fig.4B. Interestingly the rate of reduction of cytochrome *b*-563 was also inhibited by bathophenanthroline and to the same extent as the reduction of cytochrome *f* as measured from the slopes indicated in the figure. These results suggest that the reduction of cytochrome *b*-563 is linked to the reduction of cytochrome *f* in accordance with the conclusion reached by others (R. K. Chain and R. Malkin; A. Telfer and J. Barber, unpublished) using continuous light. The results do not agree with those in [17,18] using intact chloroplasts which were partially inhibited by DCMU. However, partial inhibition means that some non-cyclic electron flow is still going on, making a distinction between cyclic and non-cyclic electron flow very difficult.

4. Conclusion

The observed kinetic behaviour of cytochromes *b*-563 and *f* under cyclic conditions seems to be inconsistent with the linear arrangement:

Ferredoxin → *b*-563 → Plastoquinone → *f*

and tends to favour a scheme in which cytochrome *b*-563 participates in a protonmotive Q-cycle, an example of which is depicted in fig.5A. However, since we were unable to obtain evidence for the release of >1 H^+/e^- transferred from plastoquinol to P700 we must seek other explanations for the behaviour of these cytochromes. Extracting the following reactions from the scheme in fig.5A:

$$PQH^{\cdot} + b\text{-}563^{ox} \rightarrow PQ + b\text{-}563^{red} + H^+ \quad (1)$$

$$H^+ + PQH^{\cdot} + b\text{-}563^{red} \rightarrow PQH_2 + b\text{-}563^{ox} \quad (2)$$

and adding them together we obtain the net reaction:

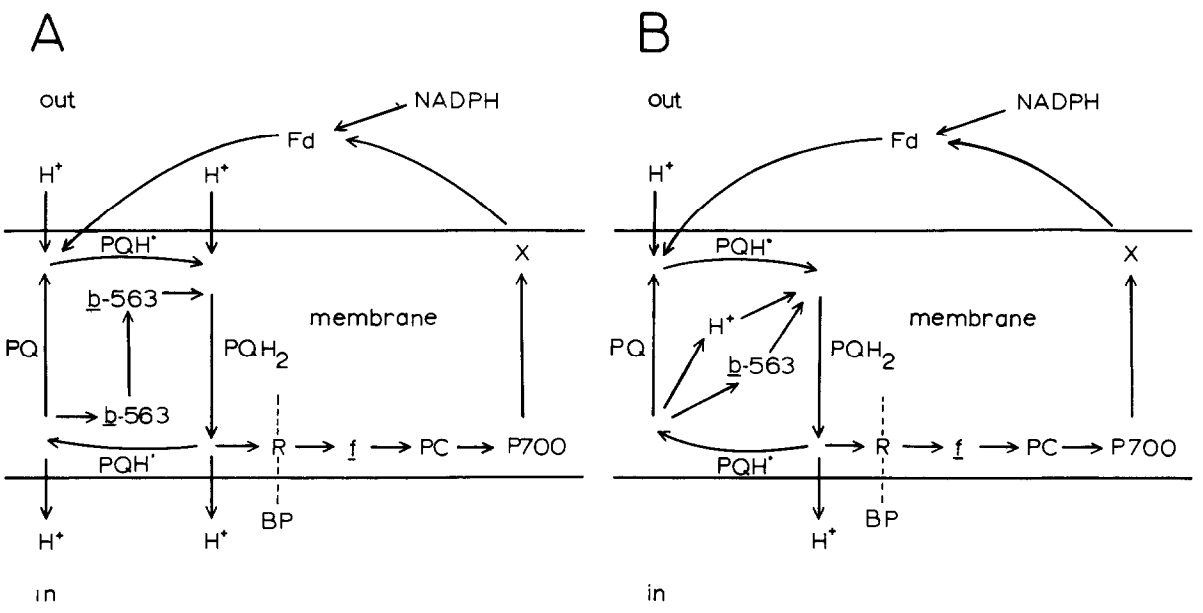

Fig.5. (A) An example of a protonmotive Q-cycle involving cytochrome *b*-563. The figure is redrawn from a model in [3]; (B) an alternative scheme in which the reactions involving cytochrome *b*-563 occur in a non-vectorial way. PQ, plastoquinone; Fd, ferredoxin; R, Rieske iron–sulphur centre; PC, plastocyanin; BP, bathophenanthroline.

$$2\ PQH^{\cdot} \rightarrow PQH_2 + PQ \qquad (3)$$

which is simply the dismutation of the plastosemiquinone radical. The reactions (1) and (2) are analogous to the catalytic mechanism proposed for the enzyme superoxide dismutase [25] which catalyses the reaction:

$$2\ H^+ + 2\ O_2^{\cdot -} \rightarrow H_2O_2 + O_2 \qquad (4)$$

We therefore propose that cytochrome *b*-563 acts as a plastosemiquinone dismutase through reactions (1) and (2) under our conditions (see fig.5B) and is not involved in the vectorial transport of protons.

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