Biochimica et Biophysica Acta 932 (1988) 33–42
Elsevier

BBA 42699

A critical examination of the supposed variable proton stoichiometry of the chloroplast cytochrome *bf* complex

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(Received 27 July 1987)

Key words: Cytochrome *bf* complex; Proton pumping; Electrogenic reaction; Electron transfer; (Pea chloroplast)

Measurements of the proton/electron stoichiometry of the chloroplast cytochrome *bf* complex are critically examined. The *bf* complex may cause a charge movement across the membrane for each quinol oxidised under a wide variety of conditions, including those of high d.c. light intensity and in the presence of a flash-generated membrane potential. The electrogenic reaction associated with turnover of the complex is the same size regardless of whether endogenous plastoquinol or added duroquinol is the reductant. Turnover of the cytochrome *bf* complex is a two-step process. The first half of the cycle is less electrogenic than the second half. There is no convincing evidence to date that the cytochrome *bf* complex can turn over without an associated electrogenic component under physiological conditions.

Introduction

Many early investigations of the steady-state proton/electron stoichiometry during chloroplast electron transport revealed that one proton was translocated into the intra-thylakoid space for each electron which was transferred from Photosystem II to Photosystem I (reviewed in Refs. 1 and 2). Direct measurements of protons liberated internally after flash activation tended to corroborate this view (3,4). A ratio of 1 was consistent with the role of plastoquinone as a transmembrane hydrogen carrier and with the cytochrome *bf* complex acting only as a plastoquinol-plastocyanin oxidoreductase. Furthermore, measurements of the membrane potential-indicating carotenoid bandshift (P-518) did not originally reveal any electrogenic component which correlated with cytochrome *bf* turnover [4].

Several subsequent developments promoted a reconsideration of this view that the cytochrome *bf* complex functioned only as an oxidoreductase. The first of these was the observation by Joliot and Delosme [5] of a slow phase of the P-518 in intact algal cells, and they considered the possibility that it might represent an additional charge separation across the membrane, as in the analogous bacterial system [6]. This slow electrogenic reaction was later observed in isolated chloroplasts and could be correlated with turnover of the cytochrome *bf* complex [7–9], although other explanations of its origin were also suggested [10]. It also appeared that a slow electrogenic reaction might only be associated with turnover of the cytochrome *bf* complex under conditions which favoured cyclic electron transfer [11].

A second development was the formulation of

Abbreviations: o site, the site of quinol oxidation; r site, the site of quinone reduction (often termed the i site); b_L, the haem group of cytochrome *b* which is thought to be associated with centre o; b_H, the haem group of cytochrome *b* which is thought to be associated with centre r, DCMU, 3-(3-,4-dichlorophenyl)-1,1-dimethylurea.

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the Q-cycle model of turnover of the cytochrome bc_1 and *bf* complexes which predicted that their redox cycle involved a transmembrane electron transfer in addition to the oxidoreductase activity; this additional reaction would result ultimately in the transfer of an additional proton across the membrane [12]. At the same time, a number of reports appeared which indicated that the proton/electron stoichiometry for electron transfer between the photosystems could be 2 under conditions of low light intensity [13,14] or during repetitive flashes in the presence of valinomycin [15]. The general view which has since evolved is that the cytochrome *bf* complex has a facultative proton pump, probably operating only at low light intensity [13,14] and/or at low membrane potential [16,17].

In this report I examine the evidence that the proton-pumping reaction of the cytochrome *bf* complex is facultative, and suggest instead that the complete catalytic cycle may always be coupled to a transmembrane electron transfer and hence to the translocation of an additional proton under any of the conditions which it is likely to encounter physiologically.

Materials and Methods

Chloroplast preparation. Broken chloroplasts were prepared from 10–15-day-old pea seedlings (variety Meteor) which had been grown at 20–25°C for 10–15 days with 8 h per day of fluorescent tube and tungsten bulb light at around 10 W/m^2. Preparation and storage of chloroplasts were as previously described [18].

Steady-state H^+/e^- ratio. The ratio of protons appearing inside the thylakoid/electrons transferred in the presence of ferricyanide was estimated by the method of Fowler and Kok [13], except that external proton concentrations were monitored with the pH-sensitive dye phenol red [19], rather than with an electrode. The initial rate of proton efflux on switching off the actinic light was determined by computer-fitting of a single first-order decay to the data; initial rate was then (rate constant × extent). The steady-state electron-transfer rate was taken to be the same as the steady state net proton production rate in the d.c. light [13]. It was estimated by linear regression analysis of the data accumulated in the 8 s immediately prior to switching off the actinic beam.

Since the steady-state rate of proton accumulation inside the chloroplasts in the light should be equal to the initial proton efflux observed on switching off the actinic beam, the ratio of protons appearing inside/electrons transferred (H^+/e^-) is given by the ratio [13]:

$$\frac{\text{initial proton efflux rate on switching off actinic beam}}{\text{steady-state proton consumption rate}}$$

Electrochromic shift. The electrochromic shift of the carotenoids in response to membrane potential [4] was measured at 518 nm. The measuring beam shutter was generally opened 50 ms before flash activation. This avoided possible actinic effects of the measuring beam.

Internal proton release. Internal proton release was estimated from spectral changes of the membrane permeable indicator neutral red at 524 nm [3], and with bovine serum albumin added to prevent pH changes in the external medium. Data representing internal pH changes were taken to be the changes at 524 nm with 10 μM neutral red added minus changes at 524 nm in the absence of neutral red.

Cytochrome deconvolution. Under conditions where no membrane potential was generated, cytochrome changes were estimated by accumulation of data at 563, 554, 542 and 575 nm and subsequent deconvolution by matrix arithmetic of spectral changes due solely to cytochrome *b*-563, *f*, plastocyanin and P-700, as described previously [20]. When a carotenoid bandshift was also present, data were accumulated at the same four wavelengths, but the very rapid P-700 changes were ignored and a 4×4 matrix deconvolution was performed to provide optical changes from cytochrome *b*-563, *f*, plastocyanin and carotenoid bandshift [21].

Simulations. All simulations were iterative and accuracy was checked by decreasing the iteration increment by a factor of 2 until a constant result was obtained. Further details are presented in the appropriate figure legends.

Results and discussion

Steady-state H^+/e^- ratio

Ratios of protons appearing inside the

thylakoid/electrons transferred were measured as outlined in Materials and Methods. Typical results of the measurement of steady-state H^+/e^- ratios with ferricyanide as terminal acceptor are shown in Fig. 1. It appears as if the H^+/e^- ratio decreases from 3.06 ± 0.17 ($n = 10$) at low light intensity (2 W/m^2) to 1.99 ± 0.29 ($n = 11$) at high light intensity (60 W/m^2). The data both at low and at high light intensities are in good agreement with those of Fowler and Kok [13] and of Rathenow and Rumberg [14]. Only one report [22] has suggested that the higher stoichiometry occurred at high light intensity, although no original data were presented for detailed scrutiny and so cannot be considered further here. The interpretation of these data has generally been that a ratio of three represents one proton released by water oxidation, one translocated by redox cycling of plastoquinone and an additional proton translocated during turnover of the cytochrome *bf* complex. Decrease of the ratio to 2 has been taken to indicate loss of protonmotive activity of the cytochrome *bf* complex.

In the experiments considered here, the light intensity necessary (Fig. 1 and Refs. 13 and 14) to lower the H^+/e^- stoichiometry fully is in excess

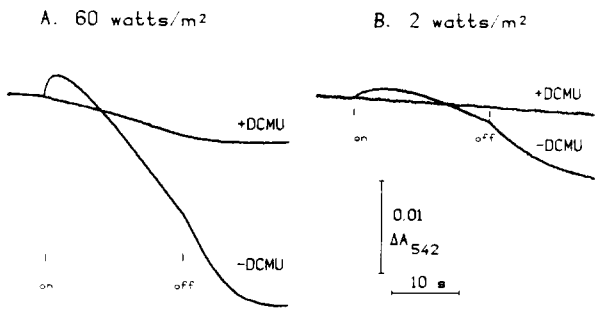


Fig. 1. Measurement of the steady-state H^+/e^- ratio in isolated chloroplasts. Broken chloroplasts were resuspended to a final chlorophyll concentration of 25 μg/ml in 0.4 M sucrose and 50 mM NaCl at a final pH of 7.3. 1 μg/ml valinomycin, 1 mM potassium ferricyanide and 25 μM phenol red were then added. The absorbance at 542 nm was monitored and actinic light was provided by a slide projector filtered with an infra-red and an RG635 long-pass filters. The intensity was adjusted with neutral density filters to either 60 W/m^2 (high light) or 2 W/m^2 (low light). Identical experiments with fresh chloroplast samples were performed with 10 μM DCMU present throughout. Data are unaveraged from a single experiment in each case.

of that needed to give maximal rates of electron transfer. For example, in the experiments of Fig. 1 the electron-transfer rate when measured independently with an oxygen electrode was saturated at around 10 W/m^2. At this light intensity, the H^+/e^- ratio was still close to 3. Only at higher intensities did the ratio appear to fall. Scrutiny of the data of Fowler and Kok (Fig. 6 of Ref. 13) reveals that this could also be the case in their experiments, if one assumes that the electron-transfer rate is actually reflected in the value of the initial dark efflux rate (their $R_{off}/3$ value). However, Fig. 1 also illustrates that high intensities promote a DCMU-insensitive proton production. This reaction causes an overestimate of the electron-transfer rate and hence an underestimate of the H^+/e^- ratio. By allowing for the DCMU-insensitive component, the calculated H^+/e^- ratio was unchanged at 3.06 ± 0.12 ($n = 3$) at 2 W/m^2, but rose to 2.81 ± 0.36 ($n = 5$) at the higher actinic intensity of 60 W/m^2. This explanation could also account for the Fowler and Kok data, since it is only their steady-state proton production rate, and not their dark efflux rate, which increases as the light intensity is increased to high values.

A number of controls were performed: (i) the DCMU-insensitive component required chloroplasts; (ii) essentially identical data were obtained with a pH electrode instead of phenol red; (iii) addition of carbonic anhydrase did not alter the H^+/e^- ratios, hence ruling out the possibility that a slow buffering by CO_2/bicarbonate equilibration might interfere to different extents with fast and slow proton changes; (iv) the ratio of internal/external buffering capacity in the phenol red experiments was less than 0.05 as judged by the size of pH overshoot with a pulse of acid or alkali.

The single turnover data of Fowler and Kok [13] had indicated that the extra proton was always translocated after a saturating flash. They also noted that an extra coupling site might be present on the acceptor side of Photosystem I which was utilised if methyl viologen, rather than ferricyanide, were the terminal acceptor. The data of Fig. 2 with ferricyanide as acceptor indicate that the proton uptake/flash is only 1.5 ± 0.1 times greater with methyl viologen as acceptor than with ferricyanide. Addition of superoxide dismutase did not increase the number of protons

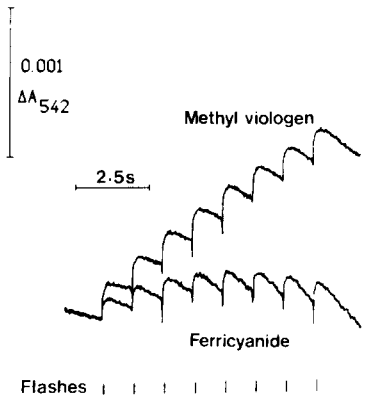


Fig. 2. Proton uptake during a train of flashes in isolated chloroplasts. Broken chloroplasts were resuspended to 25 μg/ml in 0.4 M sucrose, 50 mM NaCl, and 1 μg/ml valinomycin. The acceptor was either 1 mM potassium ferricyanide or 100 μM methyl viologen. The data represent the absorbance change at 542 nm of a sample containing 25 μM phenol red minus the absorbance change at 542 nm of a control sample with no phenol red. Activation was by a train of eight near-saturating red flashes at 1 Hz. Data are the average of 32 cycles with a time constant of 20 ms, and 5 s dark adaptation was allowed between each flash train.

associated with reduction of methyl viologen, indicating that these chloroplasts contain sufficient endogenous superoxide dismutase for full conversion of superoxide into hydrogen peroxide on these time scales (Polle and Junge [23] have shown that this is not always so). The ratio of 1.5 is the expected result if the only additional effect with methyl viologen is that an extra scalar proton is ultimately consumed by oxygen reduction during methyl-viologen reoxidation.

Measurement of the data of Figs. 3 and 4 of Fowler and Kok [13] also indicate no extra proton-translocating site with methyl viologen, i.e., the number of protons consumed/flash with methyl viologen was only 1.5-times the number consumed/flash when ferricyanide was acceptor. However, in the text of their article they note that the averaged results of many experiments indicated a doubling of proton uptake/flash with methyl viologen as acceptor, and so deduced the presence of an extra coupling site. Clearly, some variable factor was present with methyl viologen

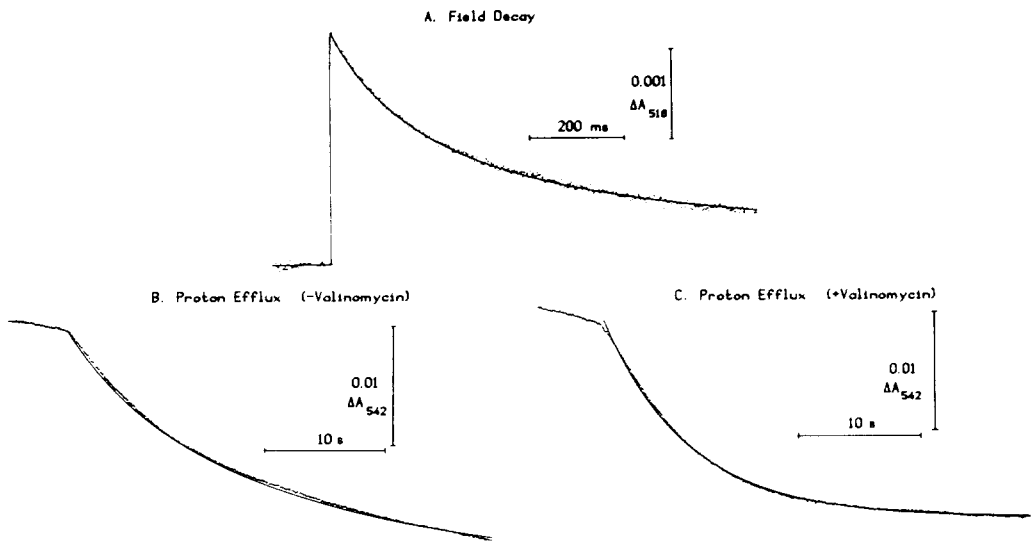


Fig. 3. Simulation of decay of the electric field and pH gradient in isolated chloroplasts. (A) Broken chloroplasts were resuspended to a final chlorophyll concentration of 75 μM in 0.4 M sucrose, 40 mM KCl, 0.5 mM EDTA and 10 mM potassium phosphate at pH 7.25. 4 mM ammonium chloride was then added. In order to avoid a large slow rise of the P-518, the sample was preilluminated with 200 ms of far-red light, followed by a dark adaptation of 4 s. Absorbance changes following a near-saturating red flash were monitored at 518 nm. Data are the average of eight sweeps, recorded with a time constant of 5 ms. The simulation assumes a total signal of 0.00255 ΔA with a second-order decay constant of 1600 $A^{-1} \cdot s^{-1}$. The small slow rise of the P-518 under these conditions (c.f. Fig. 7A(ii)) occurred in the first 10–20 ms of this trace and does not significantly affect the analysis. (B) Chloroplasts were resuspended to a final chlorphyll concentration of 25 μg/ml in 0.4 M sucrose and 50 mM NaCl at pH 7.3. 25 μM phenol red and 100 μM methyl viologen were then added. The sample was then illuminated for 20 s with d.c. light filtered with infra-red and RG635 filters. The decay of the proton gradient produced by this illumination was monitored when the actinic beam was switched off as changes in the protonation state of phenol red at 542 nm. The simulation assumes at total signal of 0.0255 ΔA and a second-order decay constant of 2.6 $A^{-1} \cdot s^{-1}$. (C) As for (B), except that 1 μg/ml of valinomycin was also present. The simulation assumes a total signal of 0.0172 ΔA and a first-order decay constant of 0.143 s^{-1}.

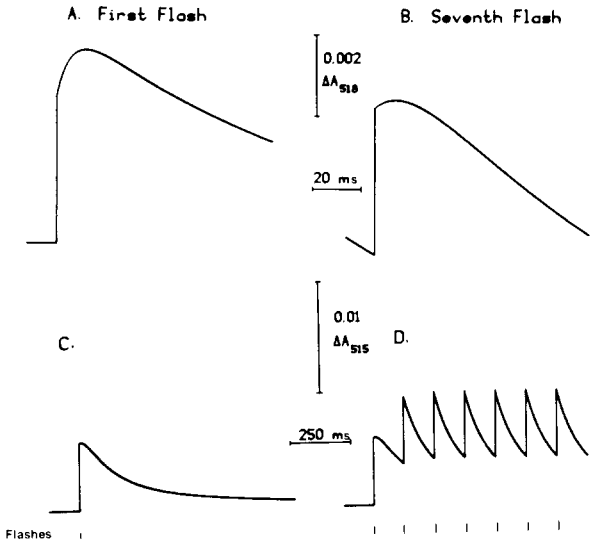


Fig. 4. Simulated behaviour of the electrochromic shift after single and multiple flash sequences. In (A) and (B), simulation conditions were as follows: ratio of slow phase/fast phase = 0.52; one charge separation produces a change of 0.0016 *A*; decay is first order with a rate constant of 9.2 s^{-1}. In (A), the slow rise is first order with $k = 160\ s^{-1}$. In (B), the slow rise is first order with $k = 64\ s^{-1}$ and six preflashes have been given at 100 ms intervals. The simulations are intended to represent conditions close to those of Fig. 7 of Ref. 16. Although the simulated preflash slope in Fig. 4B is absent in Ref. 16, it must inevitably have also been present in the original data and omitted for clarity of presentation. In (C) and (D), simulation conditions were: ratio of slow phase/fast phase = 0.5; one charge separation produces a change of 0.003 *A*; decay is second order with a rate constant of 1650 $A^{-1} \cdot s^{-1}$. The slow rise is first order with $k = 23\ s^{-1}$. In (D), flashes are given every 125 ms. Simulations are intended to represent a situation close to that in Ref. 17. In all cases, the simulations assumes a full slow rise after every flash.

as acceptor, perhaps some cyclic electron transport via superoxide anion or reduced viologen, or perhaps there were problems associated with calibration, as discussed previously by Saphon and Crofts [24]. It seems most likely that this uncontrolled variable was responsible for the apparent appearance of an extra coupling site when methyl viologen was present.

The effect of membrane potential on the slow phase of the carotenoid bandshift

It has been claimed that the slow phase of the carotenoid bandshift is lost after several successive rapid flashes. The suggestion has been made that the membrane potential produced in the first flash(es) causes the *bf* complex to function non-electrogenically [16,17,25,26].

I have attempted to examine such data quantitatively. It should firstly be noted that in isolated chloroplasts under some conditions the decay of the P-518 can often empirically be close to second order in nature (Fig. 3A). This is also true for the decay of the proton gradient in the absence of valinomycin (Fig. 3B). Addition of valinomycin promotes a more first-order decay of protons (Fig. 3C). The possible theoretical basis of such kinetics will not be examined here. In any case, as pointed out by Schmid and Junge [27], detailed analysis is hampered by the fact that the chloroplast population will be somewhat heterogeneous; examination of Fig. 3 shows that the fit to a single process is imperfect, probably due to such heterogeneity. Nevertheless, awareness of such second-order behaviour of the decay of the P-518 is relevant to analyses of whether a slow rise of the P-518 is still present after several rapid flashes. By assuming first-order decay, the rate of decay after several flashes (and hence the amount of slow phase of the P-518 rise) can be seriously underestimated if the decay is actually closer to second order.

Fig. 4 presents an iterative simulation of the expected behaviour of the P-518 change during multiple flashes. In Fig. 4A and B, the simulation assumes: (i) a slow rise of the P-518 after every flash and (ii) a first-order decay. The figure shows simulated behaviour after the first flash (Fig. 4A) and after a flash in a steady-state condition where flashes are given at a frequency of 10 Hz (Fig. 4B). The slow rise of the P-518 has apparently been severely diminished in the multiple-flash situation. The simulations are for conditions identical to those of Bouges-Bocquet (Fig. 7 in Ref. 16) and match the data quite closely. These data of Bouges-Bocquet have been widely quoted as evidence of the loss of the slow electrogenic reaction; the simulations show that this is not the case and that a full slow phase is occurring at both flash frequencies. A first-order decay has been used in the simulation, since the experiments were with intact cells, and these appear to exhibit a first-order decay. A second-order decay would hide even further a slow rise which was occurring during the rapid flashing. In Fig. 4C and D, the simulations

assume: (i) a slow rise of the P-518 after every flash and (ii) a second-order decay. In Fig. 4C, simulated behaviour for a single flash is shown; in Fig. 4D is simulated behaviour for a train of flashes at 125 ms intervals. It can be seen that the slow rise of the P-518, which is still present, has apparently disappeared after one flash. These simulations are almost identical to the data of Van Kooten et al. [17], who assumed that the slow rise of the P-518 had actually disappeared after the first flash. The recent data of Hope and Matthews [26] are similar.

Internal proton release under oxidising conditions

The experiments of Ausländer and Junge [3] and of Witt [4] indicated that an equal number of protons wer released by the oxygen-evolving complex and by plastoquinol oxidation after a single turnover flash of chloroplasts which had initially an oxidised quinone pool. They concluded that quinol oxidation by the cytochrome *bf* complex did not result in translocation of an extra proton across the membrane. The same results have not been obtained by all workers, however. For example, Velthuys [28] was able to detect an additional proton release inside associated with the oxidation of plastoquinol. Data from Hope and Matthews [29] showed that there were two phases of slow proton release of approximately equal magnitude; only the faster of these correlated with turnover of the cytochrome *bf* complex and because of this they concluded that the *bf* complex did not translocate an extra proton under their conditions of measurement.

Some consideration is necessary of the events expected after a single turnover flash when the quinone pool is initially oxidised. Assuming the Photosystems II to be randomised (i.e., equal amounts of Q_B and Q_B^-), then one quinol will be produced for every two active Photosystems II. These quinols will be oxidised by the cytochrome *bf* complexes to produce two protons per quinol inside the thylakoid. Statistically, most of the cytochrome *bf* complexes will have been hit by only a single quinol and so will have ended up in the state $(b_L b_H^-)$. Normally, this state requires a further turnover of the complex in order to regenerate plastoquinol in the pool. The halftime of cytochrome *b*-563 reoxidation in the state $(b_L b_H)^-$

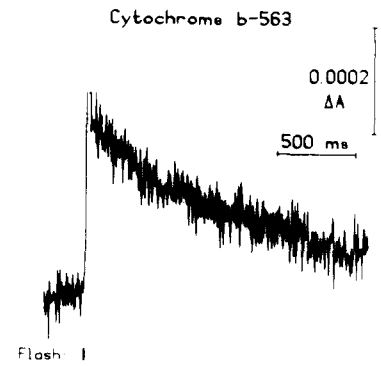


Fig. 5. Decay of the $(b_L b_H)^-$ state of the cytochrome *bf* complex when the plastoquinone pool is oxidised. Chloroplasts were resuspended to 75 μg/ml in 0.4 M sucrose, 40 mM KCl, 0.5 mM EDTA and 10 mM potassium phosphate buffer at pH 7.25. 1 mM potassium ferricyanide and 0.3 μM nonactin were also added. Before each flash the quinone pool was oxidised with 1 s far red actinic d.c. light at 60 W/m^2 followed by 10 s dark adaptation. A saturating white flash was used. Ten recordings were made at 563, 554, 575 and 542 nm and cytochrome *b*-563 changes were then deconvoluted as described in Materials and Methods.

when the quinone pool is oxidised and in the absence of another catalytic turnover is around 500 ms (Fig. 5). Overall then, flash activation of turnover commencing with an oxidised plastoquinone pool would be predicted to produce a biphasic slow release of protons associated with plastoquinol oxidation. The magnitude of the faster phase will be equal to the number of protons liberated by the oxygen-evolving enzyme; regeneration of plastoquinol by half-reduced cytochromes *b*-563 and release of further protons would normally be rather slower. It may be noted that even the data of Ausländer and Junge [3] and of Witt [4] show signs of such a slow phase of extra proton release.

It is concluded that none of the data on internal proton release after a flash (under conditions where the quinone pool is initially oxidised) demonstrate convincingly that the cytochrome *bf* complex can turn over without transmembrane movement of the additional proton.

Internal proton release under reducing conditions

Under reducing conditions with duroquinol as substrate, it has been demonstrated that a flash

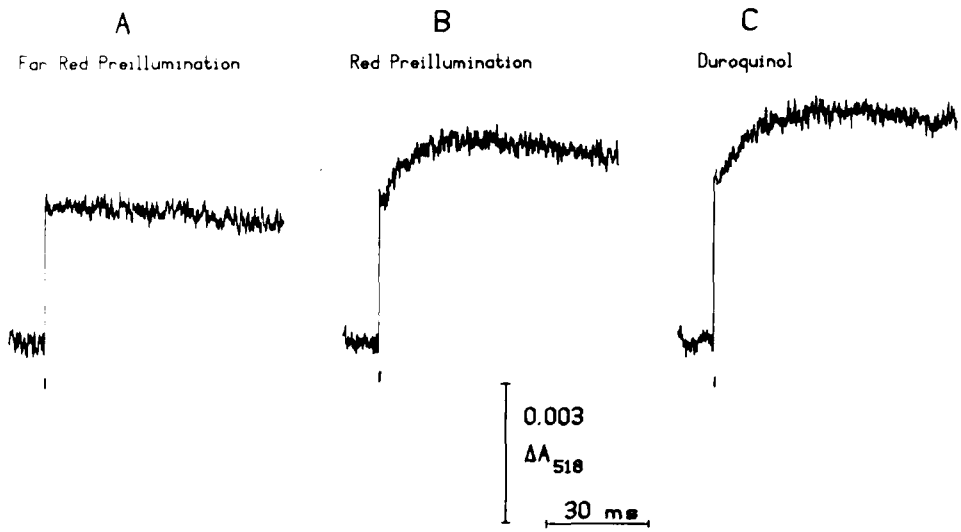
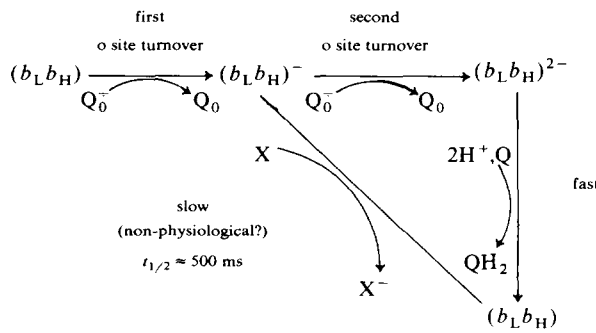


Fig. 6. P-518 responses in intact chloroplasts. Chloroplasts were resuspended to 75 μg/ml in 0.4 M sucrose, 40 mM KCl, 0.5 mM EDTA and 10 mM potassium phosphate buffer at pH 7.25. 4 mM ammonium chloride was also added. Other conditions were. (A), 1 s far-red actinic d.c. light at 60 W/m^2 followed by 10 s dark adaptation before each flash; (B), 1 s red actinic d.c. light at 60 W/m^2 followed by 10 s dark adaptation before each flash; (C), 100 μM duroquinol. Each trace is the average of eight recordings at 518 nm.

causes two protons to be released internally, and one to be taken up externally per electron passing from quinol to Photosystem I acceptors [30]. Experiments of the type shown in Fig. 7D showed that, in the absence of duroquinol, the same number of protons were released internally when endogenous plastoquinol was generated via Photosystem II by d.c. preillumination. In both cases, proton release correlates kinetically with rereduction of Photosystem-I acceptors. A slow phase corresponding to one additional charge separation accompanies this proton release (Fig. 6). In order to demonstrate the slow electrogenic reaction with endogenous plastoquinol as substrate, it was necessary to ensure that the internal pH had not fallen too low during the preillumination period. A low internal pH inhibits plastoquinol oxidation by the cytochrome *bf* complex and causes the slow rise of the P-518 to be hidden by the decay kinetics. By controlling this factor, however, no condition could be found in which a slow electrogenic reaction did not accompany turnover of the cytochrome *bf* complex and the slow P-518 was the same size regardless of whether the reductant was endogenous plastoquinol or externally added duroquinol (Fig. 6).

Electrogenicity of the first half of the bf-complex reaction cycle

By flash generating plastoquinol by Photosystem II, after far-red preillumination to oxidise the quinone pool, a significant fraction of the cytochrome *bf* population will oxidise only one plastoquinol and so only complete one half of the reaction cycle:



The resulting half-reduced cytochrome *b*-563 reoxidises only slowly ($t_{1/2} \approx 0.5$ s) in the absence of another catalytic turnover when the plastoquinone pool is oxidised. The experimental data on internal proton generation, the cytochrome *b*-563 reduction, and associated P-518 changes for such a situation are illustrated in Fig. 7. It should

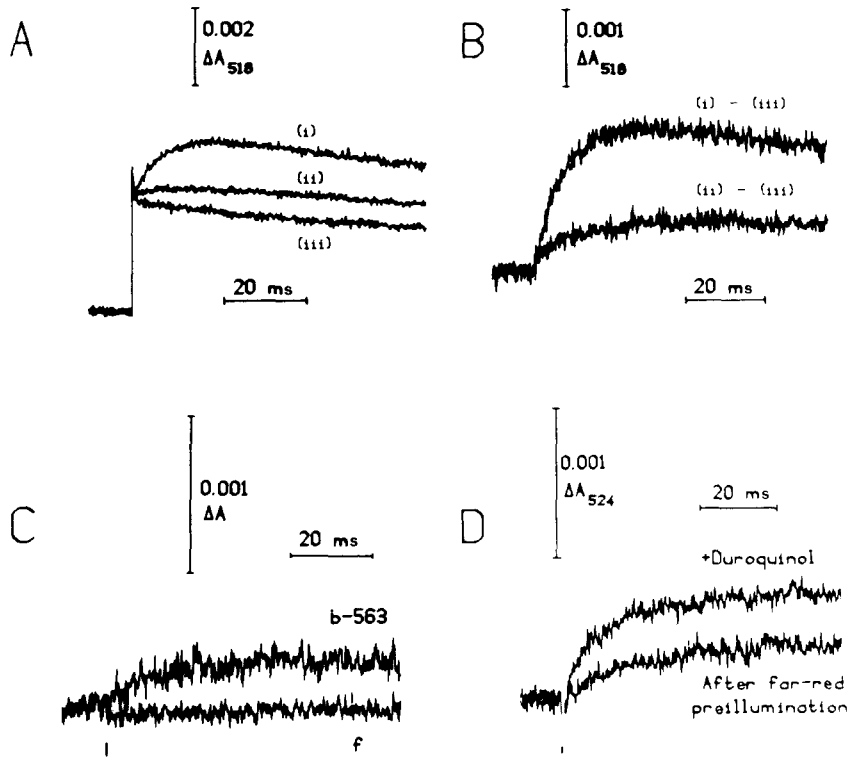


Fig. 7. Cytochrome, electrochromic and pH_{in} changes in broken chloroplasts. Broken chloroplasts were resuspended to 75 μg/ml in the same buffer as Fig. 5, but also containing 4 mM ammonium chloride to prevent a low internal pH and 1 mM methylbenzoquinol to ensure that donors to Photosystem I became fully rereduced between flashes. This latter precaution ensured that the size of the fast P-518 was equal for all traces. Data were obtained as described in methods. Amplifier time constant was 1 ms throughout. In (A), conditions were: (i) 0.5 mM duroquinol; (ii) 1 s far-red actinic d.c. light at 60 W/m^2 followed by 10 s dark adaptation before each flash; (iii) 0.5 mM duroquinol was 1 μg/ml stigmatellin. Data are the average of 20 recordings with 10 s dark adaptation between each flash. Traces in (B) are the slow-phase components of these same data obtained by computer subtraction. Data in (C) represent cytochrome changes after a flash. 1 s far-red actinic d.c. light at 60 W/m^2 followed by 10 s dark adaptation was given before each flash. Data in (D) are the internal proton chnges as monitored by neutral-red changes. Broken chloroplasts were resuspended to 75 μg/ml in 0.4 M sucrose, 50 mM NCl and 1 mg/ml bovine serum albumin at pH 8.3. Conditions were either (i) 0.5 mM duroquinol or (ii) 200 ms far-red actinic d.c. light at 60 W/m^2 followed by 4 s dark adaptation before each flash. Data are the average of 20 recordings with 4 s dark adaptation between each flash. By using red instead of far-red light, data identical to that in the presence of duroquinol were obtained (results not shown).

be noted that before the flash, after dark adaptation, cytochrome *b*-563 was essentially fully oxidised even in the presence of duroquinol, a situation consistent with our own estimates of the E_m values of the *b*-563 haems [31]. The sample contained 80 nM active Photosystem II and 80 nM cytochrome *bf* complex. Statistical calculation as in Ref. 21 indicated that, after flash generation of 40 nM plastoquinol (from the 80 nM Photosystem II present, since an equal amount are in the Q_B and the Q_B^- states before the flash), one would expect (ignoring those complexes which oxidise more than two plastoquinols) the *bf* complex population to turn over as follows:

number of quinols oxidised	amount of *bf* complex
0	49 nM
1	24 nM
2 or more	7 nm

The amount of cytochrome *b*-563 which is observed to be stably reduced is close to this predicted value of 24 nM (Fig. 7C). No cytochrome *f* turnover is seen, since it will be rapidly reoxidised by the $P\text{-}700^+$. The number of protons released rapidly inside (Fig. 7D) is close to half the number released after a flash in the presence of duroquinol

(although in our experiments we were unable to observe any very rapid proton release from Photosystem II for reasons which are as yet unclear). This again is a reflection of the fact that only 0.5 plastoquinol/Photosystem II are generated by flash activation of Photosystem II, and hence only around 0.5 quinols/*bf* complex are subsequently reoxidised by the *bf* complex, whereas flash activation in the presence of duroquinol causes the oxidation of around one quinol/*bf* complex. The electrogenicity associated with oxidation of the Photosystem II-generated plastoquinol is around 40% of that associated with the slow phase in the presence of duroquinol (Fig. 7A and B), i.e., 0.4×80 nM = 32 nM charges translocated during the slow rise. Assuming that the 7 nM *bf* complex, which turns over twice or more, translocates two charges/complex means that the 24 nM complex which turns over only to the half-reduced state is responsible for 18 nM equivalents of charge separation. Since virtually all of the stranded electrons are observed on the cytochrome *b*-563, the distribution of one electron between b_H and Q_r (in this state where the iron sulphur centre and cytochrome *f* are oxidised) must be much in favour of its being on cytochrome *b*-563. One can conclude that electron transfer from Q_0^- to b_H crosses approx. 18/24 or 0.75 of the membrane dielectric. This is similar to previous estimates for the cytochrome *bf* [32] and bacterial bc_1 [33] systems. Additionally, it is unlikely that a proton is taken up onto b_H^- from the r side, since this would then comprise a full charge separation across the membrane. This would be consistent with the reported minor pH-dependency of the midpoint potential(s) of cytochrome *b*-563 [31].

The above is meant only as a qualitative demonstration that an electron travelling from centre *o* to cytochrome b_H probably does not produce a complete charge separation across the membrane, in agreement with previous more quantitative estimates [32,33]. Most importantly in the present context, this fact should be considered together with the observation that only 0.5 plastoquinol per Photosystem II will be formed in chloroplasts when the dark adaptation between flashes is less than a few minutes. These considerations lead to the conclusion that the observation of only small slow phases of P-518 in experiments such as that of Fig. 7A(ii) is entirely consistent with electrogenic functioning of the cytochrome *bf* complex.

Conclusions

The predominant view of the functioning of the cytochrome *bf* complex is that it has a facultative electrogenic (and proton-pumping) reaction, and that this facility does not normally operate under conditions of high light or of even moderate membrane potential. Although the experiments and considerations of the present manuscript do not address all aspects of experimental data on the topic (for example, the data on proton consumption in the absence of valinomycin [15,25]), I have attempted to reopen the question and to suggest that there are no data which convincingly demonstrate that the cytochrome *bf* complex can operate without its electrogenic component under any of the conditions which it might encounter physiologically.

Previously, several models had been put forward which suggest how the cytochrome *bf* complex might turn over without an associated charge separation. All of these postulate that the semiquinone produced at centre *o* can have a fate alternative to that of reduction of haem b_L. These fates could be:

$$Q_0^- + FeS \rightarrow Q_0 + FeS^- \quad [34]$$
$$Q_0^- + b_L^- + 2H^+ \rightarrow QH_2 + b_L \quad [35\text{–}37]$$
$$Q_0^- + H^+ \rightarrow QH_0^{\cdot} \rightarrow QH_r^{\cdot} \quad [21]$$

Only the first two of these could cause a loss of the extra proton translocation during multiple turnovers. The third of these processes probably only occurs under the artificial condition of very low redox potential when cytochrome b_L is reduced, and after one turnover would allow the complex to operate with a normal reaction cycle [21]. If it is indeed true that the cytochrome *bf* complex always translocates a proton under physiological conditions, then it follows that the first two reactions do not occur at any physiological relevant rate, as is the case for the analogous mammalian and bacterial bc_1 complexes. In addition, it would also become necessary to reevaluate the accepted values of the operative ATP/$2e^-$, the ATP/H^+ and the NADPH/ATP

ratios which determine the energetic balance of carbon fixation.

Acknowledgements

The work was funded by the Venture Research Unit of British Petroleum plc and the SERC. It benefitted from the able technical assistance of S.A. Madgwick and from useful discussions with Drs. Bendall, Hope, Junge, Mitchell, Moss, Ort, West and Whitmarsh.

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