Biochimica et Biophysica Acta 894 (1987) 189–197
Elsevier

BBA 42647

The effect of pre-reduction of cytochrome *b*-563 on the electron-transfer reactions of the cytochrome *bf* complex in higher plant chloroplasts

David A. Moss * and Peter R. Rich

Glynn Research Institute, Bodmin, Cornwall (U.K.)

(Received 4 February 1987)
(Revised manuscript received 30 July 1987)

Key words: Cytochrome *bf* complex; Cytochrome bc_1 complex; Cytochrome *b*-563; Electron transport; Q-cycle; (Pea chloroplast)

An investigation has been made of the effects of pre-reduction of cytochrome *b*-563 on electron transfers through the cytochrome *bf* complex. It has been found that in a system in which anthraquinone-2-sulphonate or anthraquinone-2,6-disulphonate is used as redox buffer, a lipid-soluble mediator must also be present to allow sufficiently rapid equilibration of cytochrome *b*-563 with the ambient potential. We have found that 1 μM benzyl viologen gives full equilibration of cytochrome *b*-563 in less than 30 s, while minimizing the side reactions that have been observed with alternative mediators. Pre-reduction of cytochrome *b*-563 did not prevent turnover of site o (quinol-oxidising site of the cytochrome *bc* complex), even with fast repetitive flash activation. The site o reaction was accompanied by rapid, 2-nonyl-4-hydroxyquinoline *N*-oxide-sensitive oxidation of cytochrome *b*, and by a slow carotenoid bandshift. These results are discussed in conjunction with related results from the cytochrome bc_1 complex; Q-cycle models are considered in which the semiquinone at site o either can reduce an oxidant other than cytochrome *b*-563, or can migrate to site r (quinone-reducing site of the cytochrome *bc* complex). Of these possibilities, only the migration of the neutral semiquinone, QH, to site r is compatible with all of the data from the cytochrome *bf* and bc_1 complexes. Such a scheme would not be compatible with the semiquinone cycle proposed by Wikström and Krab ((1986) J. Bioenerg. Biomembr. 18, 181–193).

Introduction

Electron transport from quinol through the cytochrome *bc* complex in photosynthetic and respiratory electron transport chains exhibits several features that are difficult to account for in terms of a linear sequence of the components involved. Amongst these are the phenomenon of oxidant-induced reduction of cytochrome *b* [1], the presence of two quinone-binding sites (denoted o and r) (reviewed in Ref. 2), and a H^+/e^- stoichiometry of 2 (reviewed in Ref. 3).

* Present address: Institut für Biophysik der Universität Freiburg, Freiburg, F.R.G.

Abbreviations: AQ, 9,10-anthraquinone; AQS, 9,10-anthraquinone-2-sulphonate; AQSS, 9,10-anthraquinone-2,6-disulphonate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HNQ, 2-hydroxy-1,4-naphthoquinone; NQNO, 2-nonyl-4-hydroxyquinoline *N*-oxide; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)ethyl)glycine; site o, quinol-oxidizing site of the cytochrome *bc* complex; site r, quinone-reducing site of the cytochrome *bc* complex; Chl, chlorophyll; PS, Photosystem.

Correspondence: P.R. Rich, Glynn Research Institute, Bodmin, Cornwall, PL30 4AU, U.K.

These observations are the basis of the Q-cycle model of cytochrome *b-c* complex function [4]. The central features of the model are that the reduction of cytochrome *b* occurs during the consecutive oxidation of QH_2 at site o of the cytochrome *b-c* complex, and that cytochrome *b* is subsequently reoxidized by a quinone species at site r, the electron-transport pathway $Q_0^- \rightarrow b \rightarrow Q_r$ being transmembrane.

It is clear that this model predicts the electrogenic reaction to be dependent on the availability of oxidized cytochrome *b*. If the oxidation of cytochrome *b* is blocked by the inhibitor antimycin, it is found after several turnovers of the *b-c* complex that the electrogenic reaction and the reduction of the Rieske Fe-S centre have been inhibited [5]; this, in terms of the Q-cycle, represents the failure of reduced cytochrome *b* to remove Q_0^-. The same effects would be expected if cytochrome *b* were instead to be chemically reduced prior to the experiment.

There have been several recent reports of experiments designed to test this prediction in chloroplasts, in which the slow phase of the electrochromic bandshift (slow ΔA_{518}) provides a convenient means of observation of the electrogenic reaction [6]. However, these studies differed as to the signals that could be observed under reducing conditions. In one case, a slow ΔA_{518} was observed, accompanied by rapid oxidation of cytochrome *b*-563 [7]; in a second, a slow ΔA_{518} with no rapid *b*-563 oxidation [8]; and in a third, no slow ΔA_{518} [9].

This latter discrepancy has been shown to result from the use of 2-hydroxy-1,4-naphthoquinone (NHQ) as a redox mediator [10]. In the presence of HNQ, attenuation of the slow ΔA_{518} occurs at low potential; but the dependence of the midpoint potential of the titration of this effect on the concentration of HNQ implies that the attenuation is due to a direct effect of the reduced form of HNQ [11]. On the other hand, equilibration of cytochrome *b*-563 with the ambient redox potential would be expected to be slow in the absence of a low potential hydrophobic mediator; this raises some serious doubts as to the true redox poise of cytochrome *b*-563 during some of these experiments, especially where signal averaging has been used without a long dark-adaptation period between flashes. This point is of particular importance since an autocatalytic mechanism could explain the oxidation of cytochrome *b* if the pre-reduction of the cytochrome was incomplete, as has been pointed out by Crofts [12].

In the present work we have re-investigated the effects of low ambient potential on electron transfers through the cytochrome *b-f* complex in isolated thylakoid membranes, paying particular attention to the question of redox mediation to cytochrome *b*-563.

Materials and Methods

Chloroplasts were isolated from 10–14-day-old pea seedlings as described by Moss and Bendall [13].

The reaction mixture for the experiments reported here was as follows: 330 mM glucose, 50 mM Tricine (pH 8.0, approx. 400 units catalase/ml (1 unit decomposes 1 μmol H_2O_2 per min at pH 7.0, 25 °C and 10.3 mM H_2O_2)), approx. 24 units glucose oxidase (1 unit oxidizes 1 μmol β-D-glucose per min at pH 5.1 and 35 °C, O_2-saturated reaction), 0.3 μM nonactin, 10 μM DCMU, 100 μM anthraquinone-2-sulphonate (AQS) or anthraquinone-2,6-disulphonate (AQSS), chloroplasts at 75 μg chlorophyll/ml. The space above the reaction mixture was continuously flushed with N_2, and the reaction mixture had been photochemically reduced to −275 mV (AQS) or −215 mV (AQSS) versus S.H.E. before the addition of the chloroplasts [13]. Other additions were as reported in the figure legends.

The methods for measurement of the kinetics of flash-induced absorbance changes, and for the deconvolution of individual components, were as described previously [14]. The flash intensity was over 90%-saturating at 75 μg Chl/ml, yielding approximately one oxidized P-700 per *b-f* complex (there being a slight excess of PS I over *b-f* complex). One essential feature of the method was that between flashes the photomultiplier was illuminated with an LED balanced to the same intensity as the measuring beam (0.15 $mW \cdot m^{-2}$, unless otherwise stated). This allowed us to delay opening the measuring beam shutter until at most 100 ms before the flash was triggered; without this

precaution the effect of the measuring beam was to keep cytochrome *b*-563 partially oxidized.

Results

Chemical reduction of cytochrome b-563

As stated above, we have paid particular attention to the question of re-reduction of cytochrome *b*-563 between flashes. AQS is a very poor mediator to cytochrome *b*-563, even at the relatively high concentration used here [15,16]; to achieve complete re-equilibration between flashes in a multiple flash experiment, it is necessary to add a lipophilic mediator. However, it has been shown that mediators such as HNQ and AQ inhibit the slow ΔA_{518} at low ambient potential [11]. We have identified the mechanism of this inhibition; Fig. 1 shows that either mediator catalyzes a rapid reduction of cytochrome *f*, which bypasses the site of action of stigmatellin. In the absence of stigmatellin, this process would compete with plastoquinol for oxidizing equivalents arising from flash activation of PS I. Since both of these compounds catalyze high rates of DBMIB-sensitive cyclic phosphorylation [17,18], we must conclude that the movement of the reduced mediator across the membrane is slow, and that the bypass that we observe in a flash experiment depends on the accumulation of a pool of reduced mediator inside the thylakoid during the dark incubation between flashes.

Fig. 2 shows that with longer dark incubation times between flashes, a bypass was observed even when only 100 μM AQS was present. However, if AQS was replaced by AQSS, no bypass was observed under the same conditions. We conclude from these observations that AQS itself can slowly penetrate the thylakoid membrane, but that this does not occur with the more highly charged disulphonate. With either redox buffer the addition of benzyl viologen, in contrast with AQ and HNQ, did not accelerate the rate of stigmatellin-insensitive reduction of cytochrome *f*; but this mediator did catalyze the equilibration with the ambient potential of at least 98% of the dithionite-reducible cytochrome *b*, as judged by difference spectra in the α-band region (not shown).

The important issue, however, is that the rate of dark re-reduction of cytochrome *b*-563 by the mediator is sufficiently high for the cytochrome to

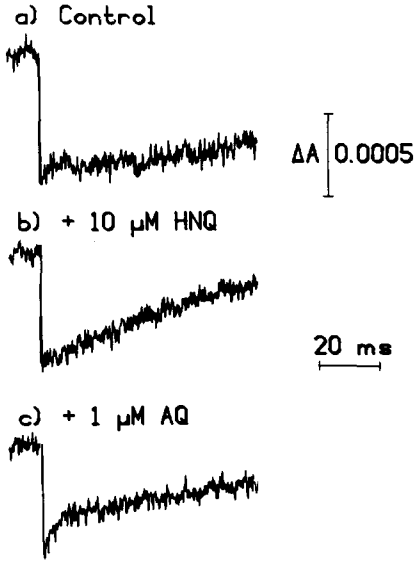


Fig. 1. Effect of lipophilic mediators on flash-induced redox changes of cytochrome *f*. The reaction mixture was as described in Materials and Methods, with AQS as redox buffer and AQ or HNQ added as shown. Cytochrome *f* redox changes were measured as described in Materials and Methods. The data are the average of four flashes, each preceded by 5 s dark incubation. 1 μM stigmatellin was present throughout.

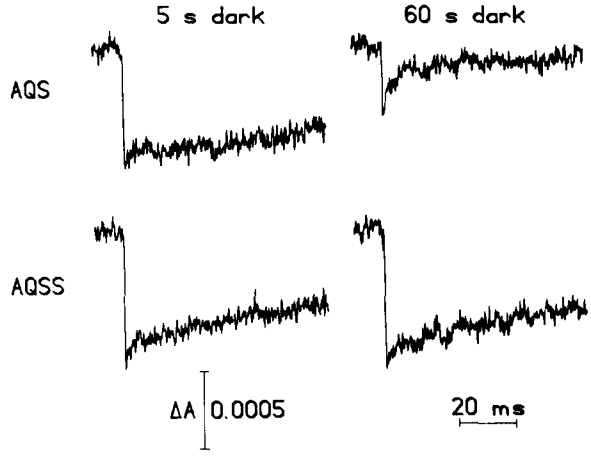


Fig. 2. Effect of dark incubation time on the amplitude of photoxidation of cytochrome *f*. The reaction mixture was as described in Materials and Methods, with AQS or AQSS as redox buffer as shown. 1 μM benzyl viologen was added to the AQSS system. The data are the average of four flashes, each preceded by the dark incubation period shown. 1 μM stigmatellin was present throughout.

re-equilibrate with the ambient potential between flashes in experiments involving signal averaging. The re-reduction of cytochrome *b*-563 following flash oxidation may be monitored continuously in the presence of 1 μM benzyl viologen, given a sufficiently weak measuring beam (0.01 $\mu W \cdot m^{-2}$). In the experiment shown in Fig. 3a cytochrome *b*-563 has been oxidized by ten flashes at a frequency of 21 Hz. The kinetics of the events in the first few seconds after the flashes were complicated by the high time constant used (0.5 s); but after 2.36 s the spectrum of the signal was that of cytochrome *b*-563 (Fig. 3c), and the subsequent recovery showed single exponential kinetics with a half-time of around 5 s. When the experiment was repeated in the presence of the site r inhibitor NQNO [19], the kinetics became more complicated; however, the re-reduction was essentially complete in 30 s (Fig. 3b). This value was taken as sufficient for dark re-equilibration, particularly since any slight actinic effect of the measuring beam would result in this being an overestimate of the time required for re-equilibration in the dark.

Electron transfer reactions with pre-reduced b-563

The Q-cycle in its classical form predicts that pre-reduction of cytochrome *b*-563 will impair the removal of Q^- from site o, resulting in an inhibition of the reaction $QH_2 \rightarrow f$ on subsequent turnovers. The result shown in Fig. 4 contradicts this prediction; in repetitive flashes spaced 45 ms apart, a rapid cytochrome *f* re-reduction occurred after each flash. One interpretation of this result may be that alternate flashes result in the oxidation of QH_2 or Q^- at site o; but such a model would not account for the slow ΔA_{518} that we have observed under similar conditions (see below). We conclude from these experiments that pre-reduction of cytochrome *b*-563 does not prevent the occurrence of an electrogenic reaction that involves the removal of Q^- from site o. However, it will be noted that the re-reduction of cytochrome *f* was significantly slower on the first flash than on subsequent flashes (see also Fig. 9), from which we infer that the removal of Q_0^- was rate-limiting when cytochrome *b*-563 was pre-reduced.

A related prediction of the classical Q-cycle is that when cytochrome *b*-563 is fully reduced, the absence of a $Q_0^- \rightarrow b$ reaction will mean that there is no production of free Q to oxidize cytochrome *b*-563 via site r; thus, no oxidation of cytochrome *b*-563 will be observed. Once again, our observations contradict the theoretical expectations. Fig. 5 shows that in a sample with pre-reduced cytochrome *b*-563, the re-reduction of cytochrome *f* following a flash was accompanied by rapid oxidation of approximately one cytochrome *b*-563

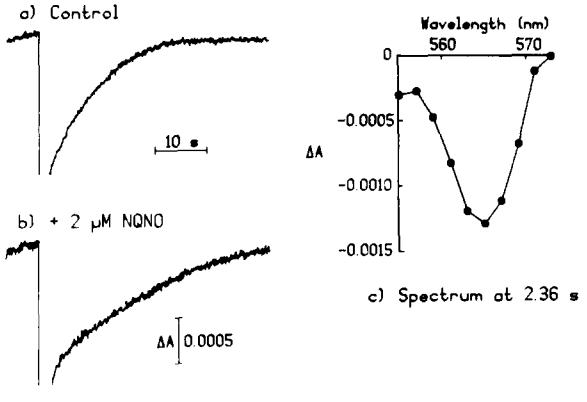


Fig. 3. Photo-oxidation and re-reduction of cytochrome *b*-563 in the presence of 1 μM benzyl viologen. Since the contributions of other components relax sufficiently rapidly so as not to be visible on this time scale, cytochrome *b*-563 was measured at 563 nm only. (a) Reaction mixture as described in Materials and Methods, with AQS as redox buffer and benzyl viologen as mediator. (b) as (a), plus 2 μM NQNO. (c) Spectrum of the signal shown in (a), 2.36 s after photo-oxidation.

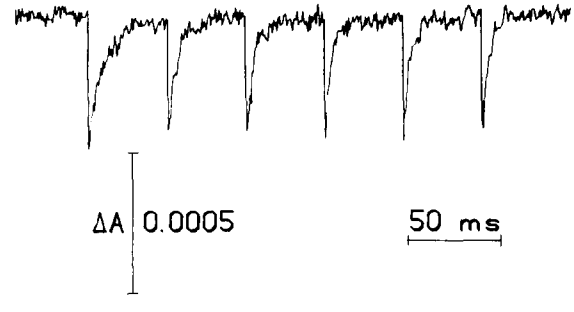


Fig. 4. Photo-oxidation and re-reduction of cytochrome *f* in repetitive flashes at 21 Hz, with pre-reduced cytochrome *b*-563. The reaction mixture was as described in Materials and Methods, with AQSS as redox buffer and 1 μM benzyl viologen as mediator; data are the average of eight flash trains, each preceded by 60 s dark incubation to allow re-reduction of cytochrome *b*-563.

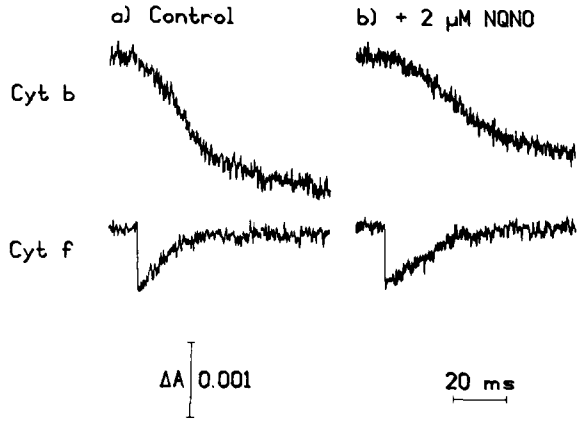


Fig. 5. Flash-induced redox changes of cytochromes *b*-563 and *f* with prereduced cytochrome *b*-563. The reaction mixture was as for Fig. 4, with NQNO added as shown. Data are the average of four flashes, each preceded by 60 s dark incubation.

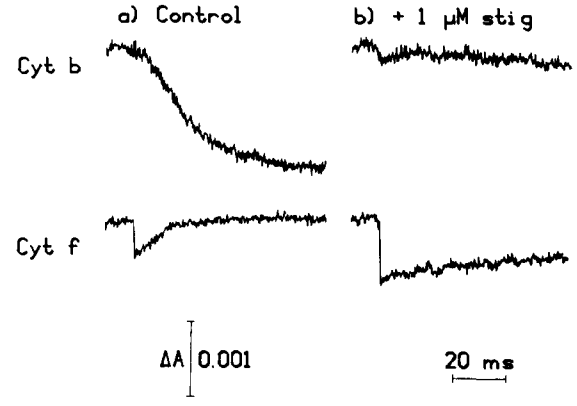


Fig. 6. The effect of stigmatellin on flash-induced redox changes of cytochromes *b*-563 and *f* with pre-reduced cytochrome *b*-563. Procedure was as described in the legend to Fig. 5, but with eight averages.

per oxidizing equivalent, in good agreement with the data of Joliot and Joliot [7], Again, in good agreement with these authors, we found that reducing the flash intensity to around 40% saturation (as judged by the amplitude of cytochrome *f* oxidation) resulted in a proportionate decrease in the amplitude of cytochrome *b*-563 oxidation; the half-times of cytochrome *f* re-reduction and of cytochrome *b*-563 oxidation were unaffected (not shown).

The effect of 2 μM NQNO was to inhibit the rate of *b*-563 oxidation. However, the inference that *b*-563 oxidation was via site *r* is not straightforward, since Fig. 5 also shows a clear effect of NQNO on the kinetics of cytochrome *f* re-reduction, as found by Jones and Whitmarsh [19]. Because of the multiphasic kinetics of the data, it would not be meaningful to compare the effects of NQNO at the two sites by means of simple parameters such as $t_{1/2}$ or initial rate. Furthermore, we cannot determine whether the two effects were entirely independent, or whether the inhibition at site r was a secondary result of inhibition at site o, or vice versa. We must conclude that, in the absence of a tight-binding and specific site r inhibitor, we are not able to exclude the possibility that cytochrome *b*-563 oxidation was via site o; though, on the basis of current understanding of the *b*-*f* complex mechanism, we favour oxidation via site r. This point will be considered further in the Discussion. Fig. 6 shows that cytochrome *b* oxidation was completely inhibited by the site o inhibitor stigmatellin, while the amplitude of cytochrome *f* oxidation increased significantly. The latter effect was presumably due to an increase in the midpoint potential of the Rieske centre in the presence of stigmatellin, as reported by Von Jagow and Ohnishi [20].

Crofts [12] has suggested that the oxidant-induced oxidation of cytochrome *b*-563 observed in experiments of this sort may be attributable to an autocatalytic effect of a small proportion of oxidized complexes, rather than to a reaction of the fully reduced complex; he points out that such a mechanism would give rise to a lag in the kinetics of cytochrome *b* oxidation. A small lag is indeed apparent in the data of Fig. 5; if Crofts' explanation were correct, the lag would be expected to become more pronounced with more complete pre-reduction of *b*-563. The data presented in Fig. 7 result from an experiment identical to that of Fig. 5, but with AQS as redox buffer rather than AQSS, giving an ambient potential 60 mV lower. The amplitude of the signals was smaller in the presence of AQS, because of the bypass effect referred to above; but, in other respects, the result was the same as that obtained at the higher potential, except perhaps for some lessening of the lag in the *b*-563 kinetics. We take this as showing that this oxidant-induced *b*-563 oxidation was indeed a reaction of the fully reduced complex, rather than resulting from an autocatalytic process

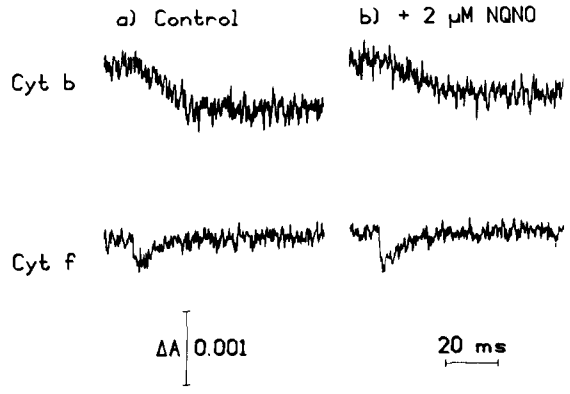


Fig. 7. Flash-induced redox changes of cytochromes *b*-563 and *f* with prereduced cytochrome *b*-563. Procedure was as described in the legend to Fig. 5, but with AQS as redox buffer. Data are the average of eight flashes, each preceded by 30 s dark incubation.

of quinone production by partially oxidized complexes.

Fig. 8 shows cytochrome *b*-563 oxidation in rapid repetitive flashes. With the second flash, the oxidation began to be preceded by a transient

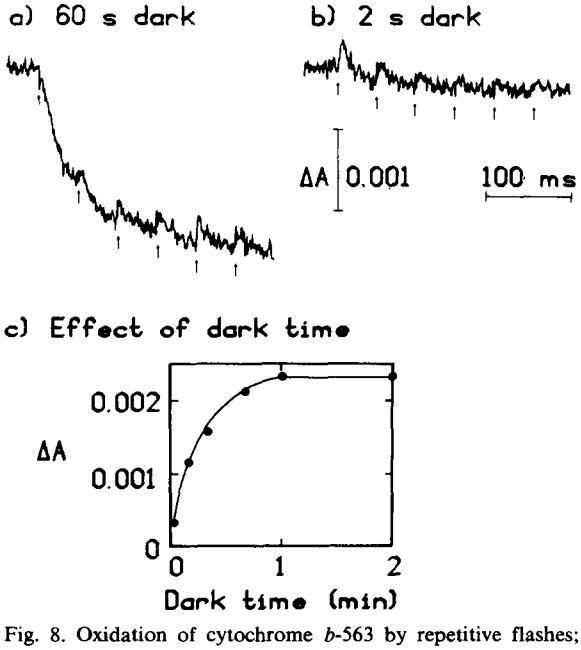
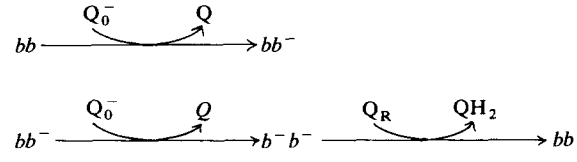


Fig. 8. Oxidation of cytochrome *b*-563 by repetitive flashes; effect of dark incubation time. The procedure was as for Fig. 4, but with four averages and dark incubation time varied as shown. (a, b) Kinetics of cytochrome *b*-563 oxidation; (c) amplitudes (270 ms after the flash) of a series of traces of the form of those shown in (a) and (b), plotted as a function of dark incubation time.

reduction phase, increasing in extent with each flash until a point was reached where there was no net change in the redox state of the cytochrome. The total extent of the multiple flash *b* oxidation decreased if cytochrome *b*-563 was partially oxidized before the experiment (Fig. 8), implying that the same steady-state *b* poise was attained in rapid flashing regardless of the start point. The maximum amplitude observed when cytochrome *b*-563 was fully pre-reduced was 1.5 cytochrome *b* haems oxidized per complex. We have shown previously [14] that the same steady-state was attained in repetitive flashes or dc illumination when the start point was full oxidation of cytochrome *b*-563, and have interpreted the observation in terms of the following scheme for the oxidant-induced reduction/oxidation of cytochrome *b*-563 at an individual complex;



Slow ΔA_{518} with pre-reduced b-563

Fig. 9 shows the result of an experiment in which we have measured the kinetics of cytochromes *b* and *f* and of the slow ΔA_{518} in the absence of any ionophore or uncoupler. We have varied the redox poise of cytochrome *b*-563 by varying the dark incubation time between flashes; on the basis of Fig. 8c, 5 s dark incubation would result in 50% reduction of cytochrome *b*-563, while 60 s would be sufficient for full equilibration.

The effect that this had on the slow ΔA_{518} was to change its rise rate. At the shorter dark incubation time the signal had a $t_{1/2}$ of 6 ms, and correlated tolerably well with the kinetics of cytochrome *f* re-reduction ($t_{1/2} = 7$ ms). When sufficient dark time was allowed for full re-equilibration, these values increased to 16 and 11 ms, respectively. In the case of the slow ΔA_{518}, the $t_{1/2}$ was almost certainly an underestimate, since the overlapping decay of the signal would limit the observable maximum amplitude; we therefore consider that pre-reduction of cytochrome *b*-563 resulted in a slow ΔA_{518} significantly slower than cytochrome *f* re-reduction, and probably corre-

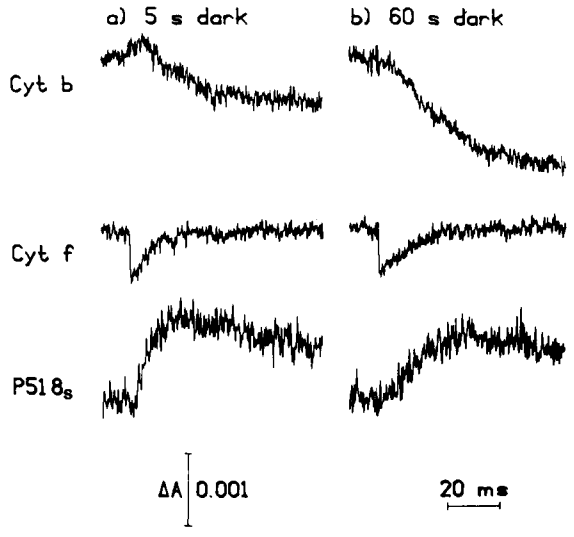


Fig. 9. The effect of dark incubation time on the flash-induced kinetics of cytochrome *b*-563, cytochrome *f* and the slow ΔA_{518}. The reaction mixture was as for Fig. 4, but without nonactin. The cytochrome data were deconvoluted as described in Materials and Methods, but with the inclusion of the following relative extinction coefficients for the carotenoid bandshift; 1 at 542 nm, 0.565 at 554 nm, 0.5 and 563 nm, 0.315 at 575 nm. These values were substituted for the P-700 values in the extinction coefficient matrix [14]. The P-518$_s$ data are the difference between the ΔA_{518} in the presence and absence of 1 μM stigmatellin. Data are the average of four flashes, each preceded by a dark incubation as shown.

lating with cytochrome *b* oxidation ($t_{1/2} = 21$ ms). An iterative computer simulation of the original ΔA_{518} data has been found to support this point of view (not shown).

The amplitude of the slow ΔA_{518} did not appear to be affected by the redox poise, since from 50 ms after the flash the two traces were superimposable. In particular, the data show clearly that full pre-reduction of cytochrome *b*-563 does not result in an increase in the amplitude of the slow ΔA_{518}. The significance of this observation will be considered below. The amplitude of the fast ΔA_{518} was approximately equal to that of the slow ΔA_{518} (not shown).

Discussion

The data reported here have shown that the behaviour of the cytochrome *b-f* complex differs from that predicted by the classical Q-cycle model in that neither cytochrome *f* re-reduction nor cytochrome *b* oxidation nor the slow ΔA_{518} is dependent on the presence of oxidized cytochrome *b*-563. Joliot and Joliot [7] have reported similar observations; the most significant difference between their data and ours was the effect of NQNO. In their hands, NQNO slightly stimulated the rate of *b*-563 oxidation at low potential, while at higher potentials they observed a marked inhibition. In collaborative work with Joliot and Joliot (unpublished data), we have established that in our system, the small inhibition of the rate of cytochrome *b* oxidation shown in Fig. 5 is the consistent effect of NQNO under all conditions. The difference with respect to the effect of NQNO on the partially reduced complex is more dramatic than that with respect to its effect on the fully reduced complex. The possibility cannot be excluded that this represents a genuine difference between the isolated higher plant chloroplasts that we have used, and the intact algal cells used by Joliot and Joliot [7].

Thus, while Joliot and Joliot have proposed an alternative to the Q-cycle based on oxidation of cytochrome *b*-563 via site o in order to account for their observations [7], we will consider alternative models which remain Q-cycles (by which we mean those in which QH_2 carries the'extra' proton across the membrane). The present data can be accommodated within the Q-cycle by the inclusion of either of two additional reactions: oxidation of the semiquinone at site o by a species other than cytochrome *b*-563; or movement of the semiquinone from site o to site r.

Without proposing the existence of an entirely new component, only two possibilities exist for an oxidant for Q_0^- under the conditions reported here; a reoxidized Rieske Fe-S centre, or a second semiquinone. The possibility that the Rieske centre oxidizes Q_0^- would require sufficient 'double hits' of oxidized plastocyanin molecules on cytochrome *b-f* complexes to account for the observed amplitude of cytochrome *b*-563 oxidation. If we assume (a) that the oxidized plastocyanin molecules and the *b-f* complexes are fully mixed in the thylakoid membrane, and (b) that the time during which a plastocyanin molecule is occupying a binding site on a *b-f* complex is negligible compared to the time spent diffusing, then it is simple to show that the proportion of complexes experiencing zero hits (P_0) and the proportion receiving a single hit (P_1)

are, respectively;

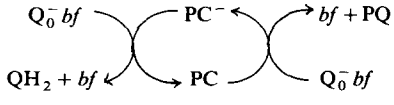
$$P_0 = \left(\frac{BF-1}{BF}\right)^{PC}$$

$$P_1 = \left(\frac{BF-1}{BF}\right)^{PC-1} \cdot \left(\frac{PC}{BF}\right)$$

where BF = number of reduced cytochrome *b-f* complexes involved and PC = number of oxidized plastocyanins involved.

Since (in a saturating single-turnover flash) $PC \approx BF$ and is very large, it will be found that $P_0 = P_1 = 1/e = 0.368$. Thus, the proportion of complexes experiencing two or more hits will be $1 - 2 \cdot 0.368 = 0.264$; and since at each of these complexes two cytochromes *b*-563 will be oxidized, the oxidation of 0.528 cytochrome *b*-563 per oxidizing equivalent would be expected by this mechanism. By contrast, our own data and those of Joliot and Joliot [7] show the oxidation of close to 1 cytochrome *b*-563 per oxidizing equivalent even under conditions where, due to the sub-saturating flash, very few multiple turnovers would be expected.

However, this treatment considers electron transfer from QH_2 to plastocyanin as an irreversible process; a reverse of this reaction, with a $Q_0^- \rightarrow$ FeS reaction, would result in a plastocyanin-mediated dismutation of pairs of Q_0^- as follows;



We would argue against such a possibility on the grounds that it would imply a second-order relationship between the amount of Q_0^- produced by the flash and the rate of cytochrome *b*-563 oxidation, while our data and those of Joliot and Joliot [7] show a half-time independent of flash intensity. The same argument applies to any more direct process of Q_0^- dismutation; but this possibility cannot be completely excluded, particularly in the light of the suggestion that cytochrome *b-c* complexes function as dimers [21].

A Q-cycle based on the movement of a semiquinone anion across the membrane was first proposed by Kroger [22], and has recently been invoked as an explanation of observations in mitochondria comparable to the present data [23,24]. To account for the rather unaesthetic presence of two alternative electrogenic reactions in the cytochrome *b-f* complex, it could be argued that the movement of Q^- is an abnormal process that occurs only under the very extreme experimental conditions used here. On the other hand, it could be argued that Q^- movement is the only electrogenic process that occurs in the *b-f* complex, the observed flash-induced redox changes of cytochrome *b*-563 representing electron sharing with the semiquinone bound at either site. In one version of the mobile Q^- model, the semiquinone cycle [24], it is proposed that the two alternative electrogenic reactions are used in alternate turnovers of the complex. A further possibility would be that it is the neutral semiquinone, QH, that moves between the two sites. This would still result in an electrogenic reaction when the cytochromes *b* are pre-reduced, since electrogenic oxidation of the cytochrome *b* would follow.

In considering the relative merit of these alternative schemes, it is important to note that there are a number of relevant experiments which are feasible in the case of the cytochrome *b*-c_1 but not the *b-f* complex. Such experiments have yielded the following results: (a) In the presence of antimycin, both cytochromes *b* can be reduced by successive turnovers at site o [25]; the reduction of *b*-562 is electrogenic [26]. (b) In the presence of myxothiazol, cytochrome *b*-562 can be reduced by quinol via site r; this reaction is antimycin-sensitive [2]. (c) In the presence of antimycin and after a few turnovers to reduce the cytochromes *b*, no rapid reduction of cytochrome c_1 can be observed [27,28].

The first two observations confirm that an electrogenic pathway exists from site o to site r via at least one of the cytochromes *b*. The last shows that when cytochrome b_0 is pre-reduced and antimycin is present, no pathway exists for removal of semiquinone from site o; this would seem to imply that any such pathway that exists in the absence of antimycin involves site r, which would be more consistent with models based on semiquinone movement than with those based on oxidation of the semiquinone by the Rieske centre.

Furthermore, if Q^- movement were occurring

in the case of the *b-f* complex, the slow ΔA_{518} would increase in amplitude with increasing dark time in the experiment shown in Fig. 8, approaching twice its normal size as cytochrome *b*-563 approached full pre-reduction. This is because the transmembrane movement of the anionic semiquinone and the subsequent oxidation of cytochrome *b*-563 would both contribute a full electrogenic reaction. It is clear that this is not the case; the data of Fig. 9 are consistent with a model based on QH movement, and may be consistent with one based on double hits, but are not consistent with one based on Q^- movement. It follows that the only one of the schemes suggested here that is applicable to both the *b*-c_1 and the *b-f* complexes is movement of QH from site o to site r.

It should be emphasised that this is not the result predicted by the semiquinone-cycle hypothesis of Wikstrom and Krab [24]. In order to reconcile the observed H^+/e^- stoichiometry of 2 with a model involving transmembrane semiquinone movement under physiological conditions, it is essential that the mobile species is Q^-, which our observations under reducing conditions render unlikely; movement of QH would yield a H^+/e^- stoichiometry of 1. Thus, QH movement may provide a explanation for the reports in the literature of a H^+/e^- stoichiometry for the cytochrome *b-f* complex of 1 under certain conditions (reviewed in Ref. 3). However, recent findings have raised serious doubts concerning the evidence for variable H^+/e^- stoichiometry in the *b-f* complex (Rich, P.R., unpublished results); therefore, the possibility must be considered that semiquinone movement occurs only in complexes with fully reduced cytochrome *b*-563, preventing this from being an inactive state, and does not occur sufficiently frequently to yield a significant decrease in the H^+/e^- stoichiometry under physiological conditions.

Acknowledgements

The authors would like to thank the Venture Research Unit of British Petroleum plc for financial support. We would also like to acknowledge the helpful discussions we have had with Peter Mitchell, Pierre Joliot, Anne Joliot and Ian West, and the technical assistance of Sonia Clarke and Sally Madgwick.

References

1 Wikstrom, M.K.F. and Berden, J.A. (1972) Biochim. Biophys. Acta 283, 403–420
2 Von Jagow, G. and Link, T.A. (1984) in Biomedical and Clinical Aspects of Coenzyme Q (Folkers, K. and Yamamura, Y., eds.), Vol. 4, pp. 87–98, Elsevier, Amsterdam
3 Rich, P.R. (1984) Biochim. Biophys. Acta 768, 53–79
4 Mitchell, P. (1976) J. Theor. Biol. 62, 327–367
5 Slater, E.C. (1973) Biochim. Biophys. Acta 301, 129–154
6 Joliot, P. and Delosme, R. (1974) Biochim. Biophys. Acta 357, 267–284
7 Joliot, P. and Joliot, A. (1986) Biochim. Biophys. Acta 849, 211–222
8 Girvin, M.E. and Cramer, W.A. (1984) Biochim. Biophys. Acta 767, 29–38
9 Giorgi, L.B., Packham, N.K. and Barber, J. (1985) Biochim. Biophys. Acta 806, 366–373
10 Moss, D.A. and Bendall, D.S. (1984) in European Bioenergetics Conference Short Reports, Vol. 3A, pp. 151–152, ICSU Press, Miami, FL
11 Moss, D.A. and Bendall, D.S. (1986) Biochem. Soc. Trans. 14, 57–58
12 Crofts, A.R. (1986) J. Bioenerg. Biomembr. 18, 437–445
13 Moss, D.A. and Bendall, D.S. (1984) Biochim. Biophys. Acta 767, 389–395
14 Rich, P.R., Heathcote, P. and Moss, D.A. (1987) Biochim. Biophys. Acta 892, 138–151
15 Cox, R.P. (1979) Biochem. J. 184, 39–44
16 Moss, D.A. and Rich, P.R. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. II, pp. 461–464, Martinus Nijhoff, Dordrecht
17 Binder, R.G. and Selman, B.R. (1980) Biochim. Biophys. Acta 590, 212–222
18 Binder, R.G. and Selman, B.R. (1980) Biochim. Biophys. Acta 592, 314–322
19 Jones, R.W. and Whitmarsh, J. (1985) Photobiochem. Photobiophys. 9, 119–127
20 Von Jagow, G. and Ohnishi, T. (1985) FEBS Lett. 185, 311–315
21 De Vries, S., Albracht, S.P.J., Berden, J.A. and Slater, E.C. (1982) Biochim. Biophys. Acta 681, 41–53
22 Kroger, A. (1976) FEBS Lett. 65, 278–280
23 Rich, P.R. and Wikstrom, M.K.F. (1986) FEBS Lett. 194, 176–182
24 Wikstrom, M.K.F. and Krab, K. (1986) J. Bioenerg. Biomembr. 18, 181–193
25 Meinhardt, S.W. and Crofts, A.R. (1983) Biochim. Biophys. Acta 723, 219–230
26 Glaser, E. and Crofts, A.R. (1984) Fed. Proc. 43, 1877
27 Slater, E.C. (1973) Biochim. Biophys. Acta 301, 129–154
28 Alexandre, A. and Lehninger, A.L. (1984) Biochim. Biophys. Acta 767, 120–129