Biochimica et Biophysica Acta, 975 (1989) 96–103
Elsevier

BBA 42997

Proton uptake by the chloroplast cytochrome *bf* complex

A.B. Hope [1] and P.R. Rich [2]

[1] *School of Biological Sciences, Flinders University, Bedford Park (Australia) and* [2] *Glynn Research Institute, Bodmin (U.K.)*

(Received 22 December 1988)

Key words: Cytochrome *bf* complex; Proton/electron ratio; Proton translocation; Chloroplast; (Pea)

The proton uptake, ΔH_n^+, associated with turnover of the quinone reduction site (here termed the Q_n site – see abbreviations) of the chloroplast cytochrome *bf* complex has been kinetically resolved. It was approximately correlated with cytochrome *f* rereduction in a range of conditions. ΔH_n^+ was inhibited by the quinol oxidation site (here termed the Q_p site – see abbreviations) inhibitors stigmatellin and DBMIB, but was unaffected by NQNO, an effector of the quinone reduction site (the Q_n site), or by antimycin A. At external pH of 6 and 8, ΔH_n^+ was equivalent to about one proton per Photosystem I per flash, i.e., each turnover of the complex caused a proton uptake. At intermediate pH values on the first flash this value was lower, but it rose to around 1 on subsequent flashes, provided that they were given sufficiently rapidly. Comparable measurements were made in the absence of nonactin so that a transmembrane potential difference was present during proton uptake. This potential, of the order of 30–50 mV, had little effect on the extent of ΔH_n^+.

Introduction

The reaction cycle of the chloroplast cytochrome *bf* complex can involve an electrogenic electron transfer across the membrane. This results in quinone reduction, with uptake of protons from the lumen. Overall, the result is the net translocation of additional protons across the thylakoid membrane for each electron which is passed on to Photosystem I donors [1]. This protonmotive property makes the *bf* complex similar to the bc_1 complexes of mitochondria and photosynthetic bacteria. However, it is widely thought that, in contrast to the tight coupling of the bc_1 complexes, those reactions in cytochrome *bf* complex which are associated with proton translocation are facultative, and that under most conditions the reaction cycle is not coupled to proton translocation [2]. A number of models which could produce this effect have been described [1,2]. However, the evidence for such a view has recently been reexamined [3] and the possibility has been raised that a net proton-translocating reaction cycle might be the rule, rather than the exception.

The Q-cycle has proved to be a useful model of the protonmotive action of the complexes [4]. It requires two different sites for interaction with quinones, one of which, the Q_p site, oxidises quinol and causes proton release to the intrathylakoid, positive, aqueous phase. The site has been well characterised with respect to specific inhibitors [5], and its required vectorial location, at least in chloroplasts, has been confirmed by the sensitivity of the quinol oxidation to lumenal pH and by the observation of proton release into the lumen which is associated with quinol oxidation [6,7].

The second type of site, the Q_n site, has been more difficult to characterise. It is required to be in protonic contact with the negative aqueous phase so that protons are taken up from this phase when cytochrome *b*-563 is reoxidised by quinone [4]. In mitochondria, identification of the site has been aided by its quinol-quinone transhydrogenase activity [8], by its Q_p site-independent quinol-cytochrome *b* oxidoreductase activity [9,10], and

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide; DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea; PMS, 5-methylphenazinium methosulphate (phenazine methosulphate); Q_p site, the quinol oxidation site of the cytochrome *bf* complex which is in contact with the positive aqueous intrathylakoid phase (also termed the Q_z or Q_o site in bacterial and mitochondrial systems); Q_n site, the quinone reduction site of the cytochrome *bf* complex which is in contact with the negative aqueous extrathylakoid stromal phase (also termed the Q_i site in bacterial and mitochondrial systems); Q_B (site), the (binding site of the) secondary quinone acceptor of Photosystem II; ΔH_n^+, proton uptake from the stromal side of the membrane caused by the reactions of the quinone reduction site, Q_n, of the cytochrome *bf* complex; ΔH_{PSI}^+, proton uptake from the stromal side of the membrane caused by reoxidation of the acceptor side of Photosystem I via methyl viologen by molecular oxygen to produce hydrogen peroxide.

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

by the sensitivity of the site to specific antibiotics such as antimycin A [11]. In the cytochrome *bf* complex, this transhydrogenase activity is absent [12] and the quinol-cytochrome *b* oxidoreductase activity is difficult to assay [12]; the reason for both is probably the low mid-point potentials of the cytochrome *b*-563. In addition, the site is entirely insensitive to antimycin A [1].

The introduction of NQNO was an important contribution, since it appeared to act like antimycin A on the Q_n sites of both mitochondrial bc_1 [13] and chloroplast cytochrome *bf* [14,15] complexes. In both it promotes additional 'oxidant-induced reduction' of *b*-563, but in chloroplasts it does not inhibit steady-state electron flux through the enzyme [15], a finding which had strengthened the view that turnover of the Q_n site of the cytochrome *bf* complex (and hence proton translocation) might be facultative.

One area which initially led to the identification of protonmotive action of the *bf* complex was measurement of proton uptake activated by continuous light [16–18] or by single turnover flashes [7,19–24]. Analogous studies have been made of the bacterial chromatophore system where kinetic separation of proton uptake by the reaction centres and by the bc_1 complex was possible [25–27]. In this report we show that kinetic resolution of the proton uptake by the Q_n site of the cytochrome *bf* complex is also possible. These measurements provide further insight into the catalytic cycle of the enzyme, the range of conditions in which proton translocation occurs, and the effects of inhibitors on the Q_n site.

Materials and Methods

Chloroplast preparation

Pea plants were grown in a growth chamber at 20–25°C, in an 8/16 h light/dark regime. The illuminance was about 50 μmol quanta $\cdot m^{-2} \cdot s^{-1}$. Class C chloroplasts were prepared as already described [28] and stored in liquid nitrogen in resuspension medium plus 5% dimethylsulphoxide, or kept on ice for more immediate use. There was little difference in results from either source of chloroplasts.

Proton uptake measurements

The dye methods developed by Junge et al. were employed [29]. Proton uptake was monitored in a medium of 0.4 M sucrose and 50 mM KCl, containing in addition 10 μM DCMU, 0.1 μM nonactin, 100 μM methyl viologen, 0.5 mM sodium azide and 0.5 mM duroquinol at 23°C. pH was kept within ± 0.05 of required value. In all cases except Fig. 1A, superoxide dismutase was also present at 10 μg/ml (Sigma, from bovine erythrocytes; specific activity of 3000 U/mg). Light flashes of half-peak-width duration about 5 μs, were delivered from a xenon flashlamp system and filtered with a Schott glass RG630 red filter. A baseline in the absence of pH indicator was first recorded at the appropriate wavelength, a number of responses being averaged (see figure legends). Indicator dye was then added and the measurement was repeated. The baseline was subtracted from the data with indicator to give the optical change due to flash-induced pH changed. The dye used was Bromocresyl purple (pH 6–7; 575 nm), Phenol red (pH 7–8; 560 nm) or Cresyl red (pH 7.5–9; 575 nm) at concentrations between 20 and 100 μM.

Cytochrome kinetics

Measurements of cytochrome *f* kinetics were made in a medium similar to that described above, using flash-induced absorbance changes at the wavelengths 542, 554, 563 and 575 nm. The individual changes due to cytochrome *b*-563, cytochrome *f*, P700 and plastocyanin were then obtained from matrix deconvolution using the matrix coefficients given in Ref. 30. Data were generally the average of ten recordings at each wavelength, with a response time-constant of 1 ms and a measuring beam bandwidth of about 1.5 nm. The results represent the absorbance change at one wavelength of a single component, in this case of cytochrome *f* absorbance change at 554 nm.

Carotenoid bandshift measurements

The absorbance changes due to the flash-induced electrochromic shift [31] in carotenoids and/or chlorophyll *b* were measured at 518 nm in a suspension of chloroplasts equivalent to 50 μM chlorophyll, in a medium as above but lacking nonactin. An average of ten runs was recorded, with a time-constant of 1 ms and measuring bandwidth 4 nm. The slow phase of the electrochromic signal was taken as the difference between a control response and that of the same chloroplast suspension to which had been added 2 μM stigmatellin and 1 μM phenazine methosulphate (PMS) (c.f. Refs. 15, 32).

Results

Effects of superoxide dismutase on proton uptake

Fig. 1 shows the signal due to proton uptake in the absence of Photosystem II turnover, and the effect on the kinetics of uptake of added superoxide dismutase. Without superoxide dismutase, the rise is roughly monophasic, with a half-time of around 100 ms. A general acceleration of the proton uptake associated with the autoxidation of reduced methyl viologen was first described by Polle and Junge [33]. Here the signal in the presence of superoxide dismutase becomes biphasic, with approximately half of its extent 'rapid' and complete in around 10 ms and the remainder occurring over the next 200 ms.

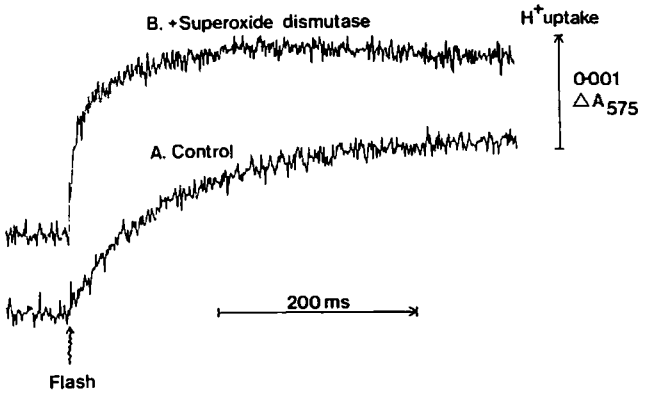

Fig. 1. The effects of superoxide dismutase on the kinetics of proton uptake by broken chloroplasts. Chloroplasts were resuspended to 20 μM chlorophyll in a medium of 0.4 M sucrose and 50 mM KCl, and containing 10 μM DCMU, 0.1 μM nonactin, 100 μM methyl viologen, 0.5 mM sodium azide and 0.5 mM duroquinol. The pH was adjusted to 7.55. A control signal in the absence of Cresol red was subtracted from that obtained in the presence of 30 μM Cresol red. A, control; B, plus 10 μg/ml (30 U/ml) superoxide dismutase. Each trace is the average of 40 recordings (taken in batches of 20 on fresh samples); 5 ms time constant; 7 s dark adaptation before each flash.

Effects of stigmatellin plus PMS on proton uptake

Under out experimental conditions where Photosystem II is inhibited with DCMU, we expect two reactions to contribute to the observed proton uptake. The first of these is caused by reoxidation of Photosystem I acceptors by the added methyl viologen (MeVi). The reduced viologen is reoxidised by molecular oxygen to produce superoxide anions which in turn dismute, with the uptake of protons from the external medium to produce oxygen and hydrogen peroxide. The net result is the uptake of one proton from the external medium for each successful charge separation in Photosystem I:

$$2 \text{ reduced PS I acceptors} + 2 \text{ MeVi} \rightarrow 2 \text{ PSI} + 2 \text{ MeVi}^-$$

$$2 \text{ MeVi}^- + 2 \text{ O}_2 \rightarrow 2 \text{ MeVi} + 2 \text{ O}_2^-$$

$$2 \text{ O}_2^- + 2 \text{ H}^+_{\text{stroma}} \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$$

It is this latter reaction which is accelerated by superoxide dismutase to produce the change in kinetics of proton uptake as seen in Fig. 1, as discussed by Polle and Junge [33]. In the presence of duroquinol, however, charge separation in Photosystem I will also cause oxidation of plastocyanin, followed by electronic turnover of the cytochrome *bf* complex as this plastocyanin is rereduced. If the reaction cycle of the cytochrome *bf* complex is protonmotive, then additional proton uptake from the external medium is expected to occur. This additional proton uptake is expected to be sensitive to inhibitors of turnover of the cytochrome *bf* complex, such as stigmatellin.

Added stigmatellin plus PMS did indeed decrease flash-induced proton uptake to a signal that was, except for a small slow fraction, 'rapid' (Fig. 2). Essentially the same result was obtained with sigmatellin alone. However, throughout the experiments described here, PMS was added together with stigmatellin in order to ensure that electron donors to $P700^+$ were completely rereduced between flashes. It had the advantage that any possible residual *bf*-mediated oxidation of plastoquinol was completely prevented by having a rapid pathway for chemical rereduction of plastocyanin (with PMS acting as an rapid redox mediator between duroquinol

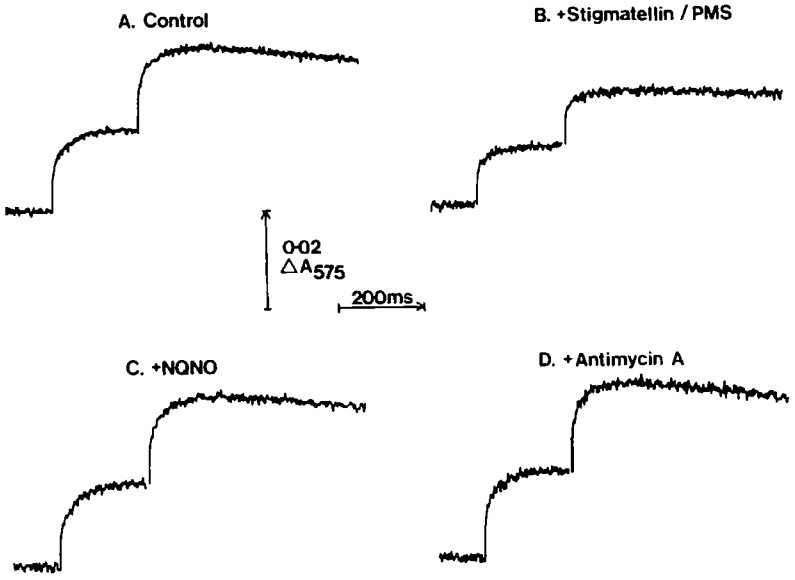

Fig. 2. Effects of inhibitors on flash-induced proton uptake. Chloroplasts were resuspended to 20 μM chlorophyll in a medium identical to that of Fig. 1B. pH was adjusted to 7.6 for all experiments. Data shown have had the signal in the absence of Cresol red subtracted. Conditions were: A, control; B, +2 μM stigmatellin and 1 μM PMS; C, plus 1 μM NQNO; D, plus 2 μM antimycin A. Data are the averages of 30 recordings, each taken in batches of 10 on fresh samples. Time constant was 20 ms with 7 s dark adaptation before each flash.

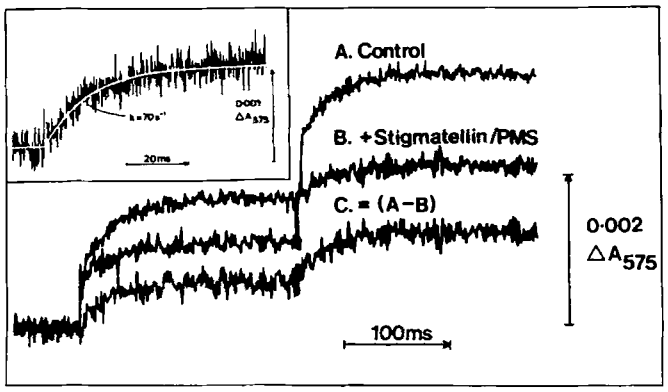

Fig. 3. Kinetic resolution of the stigmatellin/PMS-sensitive proton uptake. Chloroplasts were resuspended to 20 μM chlorophyll in a medium identical to that of Fig. 1B. pH was adjusted to 7.6 for all experiments. Data shown have had the signal in the absence of Cresol red subtracted. Conditions were: (A) control; (B) plus 2 μM stigmatellin and 1 μM PMS; (C) (A) minus (B), i.e., stigmatellin/PMS-sensitive proton uptake. Data are the averages of 30 recordings, each taken in batches of 10 on fresh samples. Time constant was 5 ms with 7 s dark adaptation before each flash; and measuring beam bandwidth was 2 nm. In the inset, the experiment of (C) was repeated at pH 7.65, but with a time constant of 0.5 ms, a bandwidth of 4 nm and with 50 averages (taken in batches of 10 on fresh samples) for each condition. A simulated first-order rise of $k = 70\ s^{-1}$ is included for comparison.

and plastocyanin and $P700^+$), and also allowed $P700^+$ to rereduce completely between flashes and so ensured that a full one electron per flash and per P700 reached viologen, even in those experiments which involved multiple flashes. Observations showed that in the presence of 0.5 mM duroquinol with 2 μM stigmatellin/1 μM PMS, $P700^+$ was rereduced between flashes given at up to 10 Hz.

By subtracting the stigmatellin/PMS-resistant signal (e.g., Fig. 2B) from control signals (all with pH-unspecific responses subtracted), the size and shape of the stigmatellin/PMS-sensitive proton uptake was revealed. It is seen from Fig. 3 (inset) that this uptake was very nearly first-order, with a rate constant of 70 s^{-1} at pH 7.65.

Fig. 2 also shows that NQNO at 1 μM or antimycin at 2 μM was completely without inhibitory effect on proton uptake.

pH-dependence of stigmatellin / PMS-sensitive proton uptake

When the kinetics of cytochrome *f* re-reduction and stigmatellin/PMS-sensitive proton uptake were compared, they were found to be correlated, but not identical, over a range of externally adjusted pH values (Fig. 4). The external pH-dependence of the size of stigmatellin/PMS-sensitive proton uptake on the first flash, expressed as a proportion of the stigmatellin-insensitive uptake, is shown in Fig. 4 (inset); there was a minimum of about 0.55 in this ratio at pH 7.2, while it rose to 0.8–0.9 at pH values larger or smaller than 7.2.

Because this stigmatellin/PMS-sensitive proton uptake is clearly associated with turnover of the cytochrome *bf* complex, we refer to it as ΔH_n^+. The proton uptake which remains insensitive to stigmatellin/PMS is that associated with reoxidation of reduced methyl viologen [33] and is termed ΔH_{PSI}^+.

Effect of dark time between flashes

The extent of stigmatellin/PMS-sensitive proton uptake, ΔH_n^+, upon the first flash was compared with that

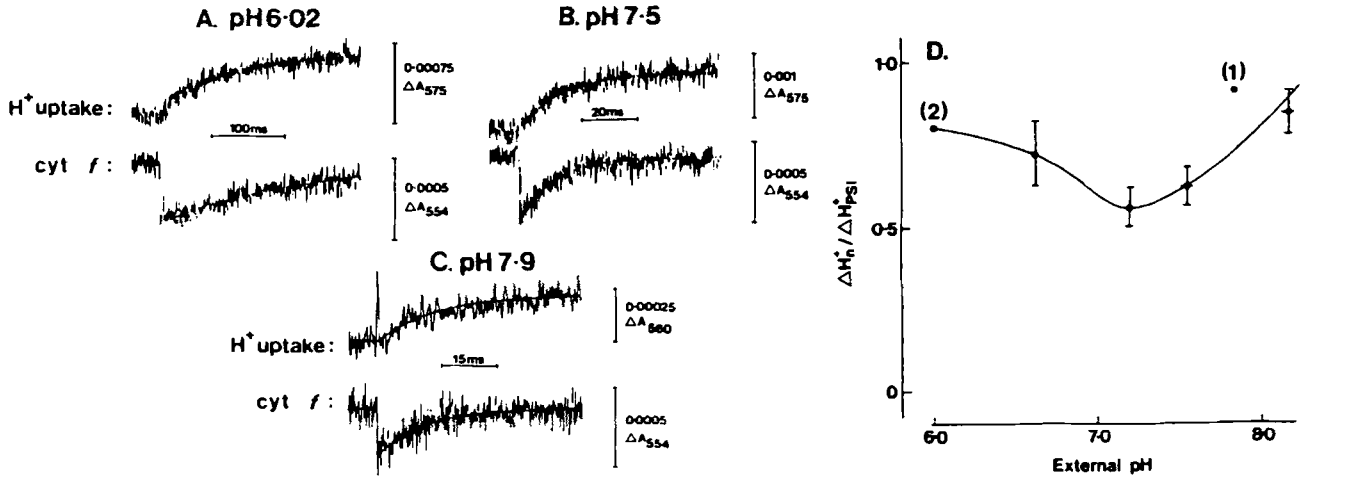

Fig. 4. pH-dependence of the kinetics of stigmatellin/PMS-sensitive proton uptake and of cytochrome *f* rereduction. Chloroplasts were resuspended to 20 μM chlorophyll for proton uptake measurements in a medium identical to that of Fig. 1B. Proton uptake was measured using: (A) 30 μM Bromocresol purple at pH 6.0 (data average of 20 at 1 ms time constant); (B) 30 μM Cresol red at pH 7.5 (average of 50 recordings at 0.5 ms time constant); (C) 20 μM Phenol red at pH 7.9 (average of 50 recordings at 0.5 ms time constant, followed by digital smoothing). Cytochrome *f* was monitored as described in Materials and Methods in the absence of any pH indicator. For comparison, first-order curves are plotted over the data of: at pH 6.0, 12 s^{-1} (H^+ uptake) and 6 s^{-1} (*f* rereduction); at pH 7.5, 70 s^{-1} (H^+ uptake) and 70 s^{-1} (*f* rereduction); at pH 7.9, 65 s^{-1} (H^+ uptake) and 85 s^{-1} (*f* rereduction). (D) shows the pH-dependence of the extent of stigmatellin/PMS-sensitive proton uptake, expressed as a fraction of the stigmatellin/PMS-resistant signal.

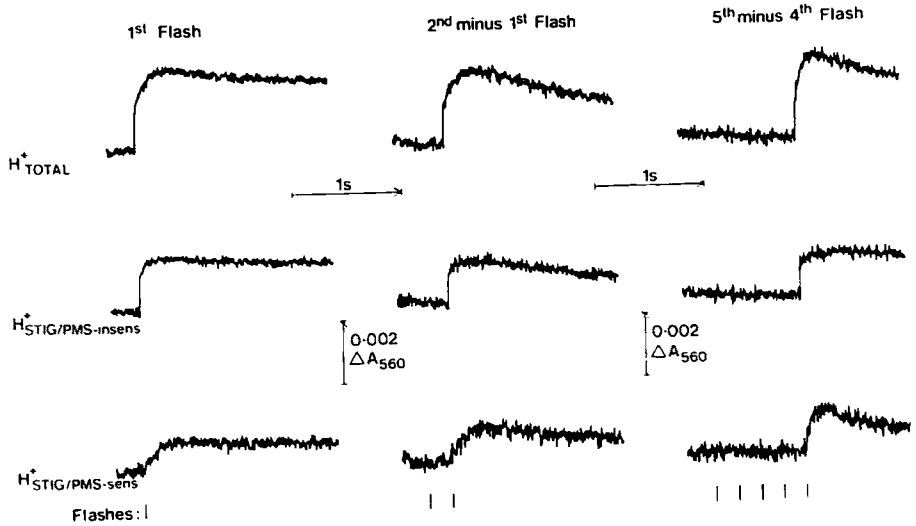

Fig. 5. The effect of flash number on the extent of stigmatellin/PMS-sensitive proton uptake. Chloroplasts were resuspended to 20 μM in proton uptake medium at pH 7.25 and Phenol red was used as the pH indicator. After 7 s dark adaptation, a series of flashes separated by 200 ms intervals were given. The experiment was repeated in the presence of 2 μM stigmatellin plus 1 μM PMS. The number of flashes was varied from one to a maximum of five in fresh samples. Proton uptake caused by flash n was obtained by subtraction of data taken with $(n-1)$ flashes from that taken with n flashes.

on subsequent flashes which were given at a frequency of 5 Hz (Fig. 5), at a pH of 7.25. It was found that the ratio $\Delta H_n^+ / \Delta H_{PSI}^+$ rapidly approached a value close to 1.0 on these subsequent flashes and did not diminish even after the fifth flash.

The effect of dark time between flashes at pH 7.2 was explored by delivering three flashes at 5 Hz, and then waiting a variable time in the dark before giving a fourth measuring flash. The ratio between total proton uptake (i.e., $\Delta H_n^+ + \Delta H_{PSI}^+$) and that insensitive to stigmatellin/PMS (i.e., ΔH_{PSI}^+) as a function of this dark time is plotted in Fig. 6. It can be seen that the system relaxed back to that with the lower ratio with a half-time of approx. 760 ms. The right-hand plot in this figure shows that it is ΔH_n^+, rather than ΔH_{PSI}^+, which decreases during this time.

pH Dependence of the slow electrochromic signal

The slow electrochromic signal was measured over a range of pH. Some results of fitting first-order rate constants to the rise and decay of the slow phase and to the decay of the stigmatellin/PMS-insensitive fast phase at pH 7.2 are shown in Fig. 7A. The variations in these fitted parameters with pH are shown in Fig. 7B. It can be seen that the extent of both the slow phase and the fast phase are essentially independent of pH between pH 6 and 8.6. Only the rise-time of the slow phase was affected by pH, as would be expected because of the known pH-dependence of the quinol oxidation reaction.

Effect of electric field on the extent of ΔH_n^+

Fig. 8 shows the result of omitting nonactin from the medium, on the extent of total proton uptake as a result

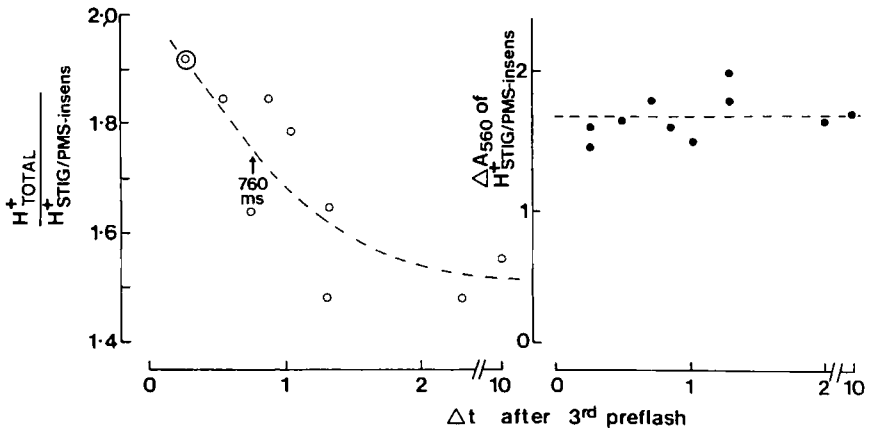

Fig. 6. The effect of dark adaptation on the extent of stigmatellin/PMS-sensitive proton uptake at pH 7.2. Chloroplasts were resuspended in proton uptake medium at pH 7.2.Three preflashes were given, followed by a dark-adaptation time, Δt, after which a fourth flash was provided. Proton uptake caused by this fourth flash was monitored with Phenol red. Data are plotted as the ratio of total proton uptake: stigmatellin/PMS-resistant uptake measured in the same sample. The extent of this stigmatellin/PMS-resistant uptake as a function of dark adaptation is also given in (B).

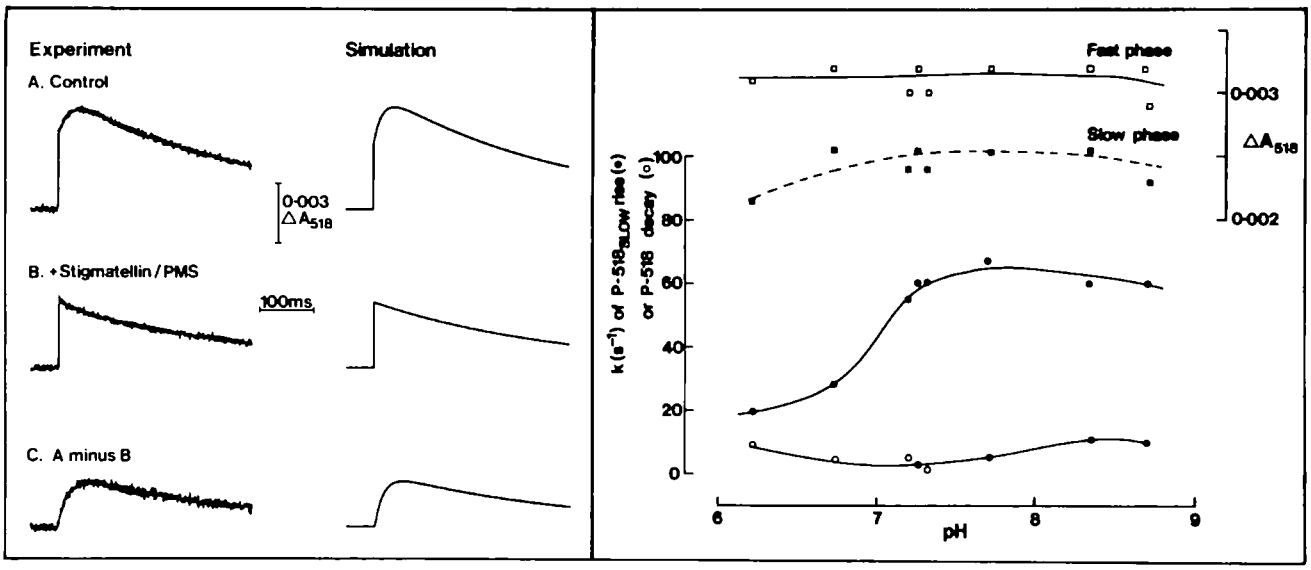

Fig. 7. The pH dependence of risetime and extent of $P518_{slow}$. Measurements were made as described in Materials and Methods using fresh chloroplasts. The rise time of the slow phase and the decay of total P518 change were assumed to be first order, so that a best fit to a single exponent could be made. This fit was reasonable, except at the extremes of pH. In (A), typical data at pH 7.2 are shown, together with the iterative simulations used. In (B), the pH dependence of the values needed for such iterations is shown.

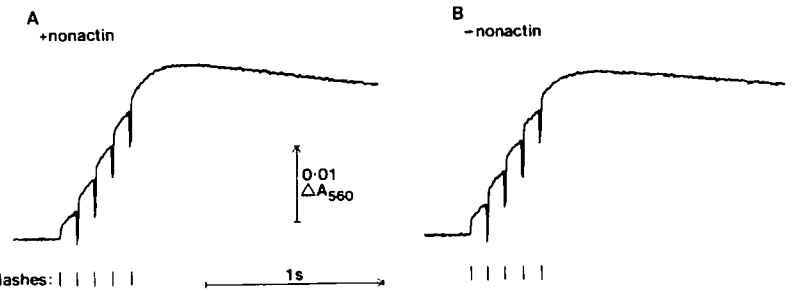

Fig. 8. The effects of nonactin on the extent of proton uptake with multiple flashes. Chloroplasts were resuspended to 20 μM in proton uptake medium at pH 7.2 and 100 μM Phenol red was used as the pH indicator. After 7s dark adaptation, a series of five flashes separated by 100 ms intervals were given. (A) Control; (B) plus 0.1 μM nonactin. Data are the average of ten recordings, taken at a measuring bandwidth of 4 nm and with a recorder time constant of 1 ms. A baseline in the absence of indicator has been subtracted in both cases.

of five flashes given at 10 Hz; the signal was depressed by about 9%. Further experiments (not shown) indicated that small decreases of both ΔH_n^+ and ΔH_{PSI}^+ contributed to this change.

Discussion

It is seen that proton uptake attributable to *bf* complex activity (ΔH_n^+) can be resolved from that attributable to reoxidation of Photosystem I acceptors (ΔH_{PSI}^+) both kinetically (though imperfectly) and as a result of the insensitivity of PS I electron transfers (and hence proton uptake due to methyl viologen autoxidation) to the quinol oxidation inhibitor [34] stigmatellin. Furthermore, the extent of ΔH_n^+ can be quantified by scaling to the assumed one proton taken up per P700 per flash as a result of electron throughput, which is represented by the stigmatellin/PMS-insensitive signal, ΔH_{PSI}^+.

Complete cycling of the cytochrome *bf* complex is a two-step process with the intermediate being a stable ($t_{1/2}$ of around 500 ms, at least under oxidising conditions) species with only one electron in the cytochrome *b*-563...Q_n region [3]. Proton uptake by *bf* complexes would not be expected from a single flash unless either double turnovers take place in the complexes, or, following a single turnover, the one-electron reduced complex becomes protonated via the Q_n site. We propose that at pH 7.2, such protonation is small, because the apparent p*K* suggested from the behaviour of proton uptake as a function of pH (data in Fig. 4B (inset), between pH 6 and 7.2) is about 6.6. Thus, proton uptake upon the first flash is due to double turnovers in some *bf* complexes. An upper limit for double turnovers can be calculated from a Poisson distribution of random oxidising collisions between diffusing, oxidized plastocyanin molecules and *bf* complexes. Thus, some complexes turn over not at all, some turnover once, and the rest turnover twice or more as a result of multiple reductions of iron-sulphur centres by quinol. For a ratio of 0.7 *bf* complexes to P700 reaction centres, as mea-

sured for these chloroplasts, this calculation predicts that, during the (1/0.7) turnovers of *bf* complexes, 0.48 double turnovers occur. Hence the ratio ($\Delta H_n^+ / \Delta H_{PSI}^+$) would be $(2 \times 0.48)/(1/0.7) = 0.67$. The observed ratio of 0.55 at pH 7.2 is somewhat lower than this estimate, possibly indicating that complete randomisation is not achieved.

In earlier experiments under oxidising conditions [7,22], any uptake due to double turnovers would have been submerged in the total, composed of uptake by Q_B^{2-} in Photosystem II together with an amount due to *bf* complexes, much smaller than 0.55 because of the lower quinol concentration generated by single, infrequent flashes in these conditions.

From the shape of the curve in Fig. 4B, the effect of pH on the extent of proton uptake upon the first flash is due to a combination of at least two factors. As we have postulated, between pH 6.0 and 7.2 the uptake is increased above that due to double turnovers by protonation of the one-electron-reduced intermediate, $(b\text{-}563 \ldots Q_n)^-$. However, ΔH_n^+ also increases to 0.9 between pH 7.2 and 8.2. In order to explain the E_m/pH relations of quinone when bound at the quinone reduction site of the bc_1 complexes [35–37], it has proved necessary to postulate a protonatable group associated with the Q_n site whose pK is lowered from a value higher than 11 when the site is occupied by quinone or quinol to around 7.6 (mitochondria) or 9 (bacteria) when semiquinone occupies the site [37]. Such a group, but with rather different pK values, could account for the increase in ΔH_n^+ at high pH in our experiments – as the pH is raised above 7.2, the site would be progressively less preprotonated so that a proton is taken up when the one-electron-reduced intermediate is formed. Further experiments will be required to test this working hypothesis.

It is to be noted that the magnitude of the slow phase of the electrochromic signal did not decrease in concert with the ΔH_n^+ changes (Fig. 7B), but remained at a high constant value. This rather suggests that the protonation events described above do not contribute to the overall electrogenic charge separation, i.e., charge separation by the cytochrome *bf* complex is caused by electrogenic electron transfer, rather than electrogenic proton transfer into the Q_n site. A similar conclusion was reached by Robertson et al. [38] for the bacterial system, although an alternative possible view has been described by Konstantinov and Popova [39].

The speed of proton uptake is affected by external pH because average internal pH is to some extent lowered by reducing external pH. The quinol oxidation reaction at the p-site is known to be sensitive to pH, being slowed down as the pH is lowered below 7 [40]. It is this effect which causes the slowing of cytochrome *f* rereduction (Fig. 4) and the slowing of the rise of the slow phase of the electrochromic signal. Since proton uptake is to an extent correlated with cytochrome *f* rereduction (Fig. 4), if is concluded that the reactions at quinol oxidation sites are limiting for the whole reaction cycle occurring in the *bf* complexes.

Dark time between flashes has an effect on the size of proton uptake after the second flash at pH 7.2. We propose that the one-electron reduced complexes, which normally await a second electron and take up two protons, oxidise slowly with a characteristic half-time of about 750 ms, probably by unbinding of plastosemiquinone. It was previously concluded, from the effect of flash frequency on proton uptake attributed to *bf* activity in oxidising conditions [22,41], that the corresponding lifetime was about 300 ms. In rough agreement, Rich [3] has estimated that the one-electron state oxidises with a half-time of around 500 ms under oxidising conditions.

The lack of effect of antimycin A on ΔH_n^+ confirms the already strong evidence that it does not inhibit turnover of the Q_n site of the cytochrome *bf* complex [1]. However, the lack of effect of NQNO on proton uptake is at variance with its often-assumed action as an inhibitor of cytochrome *b*-563 oxidation, through binding at Q_n sites. As a control, we confirmed that NQNO had the effect [14,15] of increasing the extent of cytochrome *b*-563 reduction after a single turnover flash under the same conditions (data not shown). The results support the view that NQNO alters the relative midpoint potentials of cytochrome *b*-563 [42] and bound quinone such that the equilibrium is shifted to favour reduced cytochrome *b*-563 in the one-electron intermediate, hence causing increased 'oxidant-induced reduction' of cytochrome *b*-563 after a single turnover flash. However, it appears that it does not prevent double turnovers of the quinone reduction site and so has no effect on ΔH_n^+ (this work) or on steady-state electron transfer through the enzyme [15]. Previously reported effects of NQNO on proton uptake in oxidising conditions [19] were attributed to an inhibition of Q-cycle proton uptake, but may have been confused with effects on proton uptake at the Q_B site of Photosystem II.

It has previously been suggested that a transmembrane electric field may inhibit electrogenic reactions of the cytochrome *bf* complex [43,44] and might reduce or prevent proton translocation by the complex [23,41]. This was tested in the experiments described above (Fig. 8) by comparing proton uptake in the absence of nonactin (i.e., in the presence of a transmembrane field) to that in the presence of a concentration of nonactin that collapsed the field before the enzyme turned over. Only a small effect on measured ΔH_n^+ was found, and this effect was not greater than the effect on ΔH_{PSI}^+. It is concluded that a transmembrane electric field does not prevent the protonmotive action of the cytochrome *bf* complex in these experiments and that the small de-

crease of signal in the absence of nonactin is caused by a small amount of proton transfer across the membrane, driven by the field. However, it should be noted that this field was only 30–50 mV at its maximum, so that we cannot rule out the possibility that a larger field could diminish proton translocation, although it should be noted that the chloroplast in vivo is unlikely to have a transmembrane potential in excess of the values attained here.

Acknowledgements

This work is supported by the United Kingdom SERC (grant No. GR/E53941) and by the benefactors of the Glynn Research Foundation Ltd. Expert technical assistance was provided by S. Madgwick, electronic developments by Mr. A. Jeal, and figures were produced by Mr. R. Harper, Stigmatellin was a generous gift of Prof. Dr. G. Höfle and NQNO of Prof. Dr. G. von Jagow. A.B.H. warmly thanks the Glynn Research Foundation Ltd. for the opportunity of a working visit.

References

1 Rich, P.R. (1984) Biochim. Biophys. Acta 768, 53–79.
2 Cramer, W.A., Black, M.T., Widger, W.R. and Girvin, M.E. (1987) in The Light Reactions (Barber, J., ed.), pp. 447–493, Elsevier, Amsterdam.
3 Rich, P.R. (1988) Biochim. Biophys. Acta 932, 33–42.
4 Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.
5 von Jagow, G. and Link, T.A. (1986) Methods Enzymol. 126, 253–271.
6 Junge, W., Schonknecht, G. and Forster, V. (1986) Biochim. Biophys. Acta 852, 93–99.
7 Hope, A.B. and Matthews, D.B. (1984) Aust. J. Plant Physiol. 11, 267–276.
8 Linke, P., Bechmann, G., Gothe, A. and Weiss, H. (1986) Eur. J. Biochem. 158, 615–621.
9 Trumpower, B.L. (1981) J. Bioenerg. Biomemb. 13, 1–24.
10 Rich, P.R. (1983) Biochim. Biophys. Acta 722, 271–280.
11 Slater, E.C. (1973) Biochim. Biophys. Acta 301, 129–154.
12 Rich, P.R. (1989) in Highlights of Modern Biochemistry (Kotyk, A., Škoda, J., Pačes, V. and Kostka, V., eds.), pp. 903–911, VSP, Zeist.
13 Papa, S., Izzo, G. and Guerrieri, F. (1982) FEBS Lett. 145, 93–98.
14 Jones, R.W. and Whitmarsh, J. (1985) Photobiochem. Photobiophys. 9, 119–127.
15 Jones, R.W. and Whitmarsh, J. (1988) Biochim. Biophys. Acta 933, 258–268.
16 Fowler, C.F. and Kok, B. (1976) Biochim. Biophys. Acta 423, 510–523.
17 Rathenow, M. and Rumberg, B. (1980) Ber. Bunsenges. Phys. Chem. 84, 1059–1062.
18 Ivanov, B.N., Shmeleva, V.L. and Ovchinnikova, V.I. (1985) J. Bioenerg. Biomemb. 17, 239–249.
19 Hope, A.B., Birch, S. and Matthews, A.B. (1987) Aust. J. Plant Physiol. 14, 47–57.
20 Velthuys, B.R. (1980) FEBS Lett. 115, 167–170.
21 Hope, A.B. and Matthews, D.B. (1983) Aust. J. Plant Physiol. 10, 363–372.
22 Hope, A.B., Handley, L. and Matthews, D.B. (1985) Aust. J. Plant Physiol. 12, 387–394.
23 Graan, T. and Ort, D.R. (1983) J. Biol. Chem. 258, 2831–2836.
24 Hangarter, R.P., Jones, R.W., Ort, D.R. and Whitmarsh, J. (1987) Biochim. Biophys. Acta 890, 106–115.
25 Petty, K.M. and Dutton, P.L. (1976) Arch. Biochem. Biophys. 172, 335–345.
26 Petty, K. and Dutton, P.L. (1976) Arch. Biochem. Biophys. 172, 346–353.
27 Petty, K., Jackson, J.B. and Dutton, P.L. (1979) Biochim. Biophys. Acta 546, 17–42.
28 Moss, D.A. and Bendall, D.S. (1984) Biochim. Biophys. Acta 767, 389–395.
29 Polle, A. and Junge, W. (1986) Biochim. Biophys. Acta 848, 257–264.
30 Rich, P.R., Heathcote, P. and Moss, D.A. (1987) Biochim. Biophys. Acta 892, 138–151.
31 Witt, H.T. (1979) Biochim. Biophys. Acta 505, 355–427.
32 Hope, A.B. and Matthews, D.B. (1987) Aust. J. Plant Physiol. 14, 29–46.
33 Polle, A. and Junge, W. (1986) Biochim. Biophys. Acta 848, 274–278.
34 Oettmeier, W., Godde, D., Kunze, B. and Höfle, G. (1985) Biochim. Biophys. Acta 807, 216–219.
35 Ohnishi, T. and Trumpower, B.L. (1980) J. Biol. Chem. 255, 3278–3284.
36 De Vries, S., Berden, J.A. and Slater, E.C. (1980) FEBS Lett. 122, 143–148.
37 Robertson, D.E., Prince, R.C., Bowyer, J.R., Matsuura, K., Dutton, P.L. and Ohnishi, T. (1984) J. Biol. Chem. 259, 1758–1763.
38 Robertson, D.E. and Dutton, P.L. (1988) Biochim. Biophys. Acta 935, 273–291.
39 Konstantinov, A.A. and Popova, E. (1987) in Cytochromes: Molecular Biology and Bioenergetics (Papa, S., Chance, B. and Ernster, L., eds.), pp. 751–765, Plenum Press, New York.
40 Rich, P.R. (1985) Photosynth. Res. 6, 335–348.
41 Hope, A.B. and Matthews, D.B. (1988) Aust. Journ. Plant. Physiol., in press.
42 Clarke, R.D. and Hind, G. (1983) Proc. Natl. Acad. Sci. USA 80, 6249–6253.
43 Bouges-Bocquet, B. (1981) Biochim. Biophys. Acta 635, 327–340.
44 Van Kooten, O., Gloudemans, A.G.M. and Vredenberg, W.J. (1983) Photobiochem. Photobiophys. 6, 9–14.