

Calibration and time resolution of luminal pH-transients in chromatophores of *Rhodobacter capsulatus* following a single turnover flash of light: proton release by the cytochrome bc_1 -complex is strongly electrogenic

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Abstract The flash-induced proton release into the lumen of chromatophores from *Rhodobacter capsulatus* was studied with Neutral red as pH-indicator. Calibration of the acidification jump after a single flash yielded a much larger figure, at least 0.8 units, than previously thought. A slow kinetic phase of proton release (85–90% of total) was sensitive to inhibitors of the cytochrome bc_1 -complex. Its half-rise time, about 10 ms, was the same as the rise time of the electrogenic reaction in the cytochrome bc_1 -complex that was recorded by electrochromism of carotenoids. The oxidoreduction of the two b -hemes was significantly faster ($t_{1/2} \approx 3$ ms). Thus the major electrogenic event in the cytochrome bc_1 -complex is proton and not electron transfer.

Key words: Cytochrome bc_1 -complex; Ubiquinol; Proton/electron transfer; Membrane potential; Proton gradient; *Rhodobacter capsulatus*

1. Introduction

In chromatophores (i.e. membrane vesicles from certain photosynthetic bacteria) one short flash of light ($\tau < 10 \mu\text{s}$) causes the transfer of an electron within the photosynthetic reaction center (RC) from the primary donor P, a bacteriochlorophyll dimer, to the primary (Q_A) and further to the secondary bound quinone acceptor (Q_B). This very fast and electrogenic reaction is directed from the luminal (periplasmic) to the outer (cytoplasmic) side of the chromatophore membrane. A second flash causes the reduction of Q_B^- to ubiquinol. The ubiquinol molecule is then released and oxidized by the ubiquinol:cytochrome c_2 -oxidoreductase (cytochrome bc_1 -complex) near the luminal chromatophore surface. This reaction can be inhibited by myxothiazol (see [1,2] for reviews). According to the Q -cycle concept [1,3], one of the two electrons from the oxidation of ubiquinol passes through the cytochrome bc_1 -complex to cytochrome c_2 and further along the luminal surface to P^+ . The other electron moves across the membrane via two hemes b , a low-potential heme, b_l , with an absorption maximum at 566 nm and a high-potential heme, b_h , with an absorption maximum at 560.5 nm to the another ubiquinone binding center near the outer surface of the chromatophore membrane. Here molecules of the membrane ubiquinone pool are reduced to ubiquinol in an antimycin A-sensitive reaction. This ubiquinol can be re-oxidised by the myxothiazol-sensitive site; this event completes the Q -cycle. This cycle is electrogenic as the operation of the cytochrome bc_1 -complex contributes to the transmembrane voltage difference, $\Delta\phi$, and the pH-difference, ΔpH .

A single turnover flash generates a transmembrane voltage in the range of 100–200 mV. The variation between different

preparations is owed mainly to different RC/antenna bacteriochlorophyll ratios (see [2] for a review). The fast components of the voltage rise (completed in less than 1 ms) are attributable to the operation of the RC-cytochrome c_2 complex and the slower ones to the cytochrome bc_1 -complex. It has been proposed that about 60% of the electrogenic reaction in the cytochrome bc_1 -complex are due to the transmembrane electron transfer from heme b_l to heme b_h [4]. However, the formation of $\Delta\phi$ by the cytochrome bc_1 -complex proceeds more slowly ($t_{1/2} \approx 10$ ms) than the reduction of b_h ($t_{1/2} \approx 3$ ms) when the ubiquinone pool is pre-oxidized [5,6]. To explain this discrepancy it has been proposed, that the electron transfer from b_l to b_h is electrically balanced by a cotransfer of protons, and that the observed slow $\Delta\phi$ formation is caused by the subsequent back-transfer of protons across the cytochrome bc_1 -complex following the formation of ubiquinol [6,7]. The possibility of an electrogenic proton transfer is here put on a test by monitoring the kinetics of proton release in the lumen. So far the only reported kinetic trace of the luminal acidification after a single turnover flash was obtained in chromatophores of *Rhodobacter sphaeroides* which were soaked with the hydrophilic pH-indicator Phenol red at pH 6.0 [8]. It yielded a half-rise time of 20–30 ms; the generation of $\Delta\phi$ was not monitored in these experiments. Attempts to measure ΔpH at higher pH were unsuccessful because of the inability to trap sufficient amounts of other hydrophilic pH-indicators inside chromatophores. Reported estimates of the magnitude of the acidification jump that was caused by a single turnover flash ranged from <0.4 [9] to 0.003 [10] units of pH.

In chromatophores from *Rb. capsulatus* we studied luminal pH transients by the amphiphilic pH-indicator Neutral red and voltage transients by electrochromic absorption changes. We found (i) that the magnitude of the pH jump after a single flash was at least 0.8 units of pH, i.e. much greater than previously reported; (ii) that proton transfer across the cytochrome bc_1 -complex is likely the major electrogenic step.

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2. Materials and methods

Chromatophores were isolated from the cells of *Rb. capsulatus* (wild type, strain B10, grown at 30°C under high light intensity in the medium of Sistrom [11]) that were disrupted by a French-press treatment (12,000 psi) as described in [12]. Spectrophotometric measurements were performed with the set-up described in [13]. Repetitive signals (0.14 Hz) were averaged. Under the given redox conditions every flash produced one molecule of ubiquinol per 2 RCs [12] (the cytochrome bc_1 -complex over RC ratio was about 1:2 in our preparation). Absorption transients of the pH-indicator Neutral red (NR, $pK = 6.6$) were measured at 546 nm. The use of NR was instrumental for high time resolution because this surface adsorbed dye accepts rapidly protons by direct collision with the donor groups [14]. The concentration of bacteriochlorophyll in the sample was determined according to [15]. The amount of functionally active RCs and cytochrome bc_1 -complexes was estimated as in [6]. The pH of the chromatophore suspension was buffered by the membrane impermeable bovine serum albumin (BSA, 2 mg/ml). 2 mM potassium ferrocyanide was used as a redox buffer, 10 μ M N,N,N',N' -tetramethyl-*p*-phenylenediamine (TMPD) as a redox mediator (E_0 of 280–300 mV established usually in our open-air cuvette). 1 μ M oligomycin was added routinely to prevent the rapid escape of protons from the lumen through the F_1F_0 -ATP-ase; 1 mM KCN was present to prevent the oxidation of the redox-buffering system through the terminal oxidase. Antimycin A was added always along with myxothiazol to exclude the contribution from reverse reactions in the quinone-reducing center of the cytochrome bc_1 -complex [4,16,17].

3. Results and discussion

3.1. Absorption transients of NR after a single turnover flash

In the absence of Neutral red a single flash on a suspension of chromatophores from *Rb. capsulatus* caused absorbance transients at 546 nm as shown by curve a in Fig. 1. The major portion of this transient was attributable to P^+ formation and decay. The curve b in Fig. 1 represents the absorbance change measured in the presence of NR. The difference between the traces obtained in the presence and in the absence of NR was interpreted as indicating pH-transients in the lumen as previously established for thylakoids [18]. They rose rapidly and decayed slowly with $t_{1/2} \cong 150$ ms (Fig. 1, trace b-a). Similar traces which were recorded at higher time resolution (traces c and d in Fig. 1) and their difference (trace d-c in Fig. 1) showed two components of the rise: a small faster one (10–15% of the total), with $t_{1/2} \cong 150$ μ s (time-resolved trace not shown) and a larger component with $t_{1/2}$ of 9 ms.

The absorbance changes of NR were suppressed by several different pH-buffers (see e.g. trace e in Fig. 1). The relative efficiency of the buffers decreased in the following series (the concentration of buffer that caused a half decrease of the extent of absorption transients of NR at a given pH value is indicated in parenthesis): glycylglycine (1.5 mM at pH 8.1) > imidazole (2 mM at pH 8.1) > Bis-Tris-Propane (5 mM at pH 8.4) > Tris (50 mM at pH 8.4) > HEPES (60 mM at pH 8.4) > Tricine (70 mM at pH 8.2). The different sensitivity of NR absorption transients to various water-soluble buffers indicated the different accessibility of chromatophore lumen and resembled the situation observed with freshly prepared thylakoids [19]. The absorption transients of NR were enhanced by addition of the K^+ -ionophore valinomycin (Fig. 1, trace g, difference trace g-f) whereas being suppressed by the addition of the K^+/H^+ exchanger nigericin (Fig. 1, trace h). As could be seen from the difference trace j-k in Fig. 1 only the slow absorbance transients of NR were suppressed in the presence of myxothiazol and antimycin A.

The sensitivity of the absorbance transients of Neutral red

to permeating pH-buffers and to ionophores clearly showed that both phases represented pH-transients in the lumen. Only the large and slow component was myxothiazol-sensitive and therefore attributable to the oxidation of ubiquinol by the cytochrome bc_1 -complex. The small and fast component was insensitive to myxothiazol. It may be caused by a rapid deprotonation in response to the deposition of a positive charge on the luminal side after the flash. (Proton release which is caused by formation of P^+ has been previously observed with the isolated RC [20]).

3.2. $\Delta\phi$ transients after a single turnover flash

Fig. 2A shows flash-induced absorbance changes at 523 nm that are attributed to an electrochromic band shift of carotenoids [2]. The traces were obtained in the absence (trace 1) and in the presence of myxothiazol and antimycin A (trace 2), respectively. Broadly speaking, the difference between the two traces represents the electrogenic contribution of the cytochrome bc_1 -complex (Fig. 2B, dashed trace 1-2). The rise of this portion of the electrochromic transient exactly followed the rise of the slow phase of the luminal acidification (solid trace d-c). This was observed over the whole pH range from 6.5 to 9.6. The half rise time of the electrochromic transient slightly increased from 5 ms at pH 6.5 to $\cong 10$ ms at pH 9.6 (data not shown). The much faster rise of the heme b_h reduction which was measured under the same redox-conditions is presented for comparison (Fig. 2C). These observations corroborate the suggestion that the major electrogenic event in the cytochrome bc_1 -complex is the proton release into the chromatophore lumen [6,7].

3.3. Calibration of the value of the luminal acidification after a single turnover flash

In thylakoids the acidification of the lumen which is caused by a single short flash is rather small (<0.1 units of pH [18]). Thus the extent of flash-induced transients of absorption of Neutral red as function of pH resembles the first derivative of the titration curve with the maximum at pH equal to the effective pK of membrane adsorbed NR. The latter varies depending on the surface potential [18,19]. In contrast to this behaviour, in chromatophores the maximal response of NR was observed in a broader range from pH 7.0 up to about 8.5. This pattern is shown in Fig. 3 (open symbols). One straightforward interpretation of this broadening was that even a single turnover flash induced a much larger acidification of the lumen in chromatophores than in thylakoids. If so, we expected to obtain a narrower pH-profile resembling the first derivative of the titration curve after a very weak flash, exciting only 5% of the reaction centers and causing smaller pH-jump. Indeed, this was observed (filled symbols in Fig. 3, note the different scale). Thus, we mimicked the situation found in thylakoids. We fitted both sets of experimental points with the function:

$$\Delta A_{546} = C \cdot \left(\frac{10^{-(pH-\Delta pH)}}{10^{-(pH-\Delta pH)} + 10^{-pK}} - \frac{10^{-pH}}{10^{-pH} + 10^{-pK}} \right) \quad (1)$$

where pK = effective pK of NR (accounting for the effect of the surface potential on the surface pH) and C is a constant accounting for the molar extinction coefficient of NR and its effective concentration in the membrane.

A fit to the data points at low flash energy yielded the following figures: $C = 0.17$, $pK = 7.3$ and $\Delta pH = 0.07$ (Fig. 3, solid curve). The latter two values are similar to the ones found for

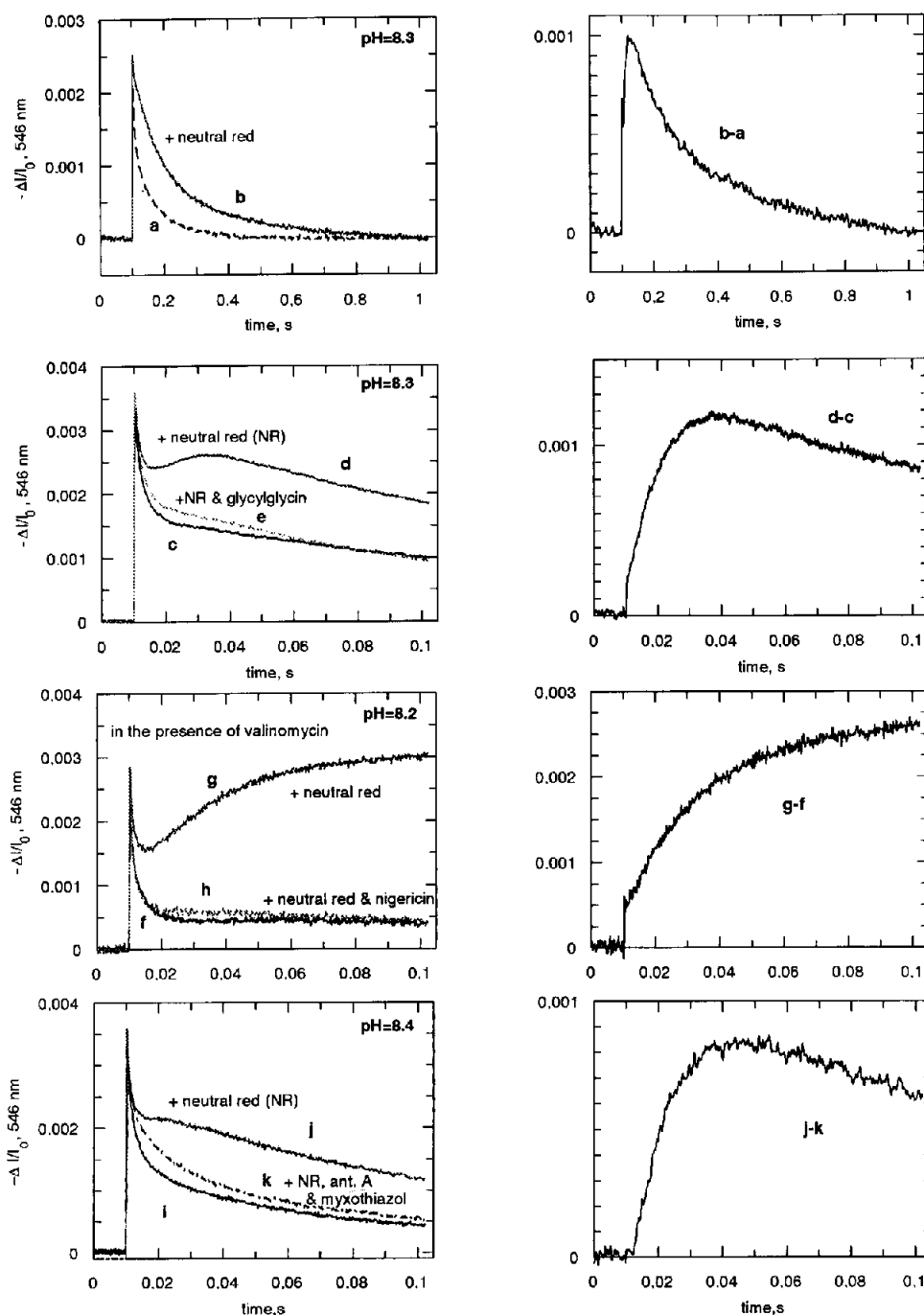


Fig. 1. Flash-induced absorption transients at 546 nm in the suspension of *Rb. capsulatus* chromatophores. a,c,i, no additions; b,d,j, +13 μM NR; f, +200 nM valinomycin; e, +13 μM NR and 10 mM glycylglycine; g, +13 μM NR and 200 nM valinomycin; k, +13 μM NR, 5 μM antimycin A and 3 μM myxothiazol; h, +13 μM NR, 200 nM valinomycin and 5 μM nigericin. Incubation medium as in section 2; 50 mM KCl was present in f–h.

thylakoids [18]. Using the same figures for C and pK to fit the experimental points obtained with a saturating flash we obtained a figure of 0.8 ± 0.2 for ΔpH (Fig. 3, dashed curve).

The redox-conditions in this work were sub-optimal for the operation of the electron transfer chain and the relative amplitude of the electrogenic reaction due to the operation of the cytochrome bc_1 -complex constituted only 65–80% of the electrogenic reaction due to one turnover of the RC at $6.5 < \text{pH} < 9.6$ (see Fig. 2). Under optimal conditions

both extents are equal [1,2]. Correspondingly, the change of the luminal pH under optimal conditions could be estimated as 1.0 ± 0.3 units of pH.

Previous estimates of the pH-jump were based on the fluorescence quenching of 9-aminoacridine [9]. They required a normalisation to the value of the internal volume of a chromatophore (v_{in}). Two extreme values of v_{in} were taken from the literature, namely 40 and 140 $\mu\text{l}/\text{mg}$ bacteriochlorophyll. Estimates of 0.28 or 0.10 units of pH resulted under conditions

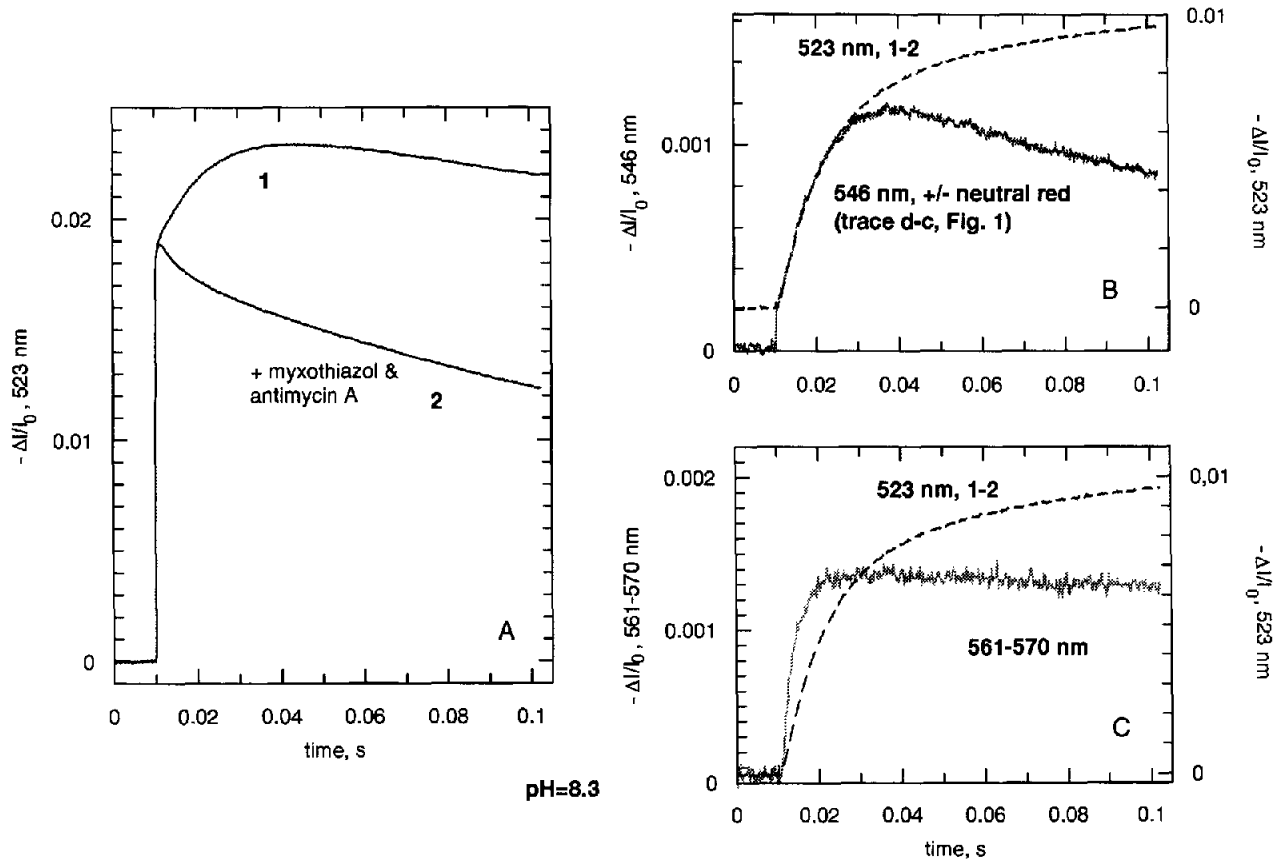


Fig. 2. Flash-induced absorption transients at 523 nm in the suspension of *Rb. capsulatus* chromatophores. (A) 1, no additions, 2, + 3 μM myxothiazol and 5 μM antimycin A. (B) Difference curve of absorption transients at 523 nm (1-2) superimposed with the difference absorption transient at 546 nm (d-c) from Fig. 1. (C) Difference curve of absorption transients at 523 nm (1-2) superimposed with the difference absorption transient 561–570 nm reflecting the heme b_h reduction and recorded under the same conditions as the trace 1 (the trace is corrected for the absorption transients of cytochromes c_1 and c_2). pH 8.3. Incubation medium as in section 2.

similar to the ones used in this work. In more recent work the value of v_{in} was estimated to be smaller, only $\approx 10 \mu\text{l}/\mu\text{M}$ bacteriochlorophyll [21]. Taking this value into account we reinterpret the results presented in [9] to indicate an acidification by about one unit, i.e. close to our own result. The extremely low value of ΔpH (0.003 units of pH) reported in [10] is difficult to explain.

Equation (2) describes the relation between the internal differential buffering capacity (β_{in}), the internal volume (v_{in}) and the amount of released protons (ΔH^+) as follows [18]:

$$\Delta\text{pH}_{in} \approx \Delta\text{H}^+ \cdot \beta_{in}^{-1} \cdot V_{in}^{-1} \quad (2)$$

In good agreement with the data from [21] the value of the internal buffering capacity β_{in} can be estimated as $\approx 4\text{--}5 \text{ mM}$ if we take the value of $10 \mu\text{l}/\mu\text{mol}$ bacteriochlorophyll for v_{in} [21] and propose that 1.6–2 protons are released after the flash per one RC (the RC/bacteriochlorophyll ratio was 1:40 in our preparation). Relating the buffering capacity to the concentration of bacteriochlorophyll one obtains a specific buffering capacity of about 1 mol/20 mol of bacteriochlorophyll which is very much alike the respective figure in thylakoids (1 mol/17 mol of chlorophyll [18]) That the pH difference in chromatophores is greater than in thylakoids is attributable to

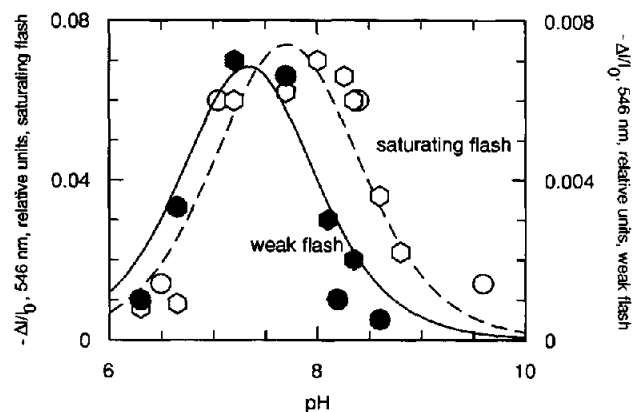


Fig. 3. pH dependence of the relative extents of the absorbance transients of NR in the suspension of *Rb. capsulatus* chromatophores induced by a saturating (open symbols) and weak (filled symbols) flash of light. The differently shaped symbols indicate different chromatophore preparations. The extent of the absorbance transients of NR measured at 546 nm were normalized to the amplitudes of absorbance changes of P^+ at 604 nm measured in the same samples after two 1 ms-spaced light flashes in the presence of 3 μM myxothiazol.

a greater density of RCs and cytochrome bc_1 -complexes (i.e. smaller antenna size).

3.4. Conclusions

Based on these data on proton release into the interior of *Rb. capsulatus* chromatophores under oxidizing conditions (E_h about 300 mV) we conclude the following:

(1) The flash-induced absorbance transients of NR in the externally buffered chromatophore suspension reflect the acidification of the chromatophore lumen;

(2) The value of ΔpH that is formed in bacterial chromatophores after a single turnover flash is about ten times larger than in thylakoids; the value of the former can reach one unit of pH under appropriate conditions;

(3) The major component of proton release into the lumen has the same half-rise time (about 10 ms) as the electrogenic reaction in the cytochrome bc_1 -complex. The reduction of heme b_h by heme b_b , on the other hand, is about three times faster. The main electrogenic reaction in the cytochrome bc_1 -complex is thus linked to proton release. This behaviour is interpreted in terms of the following reaction sequence: (a) the oxidation of ubiquinol by the cytochrome bc_1 -complex produces two electrons and two protons; (b) the protons stay bound with the cytochrome bc_1 -complex to compensate electrostatically the delivered electrons; (c) the transmembrane electron transfer from heme b_b to heme b_h is electrically silent, probably because it is accompanied by intraprotein proton rearrangement; (d) the disappearance of the additional negative charges from the cytochrome bc_1 -complex after (i) the oxidation of the cytochrome bc_1 -complex by P^+ via cytochrome e_2 and (ii) the formation of ubiquinol at the quinol-reducing site of the cytochrome bc_1 -complex causes the electrogenic proton extrusion into the lumen.

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Note added in proof

Comment to section 3.2. One might argue that the slower rate of the electrogenic reaction and proton release compared to the heme b_h reduction (Fig. 2) was caused by the contribution of two or more turnovers of the cytochrome bc_1 -complex. After a weak flash that excited only 12% of RCs and thus provided ubiquinol for only one turnover of some cytochrome bc_1 -complexes, the rate of the electrogenic reaction was not faster than after a saturating flash but slowed down to $t_{1/2} \approx 20$ ms (manuscript in preparation).