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# Proton efflux from right-side-out membrane vesicles of *Rhodobacter capsulatus* after short flashes

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**Proton release elicited by excitation of intact cells of *Rhodobacter capsulatus* strain N22 with a short flash proceeded, after a short lag, with a $t_{1/2} \approx 30$ ms. The addition of low concentrations of EDTA, a treatment which leads to enhanced permeability of the outer membrane of Gram-negative bacteria, did not increase the rate of $H^+$ efflux after a flash, but only resulted in inhibition of the extent of the reaction and a similar decline in the extent of cyclic electron transport. Proton release from vesicles prepared by treatment of intact cells of strain N22 with lysozyme and subsequent osmotic shock was faster than from intact cells. The $t_{1/2}$ value for myxothiazol-sensitive proton release was similar to the $t_{1/2}$ values for myxothiazol-sensitive cytochrome *c* re-reduction and of the myxothiazol-sensitive electrochromic absorbance change in the vesicles. However, the extents of these three processes were much lower than in intact cells because substantial quantities of cytochrome $c_2$ were lost during preparation. Addition of mammalian cytochrome *c* to these membrane preparations led to enhanced rates of reduction of reaction centre bacteriochlorophyll, proving that the vesicles were predominantly right-side-out, but not to increased rates of myxothiazol-sensitive oxidation of the cytochrome $bc_1$ complex. Proton release following flash excitation of intact cells of *Rb. capsulatus* strain MT1131 was faster than in strain N22 ($t_{1/2} \approx 10$ ms). Flash-induced $H^+$ release from intact cells of the cytochrome-$c_2$-deficient mutant, MT-G4/S4 of *Rb. capsulatus* (Daldal, F., Cheng, S., Applebaum, J., Davidson, E. and Prince, R.C. (1986) Proc. Natl. Acad. Sci. USA 83, 2012–2016) was slower ($t_{1/2}$ 23 ms) and 30% less extensive (relative to reaction centre content) than in the control strain, MT1131. In right-side-out vesicles of MT-G4/S4 the rate of proton release after a flash was faster but, as in vesicles from strain N22, the extent of the reaction was considerably less than in intact cells. These results are discussed in the context of the hypothesis that $H^+$ release after flash excitation of intact cells is restricted by immobile buffering groups in the periplasm and outer membrane (Jones, M.R. and Jackson, J.B. (1989) Biochim. Biophys. Acta 975, 34–43).**

## Introduction

*Rhodobacter capsulatus* has a cyclic electron transport chain which uses light energy to generate an electrochemical gradient of protons across the cytoplasmic membrane [1]. The proton electrochemical gradient is used to drive endergonic processes including ATP synthesis and solute transport [2]. Protons translocated across the cytoplasmic membrane following a short flash of photosynthetic light diffuse out of the bacterial cell into the surrounding medium, where the pH change may be detected using a suitable indicator dye [3,4]. However, the rate at which protons appear in the extracellular medium distal to the outer membrane of the cells is slow relative to the rate at which protons are expected to be released by the quinol oxidase ($Q_z$ or $Q_o$) site of the cytochrome $bc_1$ complex, located close to the periplasmic face of the cytoplasmic membrane [4]. It was concluded that this discrepancy can be explained by considering (a) the effect of immobile buffering groups within the periplasm and (b) the distances over which protons have to diffuse: the cytoplasmic membrane of *Rb. capsulatus* is highly invaginated and hence the periplasmic region extends to deep within the cell.

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Abbreviations: P, primary electron donor in the photosynthetic reaction centre; $bc_1$, cytochrome $bc_1$ complex (ubiquinol cytochrome *c* oxidoreductase); $Q_z$, quinol oxidase site of the cytochrome $bc_1$ complex; $Q_B$, secondary acceptor quinone of the reaction centre; $Q_c$, quinone reductase site of the cytochrome $bc_1$ complex; PMS, phenazine methosulphate; PES, phenazine ethosulphate; DAD, diaminodurene; DCPIP, 2,6-dichlorophenol-indophenol.

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However, the experiments [4] did not rule out the possibility that proton release was limited by diffusion between the $Q_z$ site and the outer face of the cytoplasmic membrane. The aim of the experiments described in this report was to distinguish between these views by measuring rates of proton efflux in cells with a permeablised outer membrane and in 'right-side-out' vesicles of the cytoplasmic membrane in which the periplasm and outer membrane have been removed. Although the chemiosmotic hypothesis is now generally adopted in the description of electron transport phosphorylation, it should be recognised that anomalies and complexities arising from proton efflux experiments with intact bacterial cells have led to alternative formulations of the energy coupling mechanism (briefly reviewed [3,3a]). The use of permeablised intact cells and of right-side-out membrane vesicles may go some way to help resolve this difficulty. In a recent report from Arata and colleagues it was found that proton release following flash excitation of right-side-out vesicles prepared from *Rb. sphaeroides* was still diffusionally restricted [5]. In that system the rate of proton release was still substantially slower than the expected maximum rate of quinol oxidation at the $Q_z$ site of the cytochrome $bc_1$ complex.

## Materials and Methods

*Rb. capsulatus* strains N22 (a green mutant of strain St. Louis, isolated by Dr N.G. Holmes, University of Bristol), MT-G4/S4 (a cytochrome-$c_2$-deficient mutant [6], provided by Dr F. Daldal, University of Pennsylvania) and MT1131 (a control strain for MT-G4/S4, also provided by Dr Daldal) were grown photosynthetically under anaerobic conditions on 'RCV medium' [7], as described [8]. Strain MT-G4/S4 was grown in the presence of 25 $\mu$g/ml kanamycin. All strains were stored in 20% glycerol at $-70$°C and cultures were routinely checked for their antibiotic sensitivity [6]. Strain N22 is sensitive to kanamycin, spectinomycin and rifampicin (unpublished data). Cells were harvested, washed by centrifugation and resuspended as a thick suspension in 10 mM $Na_2SO_4$ (for intact cell measurements) or in the appropriate medium for the preparation of vesicles (see below). The bacteriochlorophyll concentration was assayed by extraction in acetone/methanol (7 : 2) as described [9].

Right-side-out vesicles were prepared by a modification of the procedure described in [10,11]. Harvested cells were resuspended to 0.1 g wet wt/ml 0.6 M sucrose, 2.5 mM $Na_2EDTA$ and 50 mM potassium phosphate (pH 7.6) plus 0.2 mg/ml lysozyme. The solution was incubated for 40 min at 30°C, $MgCl_2$ was added to 10 mM and the solution centrifuged at 10000 $\times g$ for 30 min to give a pellet of spheroplasts. The supernatant (periplasmic fraction) was decanted and stored on ice. The pellet was resuspended with gentle homogenisation in 4 times the original volume of medium containing 2.5 mM $Na_2EDTA$ and 50 mM potassium phosphate (pH 7.6) at 30°C and incubated for 15 min. $MgCl_2$ was added to 10 mM together with a trace of DNase and the incubation was continued for a further 15 min. The solution was then centrifuged at 10000 $\times g$ for 30 min, the supernatant (cytoplasmic fraction) was decanted and stored on ice, and the pellet was washed by resuspension and centrifugation, first in 50 mM potassium phosphate (pH 7.6) ('phosphate wash') and then in 10 mM $Na_2SO_4$ ('sulphate wash'). The pellet of vesicles was resuspended in 10 mM $Na_2SO_4$.

Malate dehydrogenase was assayed monitoring NADH oxidation at 340 nm. The medium was 50 mM Tris-HCl (pH 8.0), 0.267 mM NADH and 0.2 mM disodium oxaloacetate. Succinate dehydrogenase was assayed by following DCPIP reduction at 600 nm. The medium was 0.1 M potassium phosphate (pH 7.0), 0.0167% (w/v) DCPIP, 3.33 mM KCN and 1.33 mM sodium succinate. TMAO reductase was assayed by following TMAO-dependent oxidation of dithionite-reduced methylviologen as described in Ref. 12. *c*-type cytochrome was estimated from difference spectra (dithionite-reduced minus ferricyanide-oxidised).

Flash-induced absorbance changes of whole cells and vesicles were recorded at 30°C under an atmosphere of argon (less than 3 ppm oxygen). All samples were pre-incubated under argon for 30 min with gentle stirring before flash excitation was begun. The stirrer was switched off during measurement. Experimental media are described in the figure legends. Absorbance changes were monitored as described in Ref. 4. The spectrophotometer cuvette was fitted with a Pt/calomel combination electrode for measurement of redox potential (see figure legends for mediators) and with a glass electrode for measurement of suspension pH during recordings of Cresol red absorbance changes. Absorbance changes attributed to the bacteriochlorophyll special pair (P) were recorded at 542 nm ($E_{mM} = 10.3$ $cm^{-1}$) [13]. Those attributed to *c*-type cytochrome were recorded at 552–542 nm and the electrochromic band shift attributed to changes in the transmembrane electrical potential was monitored at 503 nm. Flash-induced changes in medium pH were monitored at 587.5 nm using 100 $\mu$M Cresol red as described [4]. Suspensions of vesicles or cells in 10 mM potassium sulphate were poised at approx. pH 7.6 by the addition of small volumes of dilute HCl or KOH. Underlying buffer-insensitive absorbance changes at 587.5 nm were corrected by subtracting a trace recorded under identical conditions to the experimental trace but in the presence of buffer (10 mM potassium phosphate). The extent of the Cresol red absorbance change was calibrated by the addition of 5 $\mu$l 1 mM HCl at the end of each experiment. The total amount of photooxidisable P in the

preparations was estimated in the presence of 5 μM myxothiazol and 5 μM FCCP after multiple flash excitation; in experiments with cells the suspension was made aerobic before estimation of P [4] and in those with vesicles the $E_h$ was adjusted to a value in the region of +350 mV. Individual flashes were approx. 96% saturating under the conditions used [4].

## Results

*$H^+$ efflux after short flashes from cells of Rb. capsulatus strain N22 with permeablised outer membranes*

Following short flash excitation of intact cells of *Rb. capsulatus* strain N22 in weakly buffered media, pH changes were monitored from the absorbance change of added Cresol red. As described previously [4], $H^+$ were released from the cells after a short lag, duration approx. 1 ms, with a $t_{1/2} \approx 30$ ms (data not shown). The extent of proton release amounted to about 1.1 $H^+/P$ (see discussion in Ref. 4). Note that analysis of the rates of re-reduction of photooxidised cytochrome ($c_2 + c_1$) in the presence and absence of myxothiazol has indicated that quinol oxidation at $Q_z$ the probable site of $H^+$ release from the cytoplasmic membrane, takes place with a $t_{1/2} \approx 2.5$ ms [4]. EDTA is known to affect the integrity of the outer membrane of Gram-negative bacteria and has been used experimentally as a permeabilising agent [14]. The addition of 100 μM EDTA to a suspension of cells of *Rb. capsulatus* led to the loss of the flash-induced Cresol red signal over a period of about 1 h (data not shown). In control suspensions in the absence of EDTA the absorbance change decreased only slightly during such a period. The loss of the Cresol red signal with EDTA was due in part to an increase in the buffering capacity of the suspension but also to a real decrease in the value of $H^+/P$. In parallel experiments it was found that this concentration of EDTA also decreased the extent of the flash-induced absorbance changes attributable to P, cytochrome ($c_1 + c_2$) and the electrochemically sensitive carotenoids. Evidently incubation of cells of *Rb. capsulatus* with low concentrations of EDTA under these conditions leads to inactivation of the photosynthetic electron transport system. The reason for this was not pursued. There was no evidence of acceleration of the rate of the flash-induced Cresol red absorbance change at any time during the period of incubation with EDTA.

*$H^+$ efflux from vesicles of Rb. capsulatus strain N22*

Using protocols developed for the preparation of right-side-out vesicles from other Gram-negative organisms, membrane fractions of *Rb. capsulatus* strain N22 were prepared. Assays of marker enzymes suggest that the preparation was successful (Table I). Thus, malate dehydrogenase was almost entirely (≈ 95%) associated with the 'cytoplasmic' fractions released after osmotic shock of the spheroplasts and subsequent washing of the membrane with phosphate buffer. No further malate dehydrogenase activity was recovered after sonicating the vesicles. Succinate dehydrogenase activity was found predominantly in the membrane fraction. Trimethylamine *N*-oxide (TMAO) reductase and *c*-type cytochrome, both periplasmic proteins [12,15] were found in both the 'periplasmic' fraction and the 'cytoplasmic' fraction. This may indicate (a) that digestion of the outer cell membrane was incomplete, (b) that these proteins were weakly bound to the outer face of the cytoplasmic membrane after spheroplast preparation, or (c) that the spheroplasts retained regions of invaginated membrane in which cytochrome *c* and TMAO reductase were trapped. Since all the malate dehydrogenase was released after osmotic shock of the vesicles, it is very unlikely that the preparation contained any intact cells. Examination by electron microscopy of ultrathin sections of a vesicle pellet, positively stained with uranyl acetate and lead citrate, revealed unilamellar closed membranes, roughly circular in shape, with diameters in the range 50–150 nm. There was no evidence of any internal structure (data not shown). The question of the polarity of the vesicles is addressed below.

Short flash excitation of suspensions of the membrane preparation in the presence of Cresol red at a redox potential in the region of +100 mV led to small

TABLE I

*Distribution of marker enzymes and c-type cytochrome in fractions obtained during the preparation of membrane vesicles*

Marker enzymes were assayed as in Materials and Methods. Activities (or quantities in the case of cytochrome *c*) are expressed as percentages of the total. Key: Pp, periplasmic fraction (supernatant from EDTA/lysozyme treated cells); C, cytoplasmic fraction (supernatant from osmotically-shocked spheroplasts); $PO_4$, supernatant from vesicles washed with phosphate; $SO_4$, supernatant from vesicles washed with sulphate; M, membrane vesicles.

| Strain | Malate dehydrogenase | | | | | Succinate dehydrogenase | | | | | TMAO reductase | | | | | Cytochrome *c* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pp | C | $PO_4$ | $SO_4$ | M | Pp | C | $PO_4$ | $SO_4$ | M | Pp | C | $PO_4$ | $SO_4$ | M | Pp | C | $PO_4$ | $SO_4$ | M |
| N22 | 0 | 83 | 14 | 0 | 3 | 0 | 6 | 5 | 1 | 88 | 28 | 58 | 2 | 4 | 8 | 22 | 41 | 6 | 0 | 31 |
| MT1131 | 0 | 66 | 30 | 1 | 3 | 0 | 1 | 4 | 1 | 94 | 4 | 78 | 5 | 0 | 13 | ND | ND | ND | ND | ND |
| MT-G4/S4 | 1 | 85 | 8 | 2 | 4 | 0 | 6 | 2 | 4 | 88 | 6 | 66 | 21 | 0 | 7 | 0 | 0 | 0 | 0 | 100 |

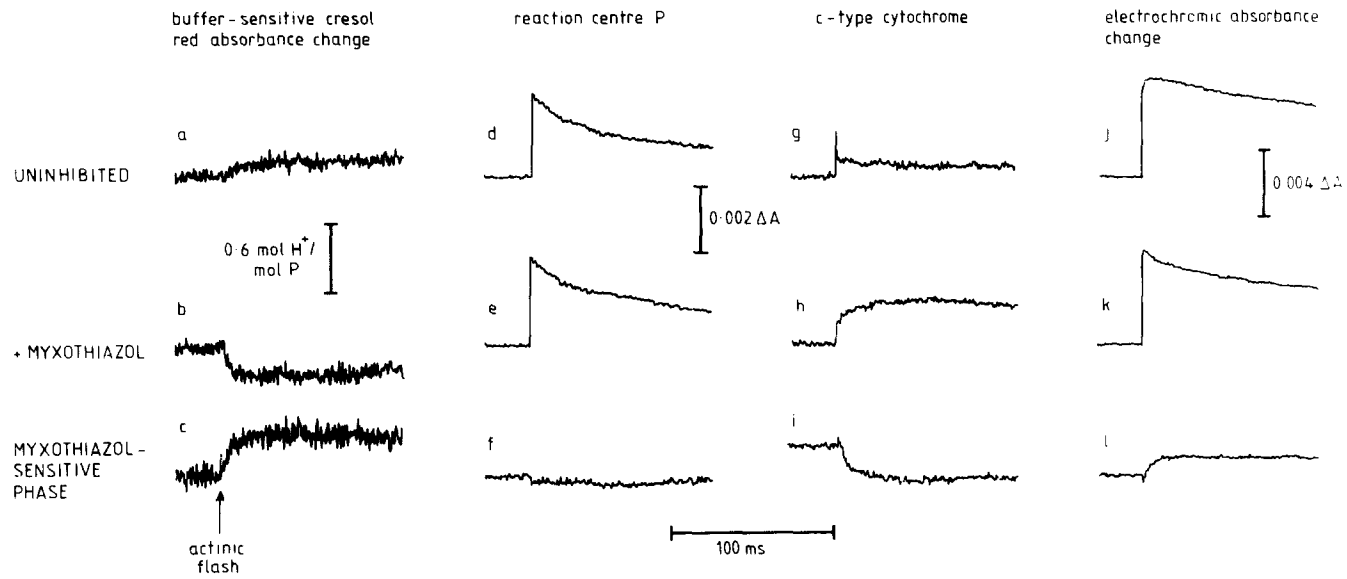


Fig. 1. Absorbance changes attributable to medium pH, redox reactions of P and cytochrome *c* and membrane potential following short flash excitation of vesicle preparations of *Rb. capsulatus* strain N22. Vesicles were suspended to 0.2 μM P in either 10 mM potassium sulphate/100 μM Cresol red (pH 7.6) (a–c) or 10 mM potassium phosphate (pH 7.6) (d–l) with 2 μM PMS, 2 μM PES and 10 μM pyocyanine. The $E_h$ was adjusted to approx. +100 mV with dilute sodium dithionite solution. In traces (a) and (b), poising of the medium pH, correction for underlying buffer-insensitive absorbance changes and calibration of the Cresol red absorbance changes were performed as described in Materials and Methods. In (b), (e), (h) and (k), myxothiazol was added to 5 μM. Traces (c), (f), (i) and (l) were obtained by subtracting data collected in the presence of myxothiazol from that collected in its absence. Traces (a) and (b) are the average of 100 sweeps, traces (d), (e), (g) and (h) the average of 16 sweeps and traces (j) and (k) the average of eight sweeps, all at a sweep frequency of 0.0167 Hz.

absorbance changes which can be partly attributed to changes in the pH of the medium. Fig. 1a shows the absorbance change at 587.5 nm in the absence of added buffer minus that recorded in the presence of 10 mM potassium phosphate (pH 7.6). It is evident that a proton release reaction of low amplitude (maximally 0.14 mol $H^+$/mol P) followed excitation by the flash. In the presence of 5 μM myxothiazol (sufficient completely to inhibit electron transport through the $bc_1$ complex [16]) the buffer-dependent absorbance change revealed a proton uptake reaction (maximally 0.23 mol $H^+$/mol P) after the flash (Fig. 1b). The myxothiazol-sensitive component, a flash-induced proton release reaction (maximally 0.34 mol $H^+$/mol P), is revealed by the subtracted data in Fig. 1c. The rate of this $H^+$ release was considerably faster ($t_{1/2} \approx 4$ ms) than in intact cells of strain N22. One reason for the much decreased amplitude of the proton release reaction, compared with that in cells, is evident from Fig. 1. (1) In vesicles poised at an $E_h = +100$ mV only a small fraction (usually approx. 20%) of P oxidised by the flash was re-reduced within 1 ms. In contrast, in strictly anaerobic cells of this strain of bacteria, more than 85% of P oxidised by a flash was re-reduced within 1 ms [4]. (2) At 552–542 nm, absorbance changes due to oxidation of *c*-type cytochrome following flash excitation of the vesicles were very small, equivalent to approx 0.2 mol/mol P, with a $t_{1/2} \approx 1$ ms in the presence of myxothiazol. The spectrum of this absorbance change had a maximum at 552 nm (relative to a reference at 542 nm). (3) The myxothiazol-sensitive (phase III) of the electrochromic absorbance change was also of low amplitude (Fig. 1l) compared with that seen in whole cells, indicating that very little charge separation through the cytochrome $bc_1$ complex took place after the flash. All these observations point to the fact that photosynthetic electron transport in the vesicles was severely restricted by loss of cytochrome $c_2$ during preparation.

In view of heterogeneity in the orientation of the membrane vesicles (see below) it was necessary to investigate whether the different populations of vesicles behaved similarly with respect to the reaction between *c*-type cytochrome and P. Thus the photooxidation of *c*-type cytochrome in the vesicles was examined in more detail. The $E_h$ dependence of the reaction after a single flash is shown in Fig. 2 (open symbols). The midpoint for attenuation of the reaction was approx. +310 mV. Further oxidation of *c*-type cytochrome was observed when subsequent flashes were fired at high frequency. The redox titration of the amount of *c*-type cytochrome oxidised after 30 closely spaced flashes is shown in Fig. 2 (closed symbols); the reaction was attenuated with a significantly lower midpoint potential (+275 mV) than that for the single flash. The total amount of photo-oxidisable *c*-type cytochrome (measured after 30 flashes) was similar to the amount of *c*-type cytochrome in the vesicles as revealed by dithionite minus ferricyanide difference spectra (not shown) and was equivalent to 0.8

mol/mol P. Fig. 2 shows that the single flash and multiple flash data can be adequately explained by a simple model in which it is assumed that all vesicles behave as though a decreased amount of cytochrome $c_2$

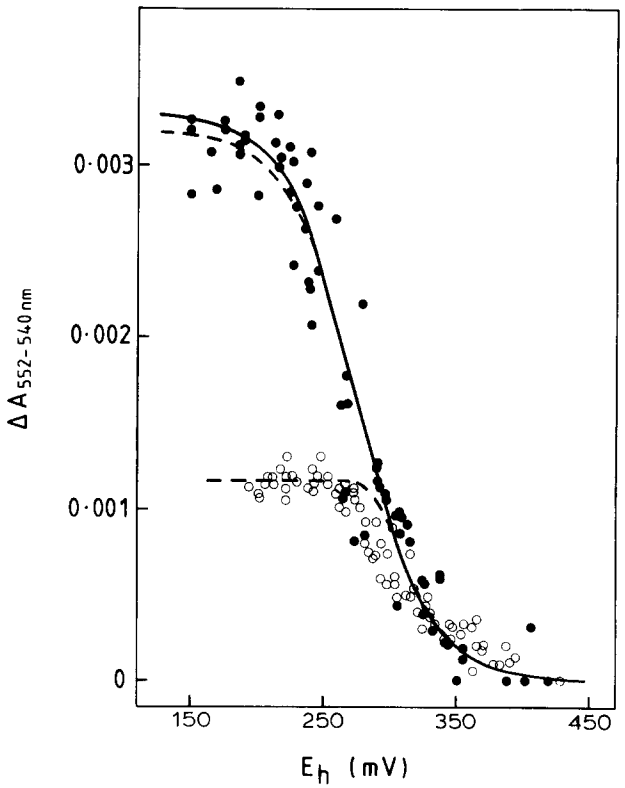


Fig. 2. Redox titration of the extent of photo-oxidation of *c*-type cytochrome in vesicle preparations of *Rb. capsulatus* strain N22 measured 100 ms after a single flash (○) or after 30 flashes fired at 40 Hz (●). Vesicles were suspended to 0.2 μM P in 10 mM potassium phosphate (pH 7.6) with 2 μM, PMS, 2 μM PES, 10 μM DAD, 5 μM myxothiazol and 5 μM FCCP. The $E_h$ was adjusted using dilute solutions of potassium ferricyanide and sodium dithionite. The solid line is the theoretical Nernst curve ($n=1$) for a component, $E_m = +275$ mV. The dashed lines are the predictions of a model constructed to illustrate the redox potential dependence of *c*-type cytochrome photooxidation in vesicles exposed to a single flash or multiple flashes. The model assumes that there is a relatively large pool of cytochrome $c_1$ ($E_m = +275$ mV, 98% of total *c*-type cytochrome), an excess of oxidised reaction centres, and a relatively small pool of cytochrome $c_2$ ($E_m = +345$ mV, 2% of total *c*-type cytochrome) which mediates electron transfer from cytochrome $c_1$ to reaction centre. This mediation occurs at a rate which has been set such that when it is maximal it gives rise to the level of *c*-type cytochrome oxidation seen after one flash at potentials below +280 mV. At potentials higher than this the rate of mediation by cytochrome $c_2$ is proportional to the amount of reduced $c_2$. The amount of *c*-type cytochrome oxidised after 30 flashes at any potential is given by the sum of the amount of reduced cytochromes $c_1$ plus $c_2$ and in this model approximates to a Nernst curve ($n=1$) with a midpoint at +275 mV. The amount of *c*-type cytochrome oxidised after one flash is given either by the number of reducing equivalents that can be transferred from the cytochrome $c_1$ pool to reaction centre pool by cytochrome $c_2$, or by the number of reducing equivalents in the cytochrome $c_1$ pool, whichever is limiting.

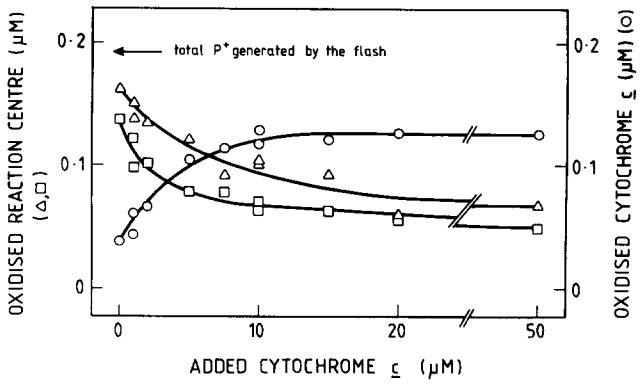


Fig. 3 The effect of horse-heart cytochrome *c* on the amount of oxidised reaction centre detected 0.5 ms (△) or 5 ms (□) after a saturating, single-turnover flash in membrane vesicles from *Rb. capsulatus* strain N22. The amount of oxidised cytochrome *c* 0.5 ms after the flash is shown (○). Vesicles were suspended to 0.2 μM P in 10 mM potassium phosphate (pH 7.6) with 2 μM PMS, 2 μM PES and 10 μM DAD. The $E_h$ was adjusted to +170 mV with sodium dithionite. Measurements of flash-induced absorbance changes were recorded as in Materials and Methods. The concentration of oxidi sed cytochrome *c* was estimated using $E_{mM} = 19$ cm$^{-1}$ [13]. Also indicated is the total amount of $P^+$ generated by a 96% saturating flash.

mediates electron transport between the $bc_1$ complex and the reaction centre (see Discussion).

Parenthetically, it may be noted that, although redox titrations of these vesicle preparations were reversible in the range +200 to +450 mV (oxidative and reductive titrations yielded similar results, see, for example, Fig. 2), this was not the case below approx. +100 mV, indicating lack of equilibrium between the electron transport components and the electrode. This problem could have arisen if the vesicles contained a large pool of a low-potential couple which reacted only slowly with the redox dyes, but it was not examined in detail.

The addition of cytochrome *c* from horse heart to a suspension of the vesicles led to rapid rereduction of $P^+$ generated by a flash and to the appearance of a signal corresponding to the rapid photooxidation of a *c*-type cytochrome (Fig. 3). Not all the $P^+$ was accessible to the added reduced cytochrome *c*. Even at high concentrations of the added cytochrome approx. 35% of the total $P^+$ was not reduced within 5 ms of the flash. In contrast, note that the addition of cytochrome *c* to our chromatophore preparations did not lead to any increase in the rate of re-reduction of $P^+$ [17]. Thus, if it is assumed that cytochrome *c* can only donate electrons to $P^+$ from the periplasmic side of the membrane [15], these data suggest that the vesicles prepared by osmotic shock (Table I) are not exclusively of a non-inverted (right-side-out) polarity. At least 35% of the reaction centres are located in inverted membranes, a further 20% are of indeterminate polarity (by this criterion) since they are reduced by endogenous *c*-type cyto-

chrome within a few milliseconds of the flash and at least 45% are located in right-side-out vesicles.

Although the addition of cytochrome *c* led to rapid re-reduction of $P^+$ and to a concomitant cytochrome *c* oxidation signal after a flash, it did not give rise to enhanced levels of electron transport through the cytochrome $bc_1$ complex in right-side-out vesicles, i.e., cyclic transport was not reconstituted. Thus, there was neither an increase in myxothiazol-sensitive cytochrome *c* rereduction nor an increase in the myxothiazol-sensitive electrochromic absorbance change after a flash. This was the case over a range of concentration of cytochrome *c* (1–20 μM) when the $E_h$ was poised between +160 and +190 mV and when the vesicles were suspended in either 10 mM potassium phosphate buffer (pH 7.6), in 50 mM glycylglycine, 10 mM KCl (pH 7.6 or in 50 mM glycylglycine (pH 7.6) (compare with Ref. 18). In summary, we could not establish conditions under which exogenous cytochrome *c* could rapidly accept electrons from the $bc_1$ complex of these right-side-out vesicles, and hence increase the extent of turnover of the $Q_z$ site with consequent enhanced $H^+$-release. In proteoliposomes containing photosynthetic reaction centres, $bc_1$ and either cytochrome *c* [19] or cytochrome [18] there is evidence of reconstituted cyclic electron transport. However, under single turnover conditions [18] it was pointed out that oxidation of the $bc_1$ complex was very slow and was probably limited by the unfavourably low ratio of oxidised to reduced *c*-type cytochrome. A similar argument may also apply to the reaction between $bc_1$ and cytochrome *c* in the right-side-out membrane vesicles described here.

*$H^+$ efflux from intact cells and right-side-out vesicles of Rb. capsulatus strains MT1131 and MT-G4/S4*

*Rb. capsulatus* strain MT-G4/S4 is deficient in cytochrome $c_2$ and, according to [6,20], is able to catalyse rapid and direct electron transport from cytochrome $c_1$ in the $bc_1$ complex to the reaction centre. This being the case it should be an ideal system for the study of $H^+$ release from the $Q_z$ site of the $bc_1$ complex in right-side-out vesicles, since cyclic electron transfer should be less affected by the loss of periplasmic components.

Flash-induced $H^+$ efflux from strictly anaerobic intact cells of *Rb. capsulatus* strain MT-G4/S4 and a control strain MT1131 are shown in Fig. 4. In strain MT1131 the extent of $H^+$ efflux (relative to the amount of P) was lower (0.75 $H^+$/P) than that found in cells of strain N22 and the-rates in different cell preparations were consistently faster (typically $t_{1/2} \approx 10$ ms). Even so, the rate of $H^+$ efflux in cells of MT1131 was considerably slower than the rate of turnover of the $bc_1$ complex (see [21]). In cells of the cytochrome-$c_2$-deficient mutant MT-G4/S4 the extent of $H^+$ efflux after a flash was generally about 30% less (relative to the amount of P) than in MT1131 and the rate somewhat

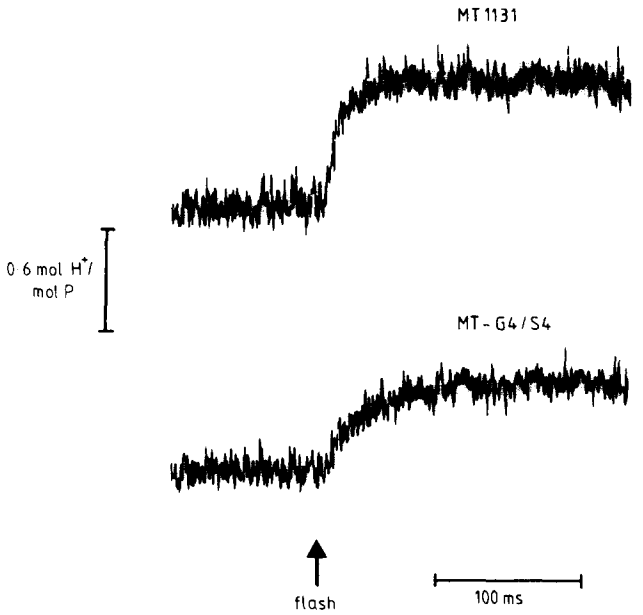


Fig. 4 Flash-induced $H^+$ efflux from intact cells of strains MT1131 and MT-G4/S4 of *Rb. capsulatus*. Cells were suspended to 0.2 μM P in 10 mM potassium sulphate/100 μM Cresol red. Poising of the medium pH (at pH 7.6), correction for underlying buffer-insensitive absorbance changes and calibration of the Cresol red absorbance changes were performed as described in Materials and Methods. Both traces are the average of 100 sweeps at a sweep frequency of 0.0167 Hz.

slower ($t_{1/2} \approx 23$ ms, see Fig. 4). The rates of electron transport through the $bc_1$ complex of intact cells of MT-G4/S4 are discussed in the accompanying paper [21]. In cells of both MT1131 and MT-G4/S4 the $H^+$-release elicited by a flash was completely sensitive to 5 μM myxothiazol (not shown). The important point here relates to experiments with vesicles prepared by osmotic shock from cells of MT-G4/S4. The distribution of marker enzymes during the vesicle preparation (Table I) was very similar to that found in strain N22. However, despite expectations [6,20], $H^+$ efflux from the vesicles after a flash, though more rapid than in cells ($t_{1/2} \approx 7$ ms), was not significantly more extensive than in vesicles of strain N22 (data not shown).

## Discussion

Treatment of intact cells of *Rb. capsulatus* strain N22 with EDTA which is often used to permeabilise Gram-negative bacteria [14], did not lead to accelerated rates of $H^+$ efflux after flash excitation. In fact, this treatment led to inhibition of cyclic electron transport and warns that uncritical use of EDTA as a permeabilising agent can lead to unwanted effects. Although there was no evidence for an accelerated rate of $H^+$ efflux at any of the tested concentrations of EDTA, this result is not meaningful in the context of the mechanism of proton translocation in bacterial cells.Experiments with vesicles

derived from spheroplasts of *Rb. capsulatus* by osmotic shock were complicated by the fact that the vesicles were of mixed polarity. That flash excitation elicited net proton release suggests that the vesicles were predominantly right-side-out (Fig. 1a). This is supported by the observation that only 35% of $P^+$ generated by a flash could not be re-reduced by high concentrations of exogenous cytochrome *c* (Fig. 3). Myxothiazol, in blocking the turnover of the $bc_1$ complex, should prevent both $H^+$-binding at the quinone reductase ($Q_c$) site and $H^+$-release from the $Q_z$ site. Therefore, although the rate of the reaction is slower than that found in chromatophores [22], $H^+$-binding by the vesicles in the presence of myxothiazol (Fig. 1b) can be attributed to protonation at the $Q_B$ site if the reaction centre in inverted membranes. The extent of this process (0.23 mol $H^+$/mol P) is also in approximate agreement with the conclusion that about 35% of the vesicles were inside-out. On this basis and assuming (see below) that the right-side-out and the inverted vesicles were equivalently depleted of cytochrome $c_2$, then the myxothiazol-sensitive pH change (Fig. 1c) is mainly the result of $H^+$-release accompanying quinol oxidation at the $Q_z$ site, with a minor contribution from $H^+$-binding at the $Q_c$ site of the $bc_1$ complex. Given the probable ratio of right-side-out to inverted vesicles, and the low level of electron flux through the $bc_1$ complex under these conditions (Fig. 1 j–l), this latter component would increase the extent of $H^+$-release by, at most, 10%.

The redox titrations of cytochrome *c* photooxidation after single and multiple flashes are consistent with the view that cytochrome $c_2$ ($E_{m7.0} = +345$ mV [13,23]) was lost from the membranes during preparation. Thus after repeated flash excitation the attenuation of oxidation of *c*-type cytochrome approximated to a Nernst curve ($n = 1$) with a midpoint potential ($E_{m7.6} = +275$ mV, Fig. 2) corresponding closely to that of cytochrome $c_1$ ($E_{m7.0} = +285$ mV [23]). This indicated that, of the two *c*-type cytochromes, cytochrome $c_1$ had by far the larger capacity for oxidising equivalents. After a single flash the extent of oxidation of *c*-type cytochrome ($E_{m7.0} = +310$ mV) was not in direct proportion to the amount of reduced cytochrome $c_1$, suggesting that under these conditions redox equilibrium was not achieved. The simplest explanation for this behaviour is that a low concentration of a higher potential component (cytochrome $c_2$) is responsible for transferring oxidising equivalents from the reaction centre to cytochrome $c_1$ under these conditions. Hence, at higher potentials (above + 300 mV) the extent of *c*-type cytochrome oxidation is limited by the available reduced cytochrome $c_1$, but at lower potentials it is limited by the rate at which cytochrome $c_2$ can transfer oxidising equivalents to the cytochrome $c_1$ pool. An illustration of this behaviour which adequately fits the data is also shown in Fig. 2.

Because of the small size of the signal and the subtractions that are necessary to reveal the component reactions, some caution must be exercised in describing the rate of $H^+$ release from the $Q_z$ site (Fig. 1c). However, a minimal conclusion is that it is much faster ($t_{1/2} \approx 4$ ms) than in intact cells of the same strain ($t_{1/2} \approx 30$ ms, [4]). Moreover, the half-time for myxothiazol-sensitive proton release (Fig. 1c) after a flash in vesicles is similar to that of myxothiazol-sensitive cytochrome *c* re-reduction (Fig. 1i) and of the myxothiazol-sensitive electrochromic absorbance change (Fig.1l). Thus, there is no apparent delay between the $H^+$ release reaction and the oxidation of quinol at the $Q_z$ site. The lag and the extended $t_{1/2}$ of $H^+$-release after flash excitation of intact cells is evidently due to the properties of the periplasm and outer cell membrane which are removed during preparation of the vesicles. This is consistent with the earlier suggestion [4] that immobile buffers in the outer layers of the cell decrease the effective diffusion coefficient for protons (see also Ref. 24). In an earlier publication [3a] it was pointed out that measurements of electron transport-linked proton efflux in suspensions of bacterial cells can be complicated by metabolically related pH changes. It was suggested [3a] that the problem may be severe after prolonged periods of electron transport such as during respiratory 'bursts' in *Escherichia coli* [25] and *Paracoccus denitrificans* [26]. After short excitation flashes in phototrophic organisms the pH change appeared to derive exclusively from electron transport [3,4]. The conclusion (above and Ref. 4) that the only kinetic barrier to proton diffusion is immobile buffer in the bacterial periplasm makes more sophisticated interpretation unnecessary, cf. Refs. 25, 26. Interestingly, it was observed in glass electrode recordings [26] that proton extrusion after respiratory bursts was faster in protoplasts than in intact cells of *P. denitrificans*, although the phenomenon was differently explained.

Somewhat different results to those described here were recently reported by Arata et al [5]. Their vesicles (of *Rb. sphaeroides*) also lost cytochrome $c_2$ during preparation and, as in those from *Rb. capsulatus* (above), about 35% of the $P^+$ generated by a flash was inaccessible to added cytochrome *c*. However, $H^+$ efflux from *Rb. sphaeroides* vesicles poised at an $E_h$ of 150 mV and at pH 6.3 was much slower ($t_{1/2} \approx 70$ ms) than cytochrome *c* re-reduction and charge separation through the cytochrome $bc_1$ complex. Furthermore, unlike in *Rb. capsulatus*, the $H^+$ release after a flash was enhanced by added cytochrome *c* while remaining sensitive to myxothiazol. The explanation advanced by Arata et al, and supported by multiple turnover experiments, was that their membrane vesicles existed as aggregrates from which proton release was limited. It is not clear why vesicles prepared from *Rb. capsulatus* and *Rb. sphaeroides* should differ in this respect. However, there

were also minor differences in the preparation procedure.

Proton efflux from *Rb. capsulatus* strain MT1131 after short flash excitation was faster than from strain N22. Within the framework of the model outlined in Ref. 4, this can be explained either by a decreased buffering capacity in the outer layers of the cells of MT1131 or by a difference in the depth of the intracytoplasmic membrane. These alternatives cannot be easily distinguished given the precision of the available measurements [4]. That flash-induced proton efflux from cells of the $c_2$-deficient mutant, MT-G4/S4, was slower and less extensive than in the control strain, MT1131, can also be explained on the basis of structural differences. However, in the case of MT-G4/S4 it seems likely that a decreased rate of electron transfer through the $bc_1$ complex in intact cells [21] is also a contributory factor to the slow rate of $H^+$ efflux. The finding that $H^+$ efflux following flash excitation of vesicles from MT-G4/S4 was not extensive may indicate that the pathway of electron transport in this organism is more complex than formerly proposed [6,20]. This possibility is explored in the subsequent report [21].

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