Rapid proton release accompanying photosynthetic electron transport in intact cells of *Rhodopseudomonas capsulata*

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The release of protons from intact cells of *Rhodopseudomonas capsulata* after either 4 μs flashes or during brief periods of continuous illumination has been measured with the indicator, cresol red. The half-time for H^+-release after a flash was 35 ms and the extent, $1H^+$ per 134 bacteriochlorophyll. Myxothiazol completely inhibited the flash-induced H^+-release and antimycin A reduced it by 37%. The proton-releasing reaction is discussed with reference to the protonmotive Q-cycle. During continuous illumination the rapid phase of H^+ release is followed by a lag and then by another period of acidification, suggesting that other protolytic reactions may be in operation.

Proton translocation *Photosynthesis* *Electron transport* *Photosynthetic bacteria*

1. INTRODUCTION

Light-induced proton efflux observed with a glass electrode in intact cells of photosynthetic bacteria [1–5] and light-induced proton uptake by topologically inverted chromatophore membranes [1,6] have been taken as evidence that the photosynthetic electron transport chain behaves as a proton translocator. The kinetics of light-driven proton uptake in chromatophores have been studied in detail using rapidly responding indicators of the external pH [7–11]. The stoichiometry, inhibitor sensitivity and redox potential dependence are consistent with a direct coupling between proton binding and electron transport although it has not yet been possible to identify unequivocally the proton binding reactions or to elaborate a mechanism of proton translocation. Proton release on the other (periplasmic) face of the membrane has been observed on a rapid time scale using pH indicators trapped inside chromatophore vesicles during preparation, although extensive use of this technique is prohibited by the fact that the indicators appear to leach slowly from the chromatophore lumen during measurement [10].

Measurements of H^+ efflux from intact cells with pH indicators have been briefly reported [7] but not on a kinetic scale. The present series of experiments was prompted by the finding that the acidification of suspensions of the chemotrophic bacteria, *Escherichia coli* and *Paracoccus denitrificans* measured with a glass electrode in response to small oxygen pulses was slower than the period of respiration as calculated from steady-state rates [12] or as measured directly with a rapidly responding oxygen electrode [13]. The conclusion from these findings was that the pH changes measured with the glass electrode are too slow to represent proton efflux compulsorily coupled to electron transport. The objective of this investigation was to discover whether rapid pH changes could be observed using pH indicators. We have used intact cells of photosynthetic bacteria which are more amenable for kinetic studies than chemosynthetic organisms. Experiments show that rapid pH changes can be observed after short light flashes or at the onset of continuous illumination. The results are consistent with the concept of electron transport-driven proton translocation although complexities in the kinetic data suggest that other protolytic reactions

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may also contribute to light-induced pH changes in intact bacterial cells.

2. METHODS

Rhodopseudomonas capsulata strain N22 was grown as described in [14]. Cells were harvested and washed in 10 mM Na_2SO_4 and stored before use as a thick suspension (about 1 mM bacteriochlorophyll) on ice for no more than 8 h. Bacteriochlorophyll was assayed by extraction with acetone/methanol [15].

Measurements of cresol red and carotenoid absorbance changes were performed under an atmosphere of argon (<3 ppm oxygen). The cells at a bacteriochlorophyll concentration of 20 μM were pre-incubated in the spectrophotometer cuvette in 10 mM Na_2SO_4, 100 μM cresol red for 30 min under argon before each experiment. Antimycin A and myxothiazol were added where shown about 5 min before the start of the experiment. The pH was adjusted during the incubation period with dilute NaOH and HCl by comparison with an identical cuvette whose contents were buffered to pH 7.8 with 10 mM sodium phosphate. Absorbance changes were measured with a rapidly-responding single beam spectrophotometer. Data were stored and averaged in a micro-computer. Exciting flashes (4 μs half-peak width) were provided by a xenon discharge tube. The cresol red change was recorded first at 587.5 nm and then, on the same sample, the carotenoid band shift was measured at 503 nm. Control experiments with added buffer showed that the absorbance changes at 587.5 nm were entirely attributable to light-induced pH changes. Cresol red does not bind significantly to intact cells of *Rps. capsulata* (unpublished).

At the end of each experiment the argon supply was disconnected and 40 μg/ml rotenone was added to the suspension to minimise pH changes arising from the respiratory activity of the cells. The cresol red change was calibrated by addition of 5 nmol HCl to the 2.5 ml of bacterial suspension. It was checked that this gave a response within the linear range of the indicator.

3. RESULTS

Excitation of anaerobic, intact cells of *Rps. capsulata* in the presence of cresol red with a 4 μs pulse of light led to an absorbance change at 587.5 nm which indicated a period of acidification. The half time for the acid production was approximately 35 ms. The electrochromic absorbance change of the endogenous carotenoid pigments showed a characteristic response (fig. 1, cf. [16]): following the flash there was a rapid absorbance change, complete in less than 0.5 ms and a slower change of approximately 3 ms half-time. The re-uptake of the protons by the cells and the decay of the carotenoid bandshift after the flash are plotted on semilogarithmic axes in fig. 2. In each case the decay is rather slow ($t_{1/2}$ > 5 s) and there is an apparent correlation between the two parameters.

The experiment in fig. 1 was recorded at a cell density equivalent to 20 μM bacteriochlorophyll which gave a good signal/noise ratio for the cresol red change. Separate experiments on the carotenoid absorbance change (not shown) demonstrated that the exciting flash was approx-

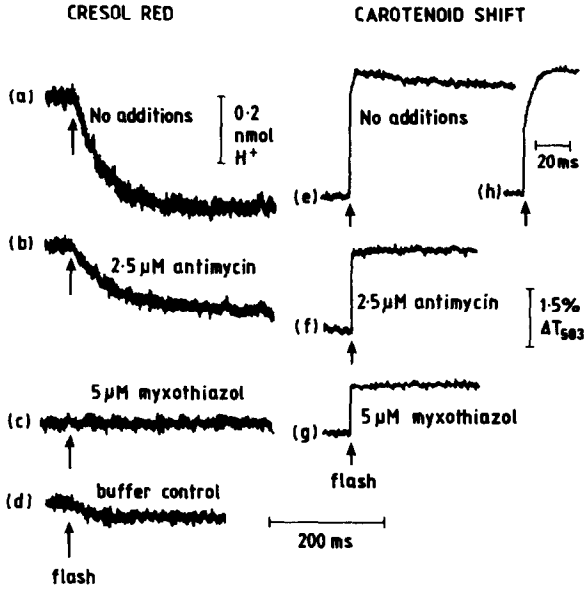

Fig. 1. Rapid H^+-release from intact cells of *Rps. capsulata* after short light flashes and accompanying carotenoid band shift kinetics. See section 2 and text. The H^+ release data are an average of 16 cresol red recordings and the carotenoid shift data are an average of 8 recordings, all at a flash frequency of 0.02 Hz. The buffer in the control trace was 10 mM Na_2PO_4. Note that trace (h) was recorded on a faster scale to show the carotenoid shift kinetics.

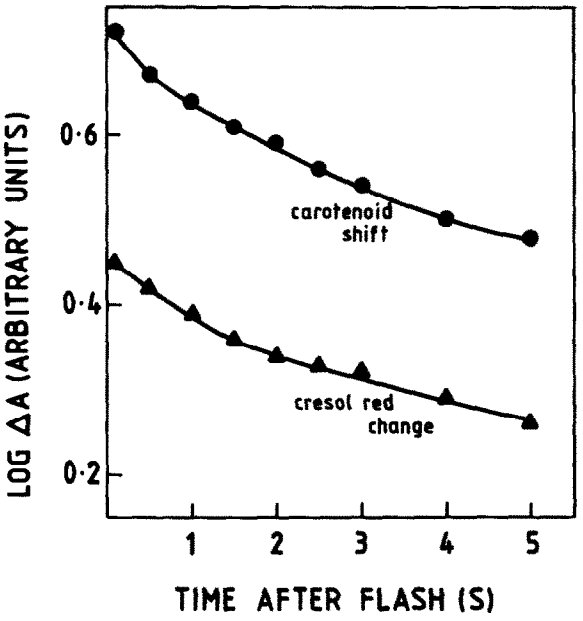

Fig. 2. Re-uptake of H^+ and dissipation of the membrane potential following short light flashes. Recorded as in fig. 1 but on the time scale shown on the lower axis.

imately 88% saturating at this concentration of bacteriochlorophyll). On this basis, the calibrated extent of the pH change produced by a saturating flash is approximately 1 H^+ per 134 bacteriochlorophyll. Similar values were observed in other preparations of intact cells.

Antimycin A is a specific inhibitor of the cytochrome b/c_1 complex [17], probably at the quinone reductase site Q_c (see [18]), and is effective at low concentrations even in intact cells of *Rps. capsulata* [16]. It reduced the extent of the flash induced proton release by about 40%. The antimycin abolished the slow phase in the generation of the electrochromic absorbance change and reduced the total extent by about 37%, in agreement with earlier data in intact cells [16] and in chromatophores [19].

Myxothiazol is also an inhibitor of the cytochrome b/c_1 complex in chromatophores from photosynthetic bacteria [20] but it is believed to operate at the ubiquione oxidase site, Q_z [19,21]. It also seems to be effective in intact cells of *Rps. capsulata* (fig. 1). At a concentration of 5 μM, myxothiazole blocked completely the H^+ release

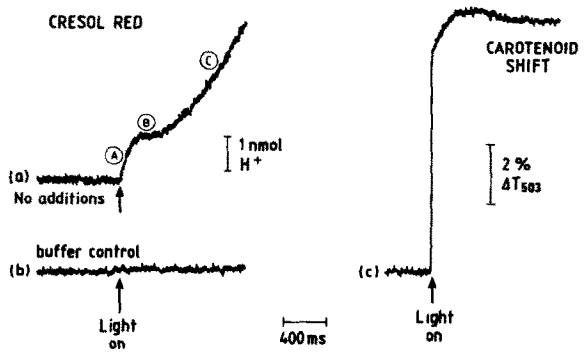

Fig. 3. Carotenoid band shift kinetics and H^+ release from intact cells of *Rps. capsulata* at the onset of continuous illumination. The traces are an average of two illumination periods spaced 2 min apart.

elicited by short flash excitation and eliminated the slow phase in the carotenoid band shift. It reduced the total extent of the carotenoid shift by about 62% more than the expected 50% inhibition for a complete block of electrogenic reactions in the cytochrome b/c_1 complex, although this could be the result of there being more than one turnover of the reaction centre during the 4 μs flash.

During a short period of continuous illumination, the cresol red-indicated changes in suspension pH were more complex (fig. 3). An initial rapid period of acidification (phase A) of half time 40 ms was followed by an apparent lag (phase B) in which the external pH remained constant. The extent of phase A amounted to approx. 1 H^+ per 37 bacteriochlorophyll. The pH then began, after about 200 ms, to rise again at a slower rate (phase C). The membrane potential at the onset of a period of continuous illumination, as revealed by the electrochromic absorbance change, rose to its full value within 200 ms and thereafter remained fairly constant. In the presence of the uncoupling agent, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone at 5 μM, the light-induced pH changes induced by continuous illumination, were totally abolished (not shown).

4. DISCUSSION

The proton release elicited by short flash excitation of intact cells of *Rps. capsulata* appears to be

directly coupled to photosynthetic electron transport. The rate of proton release ($t_{1/2}$ 35 ms) is on the same time scale as the turnover of electron transport in coupled chromatophore preparations (13 per reaction centre per sec. [22]) and in intact cells (30 per reaction centre per sec, J.F. Myatt et al., unpublished). The rapid rate of proton efflux from intact cells would be too fast to measure with a conventional glass electrode. If similar considerations apply to oxygen pulse experiments with chemotrophic bacteria then the slow proton efflux which has been observed with glass electrodes [12,13] may originate from other, possibly unrelated proton-coupled reactions of the bacterial membrane. The complexity of the reaction monitored with cresol red during short illumination periods might indeed suggest that other proton consuming or proton releasing reactions are involved. The origin of these reactions will be the subject of future investigation.

The individual rate constants of the component reactions in the electron transport chain of intact cells of *Rps. capsulata* are not known. However, with respect to current models for the cyclic electron transport chain (e.g., [18]), rapid H^+ release is probably too slow to be associated with those reactions believed to occur on the periplasmic face of the membrane from the Rieske-FeS→ cytochrome c_1→cytochrome c→reaction centre bacteriochlorophyll. Proton release is also slower than the antimycin-sensitive phase of the electrochromic absorbance change (fig. 1), which is believed to indicate the major electrogenic reaction in the cytochrome b/c_1 complex, possibly the oxidation of cytochrome b_{561} ([15] and see also [19]). A good candidate for the proton release reaction is the electrically-neutral oxidation of ubiquinol by the Rieske-FeS and cytochrome b_{566} at centre Q_z (centre o) on the periplasmic membrane face, in equilibrium with the N-phase, in the manner predicted by Crofts et al. [18], Dutton and Prince [23] and Mitchell [24].

Antimycin A appears to inhibit completely the slowest phase (phase III) of the carotenoid band shift after short flash excitation (fig. 1). However it inhibits rapid proton efflux by only 40%. This could be explained within the framework of a proton motive Q cycle [18,23,24] if the low potential cytochrome *b*-566 were oxidised but if all other components of the cytochrome b/c_1 complex, including the ubiquinone pool were largely reduced in the dark before the flash and if antimycin A is assumed to block between cytochrome *b*-561 and the Qc (Q_i) site. In this situation limited turnover of the Q_z site may be possible when the Rieske-FeS is oxidised by the flash.

The complete inhibition by myxothiazol of H^+ release from intact cells elicited by a short flash is also in broad agreement with the Q cycle model. This agent is believed to inhibit electron transport at the ubiquinol oxidase site, Qc [18,21], i.e., at the putative site for H^+ release. The elimination of the slow phase in the $\Delta\psi$, indicating carotenoid shift after a short flash, confirms that all electrogenic activity of the cytochrome b/c_1 complex is blocked by myxothiazol. More extensive inhibition of the carotenoid shift by myxothiazol than by antimycin in intact cells confirms the recent observations of Glaser and Crofts [19]. Both antimycin and myxothiazol were employed at slightly higher concentrations in the present experiments with intact cells than are necessary with chromatophore membranes but there was no indication from the decay of the electrochromic absorbance change for any uncoupling action of these inhibitors.

Preparations of chromatophores from *Rps. capsulata* grown in our standard conditions routinely have one reaction centre for approximately 100 bulk bacteriochlorophylls [25]. If this figure can be extrapolated to intact cells then the calibrated extent of proton release following a single saturating flash amounts to 0.7 H^+ per reaction centre. The Q-cycle predicts 2 H^+ released per reaction centre. The binding of 2 H^+ per reaction centre has been observed in appropriately redox-poised chromatophore suspensions [11]. Because in cells the electrochromic absorbance change did not decay appreciably during the period of H^+ release (the membrane ionic conductance is low at the small $\Delta\psi$ values generated by single flashes [16,25]) we cannot explain the low stoichiometry by invoking a co-incident electrophoretic return of H^+ across the cytoplasmic membrane. As expected, the H^+ release induced by the short flash was not enhanced in the presence of permeant ions (20 mM SCN^- and 200 μM butyltriphenylphosphonium cation were employed, not shown). A number of reasons may explain the discrepancy. (i) A proportion of the protons released at the Q_z site is not released into the bulk aqueous phase but take an

intra-membrane path to a sink within the membrane or elsewhere in the bacterial cell. This would support 'parallel' energy coupling models such as that proposed recently [26] although an additional postulate of a molecular valve would be required to apportion the proton current between membrane and bulk phase. (ii) The intracytoplasmic membrane system of *Rps. capsulata* in parts is so constricted (see [27]) that some chromatophores are electrochemically isolated and behave as discrete energy-coupling units. Cresol red does not diffuse into the lumen of these chromatophores, nor do protons diffuse out into the external medium of the cell suspension during the time of recording. (iii) Other proton consuming processes in the bacterial membrane of unknown origin compete with electron transport-driven proton release to reduce the apparent extent of the latter. In this context, phase B in the cresol red absorbance change observed during continuous illumination is interesting: the nature of the 'lag' period (fig. 3) suggests that it might arise from a combination of an alkali-going component and of the acid-going components, phases A and C. If a contribution from the alkali-going component were present also after a single flash, then the apparent extent of H^+ release would be decreased.

In summary, the kinetic properties and antimycin and myxothiazol sensitivity are consistent with rapid H^+ release upon illumination of intact cells of *Rps. capsulata* being a component of light-driven proton translocation between the bulk aqueous phases on either side of the cytoplasmic membrane. The low stoichiometry of H^+ release may indicate the existence of an additional, intramembrane (local) proton current not proceeding through the aqueous external phase but this observation can also be explained by other features of the bacterial physiology.

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