Biochimica et Biophysica Acta, 975 (1989) 34–43
Elsevier

BBA 42984

Proton release by the quinol oxidase site of the cytochrome b/c_1 complex following single turnover flash excitation of intact cells of *Rhodobacter capsulatus*

Michael R. Jones and J. Baz Jackson

Department of Biochemistry, University of Birmingham, Birmingham (U.K.)

(Received 23 September 1988)

Key words: Proton translocation; Proton diffusion; Cytochrome bc_1 complex; Photosynthetic bacteria; (*Rb. capsulatus*)

(1) The myxothiazol-sensitive reduction of cytochrome (c_1 and c_2) in anaerobic, intact cells of *Rhodobacter capsulatus* has a $t_{1/2}$ of approx. 3 ms. The myxothiazol-sensitive component of the electrochromic absorbance change under similar conditions has a $t_{1/2}$ of approx. 2.5 ms. It is concluded that the putative site for H^+-release, the quinol oxidase (Q_z) site of the cytochrome bc_1 complex, turns over with $t_{1/2} \leqslant 3$ ms. (2) Flash-induced H^+-release from intact cells as measured with pH indicator is delayed: it is preceded by a short lag and has a $t_{1/2}$ of approx. 28 ms. The extent and rate of H^+-release are almost independent of the pH of the suspension between 6.0 and 8.0. (3) The average buffering capacity of the periplasmic and outer membrane region of *Rb. capsulatus* is 55 mM · pH^{-1} at pH 7.6. The effective diffusion coefficients for H^+ and OH^- in time-dependent relaxation phenomena are decreased by immobile buffering groups (Junge, W. and Polle, A. (1986) Biochim. Biophys. Acta 840, 265–273). A schematic model is proposed in which H^+ released at the outer face of the cytoplasmic membrane are delayed by a periplasmic region of homogeneous fixed buffering groups. With reasonable assumptions about the thickness of the periplasmic region the model predicts the lag and the increased $t_{1/2}$ for H^+-release. (4) Based on independent measurements and assumptions the lower limit and upper limit for the quantity of H^+ released per quinol oxidised at the Q_z site of the cytochrome bc_1 complex in intact cells are 1.41 and 3.14, respectively. A refinement of the data used to calculate the upper limit gives a more probable H^+/QH_2 at Q_z of 1.67. (5) The application of similar assumptions to estimate the quantity of charge translocated through the cytochrome bc_1 complex per quinol oxidised at Q_z yields values of 1.03, 1.22 and 1.06 for q/QH_2 in accordance with the predictions of the protonmotive Q-cycle.

Introduction

The photosynthetic electron-transport chains of *Rhodobacter sphaeroides* and *Rb. capsulatus* are comprised of two transmembrane proteins, the reaction centre and the cytochrome bc_1 complex, together with cytochrome c_2 and ubiquinone. Operating in a cycle the electron-transport system serves to translocate protons outwards across the cytoplasmic membrane. The mechanism of electron transfer and proton translocation is not fully understood, although the protonmotive Q-cycle [1–3] is proving to be a useful working model for electron and proton movements through the bc_1 complex. Proton uptake at the cytoplasmic face of the membrane has been examined in detail using chromatophores [4,5] but proton release from the periplasmic face has not received such attention. Following single-flash excitation of intact cells of *Rb. capsulatus*, proton release into the medium can be detected with pH indicator dyes [6]. The reaction is inhibited completely by myxothiazol but only partially by antimycin which suggests that the site of proton release is the quinol oxidase (Q_z) site of the bc_1 complex. In this report we have examined two aspects of the proton release reaction in *Rb. capsulatus* in more detail.

It was noted in the earlier report [6] that proton release following a short flash was slower than the phase of the electrochromic absorbance change which is thought to indicate charge translocation through the bc_1 complex ($t_{1/2} \approx 35$ ms and $t_{1/2} \approx 3$ ms, respectively).

Abbreviations: Q_A, primary acceptor quinone; Q_B, secondary acceptor quinone; Q_c, quinone reductase site of the bc_1 complex; Q_z, quinol oxidase site of the bc_1 complex; P, P^+, reduced and oxidised reaction centre bacteriochlorophyll special pair; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; UHNQ, 3-*n*-undecyl-2-hydroxy-1,4-naphthoquinone; TMAO, trimethylamine *N*-oxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Correspondence: J.B. Jackson, Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham, B15 2TT, U.K.

Below we show that oxidation of quinol at Q_z in intact cells is essentially complete within approx. 10 ms and we discuss the reasons why proton release from the cells might be retarded. In particular we have applied the analysis of Junge and Polle [7] and Junge and McLaughlin [8] who have shown how the presence of fixed and mobile buffers can reduce the effective H^+ and OH^- diffusion coefficients and limit the rate of alkalinisation in the bulk aqueous phase from the appressed regions of stacked pea thylakoids after short-flash excitation. We find that although the periplasmic region of *Rb. capsulatus* is very thin, its buffering capacity is large and it significantly retards the rate of outward proton diffusion into the aqueous phase surrounding the cell after a flash.

Estimation of the H^+/e^- ratio of the electron-transport system is crucial to our understanding of its mechanism. In chromatophore membranes the binding of 2 H^+/e^- was recorded at neutral and low pH [4,5,9,10], although for reasons which still remain unclear the stoichiometry declined at high pH. In our earlier report of experiments on intact cells (at pH 7.6) we found that 0.7 protons were released per reaction centre following a single-turnover flash [6]. The second objective of the work described in this report was to correlate the quantity of proton release with the extent of turnover of the Q_z site. This had to be approached indirectly for the reasons discussed below. The results obtained are consistent with a stoichiometry of 2 H^+ released per quinol oxidised at the Q_z site. The extent of the proton release reaction was independent of the external pH over the range 6.0–8.0.

The data from measurements of proton release from intact cells are complemented by recordings of the electrochromic absorbance change of endogenous carotenoid pigments. The extent of the electrochromic absorbance change after a short flash is a measure of charge separation across the membrane dielectric provided that the rate of charge recombination (e.g., the return of H^+) is relatively slow and that the membrane capacitance is independent of membrane potential ($\Delta\psi$, see Ref. 11). Since many of the steps of cyclic electron transfer in *Rhodobacter* species have been kinetically characterised [3,12] and specific inhibitors are available it is possible to estimate the electrogenic contribution of individual reactions. We find here that for intact cells of *Rb. capsulatus* there is a good agreement between the extent of myxothiazol-sensitive proton release and charge transfer through the cytochrome bc_1 complex.

Accurately estimating the rate and extent of quinol oxidation in intact cells following a single-turnover flash proved problematic in this study. In obtaining such an estimate it became apparent that the effective E_h of the electron-transport system in intact cells under strictly anaerobic conditions is so low that, in a significant fraction of the reaction centres, the primary acceptor quinone (Q_A) is reduced before the flash. Consequently, the bacteriochlorophyll special pair (P) in these 'closed' reaction centres cannot donate its electron to Q_A upon photo-excitation. In addition, significant fractions of P^+ and oxidised cytochrome *c*, generated by the flash, are not re-reduced within the timescale of proton release by the cells. It proved necessary to correct for these effects in the estimation of the stoichiometry of proton translocation by the cyclic electron-transport chain.

Materials and Methods

Rb. capsulatus strain N22 was grown photosynthetically under anaerobic conditions as described in Ref. 13. Cells were harvested, washed by centrifugation and resuspended in 10 mM sodium sulphate. Cells were stored on ice before use as a thick suspension (0.5 mM bacteriochlorophyll). The bacteriochlorophyll concentration was assayed by extraction in acetone/methanol (7 : 2) as described in Ref. 14.

Flash-induced indicator dye and carotenoid absorbance changes were recorded in unstirred samples at 30 °C under an atmosphere of argon (less than 3 ppm oxygen). The experimental media are described in the figure legends. Absorbance changes were monitored in a single-beam spectrophotometer. The measuring beam was switched on (by means of a shutter) 1 s before absorbance changes were recorded. The photomultiplier signal was stored digitally using a Microlink transient capture system (Biodata, Manchester, U.K.) and averaged in an IBM AT microcomputer. Excitation flashes (less than 10 μs half-peak width) were provided by a 1 μF discharge from 1 kV through an FX201 xenon lamp (E.G. and G., MA, U.S.A.). The flash was approx. 96% saturating under the experimental conditions used. During a 30 min pre-incubation period samples were magnetically stirred under a continuous stream of argon. In experiments where the carotenoid band shift was measured (at 503 nm), indicator dye was not included in the reaction medium because absorbance changes attributable to the dye distorted the carotenoid absorbance change at this wavelength. Absorbance changes due to cresol red (pH 8.0–7.6), phenol red (pH 7.4–7.2) and chlorophenol red (pH 6.2–6.0) were measured at 587.5 nm. The data were averaged over 16 flashes (at 587.5 nm) or 4 flashes (at 503 nm) fired at a frequency of 0.017 Hz. Previous experiments have demonstrated that these indicators do not bind to intact cells of *Rb. capsulatus,* and that the absorbance change at 587.5 nm is completely quenched by the presence of buffer (10 mM phosphate) [15]. The pH of the cell suspension was monitored continuously using a pH electrode inserted through a bung in the top of the cuvette, and the pH adjusted with small additions of HCl or NaOH. At the end of each experiment stirring was resumed and 2 μg

rotenone/nmol bacteriochlorophyll was added to the sample to minimise any possible pH changes resulting from respiratory electron transport. The flash-induced pH change was then calibrated by the addition of 5 nmol HCl to the sample. Anaerobic conditions were maintained throughout the calibration.

Absorbance changes due to cytochrome *c* (c_1 and c_2) were measured by difference spectroscopy at 551–542 nm ($E_{mM} = 19\ cm^{-1}$) [16] in the single-beam spectrophotometer described above. Eight measurements were made at each wavelength and, following signal averaging, the change in absorbance due to cytochrome *c* was generated digitally by subtraction. To minimise the effect of time-dependent changes in the sample, measurements were made alternately at each wavelength.

The reaction centre content of each cell preparation was measured at 542 nm. Cells at a bacteriochlorophyll concentration of 10 μM were suspended under aerobic conditions in 10 mM sodium phosphate (pH 7.6) in the presence of 5 μM FCCP (to eliminate any possible electrochromic effects) and 5 μM myxothiazol (to prevent rapid re-reduction of photooxidised reaction centres). Samples were excited with a train of flashes fired at 10 Hz in order to photooxidise completely the reaction centres. It is stressed that reaction centre determinations in intact cell suspensions must be conducted under aerobic conditions: under anaerobic conditions the quinone pool (and, in part, Q_A and Q_B) becomes reduced preventing complete oxidation of P and the high-potential electron donors during excitation with multiple flashes in the presence of myxothiazol (unpublished observations). The extinction coefficient for P^+ at 542 nm in chromatophores of *Rb. sphaeroides* is reported to be 10.3 $mM^{-1} \cdot cm^{-1}$ [16]. On the basis of an extinction coefficient for cytochrome (c_1 and c_2) at 551–542 nm of 19 $mM^{-1} \cdot cm^{-1}$ we obtained a similar value (within 10%) for *Rb. capsulatus*: experiments were carried out on suspensions of *Rb. capsulatus* chromatophores, prepared as in Ref. 17, at a redox poise of 200 mV [18] and treated with 25 μM UHDBT (data not shown). The extinction coefficient for P^+ was calculated on the assumption that under these conditions cytochrome (c_1 and c_2) are the only electron donors to P^+ within 5 ms of flash excitation [19].

The combined buffering capacity of a volume element of the periplasmic gel and outer membrane (B_{peri}) was estimated using a pH electrode. Cells were suspended to 200 μM bacteriochlorophyll in 10 mM sodium sulphate in the presence of 2 μg rotenone/nmol bacteriochlorophyll (final volume, 8 ml). The buffering capacity of the cell suspension, determined a few seconds after the addition of 100 nmol HCl to a stirred anaerobic sample at 30 °C, was defined as B_{ext}

$$B_{ext} = -d[H_t^+]/dpH \quad (1)$$

$d[H_t^+]$ is the quantity of H^+ added (as HCl) divided by the total volume of the sample and dpH is the pH change measured 5–10 s later. The sample was then centrifuged in an MSE microfuge for 10 min and the buffering capacity of the extracellular medium (B_{med}) determined by monitoring the pH change following the addition of 10 nmol HCl to the decanted supernatant. The buffering capacity of the periplasm and outer membrane was calculated by subtraction ($B_{ext} - B_{med}$). On a unit volume basis, B_{peri} was then estimated by multiplying by the ratio of total sample volume to the volume of the periplasmic gel plus outer membrane: it was assumed that the volume of periplasm plus outer membrane constituted 20% of the total volume of the cell. The cytoplasmic volume of the cell has been calculated to be 102 μl per μmol bacteriochlorophyll [20].

UHDBT was the generous gift of Dr. P.R. Rich and UHNQ was the generous gift of Dr. R.C. Prince.

Results and Discussion

The kinetics and extent of proton release by intact cells of Rb. capsulatus

Following flash-excitation of intact cells of *Rb. capsulatus* strain N22 in an anaerobic, weakly buffered suspension in the presence of cresol red, a decrease in the absorbance at 587.5 nm was observed, indicative of proton efflux from the cells (Fig. 1a). The suitability of phenol red, cresol red and chlorophenol red as indicators of pH in experiments of the type reported here has already been established [15]. In the analysis discussed below we shall assume that the indicators report exclusively from the aqueous phase external to the bacterial cell wall. We can eliminate the possibility that the absorbance change due to the cresol red (e.g., in Fig. 1a) originates predominantly from dye within the periplasmic space for two reasons. First, the volume of the periplasmic space is small compared with the extracellular volume: assuming a periplasmic volume of 25.5 μl per μmol bacteriochlorophyll (see Materials and Methods), then in the conditions employed in Fig. 1a even complete decolourisation of cresol red in the periplasm (at a concentration of 100 μM) would yield an absorbance change less than 10% of that observed after the flash. Second, the flash-induced absorbance change attributable to the pH indicator dyes was suppressed completely in the presence of 10 mg/ml bovine serum albumin (data not shown). Since the outer membrane pore proteins exclude molecules of molecular weight greater than approx. 600 [21], it is unlikely that albumin penetrates significantly into the periplasmic region and so its buffering effect must be confined to the extracellular medium.

Improved signal averaging procedures led to the identification of a short lag period in the kinetics of proton efflux following the flash (Fig. 1a) not observed

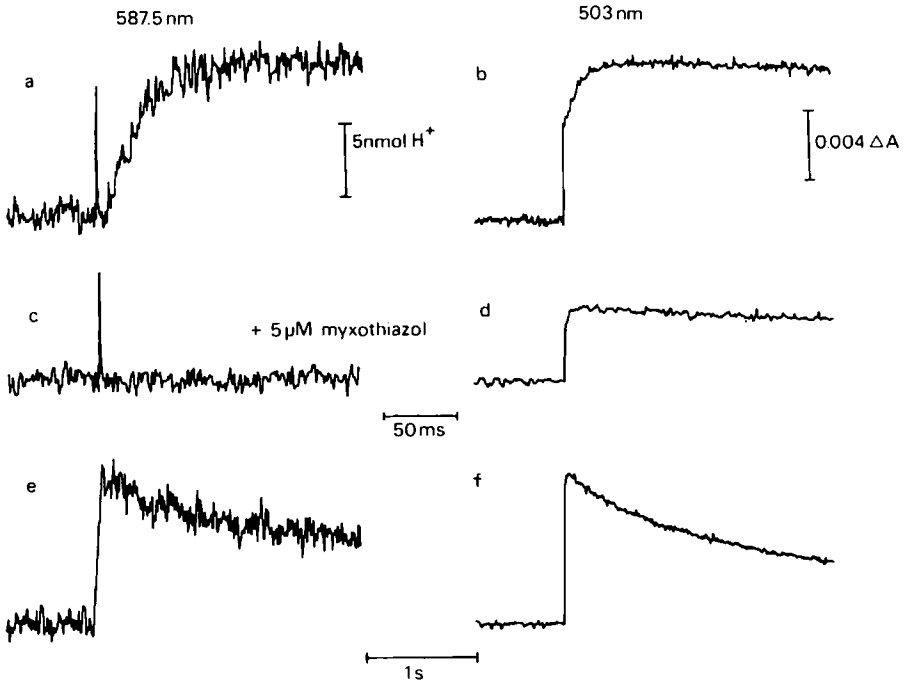

Fig. 1. (a), (c) and (e): Flash-induced proton efflux from intact cells of *Rb. capsulatus* at pH 7.6. (b), (d) and (f): Accompanying flash-induced carotenoid band shift. The rise kinetics of each change are shown in the absence (a and b) and presence (c and d) of 5 μM myxothiazol. Traces (e) and (f) show the subsequent decay kinetics in the absence of myxothiazol. Measurements were made as described in Materials and Methods. Cells were suspended to 20 μM bacteriochlorophyll in 2.5 ml 10 mM Na_2SO_4 (pH 7.6). In (a), (c) and (e) 100 μM cresol red was included.

in the earlier report [6]: the rate of efflux was maximal approx. 12 ms after the flash. In agreement with earlier findings the $t_{1/2}$ for proton release was approx. 28 ms and the reaction was completely sensitive to 5 μM myxothiazol (Fig. 1c). Proton release approached a maximum extent about 100 ms after the flash and, because the re-uptake of H^+ by the cells was slow ($t_{1/2} \approx 5$ s, Fig. 1e), the external pH remained fairly constant for the next 100 ms. The maximal extent of proton release following the flash was equal to 10.78 (±2.5 s.d. for six samples) nmol H^+ per μmol bacteriochlorophyll yielding a ratio of H^+ released per reaction centre (H^+/RC) of 1.15 (±0.24 s.d. for six samples) at pH 7.6. This ratio showed a small variation with extracellular pH (Table I). Examination of the kinetics of H^+ efflux measured with chlorophenol red at pH 6.0 revealed a lag and subsequent kinetics (not shown) that were similar to those described in Fig. 1a. The decays of the absorbance changes due to cresol red and the electrochromic band shift after the flash are shown in Fig. 1e and f. Both are very slow ($t_{1/2} > 1$ s) but the decay of the electrochromic absorbance change is slightly faster than that of the cresol red. This is consistent with the interpretation that under these conditions the decay of $\Delta\psi$ generated during the flash is partly the result of the electrophoretic return of protons back across the membrane and partly the result of the electrophoretic translocation of other ions (see also Ref. 15).

TABLE I

Maximal extent of proton efflux from intact cells of Rb. capsulatus following a single-turnover flash

Cells were suspended to 20 μM bacteriochlorophyll in 2.5 ml 10 mM Na_2SO_4, 100 μM indicator at the pH shown. Measurements were made as described in Materials and Methods. Data are presented as the mean ± standard deviation (number of replicate determinations).

pH	H^+/RC
8.0	1.28 ± 0.23 (5)
7.8	1.20 ± 0.22 (5)
7.6	1.15 ± 0.24 (6)
7.4	0.98 ± 0.22 (9)
7.2	0.79 ± 0.22 (5)
6.2	0.91 ± 0.42 (5)
6.0	0.79 ± 0.34 (6)

Rate of turnover of the Q_z site in intact cells of Rb. capsulatus

The myxothiazol-sensitive component of the electrochromic carotenoid absorbance change following short-flash excitation is a response to the generation of membrane potential by the cytochrome bc_1 complex [22–24]. This component has contributions from the oxidation of cytochrome *b*-566 by cytochrome *b*-561 and oxidation of cytochrome *b*-561 by the quinone at the Q_c site [23,24]. Under conditions similar to those employed to measure the rate of proton release from intact cells, the $t_{1/2}$ of the myxothiazol-sensitive electrochromic ab-

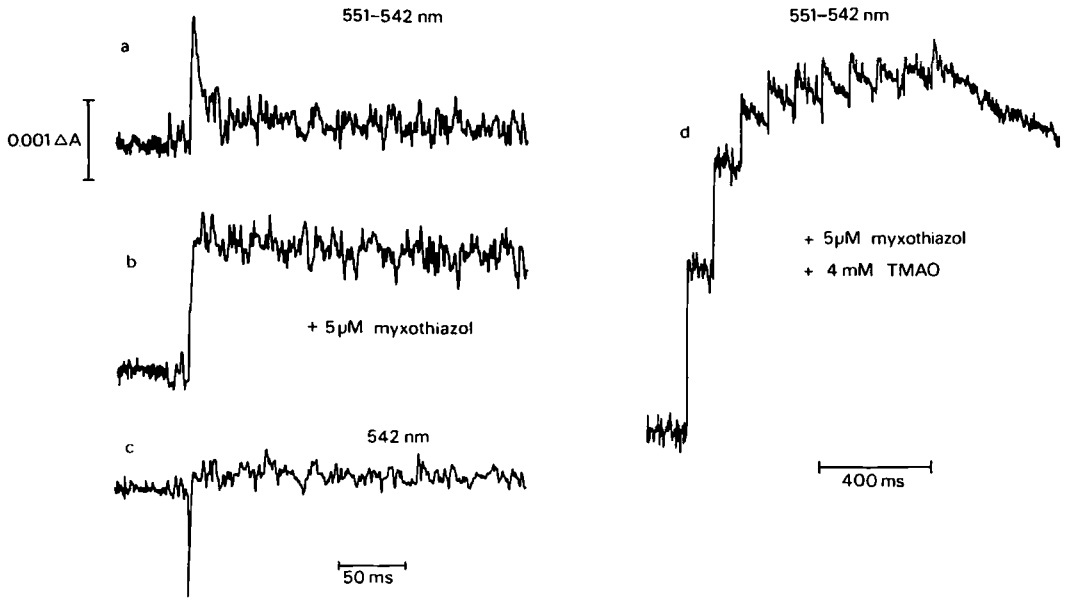

Fig. 2. Cytochrome *c* (a, b and d) and *P* (c) oxidation and re-reduction in intact cells of *Rb. capsulatus* following a single-turnover flash. Measurements were made as in Materials and Methods. Cells were suspended to 20 μM bacteriochlorophyll in 2.5 ml Na_2HPO_4 (pH 7.6). In (b) 5 μM myxothiazol was present. In (d) an anaerobic sample in the presence of 5 μM myxothiazol and 4 mM TMAO was excited with a train of 10 flashes fired at a frequency of 10 Hz. All data shown are the average of eight sweeps.

sorbance change after a flash was approx. 2.5 ms (Fig. 1b and d). According to current formulations of the Q-cycle in *Rhodobacter* [3] it would be predicted therefore that the oxidation of quinol at the Q_z site in intact cells would operate with a $t_{1/2} < 2.5$ ms.

The oxidation and subsequent re-reduction of cytochrome (c_1 and c_2) following short-flash excitation of intact cells of *Rb. capsulatus* is shown in Fig. 2a. By blocking the oxidation of quinol at Q_z, myxothiazol decreases the rate of cytochrome (c_1 and c_2) re-reduction (Fig. 2b and see Ref. 25). The $t_{1/2}$ for myxothiazol-sensitive cytochrome (c_1 and c_2) reduction is approx. 3 ms. This is an upper limit for the $t_{1/2}$ for oxidation of quinol at Q_z. In principle the rate may be faster than this if oxidation of the Rieske Fe-S centre by cytochrome c_1 is limiting, although on the basis of data with chromatophores from *Rb. sphaeroides* this seems unlikely [26].

Taken together, the electrochromic data and the data on cytochrome *c* reduction show rather convincingly that the $t_{1/2}$ for quinol oxidation at Q_z is less than 2.5–3.0 ms, at least one order of magnitude less than the $t_{1/2}$ for proton release measured with cresol red.

Buffering capacity of the bacterial periplasm: the effective diffusion coefficients for H^+ and OH^-

If there were no barrier to the free diffusion of H^+ from the putative site of release at the Q_z site, located at the periplasmic face of the cytoplasmic membrane, then the pH change recorded by the cresol red should take place at a rate which is indistinguishable from the rate of quinol oxidation at Q_z. In fact there is a short lag and a much increased half-time for proton release. Junge and Polle [7] pointed out that for time-dependent relaxations the effective diffusion coefficient for H^+ in pure water is reduced by the presence of immobile buffering groups.

$$D_{H^+}^{eff} = \frac{D_{H^+} \cdot 2.3[H^+]}{B} \quad (2)$$

where $D_{H^+}^{eff}$ is the effective diffusion coefficient for H^+, D_{H^+} is the diffusion coefficient for H^+ in pure water and B is the buffering capacity defined in Eqn. 1.

Protons released from the outer face of the cytoplasmic membrane at Q_z must traverse the gel-like matrix of peptidoglycan and associated protein which occupies the 'periplasmic space' [27], the outer membrane with its lipopolysaccharide outer leaflet and the capsule layer [28,29]. Clearly the proton diffusion pathway is heterogeneous with respect to buffer mobility, concentration and acid dissociation constant. However, in a first approximation to account for the main features of the proton efflux kinetics we have adopted a model in which it is assumed that the periplasm and bacterial cell wall behave as a homogeneous fixed buffer system. The buffering capacity of this region was estimated on the assumption that after addition of dilute HCl (see Materials and Methods) the outer layers of the bacterial cell (the periplasm, outer membrane and capsule) are permeable to protons on a timescale of several seconds whereas the cytoplasmic membrane is impermeable. The first assumption is reasonable given that the outer membrane is generally considered to be freely

permeable, via the porin proteins, to molecules with a molecular weight less than approx. 600 [21]. The observed $t_{1/2}$ for proton efflux of approx. 30 ms after a light pulse is also consistent with high proton permeability of the outer layers of the cell on a time scale of seconds. The relative impermeability of the cytoplasmic membrane to protons during the extremely low driving force experienced after a pulse of 12.5 μM HCl is also a reasonable assumption: a consequence of the non-ohmic conductance properties of chromatophore (cytoplasmic) membranes is that the half-time of relaxation after an HCl pulse ($\Delta p \approx 2$ mV) will be considerably longer than that recorded (approx. 10 s) after a single-turnover light pulse ($\Delta p \approx 50$ mV, Ref. 30).

In calculating the buffering capacity of the periplasmic regions it was necessary to correct for the buffering capacity of the extracellular medium of the bacterial suspension (see Materials and Methods). This was found to be significantly larger than the buffering capacity of the 10 mM Na_2SO_4 (0.02 mM/pH at pH 7.6), used as the suspending medium, and increased as a function of time over a period of approx. 60 min. This increase may be attributable to the release of weak acids from the cells during anaerobic incubation in the dark: the pH of anaerobic cell suspensions poised in the region pH 7–8 was seen to drift to more acid pH values at the rate of 0.01 pH unit/min. The estimated buffer capacity of the periplasm plus outer membrane region (Table II) was fairly constant in the region of pH 7–8 but increased at more acid pH.

Based on the experimentally measured buffering capacity and values of $D_{H^+} = 9.31 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1} and $D_{OH^-} = 5.3 \cdot 10^{-5}$ cm^2 s^{-1} [31], the effective diffusion coefficients within the bacterial periplasm at pH 7.6 calculated from Eqn. 2 are $D^{eff}_{H^+} = 9.8 \cdot 10^{-11}$ cm$^2 \cdot$ s^{-1} and $D^{eff}_{OH^-} = 8.82 \cdot 10^{-10}$ cm$^2 \cdot$ s^{-1}.

TABLE II

Buffering capacity of the periplasmic space, outer membrane and cell wall over the range pH 6–8

Estimates of B_{med}, B_{ext} and B_{peri} were made as described in Materials and Methods. Data are presented as the mean ± standard deviation (number of replicate determinations).

pH	B_{ext} (mM·pH^{-1})	B_{med} (mM·pH^{-1})	B_{peri} (mM·pH^{-1})
8.0	0.497 ± 0.062 (3)	0.231 ± 0.072 (3)	50 ± 6 (3)
7.8	0.488 ± 0.058 (3)	0.194 ± 0.041 (3)	58 ± 3 (3)
7.6	0.494 ± 0.146 (3)	0.213 ± 0.103 (3)	55 ± 9 (3)
7.4	0.510 ± 0.059 (3)	0.235 ± 0.074 (3)	52 ± 7 (3)
7.2	0.510 ± 0.065 (3)	0.255 ± 0.058 (3)	48 ± 8 (3)
7.0	0.575 (1)	0.358 (1)	43 (1)
6.7	0.781 (1)	0.431 (1)	69 (1)
6.4	0.862 (1)	0.508 (1)	69 (1)
6.2	0.772 ± 0.110 (3)	0.325 ± 0.039 (3)	83 ± 8 (3)
6.0	0.840 ± 0.113 (4)	0.310 ± 0.056 (4)	100 ± 8 (4)

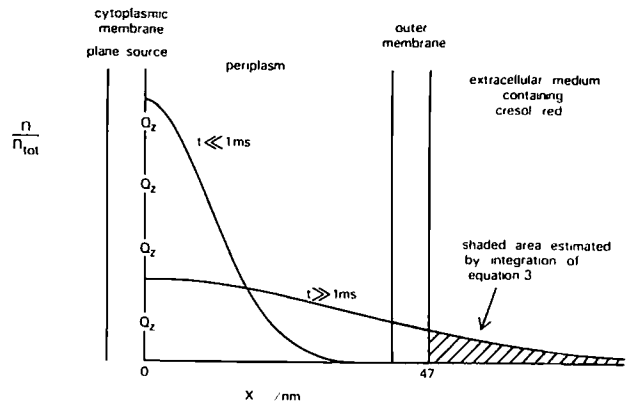

Fig. 3. Model for 1-dimensional diffusion of protons across the periplasmic region. Shown are distribution profiles of n/n_{tot} versus for a short time after the flash ($t \ll 1$ ms) when no protons have been detected in the extracellular medium, and for a longer time ($t \gg 1$ ms) when a fraction of the protons has been detected.

Delayed proton release from intact cells of Rb. capsulatus after short-flash excitation

To model the kinetics shown in Fig. 1a we shall assume that the periplasmic face of the cytoplasmic membrane is a planar source of protons (or sink for hydroxyl ions) (Fig. 3). Upon flash excitation, protons released from the Q_z sites, randomly distributed in this plane source, appear at the surface of the cytoplasmic membrane as an instantaneous burst and diffuse across the periplasmic region of homogeneous buffering capacity in a direction perpendicular to the plane of the membrane. Protons will be detected instantly by the indicator dye when they have passed a plane equivalent to the outer face of the outer membrane of the cell at a distance x from the outer face of the cytoplasmic membrane (Fig. 3). We have also assumed that all the protons released at the Q_z site are detected by the indicator dye (see General conclusions). At pH 7.6 the diffusion will be governed by $D^{eff}_{OH^-}$ and this system can be described (compare with Ref. 32) by

$$\frac{n}{n_{tot}} = \frac{1}{(\pi D^{eff}_{OH^-} t)^{1/2}} \cdot \exp\left(\frac{-x^2}{4 D^{eff}_{OH^-} t}\right) \quad (3)$$

where n/n_{tot} is the fraction of the total ion population residing in a plane at a distance x from the source at time t. Numerical integration of Eqn. 3 yields the percentage of the total ion population that has passed the plane at x as a function of time. Since the actual distance between the outer faces of the cytoplasmic and outer cell membranes is not known with certainty, x was fixed at 47 nm, a distance which is not unreasonable on the basis of electron micrographs of the organism. Fig. 4 shows the result of the simulations superimposed upon the experimental data (curve a). All curves are normalised to the same extent of proton release. Curve b shows the expected prompt release of H^+ (uptake of

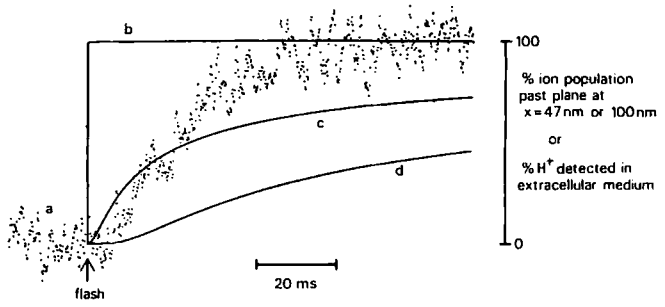

Fig. 4. Percentage of a population of ions that have past a point 47 nm (b and c) or 100 nm (d) from a plane source as a function of time. The maximal extent of the measured cresol red data (trace a) is normalised to 100% of the ion population having diffused a distance greater than 47 nm from the plane source, i.e., in our model all protons have appeared in the extracellular medium. Trace (b) shows the time-course for proton appearance in the extracellular medium when $D_{OH^-} = 5.3 \cdot 10^{-5}$ cm$^2 \cdot$s^{-1}. Traces (c) and (d) show the time-course when $D^{eff}_{OH^-} = 8.82 \cdot 10^{-10}$ cm$^2 \cdot$s^{-1}.

OH^-) when diffusion is unrestricted by buffering groups ($D_{OH^-} = 5.3 \cdot 10^{-5}$ cm$^2 \cdot$s^{-1}; $x = 47$ nm): more than 99% of the H^+ released at $t = 0$ would have crossed the plane at 47 nm within the first millisecond. Curve c shows the predicted release of protons from the cell using the diffusion coefficient effectively reduced by fixed buffers in the periplasm and outer membrane ($D^{eff}_{OH^-} = 8.82 \cdot 10^{-10}$ cm$^2 \cdot$s^{-1}) and $x = 47$ nm. In qualitative agreement with the experimental data this curve shows a lag before proton release from the outer membrane of the cell. The duration of the lag in the simulated curve (c) was rather less than that in the experimental trace (a). However, the lag is displayed more clearly in the simulated curve (d) where $D^{eff}_{OH^-} = 8.82 \cdot 10^{-10}$ cm$^2 \cdot$s^{-1} and $x = 100$ nm. Curve (c) has a value for the $t_{1/2}$ for proton release similar to that found experimentally whereas the $t_{1/2}$ for curve (d) is somewhat greater. The existence of the lag and a $t_{1/2}$ of the same order as that found experimentally is taken to be evidence supporting the view that proton release from the bacterial cells is delayed by fixed buffers in the periplasmic and outer membrane regions. Using x as a variable simulated curves could not be fitted precisely to the experimental data: reasons for this are considered under General conclusions (see below).

The extent of turnover of the Q_z site in intact cells of Rb. capsulatus and calculation of the H^+/QH_2 ratio

The extent of H^+-release after single short-flash excitation (see above) was expressed per mol of photosynthetic reaction centres or per mol bacteriochlorophyll. It is now required to estimate the extent of oxidation of quinol by the Q_z site of the bc_1 complex in order to calculate the H^+/QH_2 ratio. The extent of quinol oxidation at the Q_z site after a short flash can be measured directly in chromatophores by measuring cytochrome (c_1 and c_2) oxidation in the absence and presence of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) or 3-*n*-undecyl-2-hydroxy-1,4-naphthoquinone (UHNQ). These inhibitors block quinol oxidation at the Q_z site and also raise the mid-point potential of the Rieske Fe-S centre, preventing the re-reduction of oxidised cytochrome (c_1 and c_2) by this centre [19,26,33]. UHNQ was also reported to inhibit cytochrome (c_1 and c_2) reduction in intact cells of *Rb. capsulatus* strain MT1131 [34]. However, in our hands both UHNQ and UHDBT at 40 μM failed to give any significant inhibition of cytochrome (c_1 and c_2) re-reduction after flash excitation of *Rb. capsulatus* strain N22 either in strictly anaerobic suspensions or under aerobic conditions. The explanation for this discrepancy is not clear. Unfortunately, it means that we were unable to estimate directly the extent of oxidation of quinol at Q_z under the same conditions as H^+-release. However, we can define fairly narrow limits for the extent of turnover of Q_z from measurements of P and cytochrome (c_1 and c_2) oxidation and reduction after the flash. Essentially we have estimated the turnover of Q_z from the quantity of reducing equivalents entering cytochrome (c_1 and c_2) after oxidation by a flash under conditions equivalent to those used to record H^+-efflux from intact bacterial cells. The central assumption is that 100 ms after the flash (at which time the extent of H^+-efflux is recorded) all reducing equivalents removed from the Rieske Fe-S centre by the cytochrome *c* will be replaced by reducing equivalents derived from quinol. This is reasonable in view of the rapid rates of electron exchange amongst these components [3] and the fact that the concentrations of cytochrome (c_1 and c_2) and of reduced quinol in the Q-pool in anaerobic intact cells are both in excess of the concentration of Fe-S centre.

The lower limit for H^+/QH_2 oxidised by the bc_1 complex

The quantity of reducing equivalents withdrawn from cytochrome (c_1 and c_2) after the flash was estimated from the absorbance changes of P at 542 nm. The total amount of P in the sample was measured as described in Materials and Methods, and since the degree of saturation was known, the amount of P^+ generated during a flash could be calculated. Not all this P^+ was rapidly re-reduced by cytochrome (c_1 and c_2) after the flash (see Fig. 2c and Ref. 34): in our experiments approx. 15% of the total P remained oxidised 100 ms after the flash. This figure is a little lower than that measured by other workers [34]. Thus, of the 10.16 (±0.69 s.d. for 12 samples) nmol P per μmol bacteriochlorophyll present in the experimental system, 8.25 (±1.03 s.d. for 12 samples) nmol P^+/μmol bacteriochlorophyll was reduced by cytochrome (c_1 and c_2) within 100 ms of the flash. This figure is therefore equivalent to the total amount of cytochrome (c_1 and c_2) oxidised.

Not all of the cytochrome (c_1 and c_2) oxidised after the flash was rapidly reduced (Fig. 2a): even after 100 ms 0.63 ($\pm$0.24 s.d. for 12 samples) nmol per μmol bacteriochlorophyll of the cytochrome *c* remained oxidised. The result is that of the 8.25 nmol per μmol bacteriochlorophyll of reductant entering the reaction centre within 100 ms of the flash, 7.62 ($\pm$1.27 s.d.) nmol per μmol bacteriochlorophyll was derived from the oxidation of quinol at Q_z. From the extent of proton release in intact cells after a flash the H^+/QH_2 is therefore 1.41 ($\pm$0.40 s.d.).

We believe that this is a lower limit because we have firm evidence that a significant fraction of oxidised reaction centres in a dark anaerobic suspension of *Rb. capsulatus* are closed: Q_A appears to be partly reduced under these conditions. Experiments (Richardson, D.J. and Jones, M.R., unpublished observations) show that in anaerobic cell suspensions treated with myxothiazol both the extent of cytochrome (c_1 and c_2) oxidation and the extent of the electrochromic absorbance change after a single-turnover flash are stimulated in extent by as much as 40% by the auxiliary oxidant trimethylamine *N*-oxide (TMAO). This oxidant is believed to act as a sink for reducing equivalents and to help to poise the photosynthetic electron transport system at an optimal redox potential [35]. The pathway of electron transport to TMAO branches from the photosynthetic pathway at the level of the quinone pool [36]. Unfortunately, we cannot at present calculate from these data the degree of reduction of Q_A in the reaction centre but we can assert that the measurement of the extent of P^+ re-reduction described above is an overestimate and therefore that the H^+/QH_2 value of 1.41 constitutes a lower limit.

The upper limit for H^+/QH_2 oxidised by the bc_1 complex

The extent of myxothiazol-sensitive cytochrome (c_1 and c_2) re-reduction after a flash (Fig. 2b minus a) is equivalent to 3.43 ($\pm$1.30 s.d. for 7 samples) nmol per μmol bacteriochlorophyll. This underestimates the extent of quinol oxidation at Q_z because cytochrome (c_1 and c_2) is partially re-reduced by the Rieske Fe-S centre in the presence of myxothiazol. Hence the H^+/QH_2 ratio of 3.14 ($\pm$1.53 s.d.) based on this measurement and the measurements of proton release described above represent an upper limit.

This value of the H^+/QH_2 can be refined by calculating the quantity of oxidising equivalents that reside on the Rieske Fe-S centres following a single turnover flash in the presence of myxothiazol. The total amount of cytochrome (c_1 and c_2) which can be photo-oxidised by a train of flashes in the presence of myxothiazol and TMAO was 12.58 ($\pm$1.72 s.d. for 10 samples) nmol/μmol bacteriochlorophyll (Fig. 2d). If it is assumed that there are approx. 0.7 bc_1 complexes per reaction centre in membranes of cells grown anaerobically in the light [25,26,37–40] and that the quantities of Rieske Fe-S centre and cytochrome c_1 are equal, then the cells contain 5.47 nmol photooxidisable cytochrome c_2 and 7.11 nmol each of cytochrome c_1 and Rieske Fe-S centre/μmol bacteriochlorophyll. Based on the midpoint potentials given in Ref. 41 and assuming that these redox centres reach equilibrium a few ms after the flash (a realistic assumption in view of the measured rate constants [3]), it can be calculated that when the cytochrome *c* pool (c_1 and c_2) is oxidised to the extent of 4.06 ($\pm$1.06 s.d. for 11 samples) nmol/μmol bacteriochlorophyll (Fig. 2b), there must be an additional 3.01 nmol of oxidising equivalents per μmol bacteriochlorophyll on the Rieske Fe-S centre. Thus, at 100 ms after the flash, we recalculate that $4.06 + 3.01 - 0.63 = 6.44$ nmol QH_2 per μmol bacteriochlorophyll had been oxidised at Q_z leading to an H^+/QH_2 ratio of 1.67.

Incidentally, since we also know the quantity of P^+ that is not re-reduced within 100 ms of the flash (Fig. 2c) we can also calculate that only $4.06 + 3.01 + 1.50 = 8.57$ nmol P^+ per μmol bacteriochlorophyll was generated by the 96% saturating flash. On this basis, in agreement with the comment made above, approx. 12% of the Q_A sites in the sample of dark, anaerobic cells must have been reduced before the flash.

Calculation of the stoichiometry of q/QH_2 from electrochromic absorbance changes

The carotenoid absorbance changes at appropriate wavelengths in membranes of photosynthetic bacteria are linear indicators of membrane potential ($\Delta\psi$). Since the rate of decay of the electrochromic absorbance change is generally 2–3 orders of magnitude lower than its rate of generation [30] the extent of the absorbance change after a short flash can be used to estimate the quantity of charge translocated across the membrane dielectric during electron transport. Crystal structures confirm that the transfer of an electron from cytochrome *c* through the reaction centre to Q_A and Q_B takes place across the membrane [42]. The measured response of the electrochromic bands to single charge translocations through the reaction centre can therefore be used to estimate the quantity of charge (q) transferred through the cytochrome bc_1 complex. The kinetics of the electrochromic absorbance change in anaerobic suspensions of intact cells of *Rb. capsulatus* after a single flash in the absence and presence of sufficient myxothiazol to block completely the Q_z site of the cytochrome bc_1 complex are shown in Fig. 1b and d. The risetime of the myxothiazol-insensitive absorbance change, reflecting electron transfer from cytochrome *c* to P to $Q_{A/B}$, was too fast to be resolved by these experiments. As discussed above the $t_{1/2}$ for the myxothiazol-sensitive absorbance change, arising from

the electrogenic activity of the bc_1 complex, was approx. 2.5 ms. The amplitude of the myxothiazol-sensitive component was approx. 87% of the myxothiazol-insensitive component under conditions equivalent to those employed for the measurement of H^+-efflux and P and cytochrome *c* oxidation and reduction. Incidentally, it was noted that the extent of the myxothiazol-insensitive component decreased with prolonged anaerobic incubation, probably reflecting increased reduction of Q_A. In broad agreement with the results presented in the last section it is concluded that the extent of electron flux through the cytochrome bc_1 complex under these conditions was less than that through the reaction centre.

In attempting to estimate the quantity of charge transferred through the cytochrome bc_1 complex per quinol oxidised at Q_z (q/QH_2) we were again faced with our inability in intact cells to measure directly the denominator in the ratio. The approximations used in the previous section were used again here. Electron transfers from cytochrome *c* to P^+ and from P to Q_A or Q_B make approximately equal contributions to the development of the membrane potential by the reaction centres of chromatophores of both *Rb. sphaeroides* [22] and *Rb. capsulatus* (Jones, M.R. and Nore, B.F., unpublished observations): because re-reduction of P^+ was not complete during the period in which the electrochromic absorbance change was measured (Fig. 2c and 1b), the net charge translocation through the reaction centre had to be accordingly calculated for each estimate of the q/QH_2 of the bc_1 complex. Because of this additional calculation we lose confidence as to whether our estimates are upper or lower limits. From the data and approximations used to provide a lower limit for H^+/QH_2 the value of q/QH_2 was estimated at 1.03. Similarly from the data used to provide an upper limit for H^+/QH_2 the value of q/QH_2 was estimated at 1.22 or 1.06 after the refinement based on measured component stoichiometries. The clustering of these estimates, using different sets of assumptions and data, are consistent with a q/QH_2 of unity as predicted by the protonmotive Q-cycle.

General conclusions

The data presented above are probably consistent with a stoichiometry of 2 H^+ released in the bacterial periplasm and 1 charge translocated across the membrane through the cytochrome bc_1 complex per quinol oxidised at the Q_z site. No evidence was found for charge movement through the cytochrome bc_1 complex other than that coupled to quinol oxidation at the Q_z site. Although the technical difficulties in estimating the extent of quinol oxidation in intact cells and the degree of error on most measurements mean that the existence of additional charge translocation cannot be completely discounted, it is considered to be unlikely. The data also highlight the fact that a significant fraction of *P* is not oxidised by the flash in intact cells under strictly anaerobic conditions, a phenomenon which we attribute to partial reduction of Q_A.

The release of protons into the extracellular medium was delayed, principally due to the presence of a high concentration of fixed buffering groups in the bacterial periplasm and outer cell membrane. A simple model for proton diffusion through the outer layers of the cell predicts the experimentally observed lag in proton release and is in reasonable agreement with the kinetics of the reaction. Deviation from the simple model is expected for two main reasons. (1) The photosynthetic electron-transport apparatus of *Rb. capsulatus* is located predominantly in the intracytoplasmic membrane system of tubular extensions of the cytoplasmic membrane. Protons released from the cytochrome bc_1 complex will therefore emanate from different depths within the cell. The effect of increasing the distance between the site of proton release and the outer face of the bacterial cell wall is illustrated in Fig. 4d. (2) The buffering capacity of the lumen of the intracytoplasmic membranes, the periplasmic gel, the outer membrane and capsule layer is unlikely to be homogeneous.

As the values of the effective diffusion coefficients for H^+ (dominant diffusing species at acid pH) and OH^- (dominant at alkaline pH) are pH-dependent (Eqn. 2), the simple model also predicts an affect of extracellular pH on the proton release kinetics. Experimentally, however, we do not observe any significant effect of external pH between 6.0 and 8.0 on the rate of H^+-release. The implication of this finding is that factors other than those described in Eqn. 2 also influence the rates at which H^+ and OH^- diffuse across the periplasmic region. Junge and McLaughlin [8] have demonstrated that the presence of mobile buffer (such as mobile weak acids) can increase D^{eff} when fixed buffers are present. In our experimental system we were unable to distinguish between fixed and mobile periplasmic buffer and so have assumed for the sake of simplicity that all of the buffer groups present in this region are fixed.

A semi-quantitative way the model may provide an explanation for the very slow rate of proton release (in the region of several seconds) from intact cells of chemosynthetic bacteria after multiple-turnover oxygen pulses [43,44] and especially for the enhancement in the rate of the oxygen-dependent proton release observed after treatment of cells of *Paracoccus dentrificans* with lysozyme [45]. Clearly we should also expect an increase in the rate of proton release from cells of *Rb. capsulatus* after single-turnover flash excitation if the outer membrane and periplasmic layers were selectively removed. In our attempts to make spheroplasts of *Rb. capsulatus,* however, the integrity of the cyclic electron transport system was disrupted (Taylor, M.A. and Jones, M.R.,

unpublished observations). Arata et al. [46] have described a preparation of non-inverted vesicles from *Rb. spheroides* which elicit proton extrusion after short flashes. The kinetics of the extracellular pH change were slow ($t_{1/2} \approx 70$ ms), but multiple flash data led the authors to conclude that although proton release itself was rapid, diffusional equilibration of the released protons with the extracellular bulk aqueous phase was slow. It was suggested that their spheroplast suspension actually consisted of spheroplast aggregates, protons from deep within the aggregates taking tens of milliseconds to reach the indicator dye in the bulk solution.

Acknowledgements

This work was supported by a grant from the Science and Engineering Research Council. We are grateful to Al McEwan and Dave Richardson for useful discussion.

References

1 Mitchell, P. (1975) J. Theor. Biol. 62, 327–367.
2 Dutton, P.L. and Prince, R.C. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), Ch. 24, pp. 525–570, Plenum Press, New York.
3 Crofts, A.R., Meinhardt, S.W., Jones, K.R. and Snozzi, M. (1983) Biochim. Biophys. Acta 723, 202–218.
4 Petty, K.M., Jackson, J.B. and Dutton, P.L. (1977) FEBS Lett. 84, 299–303.
5 Petty, K.M., Jackson, J.B. and Dutton, P.L. (1979) Biochim. Biophys. Acta 546, 17–42.
6 Taylor, M.A. and Jackson, J.B. (1985) FEBS Lett. 180, 145–149.
7 Junge, W. and Polle, A. (1986) Biochim. Biophys. Acta 848, 265–273.
8 Junge, W. and McLaughlin, S. (1987) Biochim. Biophys. Acta 890, 1–5.
9 Matsuura, J.F., O'Keefe, D.P. and Dutton, P.L. (1983) Biochim. Biophys. Acta 722, 12–22.
10 Myatt, J.F. and Jackson, J.B. (1986) Biochim. Biophys. Acta 848, 212–223.
11 Jackson, J.B. and Clark, A.J. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 341–346, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht.
12 Dutton, P.L. (1985) in Encyclopedia of Plant Physiology, Vol. 19, (Staehelin, A. and Arntzen, C.J., eds.), pp. 197–237, Springer-Verlag, Berlin.
13 Cotton, N.P.J., Clark, A.J. and Jackson, J.B. (1981) Arch. Microbiol. 129, 94–99.
14 Clayton, R.K. (1963) Biochim. Biophys. Acta 75, 312–323.
15 Taylor, M.A. and Jackson, J.B. (1985) Biochim. Biophys. Acta 810, 209–224.
16 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536–556.
17 Clark, A.J., Cotton, N.P.J. and Jackson, J.B. (1983) Biochim. Biophys. Acta 723, 440–453.
18 Dutton, P.L. (1978) Methods Enzymol. 54, 411–435.
19 Bowyer, J.R., Tierney, G.V. and Crofts, A.R. (1979) FEBS Lett. 101, 207–212.
20 Clark, A.J. and Jackson, J.B. (1981) Biochem. J. 200, 389–397.
21 Flammann, H.T. and Weckesser, J. (1984) J. Bacteriol. 159, 410–412.
22 Jackson, J.B. and Dutton, P.L. (1973) Biochim. Biophys. Acta 325, 102–113.
23 Glaser, E.G. and Crofts, A.R. (1984) Biochim. Biophys. Acta 766, 322–333.
24 Robertson, D.E. and Dutton, P.L. (1988) Biochim. Biophys. Acta 935, 273–291.
25 Robertson, D.E., Davidson, E., Prince, R.C., Van den Berg, W.H., Marrs, B.L. and Dutton, P.L. (1986) J. Biol. Chem. 261, 584–591.
26 Bowyer, J.R., Dutton, P.L., Prince, R.C. and Crofts, A.R. (1980) Biochim. Biophys. Acta 592, 445–460.
27 Hobot, J.A., Carlemalm, E., Villiger, W. and Kellenberger, E. (1984) J. Bacteriol. 160, 143–152.
28 Flamman, H.T. and Weckesser, J. (1984) J. Bacteriol. 159, 191–198.
29 Benz, R., Woitzik, D., Flammann, H.T. and Weckesser, J. (1987) Arch. Microbiol. 148, 226–230.
30 Packham, N.K., Greenrod, J.A. and Jackson, J.B. (1980) Biochim. Biophys. Acta 592, 130–142.
31 Atkins, P.W. (1983) Physical Chemistry, p. 905, Oxford University Press, Oxford.
32 Bockris, J.O'M. and Reddy, A.K.N. (1970) Modern Electrochemistry, Vol. 1, p. 332, Plenum Press, New York.
33 Matsuura, K., Bowyer, J.R., Ohnishi, T. and Dutton, P.L. (1983) J. Biol. Chem. 258, 1571–1579.
34 Prince, R.C., Davidson, E., Haith, C.E. and Daldal, F. (1986) Biochemistry 25, 5208–5214.
35 Ferguson, S.J., Jackson, J.B. and McEwan, A.G. (1987) FEMS Microbiol. Rev. 46, 117–143.
36 Richardson, D.J., Kelly, D.J., Jackson, J.B., Alef, K. and Ferguson, S.J. (1986) Arch. Microbiol. 146, 159–165.
37 Prince, R.C. (1983) Biochim. Biophys. Acta 723, 133–138.
38 Prince, R.C., Bashford, C.L., Takamiya, K., Van den Berg, W.H. and Dutton, P.L. (1978) J. Biol. Chem. 253, 4137–4142.
39 Van den Berg, W.H., Prince, R.C., Bashford, C.L., Takamiya, K., Bonner, W.D. and Dutton, P.L. (1979) J. Biol. Chem. 254, 8594–8604.
40 O'Keefe, D.P. and Dutton, P.L. (1981) Biochim. Biophys. Acta 635, 149–166.
41 Cramer, W.A. and Crofts, A.R. (1982) in Photosynthesis: Energy Conversion by Plants and Bacteria (Govindjee, ed.), p. 427, Academic Press, New York.
42 Chang, C.-H., Tiede, D., Tang, J., Smith, U., Norris, J. and Schiffer, M. (1986) FEBS Lett. 205, 82–86.
43 Gould, J.M. and Cramer, W.A. (1977) J. Biol. Chem. 252, 5875–5882.
44 Gould, J.M. (1979) J. Bacteriol. 138, 176–184.
45 Hitchens, G.D. and Kell, D.B. (1984) Biochim. Biophys. Acta 766, 222–232.
46 Arata, H., Takenaka, I. and Nishimura, M. (1988) J. Biochem. 101, 261–265.