Biochimica et Biophysica Acta, 679 (1982) 138–145
Elsevier Biomedical Press

BBA 41019

THE KINETICS OF CAROTENOID ABSORPTION CHANGES IN INTACT CELLS OF PHOTOSYNTHETIC BACTERIA

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(Received July 3rd, 1981)

Key words: Membrane potential; Carotenoid absorption; Bacterial photosynthesis; (Rps. capsulata)

The kinetics of carotenoid absorption changes have been measured in intact cells of *Rhodopseudomonas capsulata* after short flash excitation. The observed changes were consistent with the thesis that they indicate the development and dissipation of membrane potential. In the generation of the absorption changes in anaerobic cells, fast (complete in 0.5 ms) and slow (half-time 3 ms) components can be distinguished. The slow component corresponds kinetically to the rate of cytochrome *c* re-reduction and is similarly antimycin sensitive. These data are similar to those observed in isolated chromatophores which have been artifically poised with redox mediators. In aerobic intact cells the kinetic profile is altered, mainly because the decay of the carotenoid change is much faster. Inhibition of respiration with KCN leads to flash-induced changes similar to those in anaerobic cells. At least two components can be distinguished in the decay of the carotenoid absorption changes in anaerobic intact cells. Only the faster decay component was inhibited by venturicidin which suggests that it corresponds to H^+ flux through the F_0F_1-ATPase during ATP synthesis. The contribution of the venturicidin-sensitive decay to the total decay was dependent upon the initial amplitude of the carotenoid absorption change produced by the flash group. This suggests that there is an apparent threshold of membrane potential for ATP synthesis. Supporting evidence was provided by the finding that venturicidin stimulated the steady-state light-induced carotenoid absorption change at high but not at low light intensities. The entire decay of the carotenoid absorption changes was stimulated by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in a manner that can be interpreted as an ionophore catalysing the dissipation of membrane potential.

Introduction

Absorption changes in the carotenoid spectrum accompanying electron-transport phosphorylation in chromatophores from the photosynthetic bacteria can, within the framework of the chemiosmotic hypothesis [1], be interpreted as a response to membrane potential [2,3]. A discussion of the possible mechanism of the response is given in Refs. 4–6. When chromatophores are energised, e.g., with light, the nature of the absorption changes suggests that the absorption maximum of a small pool of the carotenoid pigments, associated with the B800-850 light-harvesting complex, is shifted towards the red [7–12]. Estimates of membrane potential based on the carotenoid absorption changes lead, however, to larger values than those obtained from the redistribution of permeant ions and this discrepancy has yet to be satisfactorily explained [13,32].

The importance of the carotenoid shift as an experimental tool arises from the rapidity of the response. If 'electrochromism' is the underlying mechanism [4] then the lower limit of time resolution is practically unattainable. Carotenoid absorp-

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

tion changes in chromatophores have been measured within 50 ns [2]. Measurements on the millisecond time scale have been most useful in the identification of membrane potential-generating and -utilising reactions [4–6]. Following short flash excitation the carotenoid absorption bands shift in three distinct phases: phase I (complete in 50 ns) corresponds to electron transport between reaction centre bacteriochlorophyll (P) and primary ubiquinone, phase II ($t_{1/2}$ in the range 10–100 μs) corresponds to the re-reduction of oxidized P by cytochrome *c* and phase III ($t_{1/2}$ in the range 1–10 ms) corresponds to the antimycin-sensitive reaction in the cytochrome *b-c* complex [14,15]. It has been suggested that phases I and II together constitute one electrogenic reaction and phase III represents another [14]. On a longer time scale the absorption changes reverse slowly but completely. The decay processes are predominantly the result of electrophoretic ion movements – mainly protons in the absence of added ionophores [16]. The outward flux of H^+ through the F_0F_1-ATPase during ATP synthesis is reflected in an accelerated decay of carotenoid absorption changes within 100 ms after the flash [16,17].

In this communication we show that several of these processes can also be demonstrated in intact cells of the photosynthetic bacteria. The major advantage of working with intact cells is that they are less prone to artifacts of preparation than are chromatophores. Loss or damage of membrane components and inhomogeneity of vesicle populations have previously caused concern in the interpretation of chromatophore data. In the long term the carotenoid shift might provide a useful technique for investigating the relationship between bacterial physiology and membrane bioenergetics, but for the present our intentions were to characterise the response in cells grown on a minimal medium in photoheterotrophic conditions.

The work of Joliot and Delosme [18] on the pigment absorption band shifts in intact algae and of Witt and Junge (see Ref. 5) on isolated spinach thylakoids provides an interesting parallel with the present work.

Experimental Procedure

Cells of *Rhodopseudomonas capsulata* strain N22 were grown photoheterotrophically and harvested and washed as described previously [19]. The washing and resuspension medium contained 0.01 M Na_2HPO_4 adjusted to pH 7.0 with H_3PO_4. The experimental medium was similar but was supplemented with 0.03 M sodium malate (pH 7.0), 0.007 M $(NH_4)_2SO_4$. This resembles fresh growth medium lacking minor salt components. The cells were stored on ice and used within 12 h of preparation. Bacteriochlorophyll was estimated at 772 nm in acetone/methanol extracts [20].

The kinetics of the carotenoid absorption changes and cytochrome redox reactions were measured in a cross-beam, two-photomultiplier, dual-wavelength spectrophotometer as described earlier [21]. Actinic flashes, 20 μs half-peak width were fired from below the cuvette. The data were averaged as described in the figure legends. Steady-state light-induced changes were monitored in a Perkin Elmer 356 chopped dual-wavelength spectrophotometer [19].

Anaerobic experiments were carried out under argon with butyl rubber and stainless-steel gas trains (see Ref. 19). The cuvettes were not stirred. In aerobic experiments small quantities of H_2O_2 were added at intervals indicated by parallel measurements with an oxygen electrode.

Antimycin and FCCP were purchased from Sigma, London, venturicidin from British Drug Houses, Dorset, U.K. All other reagents were of analytical grade.

Results

The kinetics of the carotenoid band shift in intact cells of Rps. capsulata after short flash excitation

In the data of Fig. 1A a suspension of intact cells was allowed to become anaerobic in the dark by malate respiration under an atmosphere of argon. Upon short flash excitation there was a distinctly biphasic absorption change in the carotenoid region of the spectrum followed by a very much slower decay. Between 470 and 520 nm the spectra of the fast, slow and decay components were similar (not shown). The absorption peak in the flash-induced difference spectrum was at 503 nm and the trough at 487 nm.

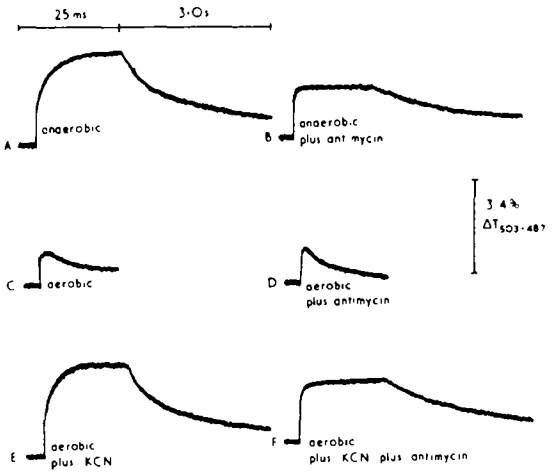

Fig. 1. Short flash-induced carotenoid absorption changes in intact cells of *Rps. capsulata*. Bacteriochlorophyll concentration 12 μM. In each experiment, A–F, the suspension was exposed to eight flashes, fired 50 s apart. The traces are averaged data for one flash. In experiments A, B, E and F, the horizontal time scale is split as shown above trace A. Experiments C and D are shown on the fast scale only. In experiments with antimycin (2.5 μM) or cyanide (2 mM), the suspension was incubated for 5 min with the inhibitor under anaerobic conditions before aerating and flashing.

These kinetics are remarkably similar to those observed in chromatophore suspensions poised at a redox potential poise of about +80 mV (pH 7) with appropriate redox mediator dyes [14,15]. In chromatophores, the fast phase has been resolved into two subsidiary components: phase I and II [14]. We have not attempted this scale of resolution in the intact cells. Most of the fast (antimycin insensitive) component is complete within 0.5 ms. The slower phase ($t_{1/2} \simeq 3$ ms in Fig. 1A) is clearly related to that described as 'phase III' in chromatophores by its kinetics and by its antimycin sensitivity (Fig. 1B). Rather more antimycin A is needed for inhibition of phase III in cells (about 1 antimycin molecule per 16 bacteriochlorophylls for 50% inhibition) than in chromatophores (about 1 per 200, see Ref. 22) but this may be due to binding or limited permeability through the bacterial outer membrane. In chromatophores phase III of the carotenoid shift has been correlated with electrogenic electron transport through the ubiquinone-cytochrome *b-c* complex [14,15]. That this interpretation may be extended to intact cells is shown by comparison of Fig. 1A and B with Fig. 2A and B. The kinetics of cytochrome *c* re-reduction following flash-induced oxidation are similar to those of the slow component in the carotenoid shift. Both processes are antimycin sensitive.

The carotenoid shift kinetics following flash activation of intact cells are different under aerobic conditions (Fig. 1C and D). Similar kinetics are not seen in chromatophore suspensions. The major difference is the greatly accelerated rate of decay which obscures any possible contribution from phase III in the rise of the shift. Fig. 2C and D reveals that cytochrome *c* is still flash oxidised in aerobic cells (at a reduced amplitude) but that the rate of re-reduction is considerably slower. Antimycin inhibition can be detected in the cytochrome *c* kinetics but not in the carotenoid data. It must be appreciated that intact cells of *Rps. capsulata* possess an active respiratory as well as a photosynthetic electron-transport chain. Under dark, aerobic conditions a considerable proton-motive force and accompanying carotenoid band shift are generated during respiration [19]. The flash-induced changes are therefore superimposed upon this dark background potential.

Respiration may be inhibited by several minutes anaerobic incubation with 2 mM KCN (data not shown). In Fig. 1E and F cells treated in this manner were returned to aerobic conditions and then flash activated. The carotenoid shift kinetics were then similar to those recorded under anaerobiosis.

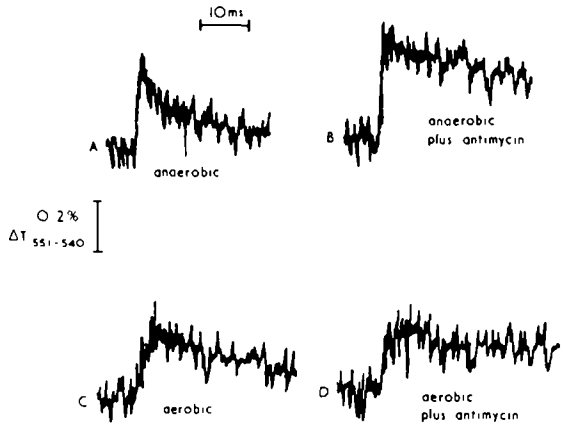

Fig. 2. Short flash-induced cytochrome *c* absorption changes in intact cells of *Rps. capsulata*. Conditions as Fig. 1 except that the bacteriochlorophyll concentration was 10 μM and each trace represents an average of 16 flashes.

Decay kinetics of the carotenoid shift in intact cells of Rps. casulata

The magnitude of the carotenoid shift in an anaerobic cell suspension may be manipulated by varying the number of flashes in a group or by inhibiting electron transport with antimycin A. Four examples are shown in Fig. 3. In Fig. 3A, untreated cells were excited by two closely spaced flashes. The total extent of the shift elicited by two flashes is close to (about 90%) the achievable maximum – more flashes (tested at a range of frequencies) produced only small increases. In both Fig. 3B and C the amplitude of the shift was only about 0.6 of that in A and it was generated by two different electron-transport patterns: in Fig. 3B, untreated cells were exposed to single flashes and in C antimycin-treated cells were excited by three closely spaced flashes. In trace D only a low amplitude of carotenoid shift was generated (0.33 of that in A) by firing single flashes at an antimycin-treated suspension.

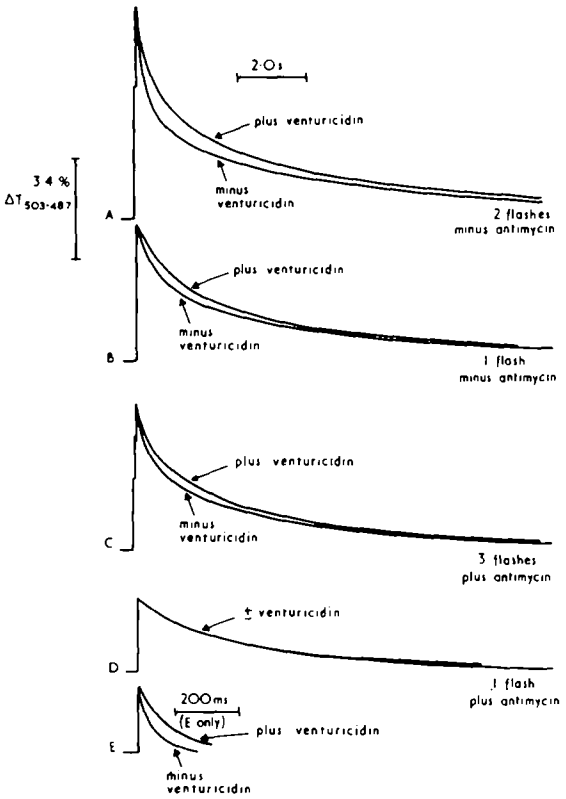

Fig. 3. The decay of the carotenoid absorption change in intact cells of *Rps. capsulata*. Bacteriochlorophyll concentration 9.4 μM. Each trace is an average of 16 sweeps, 50 s apart. Venturicidin was present where indicated, at a concentration of 5.1 μg/ml. Experiment A, excitation by double flashes 50 ms apart. B. single flash excitation. C, excitation by triple flashes 50 ms apart, suspension supplemented with 3.6 μM antimycin. D, single flash excitation in the presence of antimycin. Experiments A–D were performed anaerobically. Experiment E was performed aerobically with single flashes (absence of antimycin) on a faster time scale.

In Fig. 3, the decay profile changes distinctly throughout the series A, B/C and D. A particularly noticeable feature is that a fast decay component predominates in A, is barely noticeable in D and makes an intermediate contribution in B and C. This is made clearer from an examination of the semilogarithmic plots in Fig. 4. The decay of the shift may be approximated by the sum of two first-order processes. The proportion of the fast decay component decreases through 66, 50, 52 and 30% of the total decay for A to D, respectively. The residual fast decay component in D is much slower (2–3-fold) than that in A, B and C and is probably due to an unrelated process.

As noted above, the presence of oxygen in the intact cell suspension leads to a red-shifted carotenoid spectrum due to a steady-state respiratory membrane potential and a short flash given under these conditions elicits a further carotenoid shift which decays very rapidly. The decay kinetics are shown again in Fig. 5E for comparison with the decay in anaerobic cells (note the different time scale).

Venturicidin in intact cells of *Rps. capsulata* behaves as a specific inhibitor of the ATP synthetase [19]. In the experiment shown in Fig. 3, venturicidin

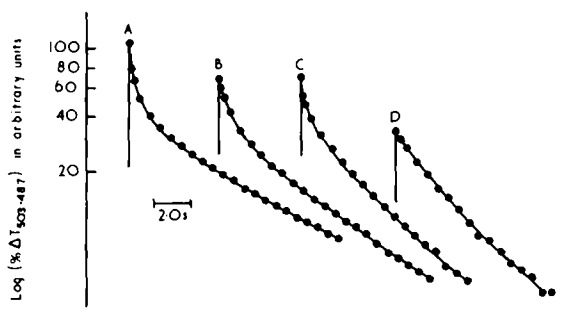

Fig. 4. Logarithmic plots of the decay of the carotenoid absorption change. Data taken from Fig. 5 (absence of venturicidin).

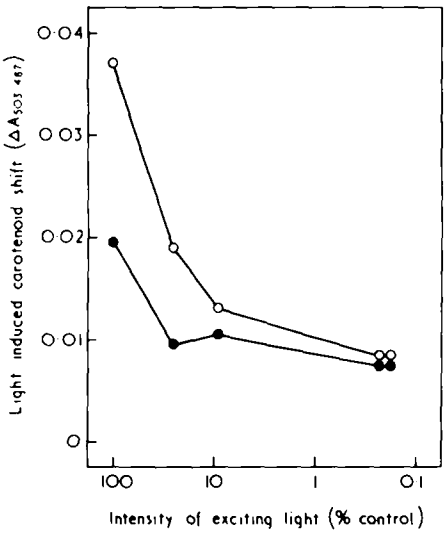

Fig. 5. The effect of venturicidin on the steady-state light-induced carotenoid absorption change as a function of light intensity. Bacteriochlorophyll concentration 10.2 μM. The intensity of the exciting light was attenuated with combinations of grey filters, the percent transmission of which was measured at 800 nm. (•) No venturicidin. (○) Venturicidin was added to a concentration of 4 μg/ml.

very effectively decelerated the decay of the carotenoid shift in trace A ($t_{1/2}$ was increased by 85%), had no effect in D and had an intermediate effect in B and C ($t_{1/2}$ increased by 40 and 35%, respectively). Log plots of the decay revealed that the decelerative effect of venturicidin was due to its decreasing the relative extent of the fast decay phase (see Ref. 19). The fast decay phase was not entirely eliminated. The rate of the slow decay phase was completely unaffected. Under aerobic conditions the entire decay of the carotenoid shift produced by a flash was slowed down by venturicidin (Fig. 3E).

There was some variation in the degree of the inhibitory response produced by venturicidin in different cell preparations. Fig. 3 shows the most common situation. The antibiotic was always observed to inhibit the decay after two closely spaced flashes in the absence of antimycin (cf. Fig. 3A). In experiments equivalent to those of Fig. 3B and C the amount of fast decay phase was variable and in about 30% of the samples investigated, venturicidin had no effect. In one experiment equivalent to that of Fig. 3D (from more than five examined) venturicidin treatment did lead to a decelerated decay of the carotenoid shift.

The extent of the carotenoid shift generated by three or four closely spaced flashes in an anaerobic suspension (not shown) is only slightly greater (about 10%) than that produced by two flashes. The decay profile is practically indistinguishable. A further seven flashes in the train did not give rise to any increase in extent of the shift, nor was the decay after ten flashes different from that after three flashes. The fast decay phase after ten flashes was venturicidin sensitive.

Since venturicidin only inhibited the decay of the carotenoid shift after a flash when a certain minimum initial amplitude was exceeded (Fig. 3), we expected a corresponding response in continuous illumination. In the experiments of Fig. 5, the amplitude of the carotenoid shift was lowered by decreasing the intensity of the exciting light. Venturicidin stimulated the carotenoid shift at high but not at low light intensities.

In chromatophores, the decay of the carotenoid shift after a flash is accelerated by ionophores such as valinomycin and FCCP [2,21]. Intact cells are not sensitive to valinomycin but the acceleration with uncoupling agents can be easily demonstrated. Fig. 6 shows how the logarithm of the half-decay time of

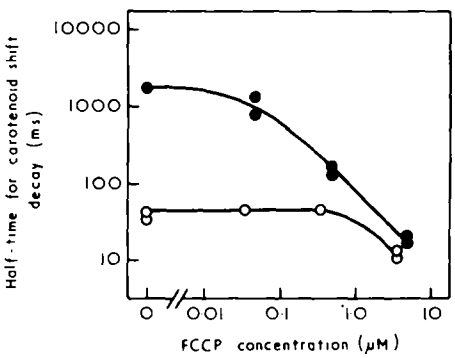

Fig. 6. The dependence of the half-time of the decay of the carotenoid absorption change after a flash on the concentration of FCCP. Conditions as Fig. 1B and E (minus venturicidin) except that the bacteriochlorophyll concentration was 14 μM. Each data point was taken from a separate experiment in which the cells were incubated with FCCP for 2 min before firing the flashes. The half-time for the carotenoid shift was estimated from traces averaged over eight passes. •, anaerobic cells, ○, aerobic cells.

the carotenoid shift depends on the logarithm of the FCCP concentration in both aerobic and anaerobic cell suspensions. A simple model [21] predicts (a) that the slope of the curve approaches a value of −1.0 when the permeability due to the ionophore is large compared with the intrinsic permeability of the membrane and (b) that the curves converge at high concentrations of ionophore independently of the intrinsic membrane permeability. The data of Fig. 6 gives a good fit to this model.

Discussion

The kinetics of membrane potential development in intact cells

In chromatophore suspensions there is an optimal redox potential poise at about +80 mV (at pH 7) at which cyclic electron transport after short flashes proceeds at a maximum rate [23,24]. This poise is achieved experimentally by incubating under anaerobic conditions in the presence of low concentrations of redox mediators and by adding trace amounts of reductant. Under these conditons, the rate of cytochrome *c* re-reduction is maximal [23,24] and the carotenoid shift is maximal in rate and extent [14, 15].

Intact cells spontaneously adopted the optimal redox poise under anaerobic conditons (Fig. 1 and 2). The cytochrome *c* oxidation-reduction reactions and the carotenoid shift kinetics were very similar to those which have been recorded earlier in chromatophores poised at about +80 mV [14,15]. In particular, we draw attention to similar reaction times of the antimycin-sensitive processes and the approximately equal contributions in both cells and chromatophores of the fast (I + II) and slow (III) phases of the carotenoid shift which have been taken to indicate the two electrogenic reactions in the electron-transport chain [14]. The cells were able to maintain this optimal poise for long periods: it was found that after 3 h of darkness and anaerobic conditions a single test flash still elicited the characteristic carotenoid response. It seems that the cells must operate a homeostatic redox mechnism in readiness for efficient utilisation of incoming light energy.

Under aerobic conditions the optimal poise was lost and cytochrome *c* re-reduction after the flash became much slower. This fact, and the lower amplitude of cytochrome *c* oxidation after a short flash, can probably be explained by the overlapping of components between the photosynthetic and respiratory chain [25]. For instance, cytochrome *c* may be oxidised either by the photosynthetic reaction centre or by an oxidase, cytochrome *b*-410 [26,27]. Inhibition of respiration with cyanide led to re-establishment of the optimal redox poise in aerobic cells (Fig. 1).

The decay of the membrane potential

The decay of the carotenoid shift after short flash excitation of bacterial cells represents the electrophoretic displacement of ions across the plasma membrane in response to the membrane potential generated by electron transport. This is supported by experiments with chromatophores [2,16,21] and by the data of Fig. 6 in which the carotenoid shift decay was accelerated by FCCP in accordance with a simple physical model for the effect of ionophores on membrane potential dissipation [21]. The electrophoretic flux of ions may be involved in the performance of useful chemical work such as the synthesis of ATP and the translocation of substrates [1]. Consistent with this view, the fast phase in the decay of the carotenoid shift probably reflects the electrophoretic flux of H^+ (inwards in cells) through the F_0F_1-ATPase during ATP synthesis. This is concluded from the experiments with venturicidin which blocked the fast decay phase. In an earlier report [19] we showed that venturicidin, an antibiotic with oligomycin-like properties, is an effective energy-transfer inhibitor in intact cells of *Rps. capsulata.*

The extent of the fast phase in the decay of the carotenoid shift was dependent upon the initial amplitude of the shift produced by the flash. The fast decay phase was only evident when a minimum initial amplitude of the shift was exceeded. It was immaterial how the minimum amplitude was reached: similar results were obtained using either triple flashes in antimycin-treated cells or single flashes in the absence of antimycin (Figs. 3 and 4). This suggests that the extent of the venturicidin-sensitive fast decay depends upon the value of the membrane potential but not upon the electron-transport route by which it was generated. We conclude that there must be a range of membrane potential, below which ATP is only slowly synthesized. A similar conclusion can be arrived at from the experiment shown in Fig. 5. At high light

intensities and membrane potentials ATP synthesis was presumably proceeding at a rapid rate. Addition of venturicidin blocks H^+ flux through the ATP synthase and therefore raises the membrane potential. At low light levels we suppose that the rate of ATP synthesis was slow and that only a relatively small proportion of the inward proton current was taking place through the F_0F_1-ATPase. Therefore, venturicidin was without effect. The experiment (Fig. 5E) on flash-induced carotenoid absorption changes under aerobic conditions can be interpreted by similar reasoning. Since these cells were respiring they were subjected to a moderately large, constant membrane potential, sufficient to drive ATP synthesis [29]. This would explain why, after a superimposed membrane potential produced by a short flash, the decay was very fast and was entirely venturicidin sensitive.

In dark, anaerobic suspensions of *Rps. capsulata* the phosphorylation potential, ΔG_p, inside the intact cells (about −35 kJ/mol, unpublished observations, assuming no nucleotide binding) is far from equilibrium with the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+} \simeq 6$ kJ/mol) [32]. Since the rate of substrate level phosphorylation from stored polysaccharide is very slow under these conditions [28], it must follow that the rate of ATP hydrolysis by the F_0F_1-ATPase is also slow. This lack of equilibrium is clearly beneficial to the cells to prevent wasteful dissipation of stored energy. We suggest that the data of Figs. 3 and 4 illustrate a steep dependence of the activity of the ATPase enzyme activity upon $\Delta\bar{\mu}_{H^+}$. By analogy with interpretations of experiments on isolated thylakoid membranes, this might imply a threshold value of $\Delta\bar{\mu}_{H^+}$ below which the ATPase is inactivated, i.e., an ATPase trigger [29], or it might reflect a high power dependence of the rate of catalysis upon $\Delta\bar{\mu}_{H^+}$ [5]. Analysis of the data in intact bacteria must, however, be approached cautiously, since on a molar basis the quantity of adenine nucleotides in the cells is only 10–20-fold in excess of the quentity of electron-transport chains (Refs. 19 and 30 and assuming 1 electron-transport chain per 100 antenna bacteriochlorophylls). Consequently, the value of ΔG_p inside the cells might rise considerably even during the period of decay following a single flash group. Of course ATP utilisation for biosynthesis will tend to restore ΔG_p and the fact that the carotenoid shift decay kinetics after ten closely spaced flashes were similar to those after two suggests that a steady state was rapidly achieved.

The carotenoid shift decay after short flash excitation of chromatophore suspensions is accelerated in the presence of ADP and P_i [16,17]. The acceleration has a similar substrate dependence and inhibitor sensitivity to ATP synthesis measured directly with luciferin/luciferase [17]. The kinetics of the accelerated portion of the decay are approximately similar to those of the venturicidin-sensitive fast decay phase in intact bacterial cells, in agreement with our general thesis. There are some differences between the response in cells and in chromatophores which we are unable to account for. For instance, in chromatophores treated with antimycin, an ADP-dependent fast phase can be routinely demonstrated [16,17] (cf. Fig. 3D). However, after multiple flashes in the absence of antimycin the fast decay phase in the chromatophores is never as extensive as it is in whole cells. Based on the flash intensity dependence we concluded that there is no evidence of a 'threshold effect' in chromatophores [31] although it is clear in intact cells. We assume that either the properties of the ATPase are subtly modified upon disruption of the cells or that crucial factors in the F_1 environment inside the cell have not been reproduced in the chromatophore suspensions.

Acknowledgement

This work was supported by a grant from the Science Research Council.

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