Biochimica et Biophysica Acta, 636 (1981) 1–8
Elsevier/North-Holland Biomedical Press

BBA 48054

PHOTOSYNTHETIC CONTROL AND ESTIMATION OF THE OPTIMAL ATP : ELECTRON STOICHIOMETRY DURING FLASH ACTIVATION OF CHROMATOPHORES FROM *RHODOPSEUDOMONAS CAPSULATA*

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(Received June 26th, 1980)
(Revised manuscript received January 12th, 1981)

Key words: ATP: electron ratio; Flash activation; Electron transport; Photosynthetic control; (Rps. capsulata)

(1) When chromatophores from *Rhodopseudomonas capsulata* Ala pho$^+$ are exposed to a train of high-frequency, saturating flashes the kinetics of the reaction centre bacteriochlorophyll absorption change enter a pseudo steady-state in which the extent of oxidation during the flashes is equal to the extent of reduction in between the flashes. The level of the pseudo steady-state is lowered by the presence of a phosphate acceptor system, raised by further addition of oligomycin, lowered by a combination of nigericin and valinomycin and raised by antimycin A. (2) In the pseudo steady-state, the extent of reaction centre bacteriochlorophyll oxidation taking place during the flash may be estimated by subtraction from the total concentration of reaction centre bacteriochlorophyll. This value is equated with the amount of electrons transported through the photosynthetic chain. Comparison with the measured ATP yield per flash in the pseudo steady-state permits calculation of the ATP : two electron ratio. The value of the ratio is 1.1 for flash frequencies between 3 and 12.5 Hz and declines at lower and higher frequencies. The ATP : two electron ratio is approximately halved in the presence of antimycin A. (3) An alternative estimate of the ATP : two electron ratio, based on the assumption that high-frequency flashes approximate to the condition of continuous illumination, was approx. 0.8.

Introduction

Chromatophores isolated from photosynthetic bacteria have been used extensively for studies on the mechanism of electron-transport phosphorylation [1,2]. In recent years, the organisms, *Rhodopseudomonas capsulata* and *Rhodopseudomonas sphaeroides* have been most commonly employed as a source of chromatophores because of their simple growth requirements, their clearly defined absorption spectra and because of the availability of useful mutant strains. However, despite apparently high rates of photophosphorylation and efficient energy coupling in chromatophores from the Rhodopseudomonads, there has to date been no report of control of the electron-transport rate by the phosphate acceptor potential even though this effect has been sought after on several occasions. In contrast, chromatophores from the related species, *Rhodospirillum rubrum* have been reported to display photosynthetic control [3,4].

In these species the pathway of photosynthetic electron transport is cyclic [5,6]. In order to measure electron-flow rates, it is necessary to interrupt the cycle using either an appropriate inhibitor and exogenous electron donors and acceptors or flashing light procedures. When a train of closely spaced flashes is fired at a chromatophore sample, the rate of relaxation of optical signals corresponding to the redox

Abbreviation: BChl, bacteriochlorophyll.

state of individual carriers has been shown to be a sensitive indication of the coupled state of the membranes [7,8]. Observing this parameter in *Rps. capsulata*, Ala pho$^+$ chromatophores, which have a rather high activity for photophosphorylation [9], we have been able to provide a simple demonstration of photosynthetic control.

Under short-flash conditions, two H^+ are taken up and probably translocated inwards across the chromatophore membrane for each electron proceeding through the cycle [10]. It has been estimated that the synthesis of one ATP molecule is accompanied by the outward flux of two positive charges [11,12]. Hence, the chemiosmotic hypothesis [13] would predict a stoichiometry of two ATP : two electron transferred. Apparent stoichiometries lower than this by an order of magnitude are, however, observed on single-flash activation [14–16]. Several thermodynamic and kinetic factors may contribute to these low estimates [14,15] among which the predominant one seems to be the limitation of the protonic potential for driving ATP synthesis. We have, therefore, attempted to measure the ratio for isolated flashes in a train of pulses fired at various frequencies. In this situation, the magnitude of the electrochemical proton gradient becomes substantially larger than that generated by a single short flash and the maximal ATP yield per flash is reached. The ATP : two electron ratio varies with flash frequency and shows an optimum value of 1.1, higher than single-flash determinations but still lower than the theoretical maximum assuming 100% efficient coupling.

Assuming that flashing light approaches the condition of steady-state light as the flash frequency is increased, we have also been also to make a crude estimate of the rate of turnover of the cycle for comparison with continuous light-induced ATP synthesis. Considering the approximations made in the latter calculation, the values of ATP : electron agree remarkably well with those obtained for isolated, single flashes in a train.

Materials and Methods

The culture and harvesting conditions for *Rps. capsulata* Ala pho$^+$ and the chromatophore preparation and storage have been described on earlier occasions [17]. Bacteriochlorophyll was measured in acetone/methanol extracts according to the method of Clayton [18].

The rate of ATP synthesis in saturating, continuous light was measured by $^{32}P_i$ incorporation according to standard procedures [17].

Electron-transport reactions were measured by conventional cross-beam spectrophotometry with near infrared actinic flashes (Wratten 88A filter) fired from below the 1×1 cm cuvette containing 1 ml of sample. The amplified signals were stored and averaged as described in the figure legends. The reaction centre bacteriochlorophyll kinetics were measured at 540 nm where, in chromatophores from this strain, interference from other components is minimal [19]. The extinction coefficient of $[BChl]_2$ at 540 nm has been determined as 10.3 $mM^{-1} \cdot cm^{-1}$ [19]. The sample routinely contained 100 mM glycyl-glycine, 10 mM $MgCl_2$, 10 mM KCl, 8 mM potassium phosphate, 0.1 M sodium succinate, 0.1% bovine serum albumin, 4 μM nigericin (to dissipate ΔpH from $\Delta\bar{\mu}_{H^+}$) and the bacteriochlorophyll concentration shown in the figure legends at a final pH of 7.75. Nigericin was shown not to affect the ATP yield during at least the first 20 turnovers [15].

Flash yields of ATP synthesis were measured in a similar medium supplemented with crude luciferase and purified lucerifin as described in Ref. 15 and the appropriate figure legends. Signals were not averaged but were recorded from signal-pulse trains.

Antimycin, valinomycin, adenine nucleotides, purified lucerifin and crude lucerifase (FLE 50) were purchased from Sigma (St Louis, U.S.A.). Nigericin was a generous gift from Dr. R.L. Hamill, Lilly Research, Indianapolis, U.S.A. Other reagents were of analytical grade.

Results

Photosynthetic control in chromatophores from Rps. capsulata

Fig. 1 shows a series of recordings of the signal due to the oxidation and reduction of reaction centre bacteriochlorophyll special dimer ($[BChl]_2$) taking place during flash train excitation of an aerobic suspension of chromatophores from *Rps. capsulata* Ala pho$^+$. Separate experiments at high redox potential, E_h = 430 mV (where cytochrome c_2 is chemically oxidized before the flashes), had shown that the chromato-

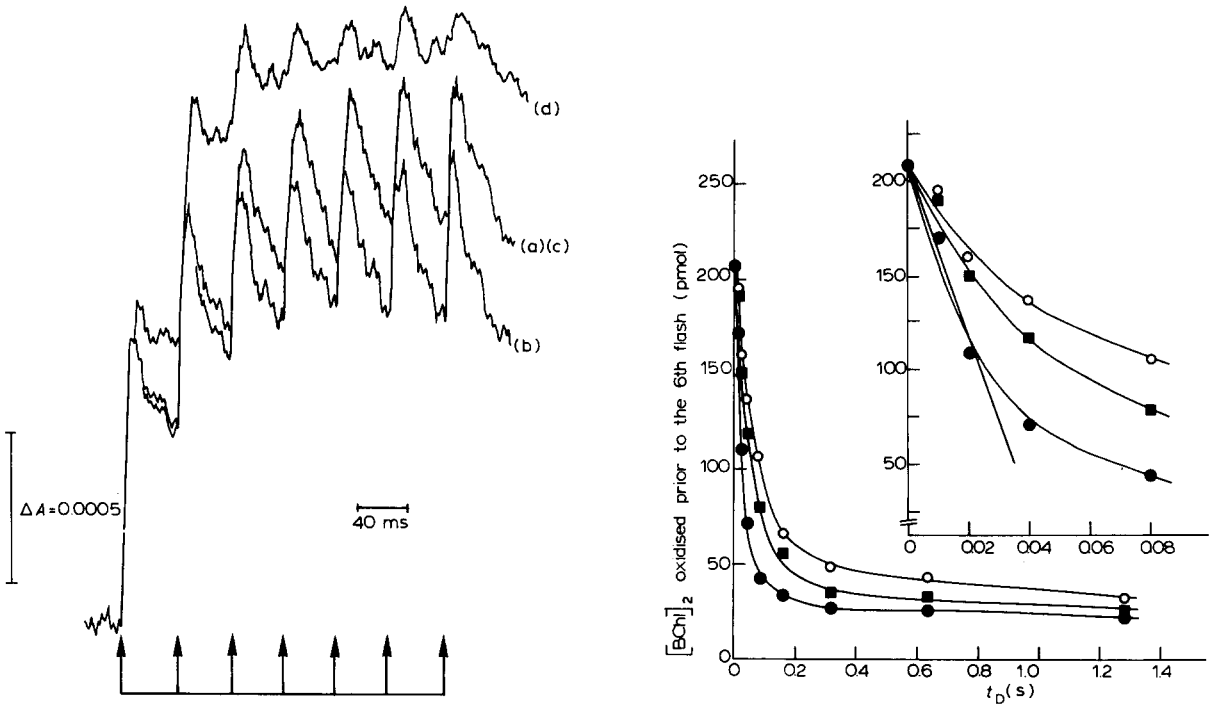


Fig. 1. $[BChl]_2$ relaxation kinetic under multipulse activation in chromatophores from *Rps. capsulata* Ala pho$^+$. The experimental media and conditions were as described in Materials and Methods. The bacteriochlorophyll concentration was 4.2 μM. Throughout the experiments 8.0 mM phosphate was present. (a) Control, no further addition; (b) plus 40 μM ADP; (c) plus 40 μM ADP, plus 10 μM oligomycin; (d) plus or minus ADP, plus 4 μM antimycin A. Each trace represents the average of eight groups of seven flashes with 50 s between the groups.

Fig. 2. The amount of $[BChl]_2$ remaining oxidized prior to the sixth flash as a function of flash frequency under phosphorylating and non-phosphorylating conditions. Each point was obtained from experiments similar to those performed in Fig. 1, except that the flash frequency in a flash train was varied. t_D is the time between flashes in a train. The $[BChl]_2$ oxidized prior to the sixth flash was measured from the kinetic traces and converted into pmol amounts using the extinction coefficient given in Ref. 19. The amount of oxidized $[BChl]_2$ at $t_D = 0$ was estimated from experiments at high flash frequency in the presence of antimycin A, or from experiments at an imposed redox potential of 430 mV (see text). The insert shows an expanded scale for short t_D and the rate used to estimate electron turnover (see text). (○) Control with 8 mM phosphate present, (●) plus phosphate plus 40 μM ADP, (■) plus phosphate and ADP plus 10 μM oligomycin.

phores were sufficiently dilute to allow more than 90% photo-oxidation of the total $[BChl]_2$ during each flash (data not shown). In Fig. 1 the microsecond reduction of photooxidized $[BChl]_2$ by cytochrome c_2 [19] is incompletely resolved. The reduction kinetics observed on the traces are partly due to the residual reaction with cytochrome c_2 on the millisecond time scale, but are mainly a result of the photosynthetic back reaction $t_{1/2} = 1.0$ s in those reaction centres deficient in cytochrome c_2 or possessing only ferricytochrome c_2 [20].

After four or five flashes in a train, the $[BChl]_2$ kinetics enter a pseudo steady-state in which the amount of oxidation during the flash is equal to the amount of reduction after the flash. This is a consequence of the cyclic nature of the electron-transport chain. During the first few flashes, the early electron donors to the reaction centre (cytochrome c_2 and J) become rapidly oxidized and the early acceptors (quinone and cytochrome *b*) become rapidly reduced. The recycling of electrons through the ubiquinone cytochrome b/c_2 oxidoreductase is rate limiting [5],

so the mean level of oxidized $[BChl]_2$ increases during successive flashes. In the pseudo steady-state the amount of reduced $[BChl]_2$ which is available for photo-oxidation depends upon the extent of electron flow that has occurred through the cytochrome b/c_2 complex between the flashes. Therefore, the redox state of $[BChl]_2$ at the instant immediately before the sixth or seventh flash gives an indication of net electron flow through the cycle during the dark time between the flashes.

In Fig. 1 it may be seen that the provision of a phosphate acceptor system leads to a relatively lower level of oxidized $[BChl]_2$, indicating that the flow of electrons has been increased. The effect is abolished by the addition of concentrations of oligomycin sufficient to inhibit light-driven ATP synthesis. Further addition of valinomycin (the medium was initially supplemented with nigericin) again leads to an accelerated rate of electron transport (cf. Refs. 7 and 8). As expected, subsequent antimycin A treatment gives rise to a greatly elevated level of $[BChl]_2^+$, due to almost complete inhibition of cyclic electron flow [21].

The dependence of the level of $[BChl]_2^+$ immediately before the sixth flash (i.e., in the pseudo steady-state) upon dark time between flashes is shown in Fig. 2. In this particular sample, the inhibition by oligomycin of the stimulation of the rate of cyclic electron transport by ADP and P_i was not complete. We did indeed observe some variations in the degree of acceptor control of electron transport in different preparations (cf. Figs. 1 and 2).

Estimation of P : 2e ratio for cyclic electron transport

The total amount of $[BChl]_2$ available for photo-oxidation in the chromatophore sample is easily determined from the absorption change at 540 nm produced by a high-frequency flash train in the presence of antimycin or in the presence of sufficient $K_3Fe(CN)_6$ to oxidise chemically cytochrome c_2 (allowance is made in the latter experiment for partial chemical oxidation of $[BChl]_2$). The two estimates agree to within 10%. The number of electrons proceeding through the cycle and driven through the reaction centre by a single flash during the pseudo steady-state (see above) can be computed by subtraction of the amount of $[BChl]_2^+$ existing immediately before the flash from the total $[BChl]_2$. Fig. 3 shows these data for a range of flash frequencies.

The above experiments were carried out in the presence of ADP and P_i. The amount of coincident ATP synthesis per flash in the pseudo steady-state was estimated by supplementing the medium with luciferin and luciferase and measuring the light emitted during a train of flashes in a parallel series of experiments over the same range of frequencies [14,15]. Since the reaction time of newly synthesised ATP with the luciferase is longer than the time between the flashes [22], it is not possible to resolve directly the ATP yield for a single, isolated flash in a train. In Fig. 3 the average yield for the sixth and seventh flashes is determined by subtraction of the total ATP produced by trains of five, six and seven flashes [14, 15].

Also shown in this figure is the ratio of ATP synthesised per two electrons transported through the reaction centre and cycle as a function of the dark time between flashes. With a dark interval between 80 and 320 ns, the P : 2e ratio was relatively constant at a value slightly greater than unity. At lower or higher flash frequencies the P : 2e ratio declined.

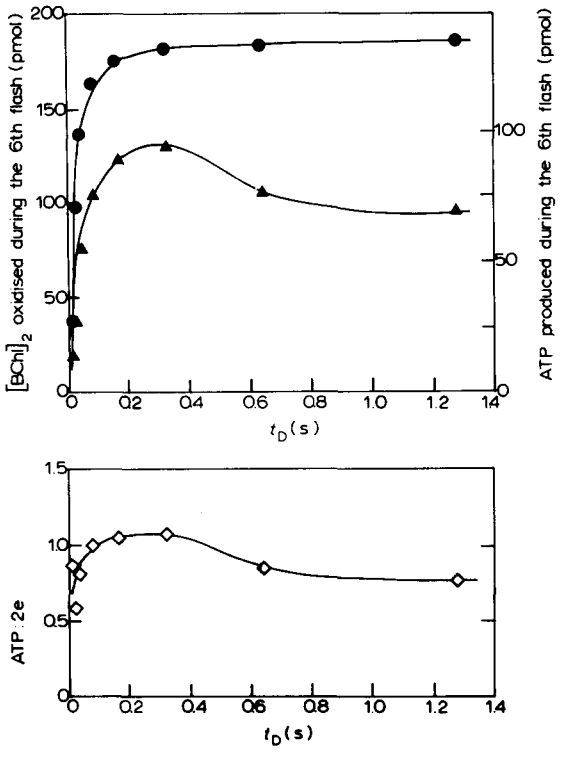
Estimation of P : 2e in the presence of antimycin A

The results from a similar series of experiments to that shown in Fig. 3, but in the presence of antimycin A, are shown in Fig. 4. The re-reduction of $[BChl]_2$ in the pseudo steady-state requires longer dark times between the flashes when electron flow through the ubiquinone-cytochrome b/c_2 oxidoreductase is blocked by this inhibitor. The recovery of reduced $[BChl]_2$ may proceed through both a 'leak' through the block and by way of the photosynthetic back reaction.

Antimycin is a very effective inhibitor of ATP synthesis per flash in the pseudo steady-state (cf. Figs. 3 and 4). Consequently, the error in determining the ATP yield during the sixth and seventh flash by the method described above is considerably larger. Nevertheless, the ATP : 2e ratio determined in the presence of antimycin was consistently between 20 and 70% of that in the absence of inhibitor.

Turnover of the electron-transport chain

Using the carotenoid absorption band shift of *Rps. sphaeroides* chromatophores as an indicator, it has been shown that the recovery of photochemistry



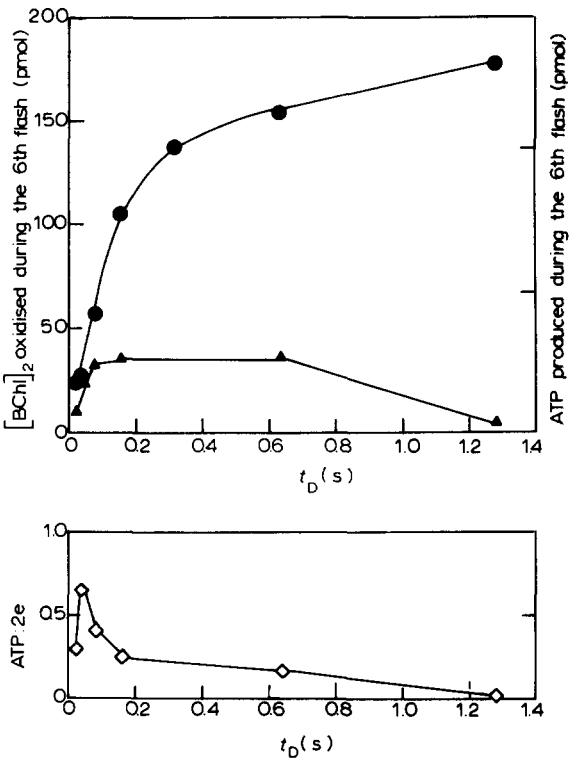


Fig. 3. The P : 2e ratio as a function of flash frequency. (•) $[BChl]_2$ oxidized during the sixth flash. The data were calculated from Fig. 2 (in the presence of ADP and phosphate) by subtracting the amount of $[BChl]_2$ remaining oxidized prior to the sixth flash at the t_D value indicated, from the total available $[BChl]_2$ (i.e., at $t_D = 0$ from Fig. 2). (▲) ATP produced during the sixth flash as described in the text. (◇) ATP : 2e ratio computed from the data above.

Fig. 4. P : 2e ratio in the presence of antimycin A as a function of flash frequency. Experiments were carried out as in Fig. 1 in the presence of antimycin, phosphate and ADP. The data were transformed as described in Figs. 2 and 3. The symbols are as for Fig. 3.

between flashes in a train has a distinctly biphasic dependence on the flash frequency [23]. The reaction centre absorption change at 540 nm in chromatophores from *Rps. capsulata* Ala pho$^+$ shows similar recovery kinetics (Fig. 2). The slower of the two phases has similar kinetics to the re-reduction of $[BChl]_2^+$ at a redox poise of 433 mV (where cytochrome c_2 is chemically oxidised) and is stimulated in extent but unmodified in rate in the presence of antimycin A. It is, therefore, probably a reflection of the photosynthetic back reaction.

The faster of the two recovery phases is accelerated by ADP and P_i (see above) or uncoupling agents and inhibited by antimycin A. After subtraction of the back reaction kinetics (Fig. 2) the fast recovery phase has a half-time of 17 ms (+ADP, P_i) and 44 ms (−ADP, P_i) and approaches a simple exponential process. These half-times and kinetic behaviour are consistent with those measured for cytochrome *c* re-reduction in chromatophores poised at a redox potential around 200 mV [24]. A redox poise between 200 and 300 mV is expected in the aerobic suspensions used here.

The relative extents of the two phases are also altered under the provision of a phosphate acceptor system. In Fig. 2 the slow phase accounts for about 28% of the total in the absence and about 17% in the presence of ADP. It appears that a smaller proportion of reducing equivalents returns to the reaction centre by way of the back reaction, when the membrane high-energy state is being dissipated during ATP synthesis. A qualitatively similar response has been ob-

served when the high-energy state is dissipated by uncoupling agents [23].

In the pseudo steady-state, we can easily compute the amount of $[BChl]_2$ which has been reduced through cyclic electron flow and which is available for photo-oxidation immediately before the sixth flash (see above). As the flash frequency is increased the pseudo steady-state should approach the conditions of continuous illumination. From the dependence of the amount of reduced $[BChl]_2$ immediately before the sixth flash upon dark time between the flashes at high frequency, we should therefore be able to estimate a value of the rate of electron turnover in steady-state light. The data of the inset Fig. 2 (in the presence of ADP and P_i) give a rate of approx. 0.71 nmol electron/nmol bulk bacteriochlorophyll per s. The rate of photophosphorylation in continuous light in this chromatophore sample was 0.28 nmol ATP/nmol bulk bacteriochlorphyll per s so the P : 2e ratio by this method is 0.78.

Discussion

The main purpose of the experiments described here is the elaboration of an experimental approach for the kinetic analysis of coupled electron flow in cyclic photosynthetic systems and consequently for the evaluation of correct ATP : 2e ratios. Since the presence of photosynthetic control phenomena can be considered the most rigorous criterion of coupled electron transfer, we have chosen for these studies *Rps. capsulata*, Ala pho^+, which show an unusually high rate of photophosphorylation (on a bacteriochlorophyll basis) for the Rhodopseudomonads [9] (up to 1500 μmol/h per mg BChl). One reason for this apparently higher rate is that the strain is deficient in light harvesting complex II and consequently has a higher than normal ratio of reaction center to antenna bacteriochlorophyll [25] (28–35 as compared to 80–200 BChl/reaction centre in other wild-type or green strains of *Rps. capsulata* and *Rps sphaeroides*). There may, however, be other factors contributing to the high rates, as suggested by the significantly higher ATP : 2e ratio observed in this strain as compared to others (Venturoli, G. and Melandri, B.A., unpublished results). One of these reasons could be the abundance of the ATP synthetase complexes in chromatophores from the Ala pho^+ strain as indicated by their high ATPase activity (100–200 μmol/h per mg BChl). Indeed, high ATP : 2e ratios during a train of flashes have been reported by Del Valle-Tascon et al. [26] also in *R. rubrum*, from which generally chromatophores with a high ATPase activity, but limited in electron flow can be prepared.

Qualititatively, the photosynthetic control phenomenon can be seen easily from experiments at fixed flash frequency (Fig. 1). Abolition of the effect by oligomycin argues strongly for the hypothesis that dissipation of the membrane-energised state during ATP synthesis is responsible for the acceleration of electron transport. The dependence on flash frequency (Fig. 2, inset) suggests an approx. 2–3-fold increase in the rate of electron transport under phosphorylating conditions, although with fresh chromatophore preparations a 4-fold acceleration was occasionally observed (not shown).

It is also quite evident from the trace of Fig. 1 that the acceleration of electron transport by addition of ADP and phosphate can be observed only after the third to fourth turnover, i.e., when a sufficiently high proton-motive force has developed. This behaviour is quite consistent with the observations by Crofts et al. [8] and Prince and Dutton [7] that the control of electron transport by the protonic gradient is exerted only after several single-turnover flashes and with the evidence [15] that the induction of maximal ATP yield per flash requires three to four turnovers under conditions of phosphate potential similar to those present in the assay ($\Delta G_p \simeq -9.5$ kcal/mol). It should be emphasised that the phenomenon of photosynthetic control is demonstrable only on the fast phase of $[BChl]_2$ re-reduction, supporting the idea that this process is a measure of coupled electron flow. The rate of the slow re-reduction phase, which we have related to the photosynthetic back reaction (see Ref. 20) is not modified by phosphorylation substrates.

The method for determination of P : 2e in Fig. 3 is fairly direct. It depends only on established procedures for the determination of ATP yield and extent of electron transport. Two factors may contribute to the decrease in value of the measured ratio as the flash frequency is decreased. Firstly, the proportion of electron flow failing to proceed through the cycle but instead returning through the photosynthetic back reaction increases at decreasing flash frequency. If 15–30% of the reaction centres are deficient in

ferrocytochrome c_2 (estimate from Fig. 2) and if the back reaction is considered to be an exponential process of half-time 1.0 s [20,27], then only 2–3% of the measured amount of electron transport takes place via the back reaction with 100 ms between the flashes but as much as 15–30% with a 1 s dark time. Secondly, the mean level of $\Delta\bar{\mu}_{H^+}$ across the chromatophore membrane is diminished in the pseudo steady-state when the dark time between flashes is long. The energetic state of the chromatophores at low flash frequencies will closely resemble that achieved during single-flash activation. The resulting decrease in the ATP yield per flash (Fig. 2 and Ref. 14) under these conditions was formerly ascribed to a dependence of the activity of the chromatophore ATPase on $\Delta\bar{\mu}_{H^+}$ [14]. Recent evidence, however, points to a simpler explanation based on the relative balance of $\Delta\bar{\mu}_{H^+}$ and phosphate potential [15]. In any case, the low values of P : 2e at low flash frequencies, i.e., at low $\Delta\bar{\mu}_{H^+}$, are consistent with the reported low values for single-flash activation [14,15].

The drop in P : 2e values at high flash frequencies is not so easily explained. It is clearly related to one of the processes limiting ATP synthesis noted in earlier report [15] and may be a result of a rate limitation of the ATP synthase.

Despite the magnitude of the experimental error discussed in Results, it is clear that the addition of antimycin leads to a considerable reduction in the measured P : 2e ratio (Figs. 3 and 4). It is generally accepted that the antimycin site, the ubiquinone-cytochrome b/c_2 oxidoreductase, represents the second electrogenic reaction in chromatophore electron transport [21]. The ATP synthase reaction is very much faster [14] than either of the pathways for electron return in the presence of antimycin A [21] (the photosynthetic back reaction and the antimycin leak), but is slow compared with the rate of electron flow through the putative first electrogenic site (cytochrome $c_2 \rightarrow [BChl]_2 \rightarrow$ ubiquinone) [19]. Antimycin would therefore be expected to lower the P : 2e ratio under the conditions of our experiment by a factor of 2 and this is not inconsistent with the data.

The accuracy of the alternative method of determining the P : 2e ratio is less certain owing to the limiting upper frequency of 100 Hz in the flash-firing system and to the poorer signal-to-noise ratio at high frequency. From a series of experiments on the same chromatophore preparation we estimate that the rate of electron transfer of 0.71 nmol electron/nmol bulk bacteriochlorophyll per s given above is accurate to within about 40%. This chromatophore sample had 1 reaction centre/35.4 bulk bacteriochlorophyll and since up to 30% of the reaction centres appear to be deficient in ferrocytochrome c_2, a 'complete' electron-transport chain turns over about 25 times/s under the aerobic conditions employed.

Two H^+ are bound to, and probably translocated by, the chromatophore membrane per one electron transported through a cycle [10]. The synthesis of one ATP molecule by the chromatophores after a single flash is accompanied by the outward translocation of two positive charges [11]. Although both of these measurements involve crucial assumptions, such as the reliability of extinction coefficients, pH indicator dyes and carotenoid shift determinations, it is unlikely that they suffer from inaccuracy due to experimental inability to assess coupling efficiency. In chemiosmotic terms, the measured e : H^+ : ATP stoichiometries therefore predict maximum ATP : 2e ratios of 2.

The experimental P : 2e ratios are consistently much lower than the maximal theoretical value expected on the basis of these stoichiometries. In fact, the measured P : 2e ratios include implicitly all losses in the efficiency of energy transduction of the real membrane. Some correction for these losses can be obtained if the degree of coupling and the mechanistic stoichiometry are evaluated with the aid of nonequilibrium thermodynamics (Eqs. 15, 17 and 18 in the review by Rottenberg [28]). Although the utilization of linear phenomenological equations under pseudo steady-state conditions in pulsed light might be questionable, these calculations, utilizing experimental data from several experiments, always indicated a degree of coupling ranging between 0.84 and 0.90 and a stoichiometry of 1.2–1.4 ATP : 2e. The corresponding efficiency in energy transduction is about 30%, a relatively low value, but which, in all cases, was found to be very close to the maximal theoretical efficiency of a system with the calculated degree of coupling.

Acknowledgements

J.B.J. is grateful to the Royal Society and the Accademia Nazionale dei Lincei for a travel grant and to

the Science Research Council for financial support. G.V., A.B.M. and B.A.M. acknowledge the financial help from Grant N.78.02186.04 from Consiglio Nationale delle Ricerche (Italy).

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