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THE INDUCTION KINETICS OF BACTERIAL PHOTOPHOSPHORYLATION

THRESHOLD EFFECTS BY THE PHOSPHATE POTENTIAL AND CORRELATION WITH THE AMPLITUDE OF THE CAROTENOID ABSORPTION BAND SHIFT

B.A. MELANDRI, G. VENTUROLI, A. DE SANTIS and A. BACCARINI-MELANDRI

Institute of Botany, University of Bologna, Bologna (Italy)

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Summary

1. ATP synthesis (monitored by luciferin-luciferase) can be elicited by a single turnover flash of saturating intensity in chromatophores from *Rhodopseudomonas capsulata*, Kb1. The ATP yield from the first to the fourth turnover is strongly influenced by the phosphate potential: at high phosphate potential (—11.5 kcal/mol) no ATP is formed in the first three turnovers while at lower phosphate potential (—8.2 kcal/mol) the yield in the first flash is already one half of the maximum, which is reached after 2—3 turnovers.

2. The response to ionophores indicates that the driving force for ATP synthesis in the first 20 turnovers is mainly given by a membrane potential. The amplitude of the carotenoid band shift shows that during a train of flashes an increasing $\Delta\psi$ is built up, which reaches a stationary level after a few turnovers; at high phosphate potential, therefore, more turnovers of the same photosynthetic unit are required to overcome an energetic threshold.

3. After several (six to seven) flashes the ATP yield becomes constant, independently from the phosphate potential; the yield varies, however, as a function of dark time (t_d) between flashes, with an optimum for t_d = 160—320 ms.

4. The decay kinetics of the high energy state generated by a long (125 ms) flash have been studied directly measuring the ATP yield produced in post-illumination by one single turnover flash, under conditions of phosphate poten-

Symbols and abbreviation: $A_p = -\Delta G'_p$, affinity for ATP synthesis; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical potential difference of protons; BChl, bacteriochlorophyll.

tial (—10 kcal/mol), which will not allow ATP formation by one single turnover. The high energy state decays within 20 s after the illumination. The decay rate is strongly accelerated by 10^{-8} M valinomycin.

5. Under all the experimental conditions described, the amplitude of the carotenoid signal correlates univocally with the ATP yield per flash, demonstrating that this signal monitores accurately an energetic state of the membrane directly involved in ATP synthesis.

6. Although values of the carotenoid signal much larger than the minimal threshold are present, relax slowly, and contribute to the energy input for phosphorylation, no ATP is formed unless electron flow is induced by a single turnover flash.

7. The conclusions drawn are independent from the assumption that a $\Delta\psi$ between bulk phases is evaluable from the carotenoid signal.

Introduction

A common approach to the study of chemiosmotic coupling of membrane-associated phosphorylation [1] has been the comparison between the extent of the protonic electrochemical potential difference across the membrane and the free energy change for ATP synthesis in systems considered in 'quasi equilibrium' conditions [2] or in a stationary state describable by linear non equilibrium thermodynamics [3—6]. Noticeably, in spite of the uncertainties still existing on the reliability of the several techniques utilized for the evaluation of the protonic gradient (e.g. Ref. 7—11), most of these studies gave results not easily reconciliable with the original Mitchell's hypothesis, but pointing to a mechanism of coupling more direct than that mediated through the activities of protons in the bulk phases (for a comprehensive review cf. Ref. 12, see however Ref. 13).

As an alternative and complementary approach, the synthesis of ATP can be studied kinetically during transient activations of the electron transport system [14—16]. In principle this type of experiments can offer informations on the time sequence of the steps of the coupled reactions; from this, notions about single molecular events can be deducted.

Bacterial chromatophores offer distinctive advantages for this type of studies, since the feeding of electrons to the cyclic coupled electron transport system can be easily controlled by single turnover activation of the primary photochemical reaction. This approach has been thoroughly utilized for studies on the electron transport coupled to proton translocation [17,18]. Also for ATP synthesis similar studies have been attempted using isotopic techniques, which were found however to be insufficiently sensitive and precise [19]. The introduction of the measure of luciferin luminescence as an assay for flash induced bacterial phosphorylation [20] has overcome most of these difficulties and has led to important contributions to our understanding of the early events of photophosphorylation by bacterial membranes [21—25]:

(a) ATP synthesis takes place already after the first turnover of the electron transport chain, although with a yield lower than that promoted by the subsequent turnovers [22,23].

(b) The synthesis of ATP is related to a transient acceleration of the decay of the field indicating absorption band shift of carotenoids [23]; two electrical charges are translocated across the membrane per molecule of ATP formed [24].

(c) ATP synthesis can be coupled also to partial reactions of the photosynthetic apparatus (e.g. at very positive redox ambient potentials in the presence of antimycin A [25].

(d) The amount of ATP formed seems to be related to the number of reducing equivalents going through the reaction center, either in chromatophores partially depleted of cytochrome c_2 [22] or inhibited by antimycin A [25]: under these conditions the level of the protonmotive force seems to be a less important factor [25].

The studies presented in this paper correlate the induction kinetics of flash induced phosphorylation in chromatophores of *Rhodopseudomonas capsulata* with the value of the free energy change for ATP synthesis. The results reported demonstrate also that the extent of the carotenoid shift measures accurately the potential ability of chromatophores to synthetize ATP; they show however that electron transfer is as well an absolute requirement for phosphorylation, also in the presence of a large preexisting membrane potential.

A preliminary report of part of this work has been presented [25a].

Materials and Methods

Chromatophores were obtained from cells of *Rps. capsulata*, strain Kb1, grown photoheterotrophically and harvested at the end of the logarithmic phase, as described previously [26].

Photosynthetic electron transfer reactions were induced by xenon flashes (10 μs half-width) of nearly saturating intensity; the flashes could be fired from 20 to 5120 ms apart. The actinic light was filtered through two layers of 88A wratten filters plus a 665 nm cut off Schott glass filter.

Light induced ATP synthesis was assayed at 30°C measuring the luciferin-luciferase luminescence, by means of the photomultiplier of a dual wavelength spectrophotometer with the monochromator slits completely closed; the photomultiplier was screened by a Corning L-96 filter. The assay medium contained in a total volume of 2 ml: 100 mM sodium glycylglycine (pH 7.75), 10 mM magnesium acetate, 0.1% bovine serum albumin, 1—8 mM inorganic phosphate, 0.2 mM sodium succinate, 20 μM ADP, 60 μM luciferin (Sigma), 1.2 mg of crude firefly lantern extract (Sigma FLE-50) and chromatophores corresponding to 10 μM BChl [21]. The luminescence was calibrated by addition of known amounts of ATP.

In experiments in which low concentrations of phosphate (1 mM) were present in the assay, purified luciferase, chromatographycally prepared by Sigma, was used to avoid the competitive inhibition by the arsenate present in the FLE 50 preparation; under these conditions the final concentration of arsenate in the assay never exceeded 10 μM, which gives negligible kinetic effects on ATP synthesis.

For the calculation of phosphate potentials at pH 7.75 at 30°C a $\Delta G'_0$ value

of 7.65 kcal/mol was used [21a]. ATP concentration was evaluated from the absolute luminescence of luciferin-luciferase, calibrated as specified in [32]; ADP concentration was calculated from the mass balance and the initial concentration of 20 μM.

Carotenoid band shift, induced by trains of single turnover flashes was monitored at 528—512 nm in parallel experiments using the same assay medium, according to Ref. 4.

Bacteriochlorophyll content was measured in acetone-methanol extracts, using the standard technique described in Ref. 27.

Experimental results

The formation of ATP by chromatophores of *Rps. capsulata* can be observed, by the sensitive luciferin-luciferase technique, also when the photosynthetic electron transfer reactions are induced by single turnover flashes of actinic light [22,23]. The experiments in Fig. 1a and 1c show typical traces of the change in luminescence of luciferin-luciferase following 1, 2, 3 and 6 flashes; the experiments were performed at pH 7.75 and in the presence of 20 μm ADP, 1 mM phosphate and 10 mM Mg^{2+}, conditions which would correspond to an affinity for ATP synthesis of —9.6 kcal/mol. As reported by other authors [22,23] photophosphorylation can be elicited also by one single flash, although the yield found is only about 45% of that observed in subsequent flashes. The maximal yield per flash obtained by us ranged between 1 ATP/350 BChl and 1 ATP/600 BChl and compares well with that obtained by others; this relatively low yield does not correlate easily with the very high rate of ATP synthesis, measured in the same preparations under continuous saturating light (500—800 μmol/h per mg BChl) (see however Ref. 25).

In Fig. 1b and 1d the amplitude of the carotenoid shift induced by flash sequences identical to those of Fig. 1a and 1c, is represented: the carotenoid signal increases additively during a train of flashes reaching a steady state extent after a few (4—6) turnovers. The average value of the carotenoid signal is also dependent on the dark time between flashes as it is evident from a comparison between Fig. 1b (t_d = 160 ms) and Fig. 1d (t_d = 2560 ms), (see also below). This behaviour of the electrochromic shift of carotenoids indicates the cooperative formation of the transmembrane electrostatic potential by the activation of multiple turnovers of the many photosynthetic units present in a single chromatophore [25,28—30].

Theoretical considerations [31], as well as experimental data [8,21,31], have indicated that the time constant for the electrostatic charging of the membrane is nearly one order of magnitude smaller than that for the formation of a transmembrane pH difference. It follows therefore that during the first turnovers of the photosynthetic electron flow, the electrochemical potential difference of protons across the membrane should be largely electrostatic, and should gradually shift to conditions in which the pH component becomes more and more important [21]. The response of the flash induced phosphorylation to the uncoupling by electroneutral or electrogenic ionophores is fully consistent with these concepts (Table I): in the presence of 10 mM KCl, nigericin (4 μM), which dissipates ΔpH by exchanging H^+ and K^+ electroneutrally, does

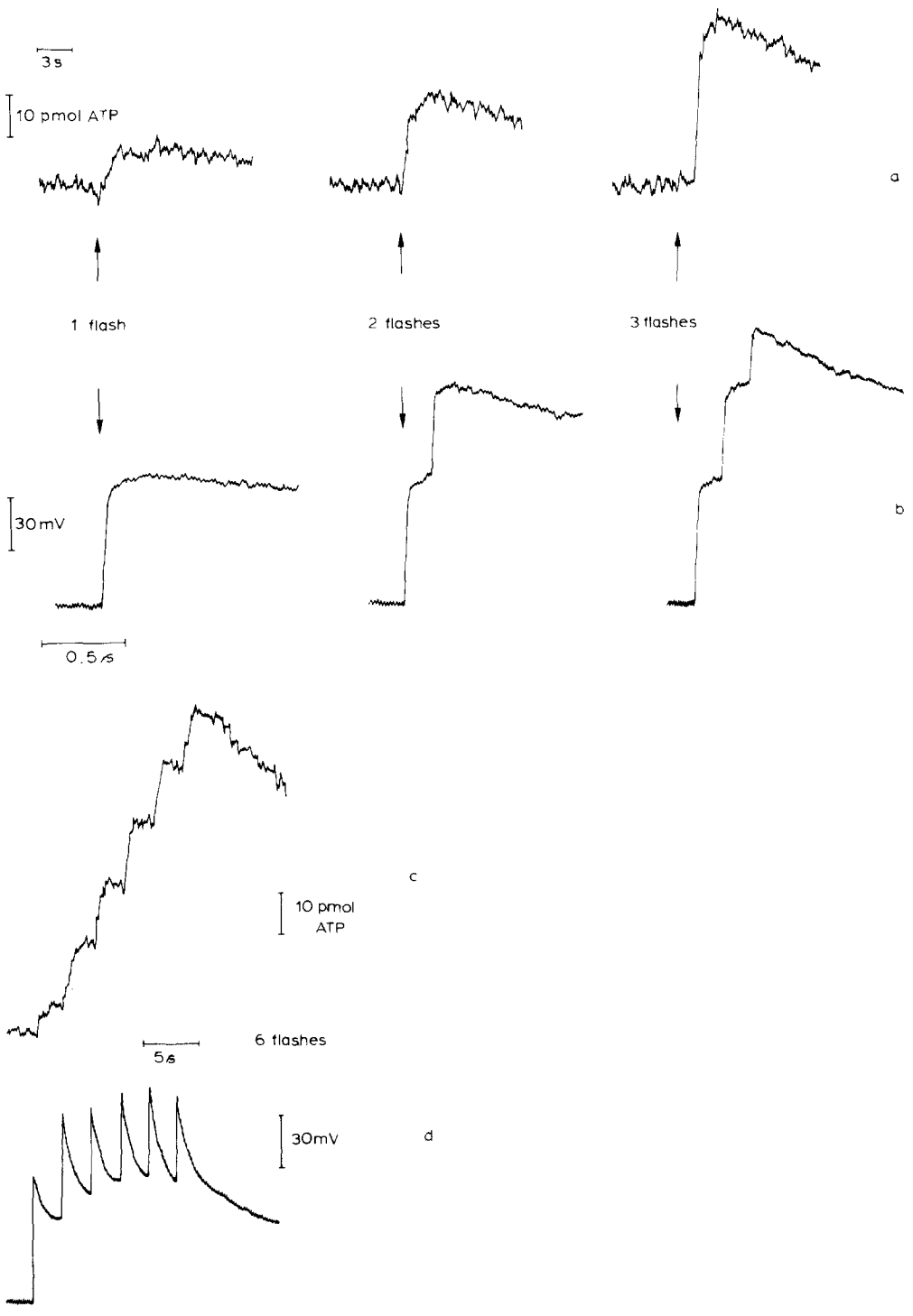

Fig. 1. ATP synthesis (a, c) and signal of carotenoids (b, d) observed varying the number of single turnover flashes and their frequencies. The assay mixture is described under Materials and Methods. Dark time between flashes: (a, b) 160 ms; (c, d) 2,560 ms. The slow response of luciferase did not allow the resolution in single turnovers of the experiment (a).

TABLE I

EFFECT OF VALINOMYCIN OR NIGERICIN IN THE PRESENCE OF 10 mM KCl ON THE YIELD OF FLASH INDUCED PHOTOPHOSPHORYLATION

Photophosphorylation was induced by trains of flashes fired 20 ms apart. Conditions as described in Materials and Methods. n.d., not determined.

Flash number	ATP yield (pmol) per flash		
	No addition	Plus nigericin (4 μM)	Plus valinomycin (4 μM)
1—3	9.6	7.6	0.0
3—7	15.6	14.8	0.0
7—12	21.6	23.0	0.0
12—18	25.3	21.5	0.0
18—27	n.d.	n.d.	14.2
27—37	n.d.	n.d.	22.5

not affect significantly the ATP yield after the first flashes. On the other hand a photosynthetic phosphorylation resistant to valinomycin (4 μM) (a uniporter for K^+ dissipating $\Delta\psi$), as found commonly under continuous illumination, becomes observable only after about 20 single turnover flashes (fired 20 ms apart) and reaches an ATP yield per flash comparable to that of the control only after about 30 flashes. In the presence of valinomycin, however, a sizeable electrostatic potential is still built up upon multiple turnover excitation, since the dissipative ion flow at 10 mM K^+ is insufficient to dissipate $\Delta\psi$ completely; on the other hand the use of higher KCl concentrations are forbidden by the sensitivity of luciferin luminescence to high ionic strength [32].

The lower ATP yield induced after the first flash, as compared to that of the subsequent flashes in a train (Fig. 1), can suggest that a minimal energetic threshold of membrane potential should be overcome in order to start ATP synthesis (see however the different interpretation suggested in Ref. 25). This working hypothesis was tested directly by controlling the affinity for ATP synthesis (A_p) during the experiments of flash-induced phosphorylation; for this purpose the adenylate kinase activity present in bacterial membrane preparations and contaminating the crude commercial firefly preparation of luciferase was utilized as a buffer enzyme system for the control of the ATP/ADP concentration ratios, either by addition of AMP (800 μM, which lowers A_p to about −8.2 kcal/mol) or by addition of exogenous ATP. The absolute concentration of ATP in the assay and the flash induced ATP synthesis were measured concomitantly monitoring the luminescence in a dual channel recorder set at low and high sensitivity respectively. Since the luminescence is not linear at the highest concentration of ATP present in the assay [33], the calibration was performed directly in the cuvette as described in Ref. 32. As shown in Fig. 2, the yield during the very first single turnovers was found to be highly dependent upon the magnitude of the phosphate potential of the system.

In sharp contrast with the observations at low A_p (−8.2 kcal/mol) for which the first flash yield was 47% of the maximum, practically no ATP was formed before the fourth flash at the highest attainable A_p (−11.5 kcal/mol).

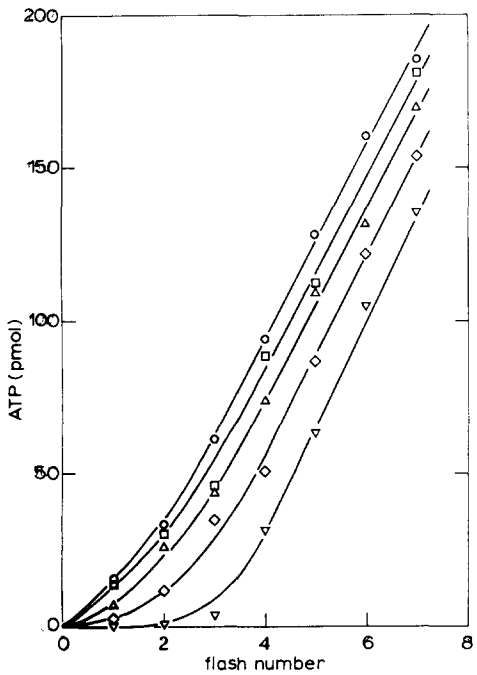

Fig. 2. The dependence of the induction kinetics of ATP synthesis upon the magnitude of the phosphate potential. ○——○, A_p = —8.3 kcal/mol ([ATP] = 0.5 μM, [ADP] = 19 μM, [P_i] = 8 mM); □——□, A_p = —9.6 kcal/mol ([ATP] = 0.45 μM, [ADP] = 19.1 μM, [P_i] = 1 mM); △——△, A_p = —9.8 kcal/mol ([ATP] = 0.65 μM, [ADP] = 18.7 μM, [P_i] = 1 mM); ◇——◇, A_p = —11.1 kcal/mol ([ATP] = 5.65 μM, [ADP] = 18.7 μM, [P_i] = 1 mM); ▽——▽, A_p = —11.5 kcal/mol ([ATP] = 12 μM, [ADP] = 20 μM, [P_i] = 1 mM). 10 mM KCl and 4 μM nigericin were present in the assay mixture. Dark time between flashes: 160 ms.

Irrespectively of A_p, however, the maximal ATP yield, obtained after several flashes, was constant in all experiments indicating that the 'steady state' rate of photophosphorylation in pulsed light is constant and independent of the phosphate potential.

This 'steady state' rate of ATP synthesis (i.e. a constant ATP yield per flash during trains of flashes), which is observed after the third or fourth flash, is on the other hand dependent upon flash-frequency [25]. In the experiment depicted in Fig. 3, the ATP yield in stationary conditions (average ATP yield after the 5th and 6th flash) was measured as a function of the dark time between flashes. The phosphate potential was controlled throughout the experiment and was about —10 kcal/mol. As shown in Fig. 3 the ATP yield per flash kept relatively constant for flashes fired about 160—320 ms apart although a slight but significative decrease was observed at higher frequencies. At lower frequencies however the flash yield rapidly decreased and practically vanished (as it was observed for a single isolated flash under the same condition for A_p) when flashes were fired every 5120 ms. This decrease in flash efficiency is probably due to the relaxation of the membrane potential during the time between flashes, caused by passive ion leaks; for sake of comparison in Fig. 3 the peak amplitude of the carotenoid spectral signal and its range of oscillation between flashes, is also reported, both as $\Delta A_{528-512}$ changes and as $\Delta\psi$.

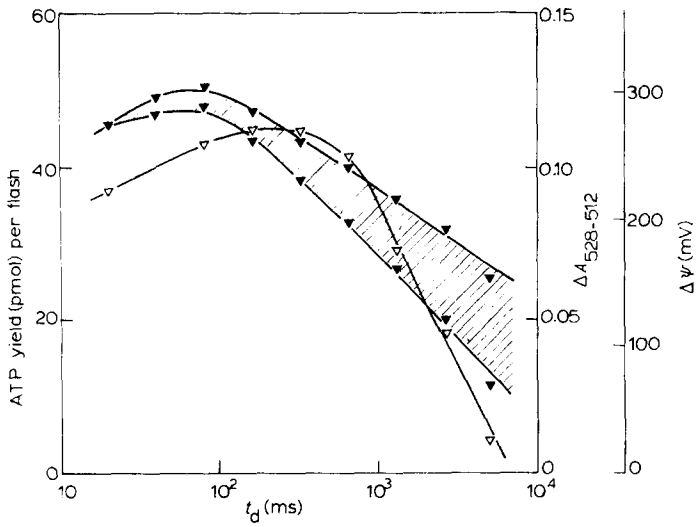

Fig. 3. The dependence of the ATP yield per flash (▽——▽) and of the carotenoid signal (▼——▼) upon the dark time between flashes (t_d) during steady state phosphorylation in pulsed light. The shaded area indicates the oscillation between the maximal and minimal values of the carotenoid signal between flashes. 10 mM KCl and 4 μM nigericin were present in the assay mixture. The phosphate potential was about —10 kcal/mol.

Indeed a maximal value of the average $\Delta\psi$ is found for dark times between flashes of 80 ms and is rapidly declining when the flash frequency is decreased.

The large difference in ATP yield after the first flash as compared with that in stationary state, observed at intermediate phosphate potentials (around —10 kcal/mol), can be utilized as a direct tool for the study of the relaxation kinetics of the light induced high energy state of the membrane. In addition to the experiments of Fig. 3, this problem was also approached by examining the dependence of the ATP yield of one single flash fired during a postillumination period. For this purpose a high energy state of the membrane was elicited by a 125 ms flash, sufficient to bring the membrane at the highest possible $\Delta\psi$ value observable in continuous light; a single 10 μs xenon flash was subsequently fired after a dark time of variable length and the ATP yield monitored (Fig. 4). All the experiments were performed in the presence of 10 mM KCl and 4 μM nigericin in order to avoid any contribution of a ΔpH induced by the 125 ms flash.

The results, shown in Fig. 5, indicated that no independent contribution to ATP synthesis by an additional single turnover can be observed if the flash is fired earlier than 200 ms after the long flash. After this time, however, ATP synthesis depends entirely upon the flash excitation and its yield is equal to the maximal attainable at the optimal flash frequency (cf. Fig. 3). If the flash is delayed further, however, the ATP yield decreases steadily until is practically zero after about 30 s. The energetic state induced by the 125 ms flash is therefore remarkably stable in time (cf. Ref. 25) and can potentially contribute to the synthesis of ATP, induced by a single turnover flash, for several seconds after photophosphorylation in postillumination has stopped.

The faster dissipation of the membrane potential by K^+ currents, promoted

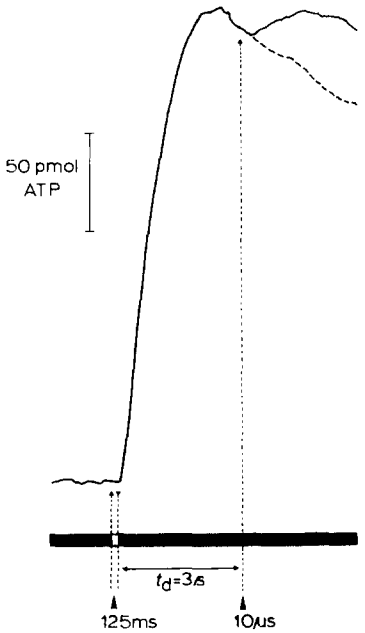

Fig. 4. A typical pattern of the luciferin-luciferase luminescence recorded during a double flash experiment. ATP synthesis was induced by a 125 ms flash followed by a single turnover flash fired after 3 s. The dotted line indicates the luminescence of the system during a control experiment in which no single turnover flash was given. 10 mM KCl and 4 μM nigericin were present in the assay mixture.

by very low concentrations of valinomycin, accelerates very effectively the decay of the energetic contribution to ATP synthesis induced by the single turnover flash. In the presence of $3 \cdot 10^{-8}$ M valinomycin and 10 mM KCl (about 1 valinomycin per 10 photosynthetic units) the postillumination effect on the flash induced phosphorylation is already negligible after 1 s of darkness (cf. Ref. 19).

The amplitude of the carotenoid signals obtained under the same experimental conditions is represented in Fig. 5; in this figure the values of the carotenoid signal observed before and after the single turnover flash are plotted as a function of the dark time between the long and the microsecond flash. As described by many authors [23,24], in the absence of ionophores and at relatively low ionic concentrations, the relaxation of the light induced carotenoid signal is relatively slow; consequently the effect of the preillumination on the single turnover induced signal can be observed for a long time (about 40 s). As expected, the addition of valinomycin strongly accelerates the decay of the membrane potential; with $3 \cdot 10^{-8}$ M valinomycin present, no postillumination effect can be observed after 1 s and the amplitude of the single turnover signal corresponds to that promoted by an isolated 10 μs flash in dark-adapted chromatophores (evaluated by calibration to be about 70 mV (cf. also Ref. 35)).

A parallelism can therefore be drawn between the behaviour of the electrochromic signal of carotenoids and the value of the ATP yield per flash during photophosphorylation in pulsed light. This parallelism is not merely circum-

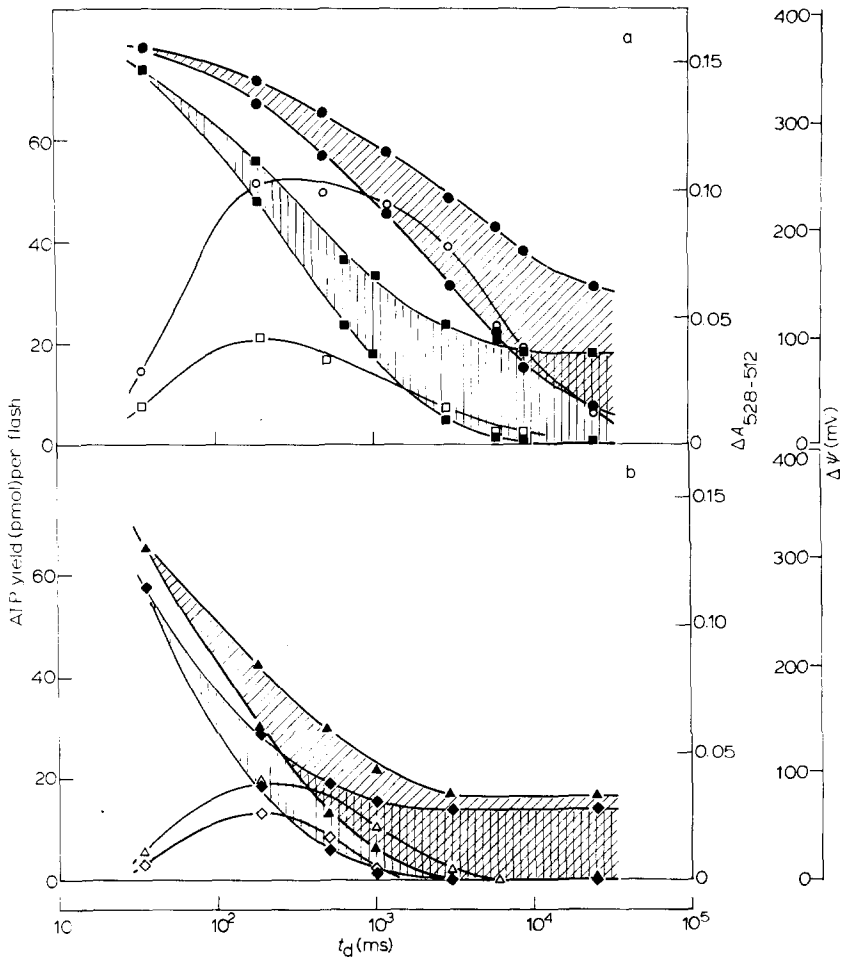

Fig. 5. The ATP yield (open symbols) and the carotenoid band shift (closed symbols) induced by a single turnover flash during a postillumination period as a function of the dark time (t_d) elapsed between the 125 ms and the 10 μs flashes. (cf. the experiment shown in Fig. 4). In addition to a control experiment (Fig. 5a, ○, ●) in which only 4 μM nigericin was present, the effect of valinomycin is also shown: (Fig. 5a, □, ■) 10 nM valinomycin; (Fig. 5b, △, ▲) 20 nM valinomycin; (Fig. 5b, ◇, ◆) 30 nM valinomycin. The phosphate potential was about −10 kcal/mol. The shaded area indicates the oscillations of the carotenoid signals observed during the postillumination period before and after the single turnover flash.

stantial but strictly quantitative. A one to one correlation between the peak values of the carotenoid signals and the ATP yield per flash is shown in Fig. 6; the data plotted have been obtained from the descending parts of Figs. 3 and 5, i.e. when energy dissipation becomes the event kinetically limiting for ATP synthesis. Although the scattering of the experimental points is rather high, a sigmoidal curve can be suggested as the correlation function between these two quantities; the validity of the carotenoid shift as a quantitative indicator of the force driving ATP synthesis results quite clearly from these data.

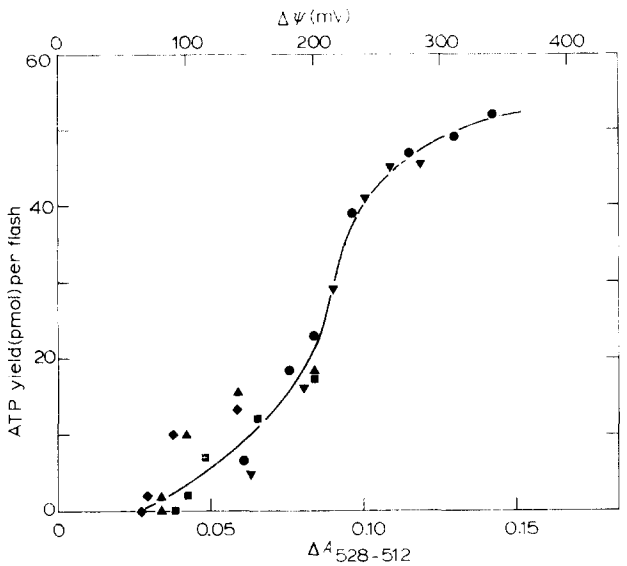

Fig. 6. Correlation between the ATP yield per flash and the carotenoid band shift amplitude. The plot summarizes all the data shown in the descending parts of the curves of Fig. 3 and Fig. 5.

Discussion

Since the time required for a complete response of the luciferin luminescence to a sudden change in ATP concentration is of the order of 400—500 ms [32], the changes in luminescence measured include both the ATP possible produced during the flash and in postillumination. However, as discussed by other authors [20,23], the rate of ATP hydrolysis by chromatophores in the dark is low enough ($t_{1/2}$ = 20 s) to justify the use of this technique for the quantitative extimation of the light-induced ATP changes.

In the present experiments we have examined the induction kinetics of photophosphorylation under controlled conditions for the affinity of ATP synthesis. In the light of thermodynamic concepts previously discussed [4,21,35,36], in fact, the onset of ATP synthesis by a photosynthetic system, during the transition from dark to light, can be considered as the inversion of a process of a reversible energy transducer, the ATPase, which, during the inversion of its flow from ATP hydrolysis to ATP synthesis, proceeds through a static head condition: a fundamental thermodynamic parameter for the analysis of such processes is therefore the free energy change for ATP hydrolysis (i.e. the negative affinity for phosphorylation, A_p), which sets in fact the energy threshold to be overcome [3—6].

The results shown in Fig. 2 clearly illustrate this situation. The data demonstrate in fact that when a high energy threshold is poised in the assay mixture (highly negative A_p) phosphorylation is not induced immediately by the first flash, but requires several turnovers of the cyclic electron flow system to reach its full rate. The number of turnovers needed is related to the affinity of the process against which the protonic force performs chemical work. This interpretation of ours differs from that proposed by Petty and Jackson [25], who

considered the involvement of an activation process of the ATP synthetase as an explanation for the differences in ATP yield observed between the first and the subsequent turnovers of the photosynthetic system.

Since the intensity of the single turnover flash used is nearly saturating, these results clearly indicate that the formation of the driving force for ATP synthesis is a cooperative process to which more than one turnover of the same photosynthetic unit can contribute. These conclusions are perfectly in line with the views of the chemiosmotic model and had been already reached in previous experiments with light pulses in the millisecond time scale [21]; in the present paper the phenomena are better resolved in time and number of turnovers of a single photosynthetic unit.

Also fully consistent with chemiosmotic coupling is the response of photophosphorylation to uncoupling by ionophores; the considerable lag induced by valinomycin in association with relatively low concentration of K^+ (10 mM) (see similar effects observed by other authors in chloroplasts [14,16,37—39] and by us in chromatophores [21]) can be taken as evidence that the prevalent component of the protonic potential difference during the very first turnovers is the membrane potential.

Therefore a quantitative study of the behaviour of flash induced ATP synthesis during the first 10—15 turnovers seems quite appropriate as an indirect approach for the investigation on the stability and intensity of the electric field established in the membrane [14,31,36]. This study is further facilitated by the marked difference in the yield of ATP produced by the first flash as compared to subsequent flashes in a train when the affinity for ATP synthesis is maintained relatively large (about —10 kcal/mol, cf. Fig. 2). Using these criteria we were able to conclude from the experiments described above that in the absence of any pH difference, an electrostatic component of the force driving ATP synthesis is considerably stable and can contribute energetically to the process of phosphorylation for at least 30 s in the dark. This electrostatic component, on the other hand, is effectively decreased and destabililized by the action of valinomycin.

A large body of experimental evidence in natural [14,40—42] and model systems [14,43] indicate clearly that the spectral shift of carotenoids senses electric field generation within or across the membrane dielectric. Doubts have been expressed, however, on the reliability of this method for quantitative thermodynamic treatments of photosynthetic energy transduction [7,9,44], especially under continuous illumination [14,45—47]. The quantitative agreement between the amplitude and decay kinetics of the carotenoid signal and the ATP yield observed was remarkably good under a large variety of experimental conditions (Fig. 6), in which the driving force can be considered essentially electrostatic, and, noticeably, also when the decay was accelerated by very low concentrations of valinomycin (one valinomycin for about ten photosynthetic units) [19]. As a whole, therefore, these experimental data indicate clearly that the extent of the carotenoid signal can be considered as a valid quantitative indicator of an electrostatic energetic state of the membrane capable of acting as an input force to the ATP synthetase. This conclusion is drawn by purely phenomenological observations and prescinds completely from any consideration about the voltage profile within the membrane, across

the membrane-water interphase or in the surroundings of individual electron carriers on the membrane surface. More specifically this deduction does not depend on the acceptance of the calibration of the light-induced shift with K^+ diffusion pulses in the dark [4,40]; this calibration in fact has only a bearing on the absolute value of the membrane potential obtained from the carotenoid signal, but not on the general correlation demonstrated in our experiments.

Recently Petty and Jackson [25] demonstrated that ATP synthesis does occur also under redox conditions in which only the charge separation between P-870 and the primary electron acceptor takes place, a reaction generally believed to span only part of the membrane dielectric. The field generated by this localized redox reaction is sensed by the carotenoids (phase I of the carotenoid signal [34]) as well as by the ATP synthetase present in the membrane. These and our own experiments therefore could indicate that the locally generated field, sensed by carotenoids, is rapidly delocalized by ion redistribution and contributes to an increase of the protonic potential difference between the bulk phases present at the two sides of the membrane, in line with an explicit assumption of the chemiosmotic hypothesis [48]. The parallel response of the ATP yield per flash and of the carotenoid signal to low concentrations of valinomycin supports this view.

If the carotenoids, on the other hand, are probing a more delocalized electric field in the surroundings of the photosynthetic reaction center, it should be assumed that the same localized field is also active at the ATP synthetase 'proton well', since the whole carotenoid signal appears to be correlated with a univocal function to the ATP generated by single turnover flashes.

As shown in Fig. 5a the field across the membrane decreases very slowly ($t_{½}$ = 5 s) and, for several seconds after the flash it exhibits values (indicated by the amplitude of the carotenoid signal or evaluated independently by the ATP yield per flash) which exceed largely the minimum threshold value necessary to drive phosphorylation. Yet already after 800 ms after the flash (or in a shorter period, given the kinetic limitations of luciferase) ATP synthesis stops and can be elicited only by a new single turnover of the electron transport chain (cf. Fig. 1c and Fig. 4 for clear examples of this behaviour). On the other hand, the field present even after phosphorylation has stopped completely, is still potentially able to contribute energetically to ATP synthesis, as demonstrated by the high ATP yield observed upon single flash excitation.

These observations evidentiate a paradoxical situation fully consistent, however, with phenomena already documented in previously published works: (a) results from our laboratory demonstrated that the inhibition of electron flow during continuous illumination resulted in a decrease in the rate of photophosphorylation, in spite of the high protonmotive force developed under the same conditions [8,31,49]; (b) observations by Jackson and coworkers 24,25,50] showed that under phosphorylating conditions only a small fraction of the decay of the carotenoid signal is accelerated: this acceleration is taken by these authors as a measure of the protonic current through the working ATP synthetase; (c) evidence presented by Del Valle-Tascon et al. (Ref 22, par. 3) demonstrated a strict correlation between the amount of ATP synthetized after a single turnover flash and the content of cytochrome c_2 in chromatophores of *Rhodospirillum rubrum*.

It seems therefore clear that in bacterial chromatophores (of *Rps. capsulata*, *Rps. sphaeroides* and *Rh. rubrum*) the coupling between electron flow and ATP synthesis is more direct than that expected on the basis of the original chemiosmotic model. In the present paper this thesis has been demonstrated and time-resolved in single turnover of the coupled system and, we believe, with a logical approach which does not rely on any assumption or calibration of other energy-linked phenomena.

These observations, at variance with a simple three phase chemiosmotic model of coupling [48], already documented for bacterial chromatophores in our previous papers [4,31,49] (and first reported in mitochondria, see Ref. 51), have been subsequently confirmed also in several respiratory systems [11,52—56]. Possible interpretations of these phenomena considered either a kinetic control of the ATP synthetase effected by redox reactions or a short range protonic coupling [4,31,51] (in line with general views of Williams [57] of an energy coupling by proton diffusion control, and discussed also by Mitchell [58]). The first possibility appears now less attractive in view of the finding that also a single turnover of a partial reaction of bacterial photosynthesis can promote ATP synthesis [25]. The second possibility, currently discussed by several authors [12,22,51], emphasizes the relevance of local proton currents possibly occurring at the water-membrane interphases [12] and facilitated by protein-protein interactions in a fluid mosaic structure of the energy transducing membranes [59].

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