THE RATES OF ONSET OF PHOTOPHOSPHORYLATION AND OF THE PROTONIC ELECTROCHEMICAL POTENTIAL DIFFERENCE IN BACTERIAL CHROMATOPHORES

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1. Introduction

We have studied the kinetic and thermodynamic parameters of photophosphorylation in bacterial chromatophores of *Rhodopseudomonas capsulata* under steady state experimental conditions, as related to the protonic potential difference (Δp) across the membrane [1–4]. The main conclusions of these studies were that under 'state 4' conditions, i.e., under conditions of static head for both Δp and phosphorylation, a general agreement exists between the affinity of the reaction of ATP synthesis (A_p) and the extent of Δp [1,3].

We observed, however, that the steady state rate of ATP synthesis was markedly affected by the rate of electron flow, when this was decreased by antimycin A or by limiting the intensity of actinic light, in spite of the fact that the value of Δp was not largely diminished under these conditions [3,4]. These observations led us to consider the possibility of short range interactions between the ATP synthetase complexes and the electron transport chains, involving the mechanism of energy transduction itself or alternatively only a control of the turnover rate of ATP synthetase.

It is clear that more detailed informations on this point would be obtained if the transient rates of ATP synthesis and of Δp formation, upon the onset of illumination, were compared. This approach requires fast and sensitive techniques for the measure of ΔpH, $\Delta\psi$ and ATP concentration. The spectroscopic methods in use in our laboratory for the measurement of ΔpH and of $\Delta\psi$ in chromatophores [3] are fast enough to allow a quantitative evaluation of Δp after a flash, before the relaxation of the protonic gradient reaches a significant extent (in preparation). The luciferine-luciferase assay, recently adapted for the continuous monitoring of photophosphorylation in bacterial chromatophores [5], offers the technical possibility for a comparative study of the transient rates of phosphorylation versus the onset of Δp.

This paper describes some preliminary results of experiments relating Δp and ATP synthesis induced by flashes of light of variable time length.

2. Materials and methods

Chromatophores from cells of *Rps. capsulata*, strain KBl, were prepared as in [4,6].

The assay mixture for measuring light-induced ATP synthesis contained in total vol. 1–3 ml: Na-glycylglycine, 100 mM (pH 8.0); Mg-acetate, 10 mM; bovine serum albumin, 0.1%; P_i, 2 mM; Na-succinate, 0.2 mM; ADP, 0.02 mM; luciferine, 0.06 mM; luciferase, 1.5–4 μg protein/ml and chromatophores corresponding to 13 μM BChl. Luciferase was purified from the FLE-50 (preparation from Sigma) by Sephadex G-100 column chromatography [7].

The assay mixture was placed in a spectrophotometric cuvette in a dual wavelength spectrophotometer and the luminescence was detected essentially as in [5]. Flashes of different duration were controlled by an electronic shutter (Compur electronic mod.1); a 5 min interval was given between each flash in order to allow a complete de-energization of the membrane.

$\Delta\psi$ and ΔpH were monitored by the carotenoid

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band shift and by the quenching of 9-aminoacridine fluorescence, respectively, as in [3], under the same assay conditions for the ATP synthesis. The response of 9-aminoacridine fluorescence quenching following a flash of light was fast enough ($t_{1/2} \cong 300$ ms) for a quantitative estimation of ΔpH after the flash, before a substantial leak of protons out of the vesicles. In the calculation of the extent of ΔpH, the apparent internal volume of chromatophore vesicles was estimated empirically by measuring the quenching of 9-aminoacridine due to artificial acid–base transitions at different initial pH values, as will be detailed elsewhere (in preparation). This procedure gives ΔpH values lower by about 0.6 pH units than those reported by using in the calculations the internal volume of chromatophore vesicles, estimated by isotopic techniques [3].

Bacteriochlorophyll content was measured in acetone–methanol extracts, following the procedure in [8].

3. Experimental results

The amount of ATP formed following high intensity flashes ranging from 2–500 ms, is compared in fig.1 with the rate of onset of ΔpH, $\Delta\psi$ and Δp, under identical experimental conditions. The amount of ATP formed is proportional to the duration of the flash; it is however not possible to assess how much ATP is formed during the flash or during the postillumination period, since the rate of appearance of luminescence is always limited by luciferase ($t_{1/2} \cong 200$ ms) [9]. ATP synthesis takes place already with the shortest flash used in this experiment (2 ms); therefore if any time lag exists at all, this must be shorter than 2 ms, a time length which corresponds in saturating light to the accumulation of 6–8 electrons in the secondary acceptor pool of a photosynthetic unit [10]. This conclusion has been undoubtly confirmed in other experiments (cf. fig.3 insert) in which the sensitivity of the technique was improved by raising the gain of the photomultiplier and by increasing the volume of the assay mixture.

In agreement with these results it can be observed in the same fig.1 that illumination for 2 ms induces already a substantial Δp, which is formed in this short time, mainly by $\Delta\psi$; only with much longer flashes (67 ms), a measurable ΔpH is formed.

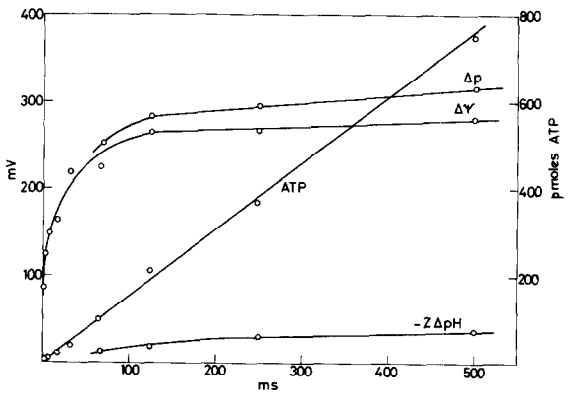

Fig.1. Time course of ATP formation and of the parameters of the electrochemical gradient of protons ($\Delta\psi$ and $-Z\Delta$pH) following single flashes of variable duration.

The effect of the ionophores nigericin and valinomycin, in the presence of 50 mM KCl, on photophosphorylation and Δp formation under similar experimental conditions is shown in fig.2. As already observed in continuous light [1,12] nigericin does not inhibit, but rather stimulates slightly ATP synthesis; nigericin moreover does not affect at all the onset of phosphorylation (fig.2a). Valinomycin on the other hand, exerts a significant inhibitory effect in the steady state (cf. [1,13] for data in continuous light) and most significantly induces a marked time lag (about 60–70 ms, if the rate in the steady state is extrapolated linearly). The measurements of Δp in the same conditions are consistent with these observations (fig.2b), since the protonmotive force increases very rapidly in the control or when nigericin is present, Δp being formed prevalently (in the control) or exclusively (in the presence of nigericin) by the membrane potential. Valinomycin, on the other hand, delays the onset rate of Δp which is, in this case, formed mainly by ΔpH, the rate of formation of which is substantially slower (in preparation).

The inhibitory effect of antimycin A, alone or in combination with valinomycin, on the initial phase of photophosphorylation is shown in fig.3. Addition of 1 μg/ml of the inhibitor (in the presence of 1 mg/ml bovine serum albumin) causes a decrease in the rate of photophosphorylation of about 67% (curve b): this inhibition is maintained proportionally constant also at the shortest flashes used (cf. fig.3 insert). In

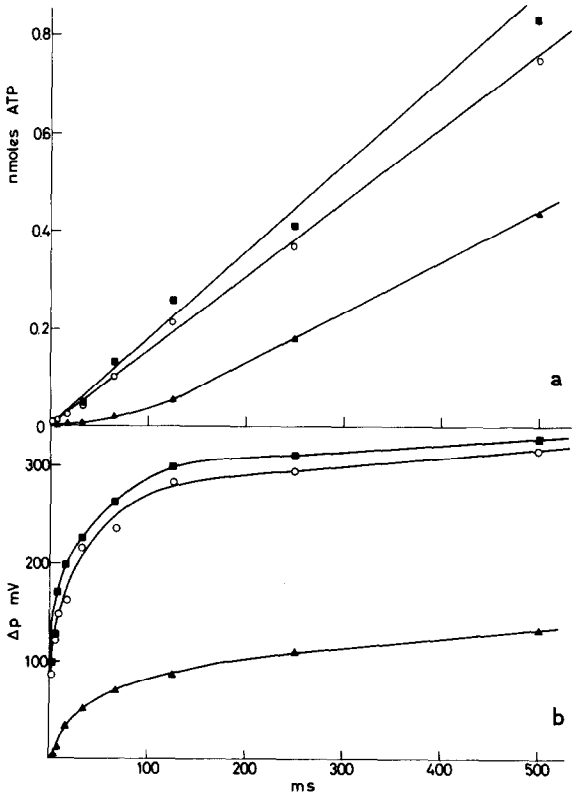

Fig.2. Time course of the formation of ATP (a) and of the protonmotive force (Δp) (b) in absence and in presence of ionophores. (o–o) No additions; (■–■) 50 mM KCl plus nigericin (1 μg/ml); (▲–▲) 50 mM KCl plus valinomycin (1 μg/ml). Conditions as in fig.1.

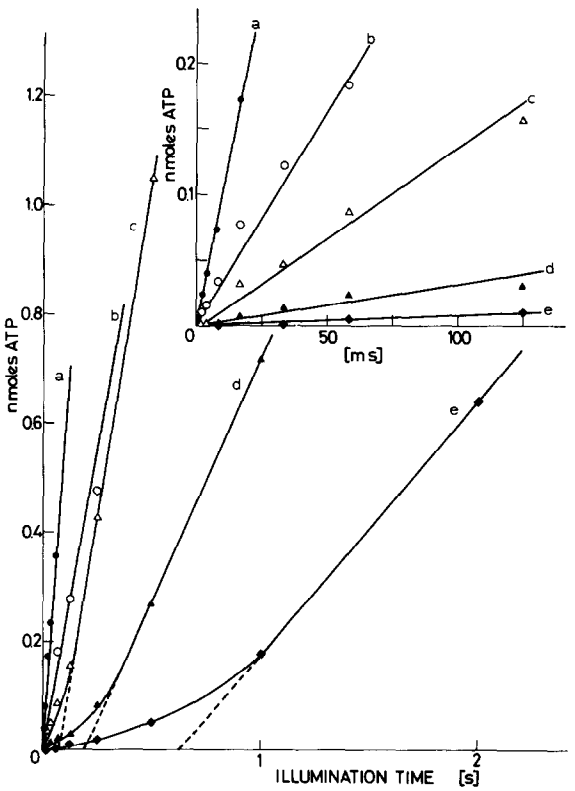

Fig.3. The effect of valinomycin and antimycin A on the time course of ATP synthesis following single flashes of variable duration. Curve (a) no additions; curve (b) in the presence of antimycin A (1 μg/ml); curve (c) in the presence of 50 mM KCl and valinomycin (1 μg/ml); curve (d) as in curve (c) plus addition of antimycin A (0.5 μg/ml); curve (e) as in curve (c) plus addition of antimycin A (1 μg/ml). The insert refers to the flashes of duration from 2–100 ms.

sharp contrast with these results, in the presence of valinomycin and 50 mM KCl, which induce a 70 ms lag (curve c) per se, antimycin largely affects the duration of the lag (curve d, e). The inhibitory effect of antimycin A is more pronounced in short flashes, as compared to the inhibition in the steady state (e.g., 0.5 μg/ml of antimycin A inhibits by 80% after a 125 ms flash, but only by 60% in steady state); consequently the lag for the onset of the maximal yield of ATP per flash (extrapolated linearly on the abscissa coordinate) increases (200 ms and 620 ms in the presence of 0.5 μg/ml and 1.0 μg/ml of antimycin, respectively).

It is clear therefore that, in the absence of a substantial membrane potential, the building up of the conditions required for the onset of a maximal rate of photophosphorylation is strictly limited by electron flow. Indeed the rate of formation of ΔpH, as monitored by 9-aminoacridine fluorescence, is also markedly delayed by antimycin A (fig.4); most noticeably a threshold value of about 1.2 pH units is apparently required for the steady state rate of ATP synthesis to be reached. With longer times of illumination, however, the values of ΔpH, in the presence of antimycin A, converge with those of the control. In spite of this large ΔpH, however, the steady state velocity of ATP synthesis appears markedly decreased by antimycin, as already documented in [2–4]; this has also been confirmed during the present experiments in indepen-

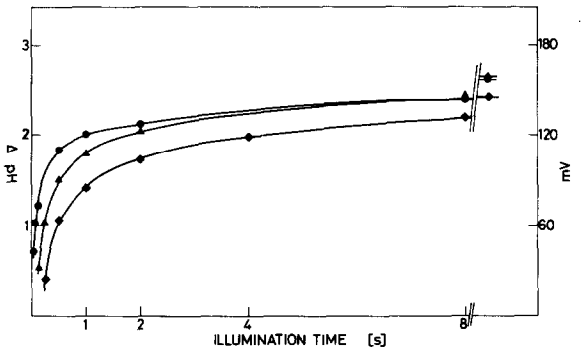

Fig.4. The onset of ΔpH, following single flashes of variable duration. (●–●) In the presence of 50 mM KCl and 1 μg/ml valinomycin; (▲–▲) in the presence of 50 mM KCl and 1 μg/ml valinomycin plus 0.5 μg/ml antimycin A; (◆–◆) in the presence of 50 mM KCl and 1 μg/ml valinomycin plus 1 μg/ml antimycin A.

dent assays performed in continuous light using ^{32}P incorporation [6].

4. Discussion

The results presented here on the formation of the membrane potential and the pH difference in bacterial chromatophores, as estimated by spectroscopic techniques, indicate that these two entities are formed at markedly different rates. The extremely fast build up of $\Delta\psi$, in comparison with ΔpH, is consistent with theoretical and empirical evaluations of the electrical capacitance [3,11,14] and buffer capacity [14] of chromatophores. The turnover number necessary to induce a 100 mV $\Delta\psi$ across the membrane has been evaluated to be about 10–15-fold smaller than that required to induce an equivalent energization in terms of ΔpH [11,14]. The situation is also similar, although less drastic, to that observed for the time course of energization of spinach chloroplasts (see, e.g., [15]). The time separation of the electrostatic and osmotic component of the protonic gradient during dark–light transition can offer new experimental criteria for discriminating between energetically or kinetically controlled phenomena in energy transduction.

The results obtained on the rate of ATP synthesis in short flashes confirm and extend the results in [16], where in *R. rubrum* chromatophores a substantial synthesis of ATP, inhibited by valinomycin, in a single 1 ms flash, was observed. For technical reasons we are not in a position of extending our studies to short single turnover flashes (5 μs) and therefore of confirming the finding in [17] that in a single turnover flash 1 ATP molecule/photooxidized reaction center is produced. It is certain, in any case, that the time lag before the onset of photophosphorylation at the full rate is, in absence of valinomycin, extremely short. Similarly short is however also the rate of formation of a substantial $\Delta\psi$, which, under our experimental conditions, is already well over 100 mV in 2 ms (fig.1 and [11]). When $\Delta\psi$ is dissipated by K^+ electrophoretic fluxes a substantial time lag is induced and photophosphorylation does not start until a relatively large ΔpH is formed. This conclusion is very similar to that drawn in chloroplasts [18–20]. Further support to this conclusion is given by the finding that inhibition of electron flow by antimycin, which cannot drastically affect $\Delta\psi$, owing to the low electrical capacitance of chromatophores, but which delays markedly the onset of ΔpH, is extremely effective in prolonging the time lag of photophosphorylation (fig.3). It is clear therefore that the significance of the time lag is an energetic one and represents the time required for the system to build up a protonmotive force sufficient to overcome the affinity for ATP synthesis under the conditions of the experiments [3]. The present data indicate that this threshold of Δp falls around 100–120 mV, in good agreement (if allowance is made of the different criteria used here for the calculation of ΔpH) with what found in continuous light in the presence of uncouplers [3], i.e., under conditions of protonic energy limitation. More work is however needed before trying and using this approach for the evaluation of the minimal H^+/ATP stoichiometry.

The main conclusion, which can be reached on the basis of the present experiments, is that the onset of a substantial protonic potential difference, electrostatic at short times and, in absence of valinomycin, equally shared between $\Delta\psi$ and ΔpH in continuous light [13], is an absolute requirement for photophosphorylation. The data indicate, however, that the maximal rate of phosphorylation, for a specific rate of electron flow, is reached at a relatively early stage, when Δp is still relatively small. The protonmotive

force reaches eventually its maximum level, which is not markedly affected by limitation of electron flow (fig.4), in contrast with the dramatic inhibition of phosphorylation (cf. curves d, e versus c, and b versus a in fig.3, and [3,4]).

The rate of phosphorylation in bacterial chromatophores seems therefore not to be controlled only by Δp but rather by a tight kinetic control of electron flow on ATP synthetase. Similar data are at present emerging in other energy transducing systems (i.e., *Paracoccus denitrificans* vesicles [21] and submitochondrial particles [22]).

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