ELECTROCHEMICAL PROTON GRADIENT AND PHOSPHATE POTENTIAL IN BACTERIAL CHROMATOPHORES

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1. Introduction

One of the obligate and basic requirements of Mitchell's chemiosmotic hypothesis [1] is the consistency of the extent of the electrochemical potential difference of protons across the membrane and the amount of energy available for ATP synthesis. An estimation of this amount of energy has been obtained by measuring the maximal affinity of the phosphorylation reaction at which no net ATP synthesis occurs (phosphate potential in state 4). Experiments of this kind, performed in membrane systems as different as mitochondria [2], chloroplasts [3] and membrane fragments from aerobic [4] and photosynthetic bacteria [5], have all yielded values ranging between 14 and 16 Kcal/mole. These values are considered by several authors to be inconsistent with a stoichiometry of $2H^+$ translocated per ATP synthesized [1] and with the size of the proton electrochemical gradient, as estimated on the basis of the distribution of permeable ionic and protonable molecules across the energized membrane, for example [6,7].

Fluorimetric and spectrophotometric methods for the evaluation of pH or potential differences across the membranes of some photosynthetic bacteria have been propos d. Specifically, work in this laboratory [8] has indicated that ΔpH in bacterial chromatophores can be estimated from the extent of the fluorescence quenching of 9-aminoacridine, employing the technique originally proposed by Shuldiner et al. [9] for chloroplasts. In addition, the presence in photosynthetic membranes of pigments undergoing spectral shifts in response to a transmembrane electric field [10] allows an evaluation of the membrane potential, which is particularly accurate in bacterial chromatophores [11].

A combination of these two techniques permits a continuous measurement of $\Delta\tilde{\mu}_{H^+}$ developing under illumination across the membrane of bacterial chromatophores and is compatible with the concurrent determination of the maximal phosphorylation potential attainable in the steady state. In this paper we present results of experiments of this kind, which demonstrate a good agreement between the extent of the proton electrochemical difference and the phosphorylation potential on the basis of the stoichiometry $H^+/ATP = 2$. This correlation holds true also when the size of the proton gradient is perturbed by addition of ionophorous antibiotics.

2. Materials and methods

Chromatophores were prepared from cells of *Rhodopseudomonas capsulata,* strain St. Louis (American Type Culture Collection 23782) grown anaerobically in the light [12]. The quenching of fluorescence of 9-amino acridine was measured at a 90° angle with a filter fluorimeter, as described [8]. Measurements of carotenoid band shift were carried out at 530–508 nm with a dual wavelength spectrophotometer in which the photomultiplier was protected from the actinic light by a Corning glass filter, no. 9782. In both instruments the actinic light was supplied by a 55 W quartz-halogen bulb screened with a Wratten 88A gelatin filter. Light intensity on the cuvette was $2.5 \cdot 10^6$ ergs/cm^2 sec. The internal volume of chromatophores was determined according to the procedure described in [8].

ΔpH was evaluated on the basis of the degree of quenching of fluorescence of 9-amino acridine and the value of the internal volume of the particles, as reported previously [8]. $\Delta\psi$ was calculated by means of a calibration curve obtained by measuring the signals produced in the dark by K^+ pulses in the presence of valinomycin [11].

Measurements of the phosphate potential were performed directly in the spectrophotometer and fluorimeter cuvette while monitoring simultaneously the carotenoid spectral shift or the quenching of 9-amino acridine. The assays contained in a final volume of 2 ml: glycylglycine buffer, pH 8.5, 40 mM; $MgCl_2$, 10 mM; sodium-succinate, 0.2 mM; KCl, 50 mM; 9-amino acridine, 4 μM; photosynthetic particles corresponding to 40–60 μg of Bacteriochlorophyll and variable amounts of ATP, ADP and inorganic phosphate. All experiments were carried out at 30°C. After 10 to 20 min the reaction was stopped by addition of 0.2 ml of cold 20% perchloric acid and centrifuged. The supernatants were neutralized accurately with KOH and the resulting precipitate discharged by centrifugation. ATP and ADP concentrations were determined enzymatically [13,14]; phosphate concentration was measured colorimetrically [15]. For the calculation of phosphate potential a standard free energy change of 8.72 Kcal/mole at pH 8.5, 0.1 M ionic strength and 10 mM $MgCl_2$ was used; this value was derived from the data of Rosing and Slater [16] corrected for temperature.

The rate of photophosphorylation was determined by measuring the incorporation of ^{32}P, phosphate into glucose 6-phosphate [12]. Bacteriochlorophyll was determined at 775 nm after extraction of the membranes with acetone: methanol [17].

3. Results and discussion

Table 1 shows the results of experiments in which the maximal potential of phosphorylation by illuminated chromatophores is compared with the extent of the $\Delta\tilde{\mu}$ H^+ measured concurrently. Under our experimental conditions, net ATP synthesis was detected when the initial concentrations of the substrates were set to give an initial $\Delta G'_{ATP}$ of 14–14.5 Kcal/mole or lower. The steady state value of $\Delta G'_{ATP}$ attained in this case was 15 Kcal/mole or slightly higher. On the contrary net ATP hydrolysis was always observed for initial $\Delta G'_{ATP}$ around 16 Kcal/mole and the final $\Delta G'_{ATP}$ levelled off at about 15.5 Kcal/mole.

Thus the maximal photophosphorylation potential of chromatophores from *Rps. capsulata,* obtainable with the intensity of illumination used in these experiments, is generally comprised between 15 and 15.5 Kcal/mole and the steady state can be reached starting from initial conditions corresponding to a lower or higher affinities, in a completely reversible way.

The values of $\Delta G'_{ATP}$ obtained correspond to 650–675 emV and would require an electrochemical potential difference of protons of at least 325–338 mV if a stoichiometry of $H^+/ATP=2$ is assumed. The value of $\Delta\tilde{\mu}_{H^+}$ measured contemporarily ranged between 400–430 mV and was therefore well adequate to drive

Table 1
Proton electrochemical gradient and maximal phosphorylation affinity in chromatophores of *Rps. capsulata*

Phosphorylation affinity											Experimental $\Delta\tilde{\mu}_{H^+}$ (mV)		
Initial conditions					Final conditions								
Substrate concentrations (μM)			ΔG′ (Kcal/mole)	$\Delta\tilde{\mu}_{H^+}$* (mV)	Substrate concentrations (μM)			$\frac{[ATP]}{[ADP]\,[P_i]}$ (M^{-1})	ΔG′ (Kcal/mole)	$\Delta\tilde{\mu}_{H^+}$ (mV)	Δψ	−ZΔpH	$\Delta\tilde{\mu}_{H^+}$
ATP	ADP	P_i			ATP	ADP	P_i						
980	108	750	14.32	310	1055	48	690	3.18×10^4	14.97	325	236	183	419
3360	63	200	16.24	352	3270	156	293	7.15×10^4	15.47	335	240	188	428
3450	85	200	16.09	349	3340	137	252	9.15×10^4	15.64	339	206	198	404

* Calculated from $\Delta G'_{ATP}$ from $H^+/ATP = 2$

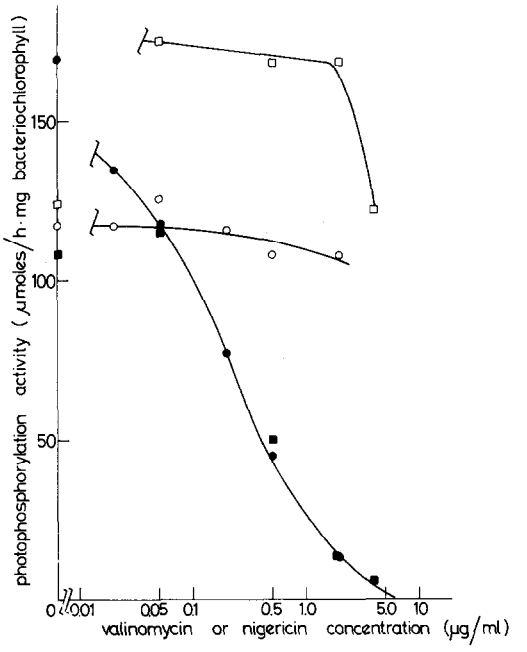

Fig. 1. The effect of nigericin and valinomycin on the rate of photophosphorylation in chromatophores of *Rps. capsulata.* (○–○–○) valinomycin alone; (□–□–□) nigericin alone; (●–●–●) 2 μg/ml nigericin plus increasing concentrations of valinomycin; (■–■–■) 2 μg/ml valinomycin plus increasing concentrations of nigericin.

photophosphorylation up to the high ATP/ADP ratios observed. These observations have been verified in a substantial number of experiments at variable substrate concentrations and in several different chromatophore preparations. The use of freshly prepared chromatophores is essential for obtaining such high values of the maximal phosphate potential and of $\Delta\tilde{\mu}_{H^+}$.

As previously reported, in the presence of high concentrations of K^+ ions, nigericin or valinomycin alone have hardly any effect on photophosphorylation of bacterial chromatophores; a marked synergistic inhibition of this activity results however after simultaneous addition of these two antibiotics [18]. Chromatophores from *Rps. capsulata* respond to ionophores in a similar way; however nigericin alone stimulates always the rate of phosphorylation well above the control rate (fig. 1).

It has also been found [18,19] that these compounds have a marked effect on the two components of the proton electrochemical difference, the antibiotic of the valinomycin class inhibiting the membrane poter tial (and consequently stimulating the pH difference) and those of the nigericin class inhibiting ΔpH (and consequently enhancing $\Delta\psi$). Measurements of the extent of these two entities in the steady state show that nigericin or valinomycin, also if added alone, affect significantly the overall value of $\Delta\tilde{\mu}_{H^+}$ since the stimulatory effect on one component of $\Delta\tilde{\mu}_{H^+}$ can never compensate completely the inhibition of the other.

Table 2 summarizes some examples of this effect: in presence of 50 mM KCl, valinomycin (at 2 μg/ml) causes a decrease of the membrane potential from over 200 mV, in the control to about 70 mV, whereas the stimulation of ΔpH corresponds to an increase of only 20 mV of the concentration component of $\Delta\tilde{\mu}_{H^+}$. The total extent of $\Delta\tilde{\mu}_{H^+}$ in this condition amounts only to about 280 mV. Conversely nigericin abolishes completely any measurable pH difference, but enhances the membrane potential only by about 30–50 mV (an experimental uncertainty of 1% quench corresponding to a ΔpH of 0.6 units or 36 mV is here assumed). Again $\Delta\tilde{\mu}_{H^+}$ is reduced to 270–300 mV.

The effect of ionophores on $\Delta\tilde{\mu}_{H^+}$ is therefore quite different from their action on the rate of phosphorylation; it is therefore of considerable interest to test the relationship between $\Delta\tilde{\mu}_{H^+}$ and the phosphate potential under these conditions. The data reported in table 2 indicate that $\Delta G'_{ATP}$ is also considerably decreased by the addition of ionophores. In the presence either of nigericin or of valinomycin net ATP synthesis is observed when the initial conditions are set to a $\Delta G'_{ATP}$ around 11.5 Kcal/mole, but net ATP hydrolysis for initial $\Delta G'_{ATP}$ as low as 14.2–14.6 Kcal/mole. The steady state value for $\Delta G'_{ATP}$ measured in these conditions ranges between 13–13.8 Kcal/mole corresponding to 282–300 mV.

The values of $\Delta G'_{ATP}$ measured in the presence of ionophores are again consistent with the extents of $\Delta\tilde{\mu}_{H^+}$. It must be noted, however, that the ratios $\Delta G'_{ATP}/\Delta\tilde{\mu}_{H^+}$, which is always around 0.8 in control experiments, are very close to 1 in the presence of ionophores. This discrepancy, which could be of fundamental meaning for an experimental test of the chemiosmotic coupling hypothesis, is currently under investigation. It is quite clear nevertheless that ionophorous antibiotics have a profound effect on phosphate potential. In fact, if our data are analyzed as-

Table 2
The effect of valinomycin or nigericin on the extent of the proton electrochemical gradient and of the maximal phosphorylation affinity in chromatophores of *Rps. capsulata*

Additions	Phosphorylation affinity											Experimental $\Delta\tilde{\mu}_{H^+}$ (mV)		
	Initial conditions					Final conditions								
	Substrate concentrations (μM)			$\Delta G'$ (Kcal/mole)	$\Delta\tilde{\mu}_{H^+}$* (mV)	Substrate concentrations (μM)			$\frac{[ATP]}{[ADP]\,[P_i]}$	$\Delta G'$ (Kcal/mole)	$\Delta\tilde{\mu}_{H^+}$ (mV)	$\Delta\psi$	$-Z\Delta pH$	$\Delta\tilde{\mu}_{H^+}$
	ATP	ADP	P_i			ATP	ADP	P_i	(M^{-1})					
Valinomycin 2 μg/ml	115	394	3000	11.57	251	393	86	2693	1.69×10^3	13.18	285	75	208	283
Valinomycin 2 μg/ml	400	23	1000	14.60	316	361	68	1045	5.09×10^3	13.87	301	71	214	285
Nigericin 2 μg/ml	122	400	3000	11.49	255	379	121	2721	1.15×10^3	12.97	282	272	0–36	272–308
Nigericin 2 μg/ml	407	44	1000	14.22	308	343	102	1058	3.18×10^3	13.58	294	266	0–36	266–302

* Calculated from $\Delta G'_{ATP}$ for $H^+/ATP = 2$.

suming a constant concentration of phosphate equal to 5 mM, they demonstrate that bacterial photophosphorylation can maintain in the steady state an ATP/ADP ratio as high as 150–450 (table 1), but that this value is decreased to about 1.5–7.5 (table 2) in the presence either of valinomycin or of nigericin.

The relevance for bacterial photophosphorylation of transmembrane pH and potential differences, suggested by the synergistic inhibition of ATP synthesis by nigericin plus valinomycin, has been confirmed experimentally by Shuldiner et al. [20] who observed in *Rh. rubrum* chromatophores a stimulation of postillumination ATP synthesis by a diffusion potential of K^+. The present experiments demonstrate, using a completely different approach, that the dissipation of $\Delta\psi$ by electrophoretic fluxes or of ΔpH by electroneutral H^+/K^+ exchanges, results in a reduction of the maximal $\Delta G'_{ATP}$ attainable in the steady state and prove therefore the strict relation existing between these two parameters and the amount of energy available for ATP synthesis.

The use of carotenoid shift for the estimation of membrane potential and that of 9-amino acridine for measuring ΔpH has been questioned by some authors [21,22]. In particular it has been suggested that only a fraction of the light induced carotenoid change is related to the onset of a membrane potential [21]. The data reported in table 2 show that a good agreement between $\Delta G'_{ATP}$ and $\Delta\psi$ or ΔpH can be observed also under conditions in which only one of these two parameters is predominant and offer therefore an independent support for the validity of these two techniques. The results obtained in the presence of nigericin indicate that, if a stoichiometry $H^+/ATP = 2$ is assumed, a membrane potential corresponding to the full extent of the carotenoid signal would be required to match the affinity of photophosphorylation determined experimentally.

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