THE PHOSPHATE POTENTIAL AND H^+/ATP RATIO IN *RHODOSPIRILLUM RUBRUM*

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1. Introduction

Rhodospirillum rubrum is a purple, non-sulfur photosynthetic bacterium capable of growing photosynthetically under anaerobic conditions. Chromatophores prepared from this organism are capable of catalyzing the synthesis of ATP and inorganic pyrophosphate upon illumination [1,2] and also exhibit ATPase and pyrophosphatase activities which can serve to drive 'energy-linked' reversed electron flow [3]. Thus in contrast to some other photosynthetic bacteria [4], the energy-coupling reactions of *R rubrum* are readily reversible. *R rubrum* chromatophores have been reported to maintain a thermodynamic phosphorylation potential of ~12–14 kcal/mol [5,6]. The phosphorylation potential, ΔG_p, is the Gibbs free energy for the ATP synthesis and is defined by the relationship:

$$\Delta G_p = -\Delta G'_o + RT \ln \frac{[ATP]}{[ADP] \cdot [P_i]}$$

where G'_o is the standard free energy for ATP hydrolysis. Using the fluorescent probe 8-anilinonaphthalene sulfonate, it was reported [5] that under these conditions the electrochemical proton gradient across the chromatophore membrane (defined by the relationship $\Delta\mu_{H^+} = \Delta\psi - 59\Delta pH$, where $\Delta\psi$ is the transmembrane potential in mV) was ~200 mV. In contrast, using flow dialysis, electrochemical proton gradients of ~100 mV, with no significant contribution from the pH gradient were reported [6]. We have extended these studies using an alternative extrinsic probe of membrane potential, the oxonol dye OX-VI, which has been described as a rapid and reliable indicator of membrane potential in a variety of photosynthetic bacteria [7], and also in submitochondrial particles [8]. OX-VI reports on the changes in membrane potential during oxidative phosphorylation and can be used to determine a 'null-point' ΔG_p at which no change in probe response is observed by the addition of adenine nucleotide mixtures of known composition [8]. The null-point ΔG_p correlated well with that for the stimulation of respiration and reversed electron transport, and also with the ΔG_p maintained in the steady state by respiring submitochondrial particles [8,9].

In the experiments reported here, the null-point titration technique has been used to explore some thermodynamic aspects of the adenine nucleotide and pyrophosphate phosphorylation reactions of *R rubrum*. We find that the magnitude of the electrochemical proton gradient is commensurate with a 'charge per ATP' ratio of 2, as originally suggested [10], rather than the higher numbers suggested [5,6].

2. Materials and methods

Rhodospirillum rubrum S1 and *Rhodopseudomonas sphaeroides* Ga chromatophores were prepared using a French pressure cell as in [11]. The dye, OX-VI, was synthesized by the method in [12]. Light-dependent absorbance changes were monitored as in [7,11]. Continuous illumination, filtered by a Kodak Wratten Gelatin Filter number 88A, was provided by an adjustable microscope lamp.

3. Results and discussion

The responses of OX-VI to continuous illumination, ATP hydrolysis and pyrophosphate hydrolysis in *R. rubrum* chromatophores are shown in fig.1. The decrease in A_{587}, an isosbestic point in the light minus dark difference spectrum of *R. rubrum*, is caused by a shift of dye absorbance similar to that observed in submitochondrial particles [8] and in other photosynthetic bacteria [7]. Pyrophosphate and ATP hydrolysis maintained dye responses significantly lower (80% and 60%, respectively) than that found during continuous illumination. This observation is in contrast to the behavior of the dye in submitochondrial particles, where ATP hydrolysis and respiration maintain dye responses of similar magnitude [8]. The results reported here suggest that the phosphorylation reactions of *R. rubrum* may only be partially reversible in the dark, being unable to match the kinetic and thermodynamic inputs of the light reaction.

The response of OX-VI to a train of saturating single turnover flashes (c.f. [7]) is illustrated in fig.2. The dye response under these conditions slightly exceeds that found during continuous illumination, probably because the pH gradient develops slowly during a train of flashes [13]. The presence of ADP and phosphate significantly diminishes the probe response to a train of single turnover flashes, and the response decays only to the level that can be maintained by ATP hydrolysis; the changes caused by the addition of ADP are completely abolished by the addition of oligomycin. This suggests that ADP was phosphorylated during the train of pulses and that subsequent hydrolysis of the ATP prevented the complete collapse of the dye response in the dark. Ventu-

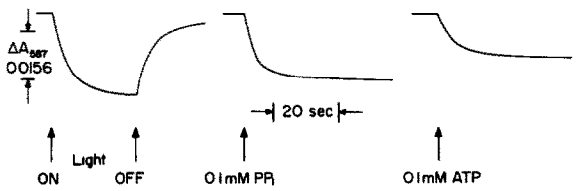


Fig.1. The energy-linked behavior of OX-VI in *R rubrum* chromatophores Chromatophores (20 μM BChl) were suspended in a medium containing 100 mM KCl, 20 mM MOPS, 1 mM $MgCl_2$, 0.5 mM ascorbate and 1.5 μM OX-VI (pH 7 0) at 23°C Illumination, 100% saturating, and additions were made as indicated

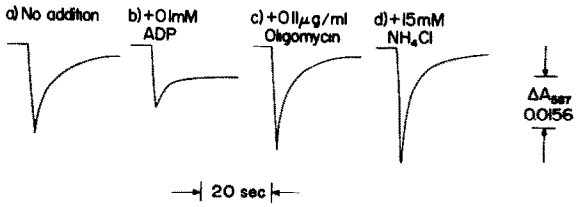


Fig.2. The effect of ADP on the energy-linked response of OX-VI in *R rubrum* chromatophores. Chromatophores (20 μM BChl) were suspended in a medium similar to that in fig.1. except that 1 mM KP_i was also present. The samples were activated by a train of 32 saturating flashes 40 ms apart. Additions were made sequentially as indicated between experiments

ricidin, oligomycin and aurovertin abolished the effect of ADP addition, consistent with their role as inhibitors of photophosphorylation [14]. An additional effect of oligomycin is evident in fig.2, where the magnitude of the dye response is larger in the presence of the inhibitor than in its absence before the addition of adenine nucleotides; an observation consistent with the suggestion that oligomycin decreases the proton permeability of chromatophore membranes [14]. The abolition of any light-dependent pH gradient by the addition of amine further increased the dye response (fig.2), implying that under these conditions energy is conserved entirely as membrane potential. Similar interconversions of pH gradient and membrane potential have been reported [5] and also in submitochondrial particles [8].

The effect of the addition of adenine nucleotide mixtures on the response of OX-VI in *R. rubrum* during continuous illumination is shown in fig.3. In the experiment reported here the illumination was attenuated to provide a dye response ~80% of maximum. By varying the ratio of ATP and ADP at constant phosphate concentration, an increase, a decrease, or no change in the probe response could be detected after the addition of nucleotides. In contrast, the addition of 0.5 mM pyrophosphate in the presence of a wide range of phosphate concentrations always increased the dye response Thus while a null-point is clearly visible in the adenine nucleotide titration, there was no evidence for its occurrence in the pyrophosphate titration. In all experiments the decay of the dye response after cessation of illumination was slow because of the presence of ATP or pyrophosphate (c.f. fig.2,3). Figure 4 illustrates the results of a

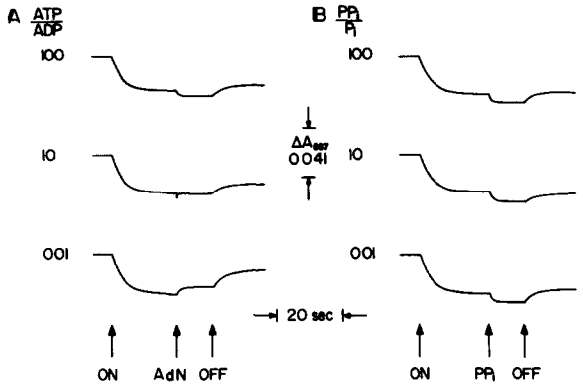
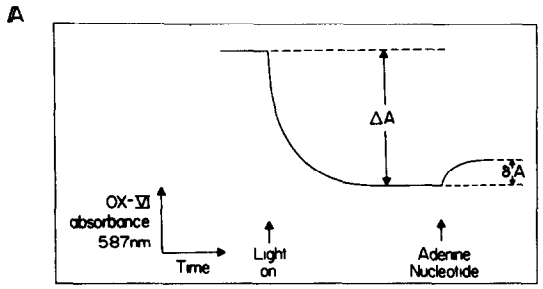
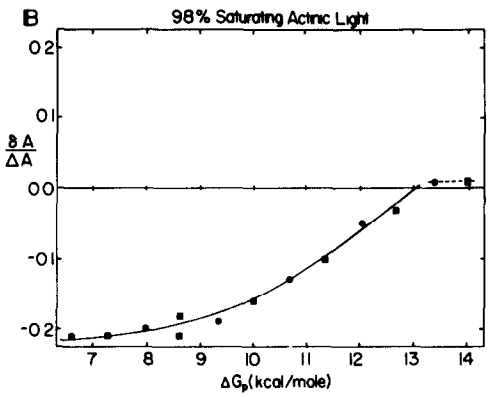


Fig.3. The effect of phosphate potential on the energy-linked response of OX-VI in *R. rubrum* chromatophores. Chromatophores (20 μM BChl) were suspended in a medium similar to that in fig.1 For traces labelled A, 3.5 mM KP_i was also present and mixtures of adenine nucleotides to final conc. 0.5 mM were added as indicated In traces labelled B mixtures of pyrophosphate and phosphate were added as indicated to give final pyrophosphate conc 0 5 mM. 80% saturing continuous illumination was provided as described in section 2





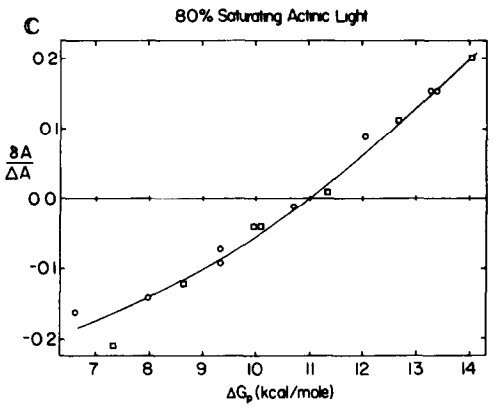


Fig 4. Phosphate potential in *R rubrum* chromatophores Chromatophores (20 μM BChl) were suspended in a medium similar to that in fig 1. The effect of the addition of adenine nucleotide mixtures as in fig.3 was analyzed according to the scheme labelled A. The fractional absorbance change $\delta A/\Delta A$ was plotted against the phosphate potential imposed by the addition of adenine nucleotides according to the relationship·

$$\Delta G_p = -\Delta G'_0 + RT \ln \frac{[ADP]}{[ADP]\ [P_i]}$$

where $\Delta G'_0$ is the standard free energy of ATP hydrolysis which has the value of 6 7 kcal/mol in the media employed here [20]. Experiments were performed in 98% saturating (closed symbols) and 80% saturating (open symbols) continuous illumination and phosphate at 3.5 mM (square symbols) and 9 8 mM (round symbols)

number of adenine nucleotide titrations similar to that presented in fig.3. The ΔG_p imposed by the addition of the adenine nucleotides is plotted on the abscissa, and the fractional OX-VI absorbance change on the ordinate. It is noteworthy that the presence of differing levels of phosphate had no effect on the magnitudes of the probe response, indicating that the dye is responding to the thermodynamic parameter, ΔG_p, rather than to the ATP/ADP ratio. In 98% saturating light the 'null-point' ΔG_p was ~13 kcal/mol whereas in 80% light it had a value of 11 kcal/mol.

The 'null-point' ΔG_p values obtained here exceed the phosphorylation potentials measured in *R. rubrum* [5], presumably reflecting differences in chromatophore preparation and experimental media However, the values of ΔG_p are slightly lower than those found in [6] and in [15,16] for *Rps capsulata* chromatophores, so our results are probably not unrealistic overestimates of the capacity of *R. rubrum* chromatophores to synthesize ATP.

The experiments reported here provide no evidence for the synthesis of pyrophosphate by *R rubrum*, despite the observation that the preparations contained a significant 'energy-linked' pyrophosphatase activity (c.f. fig.1). One possible explanation for these results may be the relative activities of the pyrophosphatase operating in its synthetic and hydrolytic modes; the pyrophosphatase activity exceeds that of the ATPase by a factor of 2 [3], whereas pyrophosphate synthesis proceeds at only ~10% of the rate of ATP synthesis at saturating light intensity [17,18]. In concert with these findings, the 'energy-linked' response of OX-VI during pyrophosphate hydrolysis exceeded that during ATP hydrolysis (fig.1). However, during continuous illumination the addition of ADP imposed a significant, oligomycin-sensitive restraint on the probe response that is not present in the presence of inorganic phosphate alone, furthermore pyrophosphate addition always resulted in pyrophosphate hydrolysis (fig.3). It is thus likely that the rate of pyrophosphate synthesis is not sufficient to cause a detectable diminution of membrane potential, in marked contrast to the synthesis of ATP, which is rapid and clearly diminishes the membrane potential.

An exact calibration of the OX-VI absorbance change with membrane potential is not available for *R rubrum* chromatophores. However, in chromatophores from the related organism *Rps. sphaeroides* the logarithm of the OX-VI response is linear with the membrane potential estimated from the carotenoid bandshift, as shown in fig.5 (and see [7]). The calibration can be used to provide an estimate of the steady state membrane potential of *R rubrum* during phosphate potential titrations and the results are summarized in table 1. Conversion of the phosphate potential into electrical units (table 1) allows a calculation of the maximum number of protons that must move across the membrane dielectric during the synthesis of ATP. In both 98% and 80% saturating light the apparent H^+/ATP ratio was close to 2 (table 1). It should be stressed that these values represent upper limits for the H^+/ATP ratio, as only the membrane potential was determined in these experiments, and no account has been taken of the additional contribution to the proton motive force provided by the light-induced pH gradient. The experiments in fig.2 indicate

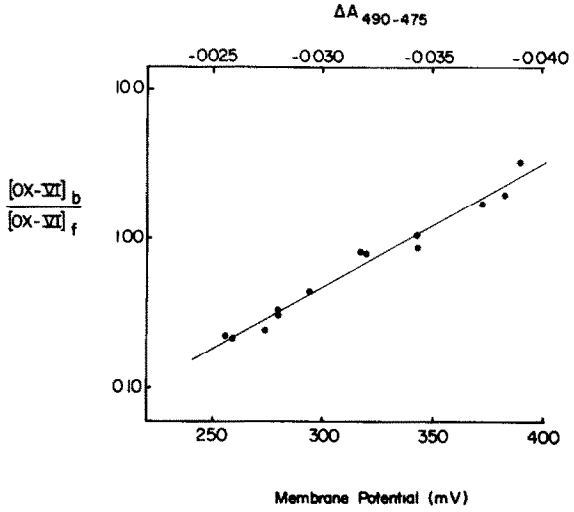


Fig 5. The light-dependent carotenoid bandshift and OX-VI response in *Rps. sphaeroides* chromatophores Chromatophores (20 μM BChl) were suspended in a medium similar to that in fig 1, except that OX-VI was present at 0 3–8 7 μM. The concentration of bound OX-VI was calculated from the ΔA_{587} assuming an extinction coefficient of $-4\ 5 \times 10^4$ M^{-1} cm^{-1} for the difference in absorbance of the bound and the free forms [7]; $[OX\text{-}VI]_f$ was then obtained by subtraction. The extent of the carotenoid bandshift was measured at 490–475 nm and this value was converted to membrane potential using the calibration with K^+ pulses in the presence of valinomycin reported [21] for these chromatophores

Table 1
ATP synthesis in *R rubrum* chromatophores

Light saturation (%)	ΔG_p (kcal/mol)	ΔG_p (mV)	Membrane potential (mV)	Apparent H^+/ATP
80	11	477	238 ± 9	2 00 ± 0.07
98	13	564	258 ± 10	2.18 ± 0.08

Chromatophores (20 μM BChl) were suspended in a medium similar to that in fig.4 and the null-point ΔG_p was estimated by the procedure illustrated in fig.4 The conversion of ΔG_p to electrical units was performed according to the relationship ΔG_p (mV) = ΔG_p (kcal/mol)/F where F is the Faraday constant (F = 23 063 kcal/V-equiv) Membrane potential was calculated from the OX-VI absorbance change according to the procedure in fig.5. The values represent the mean and standard deviation of 8 determinations The apparent H^+/ATP ratio was calculated from the phosphate potential (mV) divided by the membrane potential (mV)

some contribution from ΔpH which is collapsed by NH_4Cl. However, calculations suggest that the ΔpH is only ~12 mV which would reduce the apparent H^+/ATP ratios to 1.91 and 2.09, respectively.

In contrast to the results presented here, H^+/ATP ratios in excess of 5 have been reported [6]. It is not clear why the flow dialysis technique used [6] should provide such a low estimate of the protonmotive force. Our results are more in concert with [5] where H^+/ATP ratios between 2 and 3 were obtained, but even these values may be overestimates because they used a permeant anion to monitor membrane potential, and a calibration procedure that is not necessarily reliable under such conditions [19].

The data presented here are consistent with the original proposal of the chemiosmotic hypothesis that only 2 protons must cross the membrane per ATP synthesized [10].

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