POSSIBLE INITIAL EVENTS OF PHOTOPHOSPHORYLATION IN MEMBRANES OF *RHODOPSEUDOMONAS. VIRIDIS* AND *RHODOPSEUDOMONAS. CAPSULATA* Ala^+r

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1. Introduction

There is still controversy as to whether the initial phosphate acceptor in oxidative phosphorylation and photophosphorylation is ADP or AMP.

Ozawa et al. [1,2] have found evidence indicating that in mitochondria AMP may be the initial phosphate acceptor.

In chloroplasts Roy et al. [3] postulated that AMP is initially phosphorylated in the light. On the other hand, Smith et al. [4] by studying the early kinetics of ATP and ADP formation, conclude that in chloroplasts ADP is the first nucleotide that accepts a phosphate molecule. A similar proposal was advanced recently by Aflalo et al. [5].

In photosynthetic bacteria, Yamamoto et al. [6] and Lutz et al. [7] have described membrane bound adenine nucleotides as participants, among other possibilities, in transphosphorylations which finally lead to ATP formation. We report here on some experiments carried out in membranes of both *Rps. viridis* and *Rps. capsulata* concerned with the possible initial events of phosphate uptake in photophosphorylation.

2. Materials and methods

Rhodopseudomonas capsulata Ala^+r and *Rhodopseudomonas viridis* were grown in the same medium as previously described for *Rps. viridis* [8].

The membranes were obtained by sonicating the cells suspended in 100 mM glycylglycine buffer pH 8.0, 2.5 mM $MgCl_2$ at 70 W output in a Branson sonifier in the cold for 5 min. Cell debris were eliminated by centrifugation for 20 min at 15 000 × *g* and the membranes recovered by centrifugation at 144 000 × *g* for 90 min. The membranes were washed twice with the same buffer, and were finally resuspended in the same buffer to a concentration of 1 mg Bchl/ml.

2.1. *Photophosphorylation*

Photophosphorylation was carried out by adding the membranes to 1.0 ml of a mixture containing: 30 μmol Tricine pH 8.0, 3.7 μmol MgCl, 1.7 μmol ADP, 3.3 μmol P_i (specific activity: 7.5×10^5 cpm/μmol), 0.4 μmol of Na succinate, and 1.3 μmol phenazine methosulphate.

Incubation was at 30°C under saturating light conditions. When photophosphorylation was carried out in the presence of P_i and [β-^{32}P]ADP, 2.15 μmol of phosphate and 1 μmol ADP (usually between 2×10^5 – 6×10^5 cpm/μmol) were used.

Photophosphorylation activity was measured as described before [9].

For the analysis of the products of phosphorylation, the reaction was carried out in three times the volume of the reaction mixture described above.

2.2. *Purification of [β-^{32}P]ADP*

[β-^{32}P]ADP was recovered as a product of photophosphorylation in the presence of ^{32}P and ADP. It was separated from other contaminants by ion exchange chromatography as described below.

The zone containing maximum cpm due to ADP was adsorbed onto activated charcoal, washed twice with 2% TCA in water containing: 20 μmol/ml of unlabeled phosphate and then eluted by washing with 50% ethanol containing 1% concentrated NH_3 v/v.

The ammonia and the ethanol were evaporated

under reduced pressure and the solution was freeze-dried.

Recovery of [^{32}P]ATP was performed using a similar procedure.

Yields of 65–70% of nucleotides were currently obtained. The purity of ^{32}P-labeled nucleotide was routinely checked by paper chromatography according to Serlupi-Crescenzi et al. [10] using 1 M acetic acid/Na citrate pH 3.8 as a solvent.

2.3. *Ion exchange chromatography*

After the reaction under consideration was completed, the reaction mixture was rapidly cooled at 0°C and immediately passed through a Dowex 1 Cl column (0.7 X 22 cm). The elution was started by passing 20 ml of 10 mM HCl, then a linear gradient (60 ml) of NaCl from 0–200 mM in 10 mM HCl was started and again 1.0 ml fractions were collected. The columns were previously calibrated with the corresponding standards.

2.4. *Other analytical procedures*

Bchl was determined as described before [11], or by extraction with methanol acetone and using a millimolar extinction coefficient as previously described [12]. Radioactivity was determined in a liquid scintillation spectrometer. The solvent used was a mixture of 1 vol of Triton X-100 and 2 vol of toluene containing: 2,5-diphenyloxazole (PPO) and dimethyl 1,4-bis (2(4-methyl-5-diphenyloxazole))-benzene (dimethyl POPOP). This mixture will tolerate up to 1.0 ml of water with no appreciable quenching [13]. The amounts of ADP or ATP were determined by their absorbance at 259 nm using a millimolar extinction coefficient of 15.4 for both ADP and ATP.

2.5. *Hexokinase reaction*

The hexokinase reaction was carried out in a final volume of 3.0 ml containing: 25 μmol Tris pH 8.0, 61.6 μmol glucose, 1.5 umol $MgCl_2$ and 15 units of hexokinase and variable amounts of ATP.

The incubation was carried out at 30°C for 20 min. The reaction was stopped by rapidly cooling at 0°C and immediately processed by ion exchange chromatography as described above.

3. Results and discussion

Table 1 illustrates the products of photophosphorylation in the presence of ^{32}P and ADP in membranes of *Rps. viridis* and *Rps. capsulata* Ala$^+$r. In both cases [β-^{32}P]ADP and [^{32}P]ATP were obtained, and in a proportion which usually varied between 3–10% of [β-^{32}P]ADP and 90–97% of [^{32}P]ATP. In this case the origin of the [β-^{32}P]ADP could be explained as resulting from an ADP–P_i or and ATP–ADP exchange reaction, and the overall results are apparently in agreement with a direct addition of P_i to ADP to form ATP. If unlabeled phosphate and [β-^{32}P]ADP are used as substrates for photophosphorylation, the radioactivity distribution in P_i, ADP and ATP cannot be explained by a mechanism similar to that mentioned above.

Table 1
Photophosphorylation products obtained using [^{32}P]phosphate and ADP.

Bacterium	Percent of cpm recovered as	
	[β-^{32}P]ADP	[^{32}P]ATP
Rps. viridis	8	92
Rps. capsulata Ala$^+$r	5	95

Rates of photophosphorylation were 70 μmol ATP/h/mg Bchl for *Rps. capsulata* Ala$^+$r and 10 umol ATP/h/mg Bchl for *Rps. viridis*.

Thus, table 2 shows that while the [β-^{32}P]ADP has maintained its specific activity during the time course of the reaction, the [^{32}P]ATP obtained has increased it by a factor of 1.3 to 1.7.

If the mechanism of phosphate uptake in this process implies a direct addition of unlabeled phosphate to [β-^{32}P]ADP, the specific activity of the [^{32}P]ATP should not be higher than that of the initial

Table 2
Specific activity of the products of photophosphorylation using P_i and [β-^{32}P]ADP

Bacterium	Specific activity cpm/μmol X 10^5 recovered in		
	ADP	ATP	P_i
Rps. viridis	2.35	4.0	0.13
Rps. capsulata Ala$^+$r	5.72	7.03	0.093

The initial specific activity of [β-^{32}P]ADP for *Rps. viridis* was 2.26 X 10^5 cpm/μmol and for *Rps. capsulata* Ala$^+$r 5.66 X 10^5 cpm/μmol.

Table 3
Analysis of the ^{32}P distribution in the β- and γ-phosphates in the [^{32}P]ATP obtained by photophosphorylation.

Substrates of photophosphorylation	Bacterium	Percent cpm recovered	
		Glu-6-P	ADP
^{32}P + ADP	*Rps. viridis*	90	10
	Rps. capsulata Ala^+r	97	3
P_i + [β-^{32}P]ADP	*Rps. viridis*	47	53
	Rps. capsulata Ala^+r	37	63

[β-^{32}P]ADP. It could be lower if the ADP–P_i exchange activity was high compared to photophosphorylation.

Moreover, when the [^{32}P]ATP, product of the reaction, using either P_i and [β-^{32}P]ADP or ^{32}P and ADP, was allowed to transfer its γ-phosphate to glucose in the presence of hexokinase, the results shown in table 3 were obtained. In the case where P_i and [β-^{32}P]ADP were used, there was a substantial amount of label recovered in the glucose 6-[^{32}P]phosphate, indicating that there was a corresponding high proportion of [γ-^{32}P]ATP or double labeled [β-γ-^{32}P]ATP as a product.

When membranes of *Rps. capsulata* or *Rps. viridis* were incubated in the light or in the dark, in a reaction mixture containing [β-^{32}P]ADP, similar to that used for photophosphorylation, except that it did not contain Pi, we could not detect [^{32}P]ATP by ion exchange chromatography.

This result seems to eliminate the possibility that an unspecific adenylate kinase is responsible for the labeling pattern of ATP observed, when P_i and [β-^{32}P]ADP were used as substrates for photophosphorylation.

These results cannot be explained by a direct addition of phosphate to ADP and could be explained instead by a series of reactions, one of which, a transphosphorylation reaction between two molecules of ADP, would result in the formation of ATP and AMP, which would be rephosphorylated in the light.

The participation of AMP as primary phosphate acceptor is however not demonstrated by the experiments presented here and thus remains speculative.

We have recently reported on the isolation and purification from membranes of *Rps. viridis* of an enzyme having Mg^{2+}-stimulated ADPase activity, ADP–P_i exchange activity, and capable of restoring photophosphorylation when rebound to previously uncoupled membranes [14].

The same enzyme was also partially purified by us (AFG, NLK and NLP, unpublished experiments) from membranes of *Rps. capsulata* Ala^+r. It is possible that this enzyme could participate in a light-induced AMP phosphorylation.

The results presented here are not sufficient to build up a complete hypothesis of a possible mechanism of the early events leading to ATP formation, although they point to the possibility that a transphosphorylation reaction between two molecules of ADP is involved in this process. Moreover, they emphasize the fact that ATP is possibly not formed, at least in *Rps. viridis* and *Rps. capsulata* Ala^+r, by the simple addition of phosphate to ADP.

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