Photosynthetic Phosphorylation in a Spheroplast of a Thermophilic Cyanobacterium*

Sachio Miyairi**

National Chemical Laboratory for Industry, Yatabe, Tsukuba Gun, Ibaraki 305
(Received Sep. 17, 1983)

Spheroplasts of a Synechococcus sp. thermophilic cyanobacterium showed cyclic photophosphorylation activity of 1200 μmol ATP/mg Chl h at 45°C with ascorbate-phenazine methosulfate, and non-cyclic activity of 11 μmol ATP/mg Chl h with H2O-methyl viologen. Thylakoid membranes exhibited less than one-tenth of the spheroplasts activities in both types of photophosphorylation. The spheroplasts gave optimal cyclic phosphorylation at 55°C and lost half of the activity at 67°C for 5 min. It was suggested that the stability of the photosynthetic membranes to retain proton gradient across the membranes might be closely related to the thermostability of the photophosphorylation.

Key words: Cyanobacterium, Phosphorylation, Photosynthetic membrane, Spheroplast, Synechococcus sp., Thermophile

Introduction

Although mechanisms of photophosphorylation have been much studied, especially in higher plant chloroplasts1-4), many open questions are remained. As the thermophilic cyanobacterium possess stable photosynthetic systems which are essentially similar in mechanism to those of higher plants, they have been recently adopted for studies on photosynthesis. Photophosphorylation in membranes5,6) and living cells7) of thermophilic cyanobacteria have been studied.

Photosynthetic systems of Synechococcus sp. thermophilic cyanobacterium isolated from a hot spring have been proved to possess thermophilic and thermostable electron carriers8-11). Photosystem II extracts with high oxygen-evolving activity have recently been prepared from spheroplast membranes of this cyanobacterium12). The spheroplasts have been found to possess rather high cyclic photophosphorylation activity, and some properties of phosphorylation in the spheroplasts are described here.

Materials and Methods

A thermophilic cyanobacterium Synechococcus sp.8) given by Prof. S. Katoh, University of Tokyo, was cultivated at 50°C for 48 h in the medium of Dyer and Gafford13), supported with 5% carbon dioxide and illuminated with fluorescent lamps (Osram “L-daylight 5000”). Cells were harvested by centrifugation and washed twice in 20mM HEPES-NaOH (pH 7.8), 10mM MgCl2 and 2mM Na-K-phosphate (HMP-buffer). Spheroplasts were prepared by lysozyme-osmotic shock treatment as previously reported12). They were suspend-

Abbreviations:
DCCD, dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenol indophenol; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid; HMP-buffer, buffer containing 20mM HEPES-NaOH, 10mM magnesium chloride and 2mM Na-K-phosphate; MES, 2-(N-morpholino) ethane sulfonic acid; MV, methyl viologen; PMAA, phenylmercuric acetate; PMS, phenazine methosulfate; PS, photosystem; TPT, triphenyltin chloride.

*特許第 うん藻のスフョ ロプラストにおける光リン酸化
**宮入祥夫：工業技術院化學技術研究所 〒305 奈良県
筑波郡谷田部町東1－1
ed in a buffer of 80% (v/v) HMP containing 400mM mannitol and 20% glycerol, and stored at −70°C without any loss of photophosphorylation activity for 2 months.

Thylakoid membranes were isolated according to the previous report8) with some modifications. Cells were incubated in a medium containing 400mM mannitol, 50mM phosphate (pH 6.8) and 0.2% egg lysozyme (Serva) at 45°C for 1 h in the dark and under gentle agitation, and then collected by centrifugation, resuspended in the preparation medium containing 30% polyethylene glycol-4000, 400mM sucrose, 50mM phosphate (pH 7.5) and 10mM NaCl. After two treatments with a Ribi-press at 400 kg/cm², the suspension was centrifuged at 20000×g for 30 min to remove undisrupted spheroplasts and large fragments. Then, 1mM MgCl₂ and DNase (80 µg/ml) were added to the supernatant and incubated for 30 min at 30°C. The remaining thylakoid membranes were collected by centrifugation at 80000×g for 1 h, resuspended in the preparation medium and stored at −70°C.

Chlorophyll-a extracted from membranes with 80% acetone was determined from the absorbance at 663 nm14).

Oxygen evolution and uptake were measured with a Clark-type electrode at 45°C. Illumination was provided by light from a 250 W halogen lamp (Osram Co.) through a water filter at an intensity of 80mW/cm². The reaction mixture (2 ml) contained 50mM MES-NaOH (pH 5.5), 500mM sucrose, 20mM NaCl, 10mM MgCl₂, 1mM potassium ferricyanide and membranes (10 µg Chl) for assaying the Hill reaction. Methyl viologen (MV) photoreduction mediated by PS II and PS I was assayed in the medium containing 25mM HEPES–NaOH (pH 7.5), 1mM NaN₃, 200µM MV and the membranes. MV photoreduction mediated by PS I alone was assayed in HEPES–NaOH buffer (pH 7.5) containing 1mM NaN₃, 200 µM MV,10 µM DCMU, 2mM Na-ascorbate, 100 µM dichlorophenol indophenol (DPIP) and the membranes.

Activities of cyclic and non-cyclic photophosphorylation were measured as follows. Reaction was conducted at 45°C for 1 min, unless otherwise stated, under illumination with a 250 W halogen lamp through a red filter (RG 610) to cut light below 600nm. The light intensity in the cuvette was 20 mW/cm². The basal reaction mixture contained the membranes (30 µg Chl), 25mM HEPES–NaOH (pH 7.0), 1mM ADP, 1mM KH₂PO₄ and 5mM MgCl₂. For cyclic phosphorylation 10mM Na-ascorbate and 130µM phenazine methosulfate (PMS) were added to the basal reaction mixture to make 5 ml. For the measurement of phosphorylation depending on the non-cyclic electron flow from water to MV, 10mM NaCl and 200µM MV were added to the basal medium. The membranes were added just before the start of the illumination. The reaction was stopped by mixing with 2% trichloroacetic acid and the supernatant was assayed by the firefly bioluminescence method15). ATP assay reagent was purchased from LKB-WALLAC (Sweden).

Results and Discussion

Photosynthetic electron transport and phosphorylation activities of the spheroplasts and of the thylakoid membranes are shown in Table 1. Cyclic photophosphorylation activity of the spheroplasts was considerably high. In contrast non-cyclic photophosphorylation activity of the spheroplasts was relatively low and P/2e ratio was 0.29. Cyclic phosphorylation activity of the thylakoid membranes was only one-tenth of that of the spheroplasts. By addition of 10mM NH₄Cl or CH₃NH₂, PS I electron transport activity of the thylakoid membranes was not affected at all, while the activity of the spheroplasts was enhanced by 41 or 39%, respectively. These results indicate that the thylakoid membranes might be uncoupled (i.e. leak out protons). Non-cyclic phosphorylation activity was scarce in the thylakoid membranes, from which an interconnecting electron carrier between PS II and PS I is considered to release during the preparation process8,16). The large activity of cyclic photophosphorylation in the spheroplasts might be ascribed to the abundance of PS I reaction center and to efficient creation of H+-gradient across the membrane. Ratio of PS I and PS II reaction centers was estimated to be about 3.6 in the spheroplast membranes12). It is known that some species of thermophilic5) and non-thermophilic17,18) cyanobacteria possess large activities of cyclic phosphorylation, and that other species of cyanobacteria have a greater number of PS I reaction centers than PS II19).
Some properties of cyclic photophosphorylation in the spheroplasts are described. By omission of ascorbate, PMS or ADP from the reaction medium, the spheroplasts showed a relative activity of 21, 4 or 0% of that with the complete system. Without light, the spheroplasts exhibited a relative activity of 2% of that under illumination. The spheroplasts showed maximum activity at pH 7.0 and showed relative activities of 3, 71, 89 and 70% at pHs 5.5, 6.5, 7.5 and 8.2, respectively. 7.0 is close to the pH value of the growth medium which, initially 7.5, fell to 6.8 during the cultivation. Michaelis constants for ADP and Pi, estimated from Lineweaver-Burk's plots (figures not shown), were 21 and 36 μM at pH 7.0 in the presence of 200μM Pi and 100μM ADP, respectively. MgCl₂ was essential for the phosphorylation and optimum concentration was 5mM. NaCl and KCl were inhibitory for the reaction, presumably due to uncoupling effect of chloride ions²⁰).

Effects of various substances on the phosphorylation activity of the spheroplasts are presented in Table 2. DCMU decreased the activity a little. Basic polypeptide poly-L-lysine (Mw 130,000) and phenylmercuric acetate (PMAA) showed large inhibitory effects on the phosphorylation, though mechanisms of action of these reagents are not clear at the present stage. Ammonium chloride and gramicidin D, which decrease H⁺-gradient across the membrane, inhibited the reaction considerably. Both dicyclohexylcarbodiimide (DCCD) and triphenyltin chloride (TPT), which block H⁺-channel of CFo in higher plants, also abolished the phosphorylation in this alga. Phlorizin, a competitive inhibitor of inorganic phosphate for CF₁, totally diminished the activity. These results suggest that the mechanism of energy transfer by coupling factors (Fo, F₁) of this alga is similar to that in other photosynthetic organisms. AMP, a structural analog of ADP, also inhibited the reaction. AMP might compete with ADP for magnesium ions as well as for the catalytic sites.

Temperature dependence of the cyclic photophosphorylation was determined with the spheroplasts. Fig. 1 clearly shows that the reaction system is thermophilic. Maximum activity (1270 μmol ATP/mg Chl h) was obtained at 55°C which is also an optimum temperature for the cell growth⁹).

By heat treatment for 5 min, the phosphorylation activity of the spheroplasts was reduced steeply between

---

**Table 2** Effect of additives on cyclic photophosphorylation in the spheroplasts.

<table>
<thead>
<tr>
<th>Additives</th>
<th>Concentration</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>DCMU</td>
<td>10 μM</td>
<td>88</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>0.4 μM</td>
<td>11</td>
</tr>
<tr>
<td>PMAA</td>
<td>100 μM</td>
<td>0</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>10 mM</td>
<td>32</td>
</tr>
<tr>
<td>KCl</td>
<td>10 mM</td>
<td>74</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>1 μM</td>
<td>13</td>
</tr>
<tr>
<td>TPT</td>
<td>40 μM</td>
<td>0</td>
</tr>
<tr>
<td>DCCD</td>
<td>100 μM</td>
<td>4</td>
</tr>
<tr>
<td>Phlorizin</td>
<td>5 mM</td>
<td>3</td>
</tr>
<tr>
<td>AMP</td>
<td>5 mM</td>
<td>44</td>
</tr>
<tr>
<td>AMP⁶)</td>
<td>1 mM</td>
<td>47</td>
</tr>
</tbody>
</table>

a) KCl (10 mM) was added. b) ADP concentration was 100 μM, which was employed for the control experiment as well.
65 and 70°C, and the activity was completely lost at 80°C (Fig. 2). Half inactivation temperature was 67°C. It is apparent from the results that the phosphorylation system is thermostable. To gain insight into the mechanism of such thermostability, PS I electron transport activity of the spheroplasts was measured after heat treatment (Fig. 3). PS I was highly heat stable and more than half of the PS I activity was remained even after 80°C treatment, which suggest that PS I may not be responsible for the heat inactivation of the phosphorylation. By the addition of NH₄Cl (10mM), photosynthetic membranes heated at 50°C were uncoupled and PS I electron transport was enhanced by about 44%. The rate of enhancement caused by NH₄Cl decreased as the temperature increased (Fig. 3). By 67°C treatment, PS I activity was increased and the enhancement with NH₄Cl was only 13%. The figure shows that uncoupling of the membranes might proceed by the heat treatment between 60 and 70°C, and that PS I might begin to inactivate above 70°C. No enhancement with NH₄Cl was observed in the spheroplasts treated at 70°C, which expresses that the membranes might become irreversibly uncoupled and leaky for protons by the treatment. Though the enhancement of PS I activity with NH₄Cl was a little more sensitive to heat than the phosphorylation, the curve of the enhancement-temperature was considerably similar to the phosphorylation-temperature curve (Fig. 2 and 3). These results suggest that
uncoupling of the membranes by the heat treatment is closely related to the inactivation of the phosphorylation system. However, there remains a possibility that thermosensitivities of coupling factors might be also related to the decay of the photophosphorylation. To inspect this possibility, characterization of H+-ATPase complex might be necessary and investigations along this line are in progress.

For its thermophilic and thermostable properties, this phosphorylation system might be useful as an ATP regeneration system in applicational fields as well as a tool for studies on molecular mechanism of photophosphorylation.

Acknowledgements

A main part of the experiments was performed in Max-Volmer-Institute, Technical University of Berlin. The author gratefully acknowledges Dr. Günther H. Schatz (Max-Volmer-Institute) for valuable advice, and for critical reading of the manuscript. He wish to express his deep gratitude for the generous gift of the Synechococcus sp. and valuable advice by Prof. S. Katoh (University of Tokyo) as well as the support of this work in the Max-Volmer-Institute by Prof. H. T. Witt. He is grateful to Mr. M. Rogner, Prof. P. Gräber, Prof. G. Renger (Max-Volmer-Institute), Prof. H. Sakurai (Waseda University), Dr. A. Yamagishi and Mr. M. Namba (University of Tokyo) for helpful discussions, Mrs. R. Hachtman for technical assistance. This work was supported in part by a scholarship of the Alexander-von-Humboldt-Stiftung.

References