Biochimica et Biophysica Acta, 502 (1978) 477—485

BBA 47501

PHOTOSYNTHETIC ELECTRON TRANSPORT AND PHOSPHORYLATION REACTIONS IN THYLAKOID MEMBRANES FROM THE BLUE-GREEN ALGA *ANACYSTIS NIDULANS*

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(Received November 14th, 1977)

Summary

Thylakoid membranes were prepared from the blue-green alga, *Anacystis nidulans* with lysozyme treatment and a short period of sonic oscillation. The thylakoid membrane preparation was highly active in the electron transport reactions such as the Hill reactions with ferricyanide and with 2,6-dichlorophenolindophenol, the Mehler reaction mediated by methyl viologen and the system 1 reaction with methyl viologen as an electron acceptor and 2,6-dichlorophenolindophenol and ascorbate as an electron donor system. The Hill reaction with ferricyanide and the system 1 reaction was stimulated by the phosphorylating conditions. The cyclic and non-cyclic phosphorylation was also active.

These findings suggest that the preparation of thylakoid membranes retained the electron transport system from H_2O to reaction center 1, and that the phosphorylation reaction was coupled to the Hill reaction and the system 1 reaction.

Introduction

The photosynthetic activities of the thylakoid membranes prepared from the blue-green algae are known to be more labile than those of the higher plant chloroplasts. Mechanical disruption of the algal cells during the preparation of the membranes specifically destroys some photosynthetic activities. Lee et al. [1] suggested that the membranes prepared from the cells of *Anabaena variabilis* by sonic oscillation lacked a specific factor for the non-cyclic photo-

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCIP, 2,6-dichlorophenolindophenol.

phosphorylation. Media containing high concentrations of polyethyleneglycol [2—4] or carbohydrate [5,6] have often been used while mechanically disrupting the cells. A similar result has been reported also in the membrane preparations obtained by grinding the algal cells [7]. These reagents somehow protect the membranes during the preparation procedure and prevent the deactivation of the electron transport and phosphorylation reactions.

In order to prepare thylakoid membranes that are highly active in the electron transport and phosphorylation reactions, a lysozyme treatment followed by osmotic lysis has been employed. The membrane preparations, thus obtained from *Phormidium luridum* [7,8] and *A. variabilis* [1] show high rates of the Hill reaction as well as the cyclic and the non-cyclic phosphorylation reactions that are comparable to those in spinach chloroplasts.

Gerhardt and Santo [9] and Both [10] prepared membrane fragments from *Anacystis nidulans* by digesting the lyophilized cells with lysozyme. The membrane preparation thus obtained showed only low activities for the electron transport and phosphorylation reactions. Jansz and Maclean [6] treated the cells of *A. nidulans* with lysozyme in the presence of EDTA and prepared cell-free extracts which were highly active in the electron transport and phosphorylation reactions. They found that the activities of the preparations were destructed when the preparations were exposed to the low temperatures [6,11].

In the present study, we describe a method to prepare from *A. nidulans* thylakoid membranes that are highly active in the electron transport and phosphorylation reactions.

Methods

A. nidulans was obtained from the Algal Collection of the Institute of Applied Microbiology, University of Tokyo. The cells were grown at 28°C in Kratz and Myers' C medium [12] bubbled with air enriched with 1% CO_2. The culture at a late logarithmic phase was harvested. The algal cells were washed with 30 mM sodium potassium phosphate buffer, pH 7.0, and then with a medium containing 600 mM sucrose, 30 mM sodium potassium phosphate buffer, pH 6.8. The cells were suspended in 2 mM EDTA, 600 mM sucrose, 30 mM sodium potassium phosphate buffer, pH 6.8, and 0.1% lysozyme (Sigma Chemical Co., grade 1, 44 660 units/mg protein). The suspension was kept stirring at 30°C for 4 h in the room light. The lysozyme-treated cells were washed with a medium containing 600 mM sucrose, 5 mM $MgCl_2$ and 30 mM sodium potassium phosphate buffer, pH 6.8, and then suspended in the same medium. All of the above-mentioned procedures were performed at room temperatures. The lysozyme-treated cells when observed with a light-microscope did not form spheroplasts, but still maintained the original shape of the untreated cells. They could not be osmotically lysed. The lysozyme-treated cells were disrupted with sonic oscillation (Toyoriko, 2N-100, 100 W) at a maximum power for 4 s at 20°C. The homogenate was immediately chilled to 0°C and following procedures were performed at this temperature. After centrifugation at 8000 × *g* for 15 min to remove unbroken cells, the thylakoid membranes were collected by centrifugation at 80 000 × *g* for 60 min. The

sedimented membranes were suspended in a small amount of medium containing 600 mM sucrose, 5 mM $MgCl_2$, 10 mM NaCl, 15 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)/NaOH buffer, pH 7.0, except where otherwise noted, and used for assay of photosynthetic activities. In most cases, the membrane preparations were stored overnight at 0°C in the dark and then used for the experiments. The concentration of chlorophyll *a* was determined according to the method of MacKinney [13].

The Hill reactions with ferricyanide, DCIP and NADP were spectrophotometrically measured by following absorbance changes at 420, 600 and 340 nm, respectively, due to the reduction of these compounds under illumination with red actinic light with wavelengths from 640 to 750 nm and an intensity of $3.5 \cdot 10^6$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. The light-induced oxidation of added horse heart cytochrome *c* (Fe^{2+}) by system 1 reaction was also spectrophotometrically measured by following absorbance decrease at 550 nm due to the oxidation of the cytochrome under the red actinic illumination. Extinction coefficients of 1.0 mM^{-1} $\cdot$ cm^{-1} at 420 nm for ferricyanide, 20.9 mM^{-1} $\cdot$ cm^{-1} at 600 nm at pH 7.0 for DCIP [14], 6.3 mM^{-1} $\cdot$ cm^{-1} at 340 nm for NADP and 20.7 mM^{-1} $\cdot$ cm^{-1} at 550 nm for cytochrome *c* [15] were used in calculating rates of the reactions.

Oxygen exchange was measured by a calibrated Clark-type oxygen electrode (Yellow Springs Instruments) in the Hill reaction with ferricyanide, the Mehler reaction mediated by methyl viologen and the system 1 reaction with DCIP and ascorbate as an electron donor system and methyl viologen as an electron acceptor. White light with intensity $4.5 \cdot 10^6$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} was used for the actinic light.

Activities of cyclic and non-cyclic photophosphorylation were measured by using radioactive phosphorus according to the method of Avron [16]. The basal reaction mixture contained 10 mM NaCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 3 mM ADP, 600 mM sucrose and 50 mM Tricine/NaOH buffer, pH 7.5. The reaction was performed under aerobic conditions for 30 s under illumination with white or red light described in the above.

All measurements of electron transport and phosphorylation reactions were carried out at 25°C.

For the electron micrographs the samples were treated as follows. A pellet of the algal cells was fixed with 4% glutaraldehyde in 100 mM sodium potassium phosphate buffer, pH 7.4, for 75 min, washed by deionized water, and then fixed with 2% potassium permanganate for 3 h. Pellets of the lysozyme-treated cells and the membrane preparations were fixed with 3% glutaraldehyde in 600 mM sucrose and 100 mM sodium potassium phosphate buffer, pH 7.4, for 5 h, washed and then fixed with 2% OsO_4 for 7 h in 100 mM sodium potassium phosphate buffer, pH 7.4. The fixed samples were dehydrated with ethanol which was then replaced by propylene oxide, and were embedded in a resin mixture (Epon 812 and DER 736) [17]. Ultrathin sections were stained with 2% aqueous uranyl acetate and Reynolds solution [18], then inspected with Hitachi HL-12A electron microscope.

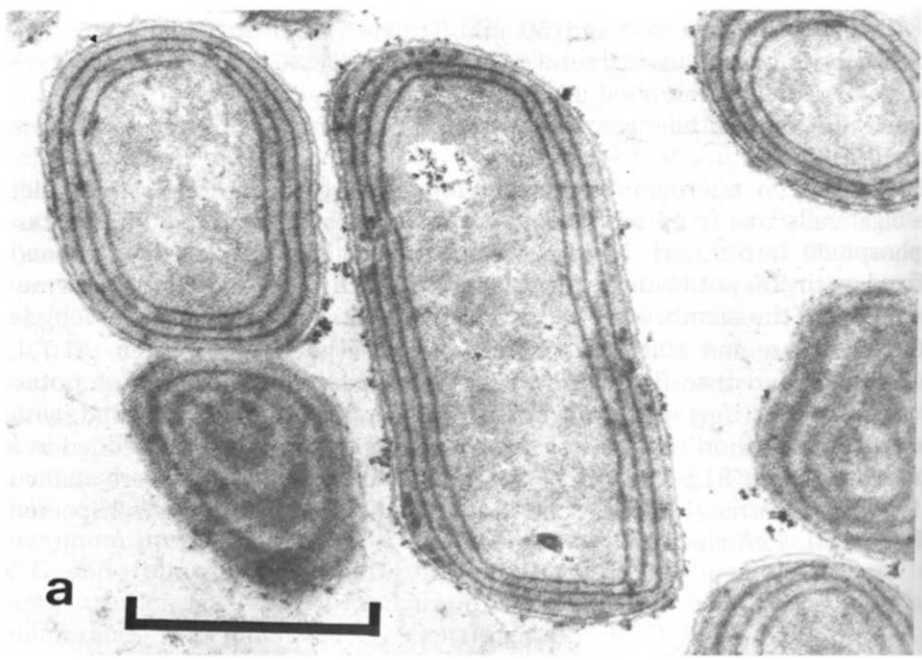
Results

Fig. 1 shows electron micrographs of the untreated cells, the lysozyme-treated cells and the thylakoid membrane preparation of *A. nidulans*. The cell wall was apparently digested by the lysozyme treatment. The thylakoid membranes prepared by this method still preserved the lamellar structures as the thylakoid membranes in the lysozyme-treated cells did. The absorption spectrum of the thylakoid membrane preparation showed major peaks at 437 and 678 nm and a minor one at 625 nm. This suggests that most of the phycobilins had been removed from the membrane preparation.

Table I shows activities of the electron transport reactions in the thylakoid membrane preparation. The activity of the Hill reaction with ferricyanide measured by the ferricyanide reduction was as high as 1000 electron μequiv. · (mg chlorophyll)$^{-1}$ · h^{-1}. A similar value was observed for the activity of the Hill reaction in the polarographic method to measure O_2 evolution. The Hill reaction was completely inhibited by DCMU.

Fig. 2 shows the effects of salts on stimulating the Hill reaction with ferricyanide. The activity of the Hill reaction was doubled at 20 mM $MgCl_2$ and $CaCl_2$ (the optimum concentration), while NaCl required 200 mM to yield a maximum effect. Similar effects of salts have been observed in the membrane preparations of *A. nidulans* [6,19] and *A. variabilis* [3].

Fig. 3 shows a curve of the pH dependence of the Hill reaction with ferricyanide. It can be noted that a sharp peak appeared at pH 7.8. These results are in a clear contrast to those reported for the membrane preparations of blue-green algae by other research groups, where the pH dependence curves were rather flat and did not show a distinct peak [1,6].



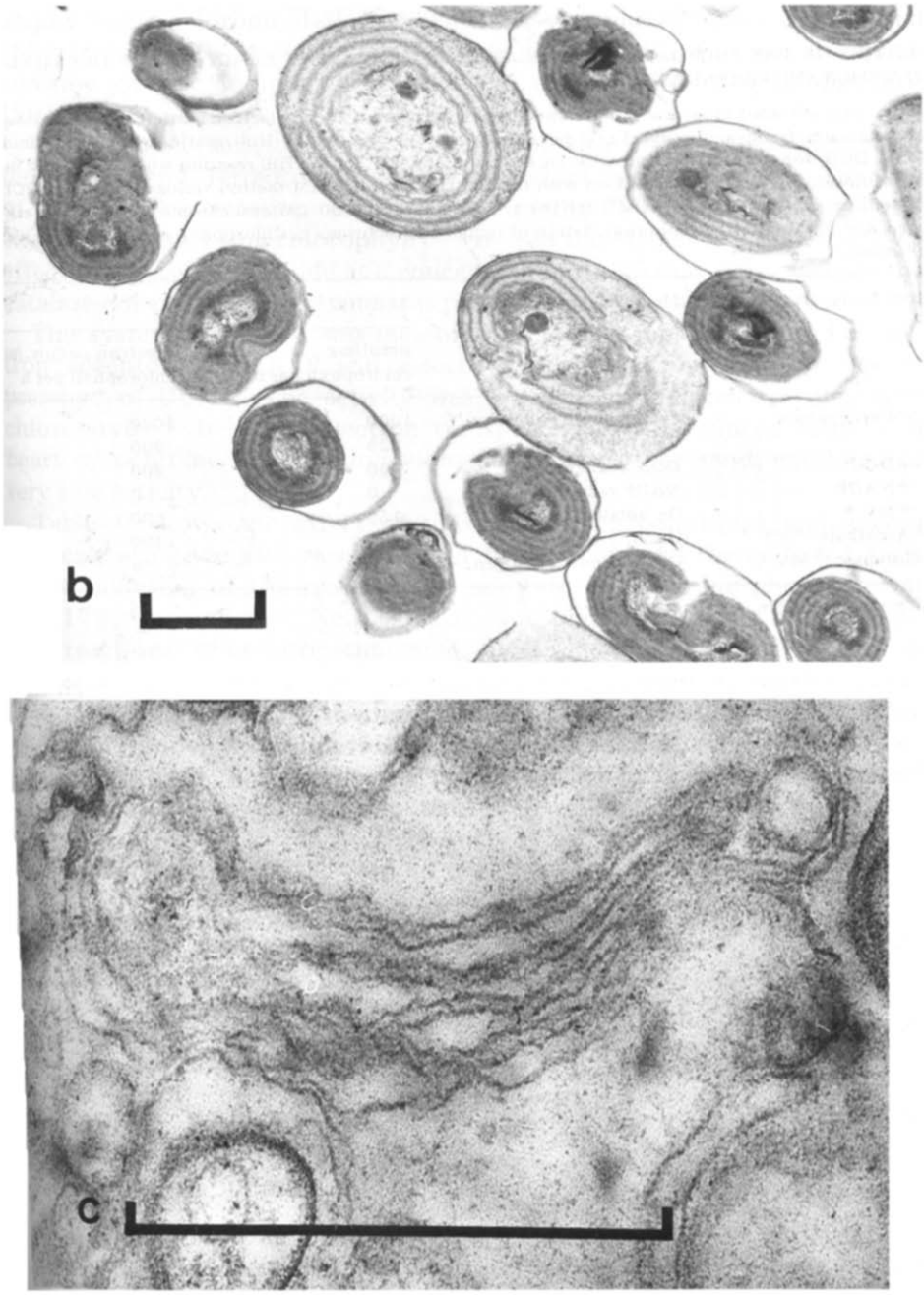


Fig. 1. Electron micrographs of untreated cells, lysozyme-treated cells and prepared thylakoid membranes of *A. nidulans*. (a) Untreated cells, (b) Lysozyme-treated cells, (c) Prepared thylakoid membranes. The bars represent 1 μm.

The light intensity dependence of the Hill reaction with ferricyanide was also studied. The reaction rate saturated at about $3 \cdot 10^6$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. Phycobilins that are major light-harvesting pigments of system 2 were depleted

TABLE I

ACTIVITIES OF THE PHOTOSYNTHETIC ELECTRON TRANSPORT REACTIONS IN THE THYLAKOID MEMBRANE PREPARATIONS

Activities were averaged in several experiments. Concentrations of added electron carriers were as follows: 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide for the Hill reaction with ferricyanide. 0.08 mM DCIP for the Hill reaction with DCIP. 0.3 mM NADP for the Hill reaction with NADP. 0.2 mM methyl viologen for the Mehler reaction with methyl viologen. 0.2 mM methyl viologen, 0.1 mM DCIP, 2 mM sodium ascorbate, 5 μM DCMU for the system 1 reaction. 30 μM cytochrome *c*, 0.2 mM methyl viologen and 5 μM DCMU for photooxidation of reduced cytochrome *c*. Chlorophyll concentrations were 3—10 μg/ml.

Electron transport	Measurement	Activity	
		μmol/mg chlorophyll per h	Electron μequiv./mg chlorophyll per h
$H_2O \rightarrow$ Ferricyanide	Ferricyanide reduction	1000	1000
	O_2 evolution	225	900
$H_2O \rightarrow$ DCIP	DCIP reduction	400	800
$H_2O \rightarrow$ NADP	NADP reduction	0	0
$H_2O \rightarrow$ MV *	O_2 uptake	150	600
DCIP-Ascorbate $\rightarrow$ MV *	O_2 uptake	1400	1400
Cytochrome *c* $\rightarrow$ MV *	Cytochrome oxidation	20	5

* MV; methyl viologen.

in the membrane preparation. This would be a reason why such a high light intensity was needed to obtain the maximum rate of electron transport.

The activities of other electron transport reactions were also measured (Table I). The Hill reaction with DCIP had an activity as high as 800 electron

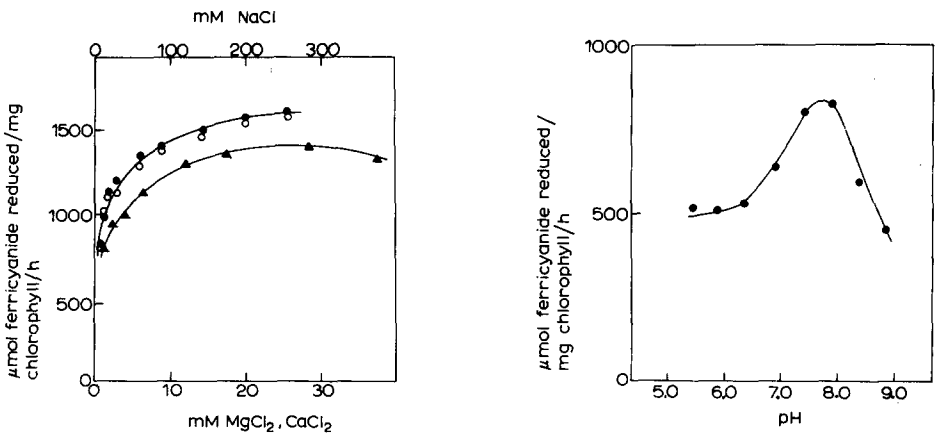


Fig. 2. Effects of salts on the Hill reaction with ferricyanide. The reaction mixture contained 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 14 μM gramicidin J, 600 mM sucrose, 10 mM NaCl, 15 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid/NaOH, pH 7.0, and indicated concentrations of salts. Chlorophyll *a* concentration was 3.8 μg/ml. The reaction was spectroscopically measured by following the reduction of ferricyanide. ○———○, $MgCl_2$; ●———●, $CaCl_2$; ▲———▲, NaCl.

Fig. 3. pH dependence of the Hill reaction with ferricyanide. Experiments were performed in 15 mM 2-(*N*-morpholino)ethane sulfonic acid/NaOH buffer below pH 6.3, in 15 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid/NaOH buffer from pH 6.9 to 7.9 and in 15 mM *N*-tris(hydroxymethyl)methylglycine/NaOH buffer above pH 8.4. Chlorophyll *a* concentration was 5.9 μg/ml. The reaction was spectrophotometrically measured by following the reduction of ferricyanide.

μequiv. · (mg chlorophyll)$^{-1}$ · h^{-1}. This rate was slightly lower than that of the Hill reaction with ferricyanide. The reduction of DCIP was not stimulated by addition of 1 mM diphenylcarbazide, an electron donor of photoreaction 2 [20].

There was no activity of the Hill reaction with NADP, unless factors such as ferredoxin and ferredoxin-NADP oxidoreductase were added. The activity of the Mehler reaction mediated by methyl viologen amounted to 600 electron μequiv. · (mg chlorophyll)$^{-1}$ · h^{-1}. In the Mehler reaction, there was no effect of potassium cyanide at a concentration of 2.5 mM. This suggests that no catalase nor peroxidase activity was present in the preparation.

The system 1 reaction was measured by adding methyl viologen as an electron acceptor, and DCIP and ascorbate as an electron donor system in the presence of DCMU. The activity was as high as 1400 electron μequiv. · (mg chlorophyll)$^{-1}$ · h^{-1}. The electron transport from the reduced form of horse heart cytochrome *c* to methyl viologen, on the other hand, exhibited only a very low activity.

Table II shows the effect of phosphorylating conditions and uncouplers in accelerating the Hill reaction with ferricyanide and the system 1 reaction. The stimulation of the reactions on addition of ADP and phosphate was 43 and 17%, respectively. Neither ADP nor phosphate alone had any effect. The reactions were also stimulated by gramicidin J and NH_4Cl, that are uncouplers of phosphorylation reactions. These findings suggest that the electron transport by the Hill reaction and the system 1 reaction are coupled to the phosphorylation reaction in this membrane preparation.

Table III shows the activities of cyclic and non-cyclic photophosphorylation reactions in the thylakoid membrane preparations. The rate of cyclic photo-

TABLE II

EFFECT OF ADP, PHOSPHATE AND UNCOUPLERS ON THE HILL REACTION WITH FERRICYANIDE AND SYSTEM I REACTION

Experimental conditions were the same as in Table I. The membranes were prepared in a medium containing 15 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid/NaOH buffer in place of phosphate buffer. Chlorophyll concentration was 3.4 μg/ml.

Addition	Electron μequiv./ mg chlorophyll per h	Relative activity
$H_2O \rightarrow$ Ferricyanide		
No addition	930	(1.00)
5 mM K_2HPO_4	870	0.94
2 mM ADP	940	1.10
5 mM K_2HPO_4 + 2 mM ADP	1330	1.43
20 mM NH_4Cl	1140	1.22
10 μM gramicidin J	1900	2.04
0.12 μM CCCP	950	1.02
DCIP and ascorbate $\rightarrow$ Methyl viologen		
No addition	1280	(1.00)
5 mM K_2HPO_4	1290	1.01
2 mM ADP	1240	0.97
5 mM K_2HPO_4 + 2 mM ADP	1500	1.17
20 mM NH_4Cl	1740	1.36
20 μM gramidicin J	1780	1.39

TABLE III

ACTIVITIES OF CYCLIC AND NON-CYCLIC PHOTOPHOSPHORYLATION REACTIONS

3 mM ADP and 5 mM K_2HPO_4, including ^{32}P at radioactivity of 6.6 μCi/ml, were added in the reaction media. In addition, 0.03 mM *N*-methylphenazonium methylsulfate, 0.026 mM DCIP and 20 mM sodium ascorbate were added for the cyclic phosphorylation. The concentrations of electron carriers for the non-cyclic electron transport were the same as in Table I. Chlorophyll *a* concentration was 13.6 μg/ml.

Electron transport	μmol ATP formed/mg chlorophyll per h
Cyclic phosphorylation	
Complete	840
Minus ascorbate	510
Non-cyclic phosphorylation	
$H_2O \rightarrow$ Ferricyanide	350
$H_2O \rightarrow$ DCIP	260
DCIP + ascorbate $\rightarrow$ Methyl viologen	250

phosphorylation mediated by *N*-methylphenazonium methylsulfate and ascorbate attained 840 μmol ATP formed · (mg chlorophyll)$^{-1}$ · h^{-1}. Ascorbate stimulated this reaction, as in the cases of other membrane preparations of blue-green algae [6,8]. The non-cyclic phosphorylation reactions coupled to the Hill reactions with ferricyanide and with DCIP and the system 1 reaction mediated by DCIP, ascorbate and methyl viologen were active, while the activities were lower than that of the cyclic phosphorylation.

Discussion

The thylakoid membranes prepared from *A. nidulans* by the lysozyme treatment in the presence of EDTA and a short period of sonic oscillation still maintained lamellar structures such as seen in the living cells. This may suggest that intactness was preserved in this preparation of thylakoid membranes. However, the membrane was devoid of the phycobilins.

The membrane preparations were highly active in the photosynthetic electron transport and phosphorylation reactions. These findings suggest that the electron transport and phosphorylation systems are also well preserved in the membrane preparation. The fact that the Hill reaction with DCIP was not stimulated by diphenylcarbazide, an electron donor to photoreaction 2, indicates that the oxygen-evolving site of electron transport was undamaged. The high activity of the Mehler reaction with methyl viologen is evidence that the electron transport components between photoreactions 2 and 1 were still in position. On the other hand, the membrane was inactive in the Hill reaction with NADP. Probably ferredoxin and/or ferredoxin-NADP oxidoreductase must have been lost during the preparation of the membranes. The presence of the photosynthetic control of the electron transport by the phosphorylating condition as well as the high rate of non-cyclic phosphorylation suggest a good preservation of integrity of the phosphorylation system also.

Binder et al. [8] prepared thylakoid membranes from *Ph. luridum* by a lysozyme treatment followed by osmotic lysis. The activities of photosynthetic electron transport and phosphorylation reactions were as high as those in the membrane preparation of *A. nidulans* in the present study.

Acknowledgments

The authors thank Dr. Y. Fujita of the Ocean Research Institute, University of Tokyo for valuable advice concerning the membrane preparations and Dr. H. Sakurai of School of Education, Waseda University and Dr. Y. Nakamura of the Isotope Center, University of Tokyo for providing the experimental facilities to use radio-isotopes. They are grateful to Dr. T. Hori of Department of Biology, Toho University for his kind technical guidance for the electron microscope. They are indebted to Mr. N. Sato in the same laboratory for taking the electron micrographs.

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