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Electron-transport reactions in cytoplasmic and thylakoid membranes prepared from the cyanobacteria (blue-green algae) *Anacystis nidulans* and *Synechocystis* PCC 6714

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Cytoplasmic and thylakoid membranes prepared from the cyanobacteria *Anacystis nidulans* and *Synechocystis* PCC 6714 were compared for their electron-transport activities. High cytochrome oxidase activity, which was sensitive to cyanide and azide, was found only in the thylakoid membranes of both strains. Activities of NAD(P)H-cytochrome *c* and succinate-cytochrome *c* oxidoreductase were low in both membranes from both strains. The NADH-cytochrome *c* oxidoreductase activity of the cytoplasmic membranes from *Synechocystis* was markedly stimulated by quinones, among which 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was the most effective. High NADH-DBMIB oxidoreductase activity, which was relatively resistant to salt washing, was found in the cytoplasmic membranes from *Synechocystis*.

Introduction

Cyanobacteria perform aerobic respiration in the dark [1–3]. There are contradicting views on the localization of the respiratory activity. Some investigators claim that the thylakoid membranes are the sites of respiratory and also photosynthetic electron-transport reactions [4–7]. Lockau and Pfeffer [8,9] did not detect membrane-bound cytochromes nor observe cytochrome oxidase activity in the cytoplasmic membranes from *Anabaena variabilis*, and inferred that there is no electron-transport system in the cytoplasmic membranes. Using a cytochemical technique, Peschek et al. [10] revealed a reductive activity in the dark in both cytoplasmic and thylakoid membranes of *A. nidulans*. They detected cytochrome oxidase activity not only in the total membrane fraction, but also in intact spheroplasts of the cyanobacterium, and suggested that both cytoplasmic and thylakoid membranes have cytochrome oxidase activity [11].

An active transport system for the uptake of D-glucose has been observed in some cyanobacteria capable of chemoheterotrophic growth, such as *Plectonema boryanum* (PCC 73110) [12], *Synechocystis* PCC 6714 [13] and *Nostoc* strain MAC [13]. Raboy and Padan [12] suggested that this uptake is driven by a proton electrochemical gradient across the cytoplasmic membrane which is produced through electron transport reactions within the cytoplasmic membrane. However, an oxygen-dependent proton efflux in the dark in *A.*

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino}ethanesulphonic acid.

variabilis [14] has been attributed to a proton-translocating ATPase rather than to a respiratory electron transport on the cytoplasmic membrane [15].

In previous studies [16,17], we isolated the cytoplasmic and thylakoid membranes from *A. nidulans* [16] and *Synechocystis* PCC 6714 [17]. The cytoplasmic membranes from *A. nidulans* contained cytochromes and plastoquinone-9 [18]. This may suggest that an electron-transport system exists in the cytoplasmic membranes of *A. nidulans*. This paper describes the activities of electron-transport reactions in the cytoplasmic and thylakoid membranes from *A. nidulans* and *Synechocystis* PCC 6714.

Materials and Methods

Preparation of cytoplasmic and thylakoid membranes and a soluble fraction

A. nidulans and *Synechocystis* PCC 6714 were grown as described previously [16,17]. Cytoplasmic and thylakoid membranes were prepared from these algal cells by a modification of the previously described method [16,17]. The cells amounting to 5 ml packed volume were suspended in 50 ml 600 mM sucrose/2 mM EDTA/5 mM Tes-NaOH buffer (pH 7.0), and incubated with 10 mg lysozyme (final, 0.02%) at 30°C for 2 h. The lysozyme-treated cells were collected by centrifugation at $3500 \times g$ for 5 min, washed with 600 mM sucrose and 20 mM Tes-NaOH buffer (pH 7.0) by resuspension and recentrifugation, and finally suspended in 30 ml of the same buffer. The cells were disrupted by passage through a French pressure cell at 40 MPa. After addition of 0.0001% (w/v) DNAase I (Sigma Chemical Co., DN-EP) and 1 mM phenylmethylsulfonyl fluoride, the homogenate was centrifuged at $5000 \times g$ for 10 min to remove unbroken cells. The supernatant was brought to a sucrose concentration of 46% (w/v) by adding 0.58 volume of the 90% (w/v) sucrose solution. An 8-ml aliquot was placed at the bottom of a centrifuge tube having an internal volume of 28 ml, overlaid with 9 ml 42%, 3 ml 30%, and 6 ml 10% sucrose solutions (w/v), and centrifuged at $300\,000 \times g$ for 4 h at 4°C in an angle rotor (Hitachi RP50T-2). All the sucrose solutions were prepared with 10 mM Tes-NaOH buffer (pH 7.0)/10 mM NaCl/5 mM EDTA. When the membranes were prepared to assay NAD(P)H-cytochrome *c*, succinate-cytochrome *c*, and NAD(P)H-DBMIB oxidoreductase activities, EDTA was omitted from the sucrose solutions. The cytoplasmic membranes formed a band in the 30% sucrose layer in the case of *A. nidulans*, or at the interface between the 10% and 30% sucrose layers in the case of *Synechocystis* PCC 6714. The thylakoid membranes remained in the 46% sucrose layer. The unbroken cells and cell walls were pelleted at the bottom. The cytoplasmic membranes and the thylakoid membranes were withdrawn from the gradient and collected by centrifugation at $300\,000 \times g$ for 1 h after three-fold dilution with 10 mM NaCl and 10 mM Tes-NaOH buffer (pH 7.0). The supernatant from the thylakoid fraction (the 46% sucrose layer) was used as the soluble fraction. In some experiments, the membranes were further washed with NaCl to dissociate water-soluble proteins by resuspension in 450 mM sucrose/400 mM NaCl/10 mM Tes-NaOH buffer (pH 7.0) and centrifugal collection at $350\,000 \times g$ for 30 min. They were then washed by resuspension in 450 mM sucrose/10 mM NaCl/10 mM Tes-NaOH buffer (pH 7.0), and collected by centrifugation as described above. They were finally suspended in 600 mM sucrose/10 mM NaCl/20 mM Tes-NaOH buffer (pH 7.0), and stored at −196°C in liquid nitrogen.

Assay of enzyme activities

Cytochrome oxidase activity was assayed using horse heart cytochrome *c* (Sigma, type VI) [19,20]. The reaction mixture was 50 mM Tes-NaOH buffer (pH 7.0)/10 mM NaCl/0.1 mM EDTA/25 μM reduced cytochrome *c* and membranes amounting to 150–250 $\mu g \cdot protein \cdot ml^{-1}$ in the case of *A. nidulans*, or 30–60 $\mu g \cdot protein \cdot ml^{-1}$ in the case of *Synechocystis* PCC 6714. The reaction was assayed in the presence or absence of 0.03% SDS. The reaction was started by adding the concentrated suspension of the membranes to the medium containing reduced cytochrome *c*, and the initial rate of cytochrome *c* oxidation was measured by following the absorbance change at 550 nm with 540 nm as a reference beam using a spectrophotometer (Shimadzu, UV300) operated in the dual-wavelength mode. In the presence of SDS, a slow

oxidation of the cytochrome *c* was observed without the membranes, and this was subtracted from the apparent changes observed in the presence of membranes; the non-enzymatic slow oxidation was about 10% of the reaction catalyzed by the thylakoid membranes. A reduced-minus-oxidized difference absorption coefficient of 18.8 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c* [19] was used when the reaction rate was calculated.

For assay of NAD(P)H-cytochrome *c* oxidoreductase activity, the reaction mixture contained 50 mM Tes-NaOH buffer (pH 7.5)/100 μM KCN/50 μM oxidized cytochrome *c* (Sigma, type VI)/100 μM NAD(P)H and membranes amounting to 30–50 μg protein · ml^{-1}. The reaction was started by adding NAD(P)H to the membrane suspension containing oxidized cytochrome *c*. The activity was measured by spectrophotometrically following the cytochrome *c* reduction as described above. Succinate-cytochrome *c* oxidoreductase activity was assayed as for NAD(P)H-cytochrome *c* oxidoreductase activity except that 100 μM NAD(P)H was replaced by 20 mM succinate.

NAD(P)H-DBMIB oxidoreductase activity was assayed in the reaction mixture containing 50 mM Tes-NaOH buffer (pH 7.5)/150 μM NAD(P)H/50 μM DBMIB. The reaction was started by adding a concentrated suspension of the membranes to a final concentration of 20–30 μg protein · ml^{-1} or a solution of the soluble fraction to a final concentration of 4 μg protein · ml^{-1}. The oxidation of NAD(P)H was measured by following the absorbance change at 340 nm using a reduced-minus-oxidized difference absorption coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$ using the spectrophotometer operated in the single-wavelength mode.

All the reactions were measured at 20°C. Protein concentration was determined as described previously [16].

Results

Low activity of cytochrome oxidase was detected in the thylakoid membranes, but none in the cytoplasmic membranes, prepared from *A. nidulans* (Table I). As previously observed by Tang and Krogmann [19] with *A. variabilis*, a low concentration of SDS markedly activated the reaction in the thylakoid membranes, but not at all in the cytoplasmic membranes (Table I). These observations suggest that the cytochrome oxidase activity is localized in the thylakoid membranes, but not in the cytoplasmic membranes. In *Synechocystis* PCC 6714, low activity of cytochrome oxidase was found in the cytoplasmic membranes, but it disappeared on addition of SDS. Cytochrome oxidase activity in the thylakoid membranes was substantially high in the absence of SDS, and was accelerated by

TABLE I

ACTIVITIES OF ELECTRON-TRANSPORT REACTIONS IN CYTOPLASMIC AND THYLAKOID MEMBRANES FROM *A. NIDULANS* AND *SYNECHOCYSTIS* PCC 6714

Activities were assayed by following spectrophotometrically cytochrome *c* oxidation for cytochrome oxidase, NADH and NADPH oxidations for NADH and NADPH oxidases, or cytochrome *c* reduction for NAD(P)H-cytochrome *c* and succinate-cytochrome *c* oxidoreductases. SDS when added, 0.03%.

Reaction	Activity (e^- nequiv. per mg protein per min)							
	A. nidulans				*Synechocystis*			
	cytoplasmic membranes		thylakoid membranes		cytoplasmic membranes		thylakoid membranes	
	without SDS	with SDS	without SDS	with SDS	without SDS	with SDS	without SDS	with SDS
Cytochrome *c* → O_2	0	0	4	39	7	0	52	68
NADH → O_2	0	–	0	–	0	–	0	–
NADPH → O_2	0	–	0	–	0	–	0	–
NADH → cytochrome *c*	1	0	4	0	5	0	2	–
NADPH → cytochrome *c*	1	0	5	3	0	–	1	–
Succinate → cytochrome *c*	0	0	0	0	0	–	1	–

SDS. As a result, the activity in the presence of SDS was high in the thylakoid membranes from both strains of cyanobacteria. The localization of the cytochrome oxidase activity in the thylakoid membranes discovered in *A. nidulans* and *Synechocystis* PCC 6714 is similar to the observation by Lockau and Pfeffer [8] in *A. variabilis.*

Fig. 1 shows that the cytochrome oxidase activity of the thylakoid membranes was inhibited by low concentrations of cyanide and azide as previously observed by Lockau [5] and Kienzl and Peschek [20]. In the case of the thylakoid membranes of *A. nidulans,* 50% inhibition occurred at 3 μM KCN and 6 mM NaN_3 in the presence of 0.03% SDS (Fig. 1A). The cytochrome oxidase activity in the absence of SDS was also sensitive to cyanide and azide with pI_{50} of 0.7 μM for cyanide and 5 mM for azide. The cytochrome oxidase activity of the thylakoid membranes of *Synechocystis* PCC 6714 showed pI_{50} of 1 μM for cyanide and 1 mM for azide in the absence of SDS (Fig. 1B).

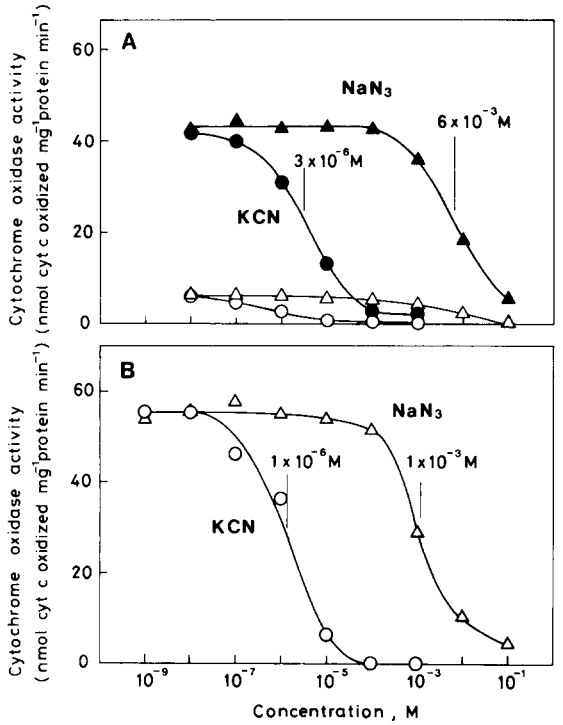

Fig. 1. Inhibition of cytochrome oxidase activity of thylakoid membranes from *A. nidulans* (A) and *Synechocystis* PCC 6714 (B) by cyanide (circles) and azide (triangles). The activity was assayed in the presence of 0.03% SDS (closed symbols) or in its absence (open symbols); SDS-induced non-enzymatic oxidation of cytochrome *c* has been subtracted in the data.

No NADH and NADPH oxidase activities were detected in either type of membrane from both strains of cyanobacteria (Table I). This contrasts with some previous reports [7,21,22] which showed substantial NADH or NADPH oxidase activities in total membrane fractions from some cyanobacterial strains. The activities in our membranes might have been lost during their intensive purification.

The cytoplasmic and thylakoid membranes were also compared with regard to their NADH-, NADPH- and succinate-cytochrome *c* oxidoreductase activities (Table I). In *A. nidulans,* NADH-cytochrome *c* and NADPH-cytochrome *c* oxidoreductase activities were low in both types of membranes, although they were higher in the thylakoid membranes. No succinate-cytochrome *c* oxidoreductase activity was detected in either membrane. In *Synechocystis* PCC 6714, NADH-cytochrome *c*, NADPH-cytochrome *c* and succinate-cytochrome *c* oxidoreductase activities were all low, with the NADH-cytochrome *c* oxidoreductase activity in the cytoplasmic membranes being the highest among them.

The NAD(P)H-cytochrome *c* oxidoreductase activities in the cytoplasmic membranes from *Synechocystis* PCC 6714 were greatly enhanced by DBMIB (Table II). This effect was more pronounced for the NADH-cytochrome *c* than the NADPH-cytochrome *c* oxidoreductase activity. Increasing the cytochrome *c* concentration from 50 to 100 μM had no effect on the reaction rates, indicating that the reduction of cytochrome *c* by reduced DBMIB is not the rate-limiting step. The rate of non-enzymatic reduction of cytochrome *c* by NADH via DBMIB was very low compared with the rate of the reaction catalyzed by the membranes (about 5%). The NADH-cytochrome *c* oxidoreductase activity in the presence of DBMIB was not inhibited by 100 μM rotenone. The NAD(P)H-cytochrome *c* oxidoreductase activities in the thylakoid membranes from *Synechocystis,* on the other hand, were very low even in the presence of 25 μM DBMIB (Table II). Other kinds of quinones also enhanced the NADH-cytochrome *c* oxidoreductase activity of cytoplasmic mem-

TABLE II

EFFECTS OF DBMIB ON NAD(P)H-CYTOCHROME *c* OXIDOREDUCTASE ACTIVITIES OF CYTOPLASMIC AND THYLAKOID MEMBRANES FROM *SYNECHOCYSTIS* PCC 6714

Activities were measured by spectrophotometrically following cytochrome *c* reduction. NAD(P)H, 150 μM; cytochrome *c*, 50 μM.

DBMIB (μM)	Activity (e^- nequiv. per mg protein per min)			
	NADH → cytochrome *c*		NADPH → cytochrome *c*	
	cytoplasmic membranes	thylakoid membranes	cytoplasmic membranes	thylakoid membranes
0	5	2	0	1
2	194	2	38	4
12	272	8	51	7
25	300	11	63	9

branes from *Synechocystis* PCC 6714, but less markedly if compared with the case of DBMIB (Table III).

The NADH-cytochrome *c* oxidoreductase activity mediated by DBMIB of the cytoplasmic membranes from *Synechocystis* PCC 6714 was further characterized by its K_m values for NADH and DBMIB. Fig. 2 shows its dependence on DBMIB concentration when NADH was at 150 μM. The reaction rate was almost saturated at about 10 μM DBMIB. The double reciprocal plot (Fig. 2, inset) gave a straight line from which the V_{max} of the

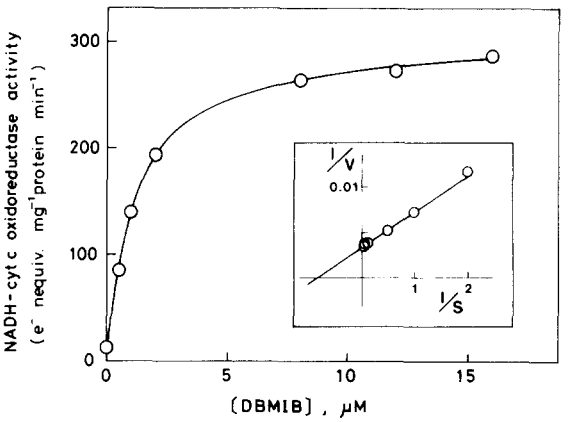

Fig. 2. Dependence of NADH-cytochrome *c* oxidoreductase activity of cytoplasmic membranes from *Synechocystis* PCC 6714 on DBMIB concentration. Activity was assayed spectrophotometrically by following cytochrome *c* reduction. NADH, 150 μM; cytochrome *c*, 50 μM. Inset, double reciprocal plot.

reaction and the apparent K_m value for DBMIB were calculated to be 310 e^- nequiv. per mg protein per min and 1.2 μM, respectively. Fig. 3 shows

the dependence on NADH concentration when DBMIB was at 12 μM. From the double reciprocal plot (Fig. 3, inset), the V_{max} of the reaction and the K_m value for NADH were calculated to be 380 e^- nequiv. per mg protein per min and 48 μM, respectively. This K_m value for NADH is much

TABLE III

EFFECTS OF QUINONES ON NADH-CYTOCHROME *c* OXIDOREDUCTASE ACTIVITY OF CYTOPLASMIC MEMBRANES FROM *SYNECHOCYSTIS* PCC 6714

Activities were measured by following cytochrome *c* reduction spectrophotometrically. NADH, 150 μM; cytochrome *c*, 50 μM; quinone, 2 μM.

Quinone	Activity (e^- nequiv. per mg protein per min)
None	5
1,4-Benzoquinone	16
Phenyl-1,4-benzoquinone	24
1,4-Naphthoquinone	54
2-Methyl-1,4-naphthoquinone	54
DBMIB	194

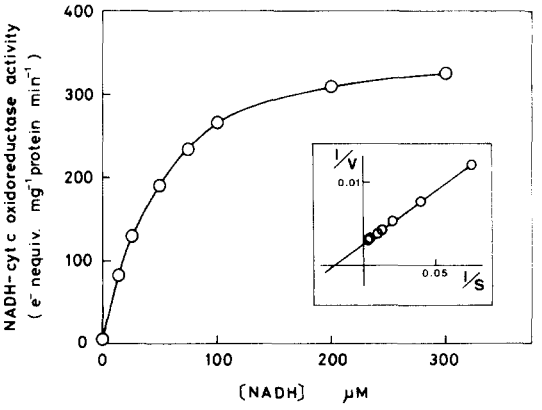

Fig. 3. Dependence of NADH-cytochrome *c* oxidoreductase activity of cytoplasmic membranes from *Synechocystis* PCC 6714 on NADH concentration. Activity was assayed as in Fig. 2. DBMIB, 12 μM; cytochrome *c*, 50 μM. Inset, double reciprocal plot.

lower than that (220 μM) reported by Sandmann and Malkin [23] for the partially purified NAD(P)H dehydrogenase prepared from the total membrane fraction from *Synechocystis* PCC 6714.

NADH and NADPH were found to be oxidized in high rates in the presence of DBMIB without cytochrome *c*; these are referred to below as NAD(P)H-DBMIB oxidoreductase activities. Both types of membranes and the soluble fractions from both strains of cyanobacteria were compared with respect to these activities (Table IV). In *A. nidulans*, the cytoplasmic and thylakoid membranes were almost equally active in both NADH-DBMIB and NADPH-DBMIB oxidoreductase activities, although the latter activity was twice as high as the former in both types of membranes. Most of the activities in the membranes were lost after washing with 400 mM NaCl. This observation suggests that the activities in the membranes were due to loosely bound water-soluble enzymes.

The cytoplasmic membranes from *Synechocystis* PCC 6714 were unique in their significantly high NADH-DBMIB oxidoreductase activity (Table IV). Their NADPH-DBMIB oxidoreductase activity was much lower. Thylakoid membranes from *Synechocystis* PCC 6714 were relatively inactive in both reactions. About 50% of the NADH-DBMIB oxidoreductase activity of the cytoplasmic membranes remained after the NaCl washing. Triton X-100 at 0.03% enhanced the NADH-DBMIB oxidoreductase activity of the cytoplasmic membranes by 30–50%, but inhibited the same activity of the thylakoid membranes by about 50% (data not shown). This last observation may suggest that the enzyme of this reaction in the cytoplasmic membranes differs from that in the thylakoid membranes. In the soluble fraction, in contrast, NADPH-DBMIB oxidoreductase activity was four times as high in *Synechocystis* and twice as high in *A. nidulans* as NADH-DBMIB oxidoreductase activity.

Discussion

Cytochrome oxidase activity measured in the presence and absence of SDS was found in the thylakoid membranes, but not in the cytoplasmic membranes, from *A. nidulans*. This agrees with the results obtained by Lockau and pfeffer [8] for *A. variabilis*, but not with the observation by Peschek et al. [11] for *A. nidulans*. Peschek et al. [11] detected cytochrome oxidase activity in spheroplasts of the cyanobacterium, and inferred that both cytoplasmic and thylakoid membranes have cytochrome oxidase activity. Although our results with isolated membrane preparations show that there is no activity of cytochrome oxidase in the cytoplasmic membranes from *A. nidulans*, the possibility that the cytochrome oxidase activity of the cytoplasmic membranes was lost during the preparation procedures cannot be excluded.

TABLE IV

NAD(P)H-DBMIB OXIDOREDUCTASE ACTIVITIES OF CYTOPLASMIC MEMBRANES, THYLAKOID MEMBRANES AND THE SOLUBLE FRACTION FROM *A. NIDULANS* AND *SYNECHOCYSTIS* PCC 6714

NaCl washing was performed by suspending the membranes in 400 mM NaCl, 450 mM sucrose and 10 mM Tes-NaOH buffer (pH 7.0) and collecting them by centrifugation. The activity was assayed spectrophotometrically by following NAD(P)H oxidation. NAD(P)H, 150 μM; DBMIB, 50 μM.

Reaction	NaCl washing	Activity (e^- nequiv. per mg protein per min)					
		A. nidulans			*Synechocystis*		
		cytoplasmic membranes	thylakoid membranes	soluble fraction	cytoplasmic membranes	thylakoid membranes	soluble fraction
NADH → DBMIB	without	68	76	250	300	19	100
	with	19	11	–	140	19	–
NADPH → DBMIB	without	140	150	520	33	20	390
	with	20	9	–	21	3	–

The cytoplasmic membranes of *A. nidulans* contain *b*-type cytochrome(s) which are different from the photosynthetic cytochromes present in the thylakoid membranes [18]. The lack of cytochrome oxidase activity in the cytoplasmic membranes from *A. nidulans* indicates that the *b*-type cytochrome(s) is not involved in a repiratory chain which terminates in cytochrome oxidase.

Low activity of cytochrome oxidase was detected in the cytoplasmic membranes from *Synechocystis* PCC 6714 when SDS was not added, but disappeared on its addition. The thylakoid membranes from the same strain revealed much higher activity of cytochrome oxidase in the absence of SDS, but was accelerated by SDS. With such low activity of cytochrome oxidase in the cytoplasmic membranes, much discussion cannot be made for the localization of the oxidase activity in the cytoplasmic membranes. It can be inferred, however, that the cytochrome oxidase in the cytoplasmic membranes, if present, differs from that in the thylakoid membranes, because they responded to SDS in different manners.

High activity of NAD(P)H-DBMIB oxidoreductase was observed in the cytoplasmic membranes from *Synechocystis* PCC 6714. This suggests that the cytoplasmic membrane binds NAD(P)H dehydrogenase. Preferential specificity of the membrane-bound enzyme to NADH and of the soluble enzyme to NADPH seems to suggest that they differ from each other. Low activity of NAD(P)H-DBMIB oxidoreductase was detected in the thylakoid membranes. Its substrate specificity and sensitivity to NaCl washing seem to suggest that it differs from either of the cytoplasmic membrane-bound or the soluble ones. Sandmann and Malkin [23] previously characterized the membrane-bound NAD(P)H dehydrogenase(s) from *Synechocystis* PCC 6714. Since they used the total membrane fraction for the assay of NAD(P)H dehydrogenase activity, the membrane-bound enzyme activity is likely to be due to a mixture of two types of NAD(P)H dehydrogenases, one bound to the cytoplasmic membranes and the other to the thylakoid membranes.

The NAD(P)H-cytochrome *c* oxidoreductase activities of the cytoplasmic membranes from *Synechocystis* PCC 6714 were very low when the quinone-type carrier was absent. Its stimulation by quinones may seem to suggest that some factor is necessary for the activity and that the exogenously added quinones substituted for it. However, this does not appear to be the case, since a considerable amount of plastoquinone-9 is present in the cytoplasmic membranes [18]. The artificial quinone analogue, DBMIB, was surprisingly more effective than the natural quinones in stimulating the reaction. Since it inhibits the light-driven electron transport at the reaction between pool plastoquinone and cytochrome b_6/f complex [24], this observation seems to suggest that the electron-transport pathway of NAD(P)H-cytochrome *c* oxidoreductase does not include this reaction. It is also possible, as in the mitochondrial electron transport [25], that DBMIB forms a short cut bypassing the cytochrome b_6/f complex, whereas it inhibits a site of the complex.

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