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Photosystem-I-driven inorganic carbon transport in the cyanobacterium, *Anacystis nidulans*

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Anacystis nidulans **grown at air levels of CO_2 accumulates high concentrations of inorganic carbon within the cells, which effluxes as CO_2 after a light period. The effect of inhibitors and electron acceptors of photosynthesis on the postillumination CO_2 burst was studied to identify the photosynthetic reaction(s) involved in the inorganic carbon transport. The following results were obtained. (i) In the presence of iodoacetamide, an inhibitor of CO_2 fixation, the CO_2 burst was greater and was insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The inorganic carbon accumulation in the presence of iodoacetamide showed an action spectrum of Photosystem (PS) I reaction. The results demonstrate that a PS-I-mediated cyclic electron flow is involved in the inorganic carbon transport. (ii) Draining of electrons to 2,5-dimethyl-*p*-benzoquinone (DMQ), *p*-nitrosodimethylaniline (PNDA), O_2 or nitrite and phenazinemethosulfate (PMS)-bypassed cyclic electron flow inhibited the CO_2 burst. This is ascribed to the inhibition of the cyclic electron flow involved in the inorganic carbon transport. (iii) The electron flow to DMQ and PNDA or PMS-bypassed cyclic electron flow produced ATP actively. Thus, the inorganic carbon transport does not proceed by ATP supply only. (iv) Uncouplers and inhibitors of ATP synthesis had a strong inhibitory effect on the CO_2 burst. From these results, we concluded that both PS-I-cyclic electron flow and ATP are required to drive the inorganic carbon transport.**

Introduction

Cyanobacteria and green algae have a mechanism to transport exogenous inorganic carbon and concentrate it internally [1–7]. The CO_2-concentrating mechanism is considered to involve an active inorganic carbon transport. Three models have been proposed to explain the transport: (i) a primary electrogenic HCO_3^- pump, (ii) H^+/HCO_3^- symport or OH^-/HCO_3^- antiport secondary to an H^+ extrusion pump [6] and (iii) $Na^+HCO_3^-$ symport secondary to a Na^+/H^+ exchange [8]. Photosynthesis supplies energy for the transporting mechanism. However, the photosynthetic reaction involved in the mechanism is not yet understood. It is assumed that photosynthesis produces ATP to drive the inorganic carbon transporting mechanism [9]. A number of papers have shown that DCMU inhibits the accumulation of inorganic carbon in cyanobacteria, suggesting a requirement of the linear electron transport [2,4,9]. Contradictory results have been obtained with high CO_2-grown cells of *Anabaena variabilis* [7,10]. The ac-

Abbreviations: PS, Photosystem; Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethyl-*p*-benzoquinone; PNDA, *p*-nitrosodimethylaniline; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; PMS, phenazinemethosulfate.

cumulation of inorganic carbon in these cells was insensitive to DCMU and showed an action spectrum of PS I reaction.

In order to identify the photosynthetic reaction(s) involved in the CO_2-concentrating mechanism, we have examined the effect of inhibitors and electron acceptors of photosynthesis on the accumulation of inorganic carbon in *Anacystis nidulans*. The inorganic carbon accumulated within *Anacystis* cells effluxes as CO_2 immediately after a light period. Such postillumination CO_2 burst has been observed in *Synechococcus* sp. [4] and *A. variabilis* [7,10]. A previous paper has shown that the total amount of CO_2 evolved as the burst is equal to the amount of inorganic carbon accumulated within the cells in the light and indicated that the CO_2 burst is a quantitative measurement of the internal inorganic carbon [11]. We report here the effect of inhibitors and electron acceptors of photosynthesis on the CO_2 burst and internal ATP levels in low CO_2-grown *Anacystis* cells together with the action spectrum for CO_2 burst generation and show that the inorganic carbon transport requires both PS I-mediated cyclic electron flow and ATP.

Materials and Methods

Gas exchange measurement

Anacystis nidulans from the algal collection of the Institute of Applied Microbiology, University of Tokyo, was grown at 30°C in 1.5 l shake flask containing Kratz–Myers' medium C [12]. The cells were aerated with air (500 μl CO_2/l). Continuous illumination was provided by fluorescent lamps (120 $\mu E \cdot m^{-2} \cdot s^{-1}$). Cells were harvested by centrifugation (3000 × *g*, 5 min) at room temperature, resuspended in 30 ml 40 mM Hepes-NaOH buffer (pH 7.0), to a Chl concentration of 3–6 μg/ml, and then placed in a reaction vessel for gas exchange measurements.

The gas exchange of the cells was measured with an open gas analysis system [11,13], which records the rate of gas exchange directly, Otherwise stated, all the measurements were done under N_2-containing 200 μl CO_2/l. The gas was led into the reaction vessel at a flow rate of 1.0 l/min. The exchanged gas was dried and then measured with an infrared CO_2 analyzer (model ZAP; Fuji Electric CO., Tokyo) and a trace oxygen analyzer (model 316; Teledyne Analytical Instrument Co., USA). The cell suspension in the reaction vessel was illuminated with orange light (1.38 $mE \cdot m^{-2} \cdot s^{-1}$). A solid glass filter (VO-52, Toshiba Kasei Co., Tokyo) was used to obtain the orange light. Interference filters (bandwidth and half-height, 12–15 nm) were used to provide various wavelengths of monochromatic light. The light source was a 650 W halogen lamp with a fan-cooled heat-absorbing filter. The light intensity was measured with a quantum sensor (L1-190S, LiCor, Inc., Lincoln, NE) or a thermocouple (model E2, Kipp en Zonen, Delft, The Netherlands).

Determination of internal inorganic carbon concentration

The amount of inorganic carbon accumulated within the cells was estimated from the total amount of CO_2 evolved in darkness after the light period, as reported previously [7,11]. The concentration of internal inorganic carbon was calculated using the sorbitol impermeable space determined according to the procedure described by Heldt and Sauer [14].

Determination of ATP and NADP

Approx. 1 ml of cell suspension was drawn rapidly from the reaction vessel into a 1.5 ml syringe containing 125 μl of 70% perchloric acid. Complete and immediate mixing of the cell suspension with the acid occurred, ensuring that rapid cellular breakage and metabolic quenching occurred. The exact volume of suspension sample was determined by weighing the syringe after removing the needle and the unquenched suspension contained therein. The extracts of cell suspensions were neutralized by adding a predetermined amount of 5 M KOH/1 M trithanolamine. The concentration of ATP in the neutralized extracts was determined by the firefly luciferase procedure using an SAI luminometer and Boehringer HS luciferin/luciferase reagents, as described by Lilley et al. [15].

NADP was extracted from cells by perchloric acid as described above. The extract was kept at room temperature at least for 5 min to decompose NADPH and then neutralized. The amount of NADP was determined by enzymatic cycling [16].

The total amount of NADP plus NADPH was determined after oxidizing intracellular NADPH as described by Takahama et al. [17].

Results

Postillumination CO_2 burst

Curves A and A′ in Fig. 1 show typical traces of the changes in CO_2 and O_2 levels, respectively, observed in the external gaseous phase of a cell suspension upon switching the light on and off. When the light was switched on there was a decline in CO_2 followed by gradual rise to a steady-state level. Switching the light off resulted in a sharp increase in CO_2 to a level higher than the initial level followed by a decline to the initial level (curve A). The O_2 concentration increased slowly upon illumination to reach a maximal level after 3 min (A′). The rate at O_2 evolution at this level was equal to the steady-state rate of CO_2 uptake. The uptake and postillumination burst of CO_2 were enhanced by iodoacetamide, an inhibitor of CO_2 fixation [18] (B), which completely abolished the CO_2-dependent O_2 evolution (B′). Thus, the accumulation of inorganic carbon proceeds even in the absence of photosynthetic CO_2 fixation or O_2 evolution.

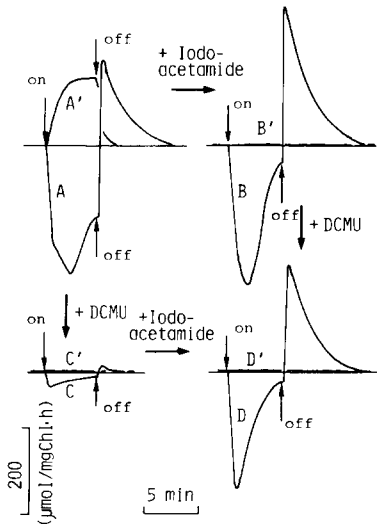

Fig. 1. Changes of CO_2 (A–D) and O_2 (A′–D′) concentrations in the gaseous phase of a cell suspension upon switching the orange light (1.38 $mE \cdot m^{-2} \cdot s^{-1}$) on and off. A, A′, no addition; B, B′, with 3 mM iodoacetamide; C, C′, with 10 μM DCMU; D, D′, with 3 mM iodoacetamide plus 10 μM DCMU.

The intracellular inorganic carbon concentration calculated from the total amount of CO_2 evolved as the burst of curve A in Fig. 1 was 63 mM (sorbitol impermeable space, 145 μl/mg Chl). A previous paper has shown that the amount of CO_2 evolved as the burst agreed with that of acid-labile ^{14}C accumulated within the cells in the light, as determined using the silicon oil centrifugation [11].

Involvement of cyclic electron flow in inorganic carbon transport

Several evidences were obtained which indicate that the inorganic carbon transport is driven by a cyclic electron flow mediated by PS I. The first evidence is the insensitivity of the CO_2 burst to DCMU in the presence of iodoacetamide (curve D in Fig. 1). The CO_2 burst showed 70% of the control activity at 10 μM DCMU. In the absence of iodoacetamide DCMU inhibited the CO_2 burst strongly (C), being consistent with the results obtained with *Synechococcus* [4] and *Coccochloris* [2]. The inhibition was relieved when iodoacetamide was added to the DCMU-treated cells. Thus, in the presence of iodoacetamide, the inorganic carbon transport does not require linear electron flow and is driven only by PS I. The results clearly demonstrate that a cyclic electron flow mediated by PS I is essential for the inorganic carbon transport.

Regarding the inhibitory effect of DCMU on the CO_2 burst in the absence of iodoacetamide, we inferred that DCMU changes the redox-state of the electron carriers, which results in the inhibition of the PS I cyclic electron flow. To test whether the redox-state of the electron transport system is affected by DCMU or iodoacetamide, we have examined the effect of these inhibitors on $NADP^+$ level in the cells under the conditions for gas exchange measurement, and the results are shown in Fig. 2. In the absence of these inhibitors, $NADP^+$ level was high in darkness and low in the light. DCMU inhibited the reduction of $NADP^+$ and kept the $NADP^+$ level high even in the light. In the presence of iodoacetamide, the $NADP^+$ level was low even in darkness and did not change on illumination. The $NADPH/NADP^+$ ratio

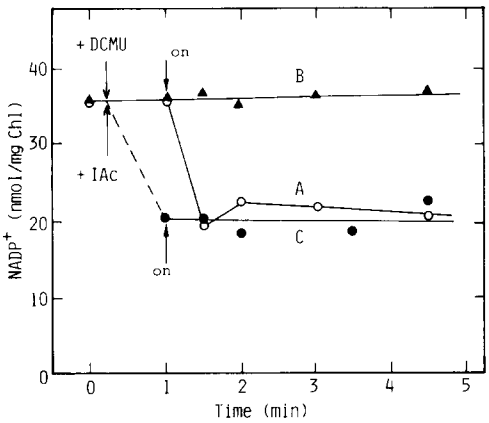

Fig. 2. Changes of $NADP^+$ level in *Anacystis* cells by illumination under anaerobic conditions ($N_2 + 200$ μl CO_2/l) in the presence or absence of inhibitors. A, no addition; B, with 10 μM DCMU; C, with 3 mM iodoacetamide (IAc).

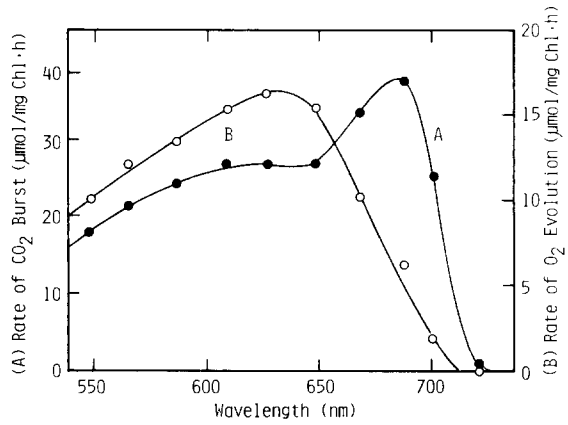

Fig. 3. Action spectra for CO_2 burst generation (A) and PNDA-supported O_2 evolution (B). These spectra were obtained by measuring the initial rate of the CO_2 burst in the presence of 3 mM iodoacetamide (A) and the steady-state rate of O_2 evolution in the presence of 0.25 mM PNDA and 3 mM iodoacetamide (B). The initial rate of the burst was calculated from CO_2 exchange profile as previously reported [7,10]. We assumed that this rate is equal to the steady-state rate of the inorganic carbon transport in the light. The rates are normalized to those at a quantum fluxes of 10 $\mu E \cdot m^{-2} \cdot s^{-1}$. The incident fluxes of monochromatic light were 78–127 $\mu E \cdot m^{-2} \cdot s^{-1}$.

measured after 3 min of illumination was 2.2, which was decreased to 0.9 by DCMU. In the presence of iodoacetamide, the ratio was 2.9, which was not affected by DCMU. These observations suggest that the PS I cyclic electron flow driving the inorganic carbon transport operates when $NADPH/NADP^+$ ratio is high, but is inhibited when the ratio is low. The inhibitory effect of DCMU in the absence of iodoacetamide can be ascribed to the inhibition of $NADP^+$ reduction which keeps the $NADPH/NADP^+$ ratio low and thereby inhibits the cyclic electron flow.

Another evidence for the involvement of PS I reaction is given by the action spectrum for inorganic carbon transport. The action spectrum for CO_2 burst generation (curve A in Fig. 3), obtained by measuring the CO_2 burst in the presence of iodoacetamide following 5 min illumination with various wavelengths of monochromatic light, showed a peak around 684 nm (due to Chl *a* in PS I), while that for PNDA-supported O_2 evolution measured in the presence of iodoacetamide (curve B) showed a broad peak around 630 nm (due to phycocyanin and allophycocyanin in PS II); the latter is essentially the same as the reported spectrum for O_2 evolution [19]. These spectra clearly show that the inorganic carbon transport is driven by PS I, being consistent with the conclusion drawn from the DCMU experiments (Fig. 1).

The third evidence which supports the involvement of PS I cyclic electron flow in inorganic carbon transport is saturation of the postillumination CO_2 burst at low light intensity (Fig. 4). The rate of CO_2 burst was half the saturation level at 80 $\mu E \cdot m^{-2} \cdot s^{-1}$, which was below the intensity, 200 $\mu E \cdot m^{-2} \cdot s^{-1}$, for half saturation of the O_2 evolution. Cyclic photophosphorylation saturates at lower light intensities than non-cyclic photophosphorylation [20]. The low light saturation of the CO_2 burst thus supports the above conclusion that the inorganic carbon transport is driven by cyclic electron flow mediated by PS I.

Inhibition of cyclic electron flow

The CO_2 burst was inhibited when the electrons were drained into DMQ and PNDA which accept electrons from plastoquinone and ferredoxin, respectively [21,22]. The inhibition occurred even in the presence of iodoacetamide (Fig. 5). *Anacystis* cells treated with iodoacetamide showed high activity of PNDA-supported O_2 evolution, indicating that the electron transport from water to ferredoxin is not affected by iodoacetamide. The catalyzer of PS-I-cyclic electron flow, PMS, also strongly

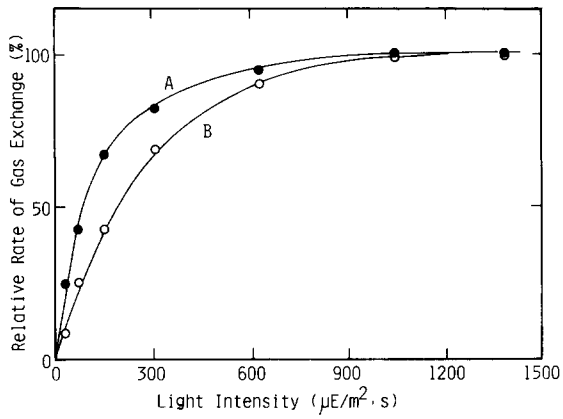

Fig. 4. Effect of light intensity on the rates of CO_2 burst (A) and O_2 evolution (B). The CO_2 burst was measured in the presence of 3 mM iodoacetamide. The initial rate of CO_2 burst and the steady-state rate of O_2 evolution at saturation levels were 203 and 210 μmol/mg Chl per h, respectively.

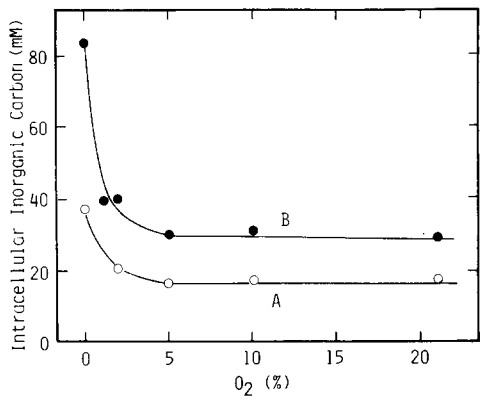

Fig. 6. Dependence of inorganic carbon accumulation on O_2 concentration in gaseous phase of a cell suspension. A, no addition; B, with 3 mM iodoacetamide.

inhibited the CO_2 burst. It is evident that the draining of electrons to DMQ and PNDA or PMS-bypassed cyclic electron flow strongly inhibit the cyclic electron flow driving the inorganic carbon transport.

A similar inhibition was effected by O_2 and NO_2^-, which accept electrons from ferredoxin (Fig. 5). Fig. 6 shows the concentrations of intracellular inorganic carbon in the light as a function of O_2 concentration in the gaseous phase, and indicates that the accumulation of inorganic carbon is much more active under anaerobic conditions than under aerobic conditions. The concentrations of intracellular inorganic carbon obtained under anaerobic conditions in the presence or absence of iodoacetamide were more than twice those, respectively, obtained at 21% O_2. Nitrite had an effect similar to O_2 on the CO_2 burst. When 5 mM $NaNO_2$ was added to iodoacetamide-treated cells, O_2 evolution occurred, and the rate of CO_2 burst became less than half the control (Fig. 5). Thus, the draining of electrons from ferredoxin to O_2 or NO_2^- also inhibits the PS-I-cyclic electron flow driving the inorganic carbon transport. The inhibition by O_2 and NO_2^- was at most 60%; in contrast, DMQ and PNDA inhibited the CO_2 burst almost completely.

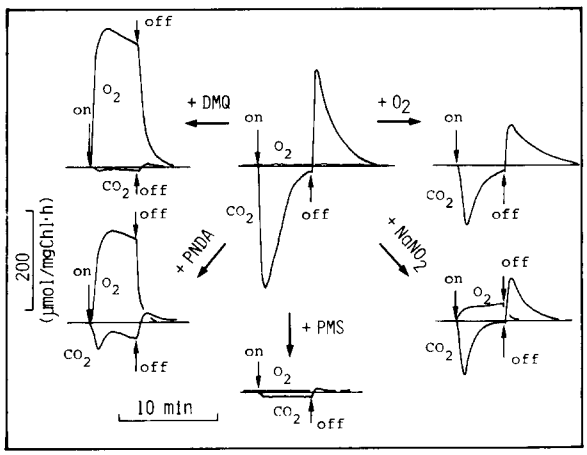

Fig. 5. Effect of DMQ, PNDA, O_2, $NaNO_2$ and PMS on the rates of CO_2 exchange and O_2 evolution in iodoacetamide-treated cells. The concentrations were: iodoacetamide, 3 mM; DMQ, 1 mM; PNDA, 0.25 mM; O_2, 21%, $NaNO_2$, 5 mM; PMS, 10 μM.

Requirement of ATP

The uncoupler, CCCP, and the ATP synthesis inhibitor, DCCD, had a strong inhibitory effect both on the CO_2 burst and O_2 evolution and lowered the ATP level significantly (Table I). Desaspidine and tributyltin had an effect similar to CCCP and DCCD, respectively. The results indicate that ATP is required for the inorganic carbon transport. When CCCP or DCCD was added to the cell suspension during illumination, CO_2 evolution occurred in the light (Fig. 7). Thus, energy is

TABLE I

EFFECTS OF INHIBITORS, ELECTRON ACCEPTORS AND MEDIATOR OF PHOTOSYNTHESIS ON ATP LEVEL, CO_2 BURST AND O_2 EVOLUTION

Sample	Iodoacetamide (3 mM)	ATP level	CO_2 burst	O_2 evolution
No addition	–	100 [b]	100 [c]	100 [d]
No addition	+	101	138	0
Dark [a]	–	24	0	0
+10 μM DCMU	–	84	8	0
	+	103	105	0
+1 mM DMQ	–	95	< 5	145
	+	100	< 5	131
+0.25 mM PNDA	–	115	< 5	156
	+	103	< 5	129
+10 μM PMS	–	120	0	19
	+	122	0	0
+10 μM CCCP	–	46	0	0
	+	18	0	0
+0.1 mM DCCD	–	24	0	0
	+	17	0	0

[a] The sample was incubated in darkness for 5 min. Other samples were illuminated for 5 min with orange light (1.38 $mE \cdot m^{-2} \cdot s^{-1}$).

[b] Values expressed in percent of the controls (153–165 nmol ATP/mg Chl).

[c] Initial rates of CO_2 burst expressed in percent of the controls (210–275 μmol CO_2/mg Chl per h).

[d] Steady-state rates of O_2 evolution expressed in percent of the controls (219–238 μmol O_2/mg Chl per h).

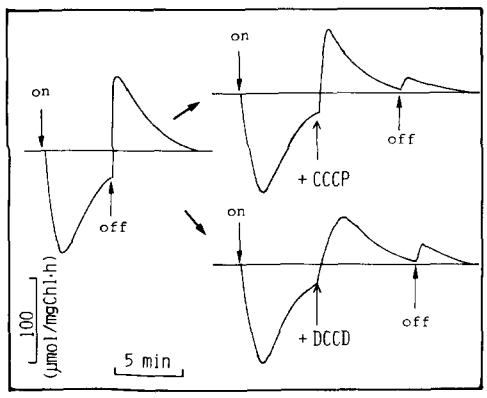

Fig. 7. Effect of CCCP and DCCD on the rate of CO_2 exchange in iodoacetamide-treated cells. CCCP (10 μM) or DCCD (0.1 mM) was added to the cell suspension in the light. The concentration of iodoacetamide was 3 mM.

required to maintain high concentration of inorganic carbon accumulated within the cells.

The electron transport from water to DMQ or PNDA is coupled to ATP formation and produced high internal ATP level in the light either in the presence or absence of iodoacetamide (Table I). The cyclic electron flow bypassed by PMS also produced ATP actively, as shown by the internal ATP level which was higher than the control value. The strong inhibition of CO_2 burst by these chemicals, in spite of the high ATP production (Table I), clearly demonstrates that the inorganic carbon transport does not proceed by ATP supply only. The results indicate that both PS-I-cyclic electron flow and ATP are required to drive the inorganic carbon transport.

Discussion

Data presented in Figs. 1, 3 and 4 clearly demonstrated that the inorganic carbon transport in *A. nidulans* is driven by cyclic electron flow mediated by PS I. The results are consistent with the previous observations on *A. variabilis*, strain M-2, that the inorganic carbon transport in this species was insensitive to DCMU and showed an action spectrum of PS I reaction [7,10]. In *A. nidulans*, the inorganic carbon transport was inhibited by DCMU (Fig. 1). However, this does not necessarily mean that a linear electron flow mediated by PS I and II is essential for the inorganic carbon transport, since DCMU did not inhibit the inorganic carbon transport when photosynthetic CO_2 fixation was inhibited by iodoacetamide. Heber et al. [23] have reported that PS-I-mediated cyclic electron flow in intact chloroplasts is under delicate redox control, and its operation requires 'poising' of the electron carriers. They also showed that DCMU could upset the 'poising' under certain conditions. Peters et al. [24] have shown that high NADPH level favors cyclic electron flow. The cyclic electron flow driving the inorganic carbon transport may also be under redox control and require high NADPH level. The low $NADP^+$ (high NADPH) level produced by iodoacetamide (Fig. 2) may be favorable for the operation of cyclic electron flow driving the inorganic carbon transport. In contrast, the high $NADP^+$ level in the presence of DCMU might be inadequate for the

cyclic electron flow. The requirement of linear electron flow in the inorganic carbon transport in *Anacystis* cells, as indicated by the DCMU inhibition of the CO_2 burst (Fig. 1), may be to produce high NADPH level required for the cyclic electron flow.

The cyclic electron flow driving the inorganic carbon transport was sensitive to electron drainage; the CO_2 burst was almost completely inhibited by draining of electrons to DMQ or PNDA and was decreased by substances such as O_2 and nitrite which accept electrons from ferredoxin (Fig. 5). Similar inhibition by O_2 or nitrite has been observed for cyclic electron flow in intact chloroplasts [23]. Thus, the electron acceptor of the cyclic pathway has a lower electron affinity than that of PNDA, O_2 or nitrite.

Regarding an ATP requirement in the inorganic carbon transport, we obtained contradictory results. The strong inhibition of CO_2 burst by uncouplers and ATP synthesis inhibitors (Fig. 7, Table I) indicated that the inorganic carbon transport is fueled by ATP. In contrast, the inorganic carbon transport did not proceed under the conditions where ATP production is active but cyclic electron flow is inhibited (Fig. 5, Table I). A compromise between these will be that both cyclic electron flow and ATP are required for the inorganic carbon transport.

The mechanism of inorganic carbon transport is poorly understood. It is unlikely that the inorganic carbon transport is driven by a change in the cytoplasmic pH, as previously described [7]. Recently, Kaplan et al. [8] proposed a model for inorganic carbon transport which involves Na^+/H^+ exchange and $Na^+HCO_3^-$ symport. Following model can be considered by modifying their model. ATP supplies energy for Na^+ efflux and cyclic electron transport is linked to H^+ influx. Since inorganic carbon transport and H^+ influx occur on the cytoplasmic membranes, we have to assume that there exists an electron transport between cytoplasmic and thylakoid membranes. Such assumption may be justified by the following results. (i) Cytoplasmic membranes of *A. nidulans* contain cytochrome(s) and plastoquinones which can be the electron carriers of the cyclic pathway [25]. (ii) When *Anacystis* cells grown at high levels of CO_2 were adapted to low CO_2 conditions, the activity of inorganic carbon transport increased several fold. The polypeptide composition of the cytoplasmic membranes changed during the adaptation but that of the thylakoid membranes and cell wall did not change [11]. Further studies are required to verify the above hypothesis.

Acknowledgements

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