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Oxygen-Dependent Proton Efflux in Cyanobacteria (Blue-Green Algae)†

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The oxygen-dependent proton efflux (in the dark) of intact cells of *Anabaena variabilis* and four other cyanobacteria (blue-green algae) was investigated. In contrast to bacteria and isolated mitochondria, an H^+/e ratio (= protons translocated per electron transported) of only 0.23 to 0.35 and a P/e ratio of 0.8 to 1.5 were observed, indicative of respiratory electron transport being localized essentially on the thylakoids, not on the cytoplasmic membrane. Oxygen-induced acidification of the medium was sensitive to cyanide and the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone. Inhibitors such as 2,6-dinitrophenol and vanadate exhibited a significant decrease in the H^+/e ratio. After the oxygen pulse, electron transport started immediately, but proton efflux lagged 40 to 60 s behind, a period also needed before maximum ATP pool levels were attained. We suggest that proton efflux in *A. variabilis* is due to a proton-translocating ATP hydrolase (ATP-consuming ATPase) rather than to respiratory electron transport located on the cytoplasmic membrane.

Cyanobacteria (blue-green algae) have two major membrane systems, namely, the cytoplasmic membrane and thylakoids (8). It has been established that thylakoids contain the photosynthetic electron transport chain. Analogous to other procaryotes, particularly photosynthetic bacteria, it may be expected that respiratory electron transport and oxidative phosphorylation are located on the cytoplasmic membrane. However, there is an interaction between respiration and photosynthesis in blue-green algae (see, e.g., references 3, 17, 28, 38, 41). In spite of several recent publications on the subject, the location of the redox chains remains controversial.

Temperature profiles of intact cells as well as of cell-free systems exhibit different breakpoints in Arrhenius plots for photosynthetic and respiratory activities of *Anabaena variabilis* (37) and *Anacystis nidulans* (27). From these experiments it was concluded that cytochrome oxidase and photosynthetic electron transport are embedded in different membrane-lipid environments. Due to the ultracytochemical evidence reported, cytochrome oxidase is thought to be located only on thylakoids of *Nostoc sphaericum* (4), but on both thylakoids and the cytoplasmic membrane of *Anacystis nidulans* (29).

By more indirect evidence such as transport processes and membrane potential measurements, it has been concluded that the cytoplasmic membrane contains respiratory electron transport activity in *Plectonema boryanum*, *Anabaena variabilis*, and *Anacystis nidulans* (32, 35, 38a). In contrast, other investigations do not indicate a location of respiratory electron transport on the cytoplasmic membrane of *Anacystis nidulans* (25), *Nostoc* sp. (strain MAC) (40), or *Anabaena variabilis* (18). To complete the inconsistencies, it has been reported for *Gloeobacter violaceus*, a thylakoid-free blue-green alga, that both photosynthetic and respiratory redox components are located on the cytoplasmic membrane (9).

All attempts to isolate cytoplasmic membranes with cytochrome oxidase activity have failed so far. A cytoplasmic membrane preparation of *Anabaena variabilis* has been described which contains neither cytochrome nor cytochrome oxidase activity (18). Recently, Murata and coworkers (23) claimed to have isolated cytoplasmic membranes from *Anacystis nidulans* which contain cytochromes but lack cytochrome oxidase activity (N. Murata, personal communication). Generally, it is difficult to prove that a membrane fraction contains cytoplasmic membranes and is devoid of contamination with thylakoids.

An oxygen-dependent proton extrusion in the dark, demonstrated in *Anabaena variabilis* and other procaryotes (39), was taken as evidence for respiratory electron transport on the cytoplasmic membrane. No detailed investigation on this proton efflux has been pursued.

We have resumed this type of study, and evidence is presented here that oxygen-dependent proton extrusion is caused by a proton-translocating ATPase rather than by a respiratory chain on the cytoplasmic membrane of *Anabaena variabilis*. Proton-translocating ATP-hydrolases (ATP-consuming ATPases) are present on cytoplasmic membranes of several organisms (34).

MATERIALS AND METHODS

Organisms and cultivation. *Anabaena variabilis* Kütz (ATCC 29413), *Anabaena cylindrica* (SAUG 1403/2 = ATCC 27899), *Nostoc muscorum* (PCC 7119 = *Anabaena* sp. ATCC 29151), *Aphanocapsa* (= *Synechocystis* sp. 6714; PCC 6714 = ATCC 27178), and *Anacystis nidulans* (SAUG 1402/1 = ATCC 27144) were grown as described previously (36) (ATCC, American Type Culture Collection, Rockville, Md.; PCC, Pasteur Culture Collection, Paris, France; SAUG, Algae Culture Collection, Göttingen, Germany). Cells were harvested in the middle or at the end of their logarithmic growth phase. Chlorophyll *a* (Chl) was determined after extraction in methanol for 5 min at 60°C, using an extinction coefficient of 80.04 mM^{-1} cm^{-1} at 663 nm.

Measurement of oxygen uptake and proton flux. A 3-ml chamber built according to Mitchell and Moyle (22), closed with a screw-cap top and equipped with a Clark-type oxygen

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† This paper is dedicated to H. A. von Stosch on the occasion of his 75th birthday.

electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio), was stirred by a magnetic stirrer. Light was excluded by a black cloth which covered the reaction chamber. The temperature was kept constant by a water bath (28°C). For technical reasons, our oxygen electrode had a lag phase of about 20 to 30 s, as seen in control experiments. Therefore, the oxygen uptake traces are not shown in this study, since they cannot be compared with the proton efflux traces; the kinetics of the pH electrode is much faster (see Discussion). On top of the reaction chamber, a gas-tight pH electrode (GK 2321C; Radiometer, Copenhagen, Denmark) was mounted through a bore in the screw-cap top of the chamber. The electrode was connected to a standard pH meter (PHM 62; Radiometer). Both oxygen uptake and pH were recorded (Table 1) with a two-channel recorder (Servogor 320, Metrawatt). By adding HCl, the rapid response of the electrode was checked: 90% of the resulting pH change was monitored after 2 s. During the assay, additions to the reaction medium were introduced by microliter syringes (Hamilton) through a small hole in the screw-cap top.

Algae were harvested by centrifugation (5 min, 3,800 × *g*), washed once in reaction buffer according to Mitchell and Moyle (22) (but containing sodium chloride [3 mM glycylglycine, 75 mM KCl, 75 mM NaCl, 5 mM $MgCl_2$, pH 6.3]), suspended in the same medium, adjusted to 300 to 500 μg of Chl ml^{-1}, and stored on ice. The activity decreased slightly during storage, but respiration, photosynthesis, and proton extrusion remained constant over a period of at least 4 h.

Oxygen was excluded beforehand by bubbling both buffer and alga suspension (20 to 30 μg of Chl ml^{-1}) with a stream of nitrogen. Algae were then allowed to remove any residual oxygen by respiration and to equilibrate with the medium in the reaction chamber. The experiment was started 15 to 20 min later by adding 200 μl of air-saturated reaction buffer. After consumption of the oxygen added by respiration and subsequent equilibration for another 10 to 20 min, a new oxygen pulse was applied, resulting in an identical proton release when corrected for dilution. After two to four oxygen pulses, the experiment was stopped. Occasionally, carbonic anhydrase was added, which had no effect, thereby excluding the possibility of a ΔpH being caused by a bicarbonate equilibrium. H^+/e ratios were calculated by the maximum rate of both H^+ efflux and oxygen uptake, both determined over 2 min, 40 s after the oxygen pulse had been given.

TABLE 1. Proton release, oxygen uptake, oxidative phosphorylation, and H^+/e ratio in *Anabaena variabilis* under the influence of several inhibitors[a]

Addition (m)[b]	% of control			
	Proton release	Oxygen uptake	Oxidative phosphorylation	H^+/e
Control	100	100	100	100
KCN, 3×10^{-3}	0	30	0	0
CCCP, 3×10^{-6}	0	100	22	0
DNP, 9×10^{-4}	51	126	35	40
DCCD, 5×10^{-5}	32	79	0	40
Nitrofen, 5×10^{-5}	68	103	87	71
Vanadate, 118 μg/ml	62	92	100	67
Fusicoccin, 10^{-5}	107	90	93	118

[a] ATP pools were determined at zero time and after 30 s (see Fig. 2). Proton release: 100% ≙ 570 nmol of H^+ mg of Chl^{-1} min^{-1}; oxygen uptake: 100 ≙ 465 nmol of O_2 mg of Chl^{-1} min^{-1}; rate of oxidative phosphorylation: 100% ≙ 468 nmol of [~ P] mg of Chl^{-1} min^{-1}; H^+/e: 100% ≙ 0.31. Values given are averages of two to six experiments. The control was averaged from 23 experiments, since each inhibitor assay was accompanied by a control measurement. Within one batch the experimental error was ±12%.

[b] CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,6-dinitrophenol.

Determination of ATP and ADP. Algae were washed once in 10 mM 2-(*N*-morpholino)-ethanesulfonic acid–KOH buffer, pH 7.5, including 5 mM $MgCl_2$, and suspended in the same buffer. Thereafter, 0.5-ml samples of algal suspension (equivalent to 40 to 80 μg of Chl ml^{-1}) were flushed with nitrogen for about 10 min in 8-ml glass vessels sealed with Suba-seal rubber stoppers (Freeman Co., Barnsley, England). The anaerobic vessels were incubated for 30 min in the dark at 30°C. After this time, the ATP level in the cell had reached a constant, low level. Oxidative phosphorylation was then started by addition of 0.5 ml of air plus 0.5 ml of air-saturated 2-(*N*-morpholino)-ethanesulfonic acid buffer (see above). ATP pool determinations were done according to reference 15 and modified according to Ernst et al. (6). A 1-ml portion of 1.4 M perchloric acid and 0.2 ml of 100 mM EDTA were added to the algal suspension. After extraction for 30 min on ice, 0.1 ml of 1.8 M triethanolamine was added and the pH was adjusted to 7.4 to 7.6 with 5 M KOH.

Precipitated potassium perchlorate and cell debris were removed by centrifugation (5 min, 8,000 × *g*), and the supernatant was analyzed for ATP by a bioluminescence assay (LKB Wallac Luminometer, model 1250; LKB Instruments). A 10-μl amount of extract was added to 200 μl of a luciferin-luciferase solution consisting of a 20-μl LKB ATP monitoring kit and 180 μl of stock solution (0.1 mM phosphoenolpyruvate, 5 mM $MgSO_4$, 12 mM K_2SO_4, 18 mM EDTA, 90 mM Tris-hydrochloride, pH 7.6). For ADP determination, 1.5 μl of pyruvate kinase (EC 2.7.1.40; Boehringer, Mannheim, Germany) was added subsequently. Finally, 5 μl of 5 μM ATP standard solution was added for calibration. P/e ratios were calculated by using maximum rates of phosphorylation and oxygen uptake.

RESULTS

Proton efflux in the dark with *Anabaena variabilis*, induced by an oxygen pulse (10 μM O_2), is shown in Fig. 1. The efflux decreased after the oxygen had been consumed by respiration. Subsequently, a slow uptake of protons was measured. The rate of proton uptake was quite variable in the range of 5 to 15% of the preceding proton efflux. It was possible to repeat the efflux by a second oxygen pulse. However, several oxygen pulses applied at 2- to 3-min intervals led to a decrease of acidification.

A lag phase of about 34 to 60 s was observed until the proton efflux reached its maximum rate. A second oxygen pulse was added immediately after the first proton efflux had come to an end, and the lag phase observed was only 5 to 10 s (Fig. 1). If the second oxygen pulse is given several minutes later, the lag phase is the same as with the first pulse (data not shown).

Subsequently, the ΔpH–time course was correlated with ATP levels in the cell. In Fig. 2, oxidative phosphorylation is shown to be dependent on an oxygen pulse. ATP formation is linear, exhibiting a short lag phase of 5 s. After 30 to 40 s, the ATP level reached a maximum. The energy-rich phosphate, calculated as 2 ATP plus ADP, is shown in the upper curve of Fig. 2. The maximum rate of oxidative phosphorylation was found to be 1,036 nmol of energy-rich phosphate mg of Chl^{-1} min^{-1}. Comparison of oxygen uptake and oxidative phosphorylation within the same culture batch resulted in P/O ratios of 0.8 to 1.5. The relevant result of these experiments is the marked difference in the kinetics of ATP

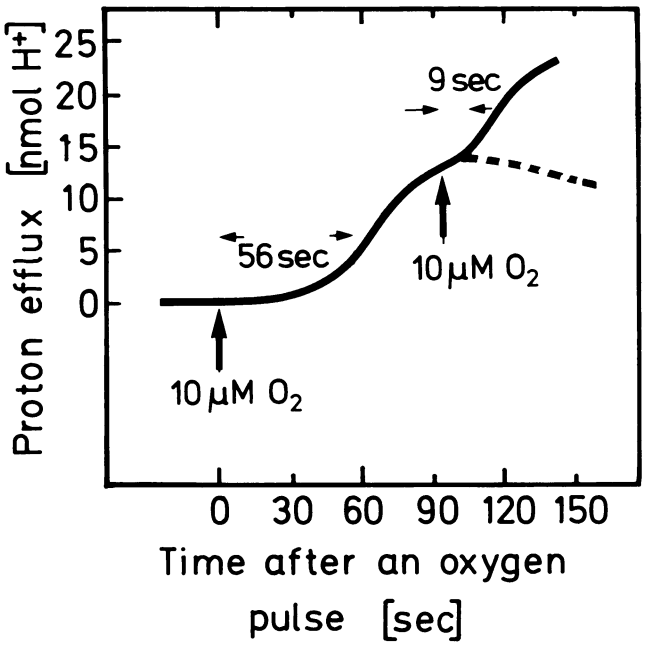

FIG. 1. Acidification of the medium after an oxygen pulse (200 μl of air-saturated buffer) given to *Anabaena variabilis*. The maximum rate of proton efflux was 383 nmol of H^+ mg of Chl^{-1} min^{-1}. Upward deflections represent proton efflux. The dotted line shows proton uptake without the second oxygen pulse given. Before the oxygen pulse, cells were preincubated anaerobically for 15 to 20 min in the medium given in the text.

formation and the time course of proton efflux (Fig. 1). Proton efflux was at its maximum when the final ATP level had been reached. Of note is that the ATP level decreased after the cells were kept anaerobically in the dark for several minutes (data not shown).

Table 2 documents H^+/e ratios of different species of blue-green algae. No major differences among the species investigated are seen. Also shown are maximum values measured occasionally. It is noteworthy that these values were never exceeded. Valinomycin (up to 10^{-5} M) had no significant effect on the rate of proton efflux. In some experiments, the amount of H^+ extruded was slightly stimulated, but in no case did the rate of proton efflux increase.

The effect of several inhibitors on proton efflux, oxygen uptake, and oxidative phosphorylation was investigated (Table 1). As the major result, inhibition of proton efflux is possible, leaving respiratory electron transport more or less unaffected. This can also be demonstrated advantageously by the H^+/e ratios given in Table 1. The ratios are lowered with these inhibitors, except for fusicoccin (see Discussion).

DISCUSSION

The pH gradient resulting from photosynthetic electron transport is consumed by an ATP synthetase producing ATP in the light. In blue-green algae, a respiratory chain is also located on the thylakoids, presumably using the same ATP synthetase (4, 18, 28, 29). Although interaction of photosynthetic and respiratory electron transport is evident (3, 17, 28, 38, 41), it is not yet clear whether the electron transport chains as a whole or certain sections or single redox proteins only are shared by both photosynthesis and respiration. Figure 3 summarizes the state of the knowledge and the data of this paper.

Apparently, cytochrome oxidase is located in a lipid environment different from that of photosynthetic electron transport, as can be concluded from discontinuities in Arrhenius plots (27, 37). This finding could indicate that respiratory electron transport is located on either the thylakoid or the plasmalemma (see Fig. 3). Model I of Fig. 3 suggests that no respiratory electron transport occurs in the plasmalemma (4, 18, 25), and protons may be translocated as indicated by an ATP-consuming plasmalemma ATPase. In contrast, model II of Fig. 3 shows a proton-translocating

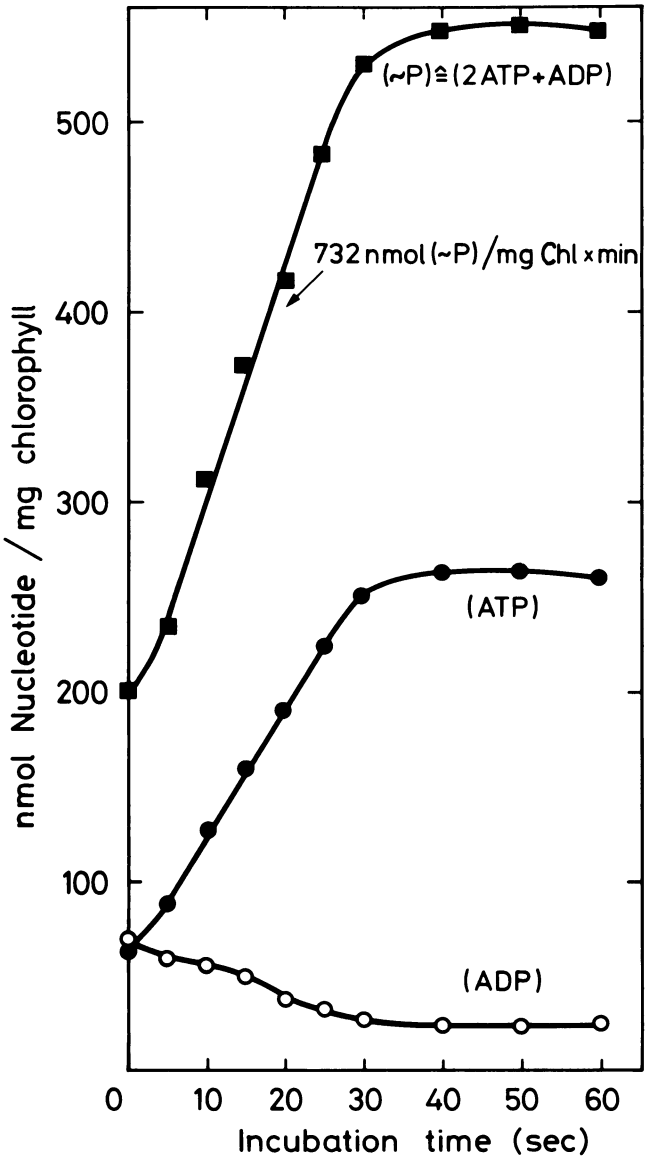

FIG. 2. Kinetics of oxidative phosphorylation. Analyses were made after an oxygen pulse given to *Anabaena variabilis* kept under anaerobiosis [suspended in 10 mM 2-(*N*-morpholino)-ethanesulfonic acid (pH 7.5), 5 mM $MgCl_2$] beforehand; at the start, oxygen was added to give a concentration of 14.4 μM. The AMP content was measured occasionally at zero time and after 60 s and accounts for the imbalance between ATP and ADP concentrations.

TABLE 2. Proton efflux of intact cells of different species of blue-green algae[a]

Species	H^+/e ratio	
	Avg	Maximum
Anabaena variabilis	0.30	0.57
Anabaena cylindrica	0.31	0.39
Nostoc muscorum	0.29	0.52
Aphanocapsa sp. 6714	0.23	0.40
Anacystic nidulans	0.35	0.48

[a] The H^+/e ratio was comparable in all species investigated. The values given are averages from 30 experiments with *Anabaena variabilis*, 6 with *Anabaena cylindrica*, 9 with *N. muscorum*, 13 with *Aphanocapsa* sp., and 7 with *Anacystis nidulans*. Within one batch, the tolerance of H^+/e ratios was ±10%. A higher variation occurred with different batches, as indicated by the maximum values.

respiratory chain on the plasmalemma with an ATP-synthesizing ATPase (24, 26, 29, 30).

An oxygen-dependent proton efflux of *Anabaena variabilis* exhibiting an H^+/e ratio of 0.27 has been reported (39) and corresponds with our findings both in extent and rate of acidification (see Fig. 1). However, neither an interpretation nor a further characterization of this proton efflux has been given as yet. In our hands, five different blue-green algae showed comparable H^+/e ratios of about 0.23 to 0.35. If proton efflux were directly dependent on electron transport, H^+/e ratios could be reliably measured only under non-phosphorylating conditions. However, if proton efflux were due to a proton-pumping ATPase, non-phosphorylating conditions would decrease rather than increase the H^+/e ratio, which is, indeed, observed (see Table 1). According to the chemiosmotic theory, this ratio turns out to be an important efficiency indicator of oxidative phosphorylation, according to the equation (22) $H^+/e = [(P/e) \times (H^+/P)]$. H^+/P (protons translocated per ATP synthesized) equals 3 as determined in either chloroplasts (33) or mitochondria (1) and was found to be 2 in *Rhodopseudomonas* sp. (2).

In Table 3, the H^+/e ratios of mitochondria and of 13 bacterial species have been compiled from the literature for comparison. Where possible, P/e ratios are given. We have calculated the H^+/P ratios according to the above equation from the data given in the first two columns (which is an approximation; see footnote *a*, Table 3). Obviously H^+/P ratios are between 2 and 4, which is in good agreement with the values given above. A very low H^+/e ratio is observed in the blue-green algae investigated. The P/e ratio, however, is comparable to that for other organisms. Obviously, in blue-green algae only a minor part of the electrons (10 to 20%) moving along the respiratory chain mediate proton efflux out of the cell. We conclude from these data that most of the respiratory electron transport is located on the thylakoid membrane. Thus, proton translocation driven by thylakoid-located electron flow cannot be measured as external acidification.

The question remains of whether proton efflux of *Anabaena variabilis* is due to an ATP-hydrolase or respiratory electron transport, both located on the plasmalemma. To answer this question, proton efflux and electron transport were tested for differential effects with inhibitors (Table 1). The effects of cyanide and the uncoupler carbonyl cyanide

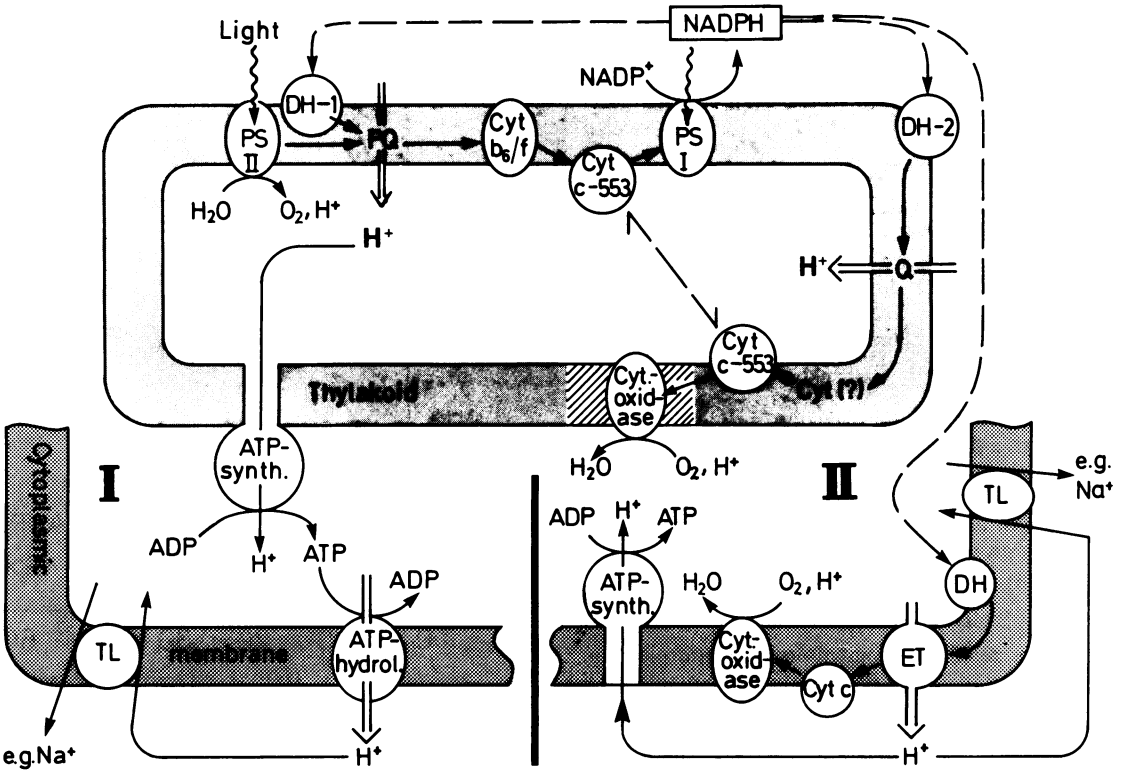

FIG. 3. Hypotheses on the interaction and localization of photosynthetic and respiratory electron transport in blue-green algae: PS, photosystem; DH-1, DH-2, pyridine-nucleotide dehydrogenases possibly of different properties: PQ, plastoquinone; Cyt b_6/f, cytochrome b_6-f complex; Cyt c-553, soluble cytochrome c_{553}; Q, unknown quinone; TL, translocators; ET, electron transport; Cyt c, unknown periplasmic cytochrome; Cyt(?), unknown membrane-bound cytochrome(s).

TABLE 3. Compilation of H^+/e ratios[a]

Organelle/organism[b]	H^+/e ratio	P/e ratio	H^+/P calculated	Reference
Mitochondria	4–6	1.5	2.7–4	8
Hydrogenomonas	3.8	1.9	2.0	5
Escherichia	3	1.53	2.0	21
Bacillus	3.3	1.68	2.0	21
Acinetobacter	2.85	1.34	2.1	21
Xanthomonas	3.65	1.53	2.4	21
Microbacterium	4.82	1.54	3.1	21
Acetobacter	1	0.54	1.8	21
Klebsiella	1.96			13
Pseudomonas	3.3			13
Micrococcus	4			39
Rhodopseudomonas	2.1			23
Anabaena	0.27			39
Anacystis	0.35[c]	1.4	0.25	24
Anabaena	0.31[c]	0.8[c]	0.40	See Table 1

[a] When cited in the references indicated, P/e ratios are given. H^+/P ratios were calculated from literature data by the equation, $H^+/P = [(H^+/e)/(P/e)]$. The data summarized from the literature were not obtained under identical conditions. Therefore, the equation gives only approximate results.

[b] For species of the genera given, see references as indicated.

[c] Our results.

m-chlorophenyl-hydrazone are in agreement with both models of Fig. 3. Interestingly, dinitrophenol inhibits proton efflux and oxidative phosphorylation, but not electron transport, as was also found for nitrofen, an ATPase inhibitor (11, 14). *N,N'*-Dicyclohexylcarbodiimide (DCCD) is a rather ambiguous inhibitor. Besides its binding to the F_1/F_0 ATPase, it has been shown that DCCD (in substantially higher concentrations) also inhibits electron transfer (e.g., see reference 31). Reed and co-workers (35) reported that DCCD did not affect the membrane potential of *Anabaena* sp. With regard to the low concentration applied in our experiment, it may be suggested that proton efflux impaired by DCCD is due to inhibition of ATP synthesis. The effect of DCCD is not yet fully clear, particularly with intact cells. Vanadate, reported to inhibit plasmalemma ATPase (18, 19), decreased the proton efflux but not oxidative phosphorylation. The weak effect of fusicoccin, which stimulated proton translocation of plasmalemma ATPase (20), cannot be evaluated. In summary, with various inhibitors, a decrease in H^+/e ratios was demonstrated together with decreased oxidative phosphorylation. This allows for the proposed model I of Fig. 3 and contradicts model II.

Furthermore, the kinetics of proton efflux and ATP synthesis in the dark favor model I. We interpret the data of Fig. 1 and 2 as follows. The ATP content is low after 20 min of anaerobiosis. After the oxygen pulse, an ATP level is built up, with a lag phase of about 5 s only. ATP synthesis in the dark is directly dependent on oxygen uptake; the kinetics of respiratory electron transport is, therefore, expressed by the kinetics of ATP synthesis (for technical reasons, electron transport cannot be measured by oxygen uptake within the first 20 to 30 s after the oxygen pulse; see Materials and Methods). As a consequence of increasing ATP, completed within 30 to 40 s, ATP-driven proton efflux is possible. After a second O_2 pulse, the ATP concentration is not as low as compared with the first pulse (Fig. 1), and the proton efflux starts with a much shorter lag phase, since the decay of ATP levels has not reached the same low value as at the start of the experiment. Thus, the existence of a proton-translocating ATP-hydrolase would fully explain our data, whereas the hypothesis of an electron transport chain on the cytoplasmic membrane is not in accordance with our findings. Similar kinetics of oxidative phosphorylation has been shown for *Anacystis* sp. (24). Comparable ATP concentrations have been reported for *Anabaena* sp. (12) and *Anacystis* sp. (24).

A proton-translocating ATPase, which can be concluded from our results, has been suggested for *Anacystis* sp. (16, 25) and *Nostoc* sp. (40). Most recently, we have obtained evidence for a vanadate-sensitive, proton-translocating ATP-hydrolase being active in the light in *Anabaena* and *Nostoc* sp., but not in *Anacystis* sp. (S. Scherer and P. Böger, FEMS Microbiol. Lett., in press).

An oxygen-induced proton efflux has been reported (26) for *Anacystis* spheroplasts which is dependent on exogenously applied reduced cytochromes. Furthermore, a periplasmic cytochrome has been claimed for *Anacystis* sp. (30). These findings have been taken as evidence that respiratory electron transport, or at least parts thereof, is located on the cytoplasmic membrane.

Our whole-cell studies have to be corroborated, of course, by characterization of isolated cytoplasmic membranes. The data presented here and those in the literature seemingly do not allow as yet for a general concept for all cyanobacteria. We may tentatively assume that filamentous and coccoid cyanobacteria have different functional cytoplasmic membranes.

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