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VANADATE AND DICYCLOHEXYLCARBODIIMIDE INSENSITIVE PROTON EXTRUSION FROM OXYGEN PULSED CELLS OF THE CYANOBACTERIUM ANACYSTIS NIDULANS

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Oxygen pulses applied to dark anaerobic suspensions of Anacystis nidulans provoked immediate acidification of the external medium. The reaction was inhibited only 75% by dicyclohexylcarbodiimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole at concentrations which completely arrested all oxidative phosphorylation. Carbonyl cyanide m-chlorophenylhydrazone eliminated the acidification of oxygen pulsed cell suspensions while ortho-vanadate and diethylstilbestrol had no effect. No lag occurred between the onset of respiration and proton extrusion. H^+/O ratios were 4.1±0.5 in the absence, and 1.9±0.4 in the presence, of dicyclohexylcarbodiimide. These results are consistent with a recently described proton-translocating aa_3-type cytochrome c oxidase (H^+/O = 1.6±0.4) in the cell membrane of A. nidulans (G.A. Peschek, J. Bacteriol. 153 (1983) 539-542).

Proton translocation coupled to electron transport in energy-transducing membranes has been studied with a wide variety of bioenergetic systems including mitochondria, chloroplasts and intact bacteria (cf. Ref. 1 for review). According to the chemiosmotic theory (2) energy stored in the proton electrochemical gradient can be converted into ATP through a membrane bound reversible H^+-ATPase (F_0F_1-ATPase); the same enzyme may build up a proton gradient across the membrane utilizing ATP formed elsewhere in the cell (3).

Cyanobacteria are unique in housing the electron transport systems of both aerobic respiration and oxygenic, plant-type photosynthesis within a prokaryotic cell (4). A major fraction of both systems is located in chlorophyll-containing intracytoplasmic membranes (ICM)[1] where common components may link

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[1] CM, cytoplasmic (cell) membrane; ICM, intracytoplasmic (thylakoid) membrane; DCCD, dicyclohexylcarbodiimide; NBD, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; Mes, 2-(N-morpholino)ethanesulfonic acid; Pipes, piperazine-N,N'-bis-(2-ethanesulfonic acid); Taps, tris (hydroxymethyl)-methyl-aminopropanesulfonic acid; Ches, 2-(N-cyclohexylamino)-ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid.

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the two systems (5-8). However, electron-transporting functions of the CM are more than likely (9-12; cf. ref. 13 for review).

To which extent CM-bound respiratory (11-13) or photosynthetic (9,10) electron transport chains, and H^+-translocating reversible (14,15) or unidirectional (16) ATPases, respectively, contribute to the observed H^+ extrusion from energized cyanobacteria (17) continues to be controversial. Moreover, bioenergetic patterns may be different in filamentous and unicellular species (13,16). Continuing our studies on respiration and dark energy metabolism of cyanobacteria (13) this paper presents evidence for ATPase independent respiration-linked H^+ extrusion from the unicellular cyanobacterium A. nidulans.

MATERIALS AND METHODS

Axenic cultures of Anacystis nidulans (Synechococcus sp.), strain 1402-1 (Göttingen, F.R.G.), were grown in modified medium D at 1 kW/m^2 warm white fluorescent light, 38°C, and a constant pH of 8.2 in a turbidostat as described previously (14). Cells were harvested during late logarithmic growth, washed twice with 40 mM Hepes/tris buffer, pH 7.4, and finally resuspended in assay media containing 100 mM KCl, 10 M valinomycin, 50 µg/ml carbonic anhydrase and 1 mM buffer of desired pH. Media were buffered with citric acid (3.2-5.5), Mes (5.5-6.7), Pipes (6.4-7.5), Hepes (7.2.-8.2), Taps (7.8-9.1), Ches (8.6-10.0) and Caps (9.7-11.0) previously titrated to the desired pH with 1 mM tris base. Total assay volume was 2.5 ml containing between 10 and 80 mg dry weight of cells. Cells pretreated with 10 mM Na_2EDTA, which increases the permeability of the cell wall of certain Gram negative bacteria towards valinomycin, gave the same results as untreated cells.

Harvested and washed cells were gently bubbled with oxygen-free nitrogen for at least 20 min in the dark. Oxygen concentration was continuously monitored with a YSI Clark type oxygen electrode, model 53. pH changes in the suspensions were followed with a Philips digital pH meter, model 9409, connected to a Goertz multi-pen recorder (18). Oxygen and pH electrodes were attached to the same assay chamber thermostatted at 35°C. 250 µl of oxygen-saturated 100 mM KCl solution were rapidly injected into anaerobic cell suspensions through the capillary inlet of the chamber giving oxygen concentrations of 0.25 to 0.3 mM O in the suspensions. Recordings were corrected for the 10% dilution due to the addition of the KCl solution. Occasionally, proton ejection was also monitored by the decrease of absorption at 560-580 nm in a Shimadzu UV-300 dual wavelength spectrophotometer using 60 µM phenol red as a pH-indicating dye (35°C; slit width 2nm; light path 1 cm; cf. Ref 18). pH changes in the medium were calibrated with standard HCl solution (Titrisol, Merck). Data from pH meter and spectrophotometer assays, respectively, were within the standard error of determination, which was within ±20%.

In control runs 250 µl 100 mM KCl solution previously saturated with N_2, instead of O_2, was added to the anaerobic cell suspensions; alternatively, O_2-saturated KCl solution was added to heat denatured cells. No pulse induced acidification of the medium was detected in these controls. Inhibitors were added between 5 and 20 min prior to oxygenation. Incubation of the cells with DCCD for up to 40 min did not increase the effect of the inhibitor above the level seen after 20 min of incubation. In case of dark/light transitions the cell suspensions were supplemented with 20 µM DCMU and kept anaerobic as described before. Illumination was provided by a 1 kW slide projector equipped with a Schott AG (Mainz, FRG) cut-off filter blocking light below 580 nm. Due

to the rather high cell densities that had to be used, photosynthesis was not light saturated under these conditions.

ATP was determined by a luciferin-luciferase assay as described previously (19,20). Proton motive forces (pmf = $\Delta\psi$ -60. ΔpH; 35°C; expressed in mV) were calculated from distribution ratios across the CM of radioactively labeled weak acids or bases (ΔpH) and lipophilic ions ($\Delta\psi$) according to established techniques (14,15). Details of these measurements will be published elsewhere. Prolonged incubation of dark aerobic and anaerobic A. nidulans at pH 3.2 and 11.0 (which were the most extreme pH values used) was not significantly deleterious to the cells as evident from unchanged respiratory and photosynthetic activities, after transfer to neutral pH, before and after the incubation.

RESULTS AND DISCUSSION

Fig. 1 shows the time course of O_2 uptake and H^+ extrusion in weakly buffered suspensions of A. nidulans following the admission of O_2 to dark anaerobic cells. Both respiration and H^+ ejection started immediately upon oxygenation. The apparent lag of O_2 uptake (Fig. 1a,c) was due to the limited response time of the oxygen electrode and/or to the time it took the O_2 to diffuse onto the surface of the electrode since extrapolation of the linear trace back to zero time (oxygenation) yields exactly the amount of O_2 initially present (0.25 mM O; cf. Fig. 1a,c). Experiments conducted in the presence of saturating amounts of DCCD (Fig. 1c,d), which completely eliminated all oxidative phosphorylation in the cells (cf. Table 1), revealed the persistence of about 25% of the respira-

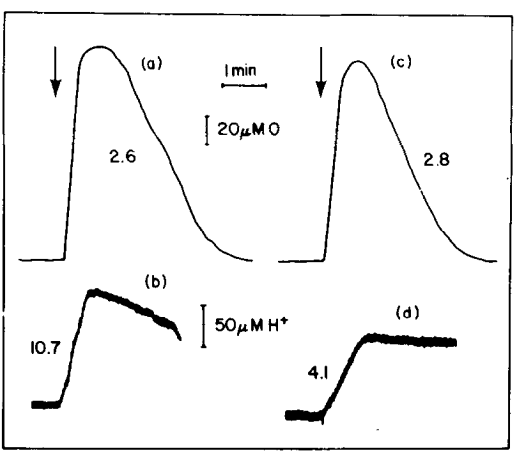


Fig. 1 Respiratory oxygen uptake (a,c) and proton extrusion (b,d) following oxygenation of dark anaerobic suspensions of A. nidulans (pH 7.4) in the absence (a,b) and presence (c,d) of saturating amounts of DCCD (1.2 nmol/mg dry weight of cells; cf. Table 1). Numbers adjacent to the traces give initial rates of oxygen uptake and proton extrusion in nmol O or H^+/min per mg dry weight of cells. Samples contained 20-23 mg dry weight of cells per ml.

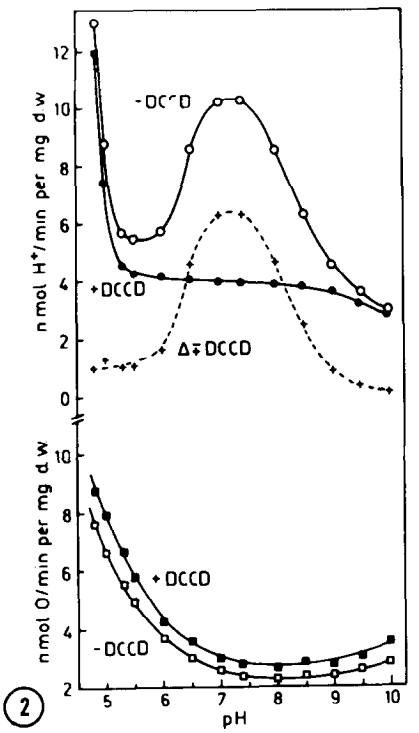
Table 1. Effect of inhibitors on proton extrusion, respiratory oxygen uptake, ATP formation and ATP levels in respiring cells of A. nidulans following oxygenation of anaerobic cell suspensions.

Inhibitor	Concentration	Proton extrusion	Oxygen uptake	ATP formation	ATP content
		nmol/min per mg dry weight			nmol/mg dry weight
None		10.5	2.6	4.1	2.6
DCCD[a]	0.12 nmol	8.7	2.5	3.1	2.3
	1.2 nmol	4.3	2.7	0.0	0.8
NBD[a]	0.12 nmol	8.5	2.6	3.0	2.2
	1.2 nmol	4.1	2.1	0.0	0.9
CCCP	10 µM	3.1	5.0	1.5	1.6
	100 µM	0.0	5.6	0.0	0.8
DES	0.1 mM	11.0	2.5	4.0	2.7
	1.0 mM	10.5	2.5	4.1	2.5
Vanadate	0.1 mM	10.8	2.6	3.9	2.6
	1.0 mM	11.2	2.5	4.0	2.7
KCN	10 µM	8.5	2.2	3.1	2.5
	1.0 mM	0.6	0.3	1.0	0.9

[a]Concentrations of inhibitors are expressed as nmol/mg dry weight of cells since the effect of the inhibitors was dependent on the cell concentration.

tion-linked H^+ efflux seen with uninhibited cells (Fig. 1b,d). H^+/O ratios around 4 and 2 in the absence and presence of DCCD are derived from Fig. 1. DCCD slightly stimulated overall rates of O_2 uptake which may be due to some regulatory balance between ICM-catalyzed O_2 uptake (tightly coupled to ATP synthesis) and CM-catalyzed O_2 uptake (also coupled to energy dependent ion transport across the membrane; cf. Refs. 19,20).

Strikingly different pH profiles were obtained for the DCCD sensitive and insensitive H+ ejection, respectively (Fig. 2), suggesting different mechanisms. Increasing O_2 uptake rates at low pH were accompanied by increasing energization of the CM (Fig. 3) which was only slightly affected by DCCD (not shown), indicating immediate energization of the CM through respiratory electron transport. The high rates of O_2 uptake at low pH were 90% inhibited by 1 mM KCN (not shown); therefore the same type of cyanide sensitive terminal oxidase (12,13,18) appears to be involved at both extremely low and physiological pH values.



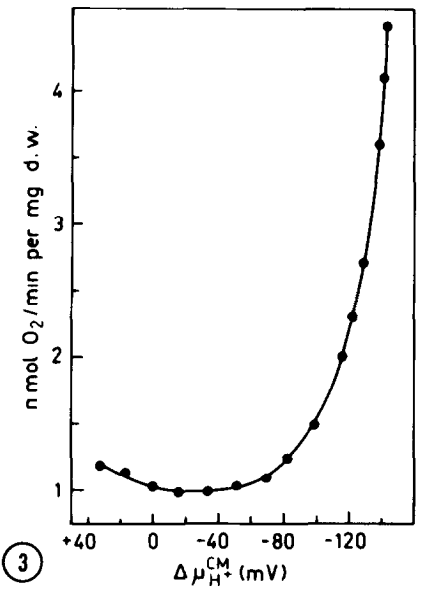


Fig. 2 pH dependence of respiration and proton ejection following the oxygenation of dark anaerobic suspensions of A. nidulans in the absence and presence of DCCD. Samples contained 10-35 mg dry weight of cells per ml. Cf. Fig. 1.

Fig. 3 Correlation between the proton motive force across the CM of, and the rate of respiratory oxygen uptake by, dark aerobic cells of A. nidulans. pH of the suspensions was between 3.2 (-120 mV) and 10.0 (+40 mV). Cf. refs. 14,15. Samples contained 20-80 mg dry weight of cells per ml.

Table 1 summarizes the effect of various inhibitors on H^+ extrusion, O_2 uptake and oxidative phosporylation in oxygenated A. nidulans, and on steady state ATP levels in respiring cells. Effects of DCCD (inhibiting F_0) and NBD (inhibiting F_1) were similar; about 25% of respiration-linked H^+ ejection remained unaffected in the presence of saturating amounts of either inhibitor. Most likely, therefore, this fraction of H^+ translocation is directly coupled to respiratory electron transport in the CM of A. nidulans (13). CCCP, which renders membranes permeable to protons, eliminated any net H^+ extrusion. DES and ortho-vanadate known to inhibit F_0-type ATP hydrolase (16,21) were without effect. Increasing concentrations of KCN shifted H^+/O ratios from 4 (10 μM KCN) to 2 (1mM KCN), at the same time strongly depressing both formation and levels of ATP in respiring cells. H^+/O ratios in the presence of KCN were the

same as in the presence of DCCD which also effected the same low ATP levels (Table 1). Since the sites of inhibition by KCN and DCCD are different these findings reinforce two different mechanisms for the H^+ extrusion from A. nidulans during transition from dark anaerobic to aerobic conditions: one mechanism (DCCD insensitive and ATP independent) appears to be directly coupled to respiratory electron transport in the CM. The second mechanism (DCCD sensitive and ATP dependent) may be mediated by a reversible H^+-translocating ATPase in the CM (dashed line in Fig. 2) which, in physiological conditions, translocates most of the protons extruded from respiring cells in the dark. Recently, the expected reversibility of the H^+ translocation catalyzed by this enzyme was shown by its capability of net ATP synthesis at the expense of an artificial proton motive force imposed across the CM of A. nidulans (14,15). In our hands the ineffectiveness of DES and vanadate seems to exclude a H^+-translocating ATP hydrolase which, however, might be present in the CM of filamentous cyanobacteria (16). For comparison, Fig. 4 shows the effect of DCCD and CCCP also on the light-induced H^+ efflux from A. nidulans. Although we did not investigate this process in more detail using inhibitors other than CCCP and DCCD (Fig. 4) the 100% inhibitory effect of DCCD clearly points to a reversible F_0F_1-ATPase. At

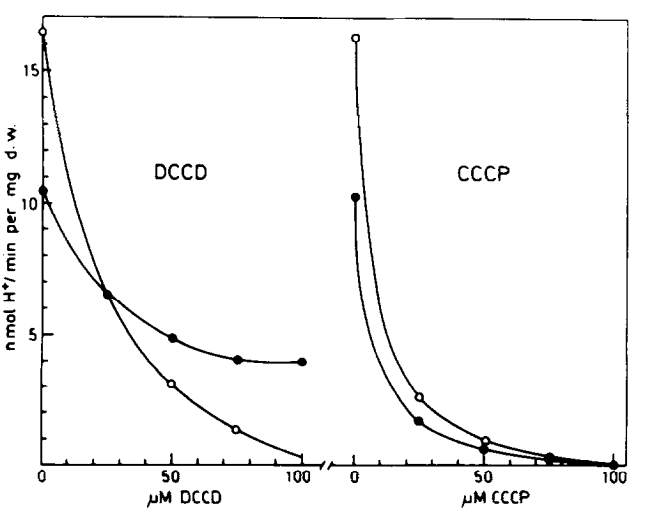


Fig. 4 Inhibition by DCCD and CCCP of the proton efflux from A. nidulans (pH 7.4) shifted from anaerobic dark to light conditions (o-o) and from dark anaerobic to aerobic conditions (•-•). Samples contained 9 mg dry weight of cells per ml.

the same time these findings exclude a major contribution by CM-bound photosynthetic electron transport (13).

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