Increased levels of cytochrome oxidase and sodium-proton antiporter in the plasma membrane of *Anacystis nidulans* after growth in sodium-enriched media

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Received 23 June 1986

Typical rates of horse heart ferrocytochrome *c* oxidation by purified plasma membrane preparations of the cyanobacterium *Anacystis nidulans* after one week of growth at low (0.03 M) and high (0.4–0.5 M) Na^+ concentrations were 15 and 170 nmol/min per mg protein, respectively. Virtually no change was observed in the cytochrome oxidase activity of isolated and purified thylakoid membranes (approx. 3–5 nmol cyt *c*/min per mg protein irrespective of Na^+ concentration during growth). Concomitantly, the rate of whole cell respiration (oxygen uptake) and the capacity for Na^+/H^+ exchange (per mg dry wt cells) by 0.4 M NaCl-grown cells were 7- and 5-fold enhanced, respectively, compared with control cells. The data are in accordance with electron transport-driven Na^+ extrusion from respiring *A. nidulans*, primarily mediated by the plasma membrane-bound cytochrome oxidase.

Respiration *Cytochrome-c oxidase* *Na^+/H^+ antiport* *Plasma membrane* *Thylakoid membrane* (Anacystis nidulans) *Cyanobacteria*

1. INTRODUCTION

A characteristic feature of all living cells is the active maintenance of low internal Na^+, but high K^+ concentrations [1]. In respiring *Anacystis nidulans* [K^+] and [Na^+] are around 150 and 5 mM, respectively [2,3]. As in other bacteria [4] the dynamic exclusion of Na^+ from the cell is achieved by a Na^+/H^+-antiporter [5–8]. The transmembrane proton gradient utilized by the antiporter may stem directly from (respiratory) electron transport in the CM [5,8–11]. We have now found that cyt oxidase levels in isolated and purified CM, but not ICM, were drastically enhanced after growth of the cells in the presence of elevated NaCl (but not KCl) and that the capacity for Na^+/H^+ exchange by NaCl-grown cells was much higher than by control cells.

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Abbreviations: CM, plasma membrane; ICM, thylakoid membrane; cyt, cytochrome; chl, chlorophyll; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DABS, diazobenzenesulfonate; GAR-HRP, goat anti-rabbit horseradish peroxidase conjugate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; EPR, electron paramagnetic resonance

2. MATERIALS AND METHODS

Axenic bath cultures of *A. nidulans* (*Synechococcus* sp.), strain 1402-1 (Göttingen, FRG) were grown at 20 $W \cdot m^{-2}$ warm white fluorescent light (as measured with a YSI 65 radiometer, at the surface of the vessels) in modified medium D (30 mM Na^+) at 35°C as described [12]. For growth at elevated NaCl concentrations, one or two days after inoculation the cultures were diluted 1 : 1 with modified medium D containing double-strength NaCl so as to give the final concentrations desired.

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00145793/86/$3.50

All media were autoclaved before use and all handling of the cultures was done aseptically in a Pathfinder laminar-flow sterile box. Cells were harvested after 1–10 days of growth by centrifugation (4000 × *g*, 10 min, room temperature), washed twice with triple-distilled water and resuspended in 5 mM NaCl, 2 mM Na_2EDTA and 10 mM Hepes/NaOH buffer, final pH 7.4 [12].

Membranes were isolated by passing lysozyme/EDTA-treated cells through a precooled French pressure cell [12,13] and a rough separation of yellow plasma membranes (CM-0) and green thylakoid membranes (ICM-0) was achieved by discontinuous sucrose density gradient flotation centrifugation [13]. Individual membrane fractions were withdrawn from the flotation gradient, pelleted, resuspended in small volumes of 10 mM Hepes/NaOH buffer (pH 7.4), and purified by recentrifugation on conventional discontinuous sucrose density gradients [12]. Membranes equilibrating at 30 and 35/42% (w/w) sucrose were designated CM-I (-II) and ICM-I (-II), i.e. plasma and thylakoid membranes, respectively, after one (two) step of purification (cf. table 1).

Identification of the yellow membrane fraction with CM was performed by labeling of intact cells, prior to breakage and membrane isolation, with a membrane-impermeant, covalently reacting protein marker, [^{35}S]DABS [14]. Radioactivity of isolated and separated membranes was determined in a Packard Tricarb 3320 liquid scintillation counter, and corrected for quenching by membrane pigments.

Oxidation of horse heart ferrocytochrome *c* (type VI of Sigma) was measured with a Shimadzu UV-300 dual-wavelength spectrophotometer at room temperature using $\Delta\epsilon(\text{red} - \text{ox}) = 19.5\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ at 550–540 nm [12]. SDS-PAGE, Western blotting, and immunological cross-reaction with antibodies against aa_3-type cyt oxidase from *Paracoccus denitrificans* [15] were performed as described for crude membranes using antibodies against subunits I and II, and GAR-HRP as the second antibody [16].

The capacity for Na^+/H^+ exchange by intact cells was assayed on dark anaerobic cell suspensions by the extent of acidification of the weekly buffered medium (1 mM Hepes/Tris buffer, pH 7.4) in response to anaerobic NaCl pulses (0.1 M final concentration) using a Philips digital PW 9409 pH meter, connected to a Goertz recorder [9]. Chlorophyll and protein were determined according to [17] and [18], respectively.

3. RESULTS

Growth curves of *A. nidulans* in the absence and presence of 0.4 M NaCl or KCl are shown in fig.1. The lag phase apparently reflects adaptation to NaCl [9]; with KCl growth proceeding as in low-salt control cultures. Harvested and washed cells grown with or without 0.4 M NaCl or KCl for one week gave normal rates of photosynthetic O_2 evolution of up to 250 μmol/h per mg chl (not shown; for respiration cf. fig.3).

Characterization of isolated CM and ICM in terms of [^{35}S]DABS labeling, chl/protein ratios, and cyt oxidase activities at different stages of purification is given in table 1. The same pattern was obtained for membranes from control and salt-grown cells. In both cases the oxidation of cyt *c* was completely inhibited by 1–2 μM KCN, and highly sensitive to NaN_3 and CO [12]. Both CM and ICM from DABS-labeled cells were devoid of cyt oxidase activity (not shown). SDS-PAGE and

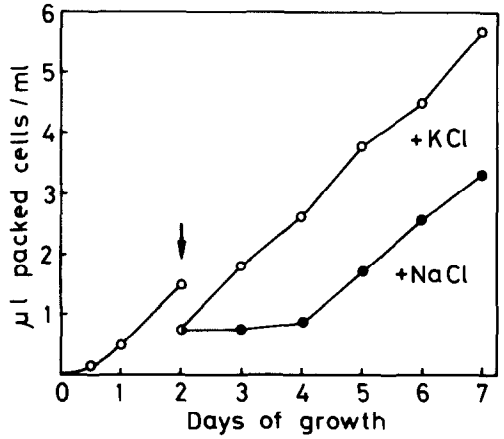

Fig.1. Photoautotrophic growth of *A. nidulans* in the absence and presence of KCl and NaCl (0.4 M final concentration). (Arrow) Dilution (1:1) of cultures with fresh medium containing 0.8 M NaCl or KCl. Growth behavior of control cultures in the absence of additional salt was the same as with KCl (not shown). Linear instead of logarithmic growth curves probably resulted from increasing light limitation (self-shadowing of cells) with progressive growth at the rather low light intensity used (cf. section 2).

Table 1

Characterization of plasma (CM) and thylakoid (ICM) membranes from *A. nidulans* in terms of chl/protein ratios, [^{35}S]DABS labeling and cyt oxidase activity during progressive purification (cf. section 2)

Membrane fraction	Chl/protein[a] (w/w)		cpm per mg protein[b]		nmol cyt *c*/min per mg protein[a]		Time (h) between harvest of cells and assay of membranes
	−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl	
CM-0	0.071	0.058	30650	28300	17 (17)[c]	70 (70)[c]	32
CM-I	0.043	0.036	63500	57950	32 (9)	142 (39)	56
CM-II	0.018	0.014	85050	76400	41 (7)	182 (33)	104
ICM-0	0.27	0.25	8500	5100	13 (13)	17 (17)	32
ICM-I	0.49	0.45	3250	2100	7 (10)	9 (12)	56
ICM-II	0.70	0.65	650	550	4 (8)	5 (9)	104
CW[d]	-	-	62500	59850	n.d.		32

[a] Determined on membranes from unlabeled cells

[b] cpm, counts per minute, corrected for quenching by membrane pigments

[c] Values in brackets indicate the activity of M-0 after storage at 4°C in the dark for periods corresponding to M-I and M-II, respectively (cf. last column of the table), thus reflecting inherent deterioration of the membrane-bound cyt oxidase activity in the course of purification and storage

[d] Pellet recovered after flotation gradient centrifugation, probably cell walls together with residual outer membrane; did not contain detectable amounts of chl

Parameters shown were determined after 5 days of growth in the absence (−) or presence (+) of 0.4 M NaCl. Membranes used were from initial flotation gradient centrifugation (-0) and from the first (-I) or second (-II) recentrifugation step (cf. section 2). n.d., not detectable

immunoblotting performed with isolated and purified CM of both control and salt-grown *A. nidulans* indicated that the cyt oxidase present in either type of cells is of aa_3-type [19] as was recently demonstrated with crude membranes [16]. Since the intensity of the immunological cross-reaction (per mg protein) in CM from NaCl-grown cells was higher than in CM from control cells (M. Trnka and G.A. Peschek, unpublished) a higher concentration of cyt oxidase in the former is likely.

Fig.2 describes the dependence of the cyt oxidase activity in CM-I and ICM-I on NaCl concentrations in the growth medium after 5 days of growth. In fig.3 cyt oxidase activities of CM-I and ICM-I, together with oxygen uptake rates of whole cells, are given in relation to the time of growth of 0.4 M NaCl-grown and control cells. The extent of H^+ extrusion from dark anaerobic *A. nidulans* pulsed with 0.1 M NaCl is shown in fig.4 using both 0.4 M NaCl-grown (+) and control (−) cells. While KCl did not elicit any effect at all the capacity for Na^+/H^+ exchange was much higher in NaCl-grown than in control cells.

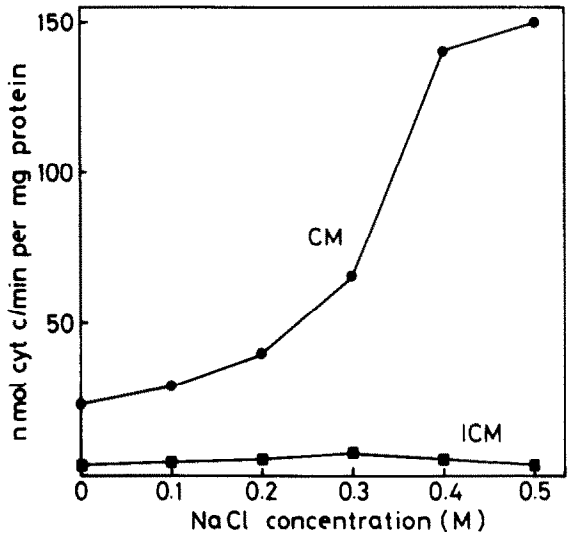

Fig.2. Oxidation of horse heart ferrocytochrome *c* by CM and ICM preparations of *A. nidulans* after 5 days of growth in the presence of various concentrations of NaCl. Data shown are mean values from 5 parallel growth experiments, standard deviations ranging within ± 15% of the corresponding mean.

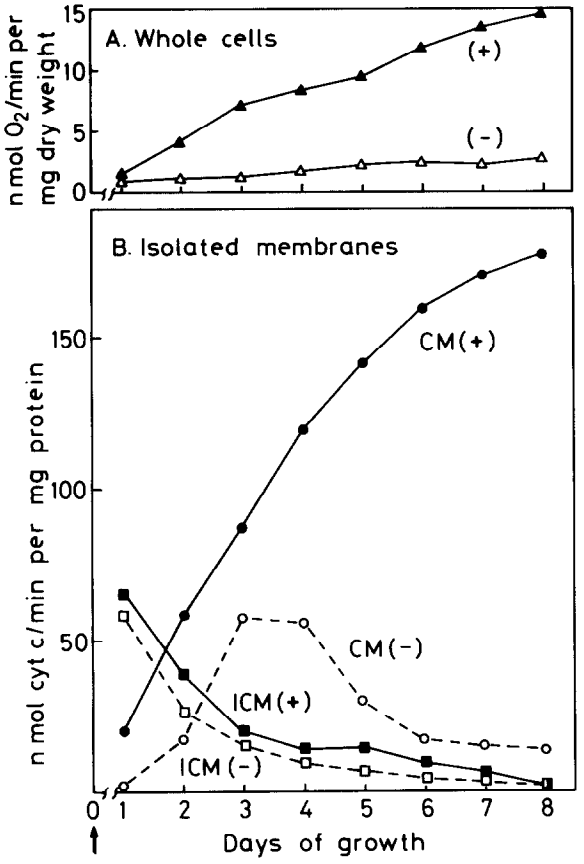

Fig.3. Oxygen uptake (A) and cyt *c* oxidation (B) by whole cells and isolated membranes, respectively, of *A. nidulans* after growth in the absence (−) or presence (+) of 0.4 M NaCl. (Arrow) Time of dilution of the growing cultures (cf. fig.1). For the polarographic determination of O_2 uptake cells were thoroughly washed free of the salt from the growth medium and finally resuspended in 30 mM Hepes/Tris buffer, pH 7.4, at 35°C [5]. Control cells (−) gave the same results as 0.4 M KCl-grown cells both with respect to whole cell respiration and cyt *c* oxidation by isolated membranes (not shown). For standard deviations of experimental data cf. fig.2.

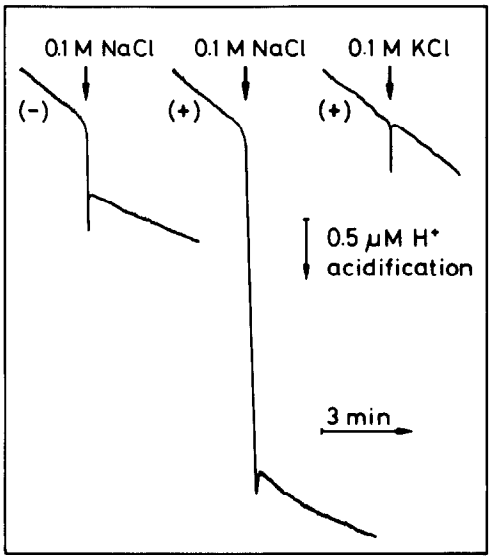

Fig.4. NaCl-induced H^+ release from *A. nidulans* after 5 days of growth in the absence (−) and presence (+) of 0.4 M NaCl as measured with a pH electrode at 35°C (cf. section 2). Cells grown with 0.4 M KCl gave the same results as control cells (not shown). (Arrows) Addition of anaerobic solutions of NaCl or KCl (final concentrations) to dark anaerobic cell suspensions (22 mg dry wt/ml throughout). pH changes were calibrated by injection of a few microliters of 0.01 N HCl (Titrisol, Merck). Results from typical experiments are shown (cf. [9]).

4. DISCUSSION

Pronounced enhancement of respiratory activity and Na^+/H^+ exchange was found with whole cells of *A. nidulans* after a few days of growth in the presence of 0.4 M NaCl. In crude membranes from a presumably identical [20] species, *Synechococcus* 6311, the levels of redox-active and EDTA-resistant copper went up 5-fold after growth in 0.5 M NaCl [9]. The intrinsically membrane-bound Cu was characterized by EPR spectrometry as a component of the cyanobacterial aa_3-type cyt oxidase [21]. Further proof of aa_3-type cyt oxidase in *A. nidulans* was obtained from immunological cross-reaction experiments using antibodies against subunits I and II, and against the holoenzyme, of *P. denitrificans* aa_3-type cyt oxidase [16,19]. From these observations it was tempting to assume that growth at high NaCl specifically enhances the cyt oxidase levels in the CM where the enzyme may be involved in providing the trans-CM ΔpH for Na^+/H^+ antiport [5,6,9,11]. In order to check this possibility it was necessary to isolate functionally intact CM [12].

To be sure that our chl-free membrane fraction ('CM-fraction') did, in fact, represent CM we used a non-penetrating protein marker, [^{35}S]DABS, for the labeling of intact cells prior to membrane isolation. Since $<1\%$ of the CM ^{35}S-activity was present in ICM the DABS did act as a largely

membrane-impermeant marker permitting clear-cut identification of our CM fraction. During progressive purification of the membranes there was a marked increase in both ^{35}S and cyt oxidase activities (per mg protein), paralleled by a decrease in chl/protein ratios, in the CM while ICM showed the opposite behavior (cf. table 1). Apparently, the initial flotation centrifugation yielded crude CM-0 and ICM-0 fractions which were still contaminated with each other. Repeated recentrifugation gradually stripped ICM-0 of intrinsically more active CM leaving less active ICM and vice versa, consistent with the observation that both CM and ICM from DABS-labeled cells were devoid of cyt oxidase activity. To which extent the rather low activities of purified ICM must be attributed to scrambled membranes remains unclear at present.

The effect of high NaCl concentrations in the growth medium of *A. nidulans* was almost entirely restricted to the CM the cyt oxidase activity of which rose dramatically while corresponding activities of isolated ICM remained fairly constant. Concomitant with the rise of CM-bound cyt oxidase levels was a roughly 10-fold enhancement of whole cell respiration which points to a truly physiological relation between both processes. Note that fully uncoupled respiration rates of approx. 3 nmol O_2/min per mg dry wt of *A. nidulans* as usually observed in the presence of 50 μM CCCP (now shown) compare well with cyt *c* oxidase activities of 50 nmol/min per mg protein typically displayed by our CM-I preparations. Thus the latter could account for nearly all of the whole cell respiration. The striking dependence on growth phase and medium salinity (Na^+) of the cyt oxidase in CM and ICM, respectively (cf. fig.3), may partly explain previous failures to detect the activity in isolated CM of *A. nidulans* [22] and other cyanobacteria [22,23].

Finally, confirming the notion that H^+-translocating respiratory electron transport (cyt oxidase) in the CM of *A. nidulans* can directly serve a Na^+/H^+ antiporter in the same membrane we measured a 5-fold increase in the capacity for Na^+/H^+ exchange by whole cells after growth in high NaCl, i.e. in cells whose CM-bound cyt oxidase activity was also drastically enhanced. From this the postulated functional link between the cyt oxidase and the Na^+/H^+ antiporter in the CM [5,6,9] is highly suggestive.

ACKNOWLEDGEMENTS

This work was supported by grants from the Austrian Science Foundation, and the Kulturamt der Stadt Wien (to G.A.P.). Thanks are due to O. Kuntner and A. Messner for skilful technical assistance.

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