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Fluorimetric pH measurement in whole cells of dark aerobic and anaerobic cyanobacteria

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9-Aminoacridine and atebrin fluorescence quenching by dark aerobic and anaerobic suspensions of *Anacystis nidulans, Plectonema boryanum* and *Gloeobacter violaceus* was determined at external pH 7–9. Individual pH values in cytosol and thylakoid compartments were calculated from the simultaneously different intracellular enrichment factors of the monovalent and the divalent base. Concomitantly, at external pH 4–7 the cytosolic pH was measured with fluorescein diacetate which is taken up and decomposed in the cell by cytosolic hydrolases. The pH-dependent fluorescence of the free fluorescein, which remains trapped in the cell, monitors the cytosolic pH. The fact that the latter was higher in aerobic than in anaerobic cells, and insensitive to saturating concentrations of dicyclohexylcarbodiimide aerobically, was taken to support the concept of a proton-translocating respiratory chain in the plasma membrane.

Respiration; Plasma membrane; Proton translocation; Fluorescence quenching; intracellular pH; Cyanobacteria

1. INTRODUCTION

Fluorescence quenching accompanying the uptake of suitable fluorescent amines by illuminated chloroplasts [1,2] and chromatophores [3] has been established as a reliable tool for monitoring the pH in cells, organelles and vesicles [4] provided the membrane-enclosed compartment is more acidic than the external medium, and provided the probe meets certain requirements such as being non-toxic, non-binding, not transported by membrane-bound carriers, etc. [4,5]. Probes are used at a concentration of a few micromolar or less [2] thus remaining without influence on the internal pH as such, even at the highest enrichment factors (e.g. c_i up to 10^4-times c_o).

Here, we decided to apply the fluorescence-quenching technique to dark aerobic and anaerobic suspensions of intact cyanobacteria so as to shed some further light on the respiratory proton translocation across the cytoplasmic membrane of cyanobacteria. Respiratory electron transport in these organisms is only poorly understood so far [6,7]. With (prokaryotic) cyanobacteria, possessing two osmotically autonomous intracellular compartments, cytosol and thylakoid, surrounded by two bioenergetically individual membranes, the CM and the ICM, it must be taken into account that, e.g. a weak amine at $pH_o > pH_i$ may be taken up simultaneously, but to different extents, into both CS and IT. Therefore, the pH_i thus calculated is a 'mixed pH_i' since actually two pH_i values exist, pH_i(CS) and pH_i(IT). Simultaneous use of a monovalent (9-amino-

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Abbreviations: CM, plasma membrane; ICM, thylakoid membrane; CS, cytosol; IT, intrathylakoid compartment; FDA, fluorescein diacetate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid

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acridine) and a divalent amine (atebrin) reveals that the intracellular accumulation is different for either amine with respect to both CS and IT thus, via two simultaneous equations, permitting mathematical resolution of pH_i(CS) and pH_i(IT) [8,9]. Moreover, following and adapting the elegant fluorimetric technique developed by Slavik [10] we measured the cytosolic pH directly from the pH-dependent fluorescence of intracellular fluorescein produced from externally applied fluorescein diacetate through cytosolic hydrolases and trapped within the cell due to negligible permeability of cell membranes to free fluorescein [11]. Results obtained by the different techniques employed were consistent and agreed with previously published data from flow dialysis using radiolabeled weak acids and bases [9] and EPR using spin-labeled probes [12].

2. MATERIALS AND METHODS

Axenic cultures of *Anacystis nidulans* (*Synechococcus* sp., strain 1402-1, Göttingen) and *Plectonema boryanum* (ATCC 27894) were grown photoautotrophically in modified medium D [13] at 10–20 W/m^2 warm white fluorescent light and 35–38°C for about 4 and 8 days, respectively [14]. *Gloeobacter violaceus* was grown similarly but in medium BG-11 [21] at 23°C and dim light (approx. 1–2 W/m^2) for 6 months. Cells were harvested by centrifugation, washed twice with 30 mM Hepes-Tris buffer (pH 7.4) and finally resuspended in 30 mM Taps (pH 8–9), Hepes (pH 7–8), Mes (pH 5–7) and citric acid (pH 3–5) previously titrated to the desired pH value with 30 mM Tris base. The following fluorescent amines were used [$\lambda_{ex}/\lambda_{em}$ (nm); pK_1/pK_2; μM final concentration]: atebrin (430/501; 7.9/10.5; 0.325), 9-aminoacridine (250/431; 9.99; 3.125) and acridine orange (258/435; –; 0.125). FDA experiments were performed essentially according to [10]; fluorescein was measured at λ_{ex} 490 and 435 nm, and λ_{em} 520 nm, using the ratio I_{490}/I_{435} for pH calibration (cf. inset to fig.3). Approx. 30 μl packed cells/ml 30 mM citric acid or Mes-Tris buffer (pH 5.5) were incubated with 70 μM FDA for 20 min at 30°C aerobically in the dark. Aliquots of 1 ml were withdrawn, pelleted in an Eppendorf centrifuge, washed four times with the desired assay buffer (pH 4–7; 1 min for each centrifugation), and finally resuspended in 1.0 ml of the buffer. 150 μl of the washed cell suspension was injected into a fluorimeter cuvette containing 3.05 ml of the same buffer. Fluorescence was measured at room temperature in a Shimadzu model RF 540 spectrofluorimeter, equipped with non-interrupting injection and cuv-o-stir rapid mixing device. Note that FDA which per se is non-fluorescent shows increasing hydrolysis (to fluorescein and acetate) at pH values above 7. Ethanolic solutions (final concentration) of CCCP (10 μM) and DCCD (50–70 nmol/μl packed cells) were added to the cell suspensions 5 and 20 min, respectively, before measurement. All data shown are means from 4–7 parallel experimental runs, each using fresh batches of cells, reproducibility being within ± 10–15% of the corresponding mean.

The following formulae were used for the calculation of pH_i(CS) and pH_i(IT) (cf. [2,4,8,9]):

General:

$$c_i/c_o = \frac{Q}{1-Q} \times \frac{1}{V_i} \quad (1)$$

Weak monovalent base (9-aminoacridine):

$$\frac{[M]_i^T}{[M]_o^T} = \frac{K' + [H^+]_c V_c + [H^+]_t V_t}{K' + [H^+]_o} \quad (2)$$

Weak divalent base (atebrin):

$$\frac{[D]_i^T}{[D]_o^T} = \frac{K_1K_2 + K_1[H^+]_c V_c + K_1[H^+]_t V_t + [H^+]_c^2 V_c + [H^+]_t^2 V_t}{K_1K_2 + K_1[H^+]_o + [H^+]_o^2} \quad (3)$$

c_i/c_o, concentration ratio 'in'/'out'; V_i, fractional volume of the osmotic compartment inside the cell; V_c, cytosolic space; V_t, intrathylakoid space; Q, fraction of amine fluorescence that is quenched after the uptake; $[M]^T$, total concentration of monovalent base; $[D]^t$, total concentration of divalent base; K', K_1, K_2, dissociation constants of protonated forms of the bases. Subscripts o, c and t refer to external medium, cytosol and thylakoid, respectively. It is seen that simultaneous applica-

tion of eqns 2 and 3 permits individual resolution of pH_i(CS) and pH_i(IT) (cf. [8]).

In case pH_i(CS) is known from FDA measurements the corresponding pH_i(IT) is calculated from pH_i(mix) (cf. fig.4) according to [9]:

$$10^{-pH_i(mix)} = 10^{-pH(IT)} \cdot V_t + 10^{-pH(CS)} \cdot V_c \quad (4)$$

Intracellular volumes were determined by the [^{14}C]inulin/^3H_2O technique [4] and from the plot ΔpH_i(mix) vs $\log[Q/(1-Q)]$ (cf. eqn 1; also cf. [15]). V_i was found to be 70, 55 and 40% of the packed cell volume for *Anacystis, Gloeobacter* and *Plectonema*, respectively, with V_i(CS) amounting to 90, 100 and 80% of total V_i as was roughly estimated from electron micrographs of thin-sectioned cells (Peschek, G.A. and Sleytr, U.B., unpublished; also cf. [16]). Note, however, that even absolute vales of pH_i(CS) and pH_i(IT) according to eqns 2–4 are fairly insensitive to variations of the V_i values.

3. RESULTS AND DISCUSSION

The time course of 9-aminoacridine, acridine orange and atebrin fluorescence quenching by dark aerobic suspensions of *Anacystis* (pH_o = 9.0), reflecting amine uptake into the cells, is shown in fig.1. The graphs are reminiscent of those obtained with illuminated chloroplasts [2] except that in case of fig.1 the pH gradient ($pH_o > pH_i$) at the boundary membrane(s) was brought about by the steady-state respiration of the cells. From fig.2 it is seen that fluorescence quenching (A) and intracellular net accumulation (B) by dark aerobic *Anacystis* at pH_o 7–9 were higher with atebrin (1) than with 9-aminoacridine (2), owing to the fact that the former behaves as a divalent and the latter as a monovalent base under the conditions used (note the different powers of $[H^+]$ in eqns 2 and 3), atebrin reaching >10-times higher intracellular enrichment factors (c_i/c_o) when compared to 9-aminoacridine, which again is similar to illuminated chloroplasts [2].

In contrast to fluorescence-quenching techniques the FDA method [10] permits immediate and individual estimation of the pH_i(CS) from a comparison with pH-dependent fluorescein fluorescence (fig.3 and inset). This is a safe and reliable method which unfortunately is applicable only

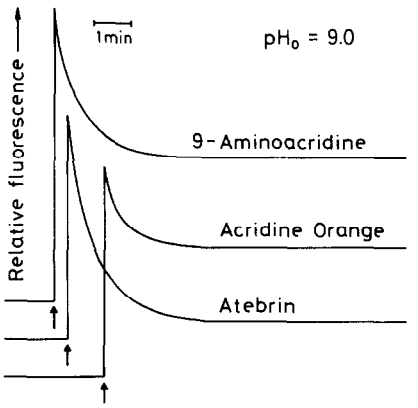

Fig.1. Time course of 9-aminoacridine, atebrin and acridine orange fluorescence quenching by dark aerobic suspensions of *Anacystis* (1.125 μl packed cells/ml) in 30 mM Taps-Tris buffer (pH 9.0) at room temperature. For details cf. section 2. Essentially similar results were obtained with *Plectonema* and *Gloeobacter* (not shown).

below pH 7 due to spontaneous FDA hydrolysis [10]. At pH 4–6 this technique was now applied to intact cyanobacteria incubated under both aerobic and anaerobic dark conditions, in both the absence and presence of saturating concentrations of DCCD which had been shown to eliminate com-

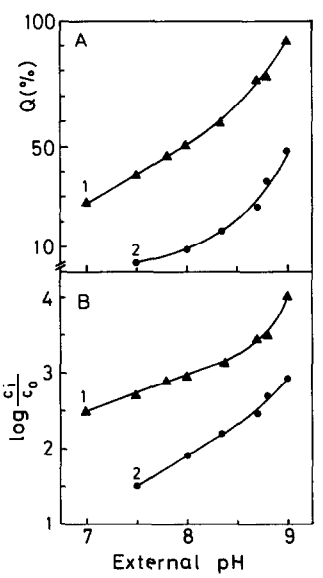

Fig.2. (A) Quenching of atebrin (1) and 9-aminoacridine (2) fluorescence by dark aerobic suspensions of *Anacystis* (1.125 μl packed cells/ml) at different pH_o values calculated from curves of the type shown in fig.1. (B) Net intracellular enrichment factors calculated from (A) by use of eqn 1 (section 2).

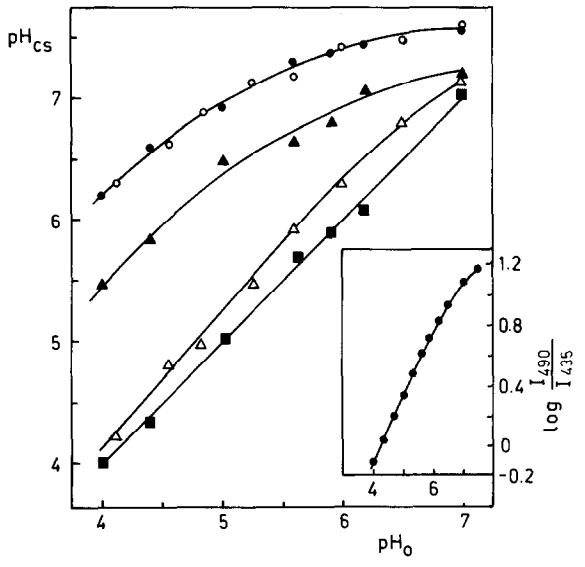

Fig.3. Cytosolic pH of dark aerobic (●,■,○) and anaerobic (▲,△) suspensions of *Anacystis* (1.4 μl packed cells/ml) at different pH_o values, in the absence (●,▲) or presence (○, △) of DCCD, and in the presence of CCCP (■), calculated from the fluorescence of intracellular fluorescein (produced from externally applied FDA) according to the pH/fluorescence calibration curve shown in the inset (cf. section 2). Qualitatively similar results were obtained with *Plectonema* and *Gloeobacter* (not shown).

pletely all oxidative phosphorylation [14,17]. The data shown in fig.3 clearly reflect a more alkaline aerobic cytosol, when respiration builds up the proton gradient across the CM through outwardly directed H^+ extrusion [8,14,17]. Whether this 'energization' of the CM is due to a CM-bound H^+-translocating ATPase, or in addition, directly to respiratory electron transport in the CM was investigated by use of the F_o inhibitor DCCD: In the presence of DCCD, when ATP synthesis by electron transport phosphorylation is blocked [8,17] and both reversible and unidirectional ATPases run out of fuel, pH_i(CS) (or ΔpH_{CS}) remained completely unaffected in aerobic, but almost collapsed in anaerobic conditions [fig.3; note the close proximity of CCCP and DCCD (anaerobic) curves]. Essentially similar results were obtained with *Plectonema* and *Gloeobacter* (not shown). This points to the occurrence of an H^+-translocating respiratory chain in the CM of certain cyanobacteria as was previously envisaged from a variety of whole-cell studies [6,7,17] and recently also from the detection of aa_3-type cytochrome oxidase (possibly H^+-translocating; cf. [18]) in isolated and purified CM preparations of *Anacystis* and other cyanobacteria ([7,19,20]; Molitor, V., Trnka, M. and Peschek, G.A., unpublished).

A formal comparison of FDA (a) and fluorescence-quenching (b) techniques, incorporating the calculation of pH_i(mix) (trace b) according to eqn 4, is given in fig.4. It is seen that both techniques applied to thylakoid-less *Gloeobacter* [21] yield fairly congruent results (since there is only one V_i in this species) while with *Anacystis* and *Plectonema* pH_i(CS), trace a, and

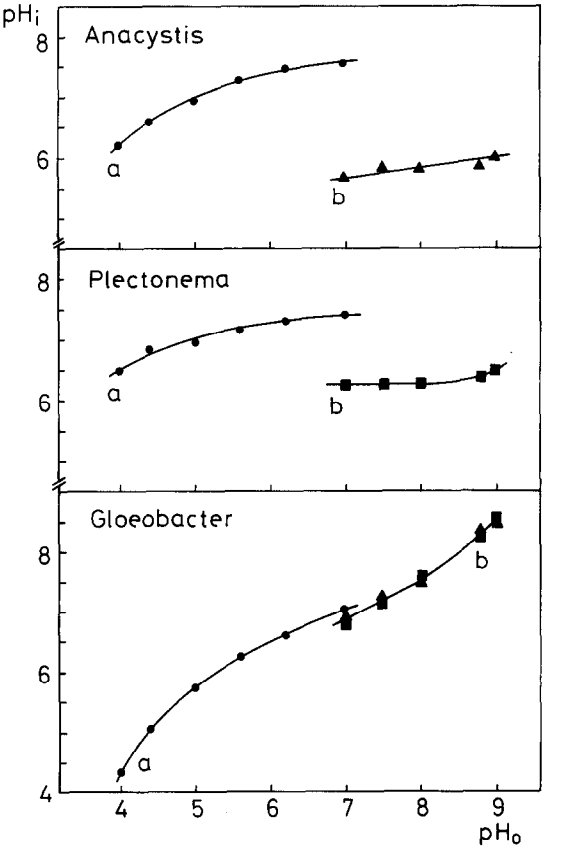

Fig.4. Intracellular pH determined from FDA procedure (pH_i(CS), trace a) and atebrin (▲) or 9-aminoacridine (■) fluorescence quenching (pH_i(mix), trace b) at different external pH values. The cyanobacteria were incubated aerobically in the dark. For details cf. section 2.

pH_i(mix), trace b, never match. Fig.4 also demonstrates that internal pH homeostasis [22] appears much more pronounced in thylakoid-containing species than in *Gloeobacter* which confirms previous results [9,12].

4. CONCLUDING REMARKS

The main goals of the present investigation were the qualitative demonstration (i) of different degrees of internal pH homeostasis in different cyanobacteria as reflected by the slope of the curve pH_i vs pH_o (fig.4); (ii) of two distinct and osmotically autonomous intracellular compartments, viz. the cytosol and the thylakoid, if present, as distinguishable by the different patterns of intracellular accumulation of monovalent and divalent bases (fig.2; eqns 2 and 3); (iii) of the applicability to cyanobacteria of the FDA technique [10,11]; and (iv) of the fact that inhibition of the coupling factor ATPase on the ICM and/or the CM under aerobic conditions does not significantly affect the transmembrane ΔpH irrespective of its actual value (fig.3), thus pointing to direct participation of proton-translocating respiratory electron transport in both CM and ICM [6–9]. These conclusions are based on the relative differential degrees of fluorescence quenching with mono- and divalent amines, at least in part reflecting amine uptake into the cell [2], in response to defined environmental parameters such as aerobiosis or anaerobiosis, extracellular pH, and inhibition of the F_0F_1-ATPase. They are therefore largely independent of the actual magnitude of the calculated ΔpH itself which, according to [23] and others (cf. [5] for review), can be quantitatively derived from fluorescence quenching with reservations only. Thus the fluorescence-quenching technique and eqns 1–3 discussed here may not always give reliable quantitative data but nevertheless render themselves a rapid and practicable tool for studying the qualitative impact of certain defined experimental conditions on the proton concentration gradient(s) across the membrane(s) of intact cyanobacteria as shown in this paper.

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