OXIDATIVE PHOSPHORYLATION IN INTACT CYANOBACTERIA

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1. Introduction

Numerous reports on ATP pools in cyanobacteria have appeared [1–3]. However, kinetic data (rates, efficiencies) rather than pool sizes should be particularly useful for characterizing the dynamic state of living cells. The only information so far available on the efficiency of oxidative phosphorylation in an intact cyanobacterium stems from a comparison of oxygen uptake and CO_2 production with incorporation of exogenous glucose into phosphorylated intermediates and storage polyglucose in *Aphanocapsa* (*Synechocystis*) 6714; this rather indirect approach yielded maximum P/O ratios of ~3 [1].

Experiments with respiratory particles derived from *Anabaena variabilis* [4] and *Anacystis nidulans* (G. A. P., unpublished; cf. [5,6]) gave P/O ratios of ~0.1 which is reminiscent of many other bacterial sytems [7]. Determination of molar growth yields [7] is difficult or impossible with most cyanobacteria showing sluggish, if any, chemoheterotrophic growth [8]. Therefore we measured initial rates of the formation of 'energy-rich' adenylate phosphate bonds (~P) and concomitant oxygen uptake by intact resting cells of *A. nidulans*, *A. variabilis*, and *Nostoc* sp. strain MAC on transition from anaerobiosis to aerobiosis. Alternatively, we used the oxygen pulse technique basically according to [9–11]. Both methods gave P/O ratios of 0.6–2.9 depending on the presence or absence of sodium in growth and/or assay media. Our results with *A. nidulans* support the conclusion that low apparent P/O ratios were caused by active sodium extrusion through the cytoplasmic membrane. Thereby the Na^+/H^+-antiporting system [12] utilizes an electrochemical proton gradient established by a proton-translocating aa_3-type cytochrome oxidase ([13–19]; G. A. P., unpublished) without intervening ATP production. A scheme relating oxidative phosphorylation and sodium extrusion to the sites of respiratory electron transport and, in particular, the terminal oxidase is discussed.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine

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2. Materials and methods

Axenic cultures of *Anacystis nidulans* (*Synechococcus* sp.), *Anabaena variabilis* ATCC 29413, and *Nostoc* sp. strain MAC were grown photoautotrophically at 38°C in a turbidostat as in [6,16]. Cells were maintained in the late logarithmic growth phase to ensure accumulation of intracellular polyglucose [1] which was used as the endogenous respiratory substrate in our measurements. Na^+-depleted cells were obtained by exchanging, during mid-logarithmic growth, the normal Na^+-containing medium (~30 mM Na^+) for a medium that contained K^+ salts only (except for Na^+ from trace minerals). After growth for ⩾10 generations the cyanobacteria were harvested, washed twice with sterile growth medium (carbonate omitted), and suspended in 30 mM Hepes buffer brought to pH 7.5 with either NaOH or KOH. Oxygen uptake was followed with a Clark-type oxygen electrode [13]. All assays were performed at 35°C and pH 7.5, and cell suspensions always contained 30–50 mg dry wt cells/ml.

Adenylates were extracted with EDTA/trichloroacetic acid [20,21]. The time required for quenching the cells (<0.5 s, cf. [20,21]) was negligible as compared to adenylate turnover rates which, in dark resting cells of cyanobacteria, were ⩾4–5-times lower

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than those reported for typically heterotrophic bacteria (cf. fig.1 with fig.1 of [11]). After exhaustive extraction with ether, the neutralized and centrifuged extracts were assayed for ATP by the firefly method [20,21] using a Biolumat Photon Counter. ADP and AMP were enzymatically converted into ATP [20] in aliquots of the same quenched cell suspension and determined as ATP. Energy-rich phosphate (~P) was taken to equal 2 [ATP] + [ADP] [9,22]. Intracellular sodium was determined by quantitative flame photometry using a Perkin-Elmer atomic absorption spectrophotometer, model 300. After incubation with NaCl, the cells were quickly separated from the suspension medium by centrifugal filtration (Eppendorf Centrifuge, model 5412), cuvettes containing successive layers of cell suspension (50 μl), Silicon oil, type SF 50 (General Electric, USA; 35 μl), and 30% H_2SO_4 (20 μl) as a quencher. Anaerobic conditions were achieved by sparging with argon, and maintained by layering paraffin oil (40 μl) on top of the suspensions. Care was taken to conduct all experiments in strict darkness or green light of low intensity. All data shown are means of ⩾5 representative determinations, deviations usually ⩽5–10% of the corresponding mean. The chemicals used were of the highest purity grade commercially available. Purity of cyanobacterial cultures was always carefully checked under a phase contrast microscope; contaminated cultures were discarded.

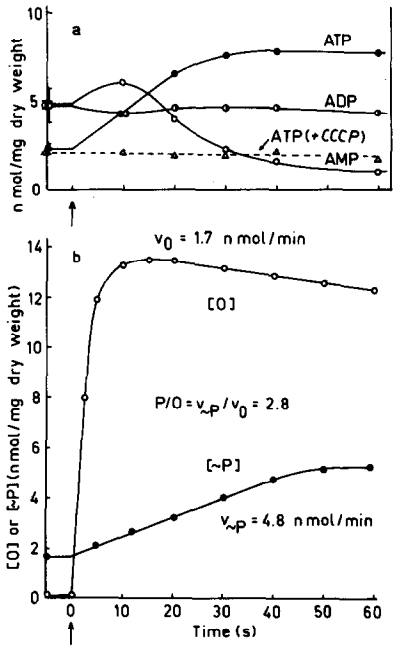

Fig.1. Kinetics of adenylate levels (a) and of coupled O_2 uptake and ~P formation (b) in whole cells of cyanobacteria on transition from anaerobiosis to aerobiosis. Arrow indicates aeration of previously anaerobic suspensions. Relative changes of the individual adenylates typically reflected the action of adenylate kinase in the cells [22]: (a) Na^+-depleted *Anacystis*; (b) Na^+-depleted *Nostoc* cells (cf. section 2). No difference between Na^+-rich and Na^+-depleted cells was observed in (a) while only Na^+-rich cells gave a pronounced enhancement of post-anaerobic O_2 uptake (cf. table 1). (△---△) ATP levels in presence of 50 μM CCCP; ~P = 2 [ATP] + [ADP].

3. Results

Fig.1a shows changes of the pool sizes of ATP, ADP and AMP in whole cells of Na^+-depleted *A. nidulans* passing from anaerobic to aerobic conditions. All of the oxygen-induced increase of ATP was due to oxidative phosphorylation as seen from the action of CCCP (——). The shape of the adenylate curves was the same with all 3 cyanobacteria (not shown) and reflected the action of adenylate kinase [22]; hence 'energy-rich phosphate' (~P) had to be expressed as 2 [ATP] + [ADP] [22]. Rates of ~P formation and respiration in Na^+-depleted *Nostoc* are given in fig.1b; a P/O ratio of 2.8 is derived. Oxygen uptake by *Anacystis* cells grown and assayed in Na^+-rich media was found to increase dramatically after anaerobiosis (table 1) while adenylate levels responded normally (not shown, cf. fig.1a). The post-anaerobic enhancement of O_2 uptake was saturated after 15–20 min anaerobiosis (not shown); it was much smaller in Na^+-free conditions (table 1). Also the effect was less pronounced with (facultatively chemoheterotrophic) *Anabaena* and *Nostoc* (table 1).

On addition of 50 or 100 mM NaCl to cells respiring

Table 1
Rates of respiratory oxygen uptake (nmol O . min^{-1} . mg dry wt^{-1}) before (A) and after (B) 20 min anaerobiosis

Assay medium (buffer)	*Anacystis*		*Anabaena*		*Nostoc*	
	A	B	A	B	A	B
Na^+	3.2	17.6	7.9	15.2	9.2	12.1
K^+	1.3	1.8	6.3	7.0	7.8	9.0

Na^+-depleted cells were suspended in 30 mM Na^+ or K^+/Hepes buffer. Anaerobic conditions were achieved by sparging the suspensions with O_2-free N_2 for 20 min

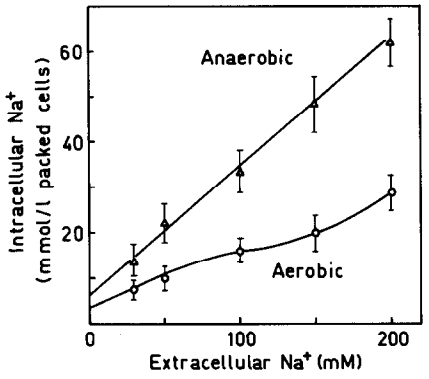

Fig.2. Dependence of intracellular Na^+ levels in *A. nidulans* on extracellular [Na^+] in anaerobic and aerobic dark conditions. Measurements performed as in section 2. Cells were grown in normal medium (containing ~30 mM Na^+) and assayed in Tris/Hepes buffer (pH 7.5) to which the desired [NaCl] was added. Equilibration between external and internal sodium was allowed to proceed for 20–30 min at 35°C in the dark.

in 30 mM Tris–Hepes buffer (pH 7.5) the resulting increase in O_2 uptake was <50% of the post-anaerobic increase observed with cells in Na^+ buffer of corresponding molarities ('salt respiration', not shown; cf. [23]). In fig.2 Na^+ accumulation within *A. nidulans* was much more pronounced anaerobically than aerobically, thus reflecting the different energy states (cf. fig.1).

Therefore, if one calculates P/O ratios by relating the post-anaerobic O_2 uptake to the concomitant rise of ~P (fig.1, table 2) the presence of sodium will result in seemingly lowered coupling efficiencies, and similarly low P/O ratios were measured by the direct oxygen pulse method ([9]; not shown). However, in both types of experiments efficiencies as high as 2.9 were measured provided Na^+-depleted cells were assayed in K^+ (or Tris)/Hepes buffer (table 2, fig.1b).

4. Discussion

Our results demonstrate that sodium markedly raises the energy costs of metabolism in dark resting cells of cyanobacteria. Generally, living cells discriminate between Na^+ and K^+, the former being excluded from, the latter being accumulated in, the cell. By way of passive diffusion Na^+ rather rapidly appeared to enter the cyanobacterial cells, particularly of *A. nidulans* ([12,24]; cf. table 2, fig.2). This may not be the case with K^+ [12,24]. Thus energy must continuously be expended for active Na^+ extrusion so as to maintain the 'cation-regulated state' [24]. Consequently, elevated [Na^+] in the environment would result in an increased need for energy of maintenance. This might eventually lead to 'obligate' phototrophy even in a tightly coupled respiring cell provided the rate of respiratory electron transport is substantially lower than the rate of photosynthetic electron transport which, in fact, appears to be true of most cyano-

Table 2
Changes of adenylate levels in whole cells of *A. nidulans* during the first 5 s of transition from anaerobiosis to aerobiosis

Exp.	ATP	ADP	AMP	~P	O	P/O
1	6.0	−1.2	−4.8	10.8	−17.4	0.6
2	5.8	0.6	−6.4	12.2	−6.0	2.0
3	1.3	2.4	−3.7	5.0	1.7	2.9

Concomitant oxygen uptake (O) and calculated P/O ratios are also shown. Rates expressed as nmol . min^{-1}. mg dry wt^{-1}: no sign, increase; negative sign, decrease; (1) normally grown cells (~30 mM Na^+) assayed in 30 mM Na^+/Hepes buffer; (2) normally grown cells assayed in 30 mM K^+/Hepes buffer; (3) Na^+-depleted cells assayed in 30 mM K^+/Hepes buffer; ~P = 2 [ATP] + [ADP]. Similar results were obtained with *Anabaena* and *Nostoc*, and the post-anaerobic rise of [ATP] was always abolished by 50 μM CCCP (not shown; cf. fig.1a)

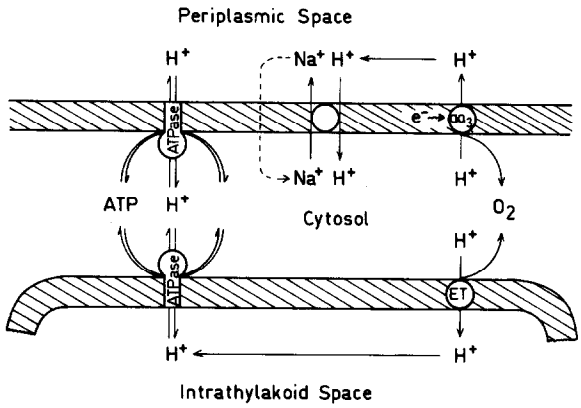

Fig.3. Scheme of H^+ and Na^+ movements, ATP production and consumption, and oxygen reduction in the cytoplasmic and/or the thylakoid membranes of *Anacystis nidulans*: ET, respiratory and/or photosynthetic electron transport in the thylakoid membrane; aa_3, cytochrome oxidase [13–16] functioning as a proton pump (G. A. P., unpublished; cf. [29]) and present in both thylakoid (not shown) and cytoplasmic membranes [17–19,28]; $e^-\rightarrow$, donation of electrons to the cytoplasmic membrane-bound terminal oxidase through hitherto unknown intermediates; – – →, passive influx of sodium.

bacteria [4,8,25,26]. Table 1 suggests the deleterious effect of Na^+ is much smaller in (facultatively chemoheterotrophic) *Anabaena* and *Nostoc* as compared to (obligately phototrophic) *Anacystis*. Intrinsically different permeabilities of the cytoplasmic membrane towards Na^+ might offer an explanation. Halophilic and halotolerant species may apply a different strategy to sodium, and it would be interesting to study the post-anaerobic enhancement (if any) of O_2 uptake in such organisms. Yet, be it noted that typically halophilic cyanobacteria such as *Oscillatoria limnetica* and *Aphanothece halophytica* are known to be strict phototrophs [27].

In [12] a cytoplasmic membrane-bound ATPase involved in Na^+ extrusion from *A. nidulans* through a Na^+/H^+ antiporter, ultimately powered by ATP from thylakoid-bound electron transport, was favoured. Supplementary to [12] and in agreement with [17–19,28] we suggest a more economic mechanism (fig.3). According to our hypothesis, respiratory electron transport in the cytoplasmic membrane participates in maintaining a proton electrochemical gradient across the cytoplasmic membrane of dark aerobic *A. nidulans*. A proton-translocating aa_3-type cytochrome oxidase ([13–19,30]; G. A. P., unpublished; cf. [29]) is involved in the build-up of the gradient which, in this way, can be formed without participation of ATP (fig.3). Cytoplasmic membrane-bound respiratory electron transport has been implicated also for *A. variabilis* [31,32] and *Plectonema boryanum* [33] from measurements of K^+ uptake [31], Arrhenius discontinuities of electron-transport activities [32] and proton electrochemical gradients [33], respectively.

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