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PHOSPHORYLATION AND NITROGENASE ACTIVITY IN ISOLATED HETEROCYSTS FROM *ANABAENA VARIABILIS* (ATCC 29413)

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Adenylate-pool composition, energy charge, and nitrogenase activity were examined in isolated heterocysts from *Anabaena variabilis* (ATCC 29413). ATP formation was detected as a light- or oxygen-induced increase in ATP concentration. No cofactors or substrates had to be added for photophosphorylation to occur, whereas oxidative phosphorylation was dependent on hydrogen and oxygen (Knallgas reaction). The increase in ATP concentration was reflected by a decrease in AMP concentration, accompanied by small changes in ADP levels. Thus, a regulation of the adenylate pool by a myokinase (adenylate kinase) has to be assumed. Upon dark-light transitions, the energy charge in heterocysts increased from values below 0.4 to values approaching 0.8. High energy-charge values, reached in the light only, allowed for high rates of acetylene reduction in the presence of hydrogen. The increase in the energy charge in the dark to approx. 0.64 by addition of oxygen (5% (v/v) in the presence of hydrogen) resulted in low nitrogenase activities, generally not exceeding 1–3% of the light-induced rates. In the dark, oxygen concentrations above 10% were inhibitory to both ATP formation and acetylene reduction. Increasing light intensities led to a steep increase in energy charge followed by an increase in nitrogenase activity. Plotting enzyme activity versus energy charge, a nonlinear, asymptotic relationship was observed.

Introduction

When grown without combined nitrogen, many filamentous blue-green algae (cyanobacteria) show a differentiation into two distinct cell types: vegetative cells and heterocysts [1]. As shown by different experimental approaches, heterocysts are the major site of nitrogen fixation. A multilayered envelope, absence of an oxygen-evolving Photosystem II, and respiratory activity provide the anaerobic environment necessary for a functional nitrogenase (for a review, see Ref. 2).

Isolated heterocysts show high rates of nitrogen fixation (= acetylene reduction) only in the presence of light and hydrogen [3,4], due to the basic requirements of nitrogenase for ATP and a low-potential reductant [5]. Although it is clear that hydrogen provides electrons for acetylene reduction, the pathway of electron donation is still open to question [6–14]. The necessary ATP might be generated in the light by cyclic phosphorylation [15,16] or by noncyclic electron transport, with hydrogen as donor to Photosystem I [6]. In the dark, ATP is formed by an oxyhydrogen (Knallgas) reaction [17].

Using isolated heterocysts exhibiting high rates of acetylene reduction, we have demonstrated changes in the adenylate pools under a variety of conditions. The data presented in this study show for the first time that ATP is formed in isolated heterocysts without any artificial cofactors added.

Abbreviations Tes, *N*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid, Chl, chlorophyll

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Thereby, using this system, the in vivo relationship between energy metabolism and nitrogenase activity can be evaluated.

A preliminary report of the data has been published at the Second European Bioenergetics Conference (Lyon, France), EBEC Reports Vol 2, 1982, p 277.

Materials and methods

Culture conditions Anabaena variabilis Kutz (ATCC 29413) was grown autotrophically in mineral medium [18] at 29°C, as described previously [19]. The cultures were inoculated to give a final chlorophyll concentration of 0.4–0.6 μg Chl/ml After an initial lag phase, doubling times of about 10 h were achieved. The algae were harvested after 42–48 h at a chlorophyll concentration of 6–10 μg/ml, and exhibited a nitrogenase activity of 40–60 μmol/mg Chl per h.

Isolation of heterocysts All experimental manipulations were performed at room temperature After harvesting by centrifugation (400 × *g*, 5 min), the filaments were washed once in medium A (pH 7 6) containing (mM). Tes-NaOH, 10, $MgCl_2$, 10, sodium/potassium phosphate, 5. Thereafter, preparation was performed in septum-stoppered centrifugation tubes under anaerobic conditions All buffers were sparged with argon/hydrogen (95/5% (v/v)) or hydrogen After a 3 min centrifugation at 750 × *g*, the filaments were resuspended in incubation medium B (pH 7 1) containing (mM). sorbitol, 300; Tes-NaOH, 10; $MgCl_2$, 10; sodium/potassium phosphate, 5, in addition, it contained bovine serum albumin (0.5%) and lysozyme (1 5 mg/ml) The chlorophyll concentration of the suspension was 130–180 μg/ml.

The algal suspension was incubated at 30°C for 1 h under a hydrogen or hydrogen/argon atmosphere and stirred continuously. The lysozyme-treated cells were sedimented by short centrifugation (250 × *g*, 90 s) and resuspended in 20 ml of medium A. After a sonification period of 2–3 min in a sonic cleaning bath (Sonorex PK102, 240 W, Bandelin, Berlin), heterocysts were sedimented by centrifugation at 250 × *g* for 5 min The heterocyst preparation was washed two to three times in 20 ml medium B (without lysozyme) until it appeared essentially free from contaminating vegetative cells by microscopic inspection Dithionite was omitted from all buffers used, since no consistent beneficial effect had been observed

Reduction of acetylene. Nitrogenase activity was determined by measuring ethylene formation in the gas phase 9-ml vials closed with septum stoppers were flushed with hydrogen for 15 min and 0.5 ml of a heterocyst suspension (30–90 μg Chl/ml) was added The reaction was started by addition of 1 ml acetylene Nitrogenase activity was measured during 45 min of illumination with white light at 30°C Ethylene formation was detected by gas chromatography (Varian M940, equipped with a 2-m Porapack-R column and a flame-ionization detector) and quantitated using a Hewlett-Packard (HP 3385) integrator. The activity was evaluated from three measuring points between 10 and 45 min, since short lag phases (up to 10 min) were frequently observed

Extraction of adenylates. 0 5 ml of the heterocyst suspension (30–90 μg Chl/ml) was precipitated by addition of 0.5 ml ice-cold perchloric acid (1.4 M) and 0.1 ml EDTA (100 mM) Adenylates were extracted in ice for 30 min After addition of 0.05 ml triethanolamine (1.8 M), the samples were centrifuged at 8000 × *g* for 5 min (Eppendorf 5411), and the supernatant was either frozen and stored in liquid nitrogen or neutralized immediately with 5 M KOH

Quantitative determination of adenylates Neutralized extracts (pH 7.6) were analyzed for ATP and ADP by bioluminescence (LKB/Wallac Luminometer 1250) according to the method of Larsson and Olsson [20] Total nucleotide pool (ATP + ADP + AMP) was determined by adding to 0 27 ml of extract. first, 20 μl of a mixture containing KCl (1.9 M), $MgCl_2$ (50 mM), phospho*enol*pyruvate (5 mM), then 5 μl pyruvate kinase (5 mg/ml) and 5 μl myokinase (1 mg/ml). The myokinase suspension in $(NH_4)_2SO_4$ was centrifuged and resuspended in triethanolamine (200 mM) prior to use. The AMP assay mixture was incubated for 15 min at 30°C Control experiments with AMP standards showed that conversion to ATP was complete under these conditions when the nucleotide concentration was above 1 μM Internal standards of ATP were added to each determination.

Calculation of the ATP concentration as a func-

tion of energy charge (EC [21]) and the apparent equilibrium constant K of myokinase was performed according to the equation (cf. ref. 22):

$$[ATP] = \frac{A[(4K-1)EC+1/2]-A\sqrt{(1-4K)(EC^2-EC)+1/4}}{4K-1}$$

$$A = [ATP]+[ADP]+[AMP]$$

$$K = [ATP][AMP]/[ADP]^2$$

$$EC = \frac{[ATP]+1/2[ADP]}{[ATP]+[ADP]+[AMP]}$$

The complete set of experimental data as described in Figs 1 and 2 and Table I was repeated four times. One typical experiment was selected for presentation.

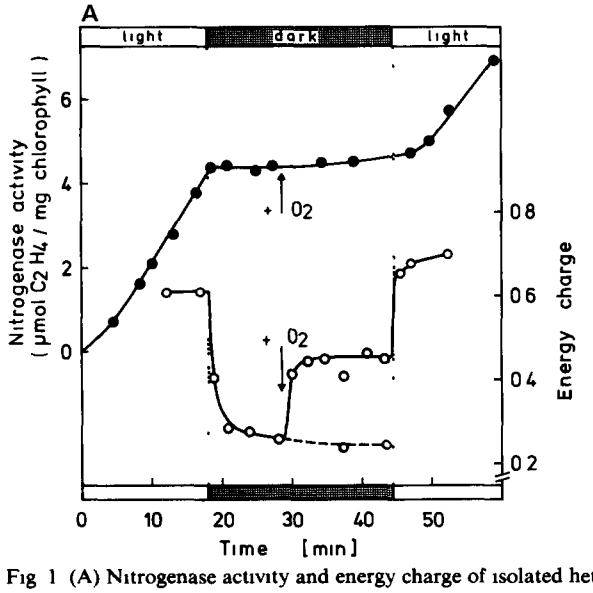
Chl a Chl. *a* was determined after methanol extraction using the extinction coefficient of Mackinney [23].

Chemicals Lysozyme (grade IV), Tes and bovine serum albumin were obtained from Sigma, Munchen. Enzymes, nucleotides, and phospho*enol*pyruvate were from Boehringer, Mannheim; other substances were of the highest analytical grade available.

Results

Phosphorylation and nitrogenase activity in heterocysts

Studies with purified nitrogenase have demon-



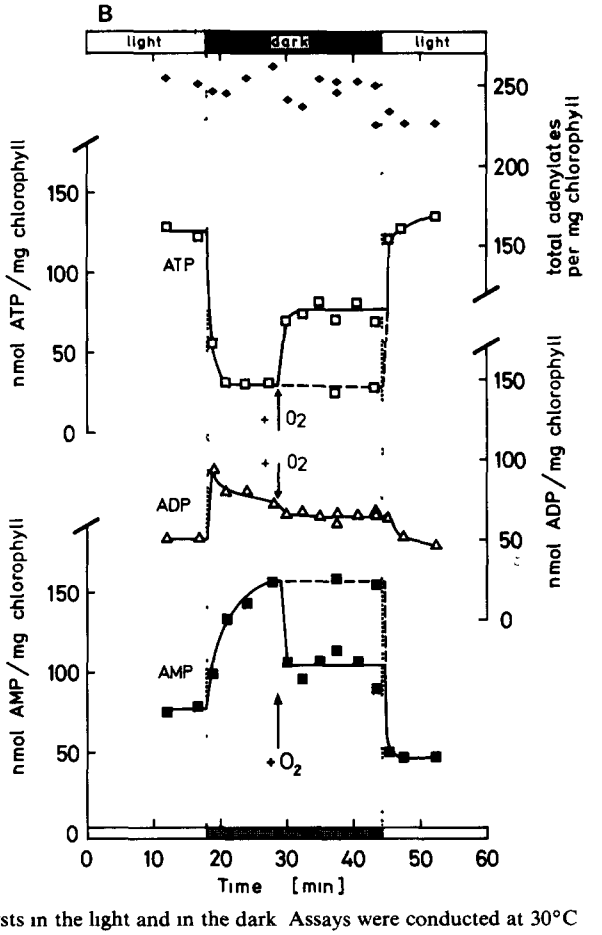


Fig 1 (A) Nitrogenase activity and energy charge of isolated heterocysts in the light and in the dark Assays were conducted at 30°C with heterocysts equivalent to 113 µg Chl/ml reaction mixture, in an atmosphere of H_2/C_2H_2 Due to the high chlorophyll concentration, the acetylene-reduction rate was partly light limited at 20 W/m² of white light as used in these experiments After 30 min, 0 45 ml (approx 5%) of oxygen was added to nine reaction vessels, as indicated, two control vials were kept anaerobic After the incubation periods, as indicated by the time scale, samples were taken from the gas phase for analysis of ethylene formation, immediately afterwards, the reaction was stopped by addition of perchloric acid for determination of adenylate concentrations The energy charge was computed from the equation given in Materials and Methods Nitrogenase activity (= ethylene formation) (●———●), energy charge (○———○), values measured in the absence of oxygen (------) (B) Concentrations of adenylates in the light and in the dark, in the absence and presence of oxygen The amounts of extracted adenylates of the experiments described in A are shown, adenylate concentrations have not been corrected for variations in the total adenylate pool (◆), ATP (□———□), ADP (△———△), AMP (■———■)

TABLE I

DEPENDENCE OF ENERGY CHARGE AND NITROGENASE ACTIVITY ON OXYGEN CONCENTRATIONS, MEASURED WITH ISOLATED HETEROCYSTS IN THE DARK

The chlorophyll concentration was 59 μg/ml, the reaction vials were flushed with hydrogen before the desired amount of oxygen was added The reaction was started by addition of 1 ml acetylene, nitrogenase activity was averaged from four measuring points taken between 10 and 70 min, for details of energy-charge determination cf Fig 1A and Materials and Methods

% oxygen	Energy charge ([ATP]+1/2[ADP]/ [ATP]+[ADP]+[AMP])	Nitrogenase activity (μmol C_2H_4/mg Chl per h)
Dark		
0	0 29	0 02
1	0 57	0 39
2	0 58	0 64
5	0 64	1 32
10	0 35	1 27
18	0 24	0 50
Light		
0	0 76	115 0

strated a specific requirement of MgATP for activity; MgADP is a competitive inhibitor, whereas MgAMP is less effective [24,25]. Using isolated heterocysts with high nitrogenase activities, we therefore analyzed the concentrations of adenylates in the light and in the dark, with and without oxygen present. All assays contained hydrogen and acetylene (90/10% v/v), allowing for simultaneous measurement of nitrogenase activity, which was hydrogen dependent [4]. A typical experiment, as shown in Fig. 1A, reveals that acetylene reduction proceeds linearly in the light, after a lag phase of several minutes. In the dark, negligible activities were found. Addition of oxygen (5%, v/v) resulted in a small, but detectable rate never exceeding 3% of the rates measured in the light (cf. Table I). Turning on the light again, a lag phase and a lower, light-induced activity was observed, however, only when oxygen was present (Fig 1A, cf. also Ref 4).

Atkinson introduced the energy charge as a measure of cell energization [21]. As shown in Fig. 1A, energy charge in heterocysts rises from 0.3 to 0.7 upon dark-light transitions under nitrogen-fixing conditions. In the presence of hydrogen, addition of oxygen (5%) also leads to a substantial increase in energy charge, but, as already mentioned, only low rates of acetylene reduction were measured. This is shown in more detail by the data summarized in Table I The optimum oxygen concentration of the oxyhydrogen reaction coupled to ATP formation was approx 5% Higher concentrations of oxygen inhibited phosphorylation and nitrogenase activity, probably due to the oxygen sensitivity of hydrogenase and nitrogenase [26]

Dark-light or anaerobic-aerobic transitions increase the ATP concentration in heterocysts due to photosynthetic or respiratory activities. The rise as well as the decay of the ATP pool is fast and completed within 1–2 min Analyzing the concentrations of the adenylates present, we found that changes in ATP concentration were mainly reflected by changes of AMP concentrations, with little variation of the ADP pool (Fig. 1B). As will be discussed later, this indicates the presence of a myokinase (adenylate kinase), balancing adenylate concentrations according to its equilibrium constant.

The data presented in Fig. 1A and B clearly show that steady states of adenine nucleotide concentrations can be established at different values of energy charge, with nitrogenase being active at high levels of ATP only A light-induced increase in the ATP pool was also observed when argon replaced hydrogen. Hence, photophosphorylation did not require hydrogen; in the absence of hydrogen, nitrogenase activities were, of course, very small (data not shown).

Nitrogenase activity and cell energization

Light-dependent nitrogenase activity was examined at different light intensities. In the experiment shown in Fig. 2A, the energy charge reached relatively high values (greater than 0.7) already in dim light (3–5 W/m^2), corresponding to only a few percent of maximum nitrogenase activity. With further increasing light intensities, little increase in energy charge was observed; saturation of the acetylene-reduction rate was reached above 50 W/m^2. Clearly, there is no linear dependence of nitrogenase activity on energy charge. This has also been observed with other

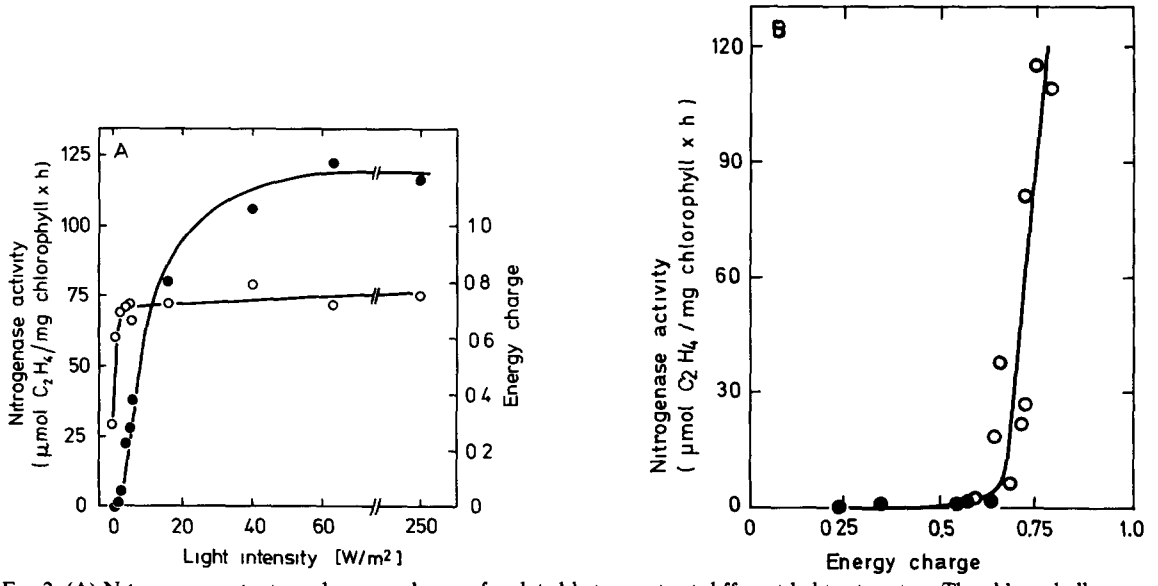


Fig 2 (A) Nitrogenase activity and energy charge of isolated heterocysts at different light intensities The chlorophyll concentration was 59 µg/ml per reaction vial, in an atmosphere of H_2/C_2H_2 Ethylene formation was followed for 30 min Then, the reaction was stopped with perchloric acid and adenylates were extracted For determination of the energy charge see Materials and Methods Light intensity was varied by neutral density filters Nitrogenase activity (●——●), energy charge (○——○) (B) Nitrogenase activity as a function of energy charge in isolated heterocysts Nitrogenase activity was varied by using different light intensities (○) (A) and different oxygen concentrations in the dark (●) (Table I) The resulting rates were plotted versus energy charge

photosynthetic, nitrogen-fixing bacteria [27].

As already mentioned, the energy charge can also be varied in the dark by different oxygen concentrations. At the maximum dark energy charge of 0.64, rates of acetylene reduction were low (Table I). When the energy charge was varied in the light or in the dark and plotted versus enzyme activity, the curve shown in Fig. 2B was obtained Variations of energy charge between 0.24 and 0.64 had little effect on nitrogenase activity, whereas at high energy-charge values, small variations of energy charge (0.7–0.8) were accompanied by large variations in the rate of acetylene reduction (increase from 10 to 100%).

Myokinase in isolated heterocysts

As described above, the energy charge is variable by different light intensities or different oxygen concentrations in the dark. The corresponding ATP, ADP and AMP concentrations were plotted as functions of the energy charge (Fig. 3). The theoretical curves (solid lines) represent the adenylate concentrations calculated from the mass action law of myokinase and the definition of energy charge based on the assumption of

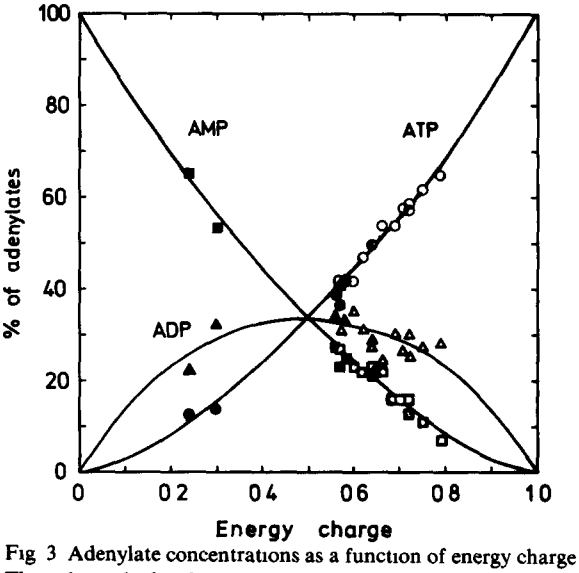


Fig 3 Adenylate concentrations as a function of energy charge The values of adenylate concentrations described in the experiments of Fig 2A and Table I were plotted as a function of energy charge The experimental data were fitted to the theoretical function $[ATP] = f(EC, K_{app})$ assuming an apparent equilibrium constant for myokinase, $K_{app} = 1\,0$ The detailed formula is given in Materials and Methods ATP (○, ●), ADP (△, ▲), AMP (□, ■), open symbols show data from the light experiment (Fig 2A), closed symbols from the dark experiment described in Table I

a constant total adenylate concentration Using the equations given in Materials and Methods, the best fit of the data points was obtained assuming an apparent equilibrium constant for myokinase (K_{app}) of 1.0. The good agreement of measured and calculated adenylate concentrations shows that the myokinase reaction was in equilibrium at different values of energy charge, irrespective of heterocyst energization by either photosynthetic or respiratory activities. It should be mentioned that variations in K_{app} of myokinase were observed with different heterocyst preparations (cf Refs. 22 and 29)

Discussion

High rates of light- and hydrogen-dependent acetylene reduction have only been reported for heterocysts isolated from *A variabilis* (ATCC 29413) [4]. Typically, dithionite, an artificial donor to nitrogenase, was without effect; addition of an ATP-generating system did not significantly stimulate acetylene reduction. It was concluded that heterocysts with low permeability to small molecules are a prerequisite for highly active preparations [4]. These criteria are also met by our preparation of heterocysts from *A. variabilis*. In addition, we find that high nitrogenase activity (60–120 μmol C_2H_2 reduced /mg Chl per h) of heterocysts is correlated with endogenous adenylate concentrations comparable to those measured in intact filaments (240–300 nmol/mg Chl) (Ref. 30, see also Ref. 29) Intact heterocysts, under nitrogen-fixing conditions, show an increase in ATP concentration upon illumination, and in the dark upon addition of oxygen. Since the adenylate-pool size remained constant and no cofactors were added, photosynthetic and respiratory phosphorylation activities are directly demonstrated by these experiments.

In heterocyst preparations with high rates of acetylene reduction, the maximum energy charge measured in the light approaches a value of 0.8. This is known to be a region of optimum regulation of energy-yielding (phosphorylation) and energy-consuming (nitrogenase) reactions [21]. The response of ADP and AMP to changes in ATP concentration upon light-dark or anaerobic-aerobic transitions suggests a regulation of energy balance by myokinase. It has been pointed out that an additional important role of myokinase in energy metabolism consists of making available the γ- and β-phosphate bonds of ATP to energy-requiring processes, even if only the γ-phosphate of ATP is used [22].

Based on the single transformation of ATP to ADP by nitrogenase activity and ADP to ATP by phosphorylation, the ADP/ATP ratio has been regarded as the major factor controlling nitrogen fixation in vivo [24]. However, the active intervention of myokinase and its regulatory action on concentrations of ATP, ADP and AMP necessitates a reinterpretation In addition, it has been reported that AMP inhibited nitrogenase [25]. As pointed out by Atkinson [21], it makes physiological sense to relate changes in enzyme activity to changes in energy charge, since all three nucleotides are present and interconvertible in living cells. When plotting nitrogenase activity against energy charge, the resulting curve has a positive slope that increases steeply at high energy-charge values (above 0.6) The shape of the curve is typical for enzymes participating in biosynthetic pathways [28]. In isolated heterocysts, the energy charge affects nitrogenase activity, especially at high energy charge values we observed large variations (10–100%) in the rates of acetylene reduction A similar correlation between acetylene-reduction rates and energy charge has been found in vitro with purified nitrogenase [25]. However, as compared to Fig 2B, the major change in nitrogenase activity occurred at energy-charge values between 0 8 and 1.0, and the curve was less steep [25]. This might be due to the in vitro experimental conditions. myokinase absent, unphysiological concentrations of adenyl nucleotides and Mg^{2+}, and electron supply by dithionite. In this respect, it is worth mentioning that in nitrogen-fixing bacteria and cyanobacteria, the membrane potential appears to regulate additionally nitrogenase activity [27,32,33]. The theory has been put forward that reductant supply to nitrogenase is dependent on an energized plasmalemma membrane, with a threshold value for $\Delta\psi$ of about -75 mV (inside negative) [27,32,33]. Currently, we are investigating the possibility whether electron supply to nitrogenase contributes to the steepness of the curve depicted in Fig. 2B.

Under nitrogen-fixing conditions, the ADP/ATP ratio in heterocysts, calculated from the experiments described above, varies between 0.4 and 0.5. According to the studies of Mortenson and Upchurch [31], this is the region of optimum electron flow through nitrogenase with greatest apparent efficiency: the number of ATP molecules hydrolyzed per electron transferred (ATP/e ratio) approaches the value of 1

The rapid decay of ATP concentration after a light-dark transition is another interesting point that should be discussed As shown in Fig. 2B, this decrease cannot be explained merely by nitrogenase activity, since nitrogenase works at relatively high energy-charge values. In intact spinach chloroplasts, similar changes of adenylate concentrations upon light-dark transitions have been observed [34] The rapid decay of the ATP pool, after light had been switched off, was explained by the ATP hydrolase activity of the activated coupling factor [35]. Upon illumination of intact chloroplasts, the coupling factor becomes both pH activated and thiol modulated [36,37] ATP hydrolysis is observed after deenergization of the thylakoid membrane following illumination [27] This would lead to a rapid drop in energy charge. Deactivation of the hydrolytic activity of the coupling factor [39], probably caused by rebinding of ADP [40], would stabilize the dark energy-charge value Rapid changes in energy charge are a sufficiently fast-acting control on ATP-consuming reactions. In heterocysts, nitrogenase would be active only at high energy input, being switched off immediately when energy supply ceased. This hypothesis, however, requires further experiments

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