Biochimica et Biophysica Acta, 1099 (1992) 226–232

BBABIO 43568

Light/dark regulation of photosynthetic enzymes within intact cells of the cyanobacterium *Nostoc* sp. *Mac*

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(Received 5 August 1991)

Key words: Thioredoxin; ATPase; Enzyme activation; Photosynthesis; Cyanobacterium; (*Nostoc*)

Light/dark regulation of several photosynthetic enzymes has been studied under near in vivo conditions using the cyanobacterium *Nostoc* sp. *Mac*. Cells were grown photoheterotrophically and rendered osmotically fragile by treatment with lysozyme. Treated cells evolved O_2 during photosynthesis at 20–80% of the rate of control cells. Enzyme activities were measured following dilution of preilluminated cells into a hypotonic assay medium. Inhibitor studies using methyl viologen, *N,N'*-dicyclohexylcarbodiimide and triphenyl tin indicate that fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, ribulose-5-phosphate kinase and NADP-linked glyceraldehyde 3-phosphate dehydrogenase are reversibly light-activated in vivo by the cyanobacterial thioredoxin system. In contrast, the protonmotive ATPase F_0-F_1-type ATPase appears to be fully active in both light- and dark-adapted cells at physiological temperatures, but is reversibly deactivated by cooling the cells on ice. No activity of NADP-linked malate dehydrogenase or glucose-6-phosphate dehydrogenase could be detected in these cells.

Introduction

The physiological function of reductive enzyme regulation has been studied by illuminating dark-adapted intact chloroplasts and protoplasts and following the changes in target enzyme activity observed after rapid osmotic lysis [1–6]. Illumination reduces thioredoxin by electron transport from Photosystem I to a ferredoxin-thioredoxin reductase [1], and consequently, several enzymes of the Calvin cycle become reductively activated, notably FBPase [2,3], sedoheptulose-1,7-bisphosphatase (SBPase) [3], ribulose-5-phosphate kinase (R5PK) [3,4] and NADP-linked glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH) [4]. Illumination also results in activation of MDH, involved in export of reducing equivalents via the malate shuttle, and inactivation of glucose-6-phosphate dehydrogenase, a major control point of the oxidative pentose phosphate pathway [4].

A general conclusion from earlier kinetic studies was that reductive modulation of these enzymes was faster than the onset of CO_2 fixation, and saturated at lower light intensities [5]. This suggested that the thioredoxin system functions as an on/off metabolic switch to prevent futile cycles occurring, for example between the oxidative and reductive pentose phosphate cycles. However, the kinetics and extent of enzyme reduction are often dependent on factors other than light intensity, for example allosteric metabolites, and a case has been made for a more dynamic function for thioredoxin in the 'fine-tuning' of metabolism to different environmental conditions [6].

In plants and green algae, thioredoxins also regulate photophosphorylation. The chloroplast protonmotive ATPase can be reduced in vitro by the thioredoxin system [7]. This involves reduction of a disulphide bridge on the γ subunit [8] which greatly enhances the activation of the enzyme by ΔpH across the thylakoid membrane [9,10]. In vivo, reductive modulation precedes CO_2 fixation in barley protoplasts and saturates at very low light intensities [11]. We concluded that it functions to increase the efficiency of ATP synthesis in the light whilst preventing wasteful ATP hydrolysis in the dark.

Like chloroplasts, cyanobacteria carry out oxygenic photosynthesis by a reductive pentose phosphate cycle

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Abbreviations: DCCD, *N,N'*-Dicyclohexylcarbodiimide; F_0-F_1, reversible protonmotive ATPase (EC 3.6.1.34); FBPase, fructose-1,6-bisphosphatase (EC 3.1.3.11); MDH, NADP-linked malate dehydrogenase (EC 1.1.1.37); NADP-GAPDH, NADP-linked glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9); NAD-GAPDH, NAD-linked glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); R5PK, ribulose-5-phosphate kinase (EC 2.7.1.19); SBPase, sedoheptulose-1,7-bisphosphatase (EC 3.1.3.37); Tricine, *N*-tris(hydroxymethyl)methylglycine; ΔpH, difference in pH across the thylakoid membrane.

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and dark degradation of carbohydrate is restricted mainly to an oxidative pentose phosphate pathway [12]. Buchanan and co-workers reported purification of both m- and f-type thioredoxins from *Nostoc muscorum* based on their ability to activate the appropriate *Nostoc* [13] enzymes in vitro. The gene for Th_m has been cloned and sequenced from *Anabaena* sp. [14] and *Anacystis nidulans* R2 [15] and found to be homologous to the bacterial and the plant Th_m sequence. Attempts to produce viable mutants by insertional inactivation of the Th_m gene failed in the phototroph *Anacystis*, suggesting that thioredoxin has an essential function in light/dark regulation of metabolism [15].

Early work based on metabolite measurements in whole cells indicated a light/dark regulation of certain enzymes much like chloroplasts [16], but few attempts have been made to directly measure these changes in enzyme activity. In one study, preillumination of cells prior to disruption in a French press did not increase the activity of FBPase, SBPase or NADP-GAPDH, nor could the latter two enzymes be activated by dithiothreitol in the disruption medium [17]. In another study, illumination of intact cells was shown to cause rapid reduction of Th_m, but from the slow reoxidation kinetics and the apparent localisation of this thioredoxin in the nucleoplasm, its role in enzyme modulation within the cell was questioned [18].

Little is known of the regulation of the cyanobacterial thylakoid ATPase in vivo. The genes for F_1 have been cloned and sequenced in several cyanobacteria [19,20]. A regulatory Cys is absent on the γ subunit compared to the plant enzyme, suggesting the enzyme would not be regulated by thioredoxin. However, isolated F_1 is a latent ATPase and dithiothreitol slightly stimulates its activation by heat [21] (but see Ref. 20). This indicates that the cyanobacterial enzyme is regulated by ΔpH and possibly by thioredoxin in vivo.

In this paper, we report a rapid method for measuring light/dark regulation of cyanobacterial enzymes in vivo based on osmotic lysis of lysozyme-treated cells [22]. The cyanobacterium *Nostoc* sp. *Mac* was chosen because it is nutritionally versatile (Carr, N.G., personal communication), and therefore of potential use in further genetic and physiological studies. It is shown that large light-activation of a number photosynthetic enzymes can be observed by this method. However, the F_0-F_1-type ATPase of cyanobacteria is already active in metabolising cells, and illumination results in little further activation.

Materials and Methods

Lysozyme treatment of cells

Nostoc sp. *Mac* was grown in liquid BG11 medium, supplemented with 5 mM glucose, on a rotary shaker (140 rpm) at 30°C in continuous white light (30 $\mu E/m^2$). Cells were harvested at a chlorophyll concentration of approx. 6 μg/ml (6–8 days growth) by centrifugation at $2860 \times g$ for 15 min. The cells were then treated with lysozyme [22] by resuspending in 50 ml of 0.5 M sucrose, 10 mM $MgCl_2$, 5 mM sodium/potassium phosphate, 2% BSA, 10 mM Mes-KOH (pH 6.9) to which was added lysozyme at 1 mg/ml. Incubation was carried out in a shaking water bath at 34°C for 1 h. The cell suspension was then centrifuged at $720 \times g$ for 90 s and the resulting supernatant discarded. A colourless supernatant was usually observed at this stage, indicating that few cells had lysed during the lysozyme treatment (lysis would have resulted in the release of phycoerythrin). The fragile lysozyme-treated cells were washed once by resuspension, centrifugation and resuspension in incubation medium lacking lysozyme and sodium/potassium phosphate but enriched with 0.5% BSA. Control cells underwent the same procedure except lysozyme was omitted from the incubation medium. Cells were stored on ice in darkness for 90 min prior to use.

Enzyme assays

Chlorophyll was quantitatively extracted by 100% methanol treatment and estimated according to Ref. 23.

Oxygen uptake and evolution were determined at a cell concentration of 50–80 μg chlorophyll/ml in 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ascorbate, 5 mM $NaHCO_3$, catalase (750 U/ml), 50 mM Hepes (pH 7.6) in an oxygen electrode (Rank Bros., Botisham, UK). The temperature was maintained at 22°C. Net oxygen uptake was measured in the dark and net oxygen evolution by illumination with red light (60 W/m^2) obtained by filtering the output of a 300 W quartz-halogen lamp through a Corning 620 nm cut-off filter. Where indicated, cells were subjected to osmotic shock by resuspending in a medium lacking sorbitol for 1 min followed by addition of sufficient 2 M sorbitol to bring the final concentration to 0.33 M.

For enzyme assays, dark-adapted cells were diluted into an isotonic activation medium containing 0.33 M sorbitol, 5 mM $MgCl_2$, catalase (750 U/ml), 30 mM Tricine-KOH (pH 8.0) to a chlorophyll concentration of 60–100 μg/ml. Methyl viologen was added as indicated. Following 3–4 min incubation in the dark, a sample (0.1 ml) was withdrawn for assay of dark-adapted enzyme activity and the remaining suspension was illuminated as above. Further samples were periodically withdrawn and introduced into 0.9 ml of hypotonic assay medium at 22°C, where rapid lysis occurred. For measurement of ATPase, the assay medium contained 2 mM ATP, 5 mM $MgCl_2$, 30 mM Tricine-KOH (pH 8.0), and the reaction was allowed to proceed for 5 min in the dark. For measurement of FBPase or SB-

Pase, the medium contained 1 mM fructose 1,6-bisphosphate or 1 mM sedoheptulose 1,7-bisphosphate in place of ATP and the assay time was 10 min. All reaction rates were linear during the assay period (results not shown). DCCD, triphenyl tin and NH_4Cl were added as indicated. All phosphatase assays were terminated by addition of 0.2 ml 20% trichloracetic acid and activity was determined from the release of P_i as previously described [9].

For measurement of 9-aminoacridine quenching, substrates were initially omitted from the assay medium and 5 μM 9-aminoacridine was included. Cells were added to approx. 2–4 μg chlorophyll/ml and quenching initiated by addition of 0.3 mM ATP as previously described [24].

Dehydrogenase activities were measured by following NAD(P)H absorbance at 340 pm in a Cecil CE545 split beam spectrophotometer. Cells were illuminated as described above, lysed in basal hypotonic medium (5 mM $MgCl_2$, 30 mM tricine (pH 8.0)) and centrifuged for 2 min in a microfuge to remove cell debris and membranes. For measurement of GAPDH, 5 mM P_i, 1 mM EGTA, 1 mM ADP and 0.5 mM NAD(P) were added, and the reaction started with 1 mM D/L-glyceraldehyde 3-phosphate. R5PK was assayed by a similar method following addition of 1 mM EDTA, 1 mM phospho*enol*pyruvate, 0.5 mM ATP, 4 U/ml pyruvate kinase, 3 U/ml lactate dehydrogenase and 0.2 mM NADH. A low rate of NADH oxidation due to soluble ATPase activity was detected and subtracted from the rate observed upon addition of 0.5 mM ribulose 5-phosphate to obtain R5PK activity.

Where indicated, error estimates are SEM from 4–6 different preparations of lysozyme-treated cells. Where no errors are given, the data represent a single determination from a typical preparation.

Results

Microscopic and biochemical integrity of lysozyme-treated cells

As far as possible, control cells were harvested and assayed by the same procedures used to produce lysozyme-treated cells. Control preparations consisted of chains of several hundred vegetative cells, and typically evolved oxygen at a rate of 60 μmol/mg Chl per h upon illumination. As shown in Table I, the rate of O_2 evolution was not significantly affected by subjecting control cells to an an osmotic shock. Similarly, the hypotonic enzyme assay procedures resulted in little observable ATPase and FBPase activity, and little release of phycobilin pigments.

Inclusion of the lysozyme treatment step affected the appearance of cells under the microscope. The chains were shorter in appearance (20–100 cells long) and were much more cohesive. Few individual spheroplasts were observed. Rates of oxygen evolution varied from 20 to 80% compared to control cells (see Table I for a typical preparation). As shown in Table I, exposure of lysozyme-treated cells to hypotonic conditions abolished photosynthetic O_2 evolution capacity, indicating that the lysozyme-treated cells were osmotically fragile. Maximal ATPase and FBPase activities were released within a few seconds of osmotic lysis. Absolute rates varied between preparations, the range for ATPase activity being 50–160 μmol/mg Chl per h. Generally, preparations with higher rates of O_2 evolution capacity also displayed higher rates of ATPase and other enzyme activities. This variation of absolute rates between experiments gave rise to the large standard errors quoted for subsequent data.

TABLE I

Effect of an osmotic shock on photosynthetic O_2 evolution by control and lysozyme-treated cells

Condition	Oxygen evolution (μmol O_2 evolved/mg Chl per h)	
	control cells	lysozyme-treated cells
Intact	60	35
Shocked	55	2

Upon centrifugation of the cells immediately after lysis, approx. 6% of the cellular phycoerythrin remained in the supernatant. This increased to 40% if lysed cells were allowed to stand for 10 min prior to centrifugation. The appearance of soluble phycoerythrin was thus slower than appearance of all enzyme activities, indicating that this protein slowly dissociates from the exposed membranes. Soluble phycoerythrin was therefore not a good marker of cellular disruption although it did appear to correlate with the number of empty cells visible under the microscope upon examination of the pellet.

Light / dark regulation of enzymes

Considerable ATPase activity was observed upon subjecting dark-adapted, lysozyme-treated cells to osmotic lysis (Fig. 1a). A small increase in activity was generally observed upon preillumination, but this was not readily reversed in the dark, nor was it affected by the presence of methyl viologen (although methyl viologen did induce in a small but general inhibition of activity). This electron acceptor was shown to completely block thioredoxin-dependent activation of CF_0-CF_1 in plant chloroplasts [25] and green algae [26] by preventing reduction of thioredoxin. At the concentration used here, methyl viologen completely abolished photosynthetic O_2 evolution by lysozyme-treated cells (results not shown).

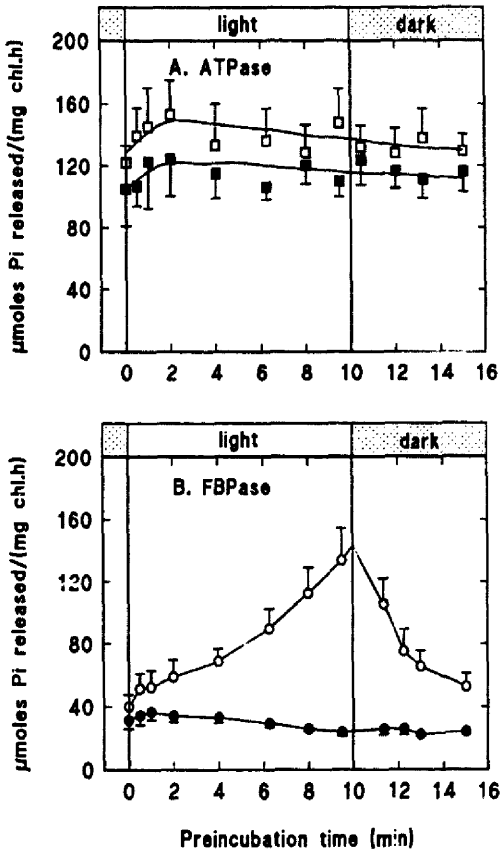

Fig. 1. Effect of preincubation in the light and dark on (a) ATPase and (b) FBPase activity observed in subsequently lysed cells. The incubation medium contained lysozyme-treated cells in the absence (□, ○) or presence (■, ●) of 0.1 mM methyl viologen. Illumination began at time 0 following 3–4 min preincubation in the dark at 22°C.

In contrast, Fig. 1b shows that preillumination induced a 2–3-fold increase in FBPase activity which was fully reversed on turning off the light. The light-induced stimulation of FBPase was completely blocked by methyl viologen. These results indicate that light/dark regulation of FBPase in cyanobacteria is analogous to that observed in chloroplasts (results not shown) and green algae [7]. The kinetics of light activation are slow, appear to be biphasic, and were probably not completed by the time illumination was terminated. Oxygen evolution on illumination of intact lysozyme-treated cells underwent an induction phase of 2–3 min typical of other photosynthetic organisms, remained constant for 10 min and thereafter gradually declined (results not shown).

Using these methods, we have examined a number of other cyanobacterial enzymes whose counterparts in chloroplasts are known to be light/dark regulated via the thioredoxin system (Table II). Both R5PK and SBPase underwent a relatively large activation upon preillumination of lysozyme-treated cells. Like FBPase, the activity change was also reversible in the dark and sensitive to methyl viologen added to the incubation

TABLE II

Effect of preillumination and methyl viologen on the enzyme activity observed following lysis of lysozyme-treated cells of the cyanobacterium Nostoc sp. Mac

Cells were either dark-adapted for 4 min or illuminated for 10 min prior to lysis. Where indicated, methyl viologen was present at a concentration of 0.1 mM during preincubation.

	Activity (µmol P_i released /mg Chl per h)			
	dark-adapted cells		preilluminated cells	
Methyl viologen:	–	+	–	+
R5PK	25 ± 8	26 ± 12	62 ± 17	29 ± 13
SBPase	8 ± 4	9 ± 3	49 ± 7	10 ± 3
NADP-GAPDH	1 ± 0.5	2 ± 0.5	5 ± 2	2 ± 1
NAD-GAPDH	21 ± 6	19 ± 3	20 ± 4	18 ± 4

medium. The NADP-linked GAP dehydrogenase was also light-regulated, though the specific activity was low, particularly when compared to the NAD-linked GAP dehydrogenase whose activity was not affected by preillumination. Of the other enzyme activities examined, no light-activation of NAD-linked malate dehydrogenase was observed, and the activities of NADP-malate dehydrogenase and glucose-6-phosphate dehydrogenase were undetectable in these cells by these methods (results not shown).

Identity of the ATPase activity

It is important to know how much of the observed ATPase activity is due to F_0-F_1-type ATPases before the significance of the above data can be assessed. We therefore examined the effects of several F_0-F_1 inhibitors including DCCD [27], triphenyl tin [28] and tentoxin [29]. The results are shown in Fig. 2a. When added to the assay medium, DCCD and triphenyl tin significantly inhibited the dark activity of the ATPase, and also appeared to abolish the small light-activation of this enzyme. Tentoxin had no inhibitory effect (results not shown). As a control, the effect of these inhibitors on FBPase was also examined. DCCD had no effect on FBPase after preincubating cells in the dark or light (Fig. 2b), but triphenyl tin did result in a small, general inhibition. comparison of Fig. 2a and b suggests that the inhibition of ATPase by triphenyl tin over and above that observed for DCCD may be a non-specific effect similar in nature to the inhibition of FBPase by triphenyl tin. The results indicate that around 50% of the observed dark ATPase activity is due to F_0-F_1-type ATPase, this being the activity sensitive to DCCD.

Fig. 3b shows additional evidence that thylakoid-bound F_0-F_1-ATPase is active in dark-adapted cells. Addition of ATP to lysed, dark-adapted cells resulted in quenching of 9-aminoacridine fluorescence. The slow

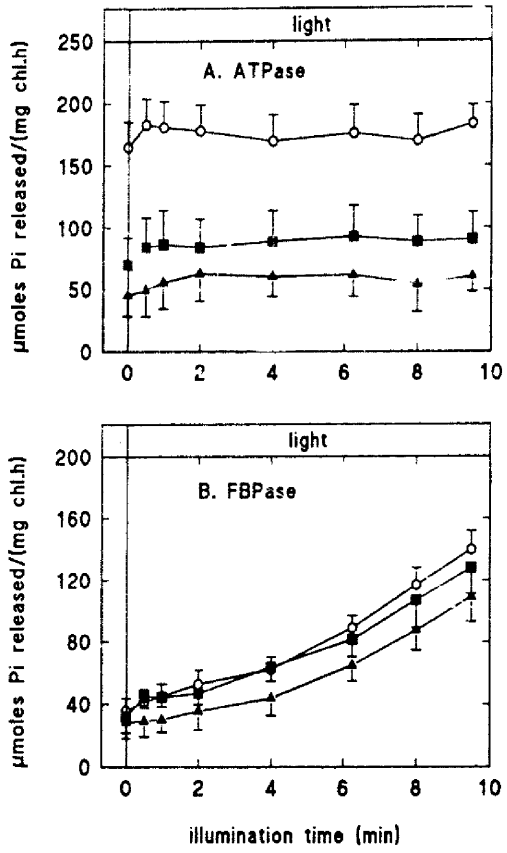

Fig. 2. Effect of F_1-F_0-inhibitors on the activity of (a) ATPase and (b) FBPase observed following lysis of preilluminated cells. The assay medium contained no inhibitor (○), 0.1 mM DCCD (■) or 10 μM triphenyl tin (▲).

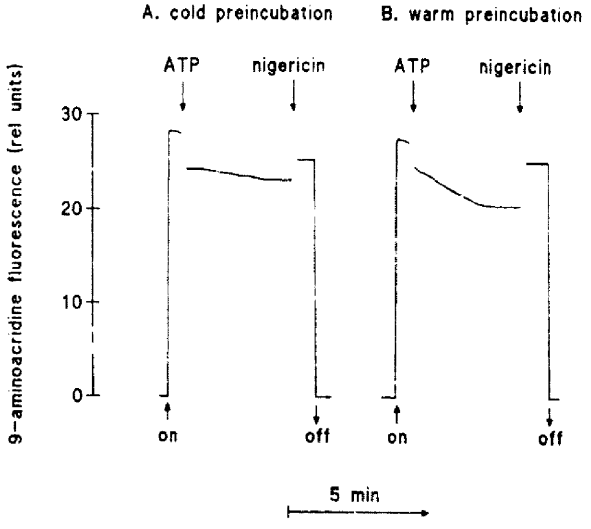

Fig. 3. ATP-dependent quenching of 9-aminoacridine fluorescence observed in lysed cells. Dark-adapted cells were stored on ice and then lysed (a) immediately, or (b) after 4 min preincubation at room temperature. Gaps in the traces occur when the photomultiplier was switched off to enable additions of ATP and nigericin to final concentrations of 0.3 mM and 1 μg/ml, respectively. The rapid, unresolved decrease in fluorescence after ATP addition is due to chemical quenching of fluorescence by nucleotides.

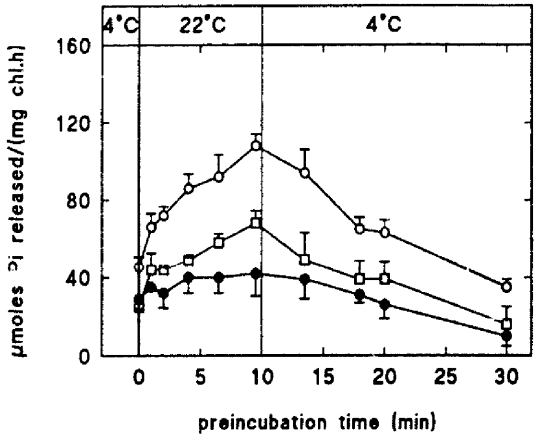

Fig. 4. Effect of warming and cooling on the activity of ATPase observed in subsequently lysed, dark-adapted cells. At time zero, 0.6 ml of dark-adapted, lysozyme-treated cells at 4°C were diluted into 1.4 ml of incubation medium at 22°C containing no other additions (○), 0.1 mM DCCD (●), or (□) 10 mM NH_4Cl. When the NH_4Cl was omitted, the assay medium contained 1 mM NH_4Cl to ensure comparable assay conditions. After 10 min at 22°C, the preincubation medium was plunged into ice and allowed to cool.

phase of quenching is fully reversed by nigericin (or NH_4Cl, not shown) indicating that the thylakoid membranes are coupled, and that F_0-F_1 is capable of generating ΔpH by protonmotive ATP hydrolysis [24].

The induction of the ATPase activity on warming

Lysozyme-treated cells were routinely stored on ice, since this resulted in better stability than storage at room temperature. In order to observe protonmotive ATPase activity, it was necessary to warm the cells for several minutes prior to lysis. As shown in Fig. 3a, cells that were immediately lysed following cold storage were much less able to support ATP-dependent quenching of 9-aminoacridine fluorescence compared to cells that had been preincubated in the dark for 4 min at room temperature prior to lysis. A similar effect of warming was also observed when total ATPase activity was assayed by P_i release. Fig. 4 shows the induction of dark-adapted ATPase on warming the cells to room temperature prior to sampling and assay. The effect is reversible on again cooling the cells. The addition of 10 mM NH_4Cl to the activation medium gave an overall reduction in activity but did not completely eliminate the warming effect. However, the DCCD-insensitive ATPase activity underwent little change in activity upon warming or cooling the cells prior to assay. Likewise, no increase in FBPase activity was observed on warming the cells prior to assay (results not shown). Only the F_0-F_1-ATPase responds to temperature at which the cells are preincubated.

Discussion

Nostoc sp. *Mac* is shown here to be a good choice for the study of light/dark regulation of metabolic

enzymes under near in vivo conditions. Lysozyme-treatment yields cells that can maintain photosynthetic O_2 evolution in isotonic media at a constant rate for at least 10 min. Exposure to hypotonic conditions abolishes O_2 evolution capacity and results in maximal release of ATPase and FBPase activity within a few seconds of exposure. This gives confidence that the assays are reporting enzyme redox states of physiological significance.

This work represents the first direct demonstration that both FBPase and SBPase are reversibly activated by light in intact cyanobacteria, although such regulation had previously been inferred from observed changes in metabolite concentrations in intact cells [19]. Prevention of the light activation by methyl viologen provides evidence that these enzymes are under the control of the thioredoxin system in vivo, confirming the suggestion from studies with purified proteins [16]. Other workers failed to demonstrate light-activation of FBPase and SBPase, although light-dependent activation of ribulose-5-phosphate kinase, and deactivation of glucose-6-phosphate dehydrogenase, were detected [20]. This failure was probably due to deactivation of the enzyme during the long time (45 min) taken to break the cells by French press, and emphasises the need for a rapid lysis and assay system for such studies.

Inhibitor studies indicate that about half of the dark ATPase activity is due to F_0-F_1-type ATPases. Although a small light-activation of the ATPase was seen, this is probably not due to reductive modulation by thioredoxin, since the changes are not dark-reversible, nor are they very sensitive to methyl viologen. This experiment supports the idea, made from sequencing data [22,24], that cyanobacterial F_0-F_1 is not a target enzyme for thioredoxin.

The high F_0-F_1-ATPase activity observed in the dark, the small activation in the first few minutes of illumination, and the effect of warming are at first sight puzzling. The cyanobacterial protonmotive ATPase was reported to be fully latent both in isolated thylakoids and in purified F_1 [23]. A reasonable explanation for all these phenomena is that F_0-F_1 is not regulated by thioredoxin, but is activated by an electrochemical potential gradient of protons across the thylakoid membrane, as are most enzymes of this class. Thus, storage on ice results in collapse of a protonmotive force which allows deactivation of F_0-F_1 to the latent form. Warming reactivates the enzyme as respiration-dependent ΔpH is re-established across the thylakoid. Our cells are observed to respire at significant rates in the dark, and respiratory electron chain activity is known to exist in the thylakoid membrane, albeit at low activity compared to photosynthetic electron transport capacity [30]. The small light-activation seen in Fig. 1a would represent the completion of the reactivation process. Further experiments are currently in progess to investigate the possible role of respiratory electron transport on ATPase activity.

Finally, the lack of control of cyanobacterial F_0-F_1 by thioredoxin compared to its counterpart in the chloroplasts of plants may reflect the different physiological demands placed on this enzyme. The thioredoxin system is often described as a switching mechanism, which either enables or disables metabolic pathways that are only required in light or in darkness [1]. Such a control mechanism would be inappropriate for the cyanobacterial F_0-F_1 which has to function both in dark respiration and photosynthetic ATP synthesis.

Acknowledgements

We thank Prof. Noel Carr, Dr. Dave Scanlan and Dr. Nick Mann of the University of Warwick for providing the culture and for fruitful discussion, and Mrs Ann Cornes and Margaret Critchlow for expert technical assistance.

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